Lysosomal acid lipase deficiency in rats: lipid analyses and lipase activities in liver and spleen

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Abstract We report the biological characterization of an animal model of a genetic lipid storage disease analogous to human Wolman's disease. Affected rats accumulated cholesteryl esters (13.3-fold), free cholesterol (2.8-fold), and triglycerides (5.4-fold) in the liver, as well as cholesteryl esters (2.5-fold) and free cholesterol (1.33-fold) in the spleen. Triglycerides did not accumulate, and the levels actually decreased in the spleen. Analysis of the fatty acid composition of the cholesteryl esters and triglcyerides showed high percentages of linoleic acid (18:2) and arachidonic acid (20:4) in both organs, especially in the liver. No accumulation of phospholipids, neutral glycosphingolipids, or gangliosides was found in the affected rats. Acid lipase activity for [^{14}C]triolein, [^{14}C]cholesteryl oleate, and 4-methylumbelliferyl oleate was deficient in both the liver and spleen of affected rats. Lipase activity at neutral pH was normal in both liver and spleen. Heterozygous rats showed intermediate utilization of these substrates in both organs at levels between those for affected rats and those for normal controls, although they did not accumulate any lipids. These data suggest that these rats represent an animal counterpart of Wolman's disease in humans. —**Kuriyama, M., H. Yoshida, M. Suzuki, J. Fujiyama, and A. Igata.** Lysosomal acid lipase deficiency in rats: lipid analyses and lipase activities in liver and spleen. *J. Lipid Res.* 1990. **31:** 1605–1612.

Supplementary key words Wolman's disease • genetic lipid storage disease • animal model • cholesteryl ester • triglyceride

Lysosomal acid lipase deficiency results in massive accumulation of cholesteryl esters and triglycerides in most tissues of the body. Two major phenotypes are expressed in humans, Wolman's disease and cholesteryl ester storage disease, both of which are congenital metabolic disorders with autosomal recessive inheritance (1–5). In the patients with Wolman's disease, the abnormality of lipid metabolism becomes clinically evident in the first weeks of life. The clinical features include hepatomegaly, malabsorption, steatorrhea, abdominal distention, and adrenal calcification, and the condition is usually fatal in the first year of life. Usually cholesteryl ester storage disease tends to run a more benign course. The principal, and sometimes only, sign is hepatomegaly, which becomes evident in early childhood or occasionally in the second decade of life. Premature atherosclerosis may be severe, and hyperbetalipoproteinemia is common. These two conditions are distinctly different phenotypes in humans, but the biochemically differences between them have not yet been clarified (1–5).

Recently, we reported a new animal model of lysosomal lipid storage disorder that developed in a colony of Donryu rats (6). The disorder was characterized by hepatomegaly, splenomegaly, and lymphadenopathy, thickening and dilation of the intestines, gradual loss of weight, and finally death from cachexia at about 120 days of age. Histopathological examination revealed large vacuoles in hepatic parenchymal cells and foamy change in Kupffer cells. There was also vacuolation of splenic cells and of histiocytes in the lamina propria of the small intestine. Cholesteryl esters and triglycerides accumulated and acid lipase activity was deficient in the liver and spleen in the affected rats. We suspected that the disorder represented an animal counterpart of Wolman's disease on the basis of the severe manifestations, short lifespan, characteristic pathological findings, and acid lipase deficiency (6). The present study was undertaken to characterize the details of the biochemical abnormalities, including the lipids that accumulated and the enzyme activities for different substrates in this animal model.

MATERIALS

The affected rats were obtained from litters of obligatory heterozygous brother-sister breeding pairs. Five affected rats, six heterozygous rats, and five normal control rats were killed and their livers and spleens were

Abbreviations: 4-MU, 4-methylumbelliferyl; HPTLC, high performance thin-layer chromatography; ACAT, acyl-CoA:cholesterol acyltransferase.

either immediately processed or quickly frozen and stored at −80°C prior to use a few days later. DEAE-Sephadex A-25 and Sephadex LH-20 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Iatrobeads (6Rs 8060) were purchased from Iatron Lab. (Tokyo, Japan), and high-performance thin-layer chromatography (HPTLC) plates (silica gel 60) were from E. Merck Co. (Darmstadt, West Germany). The radioactive substrates tri[1-^{14}C]oleoyl glycerol ([^{14}C]triolein) and cholesteryl [1-^{14}C]oleate ([^{14}C]cholesteryl oleate) were supplied by New England Nuclear (Boston, MA), and 4-methylumbelliferyl (4-MU) oleate was obtained from Koch-Light Lab. (Buckinghamshire, England). Amphoteric surfactant, N-tetradecyl-N, N,-dimethyl-3-amino-1-propanesulfonate (Zwittergent 314) was from Calbiochem-Behring Corp. (La Jolla, CA).

METHODS

Lipid analysis

Lipids were extracted from livers and spleens with 20 volumes each of the following chloroform–methanol solutions: 2:1, 1:1, and 1:2 (by volume). Neutral glycosphingolipids and gangliosides were isolated by a previously described method (7). Both lipid fractions were separated by DEAE-Sephadex A-25, acetate form, column chromatography, and were purified by Iatrobeads or Sephadex LH-20 column chromatography after mild alkaline treatment. The total cholesterol content was measured in the total lipid extract by the technique of Zak (8), and the phospholipid content was measured by determining the phosphorus content using the method of Ames and Dubin (9). Lipid-bound sialic acid in the ganglioside fraction was determined by the resorcinol-HCl method (10). Neutral lipids and glycosphingolipids were analyzed using silica gel 60 HPTLC plates with the following solvents for development: n-hexane–ethyl ether–acetic acid 80:20:2 (by volume, solvent A) for neutral lipids; chloroform–methanol–water 65:25:4 (by volume) for phospholipids and neutral glycosphingolipids; and chloroform–methanol–5 M ammonia–2% CaCl–water 60:40:4:1:4 (by volume) for gangliosides. The plates were then sprayed with copper acetate reagent for locating neutral lipids and phospholipids (11), with anthrone-sulfuric acid reagent for neutral glycosphingolipids (12), and with resorcinol-HCl reagent for gangliosides (10). The percentage distribution of free and esterified cholesterol and the components of the phospholipids were determined by scanning the developed plates with a dual-wavelength TLC densitometer (CS-910, Shimadzu Inc., Kyoto). Triglyceride content was also determined by densitometric analysis by comparison with a known amount of tripalmitate as the standard.

Fatty acid analysis

Cholesteryl esters and triglycerides were isolated from the livers and spleens of normal and affected rats by preparative TLC with solvent A. Purified individual lipids were treated with 2.5% methanolic hydrochloride at 80°C for 10 h. The resulting fatty acid methyl esters were extracted with n-hexane and separated by TLC with solvent A. Nonhydroxy fatty acid methyl esters were analyzed by gas–liquid chromatography using a 10% DEGS column (3 mm × 1 m). The methyl esters were checked using a gas chromatograph–mass spectrometer (Shimadzu QP-1000), equipped with a chemical ionization source with ammonia as the reagent gas, by monitoring the characteristic ions produced by each fatty acid derivative. The column temperature was programmed from 100° to 210°C at a rate of 10° C/min.

Enzyme assays

Frozen tissue samples from livers and spleens were suspended in a solution (1 ml/100 mg of tissue) of 1 mM EDTA, 8 mM Triton X-100, and 0.02% NaN, and then homogenized in a Teflon-glass homogenizer at 4°C (13). The tissue suspension was then centrifuged at 100,000 *g* for 1 h at 4°C. The upper lipid layer was removed and the remaining supernatant was used for the enzyme assays.

The radioactive substrates, [^{14}C]triolein and [^{14}C]cholesteryl oleate, were purified by TLC with solvent A in order to reduce background radioactivity. These purified substrates were diluted with unlabeled substrates to the final specific radioactivities of 2.71 Ci/mol for [^{14}C]triolein and 0.65 Ci/mol for [^{14}C]cholesteryl oleate. Twenty μmol of [^{14}C]triolein or 24.4 μmol of [^{14}C]cholesteryl oleate was dissolved in 2 ml of petroleum ether followed by the addition of 1.6 ml of Triton X-100. The petroleum ether was then evaporated in hot water (80°C), and 1.6 ml of distilled water was added. The substrate suspension (2 mM [^{14}C]triolein or 2.44 mM [^{14}C]cholesteryl oleate) was shaken vigorously until it dispersed and became clear. Acid lipase activities for these substrates were assayed by the method of Burton et al. (14) with slight modifications. Enzyme activities were measured in an incubation system containing 75 mM sodium acetate buffer (pH 4.4), 5 mM 2-mercaptoethanol, 0.5 mM [^{14}C]triolein, or 0.61 mM [^{14}C]cholesteryl oleate, and 250–300 μg of protein in a total volume of 200 μl. The reaction was performed for 2 h at 37°C and was stopped by the addition of 1.6 ml of isopropanol–1.5 M H_2SO_4 40:1 (by volume). Liberated free fatty acids were extracted from the reaction mixture using Burton's modification (14) of the two-step procedure of Schotz et al. (15). Distilled water (0.8 ml) and 2 ml of n-hexane were added to each tube. The solutions were mixed vigorously, and the tubes were centrifuged at 500 *g* for 5 min. Two ml of hexane was added to 0.4 ml of 0.1 M

KOH and the solution in the tubes was again mixed vigorously. Free fatty acids were extracted into the lower alkaline phase, an aliquot of which was counted in Bray's solution using an Aloka LSC-900 liquid scintillation counter.

Acid lipase activity for 4-MU oleate was assayed by the method of Warner et al. (13). The standard assay was conducted in a buffer containing 50 mM sodium acetate buffer (pH 5.0), 30 mM amphoteric surfactant (Zwittergent 314), 2.5 mM 2-mercaptoethanol, 0.5 mM EDTA, 2 mM substrate, and about 30 μg of protein, in a total volume of 250 μl. The substrate was prepared as a surfactant/ethanol-stabilized emulsion by the technique of Warner et al. (13). The reaction mixture was incubated at 37°C for 20 min, and was stopped by the addition of 1 ml of 0.1 M Tris-HCl (pH 7.6) buffer. The level of liberated 4-MU was determined using a fluorometer.

The activities of seven other lysosomal hydrolases (α-galactosidase, β-galactosidase, N-acetyl-β-glucosaminidase, α-fucosidase, α-glucosidase, β-glucosidase, and β-glucuronidase) were determined using 4-MU conjugates as the substrates. Protein content was measured by the method of Lowry et al. (16).

RESULTS

Analysis of lipids

Fig. 1 shows the TLC patterns of neutral lipids in the livers and spleens of affected rats, heterozygous rats, and normal controls. There was marked accumulation of cholesteryl esters in both organs in affected rats, while triglycerides accumulated in the liver, but not in the spleen. Quantitative determinations are shown in **Table 1**. In the livers of affected rats, total cholesterol was increased about 8.2-fold, cholesteryl esters were increased about 13.3-fold, and free cholesterol was increased about 2.8-fold in comparison to normal controls. Triglycerides were also increased about 5.4-fold. Lipid accumulation was not so great in the spleens; the increase of total cholesterol was 2-fold, that of free cholesterol was 1.33-fold, and that of cholesteryl esters was 2.5-fold. Triglycerides did not accumulate, but instead decreased in the spleens of affected rats. No accumulation of phospholipids or neutral glycosphingolipids was seen in the affected rats (**Fig. 2** and Table 1). The percentage distribution of phospholipids, which were mainly composed of sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine, was also similar to that in normal rats. Fig. 2 shows the TLC pattern of liver and spleen gangliosides in one affected rat and two control rats. A slight decrease of polysialogangliosides was noted in the liver of the affected rat, and there was no accumulation of specific gangliosides in either the liver or the spleen. The total amount of lipid-bound sialic acid was similar in both the affected rat (liver, 30.5; spleen, 63.5; μg/mg wet weight) and the two control rats (liver, 24.7 and 16.0; spleen, 78.6 and 54.9; μg/mg wet weight). Heterozygous rats did not show any accumulation of these lipids in either organ.

Table 2 shows the fatty acid compositions of cholesteryl esters and triglycerides in the liver and spleen. Linoleic acid (18:2) and arachidonic acid (20:4) levels were increased in both organs, especially in the liver of the affected rats. There was no accumulation of abnormal fatty acids in affected rats.

Enzyme activities

Acid lipase activities for three different substrates are shown in **Table 3**. All three were deficient in the livers

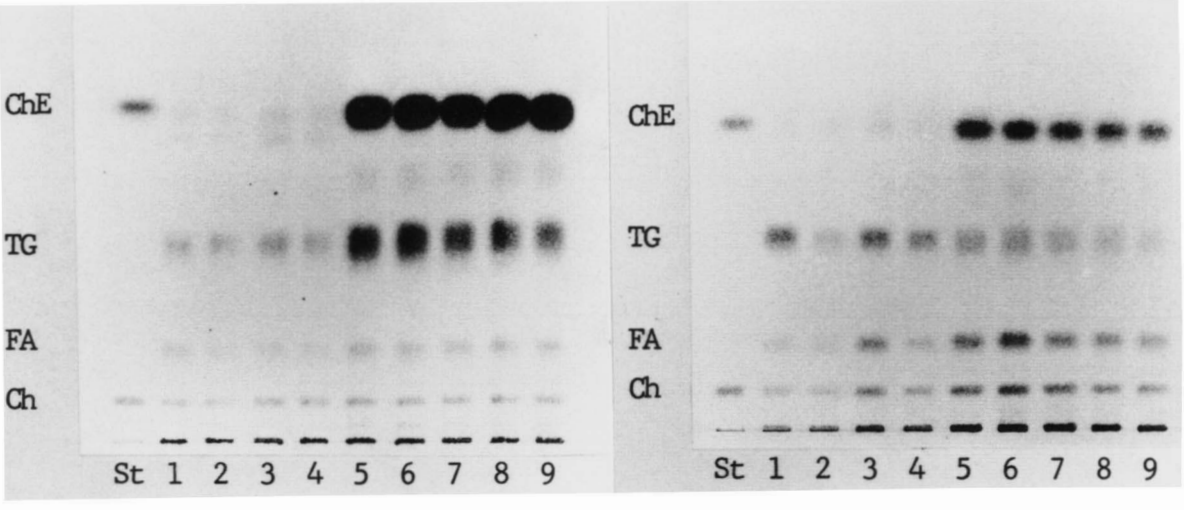

Fig. 1. Thin-layer chromatographic patterns of neutral lipids of livers (left) and spleens (right); lanes 1 and 2, control rats; lanes 3 and 4, heterozygous rats; lanes 5, 6, 7, 8, and 9, affected rats; ChE, cholesteryl esters; TG, triglycerides; FA, fatty acids; Ch, free cholesterol.

TABLE 1. Amount of cholesterol, triglyceride, and phospholipid in livers and spleens from affected, heterozygous, or control rats

Tissue	Cholesterol			Triglyceride	Phospholipid
	Total	Free	Esterified		
	μg/mg wet weight of tissue ± SD				
Liver					
Affected rats (n = 5)[a]	33.18 ± 2.28	5.62 ± 0.56	27.56 ± 1.80	87.17 ± 12.80	18.77 ± 2.21
Heterozygous rats (n = 6)	3.88 ± 0.40	1.80 ± 0.27	2.08 ± 0.29	18.04 ± 3.47	21.56 ± 2.22
Normal controls (n = 5)	4.06 ± 0.56	1.99 ± 0.44	2.07 ± 0.55	16.01 ± 2.47	23.01 ± 3.71
Spleen					
Affected rats (n = 5)	9.17 ± 0.82	2.57 ± 0.47	6.60 ± 0.73	31.30 ± 11.17	15.85 ± 1.53
Heterozygous rats (n = 6)	4.54 ± 0.47	2.32 ± 0.07	2.22 ± 0.40	55.25 ± 34.48	15.80 ± 1.96
Normal controls (n = 5)	4.61 ± 0.99	1.92 ± 0.41	2.69 ± 0.67	52.15 ± 31.88	16.00 ± 2.94

[a]Number of rats.

and spleens of affected rats. The activity for native substrates [^{14}C]triolein and [^{14}C]cholesteryl oleate was markedly decreased, while residual activity for synthetic substrate 4-MU oleate was 36.0% of the control level in the liver and 55.3% in the spleen. Heterozygous rats showed values that were intermediate between those in the affected rats and those in the normal controls for both organs.

The effects of pH on lipase activities for [^{14}C]triolein were examined in liver and spleen of an affected rat and a normal control (**Fig. 3**). The maximum activity was at pH 4.5 and minor peaks of activity were seen at a pH of 6.5 or 7.0 in the normal liver and spleen. The affected rat showed marked deficiencies of activity at pH 3.5–5.0 in both organs. The activities of the other seven lysosomal hydrolases were increased in the livers of affected rats but were nearly normal in the spleens, as shown in **Table 4**.

DISCUSSION

Brown and Goldstein and others (17–19) have established the role of lysosomal acid lipase in the cellular

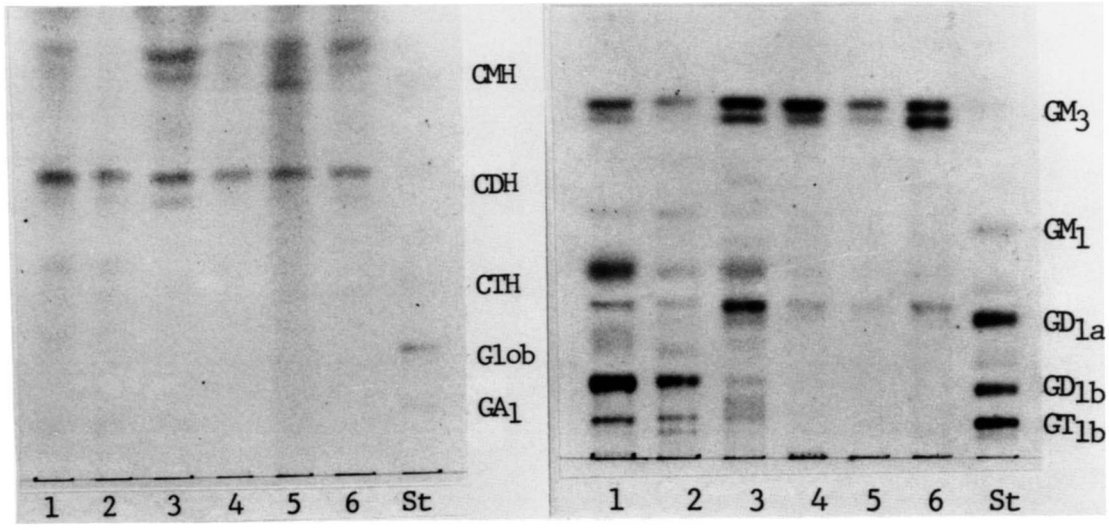

Fig. 2. Thin-layer chromatographic patterns of neutral glycosphingolipids (left) and gangliosides (right) of livers and spleens; lanes 1, 2, 4, and 5, control rats; lanes 3 and 6, affected rats; lanes 1, 2, and 3, livers; lanes 4, 5, and 6, spleens; CMH, ceramide monohexoside; CDH, ceramide dihexoside; CTH, ceramide trihexoside; Glob, globoside; GA1, asialo GM1.

TABLE 2. Fatty acid composition of cholesteryl ester and triglyceride in liver and spleen

Fatty Acid	Liver				Spleen			
	ChE		TG		ChE		TG	
	AR	NR	AR	NR	AR	NR	AR	NR
16:0	18.24	5.02	16.71	25.40	19.97	28.74	24.94	22.25
16:1	3.65	5.52	2.06	3.66	3.71	3.29	1.96	2.38
18:0	4.26	1.95	5.09	3.59	8.00	18.57	8.58	7.46
18:1	28.71	62.78	22.23	31.70	44.15	15.42	30.53	32.99
18:2	29.15	12.02	36.73	29.85	9.03	5.60	23.02	19.66
18:3	2.01	3.31	2.11	1.28	1.88	1.55	2.92	1.85
20:1	tr	tr	tr	tr	2.89	tr	0.39	1.29
20:2	tr	1.86	0.22	0.34	tr	tr	0.75	tr
20:3	0.25	1.08	0.33	0.28	0.85	1.15	1.08	0.32
20:4	9.78	1.31	4.86	1.53	1.13	0.96	1.97	0.81
20:5	0.49	1.26	1.71	0.17	tr	3.14	0.78	0.51
22:4	tr	tr	0.66	0.16	tr	tr	tr	0.05
22:5	tr	tr	1.19	0.30	tr	tr	tr	tr
Others	3.26	3.89	6.10	1.74	8.39	22.58	3.08	10.43

ChE, cholesteryl ester; TG, triglyceride; AR, affected rat; NR, normal control rat; tr, trace.

degradation of plasma low density lipoproteins (LDL). They demonstrated that LDL was taken up through a specific receptor by peripheral cells, the LDL receptor. Endocytotic vesicles containing LDL then fuse with lysosomes, and the apolipoproteins, cholesteryl esters, and other lipid constituents undergo hydrolysis by lysosomal enzymes. The lysosomal degradation of cholesteryl esters is catalyzed by acid lipase. Free cholesterol is liberated and then transferred from the lysosomes to the cytoplasm where it initiates the following three important regulatory events: *1)* reduction of cellular cholesterol synthesis through suppression of the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase; *2)* the repression of LDL receptor synthesis; and *3)* stimulation of the formation of cellular cholesteryl esters through activation of microsomal membrane-bound acyl-CoA:cholesterol acyltransferase (ACAT). Lysosomal acid lipase also hydrolyzes triglycerides. Therefore, it is one of the key enzymes in the regulation of triglycerides as well as being vital in lipoprotein and cholesterol metabolism.

Cholesteryl esters and triglycerides are the principal lipids that accumulate in the tissues of patients with Wolman's disease. Their content in the liver and spleen is elevated from a few to more than 100 times the normal level. The level of free cholesterol is also frequently increased (1). The affected rats in our model showed accu-

TABLE 3. Acid lipase activities in livers and spleens from affected, heterozygous, or control rats

Tissue	Substrate					
	[^{14}C]Triolein		[^{14}C]Cholesteryl Oleate		4-MU-Oleate	
Liver						
Affected rats (n = 5)[a]	0.30 ± 0.12	(8.1%)	0.12 ± 0.08	(19.0%)	17.67 ± 1.58	(36.0%)
Heterozygous rats (n = 6)	2.09 ± 0.55	(56.2%)	0.33 ± 0.12	(52.4%)	28.13 ± 7.06	(57.3%)
Normal controls (n = 5)	3.72 ± 1.57	(100%)	0.63 ± 0.20	(100%)	49.08 ± 18.76	(100%)
Spleen						
Affected rats (n = 5)	1.26 ± 0.30	(11.1%)	0.24 ± 0.06	(15.5%)	57.83 ± 7.58	(55.3%)
Heterozygous rats (n = 6)	8.38 ± 1.50	(74.0%)	1.08 ± 0.21	(69.7%)	87.69 ± 13.40	(83.9%)
Normal controls (n = 5)	11.33 ± 2.15	(100%)	1.55 ± 0.17	(100%)	104.55 ± 18.80	(100%)

Activities are expressed as nmol of substrate hydrolyzed per mg protein per h ± SD.
[a]Number of rats.

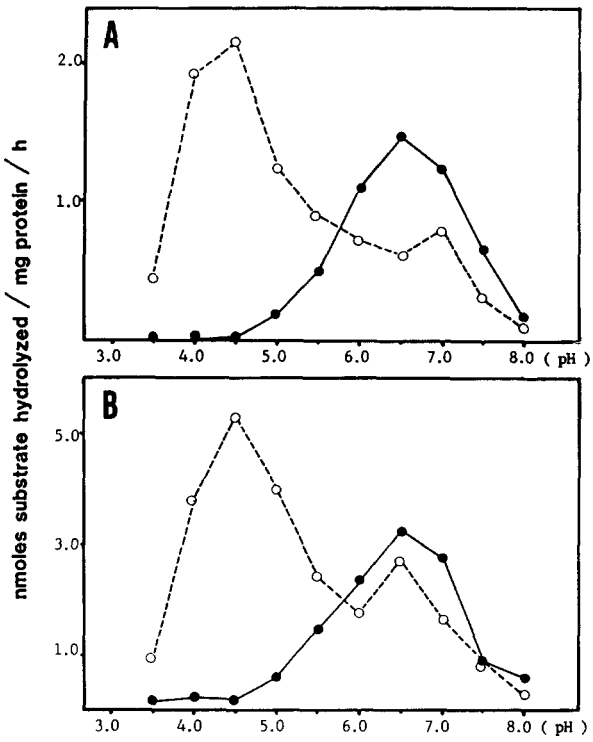

Fig. 3. The effects of pH on lipase activities towards [^{14}C]triolein in liver (A) and spleen (B); (○---○), control rat; (●—●), affected rat.

mulation of total cholesterol, both esterified and free forms, in the liver and spleen. The triglyceride level was also elevated 5.4-fold above that of the controls in the liver, but not in the spleen. Acid lipase activity for three different substrates, [^{14}C]triolein, [^{14}C]cholesteryl oleate, and 4-MU oleate, was similarly decreased in both organs. These biochemical abnormalities are quite similar to those seen in Wolman's disease in humans, except for the splenic triglyceride level. The discrepancy of a normal or decreased level of triglyceride despite the marked deficiency of [^{14}C]triolein metabolism might be due to the following: a compensatory elevation of catalytic activity by neutral lipase, a different degree of disturbance of triglyceride metabolism in the spleen, or differing severites of involvement of the heterogenous cell population of the spleen. The affected rats showed marked deficiency of lipase activity for [^{14}C]triolein at pH 3.5-5.0 in both spleen and liver, but normal activity at neutral pH in both organs. Thus, no compensatory elevation of neutral lipase activity occurred in the spleen.

The affected rats accumulated free cholesterol as well as cholesteryl esters, which suggested that their cholesterol synthesis was enhanced. This probably occurred through the activation of HMG-CoA reductase due to decreased feedback regulation of the reduction of hydrolyzed cholesterol transferred from lysosomes. Plasma cholesterol is mainly esterified to linoleic acid (18:2) in LDL, while cytoplasmic cholesterol transferred from lysosomes is esterified to oleic acid (18:1) or other C-14 to C-18 saturated or monounsaturated fatty acids by ACAT (1). The cholesteryl esters that accumulated, especially those in the livers of affected rats, contained a significantly higher percentage of linoleic acid than those in control rats. Arachidonic acid (22:4) levels were also increased in the affected rats. These results suggested that the accumulated cholesteryl esters were largely derived from the uptake of plasma LDL.

TABLE 4. Lysosomal enzyme activities in livers and spleens

Tissue	Enzyme						
	α-Gal	β-Gal	N-Ac-β-Glc	α-Fuc	α-Glc	β-Glc	β-Gluc
Liver							
Affected rats (n = 5)[a]	234 ± 21	806 ± 39	423 ± 50	403 ± 26	124 ± 20	44 ± 7	869 ± 79
Heterozygous rats (n = 6)	79 ± 19	194 ± 35	214 ± 70	82 ± 10	20 ± 3	5 ± 1	606 ± 115
Normal controls (n = 5)	102 ± 29	259 ± 105	290 ± 139	93 ± 21	19 ± 5	7 ± 3	734 ± 192
Spleen							
Affected rats (n = 5)	356 ± 38	1095 ± 108	381 ± 133	480 ± 71	195 ± 25	113 ± 16	1034 ± 108
Heterozygous rats (n = 6)	598 ± 57	1643 ± 228	514 ± 235	520 ± 61	186 ± 13	64 ± 12	1349 ± 285
Normal controls (n = 5)	607 ± 66	1668 ± 199	632 ± 150	493 ± 37	182 ± 25	71 ± 18	1320 ± 263

Activities are expressed as nmol of 4-MU liberated per mg protein per h ± SD. α-Gal, α-galactosidase; β-Gal, β-galactosidase; N-Ac-β-Glc, N-acetyl-β-glucosaminidase; α-Fuc, α-fucosidase; α-Glc, α-glucosidase; β-Glc, β-glucosidase; β-Gluc, β-glucuronidase.
[a]Number of rats.

Acid lipase has been purified or partially purified from a variety of sources, including the liver (20–24), the placenta (25), the aorta (26), and the fibroblasts (27). Both acid triglyceride lipase and acid cholesterol esterase activities from the liver were purified together (21, 22, 24). On the other hand, it has been reported that the acid cholesteryl ester hydrolase and acid triglyceride hydrolase in human mononuclear leucocytes were related to different proteins, since these activities were solubilized under different conditions (28). In the livers and spleens of the affected rats, the metabolism of cholesteryl oleate and triglycerides was proportionally decreased. These results suggested that the acid cholesteryl ester hydrolase and acid triglyceride hydrolase activities in liver and spleen actually reside in a single enzyme. The residual activity for the synthetic substrate 4-MU oleate was higher than that for natural substrates. This was probably a result of hydrolysis by phospholipases or nonspecific esterases, or else of the different levels of affinity between the synthetic and natural substrates. The changes in activity for 4-MU oleate were very sensitive, and could differentiate heterozygous rats from affected rats.

In 1974, de Duve (29) proposed that lipid accumulation in the arterial wall might possibly be the consequence of a relative deficiency of lysosomal acid lipase. The occurrence of premature atherosclerosis in patients with cholesteryl ester storage disease supports his hypothesis (1). Atherosclerosis can be induced by various pathological factors and a deficiency of lysosomal acid lipase is one of these. The biological consequences of defective acid lipase in this animal model can give us some insight into the physiological and pathological roles of this enzyme in vivo.

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