Acta Neuropathol (Berl) (1985) 66:325–336

Acta Neuropathologica

Niemann-Pick Disease Type C

Study on the Nature of the Cerebral Storage Process

M. Elleder[1], A. Jirásek[1], F. Šmíd, J. Ledvinová[2], and G. T. N. Besley[3]

[1] Hlava's Institute of Pathological Anatomy
[2] Laboratory for Proteosynthesis, Charles University Medical Faculty, Prague, Czechoslovakia
[3] Dept. of Genetic Pathology, Hospital for Sick Children, Edinburgh

Summary. A complex neuropathological study of two cases of Niemann-Pick disease (NPD) type C (NPDC) revealed some novel features in the chemical pathology of the neuronal storage. Lipid histochemistry showed the presence of a lipid which met the criteria of a neuronal glycosphingolipid. Sphingomyelin (SM) was not detected in the neurones in any of the regions examined. Lipid chemical analysis of total extracts and of partially purified lysosomal fraction of the brain cortex showed markedly increased levels of neutral ceramide hexosides especially of glucosylceramide and ceramide dihexoside (mostly of its slower band). Phospholipids were not significantly increased. Monosialogangliosides GM_2 and GM_3 were increased only slightly. The storage process displayed the well known fine structure and was accompanied by a marked secondary increase in some lysosomal enzyme activities. There was neuroaxonal dystrophy (NAD) of considerable intensity and extent. Many spheroids contained masses of degenerated organelles and neurofilaments in various proportions and displayed variable activities of acid phosphatase, nonspecific esterase and dehydrogenases. There was marked brain atrophy accompanied in one case by severe demyelination. Enzyme studies revealed partial decrease of sphingomyelinase (SMase) and beta-glucosidase activities in cultured fibroblasts, as well as lack of cathodic SMase activity on isoelectric focusing. No defects of these enzymes were found in the brain samples. The findings are regarded as significant since they indicate a biochemical defect in which SM is not primarily involved and which may thus be fundamentally different from that in type A of NPD.

Offprint requests to: M. Elleder, Hlava's 1st Institute of Pathology, Faculty of Medicine, Studničkova 2, CSSR-128 00 Prague 2, Czechoslovakia

Key words: Niemann-Pick disease type C – Neuronal storage – Sphingomyelin – Neutral glycosphingolipids – Sphingomyelinase

Introduction

The phenomenon of brain damage caused by storage as part of metabolic disturbance in Niemann-Pick disease type C (NPDC) is generally known and regarded as an integral part of the neurvisceral syndrome in typical cases of the disease (Crocker 1961; Fredrickson and Sloan 1972). However, the detailed knowledge of the prominent neurological symptomatology with all its peculiarities (Neville et al. 1973) stands in contrast to the surprisingly superficial knowledge of the quality of the neuronal storage limited practically to its structural aspects only. Thus, neuropathologists are familiar with the typical ballooning of the neurones and their processes (e. g. Anzil et al. 1973; Crocker and Farber 1958; Harzer et al. 1978; Harzer and Peiffer 1981; Norman at al. 1967; Oppenheimer et al. 1967). There have been repeated studies of the ultrastructure of neuronal lysosomal deposits, the appearance of which has been described as polymorphous cytoplasmic bodies (Elfenbein 1968), oligomembranous bodies (Harzer et al. 1978), spherical membranous sacs (Horoupian and Yang 1978) or multilamellar bodies (Pellissier et al. 1976). Attention has been devoted to a well pronounced neuroaxonal dystrophy (NAD) (Elleder and Jirásek 1981; Harzer et al. 1978; Harzer and Peiffer 1981). So far, little emerged from studies of the quality of the neuronal storage, however. There are references to strong PAS positivity in the storing neurones, while the rest of the stainings, particularly for phospholipids, are negative

(Elfenbein 1968; Hagberg et al. 1978; Harzer et al. 1978; Lowden et al. 1967; Norman et al. 1967; Pellissier et al. 1976). Others, however, report negative or questionable results with lipid histochemical methods generally (Anzil et al. 1973; Emery et al. 1972; Philippart et al. 1969; Neville et al. 1973). According to Lake (1983) the principal neuronal storage compound is a water-soluble acid oligosaccharide. So far, the results of chemical analyses all seem to show no obvious increase in SM but are sometimes suggestive of an increase in glycolipids (see Discussion). This unclear situation, however, fits well into a whole series of unelucidated problems of the chemical and biochemical pathology on NPDC (Elleder and Jirásek 1983b).

The following is a report on the results of a multi approach study of cerebral changes in two autopsies of NPDC with a view to better understanding of the neuronal storage process.

Clinical Presentation

Case 1 (P.L.)

A boy with slight hepatosplenomegaly had marked extrapyramidal hyper-hypotonia, spastic quadruparesis and vertical gaze paralysis. The EEG was slightly abnormal. There had been no seizures. The occular fundi were normal. The onset was at 1 year and death at 4 years and 8 months from bronchopneumonia.

Case 2 (R.B)

A boy had an atypical clinical course starting early after birth and characterized by rapidly increasing hepatosplenomegaly, jaundice (conjugated hyperbilirubinaemia) and altered liver function tests for the first several months of life. Then progressive neurological symptomatology developed first with hypotony, then psychomoter retardation and terminated with profound decerebration rigidity. The ocular fundi were normal. There were no fits. The EEG was diffusely abnormal. He died at 4 years with signs of bronchopneumonia.

The bone marrow in both cases contained many foam cells with a storage pattern characteristic of NPDC (Elleder et al. 1983). The liver changes were also consistent with that diagnosis and are described elsewhere (Elleder et al. 1984b). The spleens were infiltrated with foam cells and displayed about seven and eight time increases in SM, respectively.

Material and Methods

The brain was removed 5 h (case 1) and 3 h (case 2) after death and processed neuropathologically according to Spielmayer's scheme. Sections were stained with haematoxylin and eosin (HE), Nissl, Bodian and Klüver-Barrera methods. For ultrastructural examination, specimens from different regions of the cortex, brain stem, medulla oblongata and cerebellum were doubly fixed with buffered 10% paraformaldeyhde and 1% osmium tetroxide, dehydrated with acetone and embedded in Arraldite. Some specimens were examined after lipid extraction with chloroform-methanol (Elleder and Šmíd 1977). Selected specimens were fixed with digitonin after Flickinger (see Scallen and Dietert 1969). For histochemical examination, specimens from frontal cortes, thalamus, cerebellum, mid brain, medulla oblongata, and spinal cord were quenched in petrol ether cooled with a mixture of acetone and dry ice and cut into sections 12 μm thick. For lipid detection, a battery of staining methods, auxilliary extraction and blocking tests were used (Elleder 1977, 1982). The principal detection methods were as follows: autofluorescence, birefringence, phospholipid detection with iron hematoxylin (Elleder and Lojda 1973a, b), standard PAS method for detection of glycolipids and cresyl violet for detection of acidic groups. Polar lipids (with the exception of gangliosides) were detected by a modified OTAN method (Elleder and Lojda 1968). Sudan Black B was used for a polar lipids and lipopigments. Cholesterol was detected according to Schultz, and Emeis et al. (1977). As for extraction tests, alkaline hydrolysis was used for the removal of phosphoglycerides, and extraction with anhydrous acetone for the selective removal of apolar lipids (Elleder and Lojda 1971). Bromination was used to exclude possible dual bond interference (Norton et al. 1962) as well as for the possible increase of PAS positivity in neutral glycolipids (Elleder and Lojda 1972). The coupled tetrazonium reaction (CTR) was used for detection of proteins (Pearse 1968). Water soluble oligosaccharides were detected with PAS celloidin technique (Lake 1981) in non-pretreated and chloroform-methanol extracted sections.

Lipid Chemical Analysis

Frozen samples of cerebral cortex were extracted with 10 vol. of chloroform-methanol 2:1 and subsequently twice with 10 vol. of chloroform-methanol-water 1:2:0.15 mixture. The extracts were pooled and partly evaporated under nitrogen to reach the concentration of 10 ml/g wet tissue weight. Phospholipids were separated by two-dimensional TLC and measured by zone phosphorus analysis (Rouser et al. 1970). Neutral glycolipids were isolated from the total lipid extract by Florisil column chromatography (Saito and Hakomori 1971) and separated on TLC plastic sheets (Silica gel 60) or on HPTLC silica gel plates (Merck, Darmstadt, FRG) in 65:25:4 chloroform-methanol-water mixture and detected by orcinol-H_2SO_4 according to Robert and Rebel (1975). Quantitative analysis of individual fractions was done as described (Elleder et al. 1984a). Gluco- and galactocerebrosides were separated on borate impregnated silica gel plates according to Kean (1966). Gangliosides were isolated by DEAE sephadex column chromatography (Ueno et al. 1978) and separated on HPTLC. Age-matched brains from non-neurological cases served as controls.

The storage lysosome fraction was isolated from unfixed frontal cortex of case 2 (R.B.) by the procedure of Suzuki et al. (1967). First the crude lysosomal fraction was isolated by centrifugation in 0.05 M sodium ethylendiamine tetra acetic acid at $7{,}500 \times g$. It was further purified on discontinuous sucrose gradient, and three fractions were obtained in the gradient areas I, II, and III and the fourth (IV) at the bottom. Each band was further purified on continuous sucrose gradient and collected for ultrastructural, lipid and enzyme analyses. Protein was determied according to Lowry et al. (1951).

Enzyme histochemistry was done using standard aqueous media and semipermeable membrane techniques. Details were given elsewhere (Elleder et al. 1984b). For biochemical assays extracts of tissue or cultured fibroblasts were prepared and assayed basically as described by Besley and Moss (1983b). SMase activity was measured using ^{14}C labelled SM as a substrate, and beta-glucosidase activity was measured in the pres-

Table 1. Distribution and intensity[a] of neuropathological lesions

	Storage		Atrophy		NAD		Gliosis	
	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2
Cortex								
frontal	2	2	–	1	–	–	–	–
temporal	2	2	1	2	–	1	–	–
parietal	2	3	1	2	1	1	–	2
occipital	1	1	1	2	1	2	1	2
cerebellar[b]	1	1	–	1	–	–	–	–
Hippocampus	2	2	1	2	1	1	–	–
Striatum					1/2	1	–	–
large neurons	2	2	–	–				
small neurons	–	–	–	–				
Pallidum	2	2	–	–	2	2	–	–
Thalamus[c]	3	3	3	3	3	3	3	2
Hypothalamus	2	2	1	1	2	1	–	–
Substantia nigra	3	3	1	1	2	2	–	–
Mesenceph. nuclei	3	2	1	1	1	2	–	1
Pontine nuclei	2	1	–	–	2	1	–	–
Dentate nuclei	2	2	1	1	1	2	1	1
Cranial nerve nc.	3	3	–	–	1	2	–	1
Inferior olive	2	1/–	–	–	2	1	–	–
Anterior horns	3	3	1	1	2	2	2	–
Clarke's columns	3	3	–	–	3	3	2	1
Posterior horns	2	2	–	–	3	3	–	–
Spinal ganglia	3	3	–	–	–	–	–	–

[a] Evaluated semiquantitatively. *Storage:* – not detectable histologically, 1 mild distension, 2 marked distension, 3 ballooning of perikarya of most neurons; *NAD: 1* occasional spheroids, 2 frequent spheroids, 3 status globosus; *Gliosis:* 1 discernible increase in astroglial nuclei, 2 Kanzler-Arendt strongly positive, 3 glial cicatrization

[b] Present in dendrites of Purkynje cells

[c] Maximum in ventromedial part

ence of taurocholate using 4-methylumbelliferyl-beta-D-glucoside as a substrate.

Results

Structural Studies

Macroscopy. Atrophy was the only change seen with the naked eye and was greater in case 2 where it was associated with widepread depletion of myelin. Despite that, the brain weights were not very different: 778 g in case 1 (control 1,237 g) and 770 g in case 2 (control 1,141 g).

Histology. The findings in both cases were similar. The salient feature in the grey matter was widespread ballooning of the perikarya and neuronal processes. The latter frequently displayed dystrophic changes in form of fusiform or spheroidal dilatations which were either homogeneous or finely granular. In sites of pronounced storage and neuroaxonal dystrophy (NAD) there was neuronophagia of storing neurones and of spheroids. The basic neuropathological findings including semiquantitative evaluation are summarized in Table 1 and demonstrated in Fig. 1.

The *white matter* in case 1 showed pallor in myelin stains and a variable amount of gitter cells. NAD spheroids were dispersed in the parietal and temporal subcortical regions and throughout the pyramidal tract. Many spheroids were found in the cerebellar white matter and in the posterior columns. The dominating freature in case 2 was widespread demyelination in the central white matter and pyramidal tracts with many gitter cells and fibrillary gliosis. NAD was most pronounced in the posterior columns and brachia conjunctiva.

Ultrastructure

The neuronal storage vacuoles limited by a single membrane contained loose oligolamellar membranous deposits vesicular or spherical in shape sometimes with dense cores. Two peripheral dense membranes and one central lucent layer could be

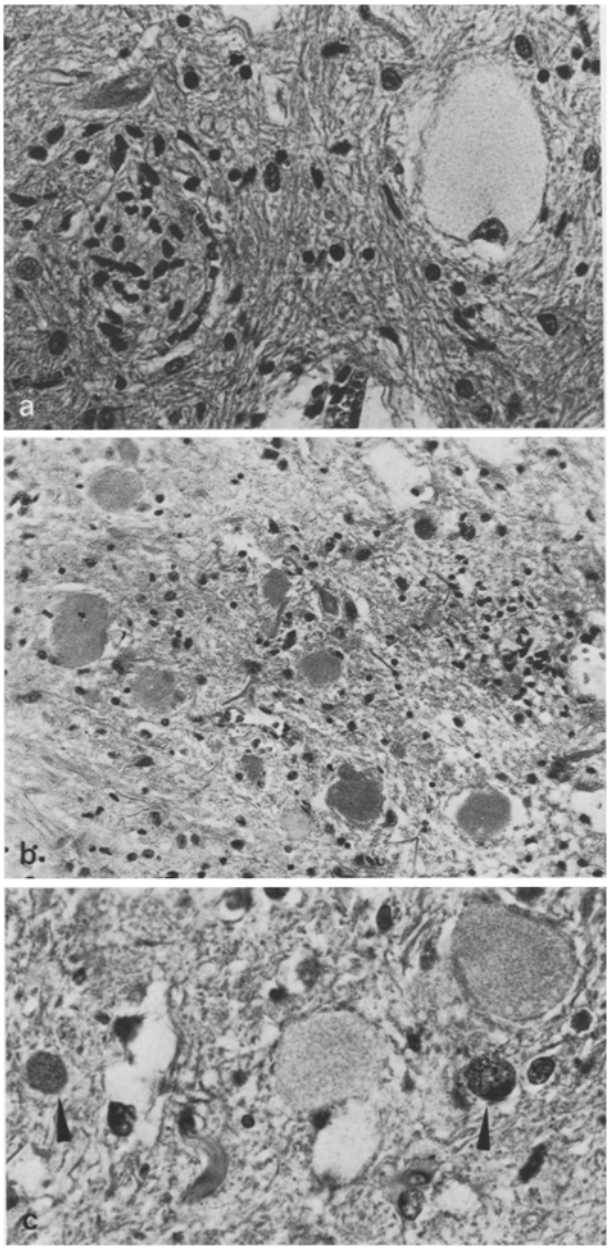

Fig. 1. a Spinal anterior horn. Neuronal storage and neuronophagia; HE, × 503. **b** Spinal posterior horn. Numerous homogeneous spheroids of NAD with resorptive reaction around some of them; HE, × 320. **c** Thalamus. Predominantly granular spheroids (*arrowheads*); HE, × 503

recognized only sometimes in the membranous deposits. Digitonin fixation left the picture unaltered. The membranous content disappeared completely in lipid extracted specimens. The pigmented neurones of the inferior olive (case 1) exhibited moderate storage. Both storage membranes and pigment were frequently found in intimate contact in the same lysosomes. The lipopigment granules were composed of a dense network of microtubules strongly resembling the inconstant bilayered structure of the storage membranous deposits. Control lipopigments of this region had the same microtubular ultrastructure. Olivary neurones of case 2 exhibited only traces of lipopigment and lipid storage.

The NAD spheroids displayed two basic structural patterns corresponding to the light microscopy. The homogenous ones were formed by the accumulation of filaments mostly thicker than 10 nm but thinner than microtubules. Exact estimation of their diameter was difficult owing to their irregular contour. The filaments were neither twisted nor paired. There was a small admixture of neurotubules. The granular spheroids were composed of masses of degenerated organelles, pleiomorphic bodies and amorphous dense substance. Both patterns were frequently seen mixted to various proportions (Fig. 2).

Lipid Histochemistry

No differences were found in the nature of the neuronal storage in various regions examined. The results are summarized in Table 2 and demonstrated in Fig. 3. There was marked neuronal deposition of a lipid which appeared to be a glycosphingolipid of predominantly neutral type not associated with SM to any detectable degree. The lipid was partly anisotropic, physically unstable, and could be partly stabilized by acetone (Fig. 3e, g). None of the techniques demonstrated cholesterol. There was no sign of lipopigment deposition in ballooned neurones. Only a slightly refractile residuum staining faintly for protein was detectable after the total lipid extraction procedure. There were no soluble oligosaccharides detectable by the PAS-celloidin method in the storing neurones. The results were the same as in conventional cryostat sections and were similarly abolished by total lipid extraction.

As for the speroids, their detectable lipid content was very low and was represented mainly by phospholipids (phosphoglycerides – in part plasmalogens, and SM). Nearly all the methods showed marked residual staining after total lipid extraction which together with strong staining for aromatic acid residues suggests the presence of a mixture of protein, polysaccharide, possibly with an admixture of lipopigment.

Gitter cells contained birefringent, apolar acetone-soluble lipid very like cholesterol ester together with some lipopigment.

The ependyma, plexus chorioideus and the vascular endothelium were without signs of storage.

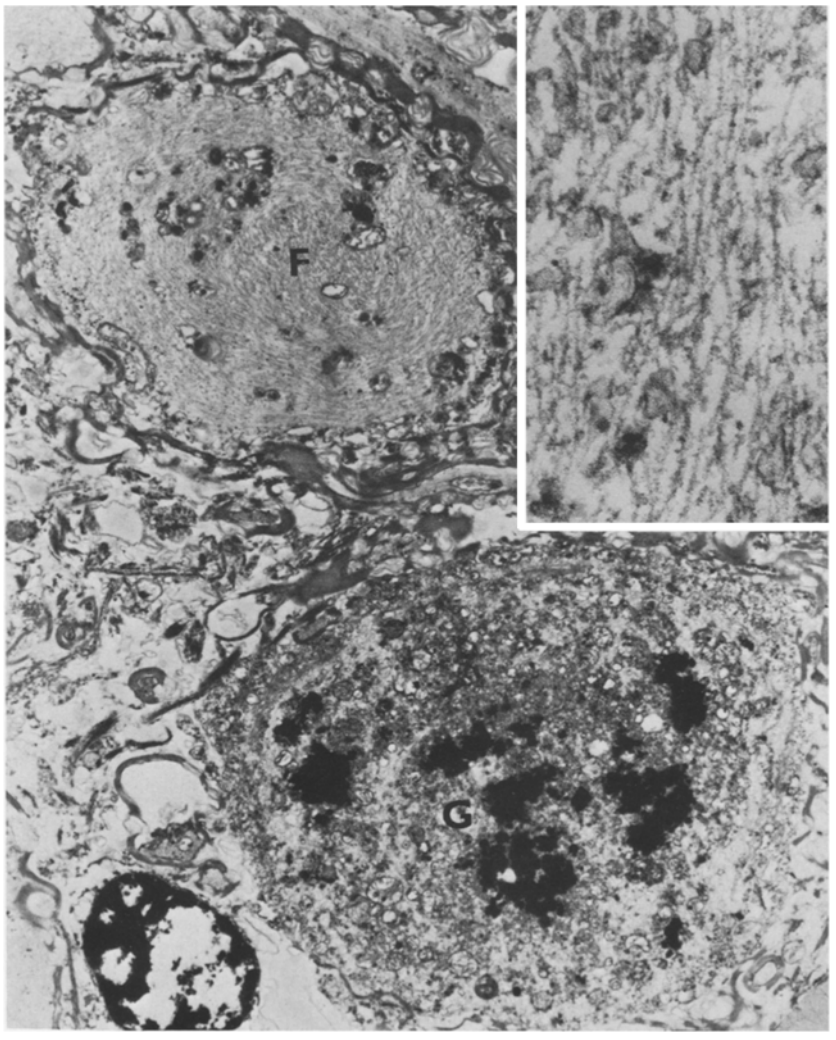

Fig. 2. Electron micrographs of predominantly granular (*G*) and fibrillar (*F*) axonal spheroids; ×12,300. *Inset*: detail of the fibrillary component; ×65,800

Chemical Analysis of Lipids

The spectrum of phospholipids is shown in Table 3. There were only minor inconstant changes consisting of a slight increase of bis (monoacylglycerol) phosphate in case 1 and a slight relative increase in SM in case 2. Marked decrease of most phospholipids in case 2 probably reflects the demyelination. Values for glycolipids are demonstrated in Table 4. The most important finding is the pronounced increment of glucocerebrosides in the ceramide monohexoside fraction and of the slower band of ceramide dihexoside. Higher ceramide hexosides displayed a minor increase of variable degree.

Increase of glycolipids paralleled the highest activities of lysosomal enzymes during the lysosomal purification procedure (Table 5). The percentage of glucocerebrosides in the ceramid monohexoside fraction of the most critical fraction II displayed an increase up to 60% against 47.6% of the total extract (see Table 4). Ultrastructurally, fraction II contained most oligomembranous bodies identical with those in the neuronal perikarya. There was, however, a still significant admixture of impurities, myelin-like fragments, dense bodies and mitchondria and unidentifiable structures, most frequent in fractions III and IV. Fraction I was small, and the utrastructure was not examined.

Gangliosides exhibited a slight increase in the GM_2 and GM_3 fractions in the cortex in both cases.

Enzyme Studies

The activities of enzymes examined histochemically in storage neurones, in the spheroids of NAD and in

Table 2. Results of lipid histochemistry in lipid storing neurons[a]

Method[b,c]	Results	Remark
PAS[d]	++/+++	Unchanged by KBr_3 and by NaOH pre hydrolysis; irrerular staining in non pre-extracted sections
Iron hematoxylin[d]	−/±	Completely negative after NaOH hydrolysis
Cresyl violet	+	Ortho-chromatically; extinguished by subsequent differentiation with 1N HCl
Plasmal	−/±	
OTAN		
without pre-extraction	+/++	
acetone pre-extraction	+/++	Unchanged by NaOH prehydrolysis
Sudan black B		
without pre-extraction	−/±	
acetone pre-extraction	−/±	
Birefringence[d]		
primary[e]	minimal	Only occassional discrete granules
secondary[f]	present	Discrete liquid spherocrystals (myelin figures) partly unstable, namely in non pre-extracted sections

[a] For the sake of clarity staining caused by lipopigment is not included
[b] Unless stated otherwise, the methods were performed after pre-extraction with anhydrous acetone (Elleder and Lojda 1971)
[c] Results after chloroform-methanol extraction were completely negative and are not included here
[d] See Fig. 3
[e] Present immediately after mounting the sections
[f] Appeared several hours or days after the sections had been mounted

gitter cells or the frontal cortex, thalamus, spinal cord, brain stem and cerebellum are given in Table 6.

The activities of SMase and beta-glucosidase appeared normal in both the brain and liver in both patients, whereas these activities were approximately 50% of normal in cultured fibroblasts. Activities in brain, expressed as nmol/h per mg protein were 19.4 (SMase) and 29.4 (beta-glucosidase) for case 1, and 19.2 (SMase) and 25.0 (beta-glucosidase) for case 2. The mean control values ± S.D were 15.4 ± 3.3 and 29.4 ± 3.5, respectively, for five control samples. On isoelectric focusing of brain extracts, no defect was observed for these enzyme profiles in the NPDC brain samples but in fibroblasts extracts from the patients there was a marked deficiency the cathodic SMase components; the beta-glucosidase profile appeared normal. These findings are typical of other cases for NPDC. Studies on the fibroblasts and liver of case 1 have been reported elsewhere (Besley and Moss 1983a, b).

Discussion

The present study offers evidence of the quality of the neuronal storage in NPDC. The histochemical conclusions (neutral glycosphingolipid) based on results obtained in adequately processed tissue with improved and revised methods and criteria established in our laboratory (Elleder 1977, 1982) and in agreement with the lipid chemical analysis in which the only significant finding was an increase in glucocerebrosides and ceramide dihexosides (probably ceramide lactoside, see below). These results and the increase of the lipids in the partially purified storage lysosomes of the cerebral cortex permits to conclude that it may be in the neurones that glycolipid storage takes place.

A discrepancy exists, however, which lacks satisfactory explanation, namely the strikingly lower intensity of staining of central neurones in NPDC brains fixed in formaldehyde (Elleder and Jirásek 1981). Destruction of the lipid by fixation has to be considered but is thought to be normally restricted to gangliosides (Suzuki 1965). Solubility seems to be another option. The negative effect of formaldehyde fixation should be noted in lipid histochemical studies and may account for some of the discrepancies reported in the literature (see Introduction).

The reports of chemical analyses in NPCD brains have a single common denominator in the absence of abvious SM storage. The rest of the results differ from each other. Some authors report an increase in neutral ceramide hexosides (Kannan et al. 1974), or mainly ceramide lactoside (Hagberg et al. 1978) or in both glucocerebroside and ceramide lactoside (Philippart et al. 1969). Monosialogangliosides GM_2 and GM_3 are sometimes reported to be the only lipids increased (Anzil et al. 1973; Harzer et al. 1978; Lowden et al. 1967; Oppenheimer et al. 1967; Pellissier et al. 1976). A normal lipid spectrum has been reported by de Leon et al. (1969) and Neville et al. (1973).

In our opinion, such discrepancies are due mainly to the diversity of methodological approach. It should be pointed out that all the fractions of neutral glycolipids can be safely evaluated only if chromatography is used after previous purification or, at least, partial purification by removing glycerophosphatides. This was confirmed by the analysis of the available

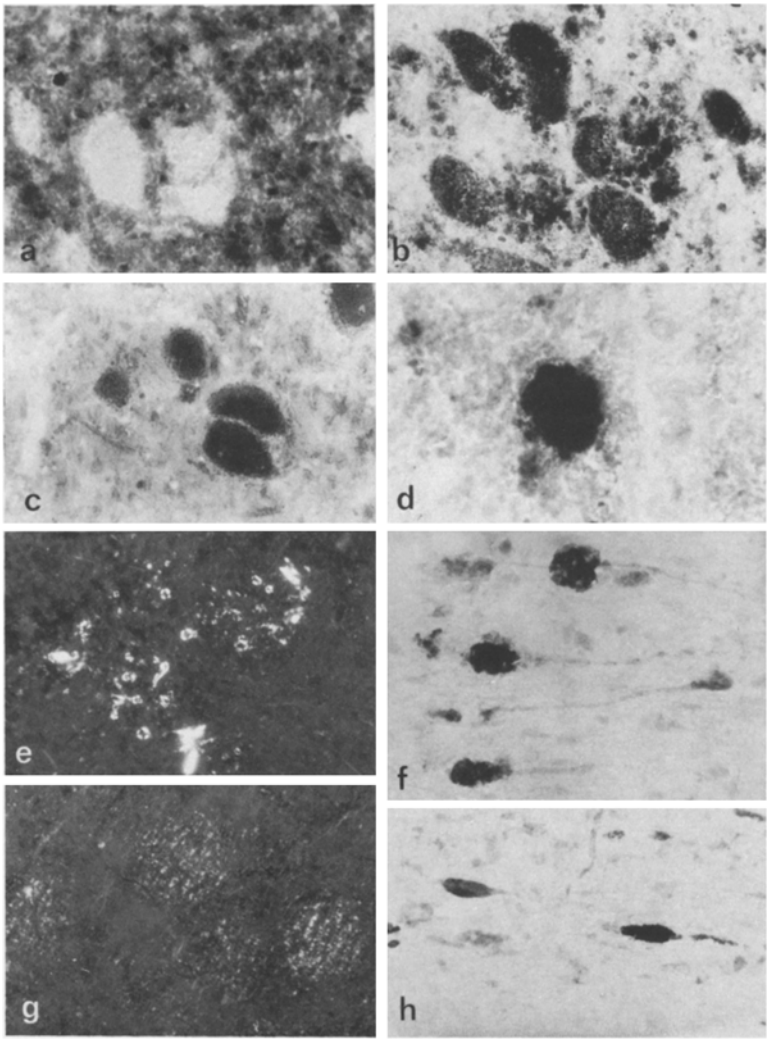

Fig. 3. a Absence of staining for SM in substantia nigra neurones. Iron hematoxylin after alkaline hydrolysis. Strong staining in the neurophile. **b** High acid phosphatase activity in storing neurones in the substantia nigra and in glial phagocytes in a neuronophagic focus. **c** Intense PAS staining in substantia nigra neurons given by glycolipid storage; ×136. **d** High acid phosphatase activity in a spheroid in the posterior spinal column at the thoracic level; ×340. **e** Unstable lipid liquid spherocrystals in neuronal bodies. Nigra. Unstained. **f** NADH tetrazolium reductase activity in cerebellar white matter NAD spheroids. **g** Stabilized lipid liquid spherocrystals after treatment with acetone (cp. with **e**). Unstained. Nigra. **h** Nonspecific esterase activity in cerebellar white matter NAD spheroides; ×221

Table 3. Composition of phospholipids in NPD type C brains. (Values are expressed in μmol of phosphorus per g wet weight. Percentage of each fraction is given in parentheses)

	Frontal cortex					Cerebellum		
	NPD type C		Controls			NPD type C		Control
Case:	P.L.	R.B	1 y	12 y	13 y	P.L.	R.B	
Age:	$4^{8}/_{12}$ y	4 y				$4^{8}/_{12}$ y	4 y	$2^{1}/_{2}$ y
LPC	0.7 (2.3)	0.4 (2.2)	0.2 (3.6)	1.3 (0.7)	0.3 (0.8)	0.1 (0.4)	0.2 (0.7)	0.1 (0.5)
PI	1.3 (4.8)	0.6 (3.3)	2.0 (5.2)	1.9 (3.1)	1.8 (4.3)	0.8 (4.1)	0.4 (1.5)	0.7 (3.1)
PS	2.4 (8.6)	1.2 (6.9)	4.4 (11.7)	5.1 (16.7)	5.7 (13.8)	1.4 (6.8)	2.5 (10.0)	2.0 (9.1)
SPH	3.1 (11.2)	4.1 (20.5)	3.4 (9.2)	4.8 (7.9)	5.3 (12.7)	2.3 (11.4)	5.4 (22.3)	3.0 (13.9)
PC	11.2 (40.3)	9.7 (55.3)	15.3 (40.7)	11.7 (34.2)	14.7 (35.3)	8.0 (39.9)	12.7 (52.0)	7.5 (34.5)
PE	8.6 (30.7)	1.4 (7.8)	12.2 (30.7)	11.1 (36.7)	13.0 (31.2)	7.2 (36.1)	3.4 (13.8)	7.2 (33.3)
BMP	0.4 (1.5)	–	–	–	–	0.2 (0.7)	–	–
PA	0.2 (0.6)	–	–	–	–	–	–	0.1 (0.2)

Abbreviations: LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; BMP, bis(monoacylglyceryl)-phosphate; PA, phosphatidic acid

Table 4. A: Composition of glycolipids in NPDC brains. B: Percentage of glucosyl- and galactosylceramide in ceramide monohexoside fraction (nmol/g wet weight)

A

	Frontal cortex			Cerebellum			Thalamus		Nc. niger	
Case:	P.L.	R.B.	Controls[a]	P.L.	R.B.	Controls[a]	P.L.	Controls[a]	P.L.	Controls[a]
GL-1a	320.0	221.7	695.3 ± 38.7	340.0	640.0	517.6 ± 27.2	460.0	436.5 ± 32.0	500.0	540.8 ± 9.7
GL-1b	173.2	184.7	794.3 ± 45.9	506.6	960.0	607.4 ± 34.2	700.0	797.6 ± 26.5	760.0	891.4 ± 15.7
GL-2a	106.6	43.3	63.6 ± 8.7	20.0	46.6	81.8 ± 5.1	46.6	60.2 ± 4.1	66.6	125.1 ± 5.1
GL-2b	604.0	450.0	1.9 ± 1.2	186.6	220.0	72.2 ± 5.6	160.0	2.8 ± 2.1	93.3	93.3 ± 9.0
Su	53.2	–	360.8 ± 15.1	126.6	20.0	21.3 ± 3.5	246.6	276.0 ± 11.9	86.6	342.5 ± 28.1
GL-3	173.2	120.0	–	20.0	6.6	–	26.6	–	–	–
GL-4	26.6	60.0	–	20.0	1.0	–	23.3	5.0 ± 2.7	2.6	9.1 ± 0.8
GM-3	133.2	613.3	–	33.3	193.3	–	–	–	–	–
GL-5	20.0	–	–	3.3	–	–	33.3	0.9 ± 0.6	1.3	–
B										
glu-cer	60.4	47.6	8.6 ± 0.9	23.5	22.0	12.2 ± 1.1	20.0	9.6 ± 0.8	16.1	8.1 ± 1.1
gal-cer	39.6	52.4	91.4 ± 3.1	76.5	78.0	87.8 ± 2.8	80.0	90.4 ± 3.1	83.9	91.9 ± 4.0

[a] Mean values ± SD in four age-matched controls

GL-1a, ceramide monohexoside (fast moving band); GL-1b, ceramide monohexoside (slow moving band); CL-2a, ceramide dihexoside (fast moving band),; GL-2b, ceramide dihexoside (slow moving band); Su, sulphatides; GL-3, ceramide trihexoside; GL-4, ceramide tetrahexoside; GM-3, ganglioside GM_3; GL-5, ceramide pentahexoside; glu-cer, glucosylceramide; gal-cer, galactosylceramide; 1,2,3,4,5 = number of sugars in the glycolipid molecule. a,b Separation according to the length of fatty acids and/or sphingosin chains

Table 5. Activities of marker lysosomal enzymes and glycolipid concentration in discontinous sucrose gradient fractions. Sample: Frontal cortex of case R.B.

	Fractions				
	I.	II.	III.	IV.	Crude fraction (7,500 *g*)
Marker lysosomal enzymes	nmol/mg protein/h				
Acid phosphatase[a]	1,500	4,300	2,170	1,768	2,509
Aryl sulphatase A[b]	127	171	9	32	61
β-Glucosidase[c]	37	124	15	38	45
β-Hexosaminidase[d]	2,250	7,333	883	1,162	3,509
Glycolipids	nmol/mg protein				
GL-1a[e]	4.15	16.5	5.0	2.0	6.0
GL-1b	–	17.3	3.0	0.5	3.0
GL-2a	10.8	5.3	0.8	0.3	2.5
GL-2b	9.1	12.5	2.5	1.5	5.8
Su	–	3.3	0.8	0.5	1.0
GL-3	–	5.5	0.3	2.8	3.8
GL-4	–	–	–	–	–
GM-3	–	12.8	5.0	3.3	23.0

Acid phosphatase[a] was determined using 0.6 mM p-nitrophenyl phosphate in sodium acetate buffer, pH 5
Aryl sulfatase A[b] was determined according to Dulaney and Moser, β-glucosidase[c] according to Peters et al., and β-hexosaminidase[d] according to Srivastava. For details see Glew and Peters (1979)
Abbreviations for glycolipids[e] see Table 4

methodological data in the papers cited. In addition, the analysis of the hexose moiety in the ceramide monohexosides appears inevitable.

In summary, our results and some of the literature data indicate that neuronal glycolipidosis should be seen as an important feature in the chemical pathology of NPDC brains. Brain glycolipid storage is in accord with the similar tendency in the visceral organs where the same glycolipid species, i.e. glucocerebroside and lactosylceramide, were found manifold increased

Table 6. Results of enzyme histochemistry

Enzyme	Neurons	NAD[a]	Gitter cells
Acid phosphatase (AM[b], SM[c])	++/+++	+/++++	+++
b-Glucuronidase (SM)	±	±	+/++
b-Galactosidase (SM) indigogenic method	+++/++++	−/++	++/+++
a-Mannosidase (SM)	±/++	±/−	−
b-Glucosaminidase (SM)	+++/++++	+/++++	+
Nonspecific esterase (AM)	−/+++[d]	±/++++[e]	±
E600 (3×10^{-5} M (SM)	±/+++[f]	±/+	±/+++[g]
Succinate dehydrogenase (AM)	−/±	+/+++	±
a-Glycerophosphate dehydrogenase (mitochondrial)	−/++	+/++++	±
NADH-tetrazolium reductase	−/±	+/++++[h]	±

[a] Neuroaxonal dystrophy (spheroids)
[b] Aqueous medium
[c] Semipermeable membrane medium (Lojda et al. 1979)
[d] Activity indirectly proportional to the degree of lipid storage
[e] See Fig. 3h
[f] Directly proportional to lipopigment deposition
[h] See Fig. 3f

(Vanier 1983). It is worth mentioning here that the so-called lactosylceramidosis (Dawson 1972; Dawson and Stein 1970) may well be explained as NPDC (Wenger et al. 1975) with enhanced visceral storage of these glycolipids (Elleder et al. 1984a). In the so-called type D of NPD which shares many features with type C and is probably its geographical variant the analysis of brain lipids showed and increase in neutral glycolipids particularly of ceramide lactoside (Rao and Spence 1977).

The reason for glycolipid storage is not clear, however, it should be borne in mind that it might be a secondary manifestation of a disorder affecting different compounds as is the case of monosialoganglioside and neutral glycolipid storage in brains in mucopolysaccharidoses (van Hoof 1973).

Comparison of brain lipid spectra in types A and C show the difference to be limited primarily to SM generally increased in type A (Brunngraber et al. 1973; Didion 1949; Greenbaum et al. 1976; Martin et al. 1972; Navarro et al. 1973; Norman et al. 1959; others reviewed by Harzer and Benz 1976). So far there has been dearth of studies of brain neutral glycolipids in NPDA. To the best of our knowledge, there are only two such reports. One, by Greenbaum et al. (1976), revealed that total extracts contain increased levels of glucosylceramide, di- and trihexoside was well as GM_2, changes identical with findings in some cases of NPDC (see above). The other, by Kamoshita et al. (1969) found a glucocerebroside as a minor lipid in the purified storage lysosomal fraction together with an admixture of GM_2 and GM_3. Increased quantities of monosialogangliosides in type A brains has been reported repeatedly (reviewed by Harzer and Benz 1976). However, the distribution of the lipid stored has as yet never been studied in type A brains. In this respect, lipid histochemistry in spite of all its limitations is particularly significant as it shows a clearly unambiguous difference in the distribution of the lipids stored in types A and C brains. In type A, there is mostly neuronal, less so vascular, SM storage, while neutral glycolipid dominates in the vascular wall (Elleder and Jirásek 1981). In type C, it is a glycolipid which is stored in the neurones, i.e. in the location different from that in type A. The need then arises to define the cellular distribution of the lipid to obtain the real picture of the state of affairs.

As regards the storage lysosome ultrastructure, the results are in perfect accordance with the published data (see Introduction). Findings regarding the neurolipofuscin fine structure and its relationship to storage will be dealt with elsewhere.

The prominent incidence of NAD in NPDC when compared with type A and other storage disorders has already been described (Elleder and Jirásek 1981). Accordingly, the intensity and extent of NAD in these two additional cases were considerable, particularly the involvement of white matter indicating affection of long myelinated axons. In many respects, the picture strongly resembled that of the idiopathic infantile form of NAD (Cowen and Olmstead 1963; Seitelberger 1975) and reflects the insufficiently stressed tendency of NPDC to develop dystrophic tissue changes. Similarly, as in infantile NAD (Elleder and Jirásek 1983a), the axonal spheroids in NPDC displayed activity of nonspecific esterase (Fig. 3h). However, the enzyme pattern in NPDC spheroids was much more heterogenous. This correlates well with the much more pleiomorphic ultrastructure of spheroids in NPDC which reflects the participation

of a variety of organelles, including neurofilaments, contrary to the relatively uniform finding of smooth membranes in infantile NAD (Jellinger and Jirásek 1971; Yagishita and Kimura 1975).

The enzyme studies will be reported separately. Although partial deficiencies of SMase and beta-glucosidase activities were indentified in these patients' fibroblast cultures, and a deficiency of cathodic SMase activity was measured on electrofocusing, no such defects were observed when studying the brain enzymes. Expression of enzyme involvement in NPCD fibroblasts (Besley 1977; Besley and Moss 1983a) may thus be a secondary phenomenon (see also Vanier et al. 1983). Nevertheless, some support of "in vivo" involvement of SMase has been provided by the demonstration of defects in SM degradation by NPDC fibroblasts of some cases (Kudoh et al. 1983; Mazière et al. 1982). Whether or not such defects occur in NPDC brains is not clear and is not supported by this or other studies (Müller and Herzer 1980).

To conclude, the present study shows the profound difference of cerebral changes between NPDA and NPDC. It is the neuronal glycolipid storage and a strong tendency to NAD in lower age groups which are the present day hallmarks of NPDC neuropathology. They are not accompanied by any detectable neuronal SM storage or by SMase or beta-glucosidase deficiencies. Conversely, the storage in NPDA can be characterized by neuronal SM storage, profound deficiency fo SMase and low incidence of NAD which is orthotopic according to Seitelberger's (1975) classification. In advanced cases there is also storage of neutral glycolipids in the walls of cerebrospinal vessels.

These differences correlate well with those found in other organs and in biochemical findings (Elleder 1983; Elleder and Jirásek 1983b). However, the nature of the underlying metabolic disorder in NPDC remains to be established and requires further investigation. Better knowledge of the chemical nature of neuronal storage may help in elucidating it.

Acknowledgement. We wish to thank Dr. V. Bradová and Miss V. Sahulová for their perfect technical assistance in lipid analyses.

References

Anzil AP, Blinzinger K, Mehraein P, Dozic (1973) Niemann-Pick disease type C: case report with ultrastructural findings. Neuropädiatrie 4:207–225

Besley GTN (1977) Sphingomyelinase defect in Niemann-Pick disease, type C, fibroblasts. FEBS Lett 80:71–74

Besley GTN, Moss SE (1983a) Studies on sphingomyelinase and beta-glucosidase activities in Niemann-Pick disease variants. Biochim Biophys Acta 752:54–64

Besley GTN, Moss SE (1983b) Sphingomyelinase studies in Niemann-Pick disease with special reference to isoelectric focusing changes in type C disease. Quoted in Elleder M, Jirásek A: Niemann-Pick disease. Report on a symposium held in Prague, September 1982. Acta Univ Carol [Med] (Praha) 29:259–267

Brunngraber EG, Berra B, Zambotti V (1973) Altered levels of tissue glycoproteins, gangliosides, glycosaminoglycans and lipids in Niemann-Pick's disease. Clin Chim Acta 48:173–181

Cowen D, Olmstead EV (1963) Infantile neuroxonal dystrophy. J Neuropathol Exp Neurol 22:175–236

Crocker AC (1961) The cerebral defect in Tay-Sachs and Niemann-Pick disease. J Neurochem 7:69–80

Crocker AC, Farber S (1958) Niemann-Pick's disease: a review of eighteen patients. Medicine (Baltimore) 37:1–96

Dawson G (1972) Glycosphingolipid levels in an unusual neurovisceral storage disease characterized by lactosglyceramide galactosyl hydrolase deficiency: lactosylceramidosis. J Lipid Res 13:207–219

Dawson G, Stein AO (1970) Lactosyl ceramidosis: catabolic enzyme defect of glycosphingolipid metabolism. Science 170:556–558

de Leon GA, Kaback MM, Elfenbein IB, Percy AK, Brady RC (1969) Juvenile dystonic lipidosis. Johns Hopkins Med J 125:62–77

Didion H (1949) Vergleichend histopathologische Untersuchungen an einem Zwillingspaar mit Niemann-Pickscher Krankheit (NPK). Frankf Z Pathol 60:194–227

Elleder M (1977) Lipid histochemistry – a critical survey. Acta Histochem [Suppl] XIX:239–265

Elleder M (1982) Lipid histochemistry. Methodological study and evaluation of its significance in diagnosis of disorders of lipid metabolism. Thesis

Elleder M (1983) Lipid histochemistry of Niemann-Pick disease. Histochem J 15:319–322

Elleder M, Jirásek A (1981) Neuropathology of various types of Niemann-Pick disease. Acta Neuropathol [Suppl] (Berlin) VII:201–203

Elleder M, Jirásek A (1983a) New enzyme findings in infantile neuroaxonal dystrophy. Acta Neuropathol (Berlin) 60:153–155

Elleder M, Jirásek A (1983b) Niemann-Pick disease. Report on a symposium held in Prague 1982. Acta Univ Carol [Med] (Praha) 29:259–267

Elleder M, Lojda Z (1968) Remarks on the "OTAN" reaction. Histochemie 14:47–64

Elleder M, Lojda Z (1971) Studies in lipid histochemistry. VI. Problems of extraction with acetone in lipid histochemistry. Histochemie 28:68–87

Elleder M, Lojda Z (1972) Studies in lipid histochemistry. VIII. Some problems of the bromination particularly in relation to lipid histochemistry. Histochemie 32:285–299

Elleder M, Lojda Z (1973a) Studies in lipid histochemistry. XI. New, rapid, simple and selective method for the demonstration of phospholipids. Histochemie 36:149–166

Elleder M, Lojda Z (1973b) Studies in lipid histochemistry. XII. Histochemical detection of sphingomyelin. Histochemie 37:371–373

Elleder M, Šmíd F (1977) Lysosomal non-lipid component of Gaucher's cells. Virchows Arch [Cell Pathol] 26:133–138

Elleder M, Hrodek J, Čihula J (1983) Niemann-Pick disease: lipid storage in bone marrow macrophages. Histochemical J 15:1065–1077

Elleder M, Jirásek A, Šmíd F, Ledvinová J, Besley GTN, Stopeková M (1984a) Niemann-Pick disease type C with enhanced glycolipid storage. Report on further case of so-

called lactosylceramidosis. Virchows Arch [Pathol Anat] 402:307–317
Elleder M, Šmíd F, Hyniová H, Čihula J, Zeman J, Macek M (1984b) Liver findings in Niemann-Pick disease type C. Histochem J 16:1147–1170
Elfenbein IB (1968) Dystonic juvenile idiocy without amaurosis. A new syndrome. Light- and electron-microscopic study of cerebrum. Johns Hopkins Med J 123:205–211
Emeis JJ, Gent van CM, Sabben van CM (1977) An enzymatic method for the histochemical localization of free and esterified cholesterol separately. Histochem J 9:197–204
Emery JM, Green WR, Huff DS, Sloan HR (1972) Niemann-Pick disease (type C). Histopathology and ultrastructure. Am J Ophthalmol 74:1144–1154
Fredrickson DS, Sloan HR (1972) Sphingomyelin lipidosis: Niemann-Pick disease. In: Stanbury JB, Wyngaarden JB, Fredrickson DS (eds) The metabolic basis of inherited disease. McGraw-Hill, New York, pp 783:807
Glew RH, Peters SP (1979) Practical enzymology of the sphingolipidoses. Liss, New York
Greenbaum M, Hoffman LM, Schneck L (1976) Ceramid hexosides in Niemann-Pick disease brain. J Neurol 213: 251–255
Hagberg B, Haltis M, Sourander P, Svennerholm L, Vanier M-T, Ljunggren CG (1978) Neurovisceral storage disorder simulating Niemann-Pick disease. A new form of oligosaccharidosis? Neuropädiatrie 9:59–73
Harzer K, Benz HU (1976) Sphingomyelinoses (Niemann-Pick'sche Erkrankung). In: Schettler G, Greten H, Schlierf C, Seidel D (Hrsg) Handbuch der Inneren Medizin, Bd VIII/4: Fettstoffwechsel. Springer, Berlin Heidelberg New York, pp 525–546
Harzer K, Peiffer J (1981) Morbus Niemann-Pick Typ C (subakute neuroviscerale Lipidose). Zur Frage der veränderten Sphingomyelinase-Aktivität im Gehirn. Arch Psychiatr Nervenkr 230:71–79
Harzer K, Schlote W, Peiffer J, Benz HU, Anzill AP (1978) Neurovisceral lipidosis compatible with Niemann-Pick disease type C: Morphological and biochemical studies of a late infantile case and enzyme and lipid assays in a prenatal case of the same family. Acta Neuropathol (Berlin) 43:97–104
Horoupian DS, Yang S (1978) Paired helical filaments in neurovisceral lipidosis (juvenile dystonic lipidosis) Ann Neurol 4:404–411
Jellinger K, Jirásek A (1971) Neuroxonal dystrophy in man: character and natural history. Acta Neuropathol (Berlin) [Suppl] V:3–16
Kamoshita S, Aron AM, Suzuki K, Suzuki K (1969) Infantile Niemann-Pick disease. A chemical study with isolation and characterization of membranous cytoplasmic bodies and myelin. Am J Dis Child 117:379–394
Kannan K, Hong-Boen Tjiong, Debuch H, Wiedemann H-R (1974) Unusual glycolipids in brain cortex of a visceral lipidosis (Niemann-Pick disease?). Hoppe-Seyler's Z Physiol Chem 355:551–556
Kean EL (1966) Separation of gluco- and galactocerebrosides by means of borate-thin-layer chromatography. J Lipid Res 7:449–452
Kudoh T, Velkoff MA, Wenger DA (1983) Uptake and metabolism of radioactively labeled sphingomyelin in cultured skin fibroblasts from controls and patients with Niemann-Picks disease and other lysosomal sorage diseases. Biochim Biophys Acta 754:82–92
Lake BD (1981) Metabolic disorderes: general considerations. In: Berry CL (ed) Pediatric pathology. Springer, Berlin Heidelberg New York, pp 617–639
Lake BD (1983) The diagnosis of ophthalmoplegic lipidosis – experience with 50 patients. Quoted in Elleder M, Jirásek A: Niemann-Pick disease. Report on a symposium held in Prague 1982. Acta Univ Carol [Med] (Praha) 29:259–267
Lojda Z, Gossrau R, Schiebler T (1979) Enzyme histochemistry – a laboratory manual. Springer, Berlin Heidelberg New York
Lowden JA, La Ramée MA, Wentworth P (1967) The subacute form of Niemann-Pick disease. Arch Neurol 17:230–237
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
Martin JJ, Philippart M, Hauwaert JV, Callahan JW, Deberdt R (1972) Niemann-Pick disease (Crocker's group A) Late onset and pigmentary degeneration resembling Hallervorden-Spatz syndrome. Arch Neurol 27:45–51
Mazière C, Mazière J-C, Mora L, Routier JD, Polonovski J (1982) In situ degradation of sphingomyelin by cultured normal fibroblasts and fibroblasts from patients with Niemann-Pick disease types A and C. Biochem Biophys Res Commun 108:1101–1106
Müller H, Harzer K (1980) Partial purification of acid sphingomyelinase from normal and pathological (M. Niemann-Pick type C) human brain. J Neurochem 34:446–448
Navarro J, Escourolle R, Berger B, Ferriere G, Bure Y, Raimbault J, Trastour J-C, Polonowski C (1973) Participation nerveuse péripherique de la maladie de Niemann-Pick. A propos d'un cas. Ann Pediat (Paris) 20:507–513
Neville BGR, Lake BD, Stephens R, Sanders MD (1973) A neurovisceral storage disease with vertical supranuclear ophthalmoplegia and its relationship to Niemann-Pick disease – a report of nine cases. Brain 96:97–120
Norman RM, Forrester RM, Tingey AH (1967) The juvenile form of Niemann-Pick disease. Arch Dis Childh 42:91–96
Norman RM, Urich H, Tingey AH, Goodbody RA (1959) Tay-Sachs disease with visceral involvement and its relationship to Niemann-Pick's disease. J Pathol Bacteriol 78:409–421
Norton WT, Korey SR, Brotz M (1962) Histochemical demonstration of unsaturated lipids by a bromine-silver method. J Histochem Cytochem 10:83–88
Oppenheimer DR, Norman RM, Tingey AH, Aherne WA (1967) Histological and chemical findings in juvenile Niemann-Pick disease. J Neurol Sci 5:575–580
Pearse AGE (1968) Histochemistry. Theoretical and applied. Churchill, London
Pellissier JF, Hassoun J, Gambarelli D, Bryon PA, Casanova P, Toga M (1976) Maladie de Niemann-Pick type "C" de Crocker. Etude ultrastructure d'un cas. Acta Neuropathol (Berlin) 34:65–76
Philippart M, Martin L, Martin JJ, Menkes JH (1969) Niemann-Pick disease. Morphological and biochemical studies in the visceral form with late central nervous system involvement (Crocker's group C). Arch Neurol 20:227–238
Rao BG, Spence MW (1973) Niemann-Pick disease type D: lipid analyses and studies on sphingomyelinases. Ann Neurol 1:385–392
Robert J, Rebel G (1975) Quantitation of neutral glycolipids by thin-layer chromatography on precoated plates. J Chromatogr 110:393–397
Rouser G, Fleischer S, Yamamoto A (1970) Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5:494–496
Saito T, Hakomori S-I (1971) Quantitative isolation of total glyco sphingo lipids from animal cells. J Lipid Res 12:257–259

Scallen TJ, Dietert SE (1969) The quantitative retention of cholesterol in mouse liver prepared for electron microscopy by fixation in digitonine-containing aldehyde solution. J Cell Biol 40:802–813

Seitelberger F (1975) General neuropathology of the degenerative diseases of the central nervous system. In: Vinken PJ, Bruyn GW (eds) Handbook of clinical neurology, vol 21, part I. North-Holland, Amsterdam, pp 43–71

Suzuki K (1965) The pattern of mammalian brain gangliosides. II. Evaluation of the extraction procedure, post mortem changes and the effect of formalin preservation. J Neurochem 12:629–638

Suzuki K, Suzuki K, Chen Gc (1967) Isolation and chemical characterization of metachromatic granules from a brain with metachromatic leukodystrophy. J Neuropathol Exp Neurol 26:537–550

Ueno K, Audo S, Yu RK (1978) Gangliosides of human, cat, and rabbit spinal cords and cord myelin. J Lipid Res 19:863–871

van Hoof F (1973) Mucopolysaccharidoses, chapt 8. In: Hers HG, van Hoof F (eds) Lysosomes and storage diseases. Academic Press, New York, pp 218–261

Vanier M-T (1983) Biochemical studies in Niemann-Pick diesease. I. Major sphingolipids of liver and spleen. Biochem Biophys Acta 750:178–184

Vanier M-T, Rousson R, Louisot P (1983) Chromatofocusing of skin fibroblast sphingomyelinase: alterations in Niemann-Pick disease type C shared by GM_1 – gangliosidosis. Clin Chim Acta 130:155–161

Wenger DA, Sattler M, Clark C, Tanaka H, Suzuki K, Dawson G (1975) Lactosylceramidosis: normal activity for two lactosyl ceramide-beta-galactosidases. Science 188:1310–1312

Yagishita S, Kimura S (1975) Infantile neuroaxonal dystrophy (Seitelberger's disease). A light- and ultrastructural study. Acta Neuropathol (Berlin) 31:191–200

Received March 9, 1984/Accepted January 22, 1985