Virchows Arch. A Path. Anat. and Histol. 385, 215–231 (1980)

Virchows Archiv A

Niemann-Pick Disease

Analysis of Liver Tissue in Sphingomyelinase-Deficient Patients

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Summary. The results of a complex analysis of liver tissue are presented (four biopsy and two autopsy samples) obtained from six patients with Niemann-Pick disease (NPD) with a gross deficiency of sphingomyelinase (SMase) accompanied by a typical increase in sphingomyelin (SM). There were five cases of NPD type A (four of them with an atypical, prolonged course) and one case of type B. By means of lipid histochemistry it was possible to demonstrate SM storage both in hepatocytes and in the reticuloendothelial system (RES) of the liver (Kupffer cells and portal macrophages) and to show in two siblings with NPD type A a so-far undescribed centrilobular storage pattern. Enzyme histochemistry revealed a secondary deficit of nonspecific esterase activity and acid β-galactosidase in liver storage macrophages and varying degrees of suppression of hepatocytic enzyme activities as a reaction to lipid storage of sudden onset. Ultrastructurally, it was possible to demonstrate cholesterol in lysosomes by using digitonin fixation, the involvement of Ito cells in lipid storage, the aggregation of storage lysosomes with certain other organelles and their occasional connections with the endoplasmic reticulum. The problems of possible lipid extraction during processing were considered as a cause of pronounced lysosomal electron-lucidity and of the ultrastructural identification of the participating lipopigment. The significance of the findings is discussed in relation to the existing classification and, particularly, to the stored lipid dilemma of cases of NPD type C.

Key words: Sphingomyelinase deficit – Niemann-Pick disease – Liver – Histochemistry – Electron microscopy.

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0340-1227/80/0385/0215/$03.40

Introduction

In the development of research on the enzymopathies of lipid metabolism the focus of interest is being shifted to the initial stages of the pathogenetic chain. The era of purely clinicopathologic studies has been succeeded by a period when interest has been focused on substrate analysis and, more recently, on deficiencies in the catalytic function of enzymes. The future era is likely to concentrate on analyzing causative disturbances in the genetic code. Despite this emphasis on the biochemical and genetic aspects in research in the 'proximal direction' of the pathogenetic chain, advances are still to be made using classical histochemical and structural methods. These techniques, though no longer the preeminent mode of diagnosis, nevertheless allow an assessment of the majority of important 'distalward' changes, and any other secondary effects they may cause, even being able to follow up the particular trait in its tissue heterogeneity. Such an approach undoubtedly allows a deeper insight into the biology of the enzymopathy concerned.

This paper reports the results of a wide range of histochemical and other analyses made on liver tissue from six patients with SMase-deficient (types A and B) cases of the Niemann-Pick disease (NPD); it attempts to show the advantages of such an approach and to assess its possible value in the study of other types of NPD.

Material and Methods

Six patients with NPD were studied, and the clinical details are given in Table 1. Specimens of liver tissue were obtained by percutaneous needle biopsy in four (cases 1–4) and at autopsy in two (cases 5–6).

The tissues were studied by routine *histology* and complex *histochemistry* in cryostat sections of unfixed tissue using a battery of lipid histochemistry methods described in detail in previous communications (Elleder et al., 1975; Elleder, 1977a) with particular stress on phospholipid detection (Elleder and Lojda, 1973a and b). Lysosomal hydrolase, dehydrogenase, and other activities were monitored in aqueous media or using the technique of semipermeable membranes (SPM) as previously described (Elleder et al., 1975). *SMase activity* (Harzer and Benz, 1973) was estimated in the liver, brain, and in peripheral leukocytes isolated from heparinized blood and repeatedly washed in saline. Leukocytes were also examined from both parents of cases 1–4. The *phospholipid spectrum* was examined according to Rouser et al. (1970). The cholesterol to sphingomyelin ratio was determined densitometrically after the chromatograms were sprayed with specific detection agents. The quantity of the two lipids and their molar ratio was ascertained using calibration with pure substances.

Electron microscopy was performed only on the biopsy specimens which were successively fixed with buffered OsO_4 and paraformaldehyde, followed by dehydration with acetone and embedding in Araldite. Part of the material was fixed according to Flickinger (see Scallen and Dietert, 1969) or examined after extraction using chloroform-methanol (Elleder and Šmíd, 1977).

Results

Histochemistry

The essential characteristics of the storage process is shown in Table 2. Histochemically, the *principal lipid stored* met the basic criteria for sphingomyelin

Table 1. Summary of clinical findings

Case Number	Age			Enlargement of			Ocular findings	General condition	Remarks
	At onset	At present	At biopsy(B) At death(D)	Liver	Spleen	Neurology			
1 Male	2 years	18 years	B 11;15 years	+++	+++	Psychomotor retardation; extrapyramidal symptomatology	Cherry red spot	Slow progression	Involvment of lung; hyperlipoprot. type IV
2 Female	1 year	11 years	B 4;8 years	+	++	Psychomotor retardation (profound)	Cherry red spot	Rapid progression	Involvment of lung involvement
3 Male	3 years	15 years	B 11;12 years	+	++	Extrapyramidal symptomatology (mild)	Cherry red spot; impaired vision	Good	Siblings; signs of lung
4 Male	3 years	19 years	B 15 years	+	++	Extrapyramidal symptomatology (mild)	Cherry red spot; impaired vision	Good	sister died when seven-years-old
5 Male	1 year		D 8 years	+++	+++	Unrelated to storage	Absent		Death in hepatic coma
6 Male	1 year		D 4 years	+++	+++	Seizures; psychomotor retardation (profound)	Absent		Death from pulmonary infection

[a] See Results

+ = Palpable 1 finger below the costal margin
++ = Palpable 3 fingers below the costal margin
+++ = Palpable more than 3 fingers below the costal margin

Table 2. Summary of the main histological and lipid histochemical findings in the liver samples

Case Number	Source[a]	Storage		Secondary findings		
		Pattern	Main lipid	Lipopigment (amount in RES)	Apolar steatosis	Other
1	B	Diffuse	SM	Moderate	Absent	Mild fibrosis
2	B	Diffuse	SM	Moderate	Discrete	Mild fibrosis
3	B	Zonal[b]	SM	Numerous	Absent	Mild fibrosis
4	B	Zonal[b]	SM	Numerous	Discrete	Mild fibrosis
5	A	Diffuse	SM	Moderate	Discrete	Cirrhosis
6	A	Diffuse	SM	Moderate	Discrete	Mild fibrosis

[a] B = biopsy; A = autopsy
[b] Involving central and various parts of midzonal regions of hepatic lobules

(SM), i.e., it was positive by the iron hematoxylin method, the staining persisting after alkaline prehydrolysis or acetone preextraction. It was extracted rapidly with chloroform-methanol. The lipid was localized in cytoplasmic vacuoles which, in case of hepatocytes, were mostly concentrated in the lysosomal peribiliary region. However, a relatively small amount of detectable lipid was repeatedly observed in larger vacuoles, a phenomenon difficult to explain as being due to ordinary triglyceride steatosis, which was merely rudimentary. The moderate PAS positivity and basophilia, both of which were extractable, were regarded as being due to the small admixture of glycolipids. Osmiophilia by the OTAN method was moderate, alkali-resistant, and was probably due mostly to SM. Pronounced birefringence of the Maltese cross type was confined to storage regions. Phosphoglycerides were demonstrable only in minor quantities in the lysosomal regions. Cholesterol was only examined chemically (see Table 3).

Distribution

SM storage was most prominent in hepatocytes and in macrophages and appeared to be inversely proportional to the amount of lipopigment present (see below). Storage in other cells was substantially less. Involvement was usually diffuse throughout the liver lobules. In two male siblings (cases 3 and 4), however, SM storage was confined to the centrilobular and intermediate zones of the lobules; the periportal zones showed no histochemically discernible storage and the change between the storage and nonstorage zones in each lobule was abrupt (Fig. 1a). The changes found in the sister of one of these patients who died in 1962 at the age of 7 were reviewed. They showed an identical picture with major storage in the central and intermediary zones of the hepatic lobule but with no detectable peripheral involvement. Typical changes of NPD were found in the lymph nodes and spleen with massive storage in the lungs and adrenal cortex. In a limited number of brain sections (frontal cortex, brain stem, cerebellum), the only detectable abnormalities were pronounced distension of Purkinje cell dendrites and vacuolization of vascular endothelium.

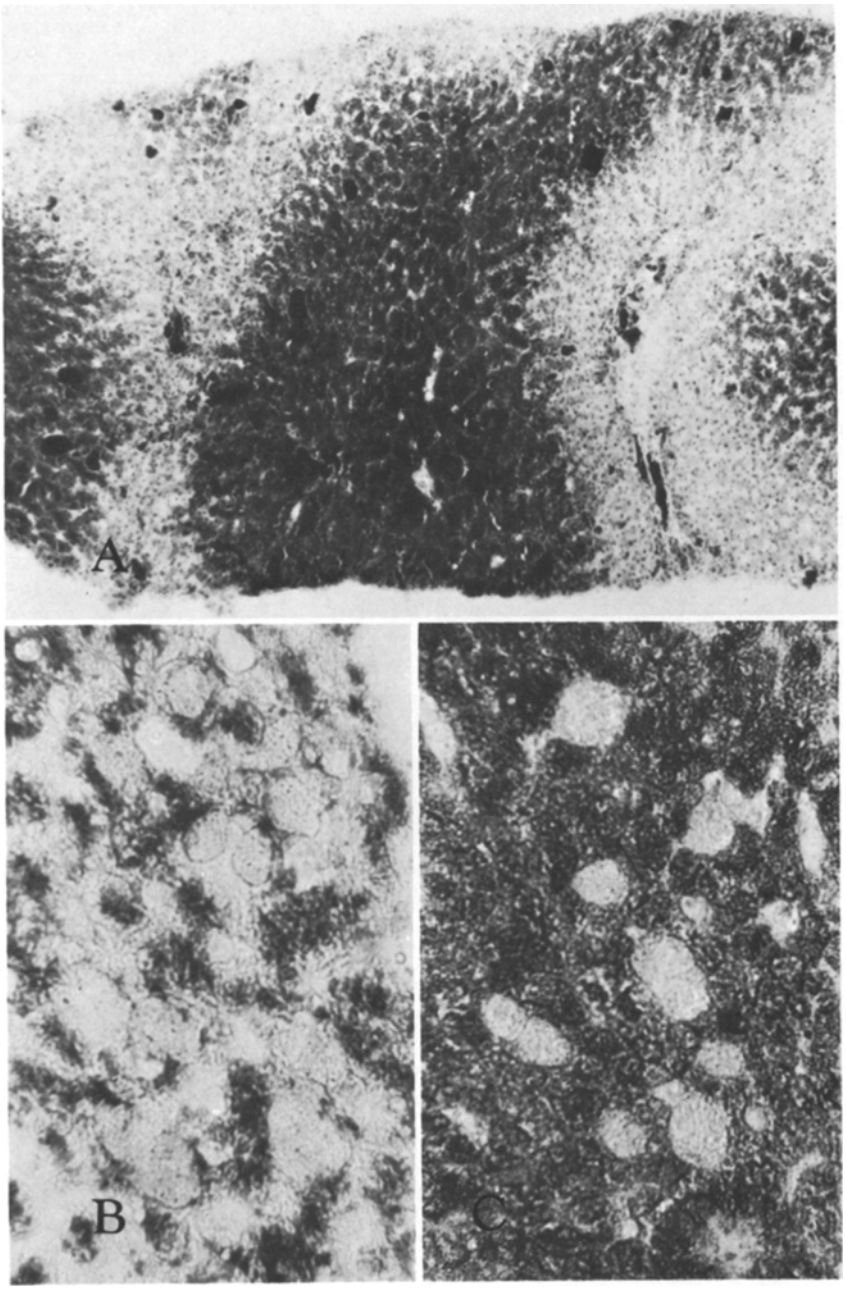

Fig. 1. A Case 3. Intense storage limited to mid and centrizonal regions of the hepatic lobule. Except for the nuclei, the bulk of staining is given by SM. NaOH-ferric hematoxylin method × 63. **B** Case 3. Centrizonal region of the hepatic lobule. Acid β-galactosidase (SPM, indigogenic technique). Strong activity in hepatocytes with moderate diffusion. The collection of storing macrophages is entirely negative. Without counterstain × 400. **C** Case 1. Neutral nonspecific esterase (aqueous medium, indigogenic technique). Strong activity in hepatocytes contrasting with total absence of activity in macrophages. Without counterstain × 400

Lipopigment, the amount of which was roughly proportional to the duration of the disease, was detected only in macrophages and the sinus lining cells. It was in the form of fine or rather coarse granules partially coalescing to form larger conglomerations. This was best seen in macrophages in cases 3 and 4 where almost spherical, mildly refringent aggregates were occasionally noted. Histochemically, this material showed all the typical properties of lipopigment by its natural color, the prominent yellow-white autofluorescence and sudanophilia, and by a number of other less specific features such as PAS-positivity, osmiophilia and orthochromasy with cresyl violet. The reaction for phospholipids was negative and that for proteins, poor. Unusual features included its relatively easy extraction with chloroform-methanol, especially after acidification or alkalization, following which all staining properties and autofluorescence disappeared. The fine granules in sinusoid endothelial cells were relatively resistant to extraction.

Chemical Analysis of Stored Lipids

The results of phospholipid and cholesterol spectrum analysis are given in Table 3.

Enzyme Activities

SMase activities in the patients and their parents are reported in Table 4.

Enzyme Activities Observed Histochemically

Hepatocytes. In *diffuse lobular involvement* (cases 1, 2, 5, 6) *lysosomal enzymes* were moderately or intensively active, the distribution of their activities showing

Table 3. Spectrum of liver phospholipids

Case Number	Total lipid P mg/g wet weigh	Percentage of distribution								Ratio chol./Sm
		SM	LBPA	PC	PE	PS	PI	LPC	DPG	
1	2.06	40.1	17.0	23.6	12.1	0.5	2.8	2.4	1.3	–
2	3.1	43.3	19.4	11.6	10.4	1.5	3.2	4.4	0.3	–
3	1.81	53.4	11.0	18.6	8.5	0.7	3.9	3.1	0.4	1:1.6
5	2.76	69.4	12.7	8.2	2.3	1.8	1.9	2.7	1.1	1:4.6
6	3.16	85.2	2.3	8.0	2.5	2.6	ND	1.6	ND	1:5.2
Control 1	0.88	6.6	ND	41.2	37.4	9.4	4.3	1.1	ND	–
Control 2	0.72	7.7	ND	38.5	42.3	7.7	2.5	1.2	ND	–

Abbreviations: SM = sphingomyelin; LBPA = lysobisphosphatidic acid; PC = phosphatidyl choline, PE = phosphatidyl ethanolamine; PS = phosphatidyl serine; PI = phosphatidyl inositol; LPC = lysophosphatidyl choline; DPG = diphosphatidyl glycerol; ND = not detected

Table 4. Sphingomyelinase activities. Values are expressed as % of the mean control activity. Methodological variation coefficient 0.10–0.25

Case Number	Patient	Source	Mother	Father	Source
1	5.6	WBC[a]	26.8	74.2[b]	WBC
2	2.6	WBC	18.2	32.2	WBC
3	4.9	WBC	45.6	37.2	WBC
4	3.0	liver[c]			
5	1.0	liver	–	–	
6	1–2	brain[c]	–	–	

[a] Peripheral white blood cells; activities in each WBC sample are given in arbitrary units related to β-hexosaminidase activity (one unit roughly corresponds to 0.1 n mol SM split/h/mg protein). The mean control activity is 11.0 ($n=5$)

[b] values in lower level of normal range; the problem of biological paternity is opened

[c] Mean control activities for the liver and brain are 2.0 ± 1.5 ($n=5$) and 3.8 ± 2 ($n=5$) n mol SM split/h/mg protein, respectively

no difference from normal tissue. Acid phosphatase and β-glucuronidase (mostly in hepatocytes) and β-hexosaminidase (mostly in mesenchymal elements) were both highly active. In the larger lipid vacuoles reaction products were localized only in the periphery. As in controls (Elleder, 1977c) α-mannosidase showed little activity. E600-resistant esterase in an aqueous medium was negative in the hepatocytes, or showed a little activity by the SPM technique. Postcoupling techniques using 6-Br-2-naphthyl glucosides (β- and α-galactosidase and glucosidase) yielded substantially more positive results than in the controls. The distribution of azodye was the same as that of the stored lipid (see below). *Alkaline phosphatase* activity was uniformly intense in the sinusoids while a more pronounced increase, compared with controls, was seen in hepatocytes towards their biliary poles. *Nonspecific esterase* activity was high as was *phosphorylase* activity when no dextran primer was used (Meijer, 1968). *Glucoso-6-phosphatase* activity was moderately decreased. *Dehydrogenase* activity (substrates: succinate, α-glycerophosphate, β-hydroxybutyrate, lactate, NADH, NADPH, glutamate, isocitrate, glucose-6-phosphate) showed no obvious differences from the controls.

In case with *regionally limited storage* (cases 3 and 4) peripheral minimal storing zones displayed normal or increased enzyme activities, particularly of lysosomal enzymes, while the central zones with intense storage showed *decreased* activities of practically all the enzymes examined. Lysosomal enzymes visualized by simultaneous azocoupling or the indigogenic technique revealed a slightly decreased activity and a somewhat less sharp localization. In the postcoupling techniques, however, maximum reaction product deposition was always found in the centrilobular regions and in macrophages. This phenomenon is due to the lipophilia of the 6-Br-2-naphthol released (Lojda, 1976) as is evident when the results for β-galactosidase obtained by this method are compared with

those given by the indigogenic method when the product is localized in hepatocytes only, throughout the lobules but with a slight preponderance in the periportal areas. As for the other enzymes, decreased activity was most prominent with β-hydroxybutyrate dehydrogenase which showed almost no activity in the lobule centers. Alkaline phosphatase activity was greater at the biliary poles of periportal hepatocytes with sinusoid activity predominating in the central zones.

Macrophages. The results were identical with both types of storage pattern. Lysosomal enzymes (acid phosphatase, hexosaminidase, glucuronidase) were invariably increased, although there was an evident inverse relationship with the amount of lipopigment accumulation. Acid β-galactosidase (indigogenic technique) and nonspecific esterase activities (even by the SPM techniques) were almost uniformly absent (Fig. 1b and c). There were only slight traces of dehydrogenase activity even with α-glycerophosphate. Only glucose-6-phosphate dehydrogenase was moderately active. Control media without substrates showed negligible results.

Ultrastructural Findings

This investigation was made only on the biopsy specimens. In standard *double-fixed specimens* the most striking finding was the small amount of structurally definable material in the markedly hyperplastic lysosomal system, most of which, particularly those in hepatocytes, were electron-lucent to a variable extent. The material present in lysosomes, though structurally pleomorphic, was of two basic types: loosely arranged or tightly packed concentric membranous formations. The material was present in and occasionally filled many lysosomes, particularly the smaller ones; the material was homogenous or granular and of varying density; it was also present in all cell types, but most prevalent in sinusoidal endothelial cells and in macrophages where it was often accompanied by vesiculare structures. A combination of both types of material as well as electron-lucent areas were usually present in each lysosome. Lysosomes of all shapes and sizes and generally with single limiting membranes could be found within individual cells. Storage lysosomes occurred as small spheroidal structures or as large, often bizarre lobulated formations with occasional smaller satellite buds at their peripheries. Sometimes there was even a gradual dissolution of the septa (and of cytoplasmic material) between two adjacent 'buds' of the conglomerate. In some cells, these formations were seen either merging directly with the slightly dilated endoplasmic reticulum or linked with each other by narrow structures of various length (Fig. 2a–c).

Very occasional invaginations of the external mitochondrial membranes or cytoplasmic vacuoles were seen in the storage lysosomes – both in macrophages and in hepatocytes (Fig. 3a). Autophagic vacuoles and membranous formations are free in the cytosol or in the dilated endoplasmic reticulum; mitochondrial crystals were only occasionally encountered. Hepatocytes often showed a conspicuous number of cytoplasmic vesicles and a blurring of the Golgi apparatus

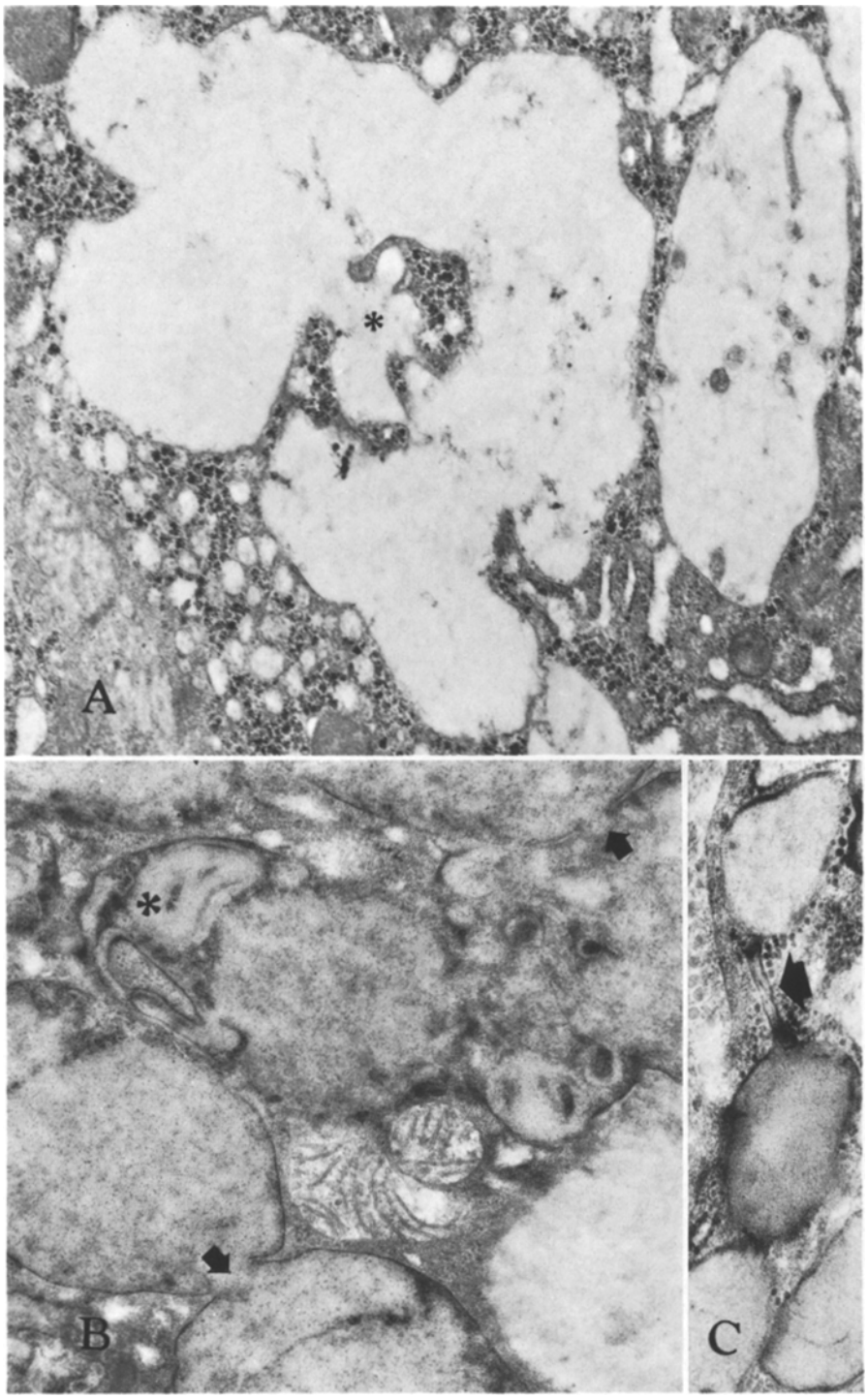

Fig. 2. A Case 1. Hepatocyte. Ultrastructure of bizarre-shaped, partly 'lobulated' storage cytosome widely communicating with dilated endoplasmic reticulum (*asterisk*) × 24,000. **B** Case 2. Hepatocyte. Group of lysosomes with several intercommunications (*arrows*). Note the highly irregular contour of one of them (*asterisk*) suggesting wide communication × 20,000. **C** Case 2. Periportal fibroblast. The connection of storing lysosomes with a structure closely resembling the endoplasmic reticulum is indicated (*arrow*) × 26,000

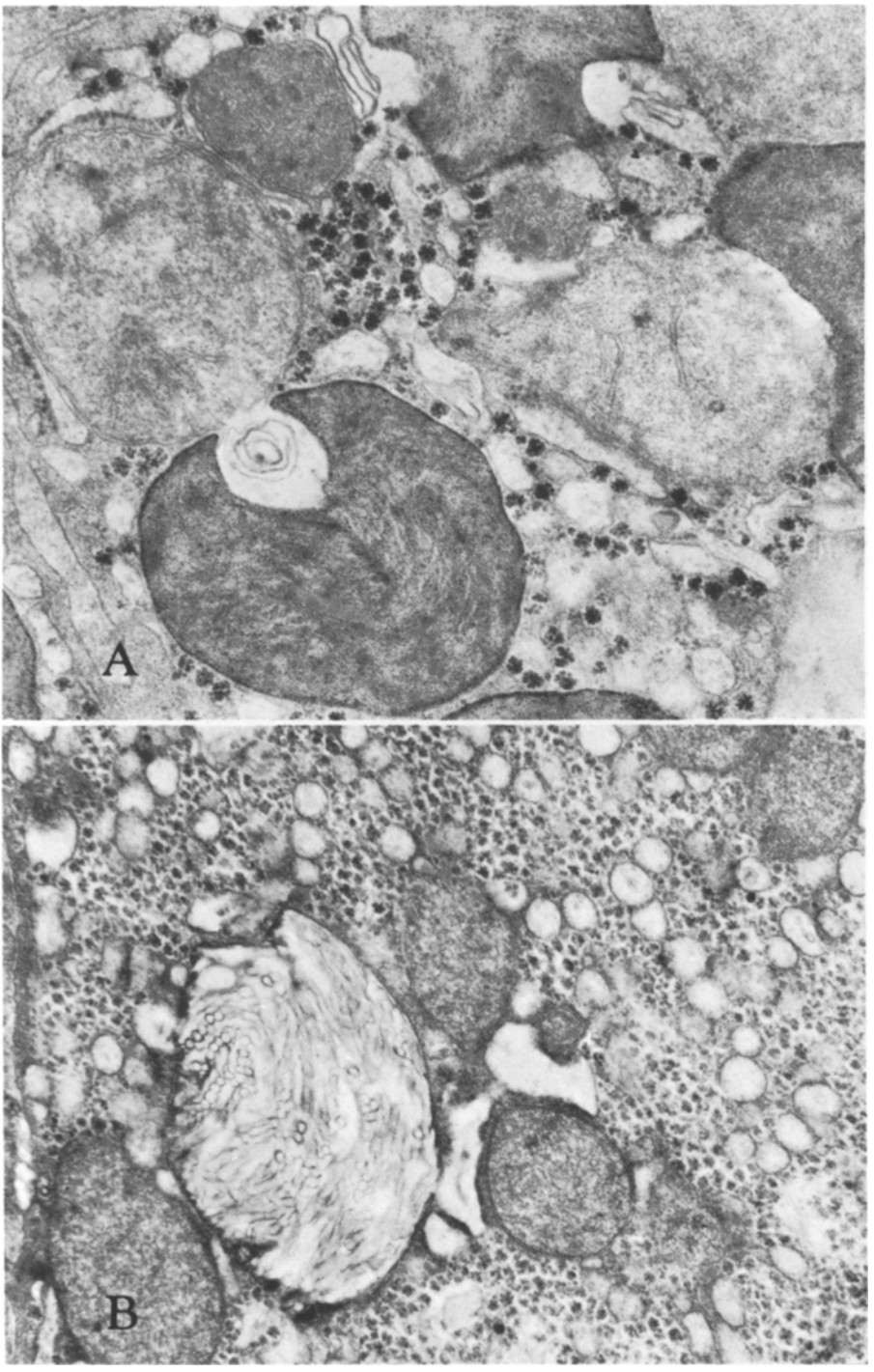

Fig. 3. A Case 2. Hepatocyte. Invagination of external mitochondrial membranes into the storing lysosome × 32,000. **B** Hepatocyte. Case 3. Digitonin fixation. Storing lysosome filled with cholesterol-digitonin crystals. Note the numerous cytoplasmic vesicles containing minute crystalline structures of similar appearance × 20,000

by locally increased cytoplasmic density. The other organelles were not visibly changed, apart from a variable reduction in their numbers.

In the lipopigment-containing sinusoidal endothelial cells and macrophages, the material was of low density and either pleiomorphic or homogeneous.

Storage distribution reflected the results of histochemistry. In addition, storage was observed in Ito cells. In areas of minimal involvement in cases 3 and 4 there were only occasional storage lysosomes in the peribiliary zones of hepatocytes.

Digitonin-fixed specimens contained characteristic cholesterol digitonin crystals (Scallen and Dietert, 1969) which either occupied the whole of the lysosomal space or were mixed with membranous or dense structures (Fig. 3b). The electron-lucent appearance of many lysosomes, however, remained unaltered.

The samples examined after *lipid extraction* with chloroform-methanol showed marked cell membrane depletion with the lysosomal system almost completely empty except for a little low density background material in a few peribiliary bodies. Lipopigment structures were not obviously altered.

Discussion

Classification. Only two cases (5 – type B; 6 – type A) met the criteria of the present NPD classification (Brady, 1978). The remaining four cases (1–4), all with neurovisceral involvement and profound SMase deficiency, were atypical of type A because of their unusually prolonged course. It is apparent that many cases are atypical both in their clinicopathological picture and often in their pattern of abnormal lipids – whether in the SMase deficiency group (Wenger et al., 1977; Harzer et al., 1978b; for others, see Elleder et al., 1978) or in the NPD types without a clearly demonstrable enzyme defect (Dunn and Sweeney, 1971; Grover and Naiman, 1971; Wiedemann et al., 1972; Neville et al., 1973; Karpati et al., 1977; Elleder et al., 1978; Harzer et al., 1978a). Thus a particular enzymatic abnormality cannot always be matched by a stereotyped phenotype. Any final definition of a given type will require exact knowledge of the genetic and biochemical lesions, which may answer the question whether the apparent variability of tissue response depends merely on slight variations in the lesion (McKusick, 1968; McKusick et al., 1972) or is due to a modification of the genetic background.

The complexity of the problem can be shown by the differences found in the *distribution of storage* in the liver lobule, an aspect not previously examined. There are frequent references to the difficulties involved in the identification of hepatocytes grossly altered by storage, and to the quantitative time-dependent changes in storage (Crocker and Farber, 1958; Schettler and Kahlke, 1967), although it is generally believed that the process of storage affects the liver diffusely. This is at odds with our findings of centrilobular storage with familial incidence (cases 3 and 4). This pattern of storage, identical in two brothers of different ages and also seen in a female sibling dying some years previously, appears to be a constant rather than transient feature of the disease in this family.

Causal interpretation is difficult. In our opinion, this is the first description of the dynamics of pathologic trait expression in the developmental chain of a cell type. A possible explanation may be related to differences in the phase of the life cycle of hepatocytes in peripheral (regeneration) and central (cytoclastic) zones. Metabolic zonal heterogeneity, particularly more intensive metabolism of lipids in the central regions or defective nutrition in areas corresponding to Rappaport's zone 3 of the primary acinus (for details see Rappaport, 1963; Bloom and Fawcett, 1975), might also account for the sudden manifestation of storage. Nevertheless, it is clear that SMase deficiency in the liver of these patients became manifest only under certain conditions appearing roughly at the intermediary zone of the anatomic lobule. The very low total activity of the enzyme (see Table 4) almost excludes the possibility of some stepwise decrease in activity being involved, although comparisons of levels of activity between the various zones might prove rewarding.

The Enzyme Deficit – Stored Lipid Relationship. In all the cases studied, *SM* was the prinicipal lipid stored. Histochemical studies demonstrated its accumulation both in *hepatocytes* and in the *hepatic RES*. Sphingomyelin was also demonstrated biochemically and histochemically in other storage organs in the cases on which an autopsy was performed (5 and 6). This was logically related to the profound SMase deficiency, which was demonstrably genetically conditioned (carrier state in parents). These findings are in accord with Brady (1978) who classified SMase-deficient cases (types A and B) as true *generalized sphingomyelinoses* affecting, to a varying degree, mesenchymal, epithelial, and neural tissues.

However, in NPD type C, not only is there no known enzyme deficiency (for details, see Harzer et al., 1977; Brady, 1978), but also, despite all progress in the methodology of lipid separation, the whole story of the stored lipid remains uncertain, a dilemma seen in the purely descriptive designations of the disease (Wiedemann et al., 1972; Neville et al., 1973; Karpati et al., 1977; Elleder et al., 1978; Harzer et al., 1978a). This appears to be due to the discrepancy between the absence of an overt SMase deficit and SM accumulation, the latter being not directly proportional to the structural storage alterations and also due to increased accumulation of other lipid species (for review, see Harzer et al., 1978a). Major SM deposits were found only in the classical RES organs (Brady, 1978). In the liver there was only a slight increase in SM with additional increases in lysobisphosphatid acid (LBPA) (Seng et al., 1971; Wiedemann et al., 1972; Debuch and Wiedemann, 1978; Elleder et al., 1978; Harzer et al., 1978a). In the brain, however, no biochemical evidence of the deposition of any type of lipid has so far been offered (Wiedemann et al., 1972; Tjiong et al., 1973; Kannan et al., 1974; Harzer et al., 1978a) other than a histochemical demonstration of peculiar focal accumulations of phosphoglyceride (Elleder et al., 1978). In our view,lipid histochemistry could make a significant contribution here (see Conclusions).

Our other findings are regarded as *secondary*, mostly regressive *changes* resulting from storage. The increased amount of cholesterol, the origin of which is still obscure Frederickson and Sloan, 1972), was variable in relation to SM, though its molar concentration was always lower. The small amounts of glycolipids in hepatocytes is also regarded as a secondary phenomenon. Chemically

and histochemically, LBPA was present in only small amounts localized mostly in the hepatocytes.

Lipopigment shows a definite tendency to increase in the longer-surviving cases (Jonas, 1966). We found it to be much more prevalent in macrophages than in hepatocytes and that it was relatively easily extracted from unfixed tissue (Elleder, 1977b). Since it is easily stained using most of the routine techniques, this might lead to some unwarranted conclusions regarding the presence of different types of lipid (Elleder, manuscript in preparation 1979). The knowledge of its quantity and distribution is of considerable significance in the interpretation of the ultrastructural findings (see below). Secondary alterations of varying degree were also observed in *enzyme activities* which depended on the lobular storage pattern and the cell type.

Hepatocytes. In specimens with a diffuse storage pattern, hepatocytic enzyme activities, particularly of lysosomal enzymes, were generally high. However, there was often little reaction product within the larger vacuoles, despite the use of the SPM technique. Canalicular and sinusoidal alkaline phosphatase activity was also increased, NPD thus being another condition showing this pattern of alkaline phosphatase activity (Hägestrand, 1975). The only enzyme with depressed activity was glucose-6-phosphatase.

In specimens with a *regional* (centrilobular and intermediate zone) storage, different results were obtained. In the peripheral and periportal areas, enzymal and lysosomal enzymal activity in particular was increased. In the intermediate and centrilobular zones, where storage became abruptly apparent, there was a variable depression of enzyme activity. This was least marked in lysosomal enzymes, nonspecific esterase, and phosphorylase and most obvious with dehydrogenases. The more diffuse localization of azodye in the simultaneous azo-coupling techniques might be a manifestation of lysosomal lability (Holtzman, 1976).

In *macrophages* the most conspicuous findings were the very marked depression or even complete absence of esterase (E600 sensitive and resistant) and acid β-galactosidase activities (indigogenic method) seen even in moderate storage cells. Other lysosomal enzyme activities were far from uniform and were very low sometimes. This was true also of α-glycerophosphate dehydrogenase activity (compare with Elleder, 1975; Elleder et al., 1975). At present there is no telling whether this depression is due to the accumulation of lipopigment, lipid, or to other organ-specific factors. Comparison with other enzymopathies is currently in progress.

Finally, storage-affected cells may show a series of secondary enzyme alterations. Examinations of more enzymes will undoubtedly help to define in more detail this acquired dystrophic condition as well as to throw more light on some of the recent interesting observations (Dacremot et al., 1974; Harzer et al., 1978a).

Electron Microscopy. Ultrastructural evaluation of the changes in NPD is generally unsatisfactory. The quantity of morphologically definable lysosomal content in NPD (Wallace et al., 1965) is strikingly small when compared with other lipidoses. Explanations of this have included the suggestions of gradual degrada-

tion (Miller and Raimann, 1972; Lejeune et al., 1973), or possible extraction of lipids during processing (Libert and Danis, 1975). Our findings suggest the latter hypothesis to be the more plausible. There is evidence of cholesterol extraction during routine processing (for review, see Sterzing and Napolitano, 1972) which might be avoided by the use of digitonin fixation. However, characteristic crystals were formed in only some lysosomes. This may have been due to imperfect penetration of digitonin, to the use of acetone (Sterzing and Napolitano, 1972), or to a possible reaction of digitonin with phospholipids (Frühling et al., 1971). We are unaware of any study dealing with the possibilities of extraction of sphingomyelin, although our preliminary observations with dichromate fixation (Elftman, 1954) suggest that it may occur.

The reliable ultrastructural analysis in NPD will be possible after a suitable technique for fixation of all the lipid monomers stored has been developed.

The presence of a certain amount of stored intralysosomal material in macrophages (Terry et al., 1954; Lynn and Terry, 1964; Kerényi et al., 1971; White and Sun, 1971; Skikne et al., 1972) is probably largely due to the presence of ceroid type lipopigment which, although undefinable ultrastructurally, may have a membranous structure (Golde et al., 1975; others in Elleder, 1977b). For this reason it is extremely useful to examine for lipopigment by parallel histochemical techniques to facilitate interpretation of the ultrastructural findings.

The ultrastructure of lipopigment will be dealt with in more detail elsewhere (Elleder, manuscript in preparation 1979). However, we should like to emphasize one of the structural variants seen here characterized by its homogenous appearance and low density (see also Chan et al., 1977).

The bizarre shapes of the storage lysosomes (see also Volk and Wallace, 1966) may be due to lysosomal fusion (see also Elleder, 1978). Another possibility is that the suspected communication of storage lysosomes with the endoplasmic reticulum (Wallace et al., 1965) might produce a gradual coalescence of segmentally dilated portions of this apparatus. The participation of the endoplasmic reticulum in the storage of SM cannot be excluded in view of the remarkably high SMase activity in the nonlysosomal fractions of hepatocytes (Weinreb et al., 1968).

However, before all these points can be resolved, a suitable method of lipid monomer fixation is needed to see if lipid storage is the only factor causing distension.

Similarly, as yet there is no reliable explanation for the peculiar way in which storage lysosomes are invaginated by other cellular organelles. Lysosomal aggregation with other organelles might be considered a type of crinophagy (Farquhar, 1969). Only Ito cells appear to participate in the storage.

Conclusions

The above results emphasize that traditional techniques, particularly histochemistry of lipids and enzymes, are useful in the investigation of NPD and can provide valuable information which cannot be evaluated by other methods.

First of all, it is a fairly sensitive storage detection process, especially in combination with ultrastructural analysis. Routine histology is not nearly as

sensitive, and negative findings, eg., in the liver, certainly cannot rule out a minor degree of storage (Wiedemann et al., 1972; Neville et al., 1973; Chan et al., 1977; Pellisier et al., 1976). Another advantage is the possibility of assessing various aspects of the heterogeneity of any of the traits followed, e.g., tissue storage gradients or differences in the distribution of individual lipids in the various cell types making up the tissue sample. This is chiefly valuable in the correct interpretation of the results of biochemical analysis. The combined approach allows much more effective localization and identification of substances than either histochemical or biochemical methods when applied alone.

This approach could be usefully applied to cases of NPD, type C group, where it might help to clarify the pathogenetically important question of stored lipid distribution, i.e., whether in this neurovisceral storage disease the SM deposition is confined solely to the RE system or is more generalized as in the SMase-deficient cases of NPD. Results of our previous study (Elleder et al., 1978) and literary data (see Discussion) speak in favor of the focal deposition pattern. For that reason, we should like to stress again the importance of studying the stored lipid distribution in the liver in each case of NPD, type C. Besides its pathogenetic significance, the proved liver RE-dependent SM storage (together with the hepatocyte-dependent LBPA deposition) would easily explain the low increment of SM which is detectable chemically (see Discussion). Proving the *focal SM deposition hypothesis* may alleviate the apparent contradistinction between the persistence of SMase activity and its substrate storage, since SM storage limited to the RE system (together with other lipids) certainly occurs in other circumstances, e.g. in thrombocytopenias (Landing et al., 1961) in the absence of SMase deficiency (Elleder and Harzer, unpublished data). Moreover, the relatively slight and often regressing visceromegaly (Harzer et al., 1978a), together with the occasionally surprising absence of detectable SM in the RES storage macrophages (Barr and Hickmans, 1956; Neville et al., 1973), might even point to a transient nature of RES sphingomyelin deposition.

Should our hypothesis be confirmed, the metabolic disorder of the epithelial and neural tissues in NPD type C could be studied more specifically. This should include, initially, the definitive determination of the cause and pathogenic significance of LBPA accumulation in the respective tissues (Elleder et al., 1978) in view of the assumption of specific hydrolase deficiency (Hostetler, personal communication, 1978) and because of a possible primary disturbance of intermediary phosphoglyceride metabolism, in which LBPA participates (Poorthuis and Hostetler, 1976; Matsuzawa et al., 1978; Poorthuis and Hostetler, 1978). Only then shall we know whether the 'unusual' character of our case (Elleder et al., 1978) was an example of biologic variation or whether the apparently atypical features were merely regular characteristics of NPD, type C, only discovered by an unusual methodological approach.

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Received June 19, 1979