Absence of functional peroxisomes does not lead to deficiency of enzymes involved in cholesterol biosynthesis

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Abstract **To unravel the conflicting data concerning the dependence of human cholesterol biosynthesis on functional peroxisomes, we determined activities and levels of selected enzymes involved in cholesterol biosynthesis in livers of *PEX5* knockout mice, a well-characterized model for human Zellweger syndrome. We found that all enzymes measured, including putative peroxisomal enzymes, are at least as active in the peroxisome-deficient Zellweger mice as in control mice, indicating that mislocalization of enzymes to the cytosol does not lead to decreased activity or degradation. Prompted by these results, we re-examined this aspect in human subjects by specific enzyme activity measurements and immunoblotting with highly specific antisera. Our results show that the previously reported deficiencies of mevalonate kinase and phosphomevalonate kinase activity in livers from human Zellweger patients reflect the bad condition of the livers, rather than mislocalization to the cytosol. Our data provide an explanation for the conflicting findings in the literature and show that great care should be taken in the interpretation of data obtained in postmortem material.**—Hogenboom, S., G. J. Romeijn, S. M. Houten, M. Baes, R. J. A. Wanders, and H. R. Waterham. **Absence of functional peroxisomes does not lead to deficiency of enzymes involved in cholesterol biosynthesis. *J. Lipid Res.* 2002.** 43: **90–98.**

Supplementary key words 3-hydroxy-3-methylglutaryl coenzyme A reductase • isopentenyl pyrophosphate isomerase • isoprenoids • mevalonate kinase • mevalonate pyrophosphate decarboxylase • *PEX5* mouse • phosphomevalonate kinase • Δ^7-sterol reductase • Zellweger syndrome

Peroxisomes are indispensable subcellular organelles involved in a number of metabolic pathways including fatty acid oxidation, ether phospholipid biosynthesis, and glyoxylate detoxification. The importance of peroxisomes in humans is stressed by the existence of a group of inherited diseases in which there is impairment in one or more peroxisomal functions. Peroxisomal biogenesis disorders [including the cerebro-hepato-renal syndrome of Zellweger (ZS), rhizomelic chondrodysplasia punctata, and infantile Refsum disease] are caused either by genetic defects in genes encoding specific peroxisomal enzymes or in *PEX* genes encoding proteins called peroxins, which are required for the biogenesis and/or functioning of the organelles (1, 2). After synthesis on free polyribosomes, peroxisomal matrix proteins carrying either a carboxy-terminal peroxisomal targeting sequence 1 (PTS1) [the tripepide (S/A/C)(K/H/R)(L/M)] or a cleavable amino-terminal PTS2 signal [a nine-amino acid, bipartite sequence (R/K)(L/V/I)X_5(H/Q)(L/A)] are translocated across the peroxisomal membrane (3, 4). A defect of one of the components (peroxines) of the peroxisomal import machinery leads to failure of protein import via the PTS1- and/or PTS2-dependent import pathway, and consequently to functional peroxisome deficiency. For example, mutations in the *PEX7* gene, which encodes the PTS2 receptor, lead to a deficiency in PTS2 import only (5). Mutations in the *PEX5* gene, encoding the PTS1 receptor, lead to a deficiency in the PTS1 import machinery but, dependent on the mutation, can also lead to a deficiency in both PTS1 and PTS2 import (6). In many cases, mislocalization of peroxisomal enzymes to the cytosol leads to degradation and/or inactivation of these enzymes. Some enzymes, however [e.g., catalase (CAT)], are stable and active in both peroxisomes and cytosol (7).

It has long been assumed that the various enzyme reactions of the isoprenoid/cholesterol biosynthetic pathway

Abbreviations: CAT, catalase; DHAPAT, dihydroxyacetonephosphate acyltransferase; DHCR7, Δ^7-sterol reductase; FPPS, farnesyl pyrophosphate synthase; GDH, glutamate dehydrogenase; GST, glutathione *S*-transferase; IPPI, isopentenyl pyrophosphate isomerase; MBP, maltose-binding protein; MPD, mevalonate pyrophosphate decarboxylase; MVK, mevalonate kinase; PMK, phosphomevalonate kinase; PTS, peroxisomal targeting sequence; RED, 3-hydroxy-3-methylglutaryl coenzyme A reductase; ORF, open reading frame; ZS, cerebro-hepato-renal syndrome of Zellweger.

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take place in the cytosol and the endoplasmic reticulum. More recent studies have indicated that at least some steps of the pathway may occur also in peroxisomes (8). This would provide an explanation for the fact that in some ZS patients hypocholesterolemia is observed (9). ZS is a fatal inherited disease caused by deficient import of peroxisomal matrix proteins. The pathogenic mechanisms underlying the extreme hypotonia, severe mental retardation, and early death associated with ZS are unknown but some of the clinical abnormalities resemble those observed in patients with inborn errors of cholesterol biosynthesis (10). Cholesterol is the major end product derived from the isoprenoid biosynthetic pathway, which produces a variety of molecules functioning in various cellular processes. Besides cholesterol these include heme A, ubiquinone, dolichol, isopentenyladenine, and farnesyl groups used for isoprenylation of proteins that function in intracellular signaling (11). Cholesterol has long been recognized as an essential component of mammalian cell membranes, and is the precursor for steroid hormones and bile acids. More recently, a crucial role for cholesterol in mammalian embryogenesis was discovered (12).

The first indication of peroxisomal involvement in the biosynthesis of isoprenoids came from immunoelectron microscopy studies by Keller and coworkers (13), who localized HMG-CoA reductase (RED) in rat liver peroxisomes. Further studies have indicated that also mevalonate kinase (MVK) (14), phosphomevalonate kinase (PMK) (15), mevalonate pyrophosphate decarboxylase (MPD) (16), isopentenyl pyrophosphate isomerase (IPPI) (17), and farnesyl pyrophosphate synthase (FPPS) (18, 19) are (partly) peroxisomal. Additional indirect support for a peroxisomal localization came from the observation that several enzymes involved in cholesterol biosynthesis contain consensus peroxisomal targeting sequences (8). With respect to cholesterol biosynthesis and ZS, however, conflicting results have been published. Whereas two groups reported decreased cholesterol biosynthesis (2–84% of control values) in fibroblasts of ZS patients (20, 21), two other groups reported cholesterol biosynthesis to be normal or even slightly increased in fibroblasts of patients with three different types of peroxisomal defects (22, 23). At the enzyme level, conflicting results have also been published. Normal activities of MVK, MPD, and IPPI were measured in ZS fibroblasts (24) in one study, but in another study decreased MVK activity was found in ZS fibroblasts (18). Furthermore, enzyme activities of RED, MVK, PMK, MPD, IPPI, and FPPS in liver homogenates of ZS patients were reported to be decreased (18). Finally, our own group previously reported a deficiency of MVK and PMK activity in three of four livers of ZS patients (24, 25), but normal activity in fibroblasts of such patients (24).

To search for additional evidence of peroxisomal involvement in isoprenoid/cholesterol biosynthesis, and to unravel the conflicting results found in different studies of cholesterol biosynthesis and ZS, we used the *PEX5* knockout mouse (26) to study the effect of peroxisome deficiency on enzymes involved in cholesterol biosynthesis. This mouse model for human ZS is a functional knockout of the murine Pxr1 gene (*PEX5*), encoding the PTS1 receptor. As a consequence, these mice are deficient in both PTS1 and PTS2 protein import, lack morphologically identifiable peroxisomes, and exhibit the typical biochemical abnormalities of ZS patients (26). In our study we examined protein levels and activities of key enzymes in isoprenoid/cholesterol biosynthesis in liver homogenates of this *PEX5* Zellweger mouse model and found normal or even slightly increased enzyme activities. Prompted by these results, we re-evaluated the previously reported deficiencies of these enzymes in livers of human ZS patients and found that these most probably reflect the condition of the liver, rather than degradation as the result of mislocalization.

MATERIALS AND METHODS

Materials

Escherichia coli Invα cells were obtained from Invitrogen (Carlsbad, CA). The pGEM-T vector was from Promega (Madison, WI). The pGEX-4T-1 vector and glutathione (GST)-agarose were from Amersham Pharmacia Biotech (Uppsala, Sweden), the pQE30 vector was from Qiagen (Hilden, Germany), and the pMAL-c2x vector and amylose-resin columns were from New England BioLabs (Beverly, MA). C_{18} Sep Pac columns were obtained from J. T. Baker (Phillipsburg, NJ). Radiolabeled [^{14}C]HMG-CoA and [^{14}C]isopentenyl pyrophosphate were from Amersham Pharmacia Biotech, [^{14}C]mevalonate was from New England Nuclear (Boston, MA), and [^{3}H]mevalonate pyrophosphate was from American Radiolabeled Chemicals (St. Louis, MO). Goat anti-rabbit antibodies conjugated with alkaline phosphatase were obtained from Bio-Rad Laboratories (Hercules, CA) and CDP-Star, the chemiluminescence substrate, was from Roche Chemicals (Grenzach-Wyhlen, Germany). All other chemicals were of analytical grade.

Liver samples

PEX5 mice and control mice (26) were killed within 5 h of birth and livers were isolated immediately, snap frozen in liquid nitrogen, and stored at −80°C. Small human liver biopsies were performed postmortem on three control subjects, eight patients diagnosed with ZS (including three aborted fetuses), and one patient with infantile Refsum disease. Control 1 (C1) had citrullinemia and died within a few days of birth. Liver was isolated within a few hours of death. Control 2 (C2) and control 3 (C3) were healthy persons who died in traffic accidents and whose livers were removed and stored in Wisconsin solution for transplantation, but were eventually rejected for transplantation. Patient 1 (P1) was diagnosed with ZS and complementation analysis performed with fibroblasts of the patient assigned this patient to complementation group 1 (*PEX1*). The patient died within 1 day of birth. Patient 2 (P2) was diagnosed with ZS, assigned to complementation group 10 (*PEX2*), and died at 4 years of age in a hospital. Patient 3 (P3) was diagnosed with ZS but complementation analysis could not be performed because of the absence of a fibroblast cell line. The patient died at home at 6 years of age. Patient 4 (P4) was diagnosed with ZS, belonged to an unknown complementation group, and died at 4 months of age (27). Patient 5 (P5) was diagnosed with ZS, was assigned to complementation group 4 (*PEX6*), and died at 4 weeks of age (27). Patients 6–8 (P6–P8) were three unrelated aborted fetuses diagnosed with ZS but of unknown complementation groups. Patient 7 (P7) was the unborn sibling of P4 and accordingly belongs to the

same (unknown) complementation group. Patient 9 (P9) was diagnosed with infantile Refsum disease, a mild variant of ZS (complementation group 1, *PEX1*). This patient is still alive and is now 9 years old. A needle biopsy of this patient's liver was taken and snap frozen immediately for various purposes. The diagnosis of ZS in the patients was made both on clinical grounds and by demonstration of elevated levels of very long-chain fatty acids in plasma and CAT immunofluorescence experiments in fibroblasts. Permission for scientific use of material was given. After isolation, all human liver samples were stored at −80°C.

Preparation of antibodies

The open reading frame (ORF) of MVK was amplified by PCR and ligated into the pGEM-T vector as described (28). The MVK ORF was released as a *Bam*HI-*Sal*I fragment and ligated in frame with the ORF of maltose-binding protein (MBP) into the *Bam*HI and *Sal*I sites of pMAL-c2x. The ORF of PMK was amplified by PCR, using primer set PMK_{4-20} (5′-cg ata gga tcc GCC CCG CTG GGA GGC GC-3′) and $PMK_{617-600}$ (5′-c gat agg tAC CTC AGC AGG CCC CAG C-3′). The primers introduce a 5′ *Bam*HI site and a 3′ *Kpn*I site (underlined). After restriction, the PCR product was ligated into the *Bam*HI and *Kpn*I sites of pQE30 and sequenced to exclude PCR-introduced mutations. The ORF was released as a *Bam*HI-*Sal*I fragment and ligated into the *Bam*HI and *Sal*I sites of pGEX-4T-1 and the *Bam*HI and *Sal*I sites of pMAL-c2x. The ORF of MPD was amplified by PCR, using primer set MPD_{4-21} (5′-gc ata gga tcc GCC TCG GAG AAG CCG CTG-3′) and $MPD_{1232-1215}$ (5′-gct atg aat tCA AGC GGC ATG CGG TCC C-3′). The primers introduce a 5′ *Bam*HI site and a 3′ *Eco*RI site (underlined). After restriction, the *Bam*HI-*Eco*RI fragment was ligated into the *Bam*HI and *Eco*RI sites of pGEX-4T-1. Subsequently, the ORF was released as a *Bam*HI-*Sal*I fragment and ligated into the *Bam*HI and *Sal*I sites of pMAL-c2x.

Plasmids containing the various ORFs were transformed into *E. coli* Invα cells. Cells were grown from a 100-fold diluted fresh overnight culture for 4 h in Luria-Bertani medium, induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and subsequently grown for an additional 2 h. Cells were lysed by sonication (twice for 15 s at an output of 8 W, with 1 min of cooling between the pulse periods).

MBP fusion proteins were purified on amylose-resin columns according to the protocol of the supplier (New England BioLabs). GST fusion proteins were purified on GST-agarose as described by Frangioni and Neel (29). The bound protein was eluted from the resin in 50 mM Tris-HCl (pH 8.0), 1% SDS, and 10 mM 2-mercaptoethanol and subsequently isolated by preparative SDS-PAGE and electroelution according to the protocol of the manufacturer (Bio-Rad).

Purified MBP-MVK, GST-PMK, and MBP-MPD were used to produce antibodies in rabbits. The crude antiserum containing anti-PMK antibodies was affinity purified on a column containing MBP-PMK fusion protein coupled to cyanogen bromide-Sepharose. The crude antiserum containing anti-MVK antibodies was affinity purified on a column containing GST-MVK coupled to cyanogen bromide-Sepharose. The crude antiserum containing anti-MPD antibodies was affinity purified in two steps. First, the antiserum was depleted of anti-MBP antibodies on a column containing MBP coupled to cyanogen bromide-Sepharose. In the next step the flowthrough of the first column was affinity purified on a column containing MBP-MPD coupled to cyanogen bromide-Sepharose.

Enzyme assays

Samples of mouse livers were homogenized in 1 ml of cold 0.9% NaCl, aliquoted, and snap frozen in liquid nitrogen. The same procedure was used for human liver samples but a second set of samples from P1, P2, P3, C1, C2, and C3 were homogenized in the presence of 10 mM DTT and incubated for 60 min at 38°C, before experiments. In addition, liver samples from *PEX5* mice, control mice, and C3 were incubated for various periods of time (0, 2, 6, and 24 h) at different temperatures (4°C, 20°C, and 37°C). After incubation, the samples were homogenized and snap frozen in liquid nitrogen. Before enzyme measurements, samples were thawed on ice and sonicated and protein concentrations were determined according to Bradford (30). Activities for the various enzymes (see below) were determined twice.

TABLE 1. Enzyme activities in liver tissue from *PEX5* knockout and control mice

	PEX5 Mice (n = 6)	Control Mice (n = 6)	*PEX5* Mice/ Control Mice	*P*
RED[a]	36.7 ± 6	18.8 ± 7	1.95	<0.001
MVK[a]	1,738.8 ± 570	1,556.8 ± 290	1.12	NS
PMK[a]	1,914.8 ± 520	1,742.1 ± 500	1.10	NS
MPD[a]	4.1 ± 1	3.4 ± 0.9	1.18	NS
IPPI[a]	415.3 ± 70	246.0 ± 45	1.69	<0.005
DHCR7[a]	25.0 ± 5	26.6 ± 10	0.94	NS
DHAPAT (n = 3)[b]	ND	5.4 ± 0.4	—	<0.005
CAT[b]	247.9 ± 35	146.2 ± 27	1.69	<0.001
GDH[b]	879.6 ± 140	1,102.3 ± 95	0.80	NS

[a] Activities in pmol/min/mg.
[b] Activities in nmol/min/mg.
ND, not detectable; NS, not significant, using Student's *t*-test ($P > 0.05$).

RED. RED was measured essentially as described by Brown, Dana, and Goldstein (31) with some modifications. Fifty microliters of liver homogenate was diluted 1:1 in phosphate buffer containing 0.1 M KP_i, 0.2 M KCl, 5 mM EGTA, 5 mM EDTA, 10 mM DTT (pH 7.1), and leupeptin (10 μg/ml). After preincubation for 10 min at 37°C with 60 μl of cofactor mix containing 0.18 M glucose 6-phosphate, 17.5 mM NADPH, 16.7 mM EDTA, and glucose-6-phosphate dehydrogenase (25 U/ml), reactions were started with 1.7 nmol of [^{14}C]HMG-CoA and 5.6 nmol of HMG-CoA in 40 μl of H_2O. After a 30-min incubation period at 37°C, reactions were terminated by adding 50 μl of 1.2 N HCl. After 30 min, the product was extracted three times with 2 ml of ethylacetate. The extracts were evaporated to dryness and loaded on a silica TLC plate. The TLC plate was developed in toluene-acetone 1:1 (v/v) and analyzed by PhosphorImaging (Molecular Dynamics, Sunnyvale, CA).

MVK. MVK was measured as described (25, 32), using [^{14}C]-mevalonate as the substrate.

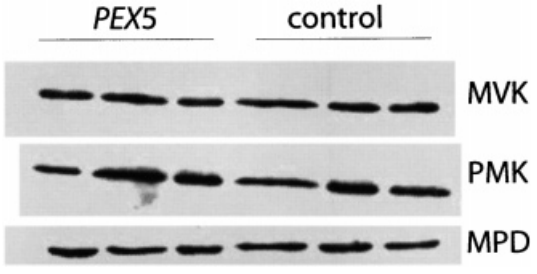

Fig. 1. Protein levels of selected enzymes in liver homogenates of *PEX5* and control mice. Fifteen-microgram samples from three *PEX5* and three control liver homogenates were separated by SDS-PAGE and immunoblotted. Protein levels were detected with highly affinity-purified antibodies (1:250) raised against MVK, PMK, or MPD.

PMK. PMK was measured as described (25), with some minor modifications. Instead of [^{14}C]phosphomevalonate, [^{14}C]mevalonate was used, which was converted to [^{14}C]phosphomevalonate by adding purified MBP-MVK. For conversion to phosphomevalonate, the same conditions were used as for MVK activity measurement.

MPD. Liver homogenate was incubated for 5 min in buffer containing 50 mM morpholinepropanesulfonic acid (MOPS, pH 7.0), 5 mM $MgCl_2$, and 5 mM ATP. Reactions were started by adding 15 pmol of [^{3}H]mevalonate pyrophosphate. After 15 min at 37°C, reactions were terminated by adding 7.5 units of alkaline phosphatase in 1 M Tris, pH 8.0. After 30 min, products and

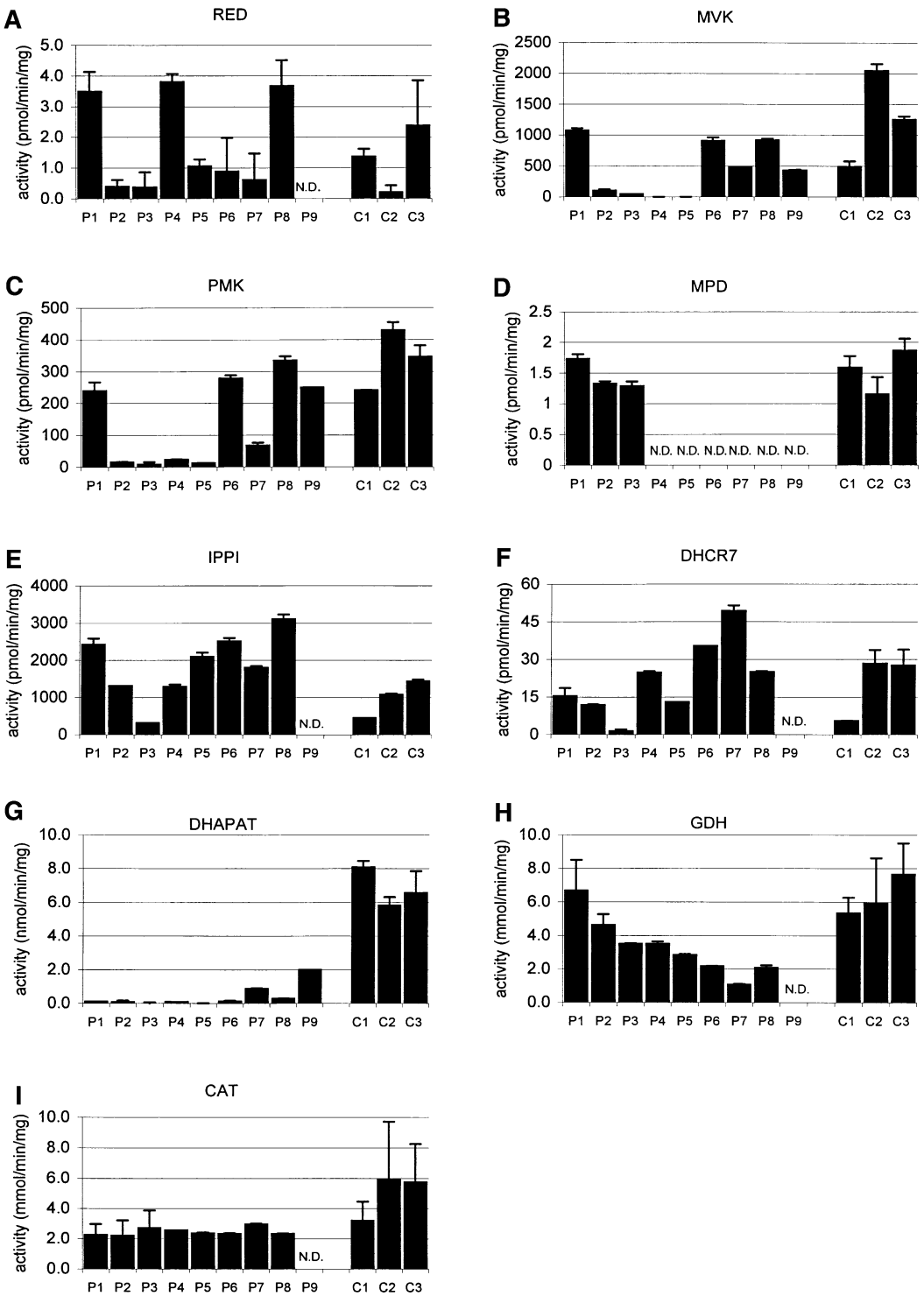

Fig. 2. Activities of selected enzymes in liver homogenates of ZS patients and control subjects. Enzyme activities of RED (A), MVK (B), PMK (C), MPD (D), IPPI (E), DHCR7 (F), DHAPAT (G), GDH (H), and CAT (I) were measured in liver homogenates of nine Zellweger syndrome (ZS) patients (P1–P9, see Materials and Methods for details) and three control subjects (C1–C3). Presented are the means of duplicate measurements. N.D., not determined

substrates were separated on a C_{18} column. Unreacted substrate was eluted first by using H_2O, followed by elution of products with methanol. Fractions were counted in a scintillation counter.

IPPI. IPPI was measured with [^{14}C]isopentenyl pyrophosphate. Liver homogenate was incubated for 20 min at 37°C after addition of a buffer containing 20 mM MOPS (pH 7.4), 2 mM DTT, 20 mM $MgCl_2$, and 2 nmol of [^{14}C]isopentenyl pyrophosphate. Reactions were terminated by adding 400 μl of hydrolyzing reagent (37% HCl-methanol, 1:3) and 1 ml of H_2O. After 30 min, NaCl was added and the product was extracted twice with 2 ml of toluene, and the extract was dehydrated with $MgSO_4$. The product was counted in a scintillation counter.

Δ^7-Sterol reductase (DHCR7). DHCR7 was measured essentially according to Honda et al (33), with some minor modifications. The composition of the standard reaction mixture was 100 mM Tris, pH 7.2, containing 0.1 mM EDTA, 1 mM DTT, 30 mM nicotinamine, 3.5 mM $NADP^+$, 30 mM glucose 6-phosphate, glucose-6-phosphate dehydrogenase (2 U/ml), BSA (0.5 mg/ml), and 25 nmol of ergosterol. Ergosterol was solubilized in a 25% solution of methyl-cyclodextrine. After 30 min at 37°C, the reaction was started by the addition of liver homogenate. The reaction was continued for 4 h at 37°C. The reaction was terminated by adding KOH (20 mg/l) in 95% ethanol containing 10 ng of epicoprostanol, which was used as an internal standard. After addition of 0.5 ml of H_2O, sterols were extracted with hexane, and converted into trimethylsilyl ether derivatives. Products were analyzed by gas chromatography-mass spectrometry, using selected ion monitoring.

Marker enzymes. Dihydroxyacetonephosphate acyltransferase (DHAPAT) was measured radiochemically as described previously (34). Glutamate dehydrogenase (GDH) (35) and CAT (18) were measured spectrophotometrically as described.

Immunoblot analysis. Equal amounts of total protein (see figure legends) were separated by SDS-PAGE and transferred onto nitrocellulose by semidry immunoblotting (36). The affinity-purified antibodies were used at a 1:250 dilution. Antigen-antibody complexes were visualized with goat anti-rabbit IgG-alkaline phosphatase conjugate and CDP-Star according to the protocol of the supplier. As a control for equal transfer of protein, each blot was reversibly stained with Ponceau S. Each immunoblotting experiment was performed at least twice.

RESULTS

Activities and levels of selected enzymes in livers of *PEX5*$^{-/-}$ Zellweger mice

To examine the effect of peroxisome deficiency on the activities of enzymes involved in cholesterol biosynthesis, we measured the activities of several enzymes involved in the presqualene segment (RED, MVK, PMK, MPD, and IPPI) and one enzyme (DHCR7) in the postsqualene segment of the isoprenoid/cholesterol biosynthesis pathway in liver homogenates of *PEX5* knockout and control mice. Similar or slightly increased activities were found for MVK, PMK, MPD, and DHCR7 in ZS mouse liver samples when compared with control mouse livers (**Table 1**). The activities for RED and IPPI were moderately but significantly increased in the ZS mouse livers. As a control for the absence of peroxisomes, DHAPAT activity was measured, and was found to be completely deficient in the livers of ZS mice. GDH activities were similar in all samples measured. CAT, the marker enzyme of peroxisomes, was increased significantly in the ZS mice. To determine whether the measured activities are a reflection of the protein levels, we performed immunoblot analysis of MVK, PMK, and MPD in the liver homogenates. Similar levels of protein were detected in both ZS and control mouse livers (**Fig. 1**).

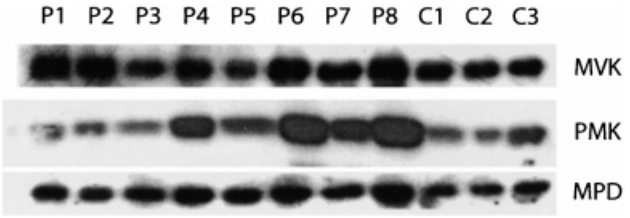

Fig. 3. Levels of selected enzymes in liver homogenates of ZS patients and control subjects. Ten-microgram samples of liver homogenates from eight ZS patients (P1–P8, see Materials and Methods for details) and three control subjects (C1–C3) were separated by SDS-PAGE and immunoblotted. Protein levels were detected with highly affinity-purified antibodies (1:250) raised against MVK, PMK, or MPD.

Activities and levels of selected enzymes in livers of human Zellweger patients

Our results with ZS mice were in contrast with a previous report on the activities of selected enzymes in human ZS liver samples, in which decreased RED, MVK, MPD, IPPI, and FPPS activities had been measured (18), and with our own report, in which decreased MVK and PMK activities had been measured (24). Prompted by our data concerning ZS mouse liver, we reinvestigated the enzyme activities in human ZS and control liver. As described previously, we measured markedly decreased activities for MVK and PMK in the two previously studied liver samples (P2 and P3) and in two additional ZS livers (P4 and P5) (**Fig. 2B** and **C**). In another postmortem ZS liver sample (P1), however, we measured similar activities as in control livers. Although no control values of MVK and PMK activities in fetuses are available, activities comparable to those of control livers were measured in the three liver samples of aborted ZS fetuses (P6–P8). Also, in the liver biopsy of the mild ZS patient (P9), normal activities could be measured. Importantly, one of the fetuses (P7) with normal activities is a sibling of P4, for whom we could measure almost no MVK and PMK activities. IPPI and MPD activities appeared similar in ZS and control liver (Fig. 2D and E), as were GDH and CAT activities (Fig. 2H and I). In addition, we measured decreased activities of RED and DHCR7 in some ZS livers (Fig. 2A and F) whereas RED, MVK, and DHCR7 were also slightly decreased in C1 (Fig. 2A, B, and F) as was RED in C2 (Fig. 2A). CAT and GDH activities were normal in all livers (Fig. 2H and I). As expected, DHAPAT was found to be deficient in Zellweger livers and normal in controls (Fig. 2G).

When we subsequently examined the protein levels of the various enzymes in the various liver samples by immunoblot analysis, we noticed no significant differences for MVK, PMK, and MPD in all samples (**Fig. 3**). These results

TABLE 2. Reactivation of MVK and PMK activity in liver homogenates by incubation with 10 mM DTT

Subject	MVK[a] −DTT	MVK[a] +DTT	MVK[a] +DTT/−DTT	PMK[a] −DTT	PMK[a] +DTT	PMK[a] +DTT/−DTT
P1	1,071.3	1,534.9	1.4	239.6	301.1	1.3
P2	98.8	394.2	4.0	15.9	43.8	2.8
P3	45.4	61.8	1.4	8.6	47.8	5.6
C1	490.0	1,674.1	3.4	240.2	209.3	0.9
C2	2,047.2	1,275.6	0.6	429.3	610.0	1.4
C3	1,250.4	1,318.5	1.1	346.5	557.8	1.6

[a] Activities in pmol/min/mg.

indicated that the deficient activities of MVK and PMK in the four ZS livers are not caused by degradation of protein but must result from inactivation of the enzymes.

Previously, Beytia and coworkers (37) reported that MVK loses its activity when stored at 4°C in the absence of β-mercaptoethanol or DTT, most probably because of oxidation of an active site cysteine. Furthermore, they showed that partial restoration of enzyme activity may occur on incubation with 10 mM DTT at 38°C for 1 h. To study whether the deficient activity in the ZS liver samples is at least partly due to oxidation of MVK and PMK, we tried to reactivate the enzyme activities by incubation at 38°C in the presence of DTT. This resulted in a partial recovery of MVK and PMK activity (**Table 2**). Partial recovery of MVK activity was also observed for C1, in which slightly decreased MVK activity was found without preincubation with DTT.

Stability of MVK, PMK, and MPD activities

Our results indicated that the deficient activity of MVK and PMK in some of the human livers is due to inactivation of the enzymes. To address this issue further, we investigated the stability of MVK, PMK, and, as a control, MPD activities, by incubation of control human liver tissue for various periods of time at different temperatures (**Fig. 4**). Normal GDH (Fig. 4A) and CAT (Fig. 4B) activity was measured in all samples although CAT activity decreased slightly after incubation for 24 h at all temperatures. In contrast, MVK activity was markedly decreased after incubation for 2 h even at a temperature of 4°C. Incubation at 20 or 37°C resulted in almost no activity of MVK even after 2 h (Fig. 4C). PMK activity slowly decreased on incubation at 4°C. After incubation at 20°C, a stronger decrease in activity was found. On incubation at 37°C almost no activity could be measured even after 2 h (Fig. 4D). In contrast, MPD activity did not decrease even after 24 h of incubation at 37°C (Fig. 4E).

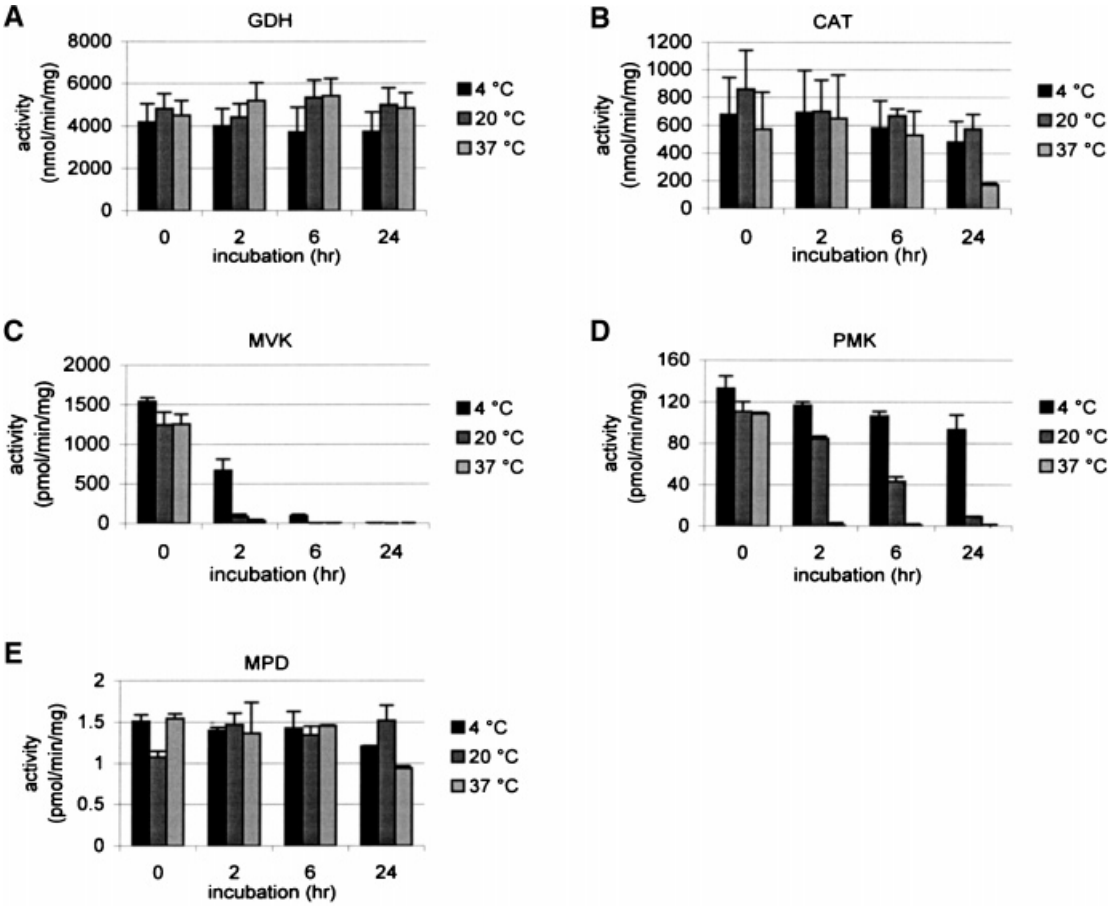

Fig. 4. Stability of enzyme activity at various temperatures. Enzyme activities of GDH (A), CAT (B), MVK (C), PMK (D), and MPD (E) measured in liver homogenates of control subject 3. Before homogenization, liver tissues were incubated for the indicated periods of time at the indicated temperatures. Presented are the means of duplicate measurements.

To determine whether there is any difference in inactivation between controls and ZS, we repeated this experiment with mouse liver samples. As in the human samples normal GDH (**Fig. 5A** and **B**) and CAT (Fig. 5C and D) activities were measured at all time/temperature points. In both the *PEX5* and the control mouse liver samples, MVK activity slowly decreased on incubation at 4°C. After incubation at 20°C and 37°C, a stronger decrease in activity was found, which resulted in almost no activity of MVK after 24 h (Fig. 5E and F). PMK activity was markedly decreased after incubation for even 2 h at temperatures of 20°C or 37°C. Incubation at 4°C resulted in almost no activity of PMK after 6 h (Fig. 5G and H). These results indicate that, as expected, there is no difference in inactivation of MVK and PMK between control and ZS liver tissue. Although we found some degradation of proteins on incubation (**Fig. 6**), this degradation does not reflect the decrease in activities.

DISCUSSION

Using liver samples of a well-defined ZS mouse model, we have demonstrated that enzymes involved in the presqualene segment of the cholesterol biosynthetic pathway are normally active or even significantly increased. Our results show that if cholesterol biosynthesis indeed partly takes place in peroxisomes, deficient peroxisomal import and the resulting mislocalization of the enzymes to the cytosol do not lead to decreased activities. This suggests that

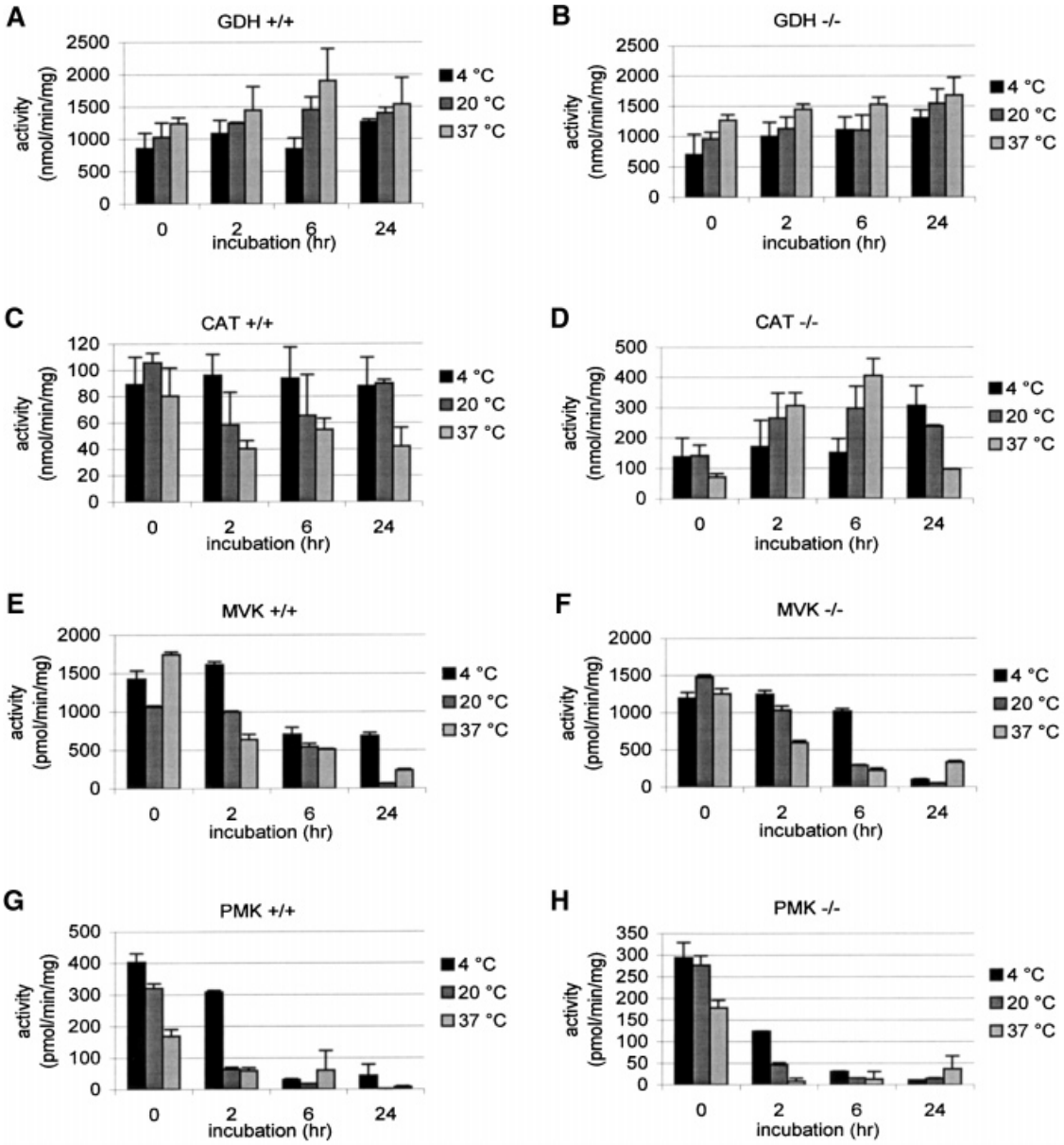

Fig. 5. Stability of enzyme activity at various temperatures in *PEX5* (−/−) and control (+/+) mice. Enzyme activity of GDH (A and B), CAT (C and D), MVK (E and F), and PMK (G and H) was measured in liver homogenates of *PEX5* and control mice. Before homogenization, liver tissues were incubated for the indicated periods of time at the indicated temperatures. Presented are the means of duplicate measurements.

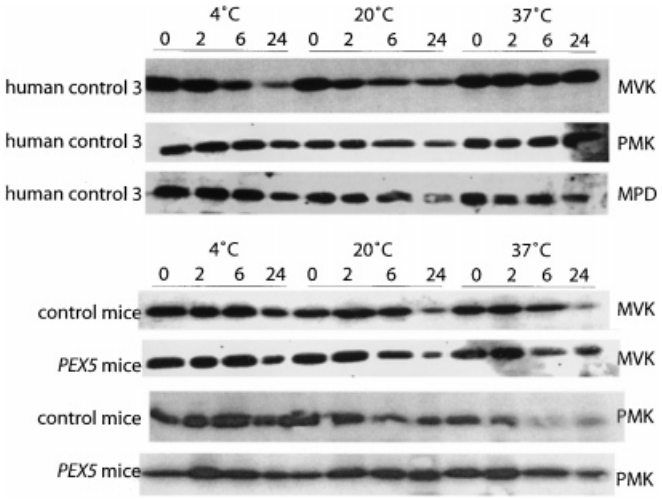

Fig. 6. Stability of protein in liver homogenates at various temperatures. Immunoblot analysis of liver homogenates from control subject 3 and of *PEX5* and control mice. Before homogenization, liver tissues were incubated for the indicated periods of time at the indicated temperatures. Ten micrograms of each liver homogenate was separated by SDS-PAGE and immunoblotted. Protein levels were detected with highly affinity-purified antibodies (1:250) raised against MVK, PMK, or MPD.

functional peroxisomes may not be required for cholesterol biosynthesis, at least in mice. Indeed, after submission of this work, an article appeared by Vanhorebeek, Baes, and Declercq (38), who published that there is no significant difference in cholesterol biosynthesis between fibroblasts of day 18.5 embryos of *PEX5* knockout mice and control mice. Also, normal levels of cholesterol and ubiquinone were found in liver homogenates of newborn *PEX5* knockout mice.

Initially, our results in mouse livers seemed to contradict the results from studies performed in human ZS livers, in which markedly decreased activities of MVK and PMK had been measured (18, 24). This deficiency was explained by protein degradation due to the mislocalization of these enzymes to the cytosol and regarded as a confirmation of their assumed peroxisomal localization. The results from our immunoblot analysis experiment with human ZS and control livers, using specific antibodies, however, showed normal levels of the enzymes. This indicates that the decreased activities are not due to mislocalization followed by degradation, but are a consequence of the lability of the enzymes. Not only did we detect no significant differences in protein levels of MVK and PMK between the human ZS and control livers, we also found enzyme activities comparable to controls in one of the five postmortem ZS livers, in the three prenatal livers, and in the needle biopsy of the liver of the patient with mild ZS. Moreover, we even found marked differences between the activities measured in liver samples of two affected siblings. Because both must belong to the same complementation group, this indicates that deficiencies do not depend on a certain complementation group.

In our article, we have provided evidence that the decrease in MVK and PMK activity observed in four livers is most probably the result of the bad condition of the livers. Using *PEX5* and control mouse liver tissue and human control liver tissue, we have shown that both enzymes are rapidly inactivated in a time- and temperature-dependent manner. This inactivation was similar in *PEX5* and control mouse liver tissue. In contrast, MPD activity is far more stable and normal activities can be measured even after an incubation for 24 h at 37°C in the human control liver. The inactivation of MVK and PMK is at least partly due to oxidation and not degradation, because normal levels of protein can be detected and partial restoration of MVK and PMK activity occurs on incubation of homogenates with DTT. The inactivation of enzymes in the liver specimens could have occurred in the time between death and autopsy. For the mice, livers were isolated and snap frozen immediately after death. In contrast, in human patients the time between death and autopsy may vary between a few hours and 1 day. The inactivation of MVK and PMK may also be due to the hepatic abnormalities commonly observed in ZS patients (27). Such abnormalities may have been more severe in P2–P5, samples that displayed decreased MVK and PMK activities. Of course, the combination of both factors may have an even stronger effect on MVK and PMK activity. These possibilities also would explain the fact that normal MVK [(24); and S. Hogenboom, data not shown] and PMK (S. Hogenboom, data not shown) activities are measured in cultured fibroblasts of ZS patients belonging to different complementation groups.

In conclusion, we have shown that previous conclusions concerning the peroxisomal localization of certain enzymes involved in cholesterol biosynthesis, made on the basis of their deficient activity in ZS, is unjustified and incorrect. It should be stated, however, that our findings do not exclude a peroxisomal localization. In fact, there is experimental evidence that certain enzymes indeed are localized in peroxisomes. Our data clearly show that great care should be taken in interpreting data of experiments based on postmortem material because it could be difficult to discriminate between primary and secondary effects, which can result in a misinterpretation of data.

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REFERENCES

1. Wanders, R. J., R. B. Schutgens, and P. G. Barth. 1995. Peroxisomal disorders: a review. *J. Neuropathol. Exp. Neurol.* **54:** 726–739.
2. Gould, S. J., and D. Valle. 2000. Peroxisome biogenesis disorders: genetics and cell biology. *Trends Genet.* **16:** 340–345.
3. Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell Biol.* **9:** 445–478.
4. Subramani, S. 1996. Protein translocation into peroxisomes. *J. Biol. Chem.* **271:** 32483–32486.
5. Motley, A. M., E. H. Hettema, E. M. Hogenhout, P. Brites, A. L. ten

Asbroek, F. A. Wijburg, F. Baas, H. S. Heijmans, H. F. Tabak, R. J. Wanders, and B. Distel. 1997. Rhizomelic chondrodysplasia punctata is a peroxisomal protein targeting disease caused by a non-functional PTS2 receptor. *Nat. Genet.* **15:** 377–380.
6. Otera, H., K. Okumoto, K. Tateishi, Y. Ikoma, E. Matsuda, M. Nishimura, T. Tsukamoto, T. Osumi, K. Ohashi, O. Higuchi, and Y. Fujiki. 1998. Peroxisome targeting signal type 1 (PTS1) receptor is involved in import of both PTS1 and PTS2: studies with *PEX5*-defective CHO cell mutants. *Mol. Cell Biol.* **18:** 388–399.
7. Singh, I., K. Kremser, B. Ghosh, A. K. Singh, and S. Pai. 1996. Abnormality in translational regulation of catalase expression in disorders of peroxisomal biogenesis. *J. Neurochem.* **67:** 2373–2378.
8. Olivier, L. M., and S. K. Krisans. 2000. Peroxisomal protein targeting and identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes. *Biochim. Biophys. Acta.* **1529:** 89–102.
9. Poll-The, B. T., J. M. Saudubray, H. A. Ogier, M. Odievre, J. M. Scotto, L. Monnens, L. C. Govaerts, F. Roels, A. Cornelis, R. B. Schutgens, R. J. A. Wanders, A. W. Schram, and J. M. Tager. 1987. Infantile Refsum disease: an inherited peroxisomal disorder. Comparison with Zellweger syndrome and neonatal adrenoleukodystrophy. *Eur. J. Pediatr.* **146:** 477–483.
10. Kelley, R. I., and R. C. Hennekam. 2000. The Smith-Lemli-Opitz syndrome. *J. Med. Genet.* **37:** 321–335.
11. Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. *Nature.* **343:** 425–430.
12. Farese, R. V., and J. Herz. 1998. Cholesterol metabolism and embryogenesis. *Trends Genet.* **14:** 115–120.
13. Keller, G. A., M. C. Barton, D. J. Shapiro, and S. J. Singer. 1985. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase is present in peroxisomes in normal rat liver cells. *Proc. Natl. Acad. Sci. USA.* **82:** 770–774.
14. Biardi, L., A. Sreedhar, A. Zokaei, N. B. Vartak, R. L. Bozeat, J. E. Shackelford, G. A. Keller, and S. K. Krisans. 1994. Mevalonate kinase is predominantly localized in peroxisomes and is defective in patients with peroxisome deficiency disorders. *J. Biol. Chem.* **269:** 1197–1205.
15. Olivier, L. M., K. L. Chambliss, K. M. Gibson, and S. K. Krisans. 1999. Characterization of phosphomevalonate kinase: chromosomal localization, regulation, and subcellular targeting. *J. Lipid Res.* **40:** 672–679.
16. Biardi, L., and S. K. Krisans. 1996. Compartmentalization of cholesterol biosynthesis. Conversion of mevalonate to farnesyl diphosphate occurs in the peroxisomes. *J. Biol. Chem.* **271:** 1784–1788.
17. Paton, V. G., J. E. Shackelford, and S. K. Krisans. 1997. Cloning and subcellular localization of hamster and rat isopentenyl diphosphate dimethylallyl diphosphate isomerase. A PTS1 motif targets the enzyme to peroxisomes. *J. Biol. Chem.* **272:** 18945–18950.
18. Krisans, S. K., J. Ericsson, P. A. Edwards, and G. A. Keller. 1994. Farnesyl-diphosphate synthase is localized in peroxisomes. *J. Biol. Chem.* **269:** 14165–14169.
19. Gupta, S. D., R. S. Mehan, T. R. Tansey, H. T. Chen, G. Goping, I. Goldberg, and I. Shechter. 1999. Differential binding of proteins to peroxisomes in rat hepatoma cells: unique association of enzymes involved in isoprenoid metabolism. *J. Lipid Res.* **40:** 1572–1584.
20. Hodge, V. J., S. J. Gould, S. Subramani, H. W. Moser, and S. K. Krisans. 1991. Normal cholesterol synthesis in human cells requires functional peroxisomes. *Biochem. Biophys. Res. Commun.* **181:** 537–541.
21. Mandel, H., M. Getsis, M. Rosenblat, M. Berant, and M. Aviram. 1995. Reduced cellular cholesterol content in peroxisome-deficient fibroblasts is associated with impaired uptake of the patient's low density lipoprotein and with reduced cholesterol synthesis. *J. Lipid Res.* **36:** 1385–1391.
22. Malle, E., K. Oettl, W. Sattler, G. Hoefler, and G. M. Kostner. 1995. Cholesterol biosynthesis in dermal fibroblasts from patients with metabolic disorders of peroxisomal origin. *Eur. J. Clin. Invest.* **25:** 59–67.
23. Appelkvist, E. L., N. Venizelos, Y. Zhang, I. Parmryd, L. Hagenfeldt, and G. Dallner. 1999. Synthesis of mevalonate pathway lipids in fibroblasts from Zellweger and X-linked ALD patients. *Pediatr. Res.* **46:** 345–350.
24. Wanders, R. J., and G. J. Romeijn. 1996. Cholesterol biosynthesis in Zellweger syndrome: normal activity of mevalonate kinase, mevalonate-5′-pyrophosphate decarboxylase and IPP-isomerase in patients' fibroblasts but deficient mevalonate kinase activity in liver. *J. Inherit. Metab. Dis.* **19:** 193–196.
25. Wanders, R. J., and G. J. Romeijn. 1998. Differential deficiency of mevalonate kinase and phosphomevalonate kinase in patients with distinct defects in peroxisome biogenesis: evidence for a major role of peroxisomes in cholesterol biosynthesis. *Biochem. Biophys. Res. Commun.* **247:** 663–667.
26. Baes, M., P. Gressens, E. Baumgart, P. Carmeliet, M. Casteels, M. Fransen, P. Evrard, D. Fahimi, P. E. Declercq, D. Collen, P. P. Van Veldhoven, and G. P. Mannaerts. 1997. A mouse model for Zellweger syndrome. *Nat. Genet.* **17:** 49–57.
27. Wanders, R. J., W. Smit, H. S. Heymans, R. B. Schutgens, P. G. Barth, H. Schierbeek, G. P. Smit, R. Berger, H. Przyrembel, T. A. Eggelte, J. M. Tager, P. D. Maaswinkel-Mooy, A. C. B. Peters, L. A. H. Monnens, J. A. J. M. Bakkeren, J. M. F. Trijbels, E. J. P. Lommen, and N. Beganovic. 1987. Age-related accumulation of phytanic acid in plasma from patients with the cerebro-hepato-renal (Zellweger) syndrome. *Clin. Chim. Acta.* **166:** 45–56.
28. Houten, S. M., G. J. Romeijn, J. Koster, R. G. Gray, P. Darbyshire, G. P. Smit, J. B. de Klerk, M. Duran, K. M. Gibson, R. J. Wanders, and H. R. Waterham. 1999. Identification and characterization of three novel missense mutations in mevalonate kinase cDNA causing mevalonic aciduria, a disorder of isoprene biosynthesis. *Hum. Mol. Genet.* **8:** 1523–1528.
29. Frangioni, J. V., and B. G. Neel. 1993. Solubilization and purification of enzymatically active glutathione *S*-transferase (pGEX) fusion proteins. *Anal. Biochem.* **210:** 179–187.
30. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
31. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1974. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 789–796.
32. Hoffmann, G. F., S. U. Brendel, S. R. Scharfschwerdt, Y. S. Shin, I. M. Speidel, and K. M. Gibson. 1992. Mevalonate kinase assay using DEAE-cellulose column chromatography for first-trimester prenatal diagnosis and complementation analysis in mevalonic aciduria. *J. Inherit. Metab. Dis.* **15:** 738–746.
33. Honda, M., G. S. Tint, S. Shefer, A. Honda, A. K. Batta, G. Xu, T. S. Chen, and G. Salen. 1998. Accurate detection of Smith-Lemli-Opitz syndrome carriers by measurement of the rate of reduction of the ergosterol C-7 double bond in cultured skin fibroblasts. *J. Inherit. Metab. Dis.* **21:** 761–768.
34. Ofman, R., and R. J. Wanders. 1994. Purification of peroxisomal acyl-CoA:dihydroxyacetonephosphate acyltransferase from human placenta. *Biochim. Biophys. Acta.* **1206:** 27–34.
35. Wanders, R. J., C. W. van Roermund, C. T. de Vries, H. Van den Bosch, G. Schrakamp, J. M. Tager, A. W. Schram, and R. B. Schutgens. 1986. Peroxisomal beta-oxidation of palmitoyl-CoA in human liver homogenates and its deficiency in the cerebro-hepato-renal (Zellweger) syndrome. *Clin. Chim. Acta.* **159:** 1–10.
36. Kyhse-Andersen, J. 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods.* **10:** 203–209.
37. Beytia, E., J. K. Dorsey, J. Marr, W. W. Cleland, and J. W. Porter. 1970. Purification and mechanism of action of hog liver mevalonic kinase. *J. Biol. Chem.* **245:** 5450–5458.
38. Vanhorebeek, I., M. Baes, and P. E. Declercq. 2001. Isoprenoid biosynthesis is not compromised in a Zellweger syndrome mouse model. *Biochim. Biophys. Acta.* **1532:** 28–36.