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Chirality of 3-Hydroxyoctadecanoic Acid from Stearoyl-CoA by Rat Liver Soluble Enzymes

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One of the transformation products formed from stearoyl-CoA by the soluble enzymes of rat liver homogenate has been identified as 3-hydroxyoctadecanoic acid. The chirality of this acid obtained from incubation of [1-^{14}C]stearoyl-CoA with the 105 000 *g* soluble enzymes of rat liver has been determined. After 10 and 20 min of incubation 70% of the L(+) and 30% of the D(−) enantiomer are formed, whereas racemic 3-hydroxyoctadecanoic acid is isolated after 90 min of incubation. These results suggest either that an epimerizing enzyme is present in the soluble fraction or that two enzymes, each specifically forming one of the two enantiomers, are present.

INTRODUCTION

It has been demonstrated recently (*1*, *2*) that the soluble fraction at 105 000 *g* of rat liver homogenate supplemented with NAD$^+$, ATP, CoA, and oxygen is capable of shortening long-chain acyl-CoA such as stearoyl- and oleoyl-CoA. Among the intermediates of the degradation process the *trans*-α,β-unsaturated, the β,γ-unsaturated, and the 3-hydroxy derivatives of the substrate have been isolated and identified in our laboratory (*2*).

The objective of this study was to establish the chirality of the 3-hydroxyacyl-CoA formed by the 105 000 *g* soluble enzymes of rat liver. These compounds have been recognized as intermediates in fatty acid oxidation, synthesis, and elongation processes (*3*); the D(−) enantiomers of long-chain 3-hydroxyacyl-CoA are the presumed intermediates in fatty acid synthesis (*4*), whereas the mitochondrial β-oxidation seems to imply the formation of both enantiomers (*5*, *6*).

[1-^{14}C]Stearoyl-CoA was incubated with the soluble fraction at 105 000 *g* of rat liver homogenate, and the radioactive 3-hydroxyoctadecanoic acid isolated after incubation was diluted with racemic nonradioactive acid obtained by chemical synthesis. By crystallization of the salt with (+) dehydroabietylamine the D(−) enantiomer of 3-hydroxyoctadecanoic acid was obtained. The optical purity of this acid was established from the specific rotation and independently confirmed on the methyl ester by a recently described nmr method (*7*) by which the enantiomeric composition can be determined on the basis of chemical shift nonequivalence of enantiomers in the presence of a chiral shift reagent, tris[3-(*tert*-butylhydroxymethylene)-*d*-camphorato] europium (III). The enantiomeric composition of the enzymatically formed 3-hydroxyoctadecanoic acid was established by comparison of the molar radioactivity of the unresolved acid with that of the resolved acid.

MATERIALS AND METHODS

Materials

[1-^{14}C]Stearic acid was obtained from Amersham, U.K.; nonradioactive stearic acid, (+) dehydroabietylamine, and sodium bisulfite adduct of palmitic aldehyde from Fluka A.G.; tris[3-(*tert*-butylhydroxymethylene)-*d*-camphorato] europium (III) from Stohler Isotope Chemicals, Innerberg, Switzerland; ATP and CoA were purchased from Boehringer, Mannheim.

Radiopurity Determinations

The radiopurity of [1-^{14}C]stearic and of [1-^{14}C]3-hydroxyoctadecanoic acids was determined by tlc-radiochromatoscanning and by radiogaschromatography of the corresponding methyl esters obtained with diazomethane. A Packard radiochromatogram scanner system, Model 7201, was used; tlc-eluting solvents were chloroform–methanol 97:3 (v:v) for methyl stearate and chloroform–methanol 95:5 (v:v) for methyl 3-hydroxyoctadecanoate. A Carlo Erba gaschromatograph Model GV connected with a Nuclear Chicago flow counter Model 4998 was used. Analysis conditions were as reported previously (*2*).

Specific Rotations

Specific rotations were calculated for acids and for the (+) dehydroabietylamine salts from the optical rotations determined at 20°C in a Perkin–Elmer polarimeter Model 141 in chloroform and toulene, respectively. The concentration of the solution was 0.8–1 %, and the length of the light path was 10 cm. The percentage of D(−)-3-hydroxyoctadecanoic acid (lit. (*8*) specific rotation = −12.2°) was calculated from the formula $\%\mathrm{D}(-) = 50 - 4.17 \times [\alpha]_{\mathrm{D}}^{20}$.

Nuclear Magnetic Resonance Spectra

A Varian XL-100 instrument was used for the analyses. The samples were prepared by dissolving 18 mg of either racemic or partially resolved methyl 3-hydroxyoctadecanoate in 0.5 ml of $CDCl_3$. After registration of the nmr spectrum, 13 mg of tris[3-(*tert*-buthylhydroxymethylene)-*d*-camphorato] europium (III) were added to the solution, and the nmr spectrum was registered after centrifugation. The chemical shifts were expressed as δ (ppm) from tetramethylsylane as internal reference. Nonequivalence was observed for the 3-proton (D, $\delta = 5.64$ ppm; L, $\delta = 5.30$ ppm) and for the O–CH_3 signals (D, $\delta = 3.92$ ppm; L, $\delta = 3.98$ ppm). The percentage of each enantiomer was determined on the basis of the peak areas of the signals.

Substrate

[1-^{14}C]Stearoyl-CoA was prepared according to Goldman and Vagelos (*9*) from [1-^{14}C]stearic acid (10^{10} dpm/mmole). No radioactivity was associated to free stearic acid, as determined by radiochromatoscanning; the radioactivity was completely associated to methyl [1-^{14}C]stearate obtained by hydrolysis of the thioester and reaction of the acid with diazomethane.

D,L-*3-Hydroxyoctadecanoic Acid*

Ethyl-D,L-3-hydroxyoctadecanoate was synthesized by the Reformatsky procedure (*10*) from palmitic aldehyde obtained by hydrolysis of the sodium bisulfite adduct in acidic ethanol. The crude material was chromatographed on a silicic acid column. Pure ethyl-3-hydroxyoctadecanoate was eluted from the column with benzene–diethyl ether (90:10, v:v). Saponification of the ester with 2 *N* KOH in 60% ethanol followed by acidification with HCl, ether extraction, and evaporation of the solvent to dryness yielded a residue which by crystallization from acetone gave pure D,L-3-hydroxyoctadecanoic acid, mp 90°, lit. 90° (*11*).

Incubation and Extraction Procedures

The soluble fraction at 105 000 *g* of rat liver homogenate was prepared as reported previously (*2*). Incubation conditions were as follows: soluble protein, 240 mg; [1-^{14}C]stearoyl-CoA (6.5 × 10^6 dpm), 0.01 m*M*; phosphate, 72 m*M*; sucrose, 44 m*M*; EDTA, 2.4 m*M*; Mg^{2+}, 5 m*M*; CoA, 0.1 m*M*; ATP, 2.4 m*M*; final volume, 65 ml. Each 750-ml Erlenmeyer flask containing the incubation mixture, except for the soluble protein, was shaken for 10 min in the air using a Dubnoff incubator and keeping the bath at 37°C to allow the temperature of the mixture to increase from 4 to 37°C. The soluble protein fraction was then added and shaking was continued for different additional times for the different incubation samples. The reaction was stopped by addition of 40 ml 2 *N* KOH in 60% methanol.

Unlabeled DL-3-hydroxyoctadecanoic acid (2 mg) was added to each sample which was then heated at 60°C for 1 hr under nitrogen. After acidification with hydrochloric acid, fatty acids were extracted with diethyl ether and transformed into the methyl esters with diazomethane.

Purification of [1-^{14}C]3-Hydroxyoctadecanoic Acid

Methyl [1-^{14}C]3-hydroxyoctadecanoate was separated from the methyl ester mixture obtained from each incubation assay by silica gel HF thin-layer chromatography (solvent chloroform–methanol, 97:3, v:v) and further purified by the same procedure using a more polar eluting solvent (chloroform–methanol, 95:5, v:v). Radiogaschromatographic analysis of some samples of methyl [1-^{14}C]3-hydroxyoctadecanoate purified as described showed a small radioactive impurity which could not be eliminated either by thin-layer chromatography with different eluting solvents or by several crystallizations of 3-hydroxyoctadecanoic acid, obtained by saponification of the ester after dilution with pure synthetic acid. The methyl ester was therefore further purified by preparative glc on a sylanized column packed with 3% SE 30 on Gaschrom Q (80–100), diameter 0.3 cm. Methyl [1-^{14}C]3-hydroxyoctadecanoate collected from the column showed by radiogaschromatography only one radioactivity peak corresponding to the single mass peak. The methyl ester was transformed into 3-hydroxyoctadecanoic acid by the usual procedure.

RESULTS

[1-^{14}C]Stearoyl-CoA was incubated under the conditions described in Materials and Methods, and [1-^{14}C]3-hydroxyoctadecanoic acid formed after 10, 20, and 90 min was isolated from the reaction mixture and purified as the methyl ester. The absence of radioactivity corresponding to 3-hydroxyoctadecanoic acid in the sample deriving from the incubation with boiled enzymes (Table 1) ensured that no radioactive compounds with chromatographic properties similar to those of methyl 3-hydroxyoctadecanoate were either present as impurities in the substrate or chemically formed during the incubation.

TABLE 1

TRANSFORMATION OF [1-^{14}C]STEAROYL-COA INTO [1-^{14}C]3-HYDROXYOCTADECANOIC ACID BY THE 105 000 *g* SOLUBLE FRACTION OF RAT LIVER HOMOGENATE

Experiment[a]	Incubation time (min)	% Transformation[b]	Crystallization of diluted [1-^{14}C]3-hydroxyoctadecanoic acid[c] × 10^{-3} (dpm/mmole)		
			Crystal I	Crystal II	Crystal III
1	10	7.5	420	405	410
2	20	10	480	473	481
3	90	15	104	106	104
4[d]	90	0.11	—	—	—

[a] Experiments were run at least in duplicate; figures represent the values of single determinations.

[b] Determined by radiogaschromatography as reported previously (2).

[c] Enzymatically formed [1-^{14}C]3-hydroxyoctadecanoic acid purified by thin-layer chromatography was diluted as follows: experiment 1, 1 300 000 dpm with 0.965 g of unlabeled D,L-3-hydroxyoctadecanoic acid; experiment 2, 1 500 000 dpm with 0.968 g; experiment 3, 1 200 000 dpm with 3.440 g.

[d] Boiled enzymes.

The purified [1-^{14}C]3-hydroxyoctadecanoic acid was diluted with unlabeled racemic 3-hydroxyoctadecanoic acid (0.7–1 g) and showed a constant molar radioactivity by crystallization from acetone (Table 1). A solution of the crystallized acid in absolute ethanol (30 mg/ml) and (+) dehydroabietylamine (1.2 moles/mole of acid) was allowed to stand at 60°C for 2 hr. The solution was then kept either at 25–28°C in an open flask or at 21°C in a closed flask until long needles formed. The different crystallization conditions caused a different enantiomeric distribution between crystals and mother liquors (Table 2). The crystals were separated from the solution by filtration, thoroughly washed with absolute ethanol, and suspended in 2 *N* KOH (50 ml/g of salt). After 1 hr at 60°C the amine was extracted with diethyl ether. The aqueous phase was acidified, and 3-hydroxyoctadecanoic acid was extracted with diethyl ether.

TABLE 2

ENANTIOMERIC COMPOSITION OF DILUTED [1-^{14}C]3-HYDROXYOCTADECANOIC ACID RESOLVED BY CRYSTALLIZATION OF THE SALT WITH (+) DEHYDROABIETYLAMINE

Experiment[a]	Crystallization temperature (°C)	$[\alpha]_D^{20}$	% Enantiomers from $[\alpha]_D^{20}$ [b]		% Enantiomers from nmr[c] peak areas of the 3-proton signals		% Enantiomers from nmr[c] peak areas of the O–CH$_3$ signals	
			D	L	D	L	D	L
1	21	−11.0°	95.0	4.2	94	6	96	4
2	21	−10.6°	94.2	5.8	95	5	95	5
3	21	−10.9°	95.4	4.6	93	7	95	5
3′	25–28	−5.7°	75	25	72	28	76	24

[a] As reported in Table 1.

[b] Calculated as reported in Materials and Methods.

[c] Of methyl[1-^{14}C]3-hydroxyoctadecanoate registered in presence of tris[3-(*tert*-buthylhydroxymethylene)-*d*-camphorato]europium (III).

Specific rotation and molar radioactivity of each sample were determined after crystallization from acetone to constant molar radioactivity to ensure complete elimination of the amine. The acid was then transformed into the methyl ester with diazomethane and subjected to nmr analysis (Table 2).

TABLE 3

ENANTIOMERIC COMPOSITION OF ENZYMATICALLY FORMED [1-^{14}C]3-HYDROXYOCTADECANOIC ACID

Experiment[a]	Molar radioactivity of [1-^{14}C]3-hydroxyoctadecanoic acid $\times 10^{-3}$ (dpm/mmole)		Enantiomeric composition[b] (%)	
	Unresolved	Resolved	D	L
1	410	271	31.4	68.6
2	481	309	29.5	70.5
3	104	104	50	50
3′	104	106	50	50

[a] As reported in Table 2.

[b] Calculated as reported in Results.

The results obtained for the different incubation samples are reported in Table 3. The enantiomeric composition of enzymatically formed [1-^{14}C]3-hydroxyoctadecanoic acid was calculated as follows:

$$0.5 \times A + 0.5 \times B = \text{molar radioactivity of unresolved acid}$$

$$\frac{C}{100} \times A + \frac{E}{100} \times B = \text{molar radioactivity of resolved acid}$$

$$\%\ \mathrm{D}(-) \text{ in the enzymatically formed acid} = \frac{A \times 100}{2 \times \text{molar radioactivity of unresolved acid}}$$

$$\%\ \mathrm{L}(+) \text{ in the enzymatically formed acid} = \frac{B \times 100}{2 \times \text{molar radioactivity of unresolved acid}}$$

where A = molar radioactivity of the D(−) enantiomer, B = molar radioactivity of the L(+) enantiomer, $C = \%$ D(−) in the resolved acid (calculated from $[\alpha]_D^{20}$ and nmr as in Table 2), $E = \%$ L(+) in the resolved acid (calculated from $[\alpha]_D^{20}$ and nmr as in Table 2).

DISCUSSION

The reaction sequence leading to the mitochondrial oxidation of long-chain fatty acids has been long established (*3*). Long-chain acyl-CoA is transformed into 2-*trans*-enoyl-CoA. Hydration of the double bond gives 3-hydroxyacyl-CoA which is dehydrogenated to 3-oxoacyl-CoA. *β*-Cleavage of these derivatives results in the loss of two carbon atoms as acetyl-CoA with the concomitant formation of the homologous acyl-CoA. The cyclic repetition of this sequence results in the complete degradation of long-chain acyl-CoA. The 3-hydroxyacyl-CoA dehydrogenase is specific for the L(+) enantiomer (*12*); however, a mitochondrial enzyme catalyzing the epimerization reaction of the D(−) to the L(+) enantiomer has been isolated (*5*). This enzyme would be involved in the *β*-oxidation of unsaturated acyl-CoA bearing a 12-*cis* double bond. Degradation of these derivatives would give a 2-*cis*-enoyl-CoA which by hydration would be transformed into the D(−) enantiomer of 3-hydroxyacyl-CoA. Epimerization to the L(+) enantiomer would make possible the utilization of this compound by the 3-hydroxyacyl-CoA dehydrogenase. Based on the fact that linoleic acid is oxidized more slowly when compared with the 9-*cis*,12-*trans*-octadecadienoic acid, it has been concluded that the epimerase reaction is rate limiting in the oxidation of octadecadienoic acids (*13*).

On the other hand, the specific hydration of 2-*trans*-enoyl-CoA has been considered. Stoffel et al. (*5*), using a 2-*trans*-enoyl-CoA hydratase isolated from rat liver mitochondria, obtained L(+)-3-hydroxydodecanoate from 2-*trans*-dodecenoyl-CoA, whereas Davidoff and Korn (*6*) obtained D(−)-3-hydroxyhexadecanoate from the incubation of 2-*trans*-hexadecenoyl-CoA with two hydratases obtained from guinea pig liver mitochondria. 3-Hydroxy acids are also formed in the oxidation of oleoyl- and stearoyl-CoA by the 105 000 *g* soluble enzymes of rat liver homogenate (*1*, *2*).

It was of interest to establish the stereospecificity of the formation of these derivatives and to identify which enantiomer is formed by the soluble enzymes. For this purpose [1-^{14}C]stearoyl-CoA was incubated with these soluble enzymes for different times, and [1-^{14}C]3-hydroxyoctadecanoic acid was isolated from the incubation mixture. Addition of NAD$^+$ was avoided, as it was demonstrated previously (*2*) that in absence of this cofactor no shortening of the carbon atom chain occurs and the 3-hydroxy derivative of the substrate accumulates. The direct determination of the chirality from the optical activity of 3-hydroxy acids isolated after incubation of acyl-CoA with the soluble enzymes was rendered impossible by the presence of these acids in the enzymatic fraction before incubation. In fact, we previously observed (*2*, *14*) that the polar fraction of fatty acids extracted after incubation of [U-^{14}C]oleoyl-CoA with these enzymes, saponification of the reaction mixture and acidification, contained in addition to the radioactive 3-hydroxyacids derived from the degradation of the substrate also nonradioactive long-chain 3-hydroxy acids of unknown origin. Therefore, a different experimental approach was utilized implying incubation of a radioactive acyl-CoA, isolation and purification of the 3-hydroxyacid formed, and determination of the enantiomeric composition of the radioactive acid based on the change of the molar radioactivity of this acid after dilution with excess of the corresponding racemic nonradioactive acid and resolution into the enantiomers. The determination of the enantiomeric composition of the [1-^{14}C]3-hydroxy-octadecanoic acids isolated from the incubation was based on the assumption that diluting the radioactive acid with nonradioactive racemic 3-hydroxyoctadecanoic acid and resolving the enantiomers by crystallization of the salt obtained from this diluted acid and (+) dehydroabietylamine, the radioactive enantiomers would cocrystallize with the corresponding nonradioactive ones.

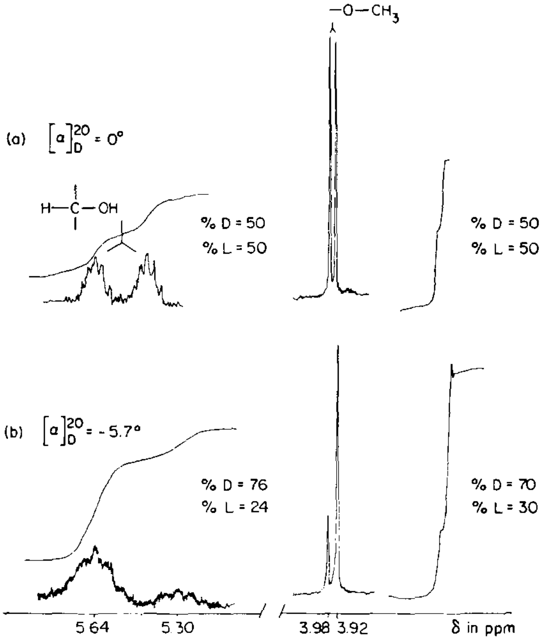

FIG. 1. Signals of the 3-proton and of the O–CH$_3$ in the nmr spectrum registered in presence of tris [3-(*tert*-buthylhydroxymethylene)-*d*-camphorato]europium (III) of methyl[1-^{14}C]3-hydroxyoctadecanoate from (a) unresolved and (b) resolved [1-^{14}C]3-hydroxyoctadecanoic acid from Expt 3 of Table 1.

As shown in Table 3 no change of the molar radioactivity was observed after partial resolution of the two enantiomers from 3-hydroxyoctadecanoic acid obtained from the 90-min incubation. The percentage of enantiomers was calculated from the specific rotation (Table 3) and from the nmr spectrum measured in presence of tris[3-(*tert*-buthylhydroxymethylene)-*d*-camphorato] europium (III) (Fig. 1). The unchanged molar radioactivity was an indication that the radioactive 3-hydroxyoctadecanoic acid was a racemic mixture of the enantiomers. A decrease of the molar radioactivity was observed instead after resolution of 3-hydroxyoctadecanoic acid derived from 10 and 20 min of incubation (Table 3). Resolution of the enantiomers gave in both cases 100% of the D(−) enantiomer. From the decrease of molar radioactivity it was calculated that the enzymatically formed 3-hydroxyoctadecanoic acid was a mixture enriched in the *L*(+) enantiomer. These data might indicate that the L(+) enantiomer is formed at first and is then epimerized to the D(−) enantiomer, resulting finally in a racemic mixture. This behaviour would be similar to that described by Stoffel et al. (5) for the mitochondrial enzymes of rat liver. However, the presence of an enzyme catalyzing the direct formation of the D(−) enantiomer in the 105 000 *g* soluble fraction cannot be excluded. Isolation of the enzymes responsible for the degradation of long-chain acyl-CoA by these soluble enzymes is in progress in our laboratories.

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