Plasmalogen biosynthesis in peroxisomal disorders: fatty alcohol versus alkylglycerol precursors

G. Schrakamp,* C. G. Schalkwijk,* R. B. H. Schutgens,† R. J. A. Wanders,† J. M. Tager, and H. van den Bosch***

Laboratory of Biochemistry,* University of Utrecht, Padualaan 8, 3584 CH Utrecht; Department of Pediatrics,† University Hospital Amsterdam, AMC, Meibergdreef 9, 1105 AZ Amsterdam; and Laboratory of Biochemistry,** University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Abstract In recent years a growing number of inherited diseases have been recognized to originate from an impairment in one or more peroxisomal functions. Since it is well established that the first two steps in the biosynthesis of plasmalogens proceed in peroxisomes, we studied the biosynthesis of plasmalogens in cultured skin fibroblasts from patients with different peroxisomal and related disorders. When de novo plasmalogen biosynthesis was studied by growing the cells in the presence of [^{14}C]hexadecanol, impaired plasmalogen biosynthesis was found in rhizomelic chondrodysplasia punctata, cerebrohepatorenal (Zellweger) syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. In all these cases, alkyl-acyl phospholipids, the precursors of plasmalogens, did not accumulate and 1-O-[9,10-$^{3}H_2$]octadecylglycerol was converted into plasmalogens with equal efficiency as in controls. This indicated that impaired de novo plasmalogen biosynthesis as measured by [^{14}C]hexadecanol incorporation was due to a deficient formation of the glycero-ether bond. Using this procedure, normal de novo plasmalogen biosynthesis was found in X-linked adrenoleukodystrophy, adrenomyeloneuropathy, X-linked chondrodysplasia punctata, adult Refsum disease, as well as in heterozygotes for Zellweger syndrome and infantile Refsum disease. The data have indicated that the average extent of the deficiency in glycero-ether bond formation is different in Zellweger syndrome, chondrodysplasia punctata, neonatal adrenoleukodystrophy, and infantile Refsum disease. — **Schrakamp, G., C. G. Schalkwijk, R. B. H. Schutgens, R. J. A. Wanders, J. M. Tager, and H. van den Bosch.** Plasmalogen biosynthesis in peroxisomal disorders: fatty alcohol versus alkylglycerol precursors. *J. Lipid Res.* 1988. **29:** 325–334.

Supplementary key words peroxisomes • human genetic disorders • ether phospholipids • hexadecanol • cultured skin fibroblasts

In recent years a new group of genetic diseases in man has been recognized, in which peroxisomal functions appear to be impaired (1–4). Peroxisomes are small subcellular organelles that are ubiquitous in mammalian cells (5). These organelles contain catalase, several oxidases, and a number of enzymes involved in other cellular functions, e.g., the oxidation of very long chain fatty acids (6, 7) and dicarboxylic acids (8), and the biosynthesis of bile acids (9) and ether lipids (10, 11). The cerebrohepatorenal (Zellweger) syndrome (1) can be considered as the prototype of the peroxisomal diseases. In liver and kidney of Zellweger patients no morphologically detectable peroxisomes could be observed (12). Subsequent biochemical investigations have confirmed the virtual absence of peroxisomes in fibroblasts of these patients and have revealed that all the cellular functions described above are impaired in Zellweger patients (1–4).

The involvement of peroxisomes in ether lipid biosynthesis was first described by Hajra and coworkers (10, 11). These authors showed that in rodent liver the first two enzymes involved in ether lipid biosynthesis, i.e., the acyl CoA:dihydroxyacetonephosphate acyltransferase and the alkyl dihydroxyacetonephosphate synthase, were located in peroxisomes. Following these observations, we discovered (13, 14) that tissues of Zellweger patients were severely deficient in plasmalogens, the major endproducts of ether lipid biosynthesis in mammals, comprising an estimated 18% of total phospholipids in man. In accordance with these findings, we then showed, by growing the cells in a medium containing [^{14}C]hexadecanol, that de novo ether lipid biosynthesis was impaired in cultured skin fibroblasts of Zellweger patients (15). These results were recently confirmed and extended by Roscher and coworkers (16, 17). However, when the Zellweger cells were cultured in a medium containing [^{3}H]octadecylglycerol, a precursor in which the ether bond is already present at the *sn*-1 position of the glycerol backbone, the incorporation of label into plasmalogens was equally effective as compared to control fibroblasts (15). These findings delineated a deficiency in the plasmalogen biosynthesis at or before the level of introducing the glycero-ether bond. Indeed, it was found that both peroxisomal enzymes involved in this process, i.e., acyl CoA:dihydroxyacetonephosphate acyltransferase (18, 19) and the alkyl dihydroxyacetonephos-

phate synthase (19, 20), appeared to be deficient in fibroblasts and tissues of Zellweger patients. Based on these and other results, postnatal and prenatal diagnostic tests for Zellweger syndrome became possible and can now be applied routinely (21–24)

Other inherited diseases in man in which peroxisomal dysfunctions are implied include adrenoleukodystrophies (25–27), adult and infantile form of Refsum disease (28–30), and chondrodysplasia punctata (31, 32). Clinical details can be found in the cited references.

There are three types of adrenoleukodystrophy, differing in age of onset, mode of inheritance, and clinical presentation. In the X-linked form of adrenoleukodystrophy peroxisomes are present (33). Biochemically this disease is characterized by a large accumulation of very long chain fatty acids and affected boys usually die during adolescence (26). Adrenomyeloneuropathy is considered to be a milder phenotypic variant of X-linked adrenoleukodystrophy (4) that becomes manifest in adults. Recent developments strongly suggest that the primary biochemical defect in these diseases is a deficiency in the peroxisomal ligase activating very long chain fatty acids (34–36). By contrast, in the neonatal form of adrenoleukodystrophy, peroxisomes are either reduced in number or undetectable (27) and a general impairment of peroxisomal functions is found. In the classic or adult form of Refsum disease (also known as phytanic acid storage disease) peroxisomes are normally found and until now the dysfunction seems to be restricted to the deficiency of phytanic acid oxidase, resulting in the accumulation of phytanic acid (30). The infantile form of Refsum disease is characterized by a general impairment of peroxisomal functions as found for Zellweger Syndrome and peroxisomes are reduced (28, 29). Peroxisomes are abundant in some hepatocytes in the rhizomelic type of chondrodysplasia punctata but deficient in others. An accumulation of phytanic acid has been described (32).

During our ongoing studies on peroxisomal disorders and related diseases we have investigated the capacity of fibroblasts of a large number of patients, heterozygotes and controls. The purpose of this study was threefold. First, to make an inventory of plasmalogen biosynthetic capacity in this group of newly recognized peroxisomal diseases. Secondly, to determine in which cases the diagnostic tests based on plasmalogen biosynthesis deficiency as developed previously for Zellweger Syndrome could be applied in the diagnosis of other peroxisomal disorders as well. Thirdly, to see whether it would become possible to classify the disorders according to severity of a single biochemical parameter such as de novo plasmalogen biosynthesis. In this study we report on the results obtained by measuring the incorporation of [^{14}C]hexadeconal and/or [^{3}H]octadecylglycerol into plasmalogens from cultured skin fibroblasts.

MATERIALS AND METHODS

Materials

[1-^{14}C]Hexadecanol (55 mCi/mmol) was obtained from IRE-Nederland Soesterberg, The Netherlands, and 1-O-[9,10-$^{3}H_2$]octadecyl-*sn*-glycerol-3-phosphocholine (84 mCi/mmol) was from Amersham International, Buckinghamshire, U.K. HPTLC-fertigplatten kieselgel 60, silica 60HR Reinst, Florisil and solvents were from Merck, Darmstadt, FRG.

Preparation of octadecylglycerol

1-O-[9,10-$^{3}H_2$]Octadecyl-*sn*-glycerol was prepared from 1-O-[9,10-$^{3}H_2$]octadecyl-*sn*-glycero-3-phosphocholine by acetolysis (37) followed by mild alkaline hydrolysis (38). The product was purified by thin-layer chromatography on silica 60HR Reinst using petroleum ether (bp 40–60°C)–diethylether–formic acid 40:60:1.5 as the developing solvent. The octadecyl glycerol was eluted from the silica with chloroform–methanol 1:1 and stored at −20°C. The compound showed a radiochemical purity of 98%.

Cell cultures

Fibroblasts of patients and controls were cultured to confluence in F10 medium (14). Then the cultures were split into two parts. The cells of one part were cultured for 3 days in fresh F10 medium supplemented with [1-^{14}C]hexadecanol (1 μCi/30 ml) or with both [1-^{14}C]hexadecanol (1 μCi/30 ml) and 1-O-[9,10-$^{3}H_2$]octadecylglycerol (0.5 μCi/30 ml). The labeled substrates were added to the medium in a small volume of hexane which was then removed by bubbling nitrogen through the medium. Control experiments (data not shown) indicated that the percentage values used to express the incorporation of label into plasmalogens (see below) were independent of the incubation time, over a period from 2 to 11 days. Cells were harvested, washed with medium, centrifuged, and stored frozen at −70°C.

The patients were all clinically and biochemically characterized. The Zellweger patients included in this study all died before they reached the age of 6 months and showed the characteristic clinical abnormalities described earlier (1). Five of the neonatal adrenoleukodystrophy patients were described by Wanders et al. (25), and are denoted as cell lines no. 1 to 4 and 6 in Figs. 1 and 2. Cell line no. 5 was described by Vamecq et al. (39), and cell line no. 7 by Kelley et al. (27). Three of the infantile Refsum patients were described by Budden et al. (40), and are denoted as cell lines no. 8, 10, and 11 in Figs. 1 and 2. The other three infantile Refsum patients, cell lines no. 9, 12, and 13, were described by Poll-Thé et al. (28).

Analytical procedures

Phospholipids were extracted from homogenized cells by the method of Bligh and Dyer (41) and separated into the individual components by two-dimensional thin-layer chromatography, with HCl treatment between the two chromatographic runs as described earlier (15). When the cells had been grown in a medium supplemented with [1-^{14}C]hexadecanol, all phospholipid spots and the long-chain aldehyde spots (derived from the alkenylgroups of ethanolamine and choline plasmalogens upon HCl treatment), after detection with iodine stain, were scraped into scintillation vials containing 10 ml of scintillation fluid and used for radioactivity measurement. When the cells had been grown in a medium supplemented with both [1-^{14}C]hexadecanol and 1-O-[9,10-$^{3}H_2$]octadecylglycerol, only the aldehyde spots were stained and scraped into scintillation vials for radioactivity measurements. In this last case the former procedure of thin-layer chromatography was optimized by using HPTLC plates (10 × 10 cm) and using chloroform–methanol–water 65:35:4 as the first developing solvent mixture. In some experiments in which cells were cultured in the presence of [1-^{14}C]hexadecanol, the acid-stable ethanolamine and choline glycerophospholipid spots (consisting of alkyl-acyl and diacyl species) were eluted from the silica gel with chloroform–methanol 1:2 (v/v) and subjected to alkaline hydrolysis (38) in order to cleave the fatty acid ester bonds. The reaction products were separated on silica 60HR plates with chloroform–methanol–conc. ammonia 100:50:12 (v/v) as a solvent mixture (15). The alkyllysophospholipids and the N-acylmethylamines were detected by iodine and the radioactivity was measured.

Protein content of homogenates was measured by the method of Lowry et al. (42).

RESULTS

We studied de novo plasmalogen biosynthesis by measuring the incorporation of [1-^{14}C]hexadecanol into the alkenyl side chain of ethanolamine and choline plasmalogens of cultured skin fibroblasts from patients with different peroxisomal or related diseases, from heterozygotes for Zellweger syndrome and infantile Refsum disease, and from controls. The results are shown in **Table 1.** The values are expressed as a percentage of total radioactivity recovered in phospholipids. The radioactivity found in diacyl phospholipids is due to [^{14}C]palmitic acid generated in the fibroblasts from [^{14}C]hexadecanol by oxidation. The incorporation of this ^{14}C-labeled fatty acid into the acyl groups of phospholipids per μg of cellular protein proceeded with equal efficiency in cells of patients and controls. The ranges of incorporation of the labeled fatty acid in individual cell cultures varied by less than a factor of 2 and these values did not differ for patients and controls. Thus the incorporation of a ^{14}C-acyl group into phospholipids can be used as an internal standard and the incorporation of a ^{14}C-labeled precursor in the alkenyl groups as a percent of total phospholipid radioactivity (Table 1) is a measure of the degree of plasmalogen biosynthesis in the different disorders.

The results from Table 1 indicate that plasmalogen biosynthesis is normal in fibroblasts from patients with X-linked adrenoleukodystrophy, adrenomyeloneuropathy,

TABLE 1. Incorporation of [1-^{14}C]hexadecanol into alkenyl chains of plasmalogens

		Alkenyl Radioactivity	
Fibroblasts	N	Plasmalogen PE	Plasmalogen PC
		percent of total radioactivity in phospholipids (mean ± SD)	
Controls	27	49 ± 10	4.7 ± 2.6
X-CDP	2	42 ± 1	3.0 ± 0.3
X-ALD	4	44 ± 16	5.1 ± 2.3
AMN	2	51 ± 4	4.7 ± 0.1
RD	4	41 ± 12	3.0 ± 1.6
IRD	6	19 ± 8	1.3 ± 0.5
NALD	7	11 ± 6	1.0 ± 0.5
ZS	21	6.6 ± 4.0	0.9 ± 0.5
RCDP	7	2.4 ± 2.6	0.5 ± 0.3
Heterozygotes			
ZS	8	49 ± 6	5 ± 2
IRD	3	43 ± 13	5 ± 3

Cells were grown in the presence of [1-^{14}C]hexadecanol and analyzed as described under Materials and Methods. Results are given as the amount of radioactivity in the alkenylchains of plasmalogens expressed as percentage of total radioactivity recovered in phospholipids. Mean values ± standard deviation for the indicated number (N) of cell lines are given. Abbreviations: PE and PC, ethanolamine and choline glycerophospholipids, respectively; X-CDP, X-linked chrondrodysplasia punctata; X-ALD, X-linked adrenoleukodystrophy; AMN, adrenomyeloneuropathy, RD, Refsum disease; IRD, infantile Refsum disease; NALD, neonatal adrenoleukodystrophy; ZS, Zellweger syndrome; RCDP, rhizomelic chondrodysplasia punctata.

Refsum disease, and X-linked chondrodysplasia punctata. About 45% and about 4.5% of total phospholipid radioactivity is present in these cases in the alkenyl chains of ethanolamine and choline plasmalogens, respectively. In contrast, in fibroblasts of patients with neonatal adrenoleukodystrophy, infantile Refsum disease, Zellweger syndrome, and rhizomelic chondrodysplasia punctata, plasmalogen biosynthesis was found to be strongly impaired. It appears from Table 1 that rhizomelic chondrodysplasia punctata is most severely deficient in plasmalogen biosynthesis (2.4 and 0.5% of radioactivity in alkenyl chains of ethanolamine and choline plasmalogens, respectively), followed by Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease in this order. Heterozygotes for Zellweger syndrome and infantile Refsum disease behave like controls with respect to their plasmalogen biosynthetic capacity. In **Fig. 1** the alkenyl radioactivity of ethanolamine plasmalogens, expressed as a percentage of total radioactivity recovered in phospholipids, is plotted for each of the individual cell lines of rhizomelic chondrodysplasia punctata, Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and control individuals that were investigated. The figure shows the ranges of hexadecanol incorporation into ethanolamine plasmalogens as observed in each group and facilitates a comparison of these groups. The data vary over quite a range for both patients and normal controls. The mean values (Table 1, lines in Fig. 1) however, for each group of patients is well distinguished from the mean value of controls. The values for rhizomelic chondrodysplasia punctata and Zellweger syndrome deviate most from that of controls, indicating that these peroxisomal diseases are most severely deficient in plasmalogen biosynthesis.

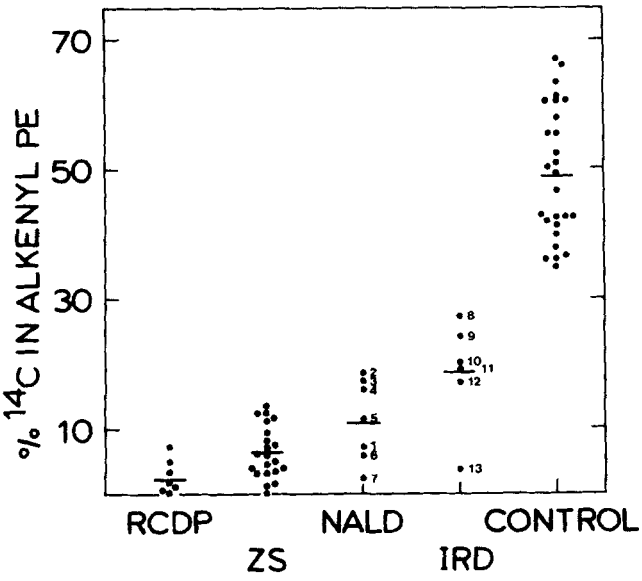

Fig. 1. Incorporation of [1-^{14}C]hexadecanol into alkenyl chains of ethanolamine plasmalogens in individual cell lines of controls and patients. The results are expressed as the percentage of total recovered radioactivity in phospholipids present in the alkenyl chain of the ethanolamine plasmalogens. Abbreviations are in the legend for Table 1. The Arabic numbers next to the values refer to individual patients as indicated in the Materials and Methods section.

In our previous studies with Zellweger fibroblasts (15) we showed that the decreased labeling of alkenyl groups in the ethanolamine and choline glycerophospholipids of Zellweger cells was not compensated for by an elevated level of the corresponding alkyl phospholipids. This finding suggested that the 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine desaturase, a microsomal enzyme that converts the alkyl bond of ether lipids into an alkenyl bond (43, 44), is not deficient in Zellweger patients. An important step in comparing the cause of plasmalogen deficiency in infantile Refsum disease, neonatal adrenoleukodystrophy, rhizomelic chondrodysplasia punctata, and Zellweger syndrome was to rule out a deficiency in the desaturating step in these diseases. In **Table 2** the distribution of radioactivity from [1-^{14}C]hexadecanol in alkenyl, alkyl, and acyl side chains in ethanolamine and choline glycerophospholipids is given for fibroblasts of infantile Refsum disease, neonatal adrenoleukodystrophy, rhizomelic chondrodysplasia punctata, Zellweger syndrome, and controls. The alkyl groups contained about 1% of the total radioactivity recovered in ethanolamine glycerophospholipids and this value was found for all disorders and for controls. In choline glycerophospholipids the alkyl chains contained about 0.3% of the total radioactivity in patients compared to 1.8% in controls. In all cases the decrease in percentage of radioactivity in alkenyl chains is compensated for by an identical increase in the percentage of radioactivity in acyl chains rather than in alkyl chains. No accumulation of alkylphospholipids in fibroblasts was found for any disease. This suggested that the desaturase is not deficient and that the plasmalogen deficiency shown in Table 1 is caused by impaired glycero-ether bond formation in all these disorders.

To obtain further support for this conclusion we studied the incorporation of alkylglycerol into plasmalogens. Before alkylglycerol enters the ether phospholipid pathway it is phosphorylated in the cells by ATP:alkylglycerol phosphotransferase (45, 46). We showed previously (15) that alkylglycerol was incorporated with equal efficiency into plasmalogens of Zellweger fibroblasts when compared to control fibroblasts. This indicated that the deficiency in Zellweger fibroblasts causing deficient plasmalogen biosynthesis can be bypassed when a precursor is offered to the cells in which the glycero-ether bond is already present. Here we extended this study to the other peroxisomal diseases. Cells were grown in the presence of both [1-^{14}C]hexadecanol and 1-O-[9,10-$^{3}H_2$]octadecylglycerol. **Table 3** gives the results obtained on the incorporation of 1-O-[9,10-$^{3}H_2$]octadecylglycerol into ethanolamine and choline plasmalogens of fibroblasts of different per-

TABLE 2. Distribution of radioactivity in alkenyl-, alkyl-, and acylchains of phospholipids

Fibroblasts	Ethanolamine Glycerophospholipids			Choline Glycerophospholipids		
	Alkenyl	Alkyl	Acyl	Alkenyl	Alkyl	Acyl
			%			
Controls	91	1.1	7.7	16.3	1.8	81.9
RCDP	9.5	1.4	89.0	1.1	0.4	98.5
ZS	17.7	1.0	83.3	0.9	0.3	98.8
IRD	35.6	0.9	63.5	1.0	0.3	98.7
NALD	29.1	1.1	69.8	1.8	0.3	97.9

Cells were grown in the presence of [1-^{14}C]hexadecanol. Lipid extracts of the number of cell lines as given in Table 3 were combined for each disorder and analyzed as described under Materials and Methods. Results are given as percentages of total radioactivity recovered in the alkenyl-, alkyl-, and acyl chains of ethanolamine and choline glycerophospholipids, respectively. Abbreviations are in the legend for Table 1.

oxisomal diseases. It is clear that the incorporation, expressed as alkenyl chain radioactivity per μg of cellular protein, is comparable in all cell lines tested including controls. These results further substantiate that the deficiency of plasmalogen biosynthesis in rhizomelic chondrodysplasia punctata, neonatal adrenoleukodystrophy, and infantile Refsum disease is at or before the introduction of the ether bond by peroxisomal enzymes, as was found earlier for Zellweger syndrome (15).

Since the above-mentioned studies had shown that alkylglycerol was incorporated into plasmalogens to comparable extents in cells of the different disorders as in control cells, it seemed attractive to use this incorporation as an internal standard, as done by Roscher et al. (16, 17). Until now we had used the incorporation of ^{14}C-labeled fatty acid into phospholipids as an internal standard by expressing the alkenyl radioactivity as a percent of total phospholipid radioactivity as a measure of plasmalogen biosynthetic capacity (Table 1). By growing the cells in a medium containing both [^{14}C]hexadecanol and [^{3}H]octadecylglycerol, the capacity for plasmalogen biosynthesis can be evaluated independent of fatty acid incorporation by measuring the ^{3}H/^{14}C ratio in the alkenyl chains of plasmalogens. An increase in this ratio compared to controls indicates a less efficient glycero-ether bond synthesis. It was hoped that by using this new procedure the standard deviations within one group of patients would become smaller than those in Table 1 and that it would perhaps become possible to classify the different peroxisomal diseases according to their residual capacity to synthesize plasmalogens. An additional advantage lies in the simplified experimental procedure, in that now only the radioactivity in the aldehyde spots on the thin-layer chromatography plates had to be measured.

Table 4 gives the ^{3}H/^{14}C radioactivity ratio in the alkenyl side chain of ethanolamine and choline plasmalogens upon incorporation of both [1-^{14}C]hexadecanol and 1-O-[9,10-$^{3}H_2$]octadecylglycerol in cultured skin fibroblasts of controls and of patients with different peroxisomal diseases. In controls, patients with X-linked adrenoleukodystrophy and adult Refsum disease, and heterozygotes of patients with Zellweger syndrome and infantile Refsum disease, the ^{3}H/^{14}C ratio is about 1.5 in the alkenyl groups of ethanolamine plasmalogen and about 1.0 in the alkenyl groups of choline plasmalogens. The ^{3}H/^{14}C ratios of the alkenyl groups of plasmalogens from infantile Refsum disease, neonatal adrenoleukodystrophy, Zellweger syndrome, and rhizomelic chondrodysplasia punctata fibroblasts were higher than those of controls, both for ethanolamine and choline plasmalogens, and the ratios increased in that order. Unfortunately, the values for the standard deviation in these experiments remained very large and did not seem to have improved when compared with those presented in Table 1. This can also be seen in **Fig. 2** in which the individual data for all cell lines of controls, rhizomelic chondrodysplasia punctata, Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile

TABLE 3. Incorporation of 1-O-[^{3}H]octadecylglycerol into plasmalogens

Fibroblasts	N	Alkenyl Radioactivity	
		PE	PC
		dpm/μg of cellular protein (mean ± SD)	
Controls	2	184 ± 22	5.1 ± 0.4
RCDP	1	192	4.1
ZS	3	196 ± 41	6.3 ± 0.8
IRD	4	250 ± 64	8.5 ± 1.2
NALD	6	222 ± 57	6.8 ± 1.9

Cells were grown in the presence of [1-^{14}C]hexadecanol and 1-O-[9,10-$^{3}H_2$]octadecylglycerol and analyzed as described under Materials and Methods. The incorporation of alkylglycerol into plasmalogens was measured as tritium radioactivity in alkenyl chains in disintegrations per minute (dpm) per μg of cellular protein. The results are given as mean ± standard deviation for the indicated number of cell lines (N). Abbreviations are in the legend for Table 1.

TABLE 4. Incorporation of [1-^{14}C]hexadecanol and 1-O-[9,10-$^{3}H_2$]octadecylglycerol into alkenyl chains of plasmalogens

Fibroblasts	N	$^{3}H/^{14}C$ Ratio in Alkenyl Chains of	
		Ethanolamine Plasmalogens	Choline Plasmalogens
		ratio (mean ± SD)	
Controls	29	1.5 ± 0.9	1.1 ± 0.7
X-ALD	8	1.0 ± 0.4	0.7 ± 0.4
RD	3	2.2 ± 1.3	1.8 ± 0.4
IRD	5	10 ± 8	3.9 ± 1.9
NALD	7	24 ± 16	7.7 ± 2.3
ZS	14	75 ± 98	7.5 ± 5.3
RCDP	5	433 ± 428	39 ± 37
Heterozygotes			
ZS	2	1.8 ± 0.3	1.2 ± 0.2
RCDP	2	1.8 ± 0.1	1.5 ± 0.2

Cells were grown in the presence of both [1-^{14}C]hexadecanol and 1-O-[9,10-$^{3}H_2$]octadecylglycerol and analyzed as described in Materials and Methods. Results are expressed as $^{3}H/^{14}C$ ratios in the alkenyl chains of the indicated plasmalogens. Abbreviations are in the legend for Table 1.

Refsum disease are plotted to visualize the ranges in a manner comparable to Fig. 1. The results of Table 4 and Fig. 2 are essentially the same as those from Table 1 and Fig. 1. Some of the peroxisomal diseases such as X-linked adrenoleukodystrophy and adult Refsum disease have normal plasmalogen biosynthesis, others are impaired in their plasmalogen biosynthesis as is the case in infantile Refsum disease, neonatal adrenoleukodystrophy, Zellweger syndrome, and rhizomelic chondrodysplasia punctata. There appears to be a considerable variation in the data for individual cell lines within a given peroxisomal disorder and a large overlap of the ranges of data for the various disorders with deficiencies in plasmalogen biosynthesis (Figs. 1 and 2). However, all individual cell lines from the latter groups that have been investigated so far yielded values lower than the range covered by controls.

DISCUSSION

In recent years the Zellweger syndrome has been described as the prototype of a severe peroxisomal disorder. The whole subcellular organelle is missing and most peroxisomal functions that have been recognized until now appear impaired. However, a normal activity for catalase and some other soluble matrix enzymes has been found (1). Several other diseases appear to be caused by peroxisomal dysfunctions as well and a new group of inheritable diseases in man, now collectively called peroxisomal disorders, has been recognized in recent years. It is of great interest to describe these peroxisomal disorders clinically and biochemically and to study the biochemical defects of each individual disorder, which is a prerequisite for the development of (prenatal) diagnosis. In addition, a comparison of the clinical and biochemical parameters of the different peroxisomal disorders and a study of their primary biochemical lesions is likely to provide information on peroxisome biogenesis and on the

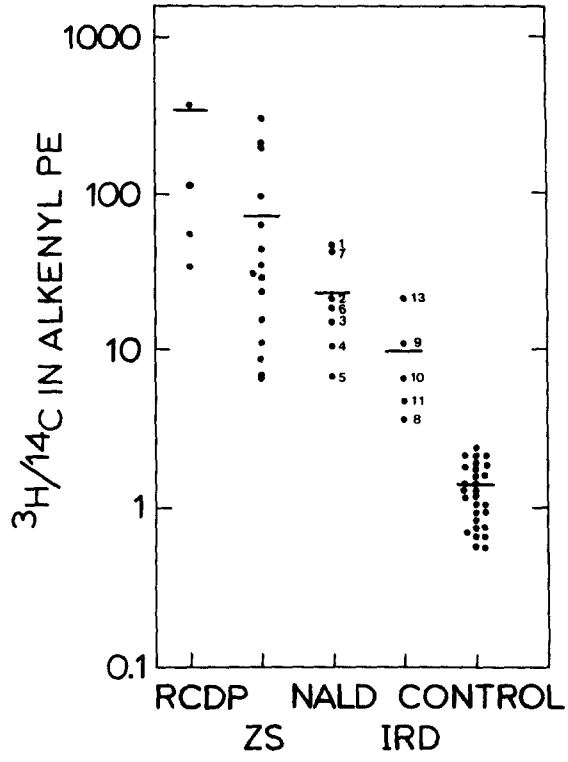

Fig. 2. Incorporation of [1-^{14}C]hexadecanol and 1-O-[9,10-$^{3}H_2$]octadecylglycerol into alkenyl chains of ethanolamine plasmalogens in individual cell lines of controls and patients. The results are expressed as $^{3}H/^{14}C$ ratios in the alkenyl chains of ethanolamine plasmalogens. Abbreviations are in the legend for Table 1. The Arabic numbers next to the values refer to individual patients as indicated in the Materials and Methods section.

relationship between the biochemical defects and the clinical features of the disorders.

In this study we investigated one of the biochemical parameters of interest in the various peroxisomal disorders, i.e., the capacity for ether lipid biosynthesis. Our hexadecanol incorporation studies have clearly shown that X-linked chondrodysplasia punctata, adrenomyeloneuropathy, Refsum disease, and X-linked adrenoleukodystrophy have a normal plasmalogen biosynthesis. In X-linked chondrodysplasia punctata no peroxisomal dysfunctions have been described so far in contrast to rhizomelic chondrodysplasia punctata. The former was only included in this study because of the related clinical features and this X-linked chondrodysplasia punctata may not belong at all to the peroxisomal disorders. In any case, ether lipid biosynthesis in this disorder as well as in adrenomyeloneuropathy proceeded normally. In cells of patients with Refsum disease and X-linked adrenoleukodystrophy, the dysfunction appears to be restricted to a single biochemical parameter, phytanic acid oxidation and the β-oxidation of very long chain fatty acids, respectively (1–4), and plasmalogen biosynthesis proceeds normally.

The other diseases we investigated, i.e., rhizomelic chondrodysplasia punctata, Zellweger syndrome, infantile Refsum disease, and neonatal adrenoleukodystrophy, were all impaired in their plasmalogen biosynthesis (Tables 1 and 4). A more careful look at the plasmalogen biosynthesis of these disorders revealed that the disturbances in all these diseases are due to the peroxisomal part of the biosynthetic process. The finding that radioactive alkyl phospholipids were not accumulating in cells grown on [^{14}C]hexadecanol suggested that the deficient plasmalogen biosynthesis was not caused by the absence of the desaturase. The finding that octadecylglycerol was incorporated into plasmalogens in comparable amounts in fibroblasts from patients and controls, confirmed this notion and proved that for all these disorders the deficient plasmalogen biosynthesis is caused by defects in the first steps of the biosynthesis. Previous studies have shown the deficiency of both peroxisomal enzymes involved in plasmalogen biosynthesis, i.e., acylCoA:dihydroxyacetonephosphate acyltransferase (18, 19) and alkyl dihydroxyacetonephosphate synthase (19, 20), in fibroblasts of Zellweger patients. Similar observations were subsequently made in fibroblasts of rhizomelic chondrodysplasia punctata (32), infantile Refsum disease (28, 29), and neonatal adrenoleukodystrophy (25) patients. Thus, in all these diseases the plasmalogen deficiency is caused by a deficiency of the same enzymes. It has been concluded from in vitro experiments with isolated subcellular fractions from laboratory animals that acyl dihydroxyacetone phosphate can be formed not only in peroxisomes but also in endoplasmic reticulum, either by glycero-3-phosphate acyltransferase (47) or by a specific dihydroxyacetonephosphate acyltransferase (10, 11, 48). Since alkyl dihydroxyacetonephosphate synthase and acyl/alkyl dihydroxyacetonephosphate reductase may also have a bimodal distribution over peroxisomes and endoplasmic reticulum (11), the data from cell fractionation experiments do not exclude the possibility that ether lipid biosynthesis could be catalyzed by endoplasmic reticulum enzymes. Our experiments on de novo plasmalogen biosynthesis in intact cells and the findings described in this report that this process is deficient in fibroblasts from patients with diseases characterized by complete or partial absence of peroxisomes clearly indicate that endoplasmic reticulum synthesis of ether lipids in vivo, if existing at all, is of minor importance. At least, it cannot compensate for the deficiencies in the peroxisomal contribution to this process.

The mean values in Table 1 and Table 4 suggest that there is a gradual difference between rhizomelic chondrodysplasia punctata, Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease with respect to the remaining capacity to synthesize plasmalogens that increases in this order. However, the incorporation of the precursor hexadecanol into plasmalogens of fibroblasts within one group of patients varied considerably and resulted in large standard deviations of the mean for that disease. As a result the ranges of the individual cell lines of the different disorders shown in Fig. 1 and Fig. 2 show a considerable overlap. To investigate to what extent these results were due to experimental imperfections, we performed the following control experiment. In a single experiment 14 cell lines of different peroxisomal diseases were grown under exactly the same conditions in a medium supplemented with [^{14}C]hexadecanol and [^{3}H]octadecylglycerol. The results obtained from this standard experiment (data not shown) were identical with those shown in Table 4 and Fig. 2. Most likely, therefore, the ranges observed in the data from individual cell lines of a given disease are caused by differences in biological activity of the individual cell lines.

The capacity for plasmalogen biosynthesis is the first biochemical feature for which such an extensive comparison between different peroxisomal disorders has been made. As shown in this study based on the mean values of the data obtained so far, the disorders rank from rhizomelic chondrodysplasia punctata, Zellweger syndrome, and neonatal adrenoleukodystrophy to infantile Refsum disease in increasing order of residual plasmalogen biosynthetic capacity. Due to the large variation in data within one disease, this sequence for the mean does not hold for individual cell lines. At the same time this means that for diagnostic purposes the data from an unknown cell line on de novo plasmalogen biosynthetic capacity alone will not permit classification to one of these peroxisomal disorders.

Another interesting question is what causes the gradual

differences in plasmalogen biosynthesis between the different disorders? In patients with rhizomelic chondrodysplasia punctata, catalase is particle-bound and peroxisomal structures are found in some hepatocytes (32) but at least two peroxisomal functions are disturbed, i.e., phytanic acid oxidation and plasmalogen biosynthesis. The primary lesion in rhizomelic chondrodysplasia punctata is not the same as in Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease as shown by complementation experiments (49). This places rhizomelic chondrodysplasia punctata in a distinct category, i.e., there is a deficiency in the enzymes involved in glycero-ether bond synthesis in the presence of peroxisomes.

In Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease, the deficiency of the enzymes involved in glycero-ether bond synthesis is accompanied by and probably caused by a pronounced reduction in the number and size of peroxisomes. Thus all biochemical features that are found to be deficient in Zellweger syndrome are also disturbed in infantile Refsum disease and neonatal adrenoleukodystrophy. It has been suggested that the primary lesion in Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease is at the level of the biogenesis of peroxisomes (49). Recently Lazarow et al. (50) hypothesized that peroxisomal membranes may be assembled in Zellweger syndrome, but that these are probably defective for the import of matrix proteins. Thus, peroxisomal proteins would be synthesized in the cytosol but many of them would be rapidly degraded. This view was based on the immunological detection of a characteristic peroxisomal integral membrane protein in membrane fragments of liver tissue from Zellweger patients. It should be mentioned, however, that if peroxisomal membranes are formed in Zellweger syndrome these are probably rudimentary structures, not only defective in the import of peroxisomal matrix proteins but most likely also in the import of the membrane-associated enzymes acyl CoA:dihydroxyacetonephosphate acyltransferase and alkyl dihydroxyacetonephosphate synthase.

More residual peroxisomal structures in neonatal adrenoleukodystrophy and infantile Refsum disease in combination with a less defective incorporation of these membrane-associated enzymes in comparison to Zellweger syndrome could explain the larger residual capacity for plasmalogen biosynthesis in these diseases. Recently, Roscher and coworkers (17) concluded that neonatal adrenoleukodystrophy can be distinguished biochemically from the Zellweger syndrome by a less impaired de novo plasmalogen biosynthesis. Although we observed a similar tendency for the mean value of the ratio of [^{3}H]alkylglycerol and [^{14}C]hexadecanol incorporation into the alkenyl chain of plasmalogens, it is also obvious from the data provided in Fig. 2 that this conclusion is untenable for each comparison of individual cell lines of these two disease states. Other investigators have also noted no or only small differences in the accumulation of saturated very long chain fatty acids in fibroblasts from neonatal adrenoleukodystrophy and Zellweger syndrome, although the accumulation of unsaturated very long chain fatty acids was somewhat higher in Zellweger syndrome than in neonatal adrenoleukodystrophy (27, 51). Similarly, immunoblot analysis of the peroxisomal β-oxidation enzymes revealed higher levels of acyl-CoA oxidase and β-ketothiolase in neonatal adrenoleukodystrophy than in Zellweger liver tissue, whereas the bifunctional enzyme was almost completely absent in both diseases (52). These differences are believed to reflect different extents of impairment of peroxisomes in neonatal adrenoleukodystrophy and Zellweger syndrome variants.

Based on these hexadecanol incorporation studies, diagnosis is possible in rhizomelic chondrodysplasia punctata and Zellweger syndrome. The results obtained with fibroblasts suggest that prenatal diagnosis of rhizomelic chondrodysplasia punctata might be equally possible using this approach as has been described for Zellweger syndrome (21, 22) but this has not yet been proven in practice. A more careful approach involving additional assays for peroxisomal functions and/or enzymes (reviewed in ref. 1 and 4), is recommended for prenatal diagnosis of infantile Refsum disease and neonatal adrenoleukodystrophy.

This work was supported financially by 'Het Prinses Beatrix Fonds' The Hague, The Netherlands and the Netherlands Organization for the Advancement of Pure Research (Z. W. O.). The authors would like to thank Miss E. Meyboom and A. Vanderput for culturing fibroblasts. The authors are grateful to Dr. N. G. Kennaway (Portland), Dr. J. Vamecq (Brussels), Dr. J. M. Saudubray (Paris), and Dr. H. W. Moser (Baltimore) for supplying us with fibroblasts from patients. Without their courtesy the comparative studies described in this paper would not have been possible to the same extent.

Manuscript received 17 July 1987 and in revised form 11 September 1987.

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