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# Peroxisomal fatty acid α- and β-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases

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## Abstract

Peroxisomes are subcellular organelles with an indispensable role in cellular metabolism. The importance of peroxisomes for humans is stressed by the existence of a group of genetic diseases in humans in which there is an impairment in one or more peroxisomal functions. Most of these functions have to do with lipid metabolism including the α- and β-oxidation of fatty acids. Here we describe the current state of knowledge about peroxisomal fatty acid α- and β-oxidation with particular emphasis on the following: (1) the substrates β-oxidized in peroxisomes; (2) the enzymology of the α- and β-oxidation systems; (3) the permeability properties of the peroxisomal membrane and the role of the different transporters therein; (4) the interaction with other subcellular compartments, including the mitochondria, which are the ultimate site of NADH reoxidation and full degradation of acetyl-CoA to $CO_2$ and water; and (5) the different disorders of peroxisomal α- and β-oxidation.

## Introduction

After their morphological identification in the early 1950s, peroxisomes were identified as truly distinct subcellular organelles by De Duve and Baudhuin [1] making use of differential and density-gradient centrifugation techniques. Because these newly discovered organelles were found to contain $H_2O_2$-producing enzymes (glycollate oxidase, urate oxidase and D-amino acid oxidase) as well as catalase, a $H_2O_2$-degrading enzyme, De Duve coined the name peroxisome. Since these pioneering studies in the 1960s not too much happened in the field of peroxisomes until the major role of peroxisomes in humans became clear in the 1980s, when two key observations on a rare genetic disease called Zellweger syndrome were made. First, Brown et al. [2] discovered defined abnormalities in the fatty acid profile in plasma from Zellweger patients with elevated levels of the very-long-chain fatty acids (VLCFAs) $C_{24:0}$ (tetracosanoic acid; 'lignoceric acid') and $C_{26:0}$ (hexacosanoic acid; 'cerotic acid'). These results immediately suggested that peroxisomes, which were known to contain a fatty acid β-oxidation system, had a major role in the β-oxidation of $C_{24:0}$ and $C_{26:0}$. Subsequent studies provided conclusive evidence for this.

The second major discovery emphasizing the importance of peroxisomes in humans came in 1983, when Heymans et al. [3] reported the deficiency of plasmalogens, a special type of phospholipid with an alkenyl group at the *sn*-1 position in tissues from Zellweger patients. Since then much has been learned about the metabolic role of peroxisomes and many functions of peroxi-

Key words: genetic diseases, membrane transport, peroxisomes.
Abbreviations used: ACOX1/SCOX, acyl-CoA oxidase 1; ACOX2/BCOX, branched-chain acyl-CoA oxidase; CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; CPT, carnitine palmitoyltransferase; CACT, carnitine/acylcarnitine translocase; D-BP, D-bifunctional protein; DHC, dihydroxycholestanoyl; L-BP, L-bifunctional protein; LCFA, long-chain fatty acid; LCS, long-chain acyl-CoA synthetase; pTH, peroxisomal thiolase; PTS, peroxisome targeting signal; RCDP, rhizomelic chondrodysplasia punctata; THC, trihydroxycholestanoyl; THCA, trihydroxycholestanoic acid; VLCFA, very-long-chain fatty acid; VLCS, very-long-chain acyl-CoA synthetase; XALD, X-linked adrenoleukodystrophy.

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[2]This paper is dedicated to the memory of our dear friend and colleague Dr Peter Vreken, who died suddenly on 23 January 2001. He contributed so much to the work described in this paper. We will continue in his spirit.

somes have been identified. Most of these functions have to do with lipid metabolism (reviewed in [4]).

In this paper we describe the current state of knowledge about the fatty acid α- and β-oxidation systems in peroxisomes, especially in relation to the various genetic diseases in which α- and β-oxidation is impaired.

## Fatty acid β-oxidation in peroxisomes

In 1969, Cooper and Beevers [5] reported their remarkable discovery of a fatty acid β-oxidation system in glyoxysomes of germinating castor-bean seedlings. The growing notion that glyoxysomes and peroxisomes were closely related organelles both belonging to the microbody family led Lazarow and De Duve [6] to study whether animal peroxisomes were also capable of fatty acid β-oxidation. These studies not only established the ability of peroxisomes to catalyse fatty acid β-oxidation but also showed that β-oxidation in peroxisomes occurs via the same mechanism as operates in mitochondria, which implies four subsequent steps: (1) dehydrogenation, (2) hydration, (3) dehydrogenation again and (4) thiolytic cleavage.

The significance of this second β-oxidation system was not immediately clear but it is now well established that the two systems differ in many respects, with distinct roles in whole-cell fatty acid β-oxidation (Figure 1). These differences include those listed in the following subsections.

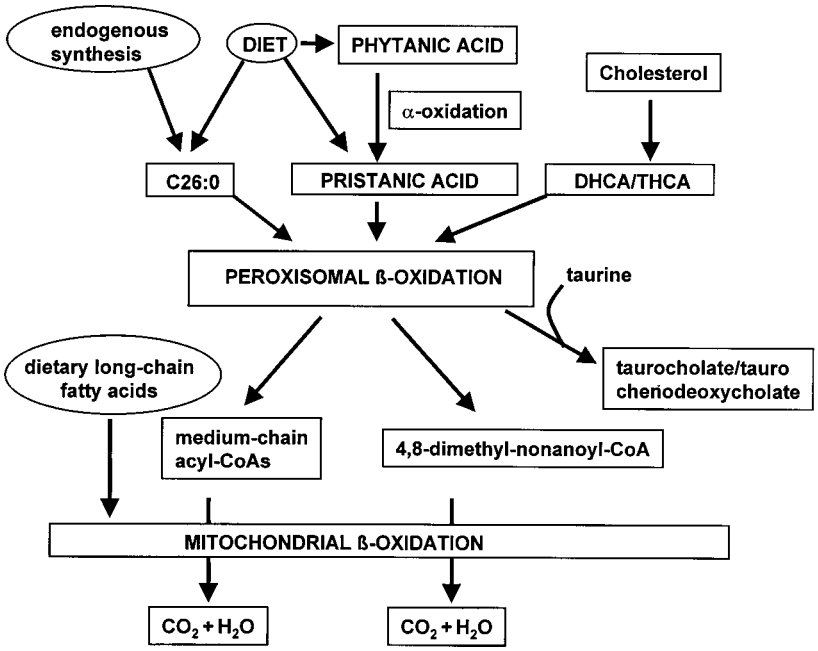
### Complete oxidation of fatty acids to $CO_2$ and $H_2O$ in mitochondria versus chain-shortening only in peroxisomes

Peroxisomes lack a Krebs cycle and cannot degrade the acetyl-CoA units produced to $CO_2$ and water. Furthermore, peroxisomes can only chain-shorten fatty acids and cannot degrade the fatty acids fully into acetyl-CoA units. For saturated fatty acids such as $C_{26:0}$ it has not been established how many cycles of β-oxidation occur in peroxisomes. In principle, β-oxidation in peroxisomes could proceed all the way down to butyryl-CoA, at which level β-oxidation stops because the two acyl-CoA oxidases known to be present in human peroxisomes (see below) show no or hardly any activity with butyryl-CoA as substrate [7]. Studies by Verhoeven et al. [8] have shown that pristanic acid undergoes three cycles of β-oxidation in the peroxisome, after which the 4,8-dimethylnonanoyl-CoA produced is transferred to the mitochondria for complete oxidation (Figure 2, upper panel).

**Figure 1**

Simplified scheme depicting the different roles of mitochondria and peroxisomes in cellular fatty acid β-oxidation

See the text for details.



### Mitochondrial versus peroxisomal fatty acid β-oxidation and ATP production

In mitochondria the first step in the β-oxidation of fatty acids is catalysed by several acyl-CoA dehydrogenases, all FAD-linked, which donate their electrons to the respiratory chain at the level of coenzyme Q [9]. In contrast, the peroxisomal oxidases, although also flavoproteins, donate their electrons directly to molecular oxygen to produce $H_2O_2$, which is subsequently decomposed to $O_2$ and water via the enzyme catalase. As a consequence, one cycle of β-oxidation in peroxisomes is at most half as efficient in terms of ATP production as is mitochondrial β-oxidation.

### Carnitine has different roles in peroxisomal and mitochondrial β-oxidation

Carnitine has an indispensable role in both mitochondrial and peroxisomal β-oxidation but at different levels. In mitochondria, carnitine is involved in the uptake of long-chain fatty acids (LCFAs) into the mitochondrial matrix via the concerted action of carnitine palmitoyltransferase (CPT) I, the carnitine/acylcarnitine translocase (CACT) and CPT II. However, in peroxisomes carnitine has no role in fatty acid uptake but does have a role in the export of chain-shortened products produced during β-oxidation of $C_{26:0}$ (Figure 2, lower panel) and pristanic acid (Figure 2, upper panel) to the mitochondria as carnitine esters.

### Regulation of mitochondrial and peroxisomal β-oxidation

Mitochondrial but not peroxisomal β-oxidation is under rapid short-term control via malonyl-CoA, the key-regulator of fatty acid β-oxidation at the level of CPT I. In contrast, despite these differences in short-term control, the long-term control of both systems shares some features because they are both induced by compounds such as fibrates and plasticizers, although the extent of induction differs [10].

### Involvement of different enzymes in mitochondrial and peroxisomal β-oxidation

Although the reactions involved in mitochondrial and peroxisomal β-oxidation are basically identical, these reactions are catalysed by different enzymes, which are in general encoded by distinct genes; there are a few exceptions in which a single gene codes for a protein directed to both peroxisomes and mitochondria. Examples are carnitine acetyltransferase (CAT) [11], $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase [12] and 2-methylacyl-CoA racemase [13–16].

### Mitochondrial and peroxisomal β-oxidation systems have different substrate specificities

From a physiological point of view, the most important difference between the mitochondrial and peroxisomal β-oxidation systems is that the two systems have different substrate specificities. Indeed, whereas the bulk of dietary LCFAs are oxidized in mitochondria rather than in peroxisomes, some fatty acids cannot be handled by mitochondria and are completely dependent on peroxisomes for β-oxidation, These include the following.

#### VLCFAs

Hexacosanoic acid ($C_{26:0}$) is exclusively oxidized in peroxisomes, as concluded from the fact that $C_{26:0}$ β-oxidation is fully deficient in fibroblasts deficient in acyl-CoA oxidase 1 but is fully normal in CPT I-deficient fibroblasts (Figure 3, lower panel). This is in line with our earlier results in which 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate, a powerful inhibitor of CPT I, was used to block CPT I [17]. Although not tested experimentally, $C_{26:0}$ β-oxidation in peroxisomes probably proceeds down to hexanoyl- and/or butyryl-CoA because the two acyl-CoA oxidases present in human peroxisomes [7] are not reactive with butyryl-CoA and are only moderately so with hexanoyl-CoA. Most probably, these CoA-esters are converted into the corresponding carnitine esters via CAT and/or carnitine octanoyltransferase (COT), followed by export of the carnitine esters to the cytosol, after which they are taken up into the mitochondria via CACT (Figure 2, lower panel). Alternatively, butyryl-CoA and/or hexanoyl-CoA undergo cleavage by one of the acyl-CoA thioesterases, followed by export of the free fatty acids to the mitochondria, activation by mitochondrial short-chain acyl-CoA synthetases and oxidation to completion.

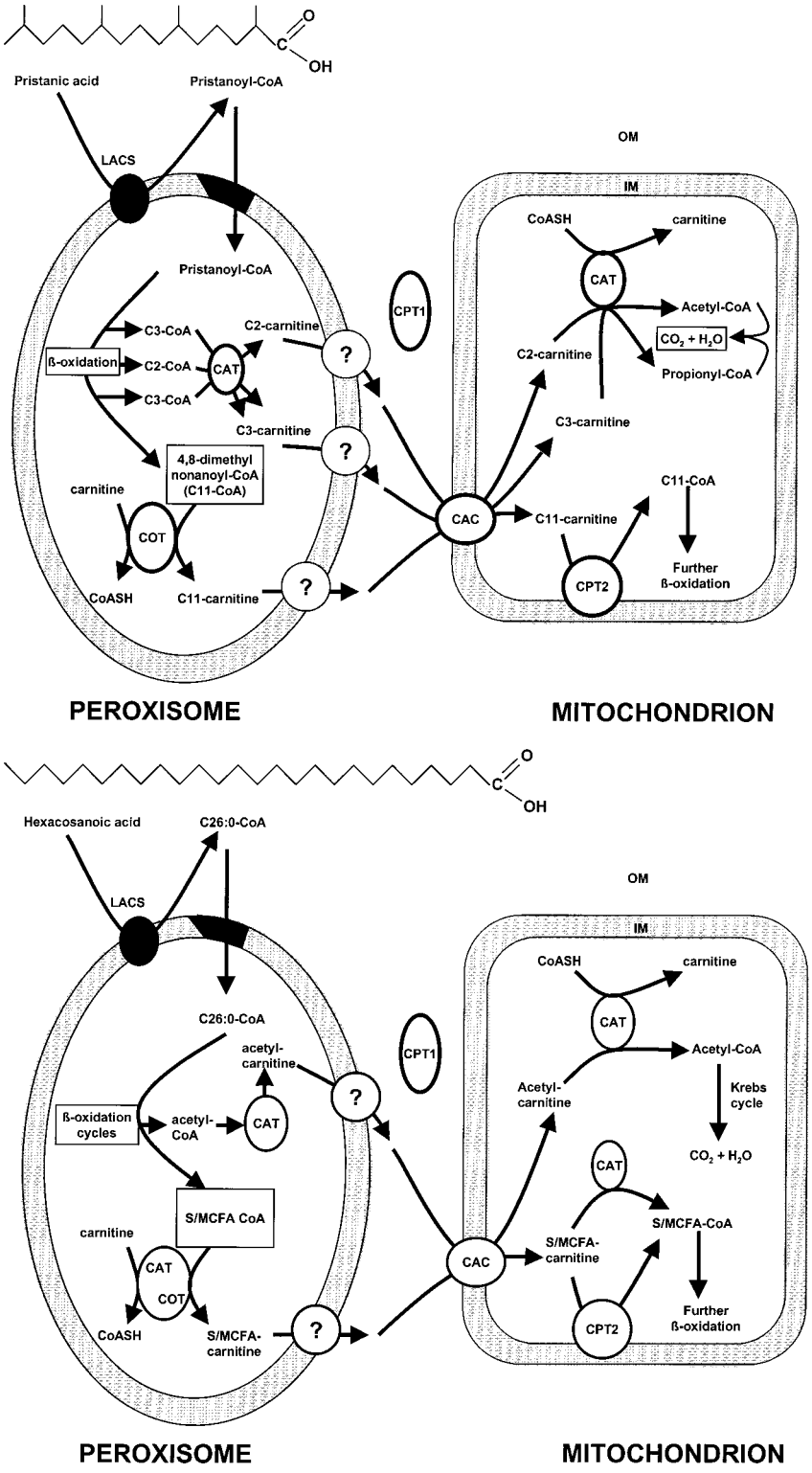
#### Pristanic acid (2,6,10,14-tetramethylpentadecanoic acid)

Pristanic acid is derived from two sources. Indeed, pristanic acid is not only the α-oxidation product of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) but is also derived directly from dietary sources (Figure 1). Oxidation of pristanic acid is completely peroxisomal, as concluded from the fact that pristanic acid β-oxidation, measured as the production of $^{14}CO_2$ plus acid-soluble products from [1-$^{14}$C]pristanic acid, is fully de-

**Figure 2**

Schematic representation of the functional interaction between peroxisomes and mitochondria in the oxidation of pristanic acid (upper panel) and hexacosanoic acid ($C_{26:0}$) (lower panel)

Abbreviations: MCFA, medium-chain fatty acid; SCFA, short-chain fatty acid; OM, outer mitochondrial membrane; IM, inner mitochondrial membrane; LACS, long-chain acyl-CoA synthetase; CAC, carnitine/acylcarnitine carrier.

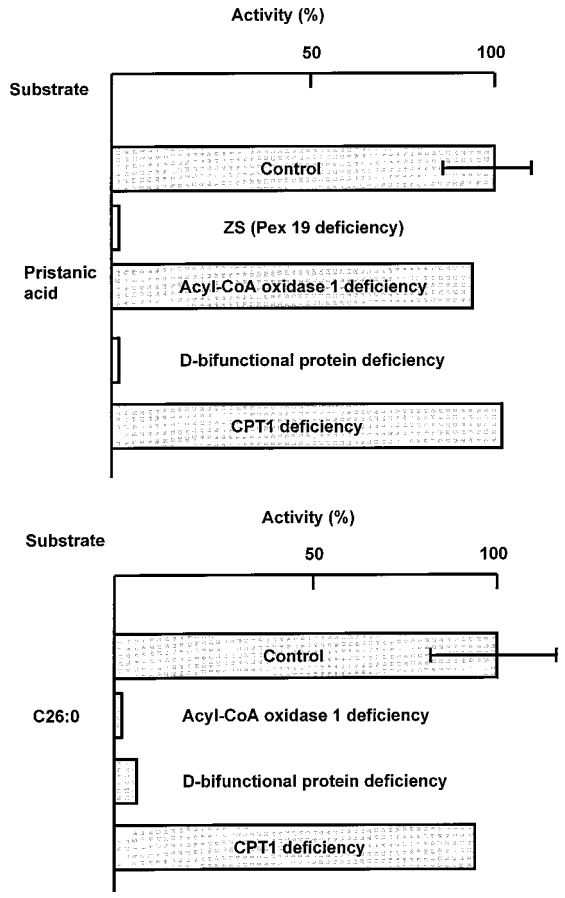


ficient in peroxisome-deficient cells, as shown in Figure 3 (upper panel), in which fibroblasts from a PEX19-deficient patient were tested [18]. Furthermore, pristanic acid $\beta$-oxidation is also fully deficient in D-bifunctional protein-deficient fibroblasts [19], whereas oxidation is fully normal in fibroblasts deficient in CPT I or very-long-chain fatty acyl-CoA dehydrogenase (Figure 3, upper panel). As described above, studies by Verhoeven et al. [8] have established that pristanic acid undergoes three cycles of $\beta$-oxidation in peroxisomes, after which the product formed (4,8-dimethylnonanoyl-CoA) undergoes conversion into its carnitine ester, most probably via COT [20], followed by export into the cytosolic space, uptake into mitochondria via CACT, retroconversion into 4,8-dimethylnonanoyl-CoA via CPT II, and oxidation to $CO_2$ and water (Figure 2, upper panel).

**Figure 3**

Whole-cell oxidation of pristanic acid (upper panel) and $C_{26:0}$ (lower panel) in control and mutant fibroblasts

The mutant fibroblasts were derived from the following patients: acyl-CoA oxidase 1 (ACOX1) deficiency, patient described in [84]; Zellweger syndrome due to PEX19 deficiency, patient described in [18]; D-bifunctional protein deficiency, patient described in [19]; CPT1 deficiency, patient described in [27].




As discussed in detail below, current evidence indicates that $\alpha$-oxidation of phytanic acid is fully peroxisomal, which implies the intraperoxisomal formation of pristanic acid. Our earlier studies had shown that the long-chain acyl-CoA synthetase (LCS) known to be present in mitochondria, peroxisomes and endoplasmic reticulum [21] also activates pristanic acid [22]. The catalytic site of the peroxisomal LCS has been found to face the cytosol, which would imply that the pristanic acid generated inside peroxisomes from phytanic acid would have to be exported out of the peroxisome, followed by activation to the CoA ester via LCS and uptake of pristanoyl-CoA into the peroxisome.

Steinberg et al. [23] recently cloned the human orthologue (*HsVLCS*) of the gene coding for the rat liver very-long-chain acyl-CoA synthetase (*RnVLCS*). The enzyme was localized to both peroxisomes and endoplasmic reticulum, and showed high activity with straight-chain and branched-chain fatty acids including pristanic acid. The authors concluded that the catalytic site of HsVLCS faces the peroxisomal matrix and not the cytoplasm and suggest that HsVLCS catalyses the intraperoxisomal activation of pristanic acid to pristanoyl-CoA.

It could well be that this newly identified synthetase (HsVLCS) [23] and the peroxisomal LCS [21] are both involved in pristanic acid oxidation but have different roles because pristanic acid is generated in two distinct subcellular compartments including the intraperoxisomal (phytanic acid $\alpha$-oxidation) and extraperoxisomal (diet) spaces. We propose that dietary pristanic acid, which enters the cell to end up in the cytosolic compartment, is activated to pristanoyl-CoA via peroxisomal LCS followed by uptake into the peroxisomal interior via an unknown mechanism possibly involving one or more of the peroxisomal half ABC transporters. In contrast, activation of intraperoxisomal pristanic acid, as generated from phytanic acid, would be catalysed by the newly identified VLCS (Figure 4).

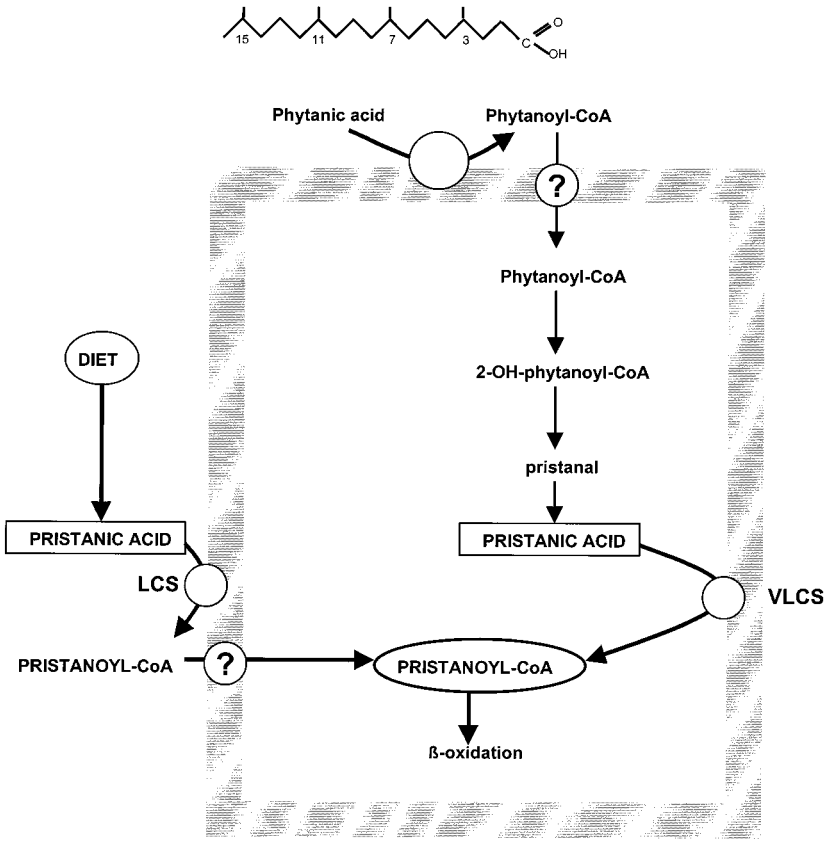
### Dihydroxycholestanoic acid and trihydroxycholestanoic acid

These two cholestanoic acids are formed in the liver from cholesterol via a complicated set of reactions and are the immediate precursors of chenodeoxycholate and cholate respectively.

**Figure 4**

Pristanic acid and differential activation by LCS and VLCS

See the text for details.




After activation at the endoplasmic reticulum membrane via a distinct synthetase, dihydroxycholestanoyl (DHC)-CoA and trihydroxycholestanoyl (THC)-CoA are transported across the peroxisomal membrane and undergo one cycle of $\beta$-oxidation in peroxisomes to produce chenodeoxycholoyl-CoA and choloyl-CoA respectively, plus propionyl-CoA. The CoA-esters are then transformed into the corresponding taurine or glycine conjugates via a specific transferase localized in peroxisomes [24] followed by export of the conjugates from the peroxisome and finally extrusion into bile after transport across the canalicular membrane (Figure 1).

### Eicosanoids

Enzymic oxygenation of polyunsaturated $C_{20}$ fatty acids leads to the formation of a broad range of compounds collectively called eicosanoids. $\beta$-Oxidation is a major mechanism for the inactivation of most of the eicosanoids including prostaglandins of the D, E and F families, prostacyclin, and certain thromboxanes, leukotrienes and hydroxyeicosatetraenoic acids (reviewed in [25]). In many cases, peroxisomes are the main site of $\beta$-oxidation.

Apart from the substrates listed above, many additional compounds undergo $\beta$-oxidation in peroxisomes including long-chain dicarboxylic acids, certain mono-unsaturated and polyunsaturated fatty acids and certain xenobiotics (reviewed in [4]).

## CPT I and the exclusive oxidation of $C_{26:0}$, pristanic acid, dihydroxycholestanoic acid and trihydroxycholestanoic acid in peroxisomes

Oxidation of $C_{26:0}$, pristanic acid and di- and trihydroxycholestanoic acid occurs predominantly if not exclusively in peroxisomes. This is especially clear for pristanic acid because the oxidation of pristanic acid is fully deficient in fibroblasts from patients suffering from classical Zellweger syndrome (Figure 3, upper panel), in which peroxisomes are deficient owing to a defect in peroxisome biogenesis. However, if pristanic acid $\beta$-oxidation is measured in fibroblast homogenates rather than in intact cells from the same patients by using a

reaction medium containing all the necessary components including pristanic acid, ATP, CoA, FAD and $NAD^+$, pristanic acid $\beta$-oxidation is not deficient, implying that mitochondria are capable of oxidizing pristanoyl-CoA. Apparently, under conditions *in vivo* in intact cells, pristanic acid is not able to enter the mitochondrion.

LCFAs normally enter the mitochondrion via the carnitine cycle, involving CPT I, CACT and CPT II. Singh et al. [26] have shown that pristanoyl-CoA is not a substrate for CPT I, which explains the findings described above. We have confirmed these important findings in the experiment of Figure 5 using the yeast *Saccharomyces cerevisiae* for expression of human CPT I [27]. The results illustrate that recombinant human CPT I shows abundant activity with palmitoyl-CoA but little, if any, activity with $C_{26:0}$-CoA, pristanoyl-CoA and THC-CoA. Taken together, these results indicate that CPT I is the main determinant in the selective oxidation of $C_{26:0}$, pristanic acid, dihydroxycholestanoic acid and trihydroxycholestanoic acid in peroxisomes.

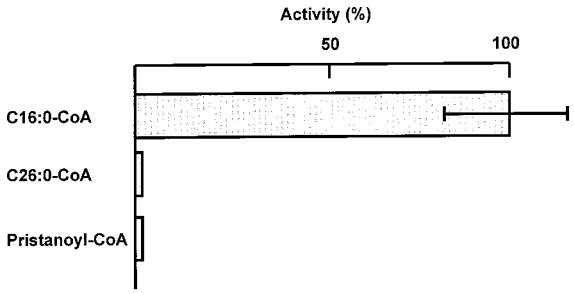
## Enzymology of the peroxisomal $\beta$-oxidation system

Studies in recent years have clearly shown that the peroxisomal $\beta$-oxidation machinery is much more complicated than originally envisaged. Indeed, it was initially thought that a single set of $\beta$-oxidation enzymes including (1) acyl-CoA oxidase, (2) bifunctional protein with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities and (3) peroxisomal thiolase catalysed the $\beta$-oxidation of fatty acids. Pioneering work by Hashimoto and co-workers led to the characterization, purification and molecular cloning of these enzymes (reviewed in [28]).

**Figure 5**

Human CPT I as expressed in *S. cerevisiae* and its reactivity with palmitoyl-CoA, $C_{26:0}$-CoA and pristanoyl-CoA

See the text for details.




The substrate specificities of these enzymes were studied in detail by Hashimoto and co-workers but included only straight-chain fatty acyl-CoAs and not pristanoyl-CoA and THC-CoA, which were only discovered as true peroxisomal substrates much later. Inclusion of these substrates in subsequent work on the peroxisomal $\beta$-oxidation enzymes rapidly led to the identification of additional enzymes as described below.

### Multiple acyl-CoA oxidases

The first step in the oxidation of straight-chain acyl-CoAs and 2-methyl branched acyl-CoAs like pristanoyl-CoA and DHC-CoA and THC-CoA is catalysed by multiple acyl-CoA oxidases, two in humans and three in the rat. The first human acyl-CoA oxidase (HsACOX1) reacts with a range of straight-chain fatty acyl-CoAs but is not reactive with pristanoyl-CoA nor with THC-CoA and can be considered as the human counterpart of the acyl-CoA oxidase identified by Osumi et al. [29]. Human peroxisomes also contain a second acyl-CoA oxidase (HsACOX2) reactive with both straight-chain and branched-chain acyl-CoAs [7]. The two acyl-CoA oxidases have been studied intensively and have been characterized at the enzyme and molecular levels. By analogy with the nomenclature used for the mitochondrial acyl-CoA dehydrogenases (very-long-chain fatty acyl-CoA dehydrogenase, medium-chain fatty acyl-CoA dehydrogenase, short-chain fatty acyl-CoA dehydrogenase, short/branched-chain fatty acyl-CoA dehydrogenase; see [9]), we propose to use the names SCOX (straight-chain acyl-CoA oxidase) and BCOX (branched-chain acyl-CoA oxidase) for the two acyl-CoA oxidases respectively (Figure 6). In the rat, pristanoyl-CoA and the two cholestanoyl-CoA esters are handled by two distinct oxidases [4].

### Two bifunctional proteins

Human (and rat) peroxisomes contain two distinct bifunctional (hydratase/3-hydroxyacyl-CoA dehydrogenase) proteins harbouring both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. The first human hydratase/3-hydroxyacyl-CoA dehydrogenase is the human counterpart of the bifunctional protein identified by Furuta et al. [30]. In recent years, a second hydratase/dehydrogenase was identified by several groups at the same time. This protein was originally discovered by Adamski et al. [31] as a 17-$\beta$-hydroxysteroid dehydrogenase.

This newly identified protein has little homology with the original bifunctional protein

identified by Furuta et al. [30] but does show homology with the various yeast multifunctional *β*-oxidation enzymes with hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. The close connection between the newly identified enzyme and the yeast multifunctional enzymes is also evident from the reaction mechanism, which involves a D-3-hydroxyacyl-CoA intermediate and not an L-3-hydroxyacyl-CoA intermediate as with the original bifunctional enzyme identified by Hashimoto and co-workers [30]. This remarkable difference between the two bifunctional enzymes inspired Hashimoto to coin the names L-bifunctional protein (LBP) and D-bifunctional protein (DBP) respectively. Alternative names are multifunctional enzymes I and II (MFE I and II), multifunctional proteins 1 and 2 (MFP1 and 2) and L- and D-peroxisomal bifunctional enzyme (L-PDE and D-PDE). In the absence of a generally agreed nomenclature, we shall use L-BP and D-BP throughout this text.

Importantly, substrate specificity studies [32–37] have shown clear differences between the two enzyme proteins. Indeed, although *both* enzymes react with the enoyl-CoAs of straight-chain fatty acids such as crotonyl-CoA ($C_{4:1}$-CoA) and hexadecenoyl-CoA ($C_{16:1}$-CoA), it is only the D-BP that is reactive with the enoyl-CoA esters of 2-methyl fatty acids such as pristenoyl-CoA and (24*E*)-3*α*,7*α*,12*α*-trihydroxy-5*β*-cholest-24-

**Figure 6**

Enzymology of the fatty acid *β*-oxidation machinery in human peroxisomes involved in the oxidation of straight-chain ($C_{26:0}$) and 2-methyl branched-chain fatty acids (pristanic acid and THCA)

D-BP is also known as MFP2, MFE II and D-PBE (see the text for details).

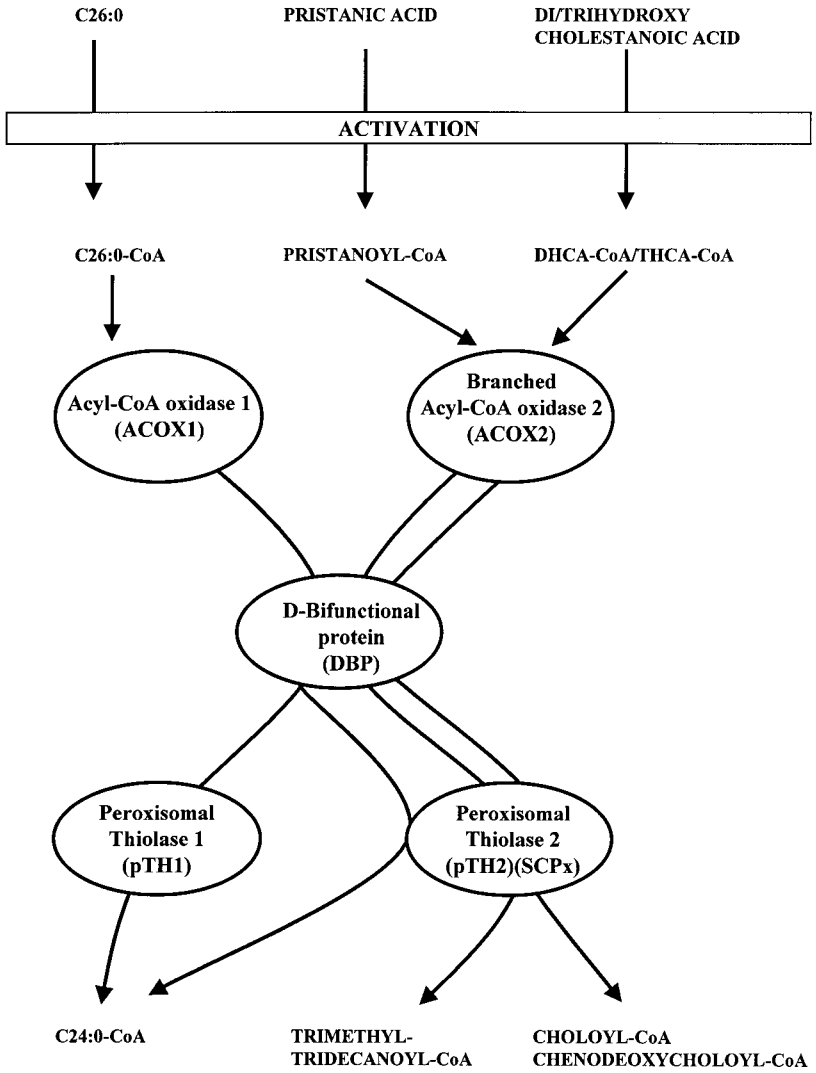


**Table 1**

**Plasma levels of VLCFAs, pristanic acid, dihydroxycholestanoic acid (DHCA) and trihydroxycholestanoic acid (THCA) in plasma from patients with a defect in peroxisomal β-oxidation and from mutant mice**

Symbols: N, normal; ↑, elevated.

| Species | Enzyme deficiency | $C_{26:0}$ | Pristanic acid | DHCA/THCA | Key references |
|---|---|---|---|---|---|
| Humans | Acyl-CoA oxidase 1 | ↑ | N | N | [84] |
| | D-BP | ↑ | ↑ | ↑† | [81–83] |
| | 2-Methylacyl-CoA racemase | N | ↑ | ↑ | [14] |
| Mice | Acyl-CoA oxidase 1 | ↑ | N | N | [49] |
| | D-BP | ↑ | ↑ | ↑ | [48] |
| | L-BP | N* | N* | N* | [47] |
| | SCPx (pTH2) | N | ↑ | – | [52] |

* R. J. A. Wanders, J. K. Reddy and co-workers, unpublished work).
† DHCA and THCA may be normal in some forms of D-BP deficiency [82].

enoyl-CoA. These results indicate that D-BP but not L-BP is indispensable for the oxidation of pristanic acid, dihydroxycholestanoic acid, trihydroxycholestanoic acid and other 2-methyl fatty acids oxidized in peroxisomes (Figure 6). As described below, our results from patients with a genetically determined deficiency of D-BP indicate that D-BP is also the main if not exclusive enzyme involved in $C_{26:0}$ β-oxidation (see also [38]).

### Multiple peroxisomal thiolases

Early studies by Miyazawa et al. [39] led to the identification of two peroxisomal 3-ketoacyl-CoA thiolases with similar properties. The two genes involved (A and B) contain 12 exons spanning 8 kb (gene A) and 9.5 kb (gene B) [40]. Gene A is constitutively expressed at a low level, whereas the transcript of gene B is hardly detectable in normal rat liver but is profoundly induced on feeding with clofibrate. In humans, there is only a single gene homologous with genes A and B [41].

Both rat and human peroxisomes also contain a second thiolase [42], which turns out to be a 58 kDa protein with both a thiolase and sterol-carrier-protein domain. Studies by Antonenkov et al. [43] and ourselves [44] have shown that the original 41 kDa thiolase (pTH1) is not reactive with the 3-ketoacyl-CoA of pristanic acid and trihydroxycholestanoic acid, whereas the 58 kDa thiolase is reactive with 2-methyl branched-chain 3-ketoacyl-CoAs. This second thiolase called peroxisomal thiolase 2 (pTH2), or alternatively SCPx or SCP2/3-ketothiolase, has been characterized at the molecular level [45,46] and is encoded by a gene spanning 80 kb and consisting of 16 exons interrupted by 15 introns. The gene has two different transcription start sites controlled by specific promoters in introns 1 and 12 respectively, and produces different transcripts. The larger transcript codes for the complete 58 kDa protein containing the thiolase (residues 1–404) and sterol-carrier-protein (residues 405–547) domains. The protein ends in -Ala-Arg-Leu at its C-terminus which ensures effective uptake into peroxisomes. The second transcript codes for a protein of 143 residues that undergoes proteolytic cleavage inside peroxisomes to produce mature SCP2 containing 123 residues.

## Resolution of the functional role of the different peroxisomal β-oxidation enzymes in peroxisomal fatty acid oxidation: lessons learned from peroxisomal patients and mutant mice

As described in more detail below, various human diseases have been described with an established defect in peroxisomal β-oxidation. This includes acyl-CoA oxidase 1 (ACOX1/SCOX) deficiency, D-BP deficiency and 2-methylacyl-CoA racemase deficiency. Table 1 lists the biochemical features observed in patients with either ACOX1/SCOX deficiency or D-BP deficiency. We measured the levels of $C_{26:0}$, pristanic acid, dihydroxycholestanoic acid and trihydroxycholestanoic acid in plasma from these two groups of patients and also studied $C_{26:0}$ and pristanic acid β-oxidation in these cells. The results for ACOX1/SCOX deficiency show elevated $C_{26:0}$ levels and a strongly decreased rate of $C_{26:0}$ β-oxidation but normal pristanic acid β-oxidation, indicating that ACOX1/SCOX has a key role in VLCFA β-oxidation but not in pristanic acid and THCA

$\beta$-oxidation, whereas the reverse is true for acyl-CoA oxidase 2 (ACOX2/BCOX) (Figure 6).

With regard to the role of D-BP in peroxisomal $\beta$-oxidation, the data in Table 1 clearly show that D-BP is not only the main, if not exclusive, enzyme involved in 2-methyl fatty acid $\beta$-oxidation but also has a key role in VLCFA ($C_{26:0}$) $\beta$-oxidation. This implies that the true function of L-BP remains elusive. These conclusions are supported by data in mice deficient in L-BP [47] and D-BP [48]. Indeed, in collaboration with Reddy and co-workers we have found that $C_{26:0}$, pristanic acid and trihydroxycholestanoic acid are normal in plasma from LBP (−/−) mice, whereas Baes et al. [48] have shown that in DBP (−/−) mice there is an accumulation of both straight-chain and branched-chain fatty acids, in line with the notion that D-BP (and not L-BP) is the enzyme involved in $C_{26:0}$, pristanic and THCA $\beta$-oxidation (Figure 6). Furthermore, in mice deficient in acyl-CoA oxidase 1 there is only the accumulation of VLCFAs such as $C_{26:0}$, in line with the scheme of Figure 6 [49].

With regard to the two thiolases, including peroxisomal thiolase 1 (41 kDa pTH1) and peroxisomal thiolase 2 (pTH2; 'SCPx/SCP2-3keto-thiolase'), the situation is less clear, especially because no human disorder has been described in which either peroxisomal thiolase 1 or thiolase 2 (SCPx) is deficient. One case of presumed peroxisomal thiolase 1 deficiency has been described [50] in which both the 41 and 44 kDa bands of peroxisomal thiolase 1 (pTH1) were found to be absent on immunoblot analysis. For several reasons we believe that the true defect in this patient is not at the level of pTH1 but at some other level. This is concluded from the fact that molecular analysis in material from the patient failed to show any mutations in the pTH1 gene (S. Ferdinandusse and co-workers, unpublished work).

Rhizomelic chondrodysplasia punctata (RCDP) type 1 is another peroxisomal disorder. In this disease the PTS2 receptor (Pex7p) is functionally inactive owing to mutations in the *PEX7* gene [51]. As a consequence all peroxisomal PTS2 proteins including (1) phytanoyl-CoA hydroxylase, (2) alkyldihydroxyacetone phosphate synthase and (3) pTH1 are mislocalized to the cytosol and rapidly degraded, which explains the deficiency of all three enzymes including pTH1 in RCDP type 1 cells. Nevertheless, the $\beta$-oxidation of $C_{26:0}$ and pristanic acid is completely normal in these cells, suggesting that SCPx is the main if not the exclusive enzyme involved in $C_{26:0}$ and pristanic acid $\beta$-oxidation. In contrast, studies in plasma and fibroblasts from the SCPx (−/−) mouse generated by Seedorf et al. [52] have shown that pristanic acid $\beta$-oxidation is deficient in SCPx (−/−) fibroblasts, whereas $C_{26:0}$ $\beta$-oxidation is fully normal [38] in line with the finding of normal $C_{26:0}$ levels in plasma from SCPx (−/−) mice. Although it is dangerous to extrapolate data from mice to humans, these results suggest that pTH2/SCPx is the key enzyme in pristanic acid $\beta$-oxidation but that pTH1 and pTH2/SCPx are both involved in $C_{26:0}$ $\beta$-oxidation. Taking all these results together, we suggest the scheme depicted in Figure 6.

### 2-Methylacyl-CoA racemase and its involvement in 2-methyl branched-chain fatty acid $\beta$-oxidation

Naturally occurring phytanic acid is a mixture of two different diastereomers (3*S*,7*R*,11*R*)- and (3*R*,7*R*,11*R*)-phytanic acid. Because $\alpha$-oxidation does not affect the hydrogen atom in the $\beta$-position, pristanic acid is similarly a mixture of two diastereomers, (2*R*,6*R*,10*R*)- and (2*S*,6*R*,10*R*)-pristanic acid. Because (mitochondrial) acyl-CoA dehydrogenases and (peroxisomal) acyl-CoA oxidases are stereospecific and act only on (2*S*)-methylacyl-CoAs, auxiliary enzymes are needed to convert (2*R*)-methyl fatty acids to the corresponding (2*S*)-fatty acids. This is brought about by so-called racemases. Studies, notably by Schmitz and Conzelmann have led to the identification of such a racemase acting on (2*R*)-pristanoyl-CoA and (25*R*)-THC-CoA. The same group purified the enzyme from rat [53] and human [54] liver and cloned the rat and mouse cDNA species, which turned out to code for proteins of 361 and 360 residues respectively [13]. Both proteins have a -Lys-Ala-Asn-Leu sequence at their C-terminal end, which is a peroxisome targeting signal. This explains the localization of racemase in peroxisomes.

We have recently identified a new peroxisomal defect [14] in a number of patients in which plasma pristanic acid, dihydroxycholestanoic acid and trihydroxycholestanoic acid were elevated with normal $C_{26:0}$, suggesting a defect in the branched-chain fatty acid $\beta$-oxidation machinery (see Figure 6). However, measurement of BCOX, D-BP and SCPx activities revealed no abnormalities, which led us to study whether the cause might be 2-methylacyl-CoA racemase deficiency. Subsequent determination of the nature of the diaster-

eomers accumulating in plasma from the patients revealed the exclusive accumulation of (25*R*)-THCA [55].

Measurement of racemase activity in fibroblasts revealed a complete deficiency of racemase. We subsequently cloned the human cDNA and identified clear-cut mutations, proving 2-methylacyl-CoA racemase deficiency [14].

We have recently found that the racemase gene codes for both the mitochondrial and peroxisomal 2-methylacyl-CoA racemase activities, which is explained by the fact that the protein has both a C-terminal peroxisome targeting signal (PTS) (-Lys-Ala-Asn-Leu in rat and mouse, -Lys-Ala-Ser-Leu in humans) as well as an N-terminal mitochondrial targeting signal. The finding that a monospecific antibody directed against recombinant enzyme was also reactive with a protein of similar size in mitochondria provided evidence in favour of the concept 'one gene, two differentially targeted proteins'. Definitive proof came when we found a complete deficiency of both the mitochondrial and peroxisomal forms of racemase in the racemase-deficient patients described in [14]. Recent studies have ascertained that the N-terminal end of the racemase protein does indeed contain mitochondrial targeting information [15].

Interestingly, we have recently found that the mitochondrial racemase also has an essential role in pristanic acid $\beta$-oxidation by catalysing the conversion of (2,6*R*)-dimethylheptanoyl-CoA into the (2,6*S*) form [16].

## Peroxisomal fatty acid $\alpha$-oxidation

Fatty acids with a methyl group at the $\beta$-position cannot undergo direct $\beta$-oxidation. This also applies to 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid), a fatty acid well known for its accumulation in patients suffering from Refsum disease, first established by Klenk and Kahlke in 1963 [56]. Studies by Steinberg and co-workers in the 1960s established that phytanic acid undergoes oxidative decarboxylation to form pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and $CO_2$. Like any 2-methyl branched-chain fatty acid, pristanic acid can undergo $\beta$-oxidation. As described above, $\beta$-oxidation starts in peroxisomes to produce 4,8-dimethylnonanoyl-CoA, which then moves to the mitochondria as the carnitine ester to be fully oxidized to $CO_2$ and water.

The mechanism by which phytanic acid is oxidatively decarboxylated to pristanic acid, called $\alpha$-oxidation, has long remained an enigma despite intense efforts, especially by Steinberg and co-workers. However, the structure of the phytanic acid $\alpha$-oxidation pathway has recently been fully elucidated. A major breakthrough was the finding by Mihalik et al. [57] of a peculiar enzyme, phytanoyl-CoA hydroxylase, localized in rat liver peroxisomes and able to convert phytanoyl-CoA into 2-hydroxyphytanoyl-CoA in a reaction involving 2-oxoglutarate, $Fe^{2+}$ and ascorbate [57]. The existence of this enzyme was subsequently confirmed in both rat liver [58] and human liver [59]. The key role of this newly identified enzyme in phytanic acid $\alpha$-oxidation soon became clear when we discovered that phytanoyl-CoA hydroxylase is fully deficient in Refsum disease [60]. We subsequently purified the enzyme from rat liver peroxisomes, microsequenced the protein and used the information obtained to clone the rat, mouse and human hydroxylase cDNA species [61,62]. This allowed us to resolve the molecular basis of Refsum disease in detail [61,63].

The phytanoyl-CoA hydroxylase that we purified from rat liver [61,62] had a molecular mass of 35 kDa, which is considerably less than the cDNA-deduced molecular mass of 38.6 kDa. Comparison of the amino acid sequences revealed that purified phytanoyl-CoA hydroxylase lacked the first 30 residues, which are probably removed by proteolytic processing inside peroxisomes. Inspection of the cDNA-deduced amino acid sequences for mouse, rat and human phytanoyl-CoA hydroxylases show the presence of typical PTS2 signals in all three sequences. This explains the primary localization of this enzyme in peroxisomes [59], and also its deficiency in Zellweger syndrome [59] and RCDP type 1 [64], in which the PTS2 receptor is functionally defective [51].

Studies by Verhoeven et al. [65] and Croes et al. [66] have resolved the subsequent structure of the pathway in which 2-hydroxyphytanoyl-CoA first undergoes cleavage to pristanal and formyl-CoA, after which pristanal is oxidized to pristanic acid via an as yet undefined aldehyde dehydrogenase.

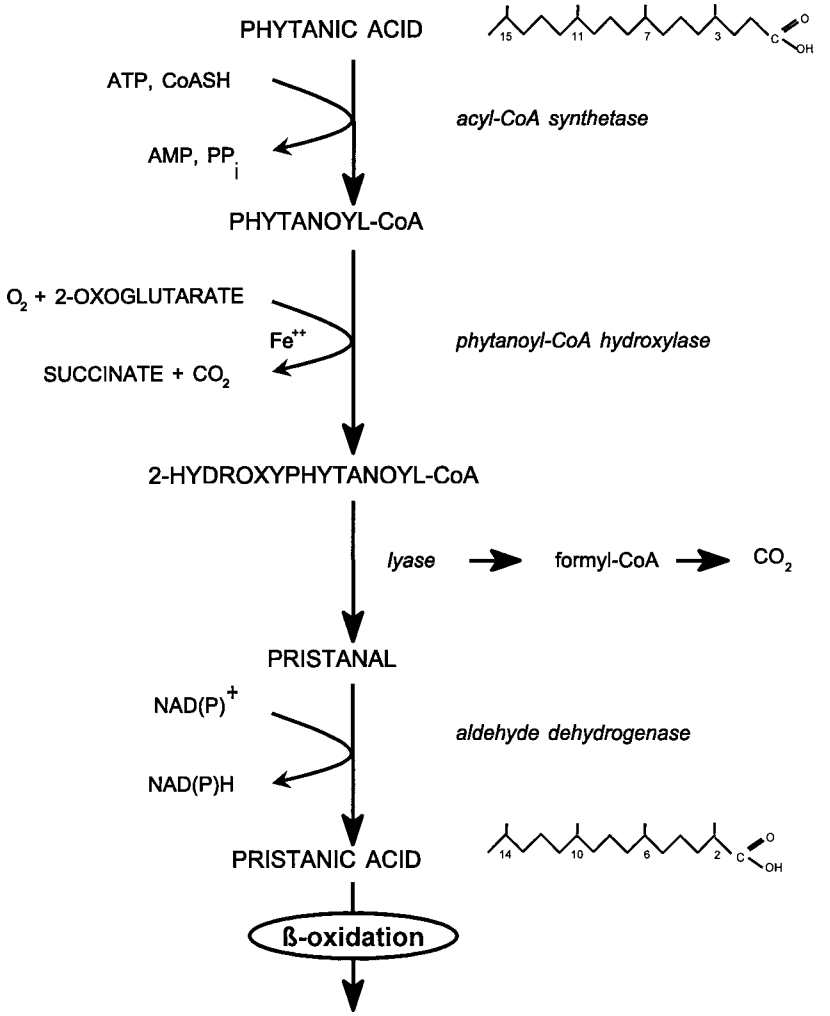
The subcellular localization of the two enzymes involved has been confusing until recently. Verhoeven et al. [67] originally reported that the conversion of 2-hydroxyphytanoyl-CoA into pristanic acid, measured as the production of pristanic acid from 2-hydroxy-phytanoyl-CoA, occurs in the endoplasmic reticulum, at least in human liver. Recent studies by ourselves and others suggest that in the intact cell phytanic acid $\alpha$-oxidation might well be fully peroxisomal

(Figure 7). This is concluded from the following observations. First, we [68] and others [69] have found that 2-hydroxyphytanoyl-CoA lyase is a peroxisomal and not a microsomal enzyme. Secondly, Foulon et al. [69] recently cloned the human 2-hydroxyphytanoyl-CoA lyase cDNA and found that the enzyme is targeted to peroxisomes via a non-consensus PTS1 signal characterized by a C-terminal tripeptide preceded by a positive charge.

The finding that phytanoyl-CoA hydroxylase and 2-hydroxyphytanoyl-CoA lyase are strictly peroxisomal makes it hard to imagine that the subsequent conversion of pristanal into pristanic acid would be non-peroxisomal. Studies by Croes et al. [66], in which use was made of the model compound 2-hydroxy-3-methylhexadecanoyl-CoA rather than 2-hydroxyphytanoyl-CoA, showed that peroxisomes, at least from rat liver, are able to catalyse the formation of both 2-methylpentadecanal and 2-methylpentadecanoic acid. We have now extended these studies by studying the subcellular localization of pristanal dehydrogenase with the true physiological substrate pristanal. The results of differential and density-gradient centrifugation experiments in rat liver have now shown that peroxisomes as well as mitochondria and microsomes contain pristanal dehydrogenase activity. Most of the total cellular activity is recovered in the microsomal fraction. Figure 8 shows an experiment in which a light mitochondrial fraction was subjected to density-gradient centrifugation with good resolution between peroxisomes, mitochondria and micro-

**Figure 7**

New, revised pathway of phytanic acid $\alpha$-oxidation in which phytanic acid is activated to phytanoyl-CoA followed by a three-step pathway involving phytanoyl-CoA hydroxylase, 2-hydroxyphytanoyl-CoA lyase and pristanal dehydrogenase

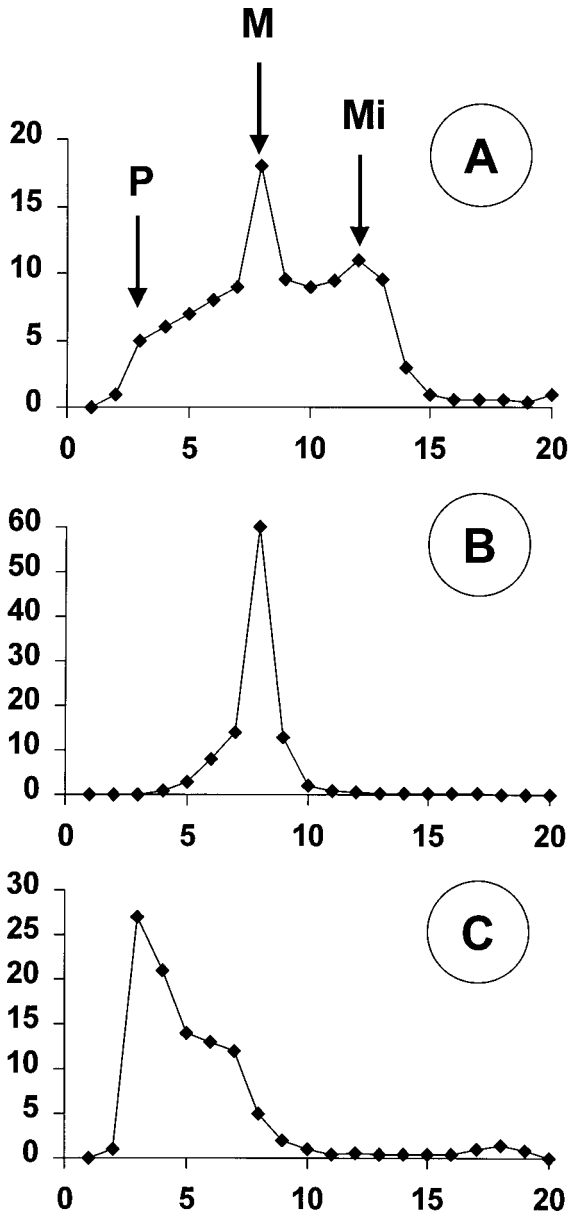


somes. The results show a trimodal activity profile for pristanal dehydrogenase, suggesting that peroxisomes harbour pristanal dehydrogenase activity. Accordingly, peroxisomes have the full capacity to α-oxidize phytanoyl-CoA to pristanic acid, followed by its intraperoxisomal activation to pristanoyl-CoA via the synthetase described by Steinberg et al. [23] and subsequent β-oxidation of pristanoyl-CoA as described above (Figure 4).

**Figure 8**

**Subcellular localization of pristanal dehydrogenase activity in peroxisomes, mitochondria and microsomes**

A rat liver light-mitochondrial fraction was prepared by differential centrifugation followed by density-gradient centrifugation on Nycodenz followed by enzyme activity measurements: (**A**) pristinal dehydrogenase; (**B**) glutamate dehydrogenase (identifying the mitochondrial fraction); (**C**) catalase (identifying the peroxisomal fraction).




The finding that peroxisomes contain endogenous pristanal dehydrogenase activity argues against the earlier suggestion that the microsomal aldehyde dehydrogenase deficient in Sjörgen–Larsson syndrome (SLS) has a key role in phytanic α-oxidation [70]. Although the isolated Sjögren–Larsson aldehyde dehydrogenase enzyme does indeed show high activity with pristanal, our results suggest that the small amount of pristanal dehydrogenase activity in peroxisomes is sufficient to catalyse the intraperoxisomal formation of pristanic acid from phytanoyl-CoA. This also agrees with the finding that phytanic acid does not accumulate in SLS patients and the oxidation of phytanic acid is fully normal in SLS fibroblasts (G. A. Jansen and co-workers, unpublished work). Figure 7 depicts the scheme of phytanic acid α-oxidation based on the analysis described above.

## Fatty acid α- and β-oxidation and the transport of metabolites across the peroxisomal membrane

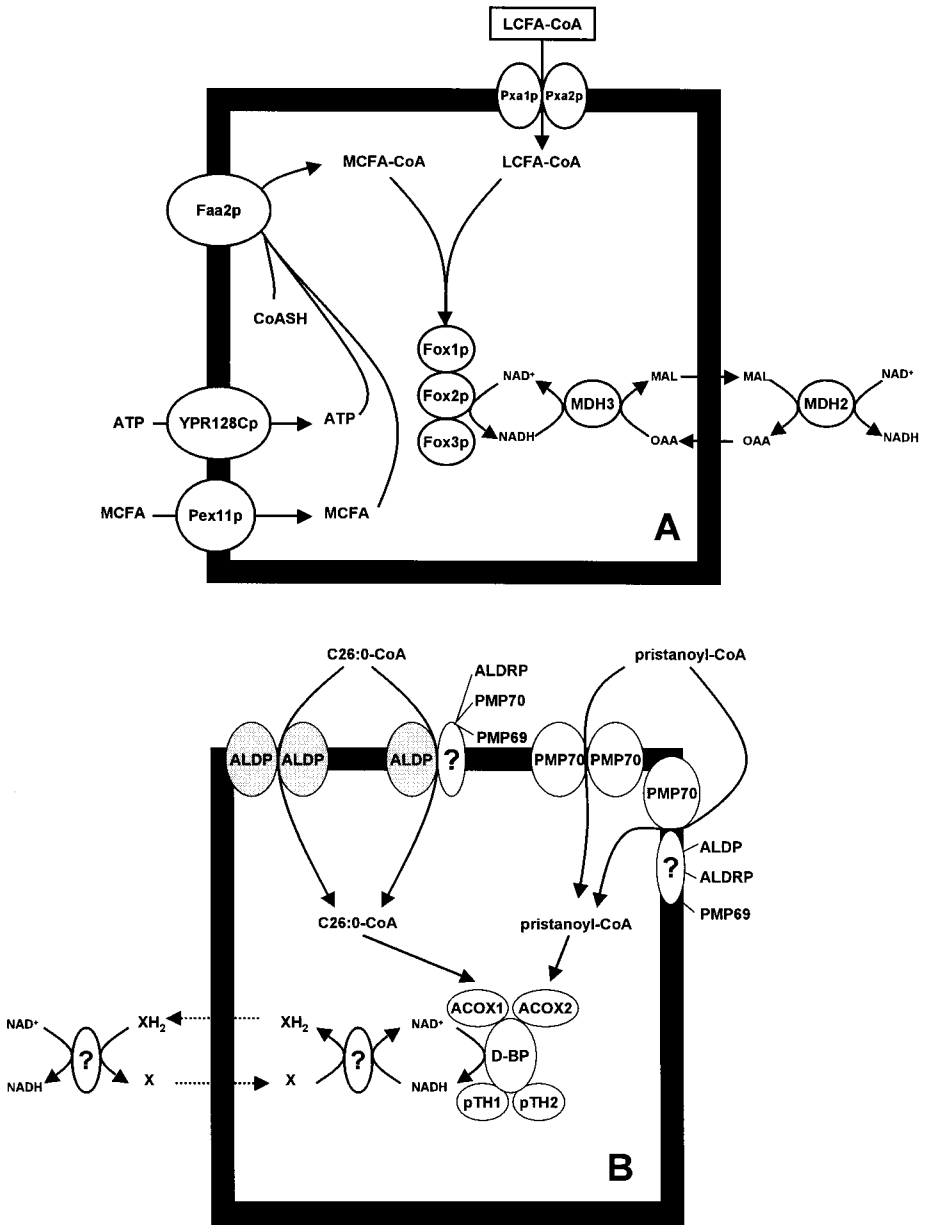
One of the main unresolved questions concerns the permeability properties of peroxisomes. It was originally thought that peroxisomes are freely permeable to low-molecular-mass compounds. This was concluded from the early studies of De Duve and Baudhuin [1] showing (1) that peroxisomes were freely permeable to sucrose and (2) that peroxisomal enzymes such as urate oxidase, D-amino acid oxidase and glycollate oxidase did not show structure-linked latency. This formed the basis for the long-held concept that the peroxisomal membrane constitutes no permeability barrier to small molecules.

Recent studies, notably in the yeast *S. cerevisiae* [71], have clearly established that at least under *in vivo* conditions the peroxisomal membrane does form a permeability barrier, which implies the existence of metabolite carriers in the peroxisomal membrane. It remains to be established why peroxisomes, in contrast with mitochondria, lysosomes and microsomes, lose their structural integrity after isolation. The concept that the peroxisomal membrane *in vivo* is truly selectively permeable is supported by the recent finding that there is a pH gradient across the peroxisomal membrane [72] that can be dissipated by uncouplers. If the peroxisomal lipid bilayer is indeed impermeable under *in vivo* conditions, specific transporters have to be present to allow

**Figure 9**

**Transport of metabolites across the peroxisomal membrane of *S. cerevisiae* (A) and higher eukaryotes (mice/humans) (B)**

See text for details. Abbreviations: MCFA-CoA, medium-chain acyl-CoA; Fox1p, acyl-CoA oxidase; Fox2p, multifunctional protein with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity; Fox3p, peroxisomal thiolase; MDH2/MDH3, cytosolic (MDH2) and peroxisomal (MDH3) malate dehydrogenase; MAL, malate; OAA, oxaloacetate. ALDP, ALDRP, PMP70 and PMP69 are the half ABC-transporters; D-BP is also known as MFP2; pTH1 and pTH2 are thiolases.




metabolite transport. Inspection of Figure 2 shows that fatty acid $\alpha$- and $\beta$-oxidation in peroxisomes requires the transport of a range of metabolites across the peroxisomal membrane. Only limited information has so far become available about these presumed transporters, although some information has recently been gained in *S. cerevisiae*. Indeed, recent studies have resolved the functional

roles of four of the presumed peroxisomal transport proteins, including Pxa1p, Pxa2p, Pex11p and YPR128cp. The available evidence indicates that Pxa1p and Pxa2p form a heterodimer, probably catalysing the transport of long-chain acyl-CoAs. Disruption of *PXA1* and/or *PXA2* is associated with decreased growth on oleate owing to a strong impairment of LCFA $\beta$-oxidation. Pex11p and YPR128cp have been found to have an essential role in medium-chain fatty acid $\beta$-oxidation rather than in LCFA $\beta$-oxidation by catalysing the transport of medium-chain fatty acids [73] and ATP [74,75] respectively.

Many more peroxisomal metabolite transporters remain to be identified, including the carriers involved in the redox shuttles catalysing the re-oxidation of intraperoxisomal NADH to $NAD^+$ [71] and the reduction of $NADP^+$ to NADPH [76] respectively (Figure 9A). Our current efforts are directed towards the identification of these presumed metabolite carriers in *S. cerevisiae*.

The situation is less clear in higher eukaryotes. Studies in recent years have led to the identification of four peroxisomal half ABC transporters including ALDP, ALDRP, PMP70 and PMP69 [4]. The function of these half transporters has not been established definitively with the exception of ALDP, which catalyses the uptake of VLCFA-CoAs such as $C_{26:0}$-CoA (Figure 9B). This has been concluded from studies on one of the peroxisomal disorders, namely X-linked adrenoleukodystrophy (XALD), which is caused by mutations in the ALD gene coding for ALDP [77]. It remains to be established whether the transport of VLCFA-CoAs by ALDP is catalysed by a homodimer or heterodimer because Aubourg and co-workers [78] have shown that the different half ABC transporters might form homodimers or heterodimers. The functions of the other three half transporters remain to be established, although PMP70 has recently been suggested [79] to be involved in the transport of 2-methylacyl-CoA esters including pristanoyl-CoA, DHC-CoA and THC-CoA on the basis of studies in mutant mice (Figure 9B).

## Human disorders of peroxisomal fatty acid $\alpha$- and $\beta$-oxidation

### Disorders of peroxisomal fatty acid $\beta$-oxidation

The following disorders of peroxisomal fatty acid $\beta$-oxidation have been identified: (1) XALD, (2) ACOX1/SCOX deficiency, (3) D-BP deficiency and (4) 2-methylacyl-CoA racemase deficiency. XALD is clinically heterogeneous, with at least six different phenotypes ranging from the severe lethal childhood cerebral form ('CCALD') to an Addison-only form with no neurological involvement. This heterogeneity can even occur within the same pedigree. In all forms of XALD there is an accumulation of VLCFAs due to an impaired oxidation of $C_{26:0}$ resulting from mutations in the ALD gene coding for the ALDP half transporter [77].

### Acyl-CoA oxidase deficiency

So far, acyl-CoA oxidase deficiency has been reported in only few patients, all of whom showed severe neurological abnormalities including early-onset seizures, hypotonia, hearing impairment and visual failure resulting from retinopathy. In these patients $C_{26:0}$ $\beta$-oxidation is deficient owing to the deficient activity of ACOX1/SCOX, which explains the elevated VLCFA levels in plasma and tissues from these patients. In line with the role of ACOX1/SCOX in peroxisomal $\beta$-oxidation (Figure 6), pristanic acid, DHCA and THCA are normal in these patients [80].

### D-BP deficiency

This has been described previously in many patients. The clinical presentation of D-BP deficiency is severe and resembles Zellweger syndrome in many respects. Abnormalities include neonatal hypotonia, craniofacial dysmorphia, seizures, developmental delay and early death in virtually all patients. Interestingly, in most cases neuronal migration is disturbed as described for Zellweger syndrome (reviewed in [80]). Importantly, we have recently found that D-BP deficiency is heterogeneous, with three distinct subgroups representing complete D-BP deficiency [81], isolated D-BP enoyl-CoA hydratase deficiency [82] and isolated D-BP 3-hydroxyacyl-CoA dehydrogenase deficiency [83] (reviewed in [80]).

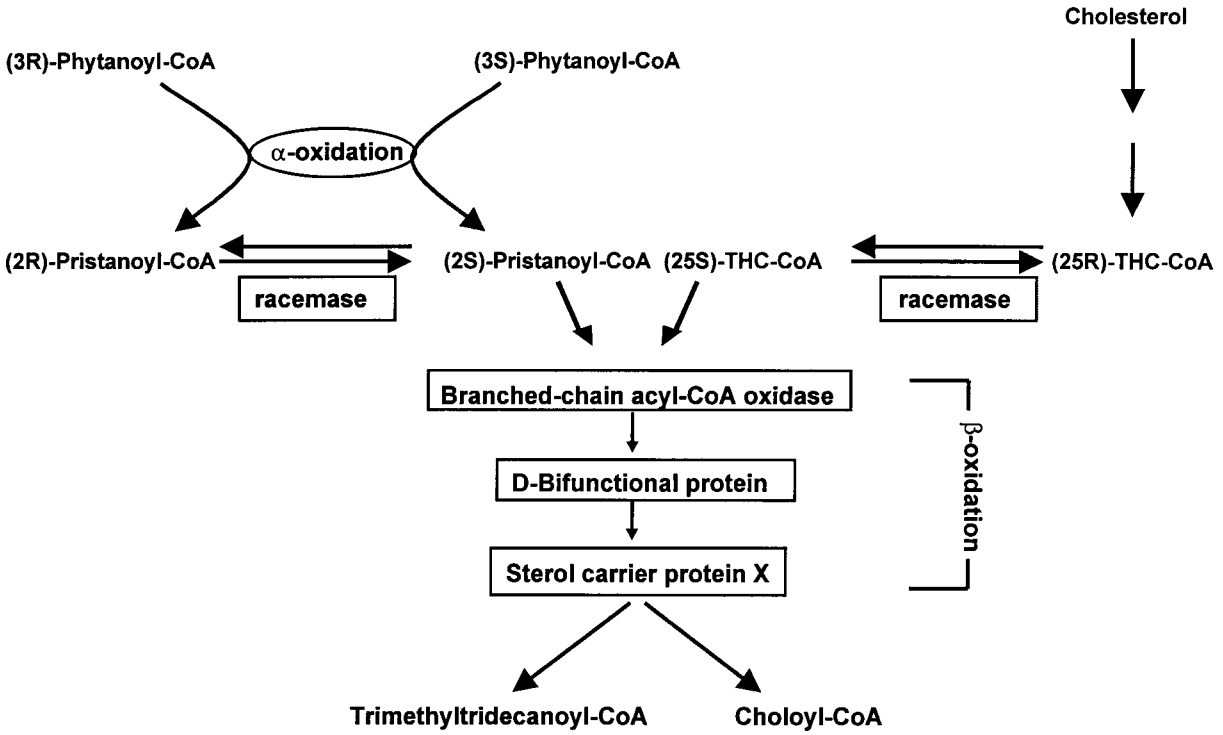
### 2-Methylacyl-CoA racemase deficiency

We recently identified a new peroxisomal defect at the level of 2-methylacyl-CoA racemase in a few patients with a late-onset neuropathy [14]. In these patients there was an accumulation of pristanic acid as well as of dihydroxycholestanoic acid and trihydroxycholestanoic acid with normal $C_{26:0}$ levels, suggesting a selective defect in 2-methyl branched-chain fatty acid oxidation (Figure 10).

**Figure 10**

Schematic representation of the steps involved in the oxidation of (3*R*)- and (3*S*)-phytanic acid as derived from dietary sources and of (25*R*)-THCA produced from cholesterol in the liver

After the activation of (3*R*)- and (3*S*)-phytanic acid to their corresponding CoA esters, they both become substrates for the peroxisomal α-oxidation system, which produces (2*R*)- and (2*S*)-pristanoyl-CoA. Because branched-chain acyl-CoA oxidase, the first enzyme of the *β*-oxidation system, can handle only the (*S*) stereoisomer, (2*R*)-pristanoyl-CoA needs to be converted by α-methylacyl-CoA racemase into its (2*S*) stereoisomer. The bile acid intermediates dihydroxycholestanoic acid and trihydroxycholestanoic acid are produced exclusively as the (25*R*) stereoisomers. To be *β*-oxidized, the CoA esters of the (25*R*) stereoisomers also need to be converted by the racemase into their (25*S*) stereoisomers.




### Disorders of peroxisomal fatty acid α-oxidation

Heredopathia atactica polyneuritiformis, generally known as Refsum disease, is the only true disorder of phytanic acid α-oxidation and is due to mutations in the gene coding for phytanoyl-CoA hydroxylase [63]. Phytanoyl-CoA hydroxylase is also deficient in other peroxisomal disorders including the peroxisome biogenesis disorders and RCDP type 1 but in these cases the deficiency is the secondary consequence of a defect in peroxisome biogenesis.

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## Microsomal malonyl-CoA-sensitive carnitine acyltransferase

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## Abstract

Liver microsomes contain two carnitine acyltransferase activities. One of these has properties closely corresponding to those of 88 kDa mitochondrial carnitine palmitoyltransferase-1 (CPT-1). Antisera against CPT-1 cross-react with an 88 kDa microsomal protein, suggesting that CPT-1 may be targeted to both microsomal and mitochondrial membranes. However, no experiments using cDNAs corresponding to CPT-1 involving *in vitro* translation with microsomes or involving *in vivo* COS-1 cell transfection provided any evidence to support this hypothesis.

Key words: microsomes, mitochondria, protein targeting.
Abbreviations used: CAT, carnitine acyltransferase; CPT I and CPT II, the overt and latent forms respectively of carnitine palmitoyltransferase (EC 2.3.1.21); EGFP, enhanced green fluorescent protein; etomoxir, 2-[6-(4-chlorophenoxy)hexyl]oxirane carboxylic acid; $N_2$ph, 2,4-dinitrophenyl.
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## Introduction: the discovery of two microsomal carnitine acyltransferases

Rat liver microsomes were first reported to contain carnitine acyltransferase (CAT) activity in 1976