Lysosome Lipid Storage Disorder in NCTR-BALB/c Mice

III. Isolation and Analysis of Storage Inclusions From Liver

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Livers of NCTR-BALB/c mice, affected by excessive accumulation of cholesterol and phospholipid, were fractionated by sucrose density gradient centrifugation. Lysosomes of very low density (ϱ = 1.05 – 1.08) were found, which by electron microscopy appeared identical to the storage inclusions seen in fixed tissues. These lysosomes could be purified about 10-fold over the original homogenate, and represented 4% of the total protein and 30–40% of the liver acid hydrolase content. The preparations were nearly free of mitochondrial, endoplasmic reticulum, and plasma membrane contamination. The lysosomes were laden with cholesterol and phospholipid. Cholesterol (>97% unesterified) accounted for half of the total lipid, and sphingomyelin accounted for another 20%. Phosphatidylcholine and phosphatidylethanolamine were also present in substantial quantities. All of the excess cholesterol and sphingomyelin of liver could be attributed to the low density lysosomes. Lysosomal acid sphingomyelinase activity, measured with a synthetic substrate, was found to be 10–60% of BALB/c mouse control levels in liver, spleen, and cerebellum, while two other lysosomal enzymes, N-acetyl-β-glucosaminidase and β-glucuronidase, were increased 2–8-fold in the same tissues. These data and the morphologic observations of the preceding paper establish that the disorder affecting NCTR-BALB/c mice is a lysosome storage disease. We propose several possible mechanisms to explain the cholesterol and phospholipid overloading of lysosomes. The specific gene defect remains to be established. (Am J Pathol 1982, 108:160–170)

IN THE PRECEDING articles of this series we described the pathologic characteristics of the NCTR-BALB/c mice affected with a genetic neurovisceral disorder and provided morphologic evidence for excessive accumulation of cholesterol and phospholipids in lysosomes of cells of many tissues.[1,2] In the present article we describe the isolation of the hepatic inclusions by sucrose density gradient centrifugation and their content in various enzymes and lipids. Our results add further support to the conclusion that the NCTR-BALB/c mice are affected by a lysosomal lipidosis, similar to certain sphingolipidoses in humans.[3] However, the precise biochemical defect producing the lysosome overloading of both cholesterol and phospholipid remains unknown, although several possibilities can be proposed and are presented here.

A preliminary communication of these investigations has been given previously.[4]

Materials and Methods

Animals

Male and female mice of the NCTR-BALB/c strain were used in these studies; they were from 60 to 75 days old, and those affected with the genetic disorder

Supported by research grants AM-21376 and HL-18157 from the National Institutes of Health and PCM-8008713 from the National Science Foundation. Dr. Fowler conducted these studies during the tenure of an Otto G. Storm Established Investigatorship from the American Heart Association.

Accepted for publication March 10, 1982.

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0002-9440/82/0810-0160$01.35

were identified by the set of symptoms described previously.[1] The mice were killed by cervical dislocation, or in some experiments by exsanguination by heart puncture after anesthesia with Metofane (Pitman–Moore, Washington Crossing, NJ).

Subcellular Fractionation

Individual livers were fractionated by differential and isopyknic centrifugation as follows. Following bleeding, the liver was quickly excised, weighed (about 0.7 g), and cut into fine pieces. The liver mince was homogenized in 3 ml of ice-cold 250 mM sucrose containing 1 mM EDTA (homogenization medium) with a Potter–Elvehjem homogenizer (Kontes Glass Company, Vineland, NJ) fitted with a Teflon pestle. A single up–down stroke of the pestle rotating at 1100 rpm was used in this step. The homogenate produced was centrifuged at 750*g* for 10 minutes in a Sorvall SS-34 rotor (Ivan Sorvall, Norwalk Conn) at 2 C. The supernatant was decanted and saved; the pellet, containing cell debris, nuclei, and unbroken cells, was homogenized again (one stroke) in 3 ml of homogenization medium and centrifuged as before. The supernatant from the second centrifugation was pooled with that from the first, and the volume was recorded (postnuclear supernatant). The pellet was also resuspended in homogenization medium to a fixed volume (nuclear fraction).

Two milliliters of the postnuclear supernatant were gently mixed with 1 ml of ice-cold 70% (wt/wt) sucrose to bring the density of the sample to 1.143 at 0 C. Two milliliters of the density-adjusted sample were layered above 1.5 ml of 1.295 density sucrose in a precooled centrifuge tube. The sample was carefully topped by an 11-ml linear sucrose gradient with density limits of 1.135–1.034 at 0 C. The preparation was centrifuged at 40,000 rpm in a Beckman SW-40 rotor (Beckman Instruments, Palo Alto, Calif) for 17 hours at 0 C. The tube contents were collected into 11 approximately equal fractions. These were weighed, the individual densities were determined by refractometry (Bausch and Lomb Precision Refractometer, Bausch and Lomb, Rochester, NY), and the volumes were calculated. The gradient fractions were then assayed for protein, cholesterol, and marker enzyme activities.[5]

The results are presented in the form of histograms.[6] The ordinate is the relative concentration of the enzyme, which is defined as the concentration in the fraction relative to the concentration the enzyme would have if it were homogeneously distributed throughout the gradient. The abscissa represents the normalized gradient volume. Pooling and averaging of several distributions were performed by computer as described by Leighton et al.[5] The method requires conversion of all the histograms to the same preset volume intervals and causes some loss in resolution. A plot of the density gradient is given separately; and, in order to facilitate interpretation, the starting conditions are also depicted on the diagrams as shaded bars.

Enzyme Assays

Marker Enzymes

N-acetyl-β-glucosaminidase and *β-glucuronidase* were assayed by measurement at 410 nm of the liberation of p-nitrophenol from their respective substrates.[7] The reaction mixture contained 1 mM substrate and 100 mM buffer (N-acetyl-β-glucosaminidase: p-nitrophenyl-2-acetamido-2-deoxy-β-glucopyranoside, sodium citrate, pH 4.8; β-glucuronidase: p-nitrophenyl-β-D-glucuronide, sodium acetate, pH 4.0), 60 mM sucrose, 0.2% Triton X-100, and enzyme (cell fraction) in a total volume of 0.5 ml. The incubation was carried out at 37 C for 30 minutes and was stopped by addition of 5 ml of a solution containing 50 mM sodium glycine, pH 10.4, 5 mM EDTA, 5% Triton X-100. *Alkaline phosphodiesterase I* was similarly determined with 1.4 mM p-nitrophenyl-thymidine-5′-phosphate as substrate in 0.5 ml medium containing 100 mM sodium glycine, pH 9.6, and 2 mM zinc acetate.[7] The incubation was carried out at 25 C for 15 minutes and stopped by addition of 2 ml of 100 mM sodium hydroxide. *Glucose-6-phosphatase* was assayed by incubation of the cell fractions in 0.5 ml of reaction mixture containing 40 mM glucose-6-phosphate, 20 mM sodium histidine buffer, pH 6.5, and 1 mM EDTA.[7] The incubation was carried out at 37 C for 30 minutes and was stopped by addition of 2.5 ml of cold 8% trichloroacetic acid. Denatured proteins were sedimented by centrifugation (1300*g* for 5 minutes), and 0.5-ml aliquots of the clear supernatants were assayed for inorganic phosphate by the method of Fiske and Subbarow.[8] *Cytochrome oxidase* was assayed by following oxidation of reduced cytochrome *c* at 550–540 nm wavelength pair.[7,9] Cell fractions were diluted 5-fold with an ice-cold solution of 1 mM EDTA, 1 mM sodium bicarbonate, 0.01% Triton X-100, and 2.0% sodium deoxycholate. Reaction was initiated by addition of 0.05 ml of the diluted sample to 3.0 ml of the reaction mixture containing 50 μM cytochrome *c* (90% reduced by addition of dithionite), 30 mM potassium phosphate buffer, pH 7.4, and 1 mM EDTA, and the rate of oxidation of cytochrome C was followed for two minutes at 28 C.

For glycosidases and phosphatases, 1 unit of activity is defined as the hydrolysis of 1 μmole of substrate per minute. One unit of cytochrome oxidase activity is expressed as 1 μmole of cytochrome *c* oxidized per minute computed from the initial rates of the reaction.

Acid Sphingomyelinase

Acid sphingomyelinase activity was determined with 2-hexadecanoyl-amino-4-nitrophenylphosphocholine as substrate.[10] The reaction mixture contained 15 mM substrate, 250 mM sodium acetate buffer, pH 5.3, 0.5% Triton X-100, 0.005% bovine serum albumin, 12.5 mM sucrose, and a diluted tissue sample in a total volume of 0.2 ml. After incubation for 60 minutes at 37 C, the reaction was stopped by addition of 5 ml 100 mM sodium glycine buffer, pH 10.5, and the release of hexadecanoyl-amino-4-nitrophenol measured at 410 nm. One unit of activity corresponds to the hydrolysis of 1 μmole of substrate per minute.

Latency of N-acetyl-β-glucosaminidase

Latent activity of N-acetyl-β-glucosaminidase was used as a measure of intactness of the lysosomes.[11] Postnuclear supernatants and lysosome-rich fractions were diluted in ice-cold 250 mM sucrose containing 1 mM EDTA and assayed for N-acetyl-β-glucosaminidase activity in the absence (free activity) and presence (total activity) of 0.2% Triton X-100 to disrupt the lysosome membranes. Assay conditions were those described above, except that the concentration of sucrose was 250 mM, 200 μg bovine serum albumin was added to the reaction mixture, and the incubation was carried out at 37 C for 15 minutes. Latency was calculated as the difference between total and free activity expressed as a percentage of total activity.

Protein

Proteins were determined by a modification of the Lowry method, utilizing the colorimetric transform of Coakley and James.[12]

Lipid Analysis

Cholesterol and phospholipid determinations on subcellular fractions were performed as described in the preceding paper.[1]

Electron Microscopy

Aliquots of the gradient fractions of density 1.05–1.08 obtained after isopycnic fractionation of affected mouse liver postnuclear supernatants were pooled and mixed 1 : 1 (vol/vol) with ice-cold 4% glutaraldehyde in 200 mM sodium cacodylate buffer, pH 7.4, and fixed for 30 minutes. The fixed samples were then collected at room temperature by filtration onto 0.05-μ-pore (VMWP) Millipore filters (Millipore Corp., Bedford, Mass) by the method of Baudhuin et al.[13] The filter with deposited sample on top was post-fixed in 1% OsO_4 in 100 mM cacodylate buffer, pH 7.4, for 30 minutes at 0 C, stained with uranyl acetate,[14] and dehydrated in graded alcohol. The filter was dissolved in propylene oxide, and the remaining sample was embedded in Epon 812. Silver sections were cut perpendicular to the bottom of the pellicle and collected on 200-mesh Formvar–carbon-coated copper grids. The sections were doubly stained with uranyl acetate and lead citrate before viewing by electron microscopy as described previously.[2]

Results

Marker Enzyme Activities

A survey of cellular enzymes in livers of affected NCTR-BALB/c mice, compared with livers of age-matched control BALB/c mice, revealed 2–3-fold increases in two acid hydrolases, N-acetyl-β-glucosaminidase and β-glucuronidase (Table 1). Alkaline phosphodiesterase I activity was the same in affected and control livers. Glucose-6-phosphatase and cytochrome oxidase activities were decreased in the affected livers; protein content was likewise decreased, whereas cholesterol content was increased 12-fold.

Subcellular Fractionation

Figure 1 shows the results of subfractionation in sucrose density gradients of the postnuclear supernatants of control BALB/c and affected NCTR-BALB/c livers. Enzyme, protein, and cholesterol recoveries from the sucrose gradients are given in Table 2. As indicated in Table 1, 60% or more of the marker enzyme activities were present in the postnuclear supernatants, except for cytochrome oxidase (25–30%). The density gradient was designed to keep most of the subcellular organelles near the initial sample layer. For control BALB/c mice (Figure 1, left), only a portion of the alkaline phosphodiesterase-I activity (plasma membrane) moved up into the gradient (density range 1.12–1.14). Cholesterol similarly partially moved into the gradient, reflecting the known association of liver cholesterol with the plasma membrane.[15]

In contrast to control livers, most of the cholesterol of affected livers traversed the entire gradient and accumulated in the first three fractions (densities 1.05–

Table 1—Marker Enzyme Activities and Cholesterol and Protein Contents of Livers of Control BALB/c and Affected NCTR-BALB/c Mice

	Content*						
	N-acetyl-β-glucosaminidase	β-Glucuronidase	Alkaline phosphodiesterase-I	Glucose-6-phosphatase	Cytochrome oxidase	Total cholesterol	Protein
Control (C)	0.863 ± 0.26 (62.3% ± 10%)†	0.338 ± 0.02 (78.3% ± 9%)	2.39 ± 0.9 (56.2% ± 15%)	15.4 ± 3 (91.3% ± 10%)	218 ± 40 (25.9% ± 7%)	1.95 ± 0.3 (80.6% ± 15%)	175 ± 30 (69.1% ± 8%)
Affected (A)	2.74 ± 0.5 (93.0% ± 5%)†	0.778 ± 0.04 (108% ± 12%)	2.53 ± 1.6 (72.7% ± 19%)	9.57 ± 1.2 (87.4% ± 6%)	129 ± 10 (31.4% ± 7%)	22.4 ± 5 (96.1% ± 8%)	136 ± 10 (74.7% ± 3%)
A/C‡	3.2	2.3	1.1	0.62	0.59	11.5	0.78

* Enzyme activities are given in units/gram liver wet weight and total cholesterol and protein are given in milligrams/gram liver wet weight. The values are expressed as the mean ± SD of four separate animals (1 male and 3 females) in each group.
† Percent of homogenate content in postnuclear supernatant.
‡ Ratio of mean contents of affected and control livers.

1.08) (Figure 1, right). It should be kept in mind that these graphs are normalized; the cholesterol content of the postnuclear supernatants of affected livers was, on an average, 14-fold greater than that present in the postnuclear supernatants of control BALB/c livers. A considerable part of β-glucuronidase, and even more of N-acetyl-β-glucosaminidase, two known lysosomal enzymes, also appeared in the first three gradient fractions. The plasma membrane marker, alkaline phosphodiesterase-I, equilibrated at the same densities as for the control BALB/c livers; and, similarly, glucose 6-phosphatase, a microsomal enzyme, and cytochrome oxidase, a mitochondrial enzyme, remained distributed near the sample layer as in the control experiments. In contrast to the controls, a small but consistent amount of protein appeared in Fractions 1-3 of gradients following subfractionation of affected livers.

The percent latency of N-acetyl-β-glucosaminidase can be used as a measure of lysosome integrity.[11] Results of latency tests shown in Table 3 indicate that intact lysosomes were present in the low-density gradient fractions. Thus, a new population of lysosomes of very low density appears in the livers of affected NCTR-BALB/c mice, and it accounts for much of the N-acetyl-β-glucosaminidase and β-glucuronidase activities present in such livers (Figure 1).

Pooled Fractions 1-3, fixed and filtered on Millipore filters, were examined by electron microscopy. Small vesicular structures, 100–400 nm in diameter, were found (Figure 2A and B), identical in appearance to the lipid inclusions observed in liver foam cells and parenchymal cells *in vivo*.[2] Only a few large inclusions, like those seen in the foam cells in fixed tissue preparations, were identified in the isolated fractions. Perhaps many of the very large inclusions were destroyed during homogenization and centrifugation. Crystal-containing structures and multilamellar bodies were rarely seen in the isolated fractions. Since latency of N-acetyl-β-glucosaminidase in Fractions 1-3 was greater than 70% (Table 3), each of the vesicles seen in Figure 2 probably represents an intact lysosome.

Composition of the Low-Density Lysosomes

Results listed in Table 4 allow an assessment of the purity of the low-density lysosomes isolated from the livers of the affected animals. From their contents in N-acetyl-β-glucosaminidase, it would appear that the

Table 2—Complementary Data on Cell Fractionation Experiments*

	Enzyme recoveries from density gradient†						
	Total Cholesterol	β-Glucuronidase	Alkaline phosphodiesterase-I	N-acetyl-β-glucosaminidase	Glucose-6-phosphatase	Protein	Cytochrome oxidase
Control	105.2 ± 21 (8)	85.9 ± 13 (6)	81.4 ± 21 (6)	104.0 ± 17 (4)	74.1 ± 15 (4)	90.3 ± 18 (8)	89.7 ± 13 (5)
Affected	102.5 ± 6 (6)	97.1 ± 15 (7)	94.7 ± 26 (6)	100.4 ± 20 (8)	98.8 ± 16 (5)	98.5 ± 8 (8)	92.7 ± 18 (6)

* Values refer to experiments described in Figure 1. Mean ± SD with number of experiments in parentheses.
† Sum of contents of gradient fractions expressed as percentage of content of postnuclear supernatant fraction.

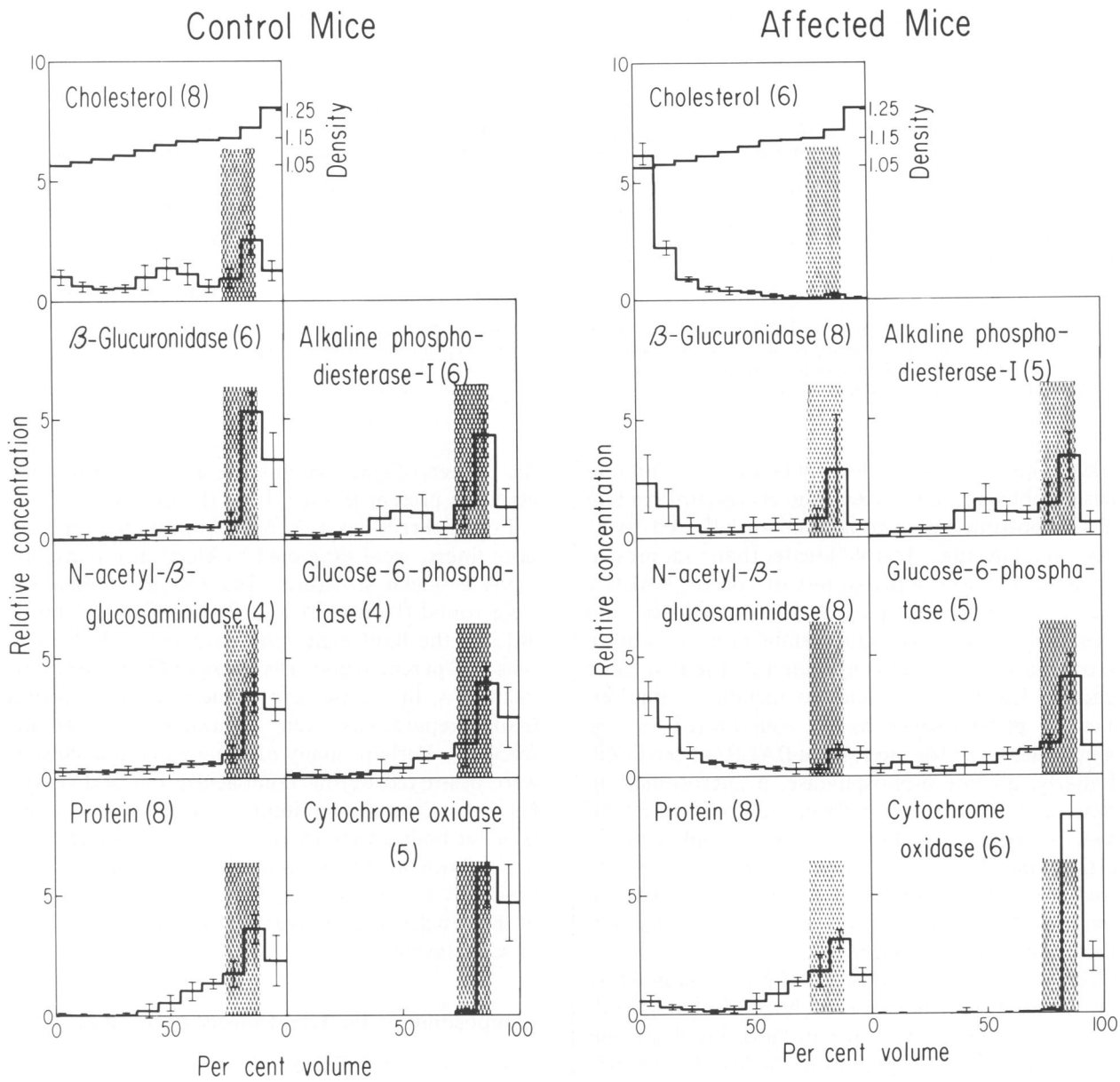

Figure 1 — Sucrose gradient centrifugation of postnuclear supernatants of livers of control BALB/c (*left*) and of affected NCTR-BALB/c (*right*) mice. The graphs present distributions of cholesterol, marker enzymes, and protein as a function of the volume recovered from the tube. Density gradient is shown by the "*staircase*" on top. The *shaded area* represents the initial position of the sample. Recoveries are shown in Table 2. The number of experiments is given in parentheses, and vertical bars represent standard deviations.

lysosomes were purified almost 10-fold, with a 40% yield. Their lower content in β-glucuronidase is readily explained by the fact that part of this enzyme is microsomal in mouse liver.[16,17] On the other hand, the low specific activities of alkaline phosphodiesterase I and of glucose-6-phosphatase indicate a low degree of contamination by plasma membranes and by endoplasmic reticulum. If the proportion of the total proteins associated with these two structures is the same in mouse as in rat liver (about 2.5% for plasma membranes, and 20% for endoplasmic reticulum),[18,19] contamination by plasma membranes amounts to $0.3 \times 2.5 = 0.75\%$, and by endoplasmic reticulum to $0.1 \times 20 = 2\%$, of the total protein of the preparation. Apparently, the pooled fractions contained no mitochondria, as indicated by their lack of cytochrome oxidase activity. Thus, the low-density lysosomes were probably more than 95% pure, a conclusion corroborated by the morphologic appearance of the fractions.

As shown in Table 4, cholesterol was concentrated almost 12-fold in the purified fractions, with a yield

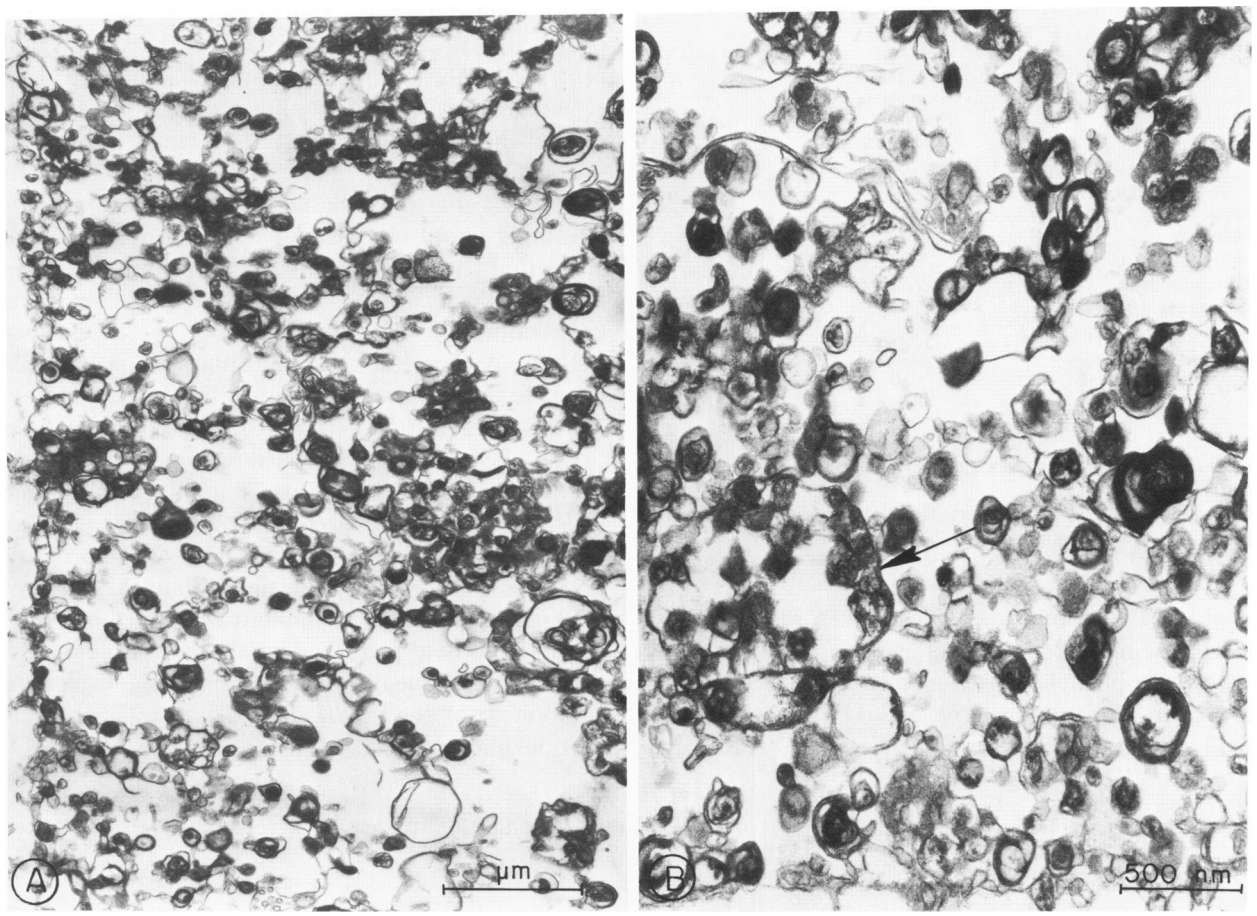

Figure 2—Electron micrograph of low-density lysosomes (pooled gradient fractions of density 1.05–1.08) isolated from livers of affected NCTR-BALB/c mice. **A**—Low-power view (× 19,000). Fixed samples were collected by Millipore filtration (filter positioned on left-hand side); random sampling of particles is seen in the vertical direction. **B**—Higher magnification (× 33,000). *Arrow* points to a large inclusion typical of structures seen in liver foam cells.[2] (With a photographic reduction of 4%)

amounting to 49% of the total cholesterol, and therefore to some 54% of the *excess* cholesterol present in the livers of the affected animals (about 90–92% of the total[1]). This cholesterol is clearly located inside the lysosomes, as shown by the morphologic evidence, and is no doubt responsible, together with the phospholipids (see below), for the low density of the particles. In other experiments, the cholesterol yield in the purified lysosomes reached almost 70% of the total (Table 5). It is very likely, therefore, that the remainder of the excess cholesterol accumulated by the affected animals is likewise intralysosomal but occurs in particles that, owing to their smaller size or higher density, do not reach the top layers of the gradient (see Figure 1).

As illustrated by the results of Table 5, the purified low-density lysosomes also contained considerable amounts of phospholipids, about equal in total

Table 3—Latency of N-acetyl-β-glucosaminidase in Low-Density Fractions Obtained From Postnuclear Supernatant of Affected NCTR-BALB/c Mouse Liver After Isopyknic Centrifugation

		Percent latency*		
	Density range	Experiment 1	Experiment 2	Experiment 3
Postnuclear supernatant		75.6	75.0	74.0
Fraction 1	1.051–1.054	93.5	84.0	84.0
Fraction 2	1.060–1.064	76.5	80.0	80.0
Fraction 3	1.071–1.077	60.0	72.0	73.0

* Difference between total activity (assayed in presence of detergent) and free activity (assayed without detergent) expressed as a percentage of total activity.

Table 4—Purification of Low-Density Lysosomes From Livers of Affected NCTR-BALB/c Mice*

	% of total liver homogenate content in pooled fractions 1-3	Relative specific content†
Protein	4.2 ± 1	1.0
N-acetyl-β-glucosaminidase	39.1 ± 5	9.4 ± 2
β-Glucuronidase	27.8 ± 8	6.8 ± 3
Alkaline phosphodiesterase-I	1.3 ± 1	0.3 ± 0.2
Glucose-6-phosphatase	0.5 ± 1	0.1 ± 0.2
Cytochrome oxidase	0	0
Cholesterol	48.8 ± 7	11.7 ± 3

* Analyses were performed on pooled gradient fractions (1-3) of density 1.05-1.08. Values are expressed as the mean ± SD of three separate experiments.

† Specific content of pooled gradient fractions per specific content in liver homogenate.

weight to the amount of cholesterol stored. In addition to the main components listed, some *bis*(monoacylglycero)phosphate was also present. No cholesteryl esters or triacylglycerols could be detected; only small amounts of glycolipids were found. Among the major phospholipids, sphingomyelin was concentrated almost 12-fold, whereas the others were concentrated only 6–7-fold. Sphingomyelin, it will be remembered,[1] is the only phospholipid found in excess in the livers of the affected animals, where its proportional concentration is increased almost as much as is that of cholesterol. Extending the above reasoning about cholesterol, we may therefore conclude that the *excess* sphingomyelin accumulated in the livers of the affected animals is most likely segregated entirely in the lysosomes, as is the excess cholesterol. As for the other phospholipids—to which must be added a small fraction of the sphingomyelin—it is interesting that they occur in the lysosomes of the affected animals in relative amounts that are very similar to their relative abundance in normal liver. The phospholipid/protein ratio, after correction for the excess sphingomyelin, is of the order of 0.95, which is only slightly higher than the value of 0.79 recorded for stripped microsomal membranes.[20] In view of these facts and of the morphologic evidence showing membranous debris as a conspicuous component of the lipid-laden lysosomes, it is likely that the phospholipids other than the excess sphingo-

Table 5—Cholesterol and Phospholipid Content of Low-Density Lysosomes From Livers of Affected NCTR-BALB/c Mice

	Content*					
Fraction	Cholesterol	Total phospholipid	Sphingomyelin	Phosphatidyl choline and lysophosphatidyl choline	Phosphatidyl ethanolamine†	Phosphatidyl serine and phosphatidyl inositol
Liver homogenate	0.121 ± 0.02 (97.7% ± 1%)‡	0.198 ± 0.02	0.054 ± 0.01 (27.2% ± 4%)§	0.078 ± 0.01 (39.3% ± 3%)§	0.051 ± 0.01 (25.8% ± 1%)§	0.014 ± 0.01 (7.2% ± 1%)§
Low-density lysosomes‖	1.542 ± 0.22 (97.5% ± 1%)‡	1.553 ± 0.21	0.630 ± 0.11 (40.5% ± 3%)§	0.450 ± 0.05 (29.5% ± 2%)§	0.338 ± 0.04 (22.2% ± 1%)§	0.102 ± 0.02 (6.7% ± 1%)§
Enrichment in lysosomes¶	12.8 ± 1	7.9 ± 1	11.7 ± 1	5.9 ± 1	6.6 ± 1	7.1 ± 1
Percent of homogenate content in lysosomes**	67.9 ± 5	41.6 ± 6	62.2 ± 6	31.0 ± 5	35.3 ± 6	38.1 ± 7

	Lipid molar ratios††				
	Cholesterol/ total phospholipid	Cholesterol/ sphingomyelin	Cholesterol/ phosphatidyl choline and lysophosphatidyl choline	Cholesterol/ phosphatidyl ethanolamine	Cholesterol/ phosphatidyl serine and phosphatidyl inositol
Liver homogenate	1.22 ± 0.1 (0.13 ± 0.01)‡‡	4.38 ± 0.3 (2.83 ± 0.7)‡‡	3.15 ± 0.6 (0.25 ± 0.02)‡‡	4.77 ± 1 (0.42 ± 0.1)‡‡	20.71 ± 6.3 (1.38 ± 0.1)‡‡
Low-density lysosomes	2.00 ± 0.2	4.81 ± 0.3	6.90 ± 1.0	9.20 ± 0.9	30.84 ± 3.7

* Expressed as milligrams per milligram protein and given as the mean ± SD of six separate experiments. Data on 3 males and 3 females were similar and were pooled.

† Some bis(monoacylglyero)phosphate present.

‡ Percent of total cholesterol.

§ Percent of total phospholipid. Phosphate recovered from TLC plates average 92%.

‖ Pooled gradient fractions (1-3) of densities of 1.05-1.08.

¶ Mean specific content of low-density lysosomes/mean specific content of homogenates.

** Lysosomes contained 5.3% ± 1% of total homogenate protein.

†† Assumed molecular weights: cholesterol, 386; sphingomyelin, 750; other phospholipids, 775.

‡‡ Lipid molar ratios for liver homogenates of six control animals.

Table 6—Sphingomyelinase and Acid Glycosidase Activities in Liver, Spleen, and Cerebellum of Control BALB/c and Affected NCTR-BALB/c Mice

Organ	Specific activity*			
	Sphingomyelinase	N-acetyl-β-glucosaminidase	β-Glucuronidase	Protein
Liver				
Control (C)	51.2 ± 32	767 ± 160	294 ± 60	189 ± 10
Affected (A)	5.3 ± 11	1866 ± 230	651 ± 170	147 ± 10
A/C†	0.10	2.4	2.2	0.78
Spleen				
Control (C)	25.2 ± 3‡	1324 ± 230	188 ± 40	130 ± 20
Affected (A)	8.7 ± 3‡	3017 ± 1140	547 ± 140	125 ± 10
A/C	0.35	2.3	2.9	0.92
Cerebellum				
Control (C)	75.0 ± 18	417 ± 80	35 ± 20	100 ± 20
Affected (A)	41.0 ± 13	3057 ± 780	80 ± 20	112 ± 10
A/C	0.55	7.3	2.3	1.1

* Enzyme activities are given in milliunits per gram tissue wet weight and protein is given in milligrams per gram tissue wet weight. The values are expressed as the mean ± SD for 7 animals (2 males, 5 females).

† Ratio of mean activity of affected and control groups.

‡ Sphingomyelinase studies, $n = 4$ (2 males, 2 females).

myelin stored in the low-density lysosomes are associated with partly degraded membrane remnants. This interpretation at the same time accounts for the excess protein found in the lysosomal compartment.

We conclude, therefore, that the contents of the low-density lysosomes consist of three main components, cholesterol, sphingomyelin, and membrane remnants, representing about 37.5%, 15.0%, and 47.5% of their dry weight, respectively. The first two represent authentic excesses, but not the membrane material, which is probably derived from a total liver content that was found to be within the normal range.[1] It is readily calculated that the amount of membrane-associated phospholipids segregated within the lysosomal compartment in this way must be on the order of 40–45% of the total, which means that the affected animals have a hepatic content in *normal*, functional cytomembranes of only 55–60% of that of control animals. This estimate is in keeping with the 40% reduction of the activities of the microsomal glucose-6-phosphatase and of the mitochondrial cytochrome oxidase recorded in Table 1.

Sphingomyelinase Activity

Shown in Table 6 are the levels of acid sphingomyelinase, a known lysosomal enzyme,[21] found in control BALB/c and affected NCTR-BALB/c liver, spleen, and cerebellum. N-acetyl-β-glucosaminidase and β-glucuronidase were also assayed in the same preparations for comparison. Sphingomyelinase activity was greatly reduced in liver and substantially decreased in spleen and cerebellum of affected NCTR-BALB/c mice. In contrast, the two lysosomal enzyme activities were elevated. The excess endogenous sphingomyelin present in the affected tissue homogenates, however, could have influenced our observed activities measured with a synthetic substrate.

Discussion

In addition to the data reported in this series, Pentchev et al[22] have recently described studies using the NCTR strain of BALB/c mice. They found progressive tissue accumulations of glucocerebroside, lactosylceramide, and gangliosides, as well as sphingomyelin and unesterified cholesterol. The former lipids, however, accumulated only in microgram amounts, in contrast to cholesterol and sphingomyelin, which both we and Pentchev et al[22] found occurring in amounts of 10–25 mg/g tissue. Pentchev et al[22] also described marked elevations in the *bis*(monoacylglycero)phosphate content of liver. We observed this phospholipid, which is a lipid possibly unique to lysosomes,[23] in both the liver homogenates of affected mice and in the isolated low-density lysosomes. It appeared in the phosphatidylethanolamine band in our quantitative thin-layer chromatographic analyses.

Abnormal levels of lysosomal enzymes in tissues accompanied the lipid overloading in NCTR-BALB/c mice. Pentchev et al[22] reported diminished activities of acid glucocerebrosidase, β-glucosidase, and sphingomyelinase in several tissues, and we have confirmed the latter finding. At the same time, several lysosomal enzyme activities were consistently elevated. We found N-acetyl-β-glucosaminidase and β-glucuronidase activity increased, a finding that agrees with the

Table 7—Comparison of Lipid Deposition in NCTR-BALB/c Mice With Human and Animal Models of Niemann-Pick Disease

Condition	A/C*			Lipid molar ratios		Acid sphingomyelinase activity
	Cholesterol	Total phospholipid	Sphingomyelin	Cholesterol/total phospholipid	Cholesterol/sphingomyelin	
NCTR-BALB/c mouse†						Deficient
Liver	9.0	1.4	8.3	1.05	4.06	
Spleen	7.7	2.3	8.8	1.30	3.87	
Niemann-Pick disease in man‡						
Type A						Deficient
Liver	5.7	3.1	37.4	0.44	1.39	
(*n* = 7)	(1.5-10.0)	(0.6-5.4)	(2.0-53.7)	(0.15-0.96)	(0.22-4.28)	
Spleen	6.1	5.3	28.2	0.65	1.07	
(*n* = 10)	(3.1-11.2)	(1.7-7.9)	(6.9-46.1)	(0.31-1.14)	(0.38-2.40)	
Type B‖						Deficient
Liver	8.0	2.9	33.2	0.50	1.00	
(*n* = 3)	(1.5-14.0)	(1.4-5.2)	(6.0-66.7)	(0.21-0.78)	(0.81-1.22)	
Spleen	2.5	5.1	31.2	0.24	0.31	
(*n* = 1)						
Type C¶						Normal or
Liver	2.9	1.2	4.3	0.48	3.4	slightly decreased
(*n* = 7)	(1.5-4.5)	(0.95-1.5)	(2.0-9.0)	(0.23-0.70)	(0.97-6.8)	
Spleen	3.5	1.8	7.2	0.96	2.23	
(*n* = 5)	(2.8-4.4)	(1.5-2.4)	(3.1-12.5)	(0.90-1.04)	(1.4-3.9)	
Foam cell reticulosis mouse**						Normal
Liver	1.1	0.8	1.1	0.56	0.70	
Spleen	1.2	0.8	0.9	1.68	1.23	
Thymus	2.9	1.9	7.1	1.00	4.36	
Drug-induced phospholipidosis in rat††						(Known inhibition of
DH-treated liver	1.1	1.5	1.4	0.08	4.49	lysosomal phospho-
Chloroquine-treated liver	0.9	1.6	0.9	0.06	5.02	lipases A and C‡‡

* Ratio of lipid content of affected versus control (normal) tissue.

† From Morris et al.[1]

‡ Calculated from Fredrickson and Sloan.[3] Values are expressed as means with ranges given in parenthesis.

§ Acute neuropathic form; ages 0.3-2.6 years.

‖ Chronic form without central nervous system involvement; ages 2.4-21 years.

¶ Subacute (juvenile) form with central nervous system involvement; ages 5.5-22 years. Nova Scotia variant not included.

** Calculated from Fredrickson et al.[26] Adachi et al[27] obtained similar values except for sphingomyelin (A/C ratio: liver, 3.0; spleen, 2.8; thymus, 33).

†† Calculated from Matsuzawa and Hostetler[28]; DH = 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane.

‡‡ Matsuzawa and Hostetler.[29]

observations of Pentchev et al[22] who, in addition, found higher levels of α- and β-galactosidase and α-L-arabinosidase activities in the affected mice.

The isolation of the liver storage inclusions by subcellular fractionation and the identification of the particles as lipid-filled lysosomes unequivocally demonstrate that the affected NCTR-BALB/c mice suffer from a characteristic lysosomal lipid storage disease. The material stored is complex, but this is often the case in lipidoses, and also in many other storage diseases. Since, however, the condition seems to be inherited as a recessive autosomal trait, this complexity must be explainable as a consequence of a single mutation, presumably expressed as a single protein defect. The example of I-cell disease[24] does, however, illustrate the fact that a number of proteins may be secondarily affected as a consequence of the primary genetic defect.

In the framework of these considerations, and taking into account the usual pathogenic mechanism underlying lysosomal storage diseases, we are tempted to correlate the intralysosomal accumulation of sphingomyelin with the marked decrease in sphingomyelinase activity. The simultaneous accumulation of cholesterol could be secondary to that of sphingomyelin, which is known to exhibit an affinity for cholesterol.[25] Indeed, abnormal deposition of cholesterol is found in the tissues of patients suffering from forms of Niemann-Pick disease in which very high accumulations of sphingomyelin are correlated with a genetic deficiency of sphingomyelinase (Table 7). As for the intralysosomal accumulation of membrane material, it could be a late phenomenon reflecting massive autophagy caused by the poor nutritional state of the animals or by the swelling of their lysosomal compartment, or by both.

This attractive explanation does not, however, account for all the facts. First, as shown in Table 7, the cholesterol/sphingomyelin ratio is distinctly higher in the affected mice than in livers of human patients suffering from sphingomyelinase deficiency. In no human case have crystals of cholesterol actually been observed within the lipid deposits. Also noteworthy is the fact that the enzyme deficiency is less marked in cerebellum (and in brain[22]), but the symptomatology is mainly neurologic. Finally, as shown by Pentchev et al,[22] the pathologic accumulation process affects certain minor glycolipids to the same relative extent as it does sphingomyelin and cholesterol. Also, at least two other lysosomal activities—glucocerebrosidase and β-glucosidase—show distinctly decreased levels in several tissues of the affected mice, in contrast to a number of other lysosomal enzymes, which are increased.[22]

If there are multiple enzyme deficiencies in the lysosomes of the affected mice, one could think of faulty processing, as in I-cell disease, or of secondary consequences of some other primary deficiency. In this respect, the intralysosomal accumulation of cholesterol deserves to be given some attention. We have observed it previously in foam cells induced in rabbits by administration of a cholesterol-rich diet.[30] In this case, the most probable explanation of the intralysosomal storage of free cholesterol is that it is the consequence of excessive endocytic intake of lipoproteins, leading to saturation of the mechanism, possibly involving a carrier-protein,[31,32] whereby cholesterol is cleared from the lysosomal compartment. A genetic deficiency of this mechanism could possibly account for the accumulation of free cholesterol in the lysosomes of the affected NCTR-BALB/c mice. In this event, however, the accumulation of sphingomyelin and of some glycolipids and the associated enzyme deficiencies would have to be viewed as secondary consequences of the accumulation of cholesterol. At present, except for the reported inhibition of sphingomyelinase activity by cholesterol,[33] there is no evidence for this hypothesis—or against it. It could be investigated with cholesterol-fed rabbits.

Although the primary genetic defect remains to be established, the NCTR-BALB/c mouse is already an attractive model for the study of lysosome storage diseases. The clinical and pathologic traits have been characterized in some detail,[1] and the selective involvement of lysosomes has been established.[2] A large productive colony of the mutant mice is available. We have described here methods for the isolation of highly pure storage inclusions from liver, permitting detailed biochemical studies of their composition. A variety of model studies for evaluating clinical treatments of storage diseases, such as enzyme replacement trials,[34-36] cell implantations,[37,38] and bone marrow or organ transplantations,[39,40] could be carried out with the NCTR-BALB/c mice under controlled laboratory conditions. Elucidation of the primary genetic defect in the NCTR-BALB/c mouse and investigation of its diverse phenotypic manifestations in various tissues will no doubt provide important new insights into lysosome physiology and function.

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Acknowledgments

The authors wish to express their gratitude to Dr. C. de Duve for his interest in this work and for his valuable suggestions in the preparation of the manuscripts. The excellent technical assistance of Charles Des Jardins and Ms. Rosa Haidar, Linda Letzig, and Aida Ramos is also gratefully acknowledged. We also express our thanks to Martha Wemple, Anna Polowetzky, and Carolyn Morris for secretarial assistance.