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Stereospecific reduction of crotonyl coenzyme A

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SUMMARY

An enzyme preparation obtained from brain tissue catalyzes the stereospecific reduction of crotonyl CoA to butyrate. Tritium from $TPNH^3$ was localized predominantly on the β-carbon of recovered butyric acid. Both this enzyme and a preparation from adipose tissue, which also catalyzes the reduction of crotonyl CoA, catalyze the synthesis of long-chain fatty acids from malonyl CoA and acetyl CoA. The significance of these findings is considered in view of the demonstration that, in long-chain fatty acids synthesized in the presence of $TPNH^3$, labeled hydrogen is localized on alternate carbon atoms beginning with the β-carbon.

Since the demonstration by Langdon in 1957 (1) that extracts of liver tissue catalyze the reduced triphosphopyridine nucleotide (TPNH)-specific reduction of crotonyl coenzyme A (CoA), the role of unsaturated intermediates in the biosynthesis of long-chain fatty acids has been the subject of considerable controversy. Partially purified enzyme systems from pigeon and rat-liver tissue that catalyze the synthesis of long-chain fatty acids appear to be unable to catalyze the reduction of crotonyl CoA with either TPNH or reduced diphosphopyridine nucleotide (DPNH) (2, 3). The situation is somewhat different with enzymes obtained from brain (4) and, as we shall demonstrate, with an enzyme preparation from adipose tissue that readily catalyzes the reduction of crotonyl CoA in the presence of TPNH. It has been postulated by Cornforth (5) that a stereospecific transfer from the reducing agent would occur at the β-carbon. It is the purpose of the present communication to present evidence consistent with this hypothesis.

EXPERIMENTAL METHODS AND PROCEDURES

The enzymes employed in these investigations were partially purified from young rat-brain tissue according to the procedure outlined in a previous communication (4) and from adipose tissue as described by Martin *et al.* (6). When crotonyl CoA was incubated with $TPNH^3$ and the enzyme obtained from brain, the radioactive reaction product was recovered in the following fashion. The reaction was stopped by heating the mixture for 2 minutes at 100°. The suspension was centrifuged, and the supernatant solution was decanted and adjusted to pH 10 with KOH. The mixture was heated at 60° for 15 minutes to hydrolyze thiol esters. The solution was then cooled and lyophylized. The residue was taken up in 0.1 ml of 2 N H_2SO_4 and extracted with 0.1 ml of ethyl acetate. The suspension was centrifuged, and the organic phase was applied to a gas-liquid chromatography column consisting of 132.5 cm of stearic acid—H_3PO_4—silicone 550 (7) on acid-washed Columnpak. The operating temperature was 130°. Carrier helium gas was saturated with water vapor, which was required for separation of very short-chain fatty acids such as formic and acetic acids. The gas flow was 50 ml per minute. The performance of the column under these conditions is illustrated in Figure 1.

Microdegradation Procedure. The component emerging from the gas-liquid chromatograph in the position of butyric acid was collected in a U-tube immersed in dry ice—ethanol. The recovered fatty acid was dissolved in ether and an aliquot was removed for counting. The remaining ether solution was evaporated in the main compartment of a multiple-dose pharmaceu-

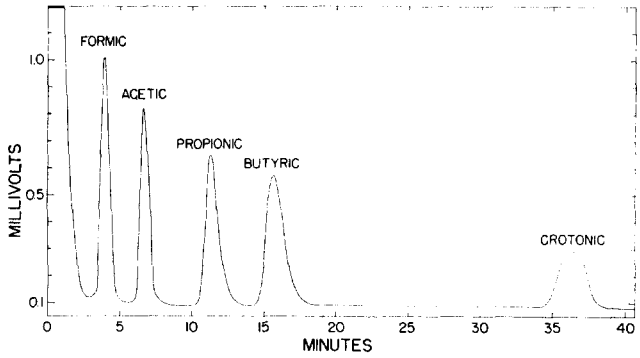

FIG. 1. Gas-liquid partition chromatographic separation of short-chain fatty acids. The conditions for the preparation and operation of the column are described in the text.

tical vial with a center well (3). To the residue were added 100 μmoles of sodium butyrate as carrier along with 50 mg of sodium azide and 0.5 ml of 100% H_2SO_4 (3). The flask was sealed and incubated with gentle shaking for 1 hour at 70°. It was then chilled in ice, and 0.4 ml of 0.2 N H_2SO_4 was injected into the center well and 2.5 ml of 9 N NaOH was injected into the main compartment. The mixture was heated for 3 hours at 90° and allowed to stand at room temperature overnight for maximal recovery of the short-chain amine. The contents of the center well were transferred to a glass-stoppered tube and neutralized with NaOH, and an aliquot was removed for counting. To the remainder was added 0.6 ml of a solution of 10% $KMnO_4$. The tube was stoppered and heated for 20 minutes at 90° to 100°. The mixture was made strongly alkaline with KOH, and the water produced in the oxidation was recovered by distillation and counted. The resulting salt was converted to the acid form for identification and purification by gas-liquid chromatography. The sequential removal of the subsequent carbon and hydrogen atoms was accomplished in the same manner.

The preparation of derivatives of coenzyme A has been described previously (3). Hexokinase, TPNH and glucose-l-H^3 were purchased commercially. Glucose-6-phosphate dehydrogenase was prepared according to Kornberg (8).

RESULTS

The reduction of crotonyl CoA in the presence of TPNH is catalyzed by enzyme preparations obtained from brain and adipose tissue (Fig. 2) (cf. [4]). These preparations also catalyze the synthesis of long-chain fatty acids from malonyl CoA and acetyl CoA (4, 6). The product of the reaction of crotonyl CoA with $TPNH^3$ was identified as butyric acid-H^3 by gas-liquid column chromatography. The radioactivity recovered in the butyric acid fraction was 22,210 cpm in the first experiment and 28,000 cpm in the second. When the radioactive butyric acid was degraded in stepwise manner, the tritium was found to be localized predominantly on the β-carbon of the butyric acid (Table 1).

TABLE 1. DISTRIBUTION OF TRITIUM IN BUTYRIC ACID-H^3

Carbon of Original Butyric Acid	Experiment 1	Experiment 2
	%	%
α	7	6
β	93	94
γ	0	0

The incubation mixtures contained 50 μmoles of potassium phosphate buffer (pH 7.0), 3 μmoles of 2,3-dimercaptopropanol, 4 μmoles of adenosine triphosphate, 4 μmoles of $MgCl_2$, 220 mμmoles of triphosphopyridine nucleotide, 3.3 μmoles of glucose-1-H^3 (specific activity 25 μc/μmole), 450 mμmoles of crotonyl CoA, dialyzed glucose-6-phosphate dehydrogenase (1 mg of protein), dialyzed hexokinase (1 mg of protein) and rat-brain fatty acid synthesizing enzyme Fraction II (4) (4 mg of protein) in a final volume of 1.5 ml. The mixture was incubated for 1 hour at 37°. The recovery, purification and sequential degradation of labeled butyric acid is described in the text.

By way of contrast, no radioactive butyric acid could be detected when acetoacetyl CoA was used as substrate. Additional experiments indicated the oxidation of only 1 mole of TPNH for each mole of acetoacetyl CoA. This reduction is presumed to have stopped at the level of β-hydroxybutyrate under the present conditions of investigation.

The reduction of acetoacetyl CoA was also observed with the adipose tissue enzyme, but at a slower rate than crotonyl CoA, whereas the converse was found with the brain enzyme. It was not determined if this difference in rates would still be apparent if the pH of the respective enzyme preparations were adjusted to comparable values.

In contrast with the rapid reduction of crotonyl and acetoacetyl CoA by both brain and adipose tissue enzymes, the reduction of D(—)-β-hydroxybutyryl CoA occurred to only a slight extent in the brain enzyme preparation and it did not appear to be reduced at all in the adipose enzyme system. No reduction of L-(+)-β-hydroxybutyryl CoA occurred in either preparation with TPNH.

DISCUSSION

The demonstration of the stereospecific reduction of crotonyl CoA to butyric acid affords direct support for the prediction (5) that labeled hydrogen would be

found predominantly on the β-carbon in the course of the enzymatic reduction of α, β-unsaturated acyl CoA compounds. Although the ratio of crotonyl CoA reductase to fatty acid-synthesizing activity was constant in the brain-enzyme preparation through purification by ammonium sulfate fractionation and calcium phosphate gel adsorption and elution,[1] the results do not necessarily indicate that crotonyl CoA reductase activity is an integral part of the fatty acid synthesizing system. This reservation must be made at this time in view of the fact that fatty acid synthesizing preparations from rat and pigeon liver do not catalyze the reduction of crotonyl CoA (2, 3). In spite of this discrepancy, it seems likely that α, β-unsaturated compounds may be intermediates in fatty acids synthesis, perhaps in the form of enzyme-bound derivatives.

A similar distribution of labeled hydrogen from DPNH has been observed in the reduction of acetoacetyl pantetheine to β-hydroxybutyric acid (9). It therefore appears that transfer of labeled hydrogen to the β-carbon can occur during both steps in the reduction of β-keto derivatives to the respective saturated compounds. These findings are consistent with the observation that tritium was found predominantly on alternate carbon atoms beginning with the β-carbon of long-chain fatty acids enzymatically synthesized in the presence of TPNH[3] (3).

Although the exact mechanism of the reduction of unsaturated acyl CoA derivatives is still obscure, the present results are compatible with reduction by direct hydride transfer from TPNH to the β-carbon. However, Lynen (10) has reported that a highly purified fatty acid-synthesizing enzyme preparation obtained from yeast has a yellow color and that fatty acid synthesis is stimulated by the presence of flavine adenine mononucleotide (FMN). A similar stimulation has been described in an enzyme prepared from *Clostridium kluwveri* (11). The enzyme prepared from brain tissue used in the present studies was completely colorless and showed no evidence of an absorption maximum in the region of 450 mμ, characteristic of flavine-containing compounds. Furthermore, treating this enzyme preparation with charcoal did not decrease its catalytic activity with regard to fatty acid synthesis nor was fatty acid synthesis increased by the addition of FMN. These observations do not necessarily exclude a stereospecific transfer from a flavine nucleotide that is tightly bound to the enzyme. If this be the case, it may be possible to resolve the controversy by experiments designed to illustrate stereospecific transfer of hydrogen mediated by a flavoprotein in a manner somewhat analogous to the recent demonstration by Drysdale *et al.* (12) of a flavine-dependent stereospecific transfer of hydrogen from DPNH to the acetylpyridine analogue of diphosphopyridine.

[1] J. D. Robinson and R. O. Brady, unpublished observations.

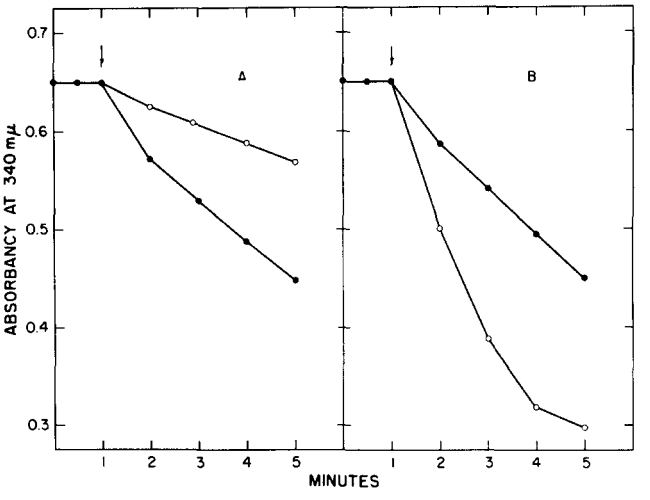

FIG. 2. Spectrophotometric demonstration of the reduction of acetoacetyl CoA (open circles) and crotonyl CoA (closed circles). A, adipose tissue enzyme; B, brain enzyme. Each cuvette contained 20 μmoles of potassium phosphate buffer (pH 7.0 in the brain enzyme experiments and pH 7.8 in the adipose tissue preparation), 1 μmole of 2-mercaptoethanol, 20 mμmoles of TPNH, and adipose tissue enzyme (step 2 (6), 240 μg of protein), or brain enzyme (80 μg of protein) in a final volume of 0.2 ml. At the arrows, 68 mμmoles of acetoacetyl CoA or crotonyl CoA were added to the respective cuvettes.

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