Peroxisomal Degradation of Branched-Chain 2-Oxo Acids[1,2]

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ABSTRACT

Branched-chain 2-oxo acids which are formed by transamination of leucine, isoleucine, and valine are metabolized by the peroxisomes from mung bean (*Vigna radiata* L.) hypocotyls. Acyl-coenzyme A (CoA) thio ester intermediates of the pathways were separated by reversed-phase high performance liquid chromatography. Retention time and cochromatography of individual acyl-CoA reference standards were used for identification of the acyl-CoA esters separated from the assay mixtures. Based on the results of identification and those of kinetic experiments, pathways of the peroxisomal degradation of 2-oxoisocaproate, 2-oxoisovalerate, and 2-oxo-3-methylvalerate are suggested.

Peroxisomes are common organelles of higher plant cells. A basic metabolic function of these organelles seems to be fatty acid degradation (7). Peroxisomes degrade by β-oxidation saturated straight chain, long-, medium- and short-chain fatty acids (8). Unsaturated fatty acids appear also to be degraded by peroxisomes. The enzymes required to link the catabolism of unsaturated fatty acids to the β-oxidation sequence have recently been demonstrated in glyoxysomes, the peroxisomes of lipid-storing nutrient tissues of seeds (2).

Glyoxysomes are involved in the conversion of reserve lipid to sucrose during germination (1). The physiological role of the peroxisomal fatty acid degrading system in non-lipid storing tissues, *i.e.* in the majority of plant tissues, has yet to be elucidated. The turnover of membrane lipids has to be considered as a source of fatty acids in non-lipid storing tissues. Unsaturated fatty acids would be the main substrates for the peroxisomal β-oxidation system. The data on the ability of glyoxysomes to degrade unsaturated fatty acids (2) support this concept.

A second physiologically important source of substrate for a fatty acid degrading system in non-lipid storing tissues can result from the degradation of branched-chain amino acids in the course of steady-state protein turnover. In *Lemna minor*, 50 to 60% of the leucine and isoleucine resulting from protein turnover is metabolized (3). Intermediates of the catabolism of leucine, isoleucine, and valine are branched-chain 2-oxo acids. The catabolism of these acids in higher plants has received very little attention up to now (12). We have recently shown that the peroxisomes but not the mitochondria from a non-lipid storing tissue are able to activate by oxidative de-

carboxylation the branched-chain 2-oxo acids which are formed by transamination of leucine, isoleucine, and valine, and that the acyl-CoAs formed are oxidized by the peroxisomal acyl-CoA oxidase (5). Further results on the peroxisomal degradation of branched-chain 2-oxo acids are presented in this paper. The studies have been restricted to intermediary acyl-CoA thio esters of the branched-chain 2-oxo acid catabolism.

MATERIALS AND METHODS

Organelle Isolation

Peroxisomes were isolated from the hypocotyls of 2- to 3-d-old, dark grown mung bean (*Vigna radiata* L.) seedlings and purified as described previously (4).

Assays

The reaction mixture (total volume 2 mL) for assaying the degradation of branched-chain 2-oxo acids contained 175 mM Tris-HCl (pH 8.5), 7.5 mM MgCl$_2$, 0.33 mM NAD$^+$, 0.6 mM CoASH, 0.1 mM branched-chain 2-oxo acid, and 60 to 160 μg peroxisomal fraction protein. After incubation at 25°C for different times, the reaction was terminated as described previously (5). Protein was determined as described elsewhere (9).

HPLC Analysis of Acyl-CoA Esters

Aliquots (250 μL) of assay mixtures were treated for and analyzed by HPLC as described previously (5). Elution of the acyl-CoA esters was monitored spectrophotometrically at 260 nm.

Reference Standards

CoASH and purchased acyl-CoA esters used as reference standards were made up as 5 mM stock solutions in water. The reference standards were chromatographed under conditions identical with those used for the chromatography of assay mixture aliquots.

Certain acyl-CoA reference standards were prepared by defined enzyme reaction(s). The preparation of 3-hydroxypropionyl-CoA from propionyl-CoA has been described previously (6). Methacrylyl-CoA and 3-methylcrotonyl-CoA were prepared using a reaction mixture which contained 175 mM Tris-HCl (pH 8.5), 1.7 nkat mL^{-1} acyl-CoA oxidase (from *Candida* sp.), and 20 μM isobutyryl-CoA and isovaleryl-CoA, respectively. The reaction mixture for preparing 2-methyl-3-hydroxybutyryl-CoA contained 175 mM Tris-HCl (pH 8.5), 115 μkat mL^{-1} crotonase (from bovine liver), and 20 μM

tiglyl-CoA. The reaction mixture for preparing 2-methylace-toacetyl-CoA contained 50 mM K-phosphate (pH 6.8), 2 mM EDTA, 0.33 mM NAD⁺, 115 μkat mL⁻¹ crotonase, 300 μkat mL⁻¹ 3-hydroxyacyl-CoA dehydrogenase (from porcine heart), 0.1% BSA, and 20 μM tiglyl-CoA. The reactions were terminated and aliquots of the reaction mixtures were treated for and analyzed by HPLC as described above. Reaction mixtures without added enzyme(s) were run as controls. After 30 min of incubation of the reaction mixtures at 25°C, the enoyl-CoAs, 2-methyl-3-hydroxybutyryl-CoA, and 2-methyl-acetoacetyl-CoA had been formed at a yield of approximately 80%, 50%, and 30%, respectively.

Chemicals

The branched-chain 2-oxo acids (2-oxoisocaproate, 2-oxo-isovalerate, 2-oxo-3-methylvalerate), acyl-CoAs, and enzymes were purchased from Sigma (Munich, FRG).

RESULTS

Reference Standards

Pathways of branched-chain 2-oxo acid catabolism have been elucidated in microorganisms and mammalian mito-chondria. Assuming that branched-chain 2-oxo acids are catabolized in the higher plant peroxisome, by using its β-oxidation system, in analogy to those pathways, certain acyl-CoAs can be proposed as intermediates of the peroxiso-mal degradation of branched-chain 2-oxo acids. The positions (retention times) of these acyl-CoAs on HPLC chromato-grams were determined using reference standards purchased or prepared by defined enzyme reaction(s). The retention times determined for the individual acyl-CoA reference stand-ards were used to identify absorbance peaks of the HPLC chromatograms of the assay mixtures.

Following HPLC separation of reaction mixtures used to prepare acyl-CoA reference standards, the HPLC elution pro-files showed absorbance peak(s) in addition to those of the controls. The number of additional peaks corresponded to the number of reaction products expected to be formed by the enzymatic reaction(s) performed. It is, therefore, assumed that the additional peaks are due to the acyl-CoAs expected to be formed during the reaction. As an example, Figure 1 shows the HPLC chromatograms of the reaction mixtures used to prepare 2-methyl-3-hydroxybutyryl-CoA and 2-methylacetoacetyl-CoA.

HPLC Chromatograms of Assay Mixtures

When complete assay mixtures were not incubated but immediately separated by HPLC, the HPLC elution profiles showed absorbance peaks below 10 min and at 13.5 min retention time. The latter peak is due to CoASH. Peaks with retention times >13.5 min were never observed for these controls. This demonstrates that the peroxisomes added to the assay mixtures did not contain detectable amounts of compounds which absorb light at 260 nm and have retention times >13.5 min, i.e. endogenous acyl-CoAs were not present in detectable amounts in the peroxisome samples.
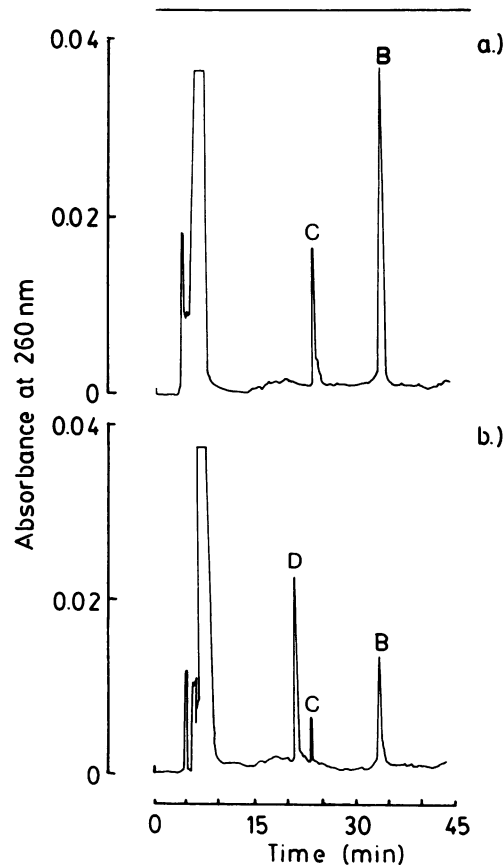

Figure 1. HPLC chromatograms showing separation of reaction mixtures for preparing the reference standards 2-methyl-3-hydroxy-butyryl-CoA (C) and 2-methylacetoacetyl-CoA (D) from tiglyl-CoA (B). The reaction mixtures were incubated for 10 min and contained (a) tiglyl-CoA and crotonase or (b) tiglyl-CoA, crotonase, and 3-hydroxy-acyl-CoA dehydrogenase. Retention times: B, 33.2 min; C, 23.0 min; D, 20.4 min.

Omission of the peroxisomes from assay mixtures resulted, after 15 min of incubation, in HPLC elution profiles identical with those of complete but unincubated assay mixtures. Sim-ilar results were obtained if NAD and/or CoASH required for the activation of branched-chain 2-oxo acids had been omitted from the assay mixture.

Figures 2, 3, and 4 show HPLC chromatograms of com-plete, incubated assay mixtures which contained 2-oxo-3-methylvalerate, 2-oxo-isovalerate or 2-oxoisocaproate as sub-strate. Prominent peaks of the HPLC elution profiles were assigned to individual acyl-CoA thio esters as given in the figure legends. Identification of these peaks was based on the retention times determined for individual acyl-CoA reference standards.

In order to confirm that an absorbance peak was due to the presumed acyl-CoA, aliquots of assay mixture and reference standard (purchased reference standard or reaction mixture used to prepare the reference standard) were mixed, and the mixture was analyzed by HPLC. This co-chromatography was performed with respect to each peak labeled in Figures 2 through 5, except for peak A of Figure 2 and peak C of Figure 3. The peak to be identified and that of the reference standard
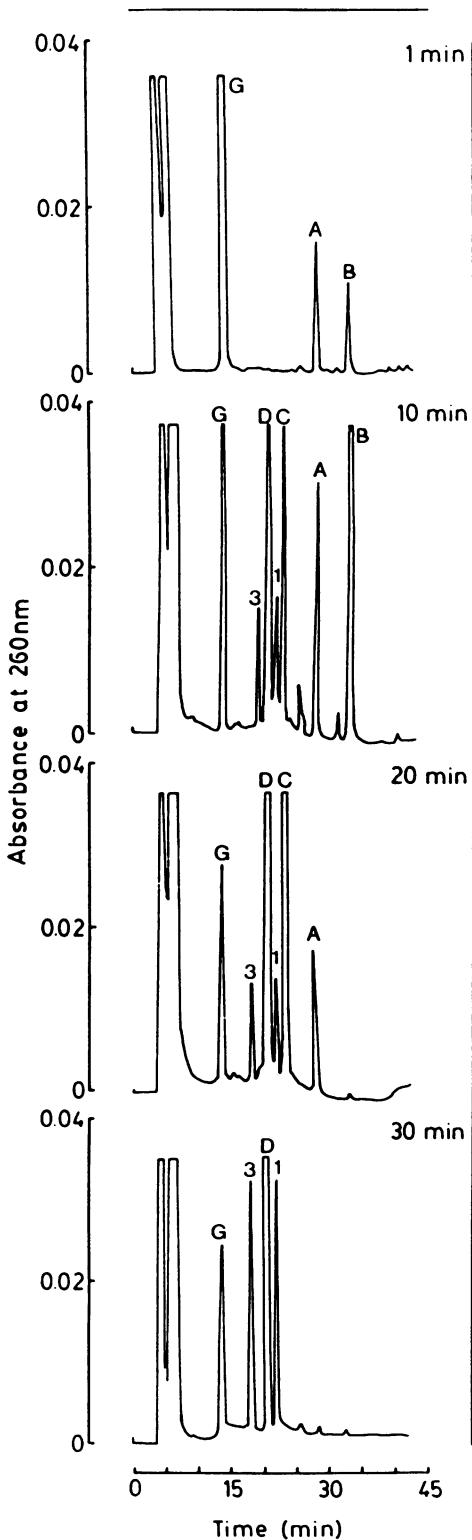
Figure 2. HPLC-chromatograms showing separation of an assay mixture which contained peroxisomes and 2-oxo-3-methylvalerate and was incubated for different times. Absorbance peak A (28.3 min) is due to 2-methylbutyryl-CoA according to Gerbling and Gerhardt (5). The retention time of the remaining labeled absorbance peaks corresponds to that of acyl-CoA reference standards as follows: B (33.2 min), tiglyl-CoA; C (23.0 min), 2-methyl-3-hydroxybutyryl-CoA; D (20.4 min), 2-methylacetoacetyl-CoA; G (13.5 min), CoASH; 1 (21.6 min) propionyl-CoA; 3 (18.4 min) acetyl-CoA.

showed always complete overlap on the HPLC elution profile.

Peak A of Figure 2 is due to 2-methylbutyryl-CoA according to (5). Peak C of Figure 3 is assumed to be due to 3-hydroxyisobutyryl-CoA. The assumption is based on the position of this peak in relation (a) to the positions of the other acyl-CoAs on the chromatogram and (b) to the relative position of 3-hydroxyacyl-CoA on other HPLC chromatograms (Fig. 2) (6). We failed to prepare a 3-hydroxyisobutyryl-CoA reference standard from isobutyryl-CoA with acyl-CoA oxidase plus crotonase.
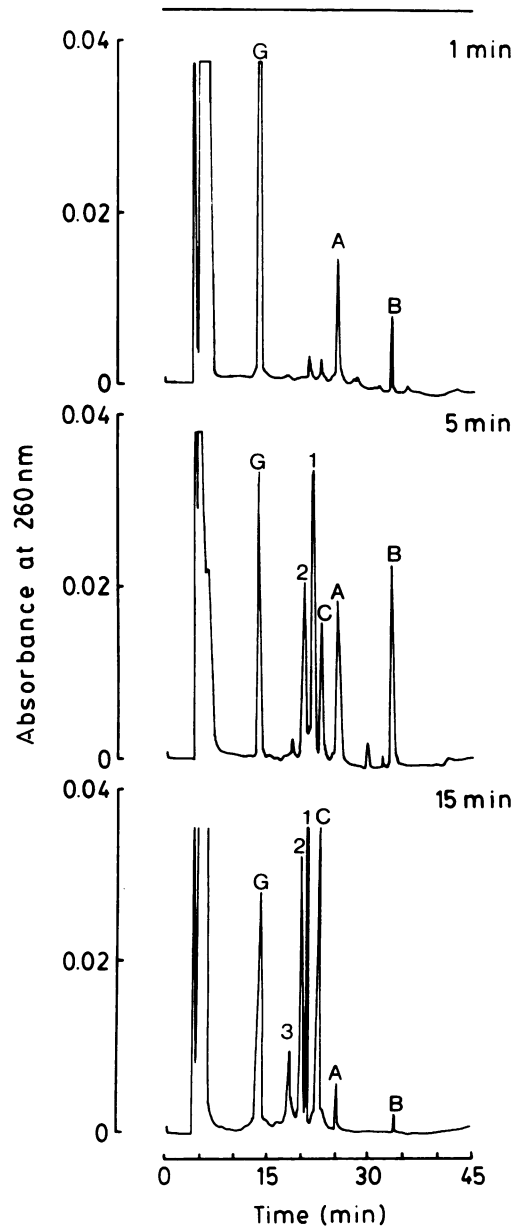

Figure 3. HPLC chromatograms showing separation of an assay mixture containing peroxisomes and 2-oxoisovalerate, and incubated for different times. The retention time of the labeled absorbance peaks corresponds to that of acyl-CoA reference standards as follows: A (25.4 min), isobutyryl-CoA; B (32.9 min), methacrylyl-CoA; G (13.5 min), CoASH; 1 (21.6 min), propionyl-CoA; 2 (20.6 min), 3-hydroxypropionyl-CoA; 3 (18.4 min), acetyl-CoA. For identification of the absorbance peak labeled C (22.8 min), see text.
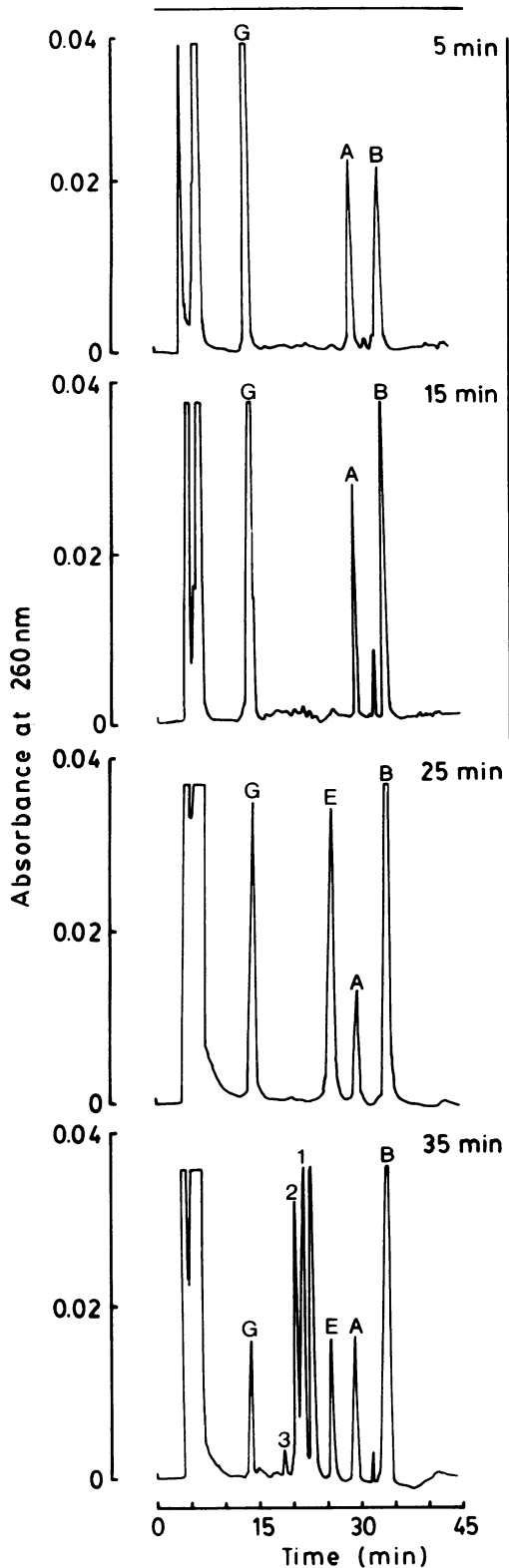
Figure 4. HPLC chromatograms showing separation of an assay mixture containing peroxisomes and 2-oxoisocaproate, and incubated for different times. The retention time of the labeled absorbance peaks corresponds to that of acyl-CoA reference standards as follows: A (29.8 min), isovaleryl-CoA; B (33.2 min), 3-methylcrotonyl-CoA; E (25.4 min), isobutyryl-CoA; G (13.5 min), CoASH; 1 (21.6 min), propionyl-CoA; 2 (20.6 min), 3-hydroxypropionyl-CoA; 3 (18.4 min), acetyl-CoA.

After 35 min of incubation of the assay mixture containing 2-oxoisocaproate as substrate, a prominent absorbance peak (not labeled in Fig. 4) appeared on the HPLC chromatogram at 22.8 min retention time. This retention time corresponds to that of peak C of Figure 3, assumed to be due to 3-hydroxyisobutyryl-CoA.

The peak pattern of the HPLC chromatograms of the assay mixtures changed during incubation (Figs. 2, 3, and 4). Pronounced absorbance peaks appearing at last on the HPLC chromatograms were those considered to represent propionyl-CoA and acetyl-CoA. This result of the kinetic studies suggests that propionyl-CoA has to be considered as a common intermediate of the peroxisomal catabolism of the branched-chain 2-oxo acids and acetyl-CoA as the ultimate end product of the pathways.

The HPLC chromatogram of the assay mixture containing 2-oxoisocaproate as substrate (Fig. 4) did not show an absorbance peak which could be assigned to HMG-CoA[3] (16.8 min retention time), a prominent intermediate of the 2-oxoisocaproate (leucine) catabolism in microorganisms and mammalian mitochondria. Since HMG-CoA is formed by an ATP requiring carboxylation of 3-methylcrotonyl-CoA and subsequent crotonase reaction, the assay mixture was supplemented with 10 mM ATP. Following incubation and HPLC separation, an absorbance peak at 16.8 min retention time was again not observed. HMG-CoA added to the assay mixture was easily detected on the HPLC chromatogram and appeared not to be metabolized during 30 min of incubation.

An absorbance peak at 16.8 min retention time (HMG-CoA) was observed on the HPLC chromatogram of the assay mixture containing 2-oxoisocaproate and ATP, when the peroxisomes as enzyme source had been replaced by a cell-free extract from mung bean hypocotyls (Fig. 5). The HPLC chromatogram of the cell free extract itself did not show an absorbance peak at 16.8 min retention time.

During incubation of assay mixtures containing 2-oxoisocaproate as substrate, a compound was formed which behaved like isobutyryl-CoA during HPLC chromatography. The formation of the supposed isobutyryl-CoA was not due to a contamination of 2-oxoisocaproate by 2-oxoisovalerate. In the presence of Triton X-100 (0.02%; v/v) which inhibits the peroxisomal acyl-CoA oxidase (9) and, therefore, branched-chain 2-oxo acid metabolism beyond oxidative decarboxylation, the formation of isovaleryl-CoA which results from the oxidative decarboxylation of 2-oxoisocaproate (5) was only observed. Isobutyryl-CoA which results from oxidative decarboxylation of 2-oxoisovalerate was not formed.

DISCUSSION

Peroxisomes of mung bean hypocotyls activate by oxidative decarboxylation the branched-chain 2-oxo acids which are formed by transamination of leucine, isoleucine, and valine (5). The acyl-CoAs generated are then oxidized by the peroxisomal acyl-CoA oxidase. As demonstrated here, further steps of branched-chain 2-oxo acid catabolism are also located in the peroxisomes (Figs. 2, 3, and 4).

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[3] Abbreviations: BCODH complex, branched-chain 2-oxo acid dehydrogenase complex; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.
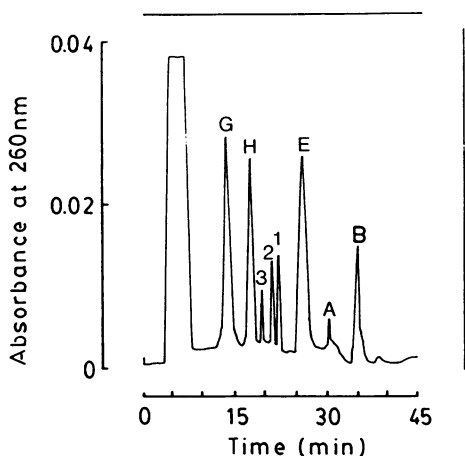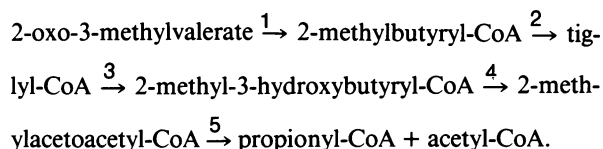
Figure 5. HPLC chromatogram showing separation of an assay mixture containing a cell free extract and 2-oxoisocaproate, and incubated for 30 min. The retention time of the labeled absorbance peaks corresponds to that of acyl-CoA reference standards as follows: A (29.8 min), isovaleryl-CoA; B (33.2 min), 3-methylcrotonyl-CoA; E (25.4 min), isobutyryl-CoA; G (13.5 min), CoASH; H (16.8 min), HMG-CoA; 1 (21.6 min), propionyl-CoA; 2 (20.6 min), 3-hydroxy-propionyl-CoA; 3 (18.4 min), acetyl-CoA.

The HPLC as performed in our investigations allows only the separation and detection of acyl-CoA thio ester intermediates of branched-chain 2-oxo acid metabolism. In order to identify these intermediates their retention times were compared with those of acyl-CoA reference standards. Correspondence of retention times was used as criterion for probable identity between intermediate and reference standard. On this basis and considering the observed changes of HPLC patterns during incubation as well as the established routes of branched-chain 2-oxo acid catabolism in microorganisms and mammalian mitochondria, pathways of the branched-chain 2-oxo acid catabolism in mung bean hypocotyl peroxisomes are suggested as outlined below.

Peroxisomal Catabolism of 2-Oxo-3-Methylvalerate

Following activation of 2-oxo-3-methylvalerate by the BCODH complex (reaction 1), the 2-methylbutyryl-CoA formed is catabolized by the β-oxidation sequence (reactions 2 through 5):

2-oxo-3-methylvalerate $\xrightarrow{1}$ 2-methylbutyryl-CoA $\xrightarrow{2}$ tig-lyl-CoA $\xrightarrow{3}$ 2-methyl-3-hydroxybutyryl-CoA $\xrightarrow{4}$ 2-meth-ylacetoacetyl-CoA $\xrightarrow{5}$ propionyl-CoA + acetyl-CoA.
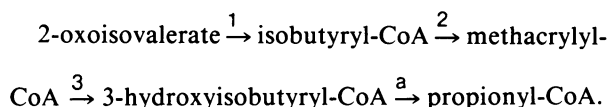
The intermediary acyl-CoAs as well as the end products propionyl-CoA and acetyl-CoA have been detected in the assay mixture containing peroxisomes and 2-oxo-3-methylvalerate (Fig. 2).

Peroxisomal Catabolism of 2-Oxoisovalerate

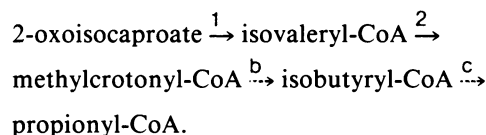From the results obtained for the assay mixture containing peroxisomes and 2-oxoisovalerate (Fig. 3), the

following pathway of 2-oxoisovalerate degradation is suggested:

2-oxoisovalerate $\xrightarrow{1}$ isobutyryl-CoA $\xrightarrow{2}$ methacrylyl-CoA $\xrightarrow{3}$ 3-hydroxyisobutyryl-CoA $\xrightarrow{a}$ propionyl-CoA.

The listed acyl-CoAs have been detected by reference standards, except 3-hydroxyisobutyryl-CoA for which a reference standard was not available. Reaction 1 is catalyzed by the BCODH complex, reactions 2 and 3 correspond to the first two reactions of β-oxidation. With reference to the catabolism of valine in heterotrophic organisms it is assumed that the reaction sequence (a) includes 3-hydroxyisobutyrate and methylmalonate semialdehyde.

Peroxisomal Catabolism of 2-Oxoisocaproate

The acyl-CoAs detected in the assay mixture containing peroxisomes and 2-oxoisocaproate (Fig. 4) can be incorporated into a pathway of 2-oxoisocaproate degradation as follows:

2-oxoisocaproate $\xrightarrow{1}$ isovaleryl-CoA $\xrightarrow{2}$ methylcrotonyl-CoA $\xrightarrow{b}$ isobutyryl-CoA $\xrightarrow{c}$ propionyl-CoA.

Reactions 1 and 2 are catalyzed by the BCODH complex and acyl-CoA oxidase, respectively. At present, there is no experimental evidence which would support or justify a specification of single reactions included in reaction sequence (b). Reaction sequence (c) corresponds to the pathway leading from isobutyryl-CoA to propionyl-CoA at the 2-oxoisovalerate degradation.

The suggested peroxisomal pathway of 2-oxoisocaproate catabolism differs from the route of 2-oxoisocaproate (leucine) degradation in microorganisms and mammalian mitochondria which metabolize methylcrotonyl-CoA to HMG-CoA. This intermediate was only detected if the peroxisomes as enzyme source of the assay mixture had been replaced by a cell-free extract. It is therefore concluded that in the intact tissue either a second, extraperoxisomal pathway of 2-oxoisocaproate degradation exists or intermediates of the peroxisomal pathway are metabolized to HMG-CoA after leaving the organelles.

The suggested pathways of peroxisomal 2-oxoisocaproate, 2-oxoisovalerate, and 2-oxo-3-methylvalerate degradation lead to propionyl-CoA. We have recently shown that the peroxisomes of mung bean hypocotyls metabolize propionyl-CoA to acetyl-CoA via acrylyl-CoA and 3-hydroxypropionyl-CoA (6), confirming the concept of propionate metabolism, in plant tissues, by a modified β-oxidation as proposed by Hatch and Stumpf (10). In mammalian mitochondria, propionyl-CoA is carboxylated. This carboxylation corresponds to that of 3-methylcrotonyl-CoA at the 2-oxoisocaproate (leucine) degradation in mammalian mitochondria. There was no indication of 3-methylcrotonyl-CoA carboxylation in the peroxisomes (see above), and this finding is supported by the peroxisomal propionyl-CoA degradation via modified β-oxi-

GERBLING AND GERHARDT

dation. The peroxisomes are apparently not able to carry out biotin-dependent carboxylations.

The absorbance peaks labeled with arabic numbers on the HPLC elution profiles shown in Figures 2 through 5, exhibit retention times identical with those of propionyl-CoA, 3-hydroxypropionyl-CoA, and acetyl-CoA, respectively. In addition, the kinetics of appearance of these peaks during incubation of the assay mixtures indicate that these absorbance peaks are due to acyl-CoAs formed towards the end of the pathways of branched-chain 2-oxo acid degradation. The HPLC chromatograms of the assay mixtures did not show, however, an absorbance peak attributable specifically to acrylyl-CoA. This intermediate of propionyl-CoA catabolism has a retention time of 33.2 min under the conditions used here for HPLC separation (6). The enoyl-CoAs formed from the branched-chain 2-oxo acids show similar retention time, *i.e.* acrylyl-CoA and these enoyl-CoAs were not separated. However, absorbance peaks at 33.2 min retention time (labeled B in the figures) which appear on the HPLC chromatograms of the kinetic experiments before the absorbance peak of propionyl-CoA is observed, are exclusively due to branched-chain enoyl-CoAs.

In summary, the data presented here indicate that the branched-chain 2-oxo acids formed by transamination of leucine, isoleucine, and valine, are catabolized by the suggested pathways to propionyl-CoA in the peroxisomes. Once propionyl-CoA is formed, it is metabolized by modified β-oxidation to acetyl-CoA as the final end product of branched-chain 2-oxo acid degradation in the higher plant peroxisome.

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