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Lipidosis with a Predominant Storage of Phosphoglycerides (Phospholipidosis Type II — Baar, Wiedemann)

A Histochemical, Chemical and Electronoptical Study of a Case

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Summary. A case of a 27 month old girl suffering from a rare form of lipidosis is described. Clinical symtoms consisted of a moderate hepatosplenomegaly and a progressive psychomotor retardation. Bioptical examination of the liver, appendix and skin revealed a pronounced lipid storage in histiocytes, hepatocytes, vascular endothelium and in peripheral nervous system. Histochemically, a generalized storage of phosphoglycerides and cholesterol was found. It was accompanied with a moderate amount of sphingomyelin and a variable amount of glycolipids (predominantly glycosphingolipids), the latter being stored mainly in the peripheral nervous system and in the vascular endothelium. Chromatographically, an increased concentration of lysobisphosphatidic acid and cholesterol could be detected. The ultrastructure of storage cytosomes was rather pleomorphic often with concentrically lamellar appearance. Further details of the investigation are described and the relation of this case to those described by Baar and Hickmans (1956) and Wiedemann *et al.* (1972) is stressed. Due to a strong evidence that this group of diseases represents a new type of phospholipid storage disease the name "Phospholipidosis Type II" (Baar-Wiedemann) or "Phosphoglyceridosis" is proposed, whereas "Phospholipidosis Type I" or "Sphingomyelinosis" should be reserved for the classical Niemann-Pick complex.

Introduction

A biochemical hallmark of lipidoses belonging to the group of lysosomal storage diseases is the sphingolipid nature of main stored polar lipids. In the case of phospholipids, only one form has been firmly established, i.e. the Niemann-Pick disease, in which sphingomyelin is stored, accompanied with a considerable amount of cholesterol (Fredrickson and Sloan, 1972). However, in 1956 a peculiar lipidosis was described in two siblings by Baar and Hickmans. Clinical and histopathological features closely resembled those of the Niemann-Pick disease, but the chemical analysis revealed a considerable accumulation of phosphoglycerides of "cephalin" type together with cholesterol, the sphingomyelin being only slightly increased. Recently, four cases of a similar disease were described by Wiedemann *et al.* (1972) in which a thorough chemical analysis of affected tissue showed a presence of lysobisphosphatidic acid and a slightly increased concentration of sphingomyelin, cerebrosides and gangliosides together with a great amount of cholesterol. According to these findings a new type of lipidosis was proposed which, due to the decreased enzymatic activity of sphingomyelinase and a moderate degree of visceral sphingomyelinosis was considered to belong nosologically to the Niemann-Pick-complex. We had recently an opportunity to study bioptical material of one case belonging apparently to the same category. The results of

histochemical, chemical and ultrastructural studies are presented in this communication.

Material and Methods

The following surgically removed tissues were obtained for examination: appendix, small excision from the liver and from the skin.

Histology. Portion of each specimen was fixed overnight with cooled Baker's formaldehyde-calcium chloride and embedded in paraffin. The sections were stained with hematoxylin-eosin.

Electron Microscopy. Small fragments of tissues were fixed overnight in a cooled cacodylate-buffered 3% glutaraldehyde, postfixed overnight with buffered 1% osmium tetroxide, dehydrated with acetone and embedded in Araldit. Ultrathin sections were stained with uranyl acetate and lead hydroxide and examined with JEM 7.

Histochemistry. The tissue were frozen rapidly in petrolether cooled with an acetone—dry ice mixture and cut as 14 μ thick cold microtome sections. The battery of *histochemical methods* is listed in Table 1.

Complementary Methods. Extraction with acetone (Elleder and Lojda, 1971) and with chloroform-methanol mixture (2:1 v/v) sometimes acidified with hydrochloric acid (room temperature, 15—60 minutes); alkaline hydrolysis for removal of phosphoglycerides(1 NNaOH, at room temperature); bromination (Elleder and Lojda, 1972a). Generally, methods for the detection of apolar lipids were carried out in unextracted and extracted sections for evaluation of a possible admixture of lipopigment or nonlipidic substances (staining persistent even after extraction with chloroform-methanol) or of polar lipids (staining abolished only after extraction with chloroform-methanol). Methods for polar lipids with an exception of the plasmal method were carried out in both acetone and chloroform-methanol extracted sections and their difference considered as the staining intensity.

The *lipopigment* was detected with Fettrot 7B, Sudan Black B and PAS methods after extraction with chloroform-methanol, with autofluorescence, acidoresistence, argentaffinity toward ammoniacal silver solution and silver methenamine (Lillie, 1965), Nile Blue sulphate and with paraldehydefuchsine after 2 minute preoxidation with acidified permanganate (Pearse, 1968). The native color was evaluated as well.

The following *enzymatic activities* were studied histochemically: alkaline phosphatase (Naphthol-AS-phosphate; Fast Blue BB), nonspecific esterase (alpha-naphthyl acetate; hexazonium pararosanilin with or without diethyl-isopropyl-fluorophosphate (E_{600}) blockade, glukose-6-phosphatase, phosphorylase (Lojda, 1966, 1970) and the method for the demonstration of peroxisomes (Novikoff and Novikoff, 1972). Dehydrogenases: succinate dehydrogenase, NADH-tetrazolium reductase and mitochondrial alpha-glycerophosphate dehydrogenase, all with an universal medium after Lojda (1965) with nitro Blue Tetrazolium as an hydrogen acceptor.

Lysosomal enzymes were studied with semipermeabile membrane technics (Lojda, 1974). In the case of postcopulative methods the sections were preextracted with cooled acetone to improve the localization. The following enzymatic activities were evaluated with simultaneous azocoupling technic with hexazonium pararosaniline and naphthol-AS-Bi derivative of a respective substrate: acid phosphatase, beta-glucuronidase, N-acetyl-beta-glucosaminidase. The postcopulative technic was employed for: beta-glucosidase, alpha-glucosidase, alpha-galactosidase and beta-galactosidase. Here, 6-Br-2-naphthyl-derivatives and Fast Blue B were employed. The last one was detected with an indigogenic technic as well (Lojda, 1973). Alpha-mannosidase activity was evaluated with 1-naphthyl-alpha-D-mannoside and hexazonium pararosaniline. Activity of acid phosphatase and the diaminobenzidine method for detection of peroxisomes were evaluated electronmicroscopically. The incubation times were 60 and 80 minutes, respectively. In the case of acid phosphatase the sections were briefly treated with a 0.05 percent ammonium sulphite after incubation and then processed as usually.

Chromatographical and chemical examination of lipids was performed in the appendix only. The tissue was homogenized in a glass homogenizer in chloroform-methanol 2:1 v/v and successively extracted with chloroform-methanol 1:2, 7:1 saturated with NH_4OH, chloroform-methanol-water-acetic acid 1:2:1:0,5. Pooled solvents were evaporated under the stream of nitrogen, redissolved in chloroform-methanol 2:1 and washed with a distilled water according

Table 1. List of methods used for detection of stored lipids

Method	Detected lipid	Reference
Birefringence	crystalline state	
Fettrot 7B (in 70% ethanol)	liquid apolar lipids phospholipids (faintly) lipopigment	Lillie (1965)
Sudan Black B (in 70% ethanol)	liquid apolar lipids phospholipids lipopigment	Lillie (1965)
Copper-rubeanic acid	free fatty acids phospholipids lipopigment	Holczinger (1959) Elleder, Lojda (1972b)
Schultze	cholesterol (free and esterified)	Pearse (1968)
Ferric haematox.	all phospholipids	Elleder, Lojda (1973a, b)
after NaOH	sphingomyelin	Elleder, Lojda (1973b)
Controlled Chromation	phospholipids (choline-bearing) cerebrosides (faintly)	Elftman (1954) Lillie, Henderson (1968)
after NaOH[a]	sphingomyelin cerebrosides (faintly)	Adams (1965) Elleder, Lojda (1973b)
Plasmal PAS	plasmalogens glycosphingolipids phosphatidylinositol lipopigment	Pearse (1968) Adams (1965) Elleder, Lojda (1972a)
After NaOH	glycosphingolipids lipopigment (?)	Adams (1965)
Cresyl violet (Hirsch-Pfeiffer)	sulphatides (brown) other acidic lipids (violet) lipopigment	Adams (1965)
OTAN	all unsaturated lipids (except gangliosides) lipopigment	
After NaOH[a]	sphingolipids (except gangliosides) lipopigment	Adams (1965) Elleder, Lojda (1968)

[a] In sections preextracted with acetone.

to Folch. The lower phase was used for evaluation of lipid phosphorus according to Barttlet, for lipid hexose with anthron and for the chromatographical examination on commercial Silicagel G plates (Merck, Darmstadt, BDR). Apolar lipids were separated with hexan: ether: acetic acid 80:20:1 mixture, the bidimmensional chromatography of polar lipids was performed according to Rouser, Simon and Kritchevsky (1969). Detection: charring with ammonium sulphate, molybdenum reagent for phospholipids, anthron reagent for glycolipids. The upper phase was used for estimation of lipid hexose only.

Results

Clinical History

M., S., a female child, was born at term after an uncomplicated pregnancy. The parents are not consanquineous and are healthy, similarly as their second child. No abnormalities were apparent until six month of age. At that time she suffered repeatedly from transient respiratory infections. At nine month she was hospitalized because of psychomotor retardation and a marked hypotony. A skeletal roentgenogman revelaed slightly "cup-like" shaped metaphyses of antebrachial bones suggestive of healing rickets. A chest roentgenogram showed enlargement of mediastinal lymphnodes which was diagnosed to be of tuberculous origin. After one year treatment with Glunizid the roentgenologic finding regressed markedly.

At twenty-seven months the increase of psychomotor retardation was apparent and a moderate hepatosplenomegaly was palpable. Hypotony, especially on lower extremities persisted unchanged.

Laboratory Data. Sedimentation rates 14/36, 16/40; red cell count 3,9 million; haemoglobin 69%; white cell count 5400 with a normal differential; GOT 40μM, on repeated examination 54,6 μM; thymol turbidity, bilirubin in serum, nonprotein nitrogen, cholesterol, electrolytes, alkaline phosphatase and serum proteins gave all normal values. However, there was a marked increase of IgA globulins (350 mg per 100 ml). Bromsulphalein excretion test was normal. Urinalysis showed no abnormalities. Electromyographic examination of tibial nerve gave somewhat lower values but still within normal range. In the ulnar nerve entirely normal values were obtained. EEG examination was free of epileptic graphoelements, but there was a slightly diffuse abnormal record. In the bone marrow puncture histiocytes suggestive of storage were observed.

The combination of psychomotor retardation and hepatosplenomegaly with the finding of cells suggestive of storage in the bone marrow led to a suspicion of lipidosis and the bioptical examination was carried out.

At three years there was a short episode of a bronchopulmonary infection with diarrhea treated succesfully with penicillin. GOT 54 μM, urinalysis (protein, glucose, keton bodies, bilirubin, aminoacids) normal. The repeated examination of bone marrow showed storage cells again, and moreover changes in red cell series consistent with hypersplenism.

Histology

The general architecture of the *liver* tissue was well preserved except for a slight portal and periportal fibrosis. Hepatocytes did not display conspicuous abnormalities in structure except for some lightness of their cytoplasm and scarse minute vacuoles. Kupffer cells were activated, many of them had a plentiful cytoplasm with a variable amount of minute granules and vacuoles. Some of them were transformed into typical foam cells. The sinus endothelium did not display evident abnormalities.

Appendix. The dominant feature was the presence of a relatively numerous population of larger storing macrophages surrounding the outer parts of lymphatic follicles (Fig. 1). A smaller amount was present in the germinal centers as well. One portion of these macrophages had cytoplasm filled with a number of fine eosinophilic granules and occasional vacuoles, others displayed a various degree of vacuolization or had a typical foamy appearance. Those in germinal centers

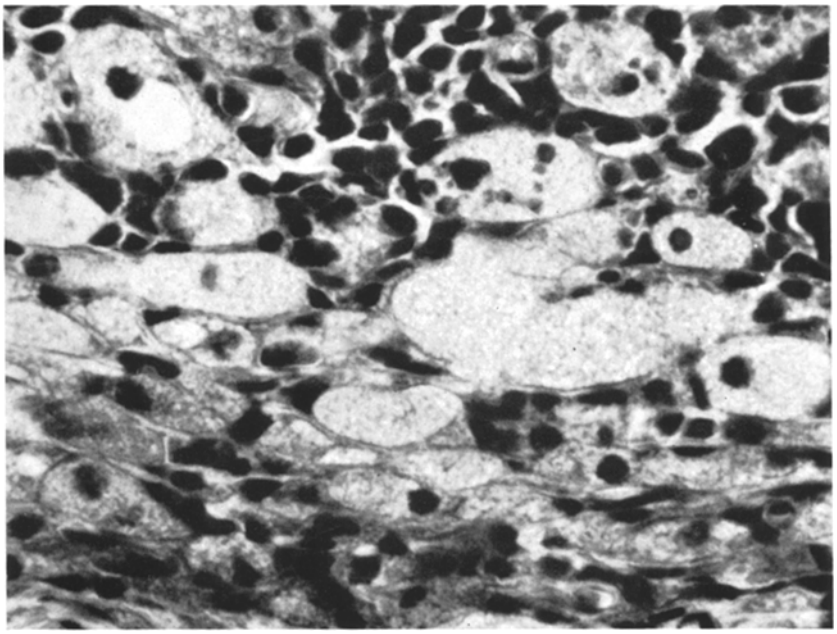


Fig. 1. Storing histiocytes on the periphery of the appendical lymphatic follicle. Paraff. HE ×350

contained typical Fleming's bodies. Other mucosal histiocytes were histologically unremarkable except for occasional vacuoles and eosinophilic granules. A variable degree of vacuolization was observed in the *vascular endothelium* (Fig. 2) most prominently in arterioles of the mucosa, submucosa and sometimes in germinal lymphatic centers. The vacuolization was seen in perivascular fusiform cells in all appendical layers. The ganglion cells and Schwann cells of both neural plexi displayed a marked balooning and a moderate degree of granularity of the cytoplasm (Fig. 3) the rest of which was either normal or condensed.

The Skin. Except for occasional discretely vacuolized perivascular cells, there was no other pathological finding.

Histochemistry

A. Lipids

Sites with histochemically detectable abnormal accumulation of lipids are listed in Table 2. Additional sites of storage, histochemically undetectable, were seen in the course of an electronmicroscopical study (*vide infra*).

1. Physical Properties of Stored Lipids. There was a great number of lipid droplets in storing macrophages giving the Maltese cros-type birefringence in the polarited light. Similar findings were in the vascular endothelium. In other sites of storage the birefringence was barely recognizable (cells of neural plexi) or entirely absent (hepatocytes). The initially discrete uniform birefringent droplets

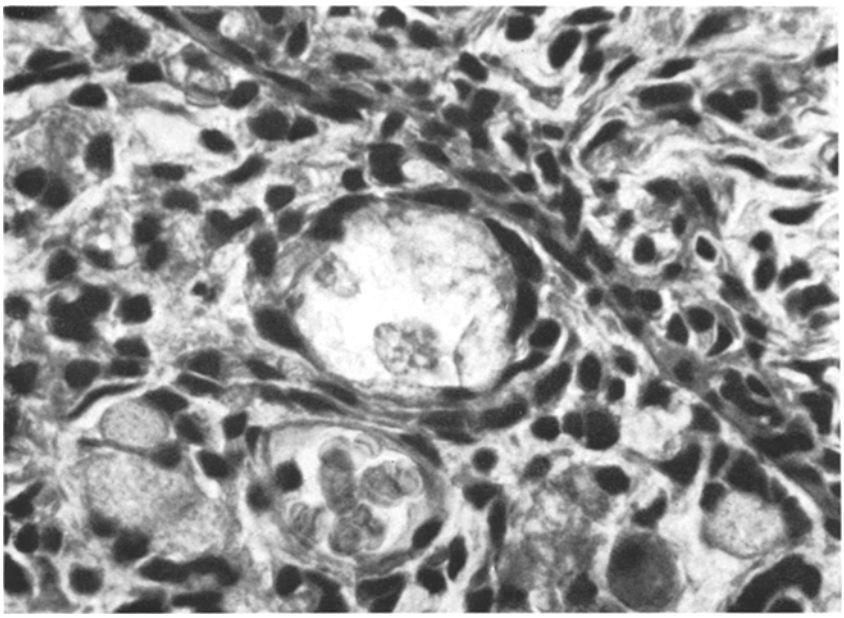


Fig. 2. Marked vacuolization of the vascular endothelium in the appendical mucosa. Paraff. HE ×350

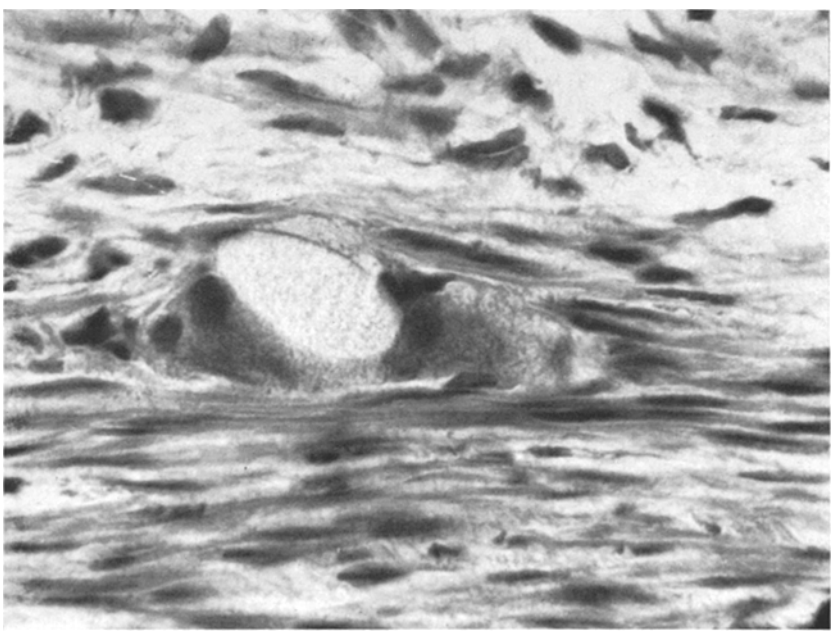
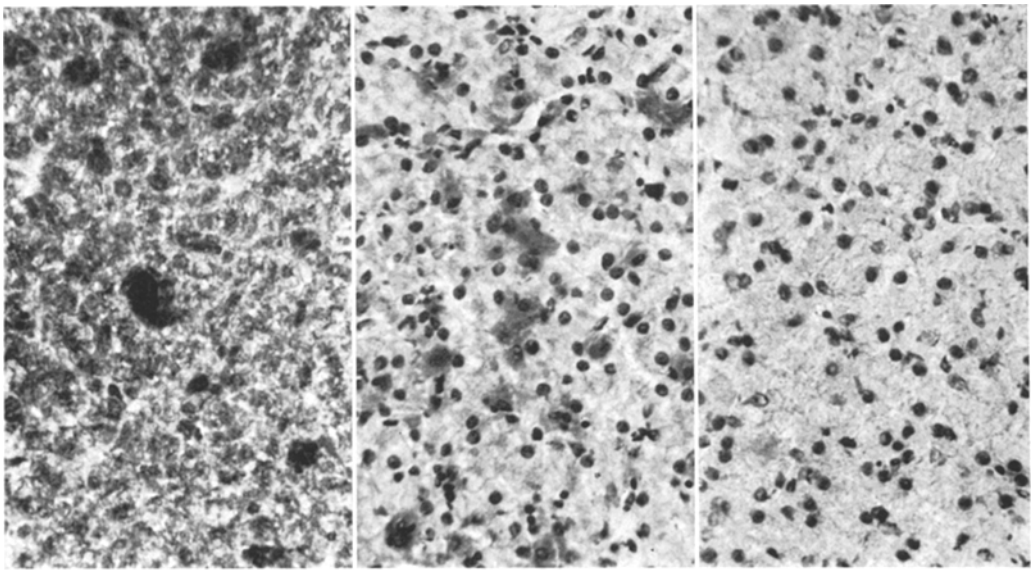


Fig. 3. Storage in the appendical neural plexus. Paraff. HE ×350



a b c

Fig. 4a—c. Cold microtome sections of the liver stained with the ferric-haematoxylin method for phospholipids. (a) After extraction with acetone, (b) after extraction with acetone and subsequent alkaline hydrolysis, (c) after extraction with chloroform-methanol. The difference of the staining intensity between (a) and (b) is given by phosphoglycerides, the difference between (b) and (c) is given by sphingomyelin. Note the abnormal granular staining of hepatocytes caused by storage of phosphoglycerides and the strong staining of Kupffer cells, partially caused by sphingomyelin storage. ×150

Table 2. Semiquantitative[a] results of the main staining methods in most conspicuous storage sites

Method	Kupffer cells	Appendical histiocytes	Hepatocytes	Neural plexi	Vascular endothelium
Schultze	++	++/+++	±	+	+/++
Ferric-haematoxylin[d]	++++	++++	+++	++/+++	+++/++++
After NaOH	+/++	++	±	+/++	++
PAS	±/+[b]	++/+++[c]	+[b]	++	+++/++++
After NaOH	±/+[b]	−	−[b]	++	+++/++++

[a] See Results.
[b] Detection carried out after removal of glycogen with diastase.
[c] After extraction with acidified chloroform-methanol.
[d] For results in the liver see Fig. 4a—c.

displayed a pronounced instability in cold microtome sections mounted into an Apathy's sirup, i.e. they became dilated and variously shaped. This was to a great part inhibited in sections mounted into gelatine or after extraction with acetone.

In addition after several days there was a further change characterized by the development of typical cholesterol plate-like crystals (absent in section preextracted with acetone) and by partial transformation of deformed myelin figures into minute spindle-shaped solid crystals. This additional change was completely inhibited in sections pretreated with 1% sublimate. The birefringence was completely abolished with an alkaline hydrolysis. The degree of polarity of the stored lipid, estimated with a modified OTAN method, will be described elsewhere (Elleder, work in progress).

2. Identification of the Stored Lipids. Liquid *apolar lipids* were detected in a form of separate phase only in hepatocytes (slight diffuse microvacuolar steatosis). The most important results of key methods are demonstrated in Table 2.

Somewhat less distinctive results were obtained with the *controlled* chromation test, which stained the stored lipid moderately. The staining was almost completely resistent to alkaline hydrolysis .*Plasmalogens* were present in a very low amount in some appendical storing histiocytes and in neural cells. The method for detection of gangliosides (Ravetto, 1964) did not work in our hands. *Cresyl violet* stained lipids in some storage cells only (histiocytes, endothelium and neural cells). The staining was weakly metachromatic and was completely prevented by alkaline hydrolysis. No sulphatides could be detected.

The *OTAN method* gave similar results as the ferric haematoxylin method but there was a stronger staining after alkaline hydrolysis in cells of neural plexi, in the vascular endothelium and in hepatic Kupffer cells. In sites with minimal storage, such as *skin* (endothelium and perivascular cells) only phospholipids sensitive to alkaline hydrolysis could be detected.

3. The *residual* (i.e. unextractable) *granules*, present in some storing cells, particularly in histiocytes of appendix, were extremely resistant to the most vigorous extraction procedures. They had the following staining and physical properties: absence of the native color, strong autofluorescence (white-yellow), moderate acidoresistance, questionable sudanophilia, negative argentaffinity with ammoniacal silver and only occasionally positive reaction with a silver methenamine. The basophilia was very weak (cresyl violet, Nile Blue sulphate, toluidine blue, Azur A). The PAS method gave moderately intensive staining which could be abolished almost entirely with an acidified chloroform-methanol only. Quite strong staining was obtained with paraldehydefuchsine. In routine histological staining they were eosinophilic.

B. Enzymatic Activities

Alkaline phosphatase was strongly active in hepatic sinusiods, otherwise its distribution and activity was normal. *Nonspecific esterase* was strongly active in hepatocytes. In storage sites, generally, its activity was low or moderate, except for some ganglion cells, which had somewhat stronger activity. It was generally E_{600}-sensitive. *Phosphorylase* had a normal activity in a bulk of hepatocytes and the predominating color was brown. In some of them, however, the activity was very low or absent. *Peroxisomes* were plentifull in hepatocytes, *Glucoso-6-phosphatase* evaluated in liver sections had normal activity and distribution. *Succinate-dehydrogenase* and *NADH-tetrazolium reductase* activities were low in storage

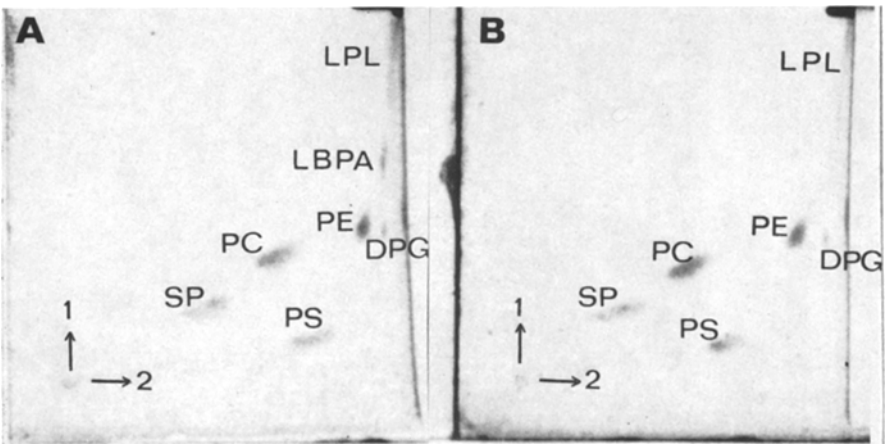


Fig. 5 A and B. Map of spots obtained by two-dimmensional TLC of polar lipids. (A) pathological specimen, (B) control specimen. 1st dimmension: chloroform-methanol-ammonia 65:25:5 2nd dimmension: chloroform-methanol-acetic-acid-water 3:4:1:1:0,5. Abbreviations: *LPL*, less polar lipids/cholesterol, trigly-cerides, etc./; *LBPA*, lysobisphosphatidic acid; *DPG*, diphosphatidyl glycerol; *PE*, phosphatidyl ethanolamine; *PC*, phosphatidyl choline; *PS*, phosphatidyl serine; *SP*, sphingomyelin

sites, the latter beying somewhat stronger. The *mitochondrial alpha-glycerophosphate dehydrogenase* was much more stronger active in storage sites especially in histiocytes.

Lysosomal enzymes were all active to a different degree. The *most active* were acid phosphatase, beta-glucuronidase and beta-galactosidase. All storing sites displayed a strong activity. N-acetyl-beta-hexosaminidase was strongly active as well with the exception of hepatocytes, which were relatively less active. *Moderate activities* were obtained with alpha-galactosidase and beta-glucosidase. *Low activity* was seen with alpha-glucosidase and alpha-mannosidase. However, these latter did not differed significantly in their activity from control sections examined simultaneously. The activity of sphingomyelinase cannot be evaluated in this laboratory.

Chromatographical and Chemical Examination

Due to a limited amount of tissue only appendix was examined. In the bi-dimmensional chromatography of *phospholipids* the only significant difference against the control sample was the presence of lysobisphosphatidic acid (Fig. 5). The spot of phosphatidylserin was questionably increased. The amount of total lipid phosphorus was slightly increased (6,2 per cent) against two control samples. No qualitative or quantitative abnormalities of *glycolipids* could be proved on repeated examination. Chromatographical examination of apolar lipids revealed a marked increase of *unesterified cholesterol*. The spots of triglycerides and free fatty acids were enlarged as well, most probably due to the presence of white adipose tissue cells in the submucosa of the pathological appendix.

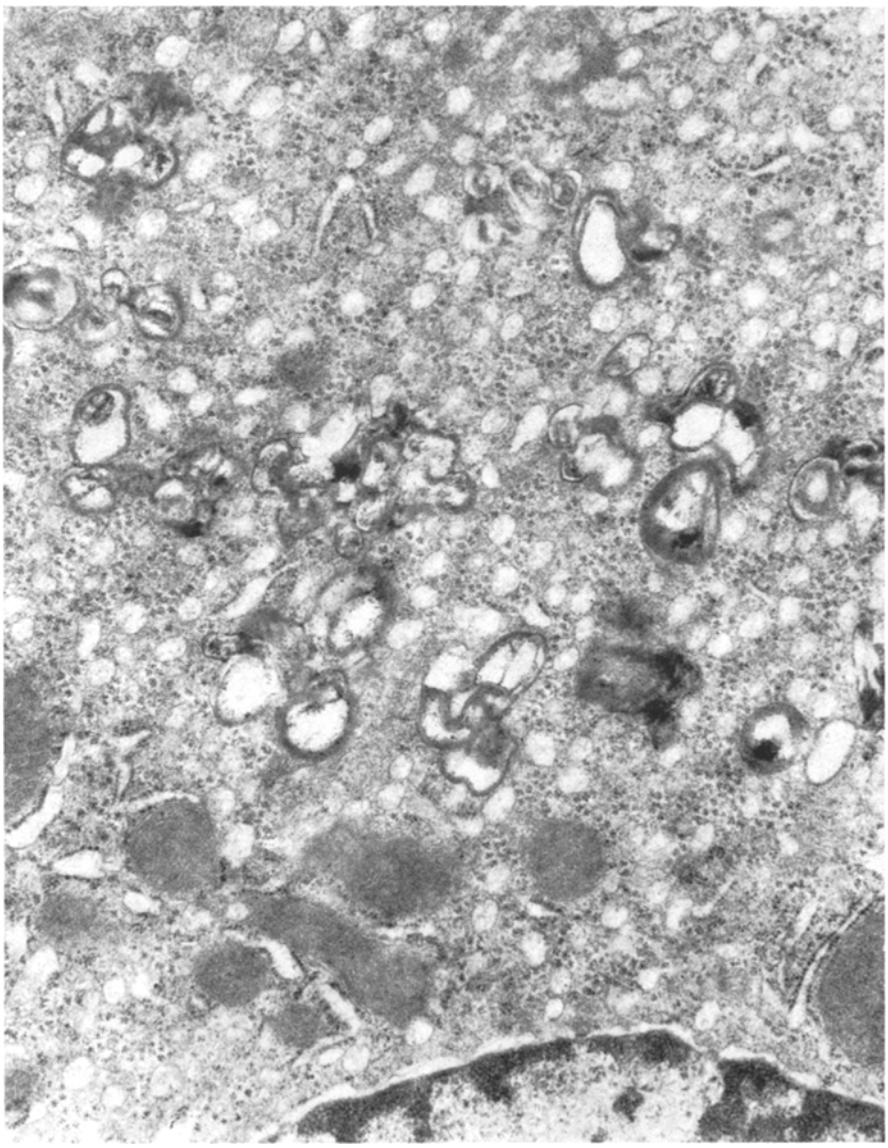


Fig. 6. Detail of the hepatocyte. Storage cytosomes and numerous minute vesicles of smooth endoplasmic reticulum. ×11 000

Ultrastructure

Generally, the storage cytosomes displayed a relatively marked degree of pleomorphism due to variations in size and degree of their coalescence and to variations in amount and internal structure of their content. Most often, concentrically arranged variously densely packed membranes could be seen. They were sometimes arranged along the single limiting membranes of the cytosomes with central parts either empty or filled with an electron-lucent material. Further variations in apparence will be mentioned individually. The product of acid

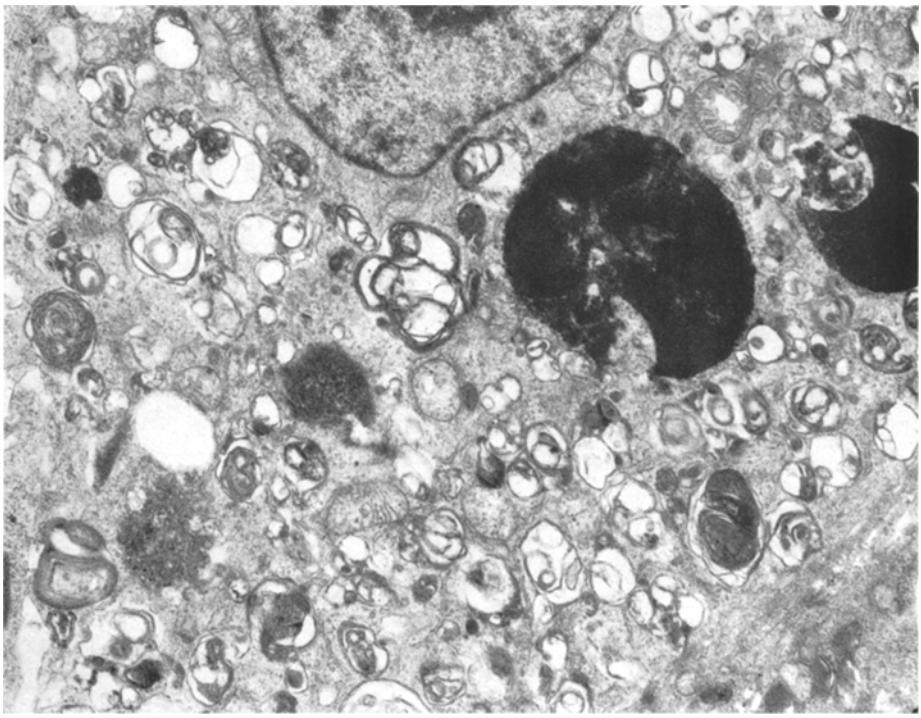


Fig. 7. Detail of the storing macrophage in the germinal lymphatic center. Digested cell residues and numerous storage cytosomes. ×3700

phosphatase activity evaluated in the liver only was localized in a majority of cytosomes, mostly on their periphery and to a "septa-like" structures in cases where coalescence occured.

The Liver. In *hepatocytes* the storage cytosomes were localized almost diffusely in the cytoplasm with a maximum in the peribiliary region. They were small with a low degree of coalescence (Fig. 6). There was a small number of typical lipofuscine bodies. Peroxisomes were not increased markedly. The smooth endoplasmic reticulum was hypertrophic in a majority of hepatocytes in a form of minute vesicles, some of which limited with a conspicuously thicker membrane.

The *Kupffer cells* were affected to a various degree. The content of storage cytosomes was much more variable than in hepatocytes due to a considerable amount of dense homogenous granules and complex structures resembling digested cell residues. A lot of them was almost empty. The degree of coalenscence was high.

Sinus endothelium and *vascular endothelium* and *perivascular fussiform cells* in the portal triads were affected similarly but to a substantially less degree.

Appendix. A small number of storage cytosomes was observed in the surface, in the glandular *epithelium* and in *argentaffin cells. Histiocytes* in the mucosal stroma and in the submucosa were constantly but variably affected by storage and very often displayed an intensive phagocytosis of erythrocytes, polynuclear leucocytes, plasmocytes and hardly identifiable mononuclear cells with storage

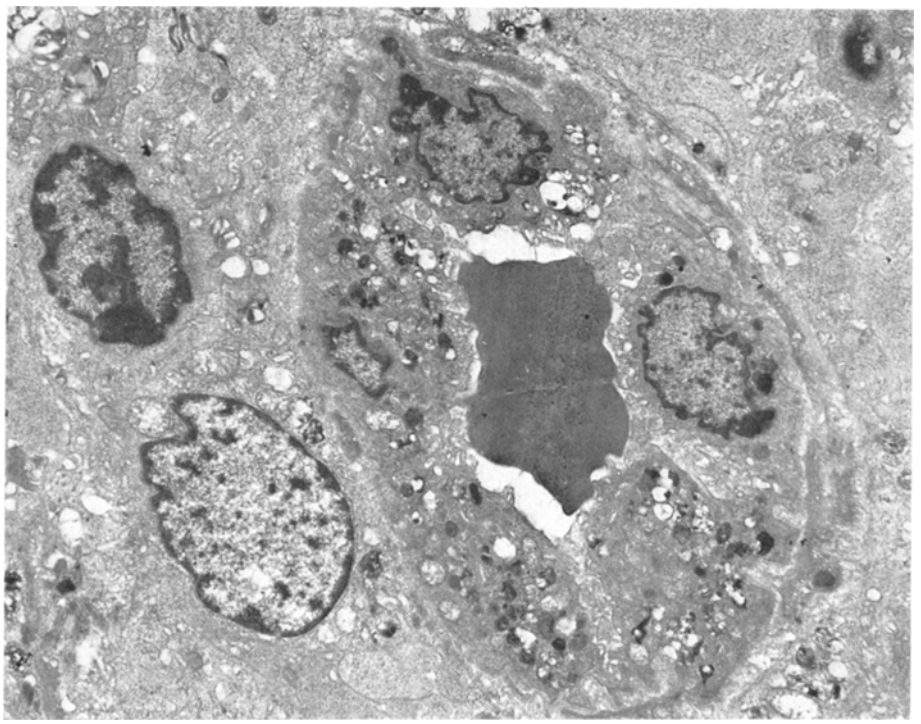


Fig. 8. Storage in the capillary endothelium of the appendical mucosa. ×2700

cytosomes in a close vicinity of the digested cellular residues. Similar finding were encountered in histiocytes of the germinal centers (Fig. 7) and on the periphery of lymphatic follicles, where small groups of them were embraced by slender processes of the nonfagocytizing reticulum, which displayed a moderate degree of storage only. Histiocytes in this location were maximally loaded with storage cytosomes often with a lamellar content, sometimes more solid and dense, representing most probably the "residual granules" seen in the light microscopy. Generally, the surface of all histiocytes examined was rich with numerous villous projections.

The *vascular endothelium* was almost constantly affected to a various degree. The storage cytosomes were small, markedly pleomorphic with a low degree of coalescence (Fig. 8.). The *perivascular fussiform* cells were affected to a less degree and their cytosomes were either almost empty or contained dense homogenous granules with occasional membranous material. The cells of *neural plexi* were diffusely or segmentally distended with numerous storage cytosomes mostly of concentric lamellar appearance. Both Schwann cells (Fig. 9) and ganglion cells were affected. No storage was seen in smooth muscle cells and in the endothelium of lymphatic vessels.

Skin. Minor degree of storage was seen in vascular endothelium and in the perivascular cells. There were no decisive pathological changes in sweat glands and in Schwann cells which could be examined.

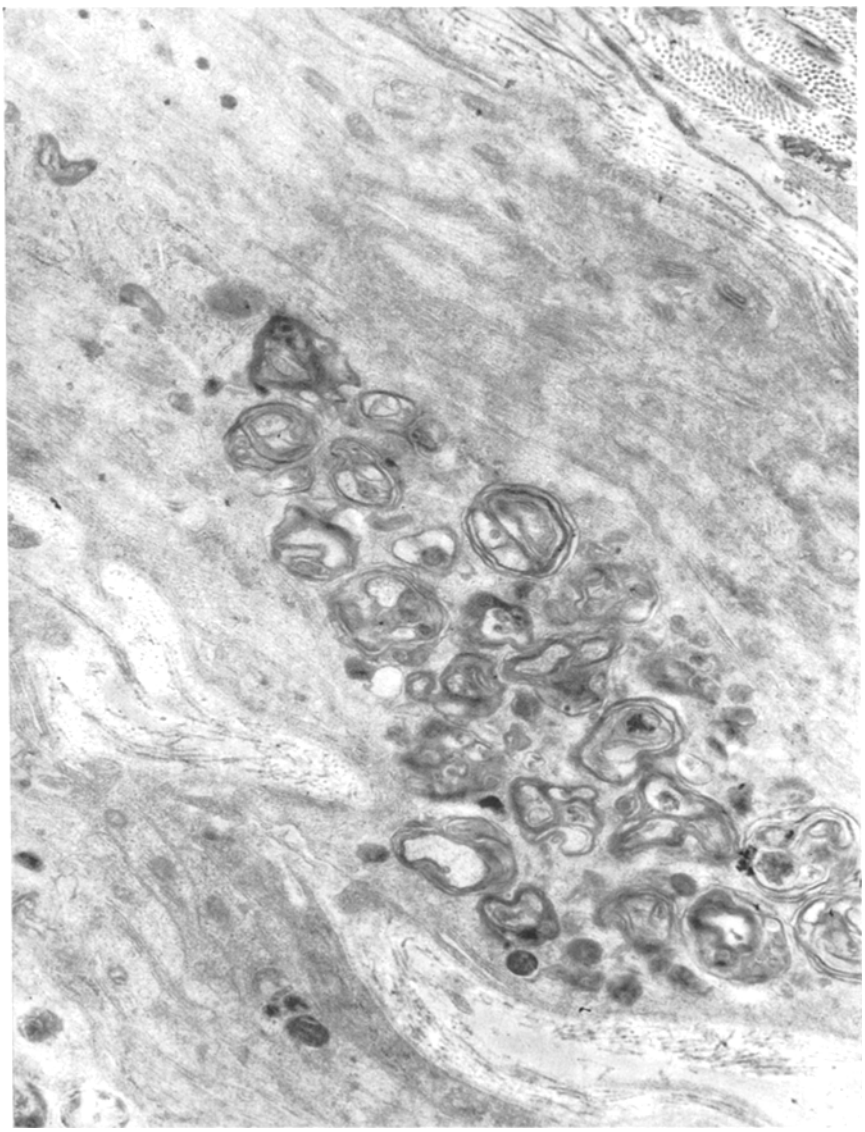


Fig. 9. Appendical neural plexus. Storage cytosomes in the cytoplasm of Schwann cell. ×2200

Discussion

1. To the Results

There are not any problems in the interpretation of results of methods for the detection of *apolar lipids*, i.e. only cholesterol could be proved to participate in the storage. However, the interpretation of seemingly discordant results of methods for the detection of *polar lipids*, especially of phospholipids, requires a short comment. In the case of the ferric-haematoxylin it was proved that only phosphorus-bearing polar lipids are stained (Elleder and Lojda, 1973a).

After substraction of an intensity of sphingomyelin staining, we get the staining intensity corresponding to phosphoglycerides, which in this case are either predominating or at least equal to intensity of sphingomyelin staining. The relatively weak staining with controlled chromation method, resistant to sodium hydroxide, could be satisfactorily explained by the fact, that this method stains only choline-bearing phospholipids (Roozemond, 1971; Elleder and Lojda, 1973a). Consenquently, the results of this method points to the presence of sphingomyelin and to an absence of a significant amount of lecithin. As for the OTAN method, it stains strongly all polar lipids with the exception of gangliosides (Elleder and Lojda, 1973a). The significantly less weakening of the staining after NaOH corresponds well with the presence of an alkali-stable glycolipid in the vascular endothelium and in neural plexi, and similarly, there is a correspondence between the alkali-sensitivity of the OTAN and PAS methods in appendical histiocytes. The only exception were the Kupffer cells, where the OTAN method was not markedly weakened by NaOH despite the very low amount of glycolipids and a high amount of phosphoglycerides detectable in them.

A painstaking problem, at least in our hands, is the differentiation between glycosphingolipids, i.e. between gangliosides and cerebrosides. The modified Bial method after Ravetto (1964) did not repeatedly work in our hands a thus the finding of a glycosphingolipid in the mentioned sites cannot be further specified. According the relatively strong staining with OTAN method and to an absence of staining with cresyl violet, we consider the presence of cerebrosides as more probable. The finding of an alkali-sensitive glycolipids is most probably due to the presence of phosphatidyl inositol (see also Adams, 1965).

The birefringence of the stored lipids is also worth mention, especially the destruction of their Maltese-cros appearance and their replacement by cholesterol plates and minute spindle-shaped solid crystals. This strongly suggest the progressive breakdown (probably enzymatic) of the polar lipids. Similar finding were encountered in the case of the splenic phospholipid steatosis consequent to thrombocytopenia (unpublished observation) but were absent in the case of the Niemann-Pick's disease (Elleder, Jirásek and Šmíd, 1974). We have no explanation for the absence of birefringence in neural storing cells.

In sum, according to the histochemical examination, the stored lipids are composed of phosphoglycerides (most probably with the exception of lecithin), sphingomyelin and cholesterol accompanied in some sites with glycolipids (mainly glycosphingolipids). We consider the histochemical approach, despite its deficiencies which are still present, as more fruitful for the detection of stored lipids, at least in this case, than the chromatographical study, due to an ability of the former to show clearly changes in individual affected cells, whereas results of the latter are necessarily influenced by the lipid composition of unaffected tissues and are, therefore, average only.

As for *residual granules* in storage cells, it appears that their staining and physical properties resemble those of lipopigment in several points (autofluorescence, sudanophilia, acidoresistance), but on the other hand, they differ from it by an absence of the native color, argentaffinity and relatively faint basophilia. Similar findings were encountered in the case of the Niemann-Pick's disease (Elleder, Jirásek and Šmíd, 1974). Their ultrastructure appears to be concentric lamellar, similarly as was described already in the case of ceroid (see Györky, Shimamura and O'Neal, 1967). Their relation to lipopigment is thus evident, but their exact nature remains to be established.

The ultrastructural findings showed before all the considerable pleomorphism of the storage cytosomes, which due to their content of acid phosphatase activity belongs to the lysosomal system. Further, additional localizations of the storage, undetectable histochemically, could be obtained.

2. To the Disease Itself

According to our results we can strongly suggest that this disease resembles in many aspects those described by Baar and Hickmans (1956) and Wiedemann *et al.* (1972). In cases described by Baar and Hickmans the marked increase of cephalin and cholesterol was reported. However, the term "cephalin" is rather vague, because except the presence of phosphoglycerides insoluble in ethanol (see Ansell and Hawthorne, 1964) it says nothing about individual species which are in play. The considerable participation of phosphatidylinositol could be inferred from the presence of a large amount of inositol in hydrolysates of lipid extract. This is in accordance with our observation of phosphoglycerids accumulation and especially with the detection of a PAS-positive and alkali-sensitive lipid in appendical histiocytes and in hepatocytes. Similarly as in cases described by Wiedemann *et al.* we have observed the increase of lysobisphosphatidic acid, cholesterol and glycolipids in tissues affected with storage. Therefore, it is highly probable that our case belongs to the same catogery as the above mentioned cases.

One of the crucial problems which remains to be unequivocally established is to determine an exact relationship of this lipidosis to the Niemann-Pick's disease. According to Wiedemann *et al.*, (1972) it represents a new type of lipidosis belonging nosologically to the Niemann-Pick-complex. It undoubtedly resembles the Niemann-Pick's disease not only histologically, but mainly by the fact that the majority of stored lipids were proved in tissues affected by the Niemann-Pick disease, i.e. not only sphingomyelin and cholesterol, but lysobisphosphatidic acid as well (Rouser *et al.*, 1968) similarly as glycolipids (Kashimota *et al.*, 1969; Kawamura, 1971) especially in the central nervous system (Jørgenson *et al.*, 1964; Pilz *et al.*, 1966). Further, a 50 per cent decrease of sphingomyelinase activity was described (Wiedemann *et al.*, 1972). Therefore, in light of the present knowledge in appears, that there are not markedly distinctive *qualitative* chemical differences between these two diseases. The main distinquishing feature seems to be rather a *quantitative* one, i.e. the preponderance of phosphoglycerides over sphingomyelin in contrast to the Niemann-Pick's disease. However, this resemblance says nothing about the relationship in enzymatic deficiency and can be superficial only.

It is very interesting that there is a considerable similarity to the "Niemann-Pick-like syndrome" described in humen treated with a coronary vasodilator 4,4′-diethylaminoethoxyhexestrol (Yamamoto *et al.*, 1971a) in which a constant increase of lysobisphosphatidic acid, cholesterol and desmosterol was proved in the liver accompanied in a majority of cases with a substantial increase of phosphatidylinositol. In other affected tissues only an increase of lysobisphosphatidic acid was found. Similar results were obtained experimentally (Yamamoto *et al.*, 1971b; Akeda, 1973).

Further, the lysobisphosphatidic acid was detected in the late infantile amaurotic idiocy (Rouser *et al.*, 1968) and a many-fold increase was proved in the lysosomes after experimental application of Triton WR-1339 (Wherrett and

Huterer, 1972). The increased concentration of this compound under variety of conditions is thought to be a reflection of an increased number of lysosomes (Wherrett and Huterer, 1972) and consenquently of secondary character. However, we still do not know exactly, whether the lysobisphosphatidic acid is the sole representant of the accumulated phosphoglycerides, or whether other members of this group are increased as well.

It seem to us that any further discussion about pathogenetic problems of the described disease would suffer from gaps in our knowledge of the normal metabolism of complex lipids and their interrelationship which could be extremely complicated especially under pathological conditions. Therefore a study of further cases is urgently needed, namely an analysis of enzymes of phospholipid metabolism complemented with chemical analysis of cellular fractions for establishment of the *definite pattern of the stored phosphoglcerides* and of their definite metabolic relation to other accumulated lipids and to the lysosomal system.

From the practical point of view, there is a growing evidence that this peculiar lipidosis must be taken into the consideration in the diagnosis of the Niemann-Pick's disease. Its routine recognition can be easily carried out histochemically by means of the ferric-haematoxylin method in the combination with an alkaline hydrolysis. According to a common practice we suggest that "Phospholipidosis Type II (Baar-Wiedemann) could be an appropriate name for this disease in contrast to the clasical Niemann-Pick's disease which could represent the Type I with six phenotypes (IA-E).

References

Adams, C. W. M.: Histochemistry of lipids. In: Neurohistochemistry, ed. by C. W. M. Adams, Amsterdam: Elsevier 1965

Akeda, S.: A study of the lipidosis induced by a coronary vasodilator, 4,4′-diethylamioethoxy-hexestrol dihydrochloride. Mie. med. J. **22**, 65–96 (1973)

Baar, H. S., Hickmans, E. M.: Cephalin-lipidosis. A new disorder of lipid metabolism. Acta med. scand. **155**, 49–64 (1956)

Elftman, H.: Controlled chromation. J. Histochem. Cytochem. **2**, 1–8 (1954)

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

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

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

Elleder, M. Lojda, Z.: Studies in lipid histochemistry. IX. The specifity of the Holzinger's reaction for fatty acids. Histochemie **32**, 301–305 (1972b)

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

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

Elleder, M., Jirásek, A., Šmíd: Niemann-Pick's disease type C. A histochemical study of the distribution and of qualitative difference of the storage process. (Prepared for publication)

Frederickson, D. S., Sloan, H. R.: Sphingomyelin lipidoses: Niemann-Pick disease. In: The metabolic basis of inherited diseases, ed. by J. B. Stanbury, J. B. Wyngaarden and D. S. Frederickson, p. 783. New York: McGraw-Hill Comp. 1972

Györkey, F., Shimamura, T., O'Neal, R. M.: The fine structure of ceroid in human atheroma. J. Histochem. Cytochem. **15**, 732–736 (1967)

Jørgensen, L., Blackstadt, Th., W., Harkmark, W., Steen, J. A.: Niemann-Pick's disease. Report of a case with histochemical evidence of neuronal storage of acid glycolipids. Acta neuropath. (Berl.) **4**, 90–106 (1964)

Kashimota, S., Aron, A. M.: Suzuki, K., Suzuki, K.: Infantile Niemann-Pick disease. A chemical study with isolation and characterization of membranous cytoplasmic bodies and myelin. Amer. J. Dis. Child. **117**, 379–394 (1969)

Kawamura, N.: Hepatic and splenic glycolipids in a case of Niemann-Pick's disease. Jap. J. exp. Med. **41**, 221–232 (1971)

Lillie, R. D.: Histopathologic technic and practical histochemistry, ed. by R. D. Lillie. New York: McGraw-Hill Comp. 1965

Lillie, R. D., Henderson, R.: A short chromic acid-haematoxylin stain for sections of formol-fixed brain and spinal cord. Stain Technol. **43**, 121–122 (1968)

Lojda, Z.: Remarks on histochemical demonstration of dehydrogenases. II. Intracellular localization. Folia morph. (Warszawa) **13**, 84–97 (1965)

Lojda, Z.: Histochemical detection of enzymes. I. part. Brno: 1966 ČSAV (in Czech.)

Lojda, Z.: Histochemical detection of enzymes. III. part. Brno: 1970 ČSAV (in Czech.)

Lojda, Z.: Histochemical methods for acid beta-galactosidase: technics for semipermeable membranes. Histochemie **37**, 375–378 (1973)

Lojda, Z.: Topochemistry of beta-glycosidases in aortas and coronary arteries of rats, guinea pigs and rabbits in normal conditions and after cholesterol feeding. Čs. Pat. **10**, 1–10 (1974) (in Czech.)

Novikoff, P. M., Novikoff, A. B.: Peroxisomes in absorptive cells of mammalian small intestine. J. Cell Biol. **53**, 532–560 (1972)

Pearse, A. G. E.: Histochemistry. Theoretical and applied, vol.1., ed. by A. G. E. Pearse. London: J. A. Churchill Ltd. 1968

Pilz, H., Sandhoff, K., Jatzkewitz, H.: Eine Gangliosidstoffwechselstörung mit Anhäufung von Ceramid-lactosid, Monosialo-ceramidlactosid und Tay-Sachs-Gangliosid im Gehirn. J. Neurochem. **13**, 1273–1282 (1966)

Ravetto, C.: Histochemical identification of sialic (neuraminic) acid. J. Histochem. Cytochem. **12**, 306 (1964)

Roozemond, R. C.: The staining and chromium binding of rat brain tissue and of lipids in model systems subjected to Baker's acid hematein technique. J. Histochem. Cytochem. **19**, 244–251 (1971)

Rouser, G., Kritchevsky, G., Yamamoto, A., Knudson, A. G., Jr., Simon, G.: Accumulation of a glycerophospholipid in classical Niemann-Pick disease. Lipids **3**, 287–290 (1968)

Rouser, G., Simon, G., Kritchevsky, G.: Species variations in phospholipid class, distribution of organs: I. Kidney, liver and spleen. Lipids **4**, 599–606 (1969)

Wherrett, J. R., Huterer, S.: Enrichment of bis-(monoacylglyceryl) phosphate from rat liver. J. biol. Chem. **247**, 4114–4120 (1972)

Wiedemann, H.-R., Debuch, H., Lennert, K., Caesar, R., Blümcke, S., Harms, D., Tolksdorf, M., Seng, P. N., Korenke, H.-D., Gerken, H., Freitag, F., Dörner, Kl.: Über eine infantil-juvenile, subchronisch verlaufende, den Sphingomyelinosen (Niemann-Pick) anzureihende Form der Lipidosen — ein neuer Typ? Z. Kinderheilk, **112**, 187–225 (1972)

Yamamoto, A., Adachi, S., Ishikawa, K., Yokomura, T., Kitani, T., Nasu, T., Imoto, T., Nishikawa, M.: Studies on drug-induced lipidosis. III. Lipid composition of the liver and some other tissues in clinical cases of "Niemann-Pick-like syndrome" induced by 4,4′-diethyl-aminoethoxyhexestrol. J. Biochem. **70** 775–784 (1971a)

Yamamoto, A., Adachi, S., Kitani, T., Shinji, Y., Seki, K., Nasu, T., Nishikawa, M.: Drug-induced lipidosis in human cases and in animal experiments. Accumulation of an acidic glycerophospholipid. J. Biochem. **69**, 613–615 (1971)

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