Stereospecificity of the Transfer of Hydrogen from Reduced Nicotinamide Adenine Dinucleotide Phosphate to the Acyl Chain in the Dehydrogenase-catalyzed Reactions of Fatty Acid Synthesis

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RICHARD E. DUGAN, LINDA L. SLAKKEY, AND JOHN W. PORTER

From the Lipid Metabolism Laboratory, Veterans Administration Hospital, and the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

Tritium from NADPH was incorporated into the fatty acids (mainly palmitic acid) synthesized from acetyl and malonyl coenzyme A by purified pigeon and rat liver fatty acid synthetases. The quantity of tritium incorporated into the product was nearly the same from the A and B forms of NADPH. These compounds were prepared from tritiated isocitric and glutamic acids, respectively. The quantity of radioactivity incorporated into fatty acids per mole of NADPH oxidized was slightly less than half the quantity of radioactivity present per mole of isocitric or glutamic acid. On the assumption that the two types of reduction in fatty acid synthesis (β-ketoacyl-enzyme and α,β-unsaturated acyl-enzyme) proceed stereospecifically, as do all other pyridine nucleotide dehydrogenase reactions, these results indicate that one reduction proceeds by the transfer of hydrogen from the A side of position 4 of the pyridine ring of NADPH, and the other reduction proceeds by transfer from the B side. This deduction was confirmed by the performance of partial reactions with model substrates. When acetoacetyl coenzyme A or S-acetoacetyl-N-acetylcysteamine was reduced to the β-hydroxybutyryl ester with fatty acid synthetase and tritiated NADPH, tritium was incorporated into the product only from the A form of the coenzyme. When S-crotonyl-N-acetylcysteamine was similarly reduced to the butyryl ester, tritium was incorporated into product only from the A form of NADPH. Thus, the reduction of β-ketoacetyl-enzyme in fatty acid synthesis results in the transfer of hydrogen from the B side of NADPH, and the reduction of α,β-unsaturated acyl-enzyme results in the transfer of hydrogen from the A side of NADPH.

The synthesis of long chain fatty acids (mainly palmitic acid) from acetyl and malonyl coenzyme A and NADPH is catalyzed by at least six to eight enzymes of vertebrates which purify to homogeneity as a multi-enzyme complex. Two of the enzymes of this complex are dehydrogenases. These enzymes catalyze the following NADPH specific reductions.

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\begin{align*}
\text{R-C-CH}_2\text{C-enzyme} + \text{NADPH} + \text{H}^+ &\rightarrow \text{OH} \quad \text{O} \\
\text{R-C-CH}_2\text{C-enzyme} + \text{NADPH} + \text{H}^+ &\rightarrow \text{OH} \\
\end{align*}
\]

In the present investigation we are concerned with the stereochemistry of the transfer of hydrogen from NADPH to the acyl chain in each of the above reactions. Previous investigations by others have established that a hydride ion is transferred from position 4 of the pyridine ring of NADPH to the β carbon of the acyl chain in each of these reactions (2). It has also been shown that a proton from water is acquired by the α carbon in the second reduction (3, 4). Furthermore, it has been established that the product of the first reduction, a β-hydroxyacyl ester, has the p configuration (5, 6).

Recent investigations by Drysdale\(^1\) have shown that yeast crotonyl-CoA reductase transfers hydrogen from NADPH to the β carbon of the α,β-unsaturated acyl ester to yield the pro R configuration of the product. If it is assumed that this finding extends to the fatty acid synthetase-catalyzed transfer of hydrogen in the second reduction of fatty acid synthesis, then a service. A preliminary report (1) of this work was presented at the 54th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April 12 to 17, 1970.

\(^1\) G. R. Drysdale, personal communication; manuscript in preparation.
determination of the side of the pyridine ring of NADPH from which tritium is transferred, in this and the first reduction of fatty acid synthesis, would establish the complete stereochemistry of hydrogen transfer in fatty acid synthesis.

In an earlier study on the transfer of hydrogen by a mammalian fatty acid synthetase it was reported (7) that tritium is incorporated into fatty acids from both the A and B forms of tritiated NADPH. In addition it was reported that more tritium was incorporated into the product from the A form. However, no conclusions were reached as to the stereospecificity of each of the reductive reactions in fatty acid synthesis.

**Experimental Procedure**

**Chemicals**—The chemicals used in these investigations were obtained from the following sources: NADP, NADPH, acetoacetyl-CoA, and (+)-isocitric acid (monopotassium salt) from Sigma; acetyl- and malonyl-CoA from P-L Biochemicals; ethylenimine, diketene, and butyric acid from Matheson Coleman and Bell (Division of the Matheson Company); thiolacetic acid and 1,2-dibromo-1,2-dihydroxybutylic acid from Aldrich; D-glutamic acid from Nutritional Biochemicals; isocitric and glutamic dehydrogenases from Boehminger Mannheim; and potassium DL-isocitrate-2-3H and DL-glutamic-2-3H acid from New England Nuclear.

**Preparation of Enzymes**—Isocitric dehydrogenase (5 mg of protein in glycine) and glutamic dehydrogenase (10 mg of protein, as a crystalline suspension in ammonium sulfate) were prepared by the methods of Hsu, Wasson, and Porter (8) and Hsu and Porter (9), respectively, and then stored at -20° in the presence of 10 mM diithiothreitol. The fatty acid synthetases assayed spectrophotometrically (10) at specific activities of 65 to 85 nmoles of palmitic acid formed per min per mg of protein.

**Preparation of Substrates**—Tritiated isocitrate, which contained 16.6 × 10⁶ dpm per nmole of the correct stereoisomer, was diluted 1,400-fold with the same stereoisomer of nonradioactive isocitrate to yield a specific activity of 11,440 dpm per nmole. The tritiated L isomer of glutamic acid (6.21 × 10⁶ dpm per nmole) was diluted 490-fold with nonradioactive L-glutamic acid to yield a specific activity of 12,650 dpm per nmole. The tritiated A form of NADPH was then synthesized in a 1-ml incubation mixture which contained tritiated isocitrate (11,400 dpm per nmole), 2 μmoles; NADP, 0.3 μmole; MgCl₂, 1 μmole; isocitric dehydrogenase, 50 μg of protein; and potassium phosphate buffer, pH 7.5, 50 μmoles. The reduction of NADP was followed to completion by measurement of light absorption at 340 nm with a Zeiss monochromator and a Gilford 200 optical density converter. In some experiments isocitric dehydrogenase was denatured at the end of an incubation by boiling for 2 min. The insoluble protein was then centrifuged from the supernatant solution. In other experiments the enzyme was removed by filtration through Sephadex G-25 (coarse) gel on a 45 × 1-cm column. Both methods completely removed isocitric dehydrogenase activity with very little loss of radioactive NADPH. The tritiated B form of NADPH was prepared by the same procedure, except that incubations were performed in a 1-ml solution containing tritiated glutamic acid (12,650 dpm per nmole), 4 μmoles; NADP, 0.3 μmole; glutamic dehydrogenase, 100 μg of protein; and potassium phosphate buffer, pH 7.5, 50 μmoles. Between one-third and one-half of the NADP in this system was reduced under these conditions. The resulting tritiated NADPH was separated from glutamic dehydrogenase as reported above.

N-Acetylcysteamine was prepared from thiolactic acid and ethylenimine according to the procedure of Kuhn and Quadbeck (11). S-Acetoacetyl-N-acetylcysteamine was prepared from N-acetylcysteamine and diketene according to the method of Lynen and Wieland (12). Recrystallization of the product from ethanol-ether gave white needles, m.p. 55-56°. These crystals assayed at 90% S-acetoacetyl-N-acetylcysteamine as determined by measurement of absorbance at 237 nm and pH 7.0 (13). S-Crotonyl-N-acetylcysteamine was synthesized from crotonyl chloride and the lead salt of N-acetylcysteamine in anhydrous benzene by the method of Kass, Brock, and Bloch (14). The product, purified by column and thin layer chromatography (14), was an oil with two absorption maxima, at 226 and 263 nm, A226:A263 = 1.74. Some of the experiments were done with S-crotonyl-N-acetylcysteamine recrystallized from benzene-petroleum ether, m.p. 61-62°. No difference in the utilization of tritiated NADPH was observed for the two preparations.

**Synthesis of Fatty Acids in Presence of A and B Forms of Tritiated NADPH**—Determination of Triterium Incorporation into Product—Long chain fatty acids were synthesized in a solution of 0.75 ml which contained acetyl-CoA, 50 nmol; malonyl-CoA, 100 nmol; pigeon or rat liver fatty acid synthetase, 100 μg of protein; potassium phosphate buffer, pH 7.0, 150 μmoles; and the A form of tritiated NADPH (made from tritiated isocitrate), 48 to 52 nmol, or the B form of tritiated NADPH (made from tritiated glutamate), 17 to 20 nmol. The oxidation of NADPH was followed spectrophotometrically at 340 nm with a Zeiss monochromator and a Gilford 200 optical density converter. Oxidation of NADPH under the above conditions was complete in approximately 1 min. The enzyme was then denatured by the addition of 0.04 ml of 60% perchloric acid and 1 ml of absolute ethanol. In one set of control experiments all conditions and components were the same as reported above except for the absence of fatty acid synthetase in the incubation mixture. In another set of controls the fatty acid synthetase was present but malonyl-CoA was omitted. The reaction time and the conditions for stopping the reaction were the same for controls as for samples. The ethanolic, acidified solutions (samples and controls) were then extracted three times with 5-ml portions of petroleum ether. The combined petroleum ether extract of each sample was washed twice with 100 ml of water. Then it was placed in a counting vial and evaporated just to dryness under a gentle stream of air. The residue was dissolved in dioxane- phosphor and assayed for radioactivity with a model 3365 Packard Tri-Carb liquid scintillation spectrometer.

**Reduction of Acetoacetyl and Crotonyl Esters with Tritiated A and B Forms of NADPH**—The reduction of acetoacetyl esters to β-hydroxybutyryl esters was carried out in an incubation mixture of 0.70 ml which contained acetoacetate-CoA, 100 nmols, or S-acetoacetyl-N-acetylcysteamine, 6.2 μmoles; NADPH (A form), 50 to 55 nmols, or (B form), 30 to 35 nmols; pigeon or rat liver fatty acid synthetase, 150 μg of protein; and potassium phosphate buffer, pH 7.0, 130 μmoles. The reduction of the crotonyl ester to the butyryl ester was effected in an incubation mixture of 0.70 ml which contained S-crotonyl-N-acetyl-

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1 This compound was a gift from Dr. Surinder Kumar.
and controls containing no ester, were spotted on Whatman No. 1 paper. The chromatogram was developed by descending chromatography with a solvent system of NH₄OH (3 N)-EDTA (pH 4.5, 0.1 M)-isobutyric acid-water 12:1:6:2:28 (16). The β-hydroxybutyryl-CoA, Kₚ 0.70, which was located with ultraviolet light, was effectively separated from the other radioactive components, isocitric acid, Kₚ 0.40, or glutamic acid, Kₚ 0.54, and the acid degradation products of NADPH, Kₚ of less than 0.50. The area containing β-hydroxybutyryl-CoA was cut out and this section of paper was eluted with water. The eluate was concentrated to 1 ml by lyophilization, then the ester was hydrolyzed by incubation for 1 hour at pH 11 to 12. The solution was acidified to pH 3 and extracted with three 1-ml portions of diethyl ether. The β-hydroxybutyric acid was converted to the methyl ester with ethereal diazomethane and then chromatographed with carrier on a Barber Colman model 10 gas-liquid chromatograph. The column (6 feet x 6 mm) was packed with 15% diethylene glycol succinate and 2% phosphoric acid on Chromosorb W (60 to 70 mesh). The chromatographic separation was performed at 100°C with an effluent argon flow of 100 ml per min. Elute fractions were trapped in a mixture of toluene phosphoric and diethyl ether in a Dry Ice-acetone bath and then assayed for radioactivity as described previously for fatty acids.

Detection and Estimation of Tritium Incorporated into N-Acetylcysteamine Esters of β-Hydroxybutyric and Butyric Acids—Incubation mixtures containing reduced N-acetylcysteamine esters, and controls, were extracted three times with 1-ml portions of chloroform. Measurements of absorbance at 263 nm of the chloroform and the water layers showed that this extraction procedure transfers the N-acetylcysteamine esters quantitatively to chloroform. The chloroform extracts were evaporated just to dryness and the residue was made alkaline with 0.5 ml of 0.1 N KOH. Carrier butyric or β-hydroxybutyric acid was added. After a 1-hour incubation at 38°C samples were acidified with HCl to pH 3. The acidified solutions (<1.0 ml) were then extracted with three 1-ml portions of diethyl ether and a measured aliquot of the ether was assayed for radioactivity in dioxane phosphor solution. The remainder of each ether extract containing β-hydroxybutyric or butyric acid was evaporated in an ice bath to a small volume. Some of the samples containing β-hydroxybutyric acid were converted to the methyl ester with ethereal diazomethane. Butyric acid and the methyl ester of β-hydroxybutyric acid were subjected to gas-liquid chromatography on a column (6 feet x 6 mm) packed with 20% Carbowax 20 M and 3% terephthalic acid on Gas chromatography support. Chromatography was performed at 115°C for butyric acid, and 105°C for the methyl ester of β-hydroxybutyric acid, with an effluent helium flow of 100 ml per min. Mass and radioactivity response were recorded using a Barber Colman model 5000 instrument or measured with a Barber Colman model 10 instrument as described in the previous section.

RESULTS

Incorporation of Tritium from A and B Forms of Tritiated NADPH into Long Chain Fatty Acids by Fatty Acid Synthetase—The incorporation of tritium from the tritiated A and B forms of NADPH into long chain fatty acids synthesized by the pigeon liver fatty acid synthetase is reported in Table I. Almost equal amounts of tritium were incorporated into fatty acids from each form of NADPH. Controls in which fatty acid synthetase or malonyl-CoA were omitted from the incubation mixture showed

### TABLE I

| Form of NADPH | Specific activity of product | Ratio of specific activity of product to specific activity of substrate |
|---------------|----------------------------|--------------------------------------------------------------------|
| A             | 5000                      | 44                                                                  |
| B             | 5250                      | 42                                                                  |

*The procedure for the synthesis, isolation, and analysis of the radioactive products formed by the pigeon liver fatty acid synthetase is given under "Experimental Procedure.

**Synthesized from acetyl- and malonyl-CoA.

***Prepared from the product formed on reduction of S-crotonyl-N-acetylcysteamine with pigeon liver fatty acid synthetase.

### TABLE II

| Compound                  | Form of NADPH | Radioactivity recovered |
|---------------------------|---------------|------------------------|
| Methyl-β-hydroxybutyrate   | A             | 80                     |
|                           | B             | 1600                   |
| Butyric acid              | A             | 2400                   |
|                           | B             | 0                      |

*The procedure for the synthesis, isolation, and analysis of the radioactive products formed by the pigeon liver fatty acid synthetase is given under "Experimental Procedure." Radioactivity in methyl β-hydroxybutyrate and butyric acid was trapped in effluent fractions emerging from the column on gas-liquid chromatography. Coincidence of radioactivity was obtained with the mass of each authentic compound.

**Prepared from the product formed on reduction of acetoacetate-CoA with pigeon liver fatty acid synthetase.

***Prepared from the product formed on reduction of S-crotonyl-N-acetylcysteamine with pigeon liver fatty acid synthetase.
Fig. 1. Gas-liquid chromatography of methyl-β-hydroxybutyrate. The radioactive β-hydroxybutyryl moiety was formed on reduction of acetoacetyl-CoA and S-acetoacetyl-N-acetylcysteamine with the B form of NADPH. The details of this experiment are given under “Experimental Procedure.” A, the methyl ester of β-hydroxybutyrate was prepared following the reduction of acetoacetyl-CoA with pigeon liver fatty acid synthetase. Chromatography of the methyl ester of β-hydroxybutyrate was effected on a diethylene glycol succinate column in a Barber Colman model 10 gas-liquid chromatograph. B, the methyl ester of β-hydroxybutyrate was prepared following the reduction of S-acetoacetyl-N-acetylcysteamine with rat liver fatty acid synthetase. The chromatographic separation of the methyl ester of β-hydroxybutyrate was effected on a Carbowax column with a Barber Colman model 5000 instrument equipped with a system monitoring for radioactivity.

Fig. 2. Gas-liquid chromatography of butyric acid. The radioactive butyryl moiety was produced by reduction of S-crotonyl-N-acetylcysteamine by pigeon or rat liver fatty acid synthetase with the A form of NADPH. The details of this experiment are given under “Experimental Procedure.” Chromatography of the butyric acid was effected on a Carbowax column. A, the reduction of S-crotonyl-N-acetylcysteamine was catalyzed by pigeon liver fatty acid synthetase. The product, butyric acid, was chromatographed on a Barber Colman model 10 gas-liquid chromatograph. B, the reduction of S-crotonyl-N-acetylcysteamine was catalyzed by rat liver fatty acid synthetase. The chromatographic separation of butyric acid was effected with a Barber Colman model 5000 instrument equipped with a unit monitoring for radioactivity.

Reduction of acetoacetyl-CoA and S-acetoacetyl-N-acetylcysteamine with the tritiated B form of NADPH by pigeon or rat liver fatty acid synthetase resulted in the incorporation of tritium into the β-hydroxybutyryl ester (Table II). The coinco-

Incorporation of Tritium into Product on Reduction of Acetoacetyl Thioesters with A and B Forms of Tritiated NADPH—

No oxidation of NADPH and no transfer of radioactivity to the petroleum ether extract.
dence of radioactivity in the product with the mass of authentic methyl β-hydroxybutyrate is shown in Fig. 1. When the A form of NADPH was used, or when acetoacetyl ester was omitted from the incubation mixture, no tritium was associated with methyl β-hydroxybutyrate on gas-liquid chromatography.

Incorporation of Tritium into Product on Reduction of S-Crotonyl-N-acetylcysteamine with A and B Forms of Trtitiated NADPH—The reduction of S-crotonyl-N-acetylcysteamine with the A form of NADPH resulted in the incorporation of tritium into the butyryl ester when this reaction was catalyzed by pigeon or rat liver fatty acid synthetase (Table II). Proof of the incorporation of tritium into product was provided by gas-liquid chromatography of the free acid (Fig. 2). Tritium was not associated with authentic butyric acid on gas-liquid chromatography of controls which contained no thioester, nor was it associated with authentic tritiated NADPH in the A form of NADPH when the B form of NADPH was used, or when acetoacetyl ester was omitted.

The A form of NADPH resulted in the incorporation of tritium into the A form of NADPH in this reaction was catalyzed by pigeon or rat liver fatty acid synthetase (Table II). Proof of the incorporation of tritium into product was provided by gas-liquid chromatography of the free acid (Fig. 2). Tritium was not associated with authentic butyric acid on gas-liquid chromatography of controls which contained no thioester, nor was it associated with authentic tritiated NADPH in the A form of NADPH when the B form of NADPH was used.

DISCUSSION

It is evident (Table I) that tritium is transferred from isocitric and glutamic acids through the two forms of NADPH into fatty acids in approximately equal molar quantities. Since all enzyme-catalyzed pyridine nucleotide hydrogen transfers are stereospecific (11-20), this would be the expected result if one reduction in fatty acid synthesis transfers hydrogen from the A side of NADPH and the other from the B side. The specific activity for tritium in the fatty acid products per mole of NADPH oxidized is near the expected 50%. Hence, these results conform with one A and one B stereospecific reduction in fatty acid synthesis.

The finding (Fig. 1 and Table II) that tritium is incorporated from the B side of NADPH in the β-ketoacyl ester reduction, but not from the A side, established that this reduction is B-stereospecific in fatty acid synthesis. The finding (Fig. 2 and Table II) that tritium is incorporated from the A side of NADPH in the α,β-unsaturated ester reduction, but not from the B side, established that this reduction is A-stereospecific in fatty acid synthesis.

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Our preliminary finding (1) of tritium incorporation into the product from the A form of NADPH in this reaction was in error due to the incomplete removal of isocitric dehydrogenase after the synthesis of the A form of NADPH. Under these conditions the tritium remaining at position 4 of the pyridine ring of tritiated NADPH (formed on oxidation of tritiated NADP) is then moved to the B side in the second reduction with isocitric dehydrogenase. This tritium is then abstracted in the reduction of another molecule of acetoacetyl thioester by NADPH. When isocitric dehydrogenase was completely removed before the reduction of the acetoacetyl thioester, no tritium was incorporated into the product β-hydroxybutyryl ester from the A form of NADPH.
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