The mitochondrial metabolism of unsaturated fatty acids with conjugated double bonds at odd-numbered positions, e.g., 9-cis,11-trans-octadecadienoic acid, was investigated. These fatty acids are substrates of β-oxidation in isolated rat liver mitochondria and hence are expected to yield 5,7-dienoyl-CoA intermediates. 5,7-Decadienoyl-CoA was used to study the degradation of these intermediates. After introduction of a 2-trans-double bond by acyl-CoA dehydrogenase or acyl-CoA oxidase, the resultant 2,5,7-decatrienoyl-CoA can either continue its pass through the β-oxidation cycle or be converted by \( \Delta^3,\Delta^2 \)-enoyl-CoA isomerase to 3,5,7-decatrienoyl-CoA. The latter compound was isomerized by a novel enzyme, named \( \Delta^3,\Delta^2,\Delta^4 \)-enoyl-CoA isomerase, to 2,4,6-decatrienoyl-CoA, which is a substrate of 2,4-dienoyl-CoA reductase (Wang, H.-Y. and Schulz, H. (1989) Biochem. J. 264, 47-52) and hence can be completely degraded via β-oxidation. \( \Delta^3,\Delta^5,\Delta^7,\Delta^2,\Delta^4,\Delta^6 \)-Trienoyl-CoA isomerase was purified from pig heart to apparent homogeneity and found to be a component enzyme of \( \Delta^3,\Delta^2 \)-enoyl-CoA isomerase, EC 5.3.3.8; referred to hereafter as enoyl-CoA isomerase), 2,4-dienoyl-CoA reductase (4-enoyl-CoA reductase (NADPH), EC 1.3.1.34), and \( \Delta^3,\Delta^5,\Delta^7,\Delta^2,\Delta^4 \)-dienoyl-CoA isomerase (referred to hereafter as dienoyl-CoA isomerase). These enzymes catalyze either the reduction or isomerization of double bonds once the double bond is close to the thioester function as the result of chain shortening. Consequently, double bonds either are reductively removed or are shifted to yield 2-trans-enoyl-CoAs, which are intermediates of the β-oxidation spiral. Polysaturated fatty acid with conjugated double bonds may yield intermediates with more extended chromophores. For example, a fatty acid with two conjugated double bonds at even-numbered positions is assumed to be chain-shortened by β-oxidation to 4,6-dienoyl-CoA and is then converted to 2,4,6-trienoyl-CoA by acyl-CoA dehydrogenase. The further metabolism of this intermediate is facilitated by 2,4-dienoyl-CoA reductase, which catalyzes the reduction of one double bond of the 2,4,6-trienoyl-CoA chromophore to yield 3,6-dienoyl-CoA (2).

The β-oxidation of a fatty acid with two conjugated double bonds at odd-numbered positions would produce 5,7-dienoyl-CoA, which may be dehydrogenated by acyl-CoA dehydrogenase to 2,5,7-trienoyl-CoA. The latter compound may be chain-shortened to 3,5-dienoyl-CoA or isomerized to 3,5,7-trienoyl-CoA by enoyl-CoA isomerase. The further metabolism of 3,5,7-trienoyl-CoA would require the action of a \( \Delta^3,\Delta^5,\Delta^7,\Delta^2,\Delta^4,\Delta^6 \)-trienoyl-CoA isomerase. Such an enzyme has not been described so far.

Since polysaturated fatty acids with conjugated double bonds (such as, for example, conjugated linoleic acid) are formed during the partial catalytic hydrogenation of fats (3) and in ruminants (4), they are constituents of the human diet, and hence, their degradation by β-oxidation deserves to be studied. It was the aim of this study to elucidate the further metabolism of 5,7-dienoyl-CoA and to identify, isolate, and characterize a suspected \( \Delta^3,\Delta^5,\Delta^7,\Delta^2,\Delta^4,\Delta^6 \)-trienoyl-CoA isomerase (referred to hereafter as trienoyl-CoA isomerase).

**EXPERIMENTAL PROCEDURES**

**Materials**—CoASH, Q-Sepharose, polybuffer exchanger 94, polybuffer 96, reactive red 120, Sepharose CL-6B, phenylmethyalsulfonyl fluoride, polyethylene glycol and an average M, of 8000, benzamide hydrochloride, acyl-CoA oxadise from Arthrobacter species, Staphylococcus aureus (Cowran strain) suspension (10%, w/v), and all standard biochemicals were obtained from Sigma. (4-Carboxybutyl)triphosphonphionium bromide, trans-2-pentenal, and lithium bis(trimethylisilyl)amide were purchased from Aldrich. Hydroxylapatite, the dye reagents for protein assays, and materials for immunoblotting, including alkaline phosphatase-conjugated goat anti-rabbit IgG, were bought from Bio-Rad. The multifunctional protein I from rat liver peroxisomes (5, 6), enoyl-CoA hydratase (cratonease) from bovine liver (7), enoyl-CoA isomerase from rat liver (6), 1,3-hydroxyacyl-CoA dehydrogenase from pig heart (9), and dienoyl-CoA isomerase from rat liver (10) were purified as described. Methyl 5-cis-octenoate (generously provided by Dr. H. Sprecher, Ohio State University) was saponified as described (11) to yield 5-cis-octenoic acid. The CoA thiosters of 5-cis-octenoic acid and 5,7-decadienoic acid were synthesized by the mixed anhydride method as described by Feng and Schulz (12) and purified by HPLC. The abbreviation used is: HPLC. The abbreviation used is: HPLC, high performance liquid chromatography.
β-Oxidation of Unsaturated Fatty Acid

 sized by a Wittig reaction using the procedure of Maryanoff et al. (15). Twenty nmol of (4-carboxybutyl)tri phenylphosphonium bromide in 10 ml of anhydrous tetrahydrofuran were combined with 42 nmol of lithium bis(trimethylsilyl)amide in 42 ml of tetrahydrofuran at 25 °C and under N₂ with stirring. After 15 min, 16 mmol of trans-2-pentenal in 10 ml of 50% v/v acetic acid. Fractions of 5 ml were collected, and the active fractions were combined and concentrated. Thereafter, the sample was developed with 12 column volumes of polybuffer 96 adjusted to pH 6.0 and 20% glycerol (buffer C), the sample was applied to a chromatofocusing column was eluted with 15 ml of buffer A. The resulting suspension was centrifuged at 13,000 g for 3 min. The supernatant was transferred to clean tubes and kept at 4 °C until assayed for dienoyl-CoA and trienoyl-CoA isomerase activities.

**Analysis and Purification of Acyl-CoA Thioesters by HPLC—Acyl-CoAs were purified or analyzed by reverse-phase HPLC on a Waters µBondapak C18, column (30 cm x 3.9 mm) attached to a Waters gradient HPLC system. The absorbance of the effluent was monitored at 254 nm. Separation of different acyl-CoA thioesters was achieved by linearly increasing the acetonitrile (91-100%) content of the 50 mM ammonium phosphate buffer (pH 5.5) from 20 to 50% at a flow rate of 2 ml/min. When acyl-CoAs were purified, the desired fraction of the effluent was collected. After evaporation of acetonitrile under vacuum, the product was concentrated by use of a Sep-Pak C18 cartridge.

**RESULTS**

**Metabolism of 5,7-Decadienoyl-CoA—The β-oxidation of polysaturated fatty acids with conjugated double bonds at odd-numbered positions is expected to produce 5,7-dienoyl-CoA intermediates. To study the metabolism of these intermediates, 5,7-decadienoyl-CoA was synthesized. The required 5,7-decadienoic acid was prepared from (4-carboxybutyldiene)triph enylphosphorane and 2-trans-pentenal by a Wittig reaction (15). Since the synthetic procedure is predicted to yield mostly the trans-isomer of the newly formed double bond, the major product is expected to be 5-trans,7-trans-decadienoic acid. The CoA derivative of this acid was obtained in pure form after converting the acid to the CoA thioester and isolating the major product by HPLC (Fig. 1A).

In a preliminary experiment, the capacity of mitochondria to oxidize fatty acids with odd-numbered conjugated double bonds was assessed. The data presented in Table I demonstrate that such fatty acids supported the respiration of coupled rat liver mitochondria at rates that were slightly lower than those obtained with the corresponding fatty acids having either non-conjugated double bonds or no double bond at all. This result suggests the presence of a mitochondrial pathway for the β-oxidation of fatty acids that have odd-numbered conjugated double bonds.

The step-by-step degradation of 5,7-decadienoyl-CoA was studied spectrophotometrically by use of purified enzymes. The spectrum of 5,7-decadienoyl-CoA (shown in Fig. 2A, spectrum 1) is characterized by a major absorbance band centered around 230 nm and a shoulder at 260 nm. These absorbances are attributed to the diene and CoA chromophores, respectiv
agrees with the conversion of an acyl-CoA to 2,4,6-decatrienoyl-CoA, which harbors enoyl-CoA isomerase activity (Fig. 1). The resultant two compounds (Fig. 1A) are assumed to be the 3-cis-isomer (minor peak) and 3-trans-isomer (major peak) of 3,5,7-decatrienoyl-CoA because both are converted to 2,4,6-decatrienoyl-CoA by a partially purified preparation of dienoyl-CoA isomerase exhibiting trienoyl-CoA isomerase activity (Fig. 1C). The reduction of 2,4,6-decatrienoyl-CoA (Fig. 1D) by NADPH in the presence of 2,4-dienoyl-CoA reductase from E. coli (Fig. 1E) yields a single product that was eluted at the same position as was the starting material (Fig. 1, compare D and E). However, the addition of crotonase to the reaction product, presumed to be 2,6-decadienoyl-CoA because the E. coli reductase catalyzes the reduction of the 4,5-double bond, produced a more polar compound, most likely 3-hydroxydec-6- enoyl-CoA (Fig. 1F). In contrast, the addition of crotonase to 2,4,6-decatrienoyl-CoA (Fig. 1D) did not produce the product of different polarity. This experiment supports the assigned structures of 2,4,6-decatrienoyl-CoA (Fig. 1D) and 2,6-decadienoyl-CoA (Fig. 1E) because 2-enoyl-CoA compounds are hydrated to a significant extent only when the 2-double bond is in conjugation with the thioester group, but not when it is part of a more extended chromophore (18).

Although 5,7-decadienoyl-CoA can be converted enzymatically to 2,4,6-decatrienoyl-CoA and further degraded after the

**TABLE I**

| Substrate                      | Rates of respiration* (nmol O₂/min/mg protein) |
|-------------------------------|-----------------------------------------------|
| 9-cis,12-cis-Octadecadienoyl-CoA (linoleoyl-CoA) | 81.4 ± 1.6 (3) |
| 9-cis,11-trans-Octadecadienoyl-CoA | 64.3 ± 1.3 (3) |
| Decanoic acid                  | 110.4 ± 3.3 (3) |
| 5-trans,7-trans-Decadienoic acid | 84.3 ± 1.8 (3) |

*Values are means ± S.D. based on the number of measurements indicated in parentheses.

Fig. 2. Spectrophotometric analysis of enzymatic conversions of 5,7-decadienoyl-CoA and its metabolites. A, spectral changes associated with the dehydrogenation of 5,7-decadienoyl-CoA (7 μM in 0.1 M potassium P₃ (pH 9.0)) by acyl-CoA oxidase (30 milliunits/ml) and isomerization of 2,5,7-decatrienoyl-CoA to 3,5,7-decatrienoyl-CoA by enoyl-CoA isomerase (0.15 μM/ml). Spectrum 1, 5,7-decadienoyl-CoA at time 0; spectrum 2, 30 s after the addition of acyl-CoA oxidase; spectrum 3, 3 or 30 min after the addition of acyl-CoA oxidase; spectrum 4, 30 s after the addition of enoyl-CoA isomerase to the sample characterized by spectrum 3; spectrum 5, 2 min after the addition of enoyl-CoA isomerase. B, spectral changes associated with the conversion of 5,7-decadienoyl-CoA to 3,5,7-decatrienoyl-CoA catalyzed by acyl-CoA oxidase and enoyl-CoA isomerase. Spectrum 1, 5,7-decadienoyl-CoA (19 μM in 0.1 M potassium P₃ (pH 8.0)); spectrum 2, 15 s after the addition of acyl-CoA oxidase (0.15 units/ml) and peroxisomal multifunctional protein I (6 μM in 0.1 M potassium P₃ (pH 8.0)). C, spectral changes associated with the isomerization of 3,5,7-decatrienoyl-CoA to 2,4,6-decatrienoyl-CoA catalyzed by a soluble extract of rat liver mitochondria or partially purified dienoyl-CoA isomerase. Spectrum 1, 3,5,7-decatrienoyl-CoA (20 μM in 0.1 M potassium P₃ (pH 9.0)); spectrum 2–4, 3, 7, and 30 min after enzyme addition, respectively. Spectrum 5, HPLC-purified 2,4,6-decatrienoyl-CoA. D, spectral changes associated with the reduction of 2,4,6-decatrienoyl-CoA by NADPH in the presence of purified 2,4-dienoyl-CoA reductase from E. coli. Spectrum 1, 2,4,6-decatrienoyl-CoA (4 μM in 0.1 M potassium P₃ (pH 8.0); NADPH (0.1 mM) was added to the sample and reference solutions and the reaction was initiated by the addition of 2,4-dienoyl-CoA reductase (0.5 μg); spectra 2–5, 1 s, 1 min, 2.5 min, and 6 min after starting the reaction, respectively.

Fig. 1. HPLC analysis of metabolites formed by enzymatic conversions of 5,7-decadienoyl-CoA. A, HPLC-purified 5,7-decadienoyl-CoA (Δ⁵): B, 3,5,7-decatrienoyl-CoA (Δ⁵,6,8): C, 2,4,6-decatrienoyl-CoA (Δ⁵,6,8,12) formed from 5,7-decadienoyl-CoA (8 nmol in 0.2 ml of 0.1 M potassium P₃ (pH 8.0)) by acyl-CoA oxidase (0.1 unit) and peroxisomal multifunctional protein I (6 μg/ml) within 10 min; C, 2,4,6-decatrienoyl-CoA (Δ⁵,6,8,12) formed from 3,5,7-decatrienoyl-CoA, described for B, by dienoyl-CoA isomerase (0.4 units); D, HPLC-purified 2,4,6-decatrienoyl-CoA after incubation without or with crotonase (0.7 units); E, 2,6-decadienoyl-CoA (Δ⁵,12) formed from HPLC-purified 2,4,6-decatrienoyl-CoA (10 nmol in 0.5 ml of 0.1 M potassium P₃ (pH 8.0)) by E. coli 2,4-dienoyl-CoA reductase (5 μg/ml) plus 0.1 mM NADPH within 5 min; F, 3-hydroxydecanoyl-CoA (3OHΔ⁶) formed from half of the sample described for E by crotonase (0.7 units) within 1 min.
The relationship between trienoyl-CoA and dienoyl-CoA isomerases was further investigated by studying their behavior during purification. The enzymes were purified from pig heart to minimize a possible interference by peroxisomal forms of these enzymes. The results of this purification effort, summarized in Table II, demonstrate the co-purification of the two enzymes. The activities of trienoyl-CoA and dienoyl-CoA isomerases remained inseparable throughout the procedure even though the dienoyl-CoA isomerase/trienoyl-CoA isomerase ratio changed from 35:1 to 143:1. The result of the last purification step, the elution of the purified enzyme from a reactive red 120 column, is shown in Fig. 5A. The elution of trienoyl-CoA isomerase from this column coincided with the appearance of dienoyl-CoA isomerase and was proportional to the amount of protein present in each fraction. Moreover, an analysis of individual column fractions by SDS-polyacrylamide gel electrophoresis revealed the presence of only one band (Fig. 5B) that corresponded to a protein with a molecular mass close to 32 kDa, which is the mass reported for dienoyl-CoA isomerase.

**DISCUSSION**

The observation that the CoA derivatives of fatty acids with two conjugated double bonds at odd-numbered positions, e.g. 9-cis,11-trans-octadecadienoyl-CoA (Fig. 6, compound I), sustain mitochondrial respiration demonstrated their degradation by mitochondrial δ-oxidation. Chain shortening of such unsaturated acyl-CoAs by β-oxidation is expected to produce 5,7-dienoyl-CoA intermediates (Fig. 6, compound II). Based on evidence obtained with monounsaturated fatty acids that have a double bond at an odd-numbered position (11), the degradation of 5,7-dienoyl-CoA is predicted to proceed by two different routes. Common to both of them would be the dehydrogenation of 5,7-dienoyl-CoA to 2,5,7-trienoyl-CoA (Fig. 6, compound III) catalyzed by one of the acyl-CoA dehydrogenases. If 2,5,7-trienoyl-CoA completes the cycle of β-oxidation, the resultant product would be 3,5-dienoyl-CoA. The further metabolism of such intermediates has been shown to require the sequential actions of dienoyl-CoA isomerase, 2,4-dienoyl-CoA reductase, and enoyl-CoA isomerase to produce 2-trans-enoyl-CoA, which can reenter the β-oxidation spiral (11). If, however, enoyl-CoA isomerase catalyzes the isomerization of the double bond from the 2,3-position to the 3,4-position, 3,5,7-trienoyl-CoA (Fig. 6, compound IV) would be formed. This isomerization would be irreversible for all practical purposes, as is the isomerization of 2,5-octadienoyl-CoA to 3,5-octadienoyl-CoA (13). The evidence presented in this report indicates that a significant fraction of the 2,5,7-trienoyl-CoA is converted to the 3,5,7-isomer even though the major portion of 2,5,7-trienoyl-CoA completes the cycle of β-oxidation and thereby bypasses the formation of a
trienoyl-CoA intermediate. The further metabolism of 3,5,7-trienoyl-CoA was hitherto unknown. The identification of trienoyl-CoA isomerase suggested a pathway for the complete degradation of 3,5,7-trienoyl-CoAs. Isomerization of 3,5,7-trienoyl-CoA by trienoyl-CoA isomerase yields 2,4,6-trienoyl-CoA, presumably in the all-trans-configuration, as established for the formation of 2,4-octadienoyl-CoA from 3,5-octadienoyl-CoA (Fig. 6, compound V). In liver mitochondria, 2,4,6-trienoyl-CoA can be reduced to 3,6-dienoyl-CoA by NADPH-dependent 2,4-dienoyl-CoA reductase (2). The complete degradation of the latter intermediate would proceed by well-established reactions that require the actions of enoyl-CoA isomerase to shift the odd-numbered double bond from carbon 3 to 2 and of 2,4-dienoyl-CoA reductase to reductively remove the even-numbered double bond.

The co-purification of trienoyl-CoA and dienoyl-CoA isomerases as well as the co-immunoprecipitation of these two enzyme activities by antibodies raised against dienoyl-CoA isomerase suggested that both enzymes reside on one protein. The demonstration that one protein, as indicated by a single band on SDS-polyacrylamide gel electrophoresis (PAGE) and stained for protein with Coomassie Brilliant Blue, exhibited both trienoyl-CoA and dienoyl-CoA isomerase activities confirmed the conclusion about the association of both enzyme activities with one protein.

Dienoyl-CoA isomerase was first isolated from rat liver mitochondria and reported to have a subunit molecular mass of 32 kDa (10). Subsequently, peroxisomes were shown to contain a form of this enzyme that cross-reacted with antibodies raised against the mitochondrial enzyme (19). The molecular cloning of dienoyl-CoA isomerase yielded a cDNA sequence that strongly suggested peroxisomal as well as mitochondrial localizations of this enzyme (20). Evidence in support of the dual subcellular localization of this protein was obtained by immunoelectron microscopy. However, the precise terminal sequences of the mature forms of dienoyl-CoA isomerase detected in mitochondria and peroxisomes have not been reported. Since the published cDNA sequence encodes a 36-kDa polypeptide, molecular masses >36 kDa reported for this enzyme (19, 21) must be those of other polypeptides. Finally, expression of a fragment of the cDNA coding for dienoyl-CoA isomerase yielded an active protein that was crystallized and analyzed by x-ray diffraction (22). The crystal structure revealed an active-site pocket that is hydrophobic except for the side chains of three acidic residues. Two of these residues, Glu-196 and Asp-204, were proposed to facilitate the proton removal from carbon 2 and the proton addition to carbon 6.

### Table II

| Step                          | Total activity | Specific activity | Activity ratio |
|-------------------------------|----------------|------------------|---------------|
|                               | DI* units      | DI units/mg      |               |
| Soluble extract               | 760            | 0.067            | 34.5          |
| PEG precipitation             | 774            | 0.278            | 38.7          |
| Q-Sepharose                   | 590            | 2.85             | 53.6          |
| Hydroxylapatite               | 363            | 4.22             | 53.4          |
| Chromatofocusing              | 174            | 11.1             | 82.8          |
| Sepharose CL-6B/reactive red  | 56             | 96               | 143           |

* DI, dienoyl-CoA isomerase; TI, trienoyl-CoA isomerase; PEG, polyethylene glycol.

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**FIG. 5.** Analyses of fractions eluted from a reactive red 120 column during the final purification step of trienoyl-CoA isomerase. A, fractions were assayed for trienoyl-CoA isomerase (■) (values were multiplied by 75), dienoyl-CoA isomerase (●), and protein (□) based on the relative densities of bands shown in B. B, fractions were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and stained for protein with Coomassie Brilliant Blue.

**FIG. 6.** Proposed trienoyl-CoA isomerase-dependent pathway for the β-oxidation of 9-cis,11-trans-octadecadienoyl-CoA (conjugated linoleoyl-CoA).
respectively, of the substrate, 3,5-dienoyl-CoA. If 3,5,7-trienoyl-CoA binds to the same active site, the proton abstraction from carbon 2 could also be facilitated by Glu-196. However, the residue involved in the protonation of carbon 8 remains to be identified.

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