Accumulation of glyceryl ether lipids in Wolman's disease[1]

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Abstract We have shown that ether-linked glycerolipids accumulated in the adrenal, liver, and spleen of a male Chinese infant with Wolman's disease; the increases were mainly in the alkyl and alk-1-enyl glycerolipids that did not contain phosphorus. Alkyldiacylglycerol accounted for a portion of the rise in the neutral alkylglycerols. The spleen also contained increased amounts of ether-linked phosphoglycerides of the alkyl and alk-1-enyl types. Organs from a Niemann-Pick patient were also included in this study; they did not show comparable rises in the content of ether-linked glycerolipids, suggesting the possibility that storage of these compounds may be characteristic of Wolman's disease, or a variant form thereof.

Supplementary key words lipid storage disease • alkyldiacylglycerol • adrenal lipids

Wolman's disease is a rare familial disorder in man, characterized by lipid accumulation in several organs and within the macrophages present in many tissues. Calcification of the involved adrenals is a consistent feature (1, 2). Cholesteryl esters account for most of the stored lipids, and there is some accumulation of triacylglycerols and free fatty acids (3–5). Tissue lipid analyses carried out on a case of Wolman's disease in Hong Kong showed high concentrations of ether-linked glycerolipids in the adrenals, liver, and spleen. The distribution of the alkyl and alk-1-enyl moieties among various lipid fractions is given in this report. Alkyldiacylglycerol was identified as one of the compounds that accumulated in the organs.

MATERIALS AND METHODS

Commercial preparations of lipids were used as reference standards. Octadecanoic acid, glyceryl-1,2-dioleyl-3-hexadecyl ether, and hexadecyl- and octadecylglycerol were obtained from Analabs, Inc., North Haven, Conn. Triolein was from Sigma Chemical Co., St. Louis, Mo., and methyl palmitate was from Applied Science Laboratories, State College, Pa. Pre-coated silica gel plates (0.25 mm layer thickness) with and without a fluorescent indicator F_{254} were obtained from E. Merck, Darmstadt, Germany. The compositions of the solvent systems used for TLC are given in **Table 1.**

Adrenal, liver, spleen, and brain specimens were taken at autopsy and either stored at −20°C or fixed in phosphate-buffered 10% formalin (pH 6.7) for several weeks before extraction of lipids with chloroform–methanol 2:1 (6). The case report on the infant (male, 51 days) who died of Wolman's disease will appear elsewhere.[2] Control cases 1 and 2 were two male infants (age 1–2 mo) who died of pneumonia; case 1 had in addition a mild fatty change in the liver. Control case 3 (female, 1 mo; cause of death, neonatal hepatitis) provided two specimens of adrenal tissue, one of which was fixed and the other frozen. Further adrenal specimens were obtained from two male adults, one of whom died of hepatocellular carcinoma (control case 4) and the other, of cerebral infarction (control case 5). These were stored at −20°C. Tissues from a case of Niemann-Pick disease (male, 6 yr) were made available for this study by Dr. C. W. Chow.

Isolation and purification of lipid components

Partial purification of alkyldiacylglycerols and of a lipid designated Y was achieved by elution of the lipids on a silicic acid column with petroleum ether containing small proportions of diethyl ether (7). A chromatographic column (18 g of silicic acid) to which 300 mg of lipids was applied, was eluted with 200 ml of 1% ether in petroleum ether, then with 4% ether in petroleum ether in successive 60, 40, 120, and 80 ml portions, and finally with 80 ml of 8% ether in petroleum ether. Neutral plasmalogens and alkyldiacylglycerols appeared in the 40 ml fraction and lipid Y was in the final 160 ml of eluate.

Preparative TLC (10 × 20 cm plates) of up to 5 mg of lipid was carried out. Solvent systems c, d, and e (see Table 1) were used for the isolation of the individual lipid classes. To locate the lipids, plates in which the fluorescent indicator was incorporated or plain plates sprayed with a solution of 0.2% 4,5-dibromofluorescein in ethanol were viewed under ultraviolet light. The bands were marked, and the adsorbent present in each zone was scraped off, transferred to a funnel with a fritted glass bottom, and eluted with diethyl ether in order to obtain the lipid.

Abbreviation: TLC, thin-layer chromatography

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TABLE 1 Comparison of the relative mobilities (R_f) of lipid X and alkyldiacylglycerol and their saponification products[a]

Solvent System[b]	Source of lipid X, from the case of Wolman's disease			Standard Lipids					
	Adrenal	Liver	Spleen	Glyceryl-dioleyl-hexadecyl Ether	Methyl Palmitate	Triolein	Octadeca-noic Acid	Hexadecyl Glycerol	Octadecyl Glycerol
a	0.19	0.19	0.19	0.17		0.10	0		
b	0.31	0.29	0.31	0.30	0.44	0.18	0.08		
c	0.47	0.46	0.46	0.47	0.50	0.41	0.03		
d	0.52	0.53	0.52	0.52	0.50	0.40	0.17		
e	0.59	0.58	0.59	0.58	0.54	0.49	0.19	0	
f	0.82	0.82	0.82	0.82		0.82	0.38	0.17	0.17
g	0.86	0.86	0.86	0.86		0.86	0.68	0.30	0.32
f[c]	0.17	0.17	0.15	0.17					
	0.38	0.38	0.39	0.39					
g[c]	0.32	0.33	0.33	0.30					
	0.68	0.66	0.70	0.68					

[a]Thin-layer chromatography on silica gel G. Specimens of lipid X were isolated by means of preparative thin-layer chromatography prior to making the comparisons of R_f values.

[b](Proportions by volume): a, hexane–diethyl ether 95:5; b, hexane–diethyl ether–acetic acid 95:5:1; c, benzene; d, hexane-diethyl ether–acetic acid 80:20:1; e, benzene–hexane–diethyl ether–acetic acid 45:50:5:1; f, hexane–diethyl ether–methanol–acetic acid 80:20:-10:1; g, diethyl ether–water 200:1

[c]After saponification. Two products were detected in each sample.

Saponification of lipids

Hydrolysis was carried out with 0.75 M KOH in 90% ethanol (22°C, 1 hr). A precise amount of 6 M HCl was added to neutralize the solution, the final pH being in no case lower than 5.

Determination of glyceryl ethers

The concentration of neutral and phosphorus-containing alkyl- and alk-1-enylglyceryl ethers in the tissues was determined by photodensitometric estimation of the products formed after $LiAlH_4$ reduction as described by Wood and Snyder (8). Separation of neutral lipids from phospholipids for these estimations was carried out on silicic acid columns using chloroform and methanol as eluents (9).

Estimations of alkyldiacylglycerol

Preparative TLC was carried out on samples consisting of 6–10 mg of total lipids. The isolated lipid was saponified, and a known proportion of the hydrolysate was applied to a thin-layer plate which was then developed in diethyl ether–water 200:1. A standard amount of alkylglycerol served as the reference compound. Alkyldiacylglycerol was determined by quantitative densitometry of the released alkylglycerol. Recoveries of 0.015–0.12 mg of glyceryl-dioleyl-hexadecyl ether by this method were quantitative.

In the two specimens of adrenal lipids from control cases 4 and 5, alkyldiacylglycerol was estimated directly on fractions from silicic acid columns. Aliquots representing 8–20 mg of total unfractionated lipids were applied to thin-layers adjacent to a known amount of glyceryl-dioleyl-hexadecyl ether, and the plates were developed in solvent system e (Table 1). The relative densities of the alkyldiacylglycerol spots were determined as follows. Chromatoplates charred with acid dichromate (8) were scanned in a Joyce-Loebl integrating densitometer (Chromoscan 200) with a thin-layer attachment. Integrator response measured with known amounts of alkylglycerol was linear up to 15 μg.

RESULTS

Attention was first drawn to the accumulation of ether lipids in the Wolman lipid specimens when TLC showed the presence of a spot (X) that had the R_f value of standard alkyldiacylglycerol. The identity of X with alkyldiacylglycerol was shown by comparison of their R_f values on chromatoplates developed in solvent systems of different polarities (Table 1). An alternative explanation, that X was a fatty acid methyl ester, was specifically excluded by virtue of its relative mobility on plates developed in solvent system b, in which methyl palmitate and alkyldiacylglycerol were clearly separated (10). Saponification of X resulted in the appearance of two major products; one migrated as free fatty acid and the other had the relative mobility of octadecylglycerol. Periodate oxidation of a specimen of the latter product from an adrenal from the patient with Wolman's disease produced chromogens reacting with fuchsin, thus confirming the identification as alkylglycerol (11).

Abnormally high concentrations of alkyldiacylglycerols were present in the tissues from the case of Wolman's disease. The adrenal contained 2.9 mg of this lipid, computed as glyceryl-dioleyl-hexadecyl ether, per g wet weight of tissue. Values of 0.2–0.5 mg/g were obtained in formalin-fixed adrenal tissue from control cases 1–3; fresh specimens of adrenal from control cases 3–5 contained 0.2 mg/g of alkyldiacylglycerol. The liver and spleen from the case of Wolman's disease contained, respectively, 0.7 and 0.5 mg/g of this lipid. The levels of alkyldiacylglycerol in the correspond-

TABLE 2 Levels of alkyl- and alk-1-enylglyceryl ether lipids in the adrenal, liver and spleen from a patient with Wolman's disease[a]

Source	Neutral Lipids	Neutral Glyceryl Ethers		Phospho-lipids	Ether-linked Phospho-glycerides	
		Alkyl Fraction	Alk-1-enyl Fraction		Alkyl Fraction	Alk-1-enyl Fraction
			mg/g wet weight tissue			
Adrenal						
Control case 1[b]	41	0.1	0.9	9	0.07	0.3
Control case 2	59	0.4	1.4	17	0.2	0.7
Wolman	230	2.9	4.6	12	0.3	0.7
Liver						
Control case 1	75	0.09	0.5	16	0.2	0.08
Control case 2	30	0.5	n.d.[c]	27	0.06	0.5
Niemann-Pick	41	0.04	0.09	85	0.06	0.3
	(65)[d]	(0.04)	(0.09)	(85)	(0.2)	(0.6)
Wolman	179	3.1	5.0	9	0.2	0.5
Spleen						
Control case 1	18	0.1	0.4	9	0.03	0.2
Control case 2	21	0.05	0.3	10	0.03	0.1
Niemann-Pick	38	0.1	0.2	70	0.05	0.3
	(55)	(0.1)	(0.6)	(68)	(0.2)	(0.5)
Wolman	54	2.9	3.9	7	0.8	1.0

[a]Glyceryl ether lipid values, expressed as octadecyl- or octadecenylglycerol, were determined by densitometry of the alkyl- or alkenylglycerols liberated by $LiAlH_4$ treatment of neutral lipid and phospholipid fractions.

[b]The control and pathological cases are described in the text under Materials and Methods.

[c]Not detected.

[d]Obtained on another portion of the same specimen stored at −20°C without fixative.

ing organs from control cases 1 and 2, and the Niemann-Pick patient, were estimated at less than 0.1 mg/g.

The relative quantities of glyceryl ether lipids found in the adrenal, liver, and spleen of the case of Wolman's disease are presented in **Table 2.** The glyceryl ether lipids accumulating in the adrenal and liver from the patient with Wolman's disease were mainly of the neutral alkyl and alk-1-enyl type. In the spleen abnormally high quantities of each of the four ether lipid fractions were found, the magnitude of these increases being at least five-fold. The previously noted rises in alkyldiacylglycerols accounted for 7–40% of the neutral alkylglycerol compounds present in the organs of the patient. The specific compounds accounting for the rise in the neutral alk-1-enylglycerol fraction were undefined. Neutral plasmalogens could not be detected in these lipid specimens. The brain from the patient with Wolman's disease was also analyzed. The total alkyl- plus alk-1-enylglycerol level (estimated by the $LiAlH_4$ method) was 1.1 mg/g wet weight tissue, compared to values of 1.1 and 1.2 in corresponding tissues from control cases 1 and 2.

The analyses of tissues from the patient with Niemann-Pick disease (Table 2) brought out two noteworthy points. The formalin-treated portions of liver and spleen showed none of the changes observed in the organs from the Wolman's disease patient. Secondly, the effect of formalin during the fixation period of the tissues was to reduce the concentration of alkyl- and alk-1-enylglycerol lipids. It is possible that the levels of glyceryl ether compounds found in the tissues from the case of Wolman's disease were underestimated. In a separate experiment, it was found that degradation of alkyldiacylglycerol occurred in formalin solution with the formation of alkylglycerol, which under certain conditions may be slightly soluble in aqueous solutions (10). Standard glyceryl-dioleyl-hexadecyl ether was applied to strips of filter paper which were then placed in neutral formalin for several days; TLC of the recovered lipid showed the presence of alkylglycerol and free fatty acid, in addition to the glyceryl ether diester.

In the course of these studies it was noted that the adrenal, liver, and spleen from the Wolman's disease patient contained significant amounts of a neutral saponifiable lipid designated Y, which occupied a position between triacylglycerols and free fatty acids on thin-layer chromatograms. As Y may be a natural constituent of the normal human adrenal, a separate study will be directed toward its identification.

DISCUSSION

Accumulations of uncommon lipids have been shown in other cases of Wolman's disease. In a study of two Wolman patients by Marshall et al. (12), TLC of liver and spleen lipids in both cases revealed unidentified bands in the vicinity of the triacylglycerols. A recent report described the presence of five compounds identified as oxygenated steryl esters in the livers of two patients with Wolman's disease (13). The study described here is the first reported case of Wolman's disease associated with increased tissue levels of ether-linked lipids (compare 2, 4, 5). Abnormally large quantities of glyceryl ether lipids have been found in several types of neoplasms occurring in man and in animals (14, 15).

The present series of analyses included measurements of the tissue levels of several other lipid classes.[2] Higher concentrations of alkyldiacylglycerols and of cholesteryl esters were found in the control adrenals as compared to the normal spleen or liver. In this case of Wolman's disease, the striking accumulation of cholesteryl esters was associated with increases in alkyldiacylglycerols in all three organs. Rises in the tissue levels of triacylglycerols and free fatty acids did not invariably accompany these changes.

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