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Enzyme Activities and Phospholipid Storage Patterns in Brain and Spleen Samples from Niemann–Pick Disease Variants: a Comparison of Neuropathic and Non-neuropathic Forms

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Phospholipid levels and enzyme activities were measured in brain and spleen samples from patients with the three major variants of Niemann–Pick disease. Accumulations of sphingomyelin and bis(monoacylglycero)phosphate were demonstrated in spleen from types A and B and group C Niemann–Pick disease, whereas only in type A Niemann–Pick brain was the sphingomyelin concentration increased. Sphingomyelinase activity was markedly deficient in type A Niemann–Pick brain and spleen but residual activity of approximately 12% of control was measured in type B Niemann–Pick brain. Normal or raised sphingomyelinase and β-glucosidase activities were measured in group C Niemann–Pick brain and spleen. Significant (17% of control) residual β-glucosidase activity was also measured in non-neuropathic Gaucher brain. Normal levels of neutral sphingomyelinase activity were measured in brain samples from the three variants of Niemann–Pick disease. Acid sphingomyelinase activity in group C Niemann–Pick brain appeared normal with respect to enzyme extraction, pH optimum (pH 5.0) and apparent K_m (approximately 0.4 mmol/L). Isoelectric focusing of brain sphingomyelinase revealed a degree of heterogeneity with activity peaks between pI 4.5 and 6.5. No defect was observed in group C Niemann–Pick brain and, although attenuated, all peaks were present in type B Niemann–Pick brain.

Niemann–Pick disease (NPD) (McKusick 25720) represents a group of inherited disorders resulting in the accumulation of sphingomyelin and certain other lipids within lysosomes, especially of the reticulo-endothelial system. Several distinct types or groups of the disease have been recognised which may be classified from A to E depending on their phenotypic expression (Crocker, 1961; Brady, 1983) or

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types 1 and 2 dependent on sphingomyelinase (EC 3.1.4.12) involvement (Elleder and Jirasek, 1983). The classical or acute neuropathic form, type A NPD, presents in early infancy with marked hepatosplenomegaly due to massive accumulation of sphingomyelin and associated lipids. Acid sphingomyelinase activity is severely depressed in all tissues including liver, spleen and brain, as well as leukocytes and cultured fibroblasts. There is a gradual progression of neurological symptoms and patients generally succumb before 3 years of age. Patients with the chronic non-neuropathic form, type B NPD, exhibit many features similar to type A NPD but are spared neurological involvement. However, the degree of lipid storage in the viscera may be somewhat less than in type A NPD (Vanier, 1983). Few studies appear to have been carried out on type B NPD brain although it has recently been reported that levels of sphingomyelin are not abnormally high despite a deficiency of sphingomyelinase activity (Vanier and Rousson, 1984). Studies on a fetus presumed to have type B NPD revealed deficient acid sphingomyelinase activity but brain sphingomyelin levels, like those from a type A NPD fetus, were in the high normal range (Wenger *et al.*, 1981).

A third group of patients have been described with a juvenile or sub-acute neuropathic form of NPD. Since this may represent a heterogeneous variant, the term group C NPD may be preferred. Patients may present in early infancy or during childhood. Early visceral problems generally give way to neurological symptoms and most patients succumb between 3 and 15 years. Although a less severe lipidosis may occur in the viscera of these patients, the pattern appears more complex, and the accumulation of certain glycosphingolipids may be equally prominent (Vanier, 1983). Sphingomyelinase activity appears essentially normal in brain, liver, spleen and leukocytes of these patients (Callahan *et al.*, 1975; Vanier *et al.*, 1980), but partially reduced, together with that of β-glucosidase in cultured fibroblasts (Vanier *et al.*. 1980; Besley and Moss, 1983). A deficiency of cathodic forms of sphingomyelinase activity has been found in fibroblasts (Besley, 1977; Besley and Moss, 1983; Vanier *et al.*, 1983) as well as in brain and liver (Callahan *et al.*, 1975). However, certain of these observations have been difficult to reproduce (Harzer *et al.*, 1977) and their significance is still not clear.

In the present communication, results are presented in an attempt to compare sphingomyelinase activities with phospholipid accumulation in brain and spleen samples from patients with the three major variants of NPD and to present the biochemical expression of type B NPD in affected brain. The significance of these results particularly with regard to the level of residual acid sphingomyelinase activity in type B brain is discussed, together with the apparently normal findings in group C NPD brains.

PATIENTS AND METHODS

Patients

A brief description of the patients studied is given below.

Case M.Z., type A NPD: This Pakistani male infant first presented at 4 months

with hepatosplenomegaly and failure to thrive. He had all the classical features of the disease and died at 20 months with cardiorespiratory and liver failure. Tissues were removed 9 h after death and stored at −40° C for 3–4 years before study. Fibroblast sphingomyelinase activity was <1% of normal.

Case J.B., type B NPD: This Czechoslovak man presented with hepatosplenomegaly and sea-blue histiocytes in bone marrow. He had no evidence of neurological involvement and lived to 56 years when he died of the disease compounded by a hepatoma. Tissues were removed 16 h after death and stored at −40° C for approximately 1 year before study. Fibroblast sphingomyelinase activity was 5% of normal, and leukocyte activity (K. Harzer, Tubingen, FRG) was 10% of normal.

Case P.L., group C NPD: This Czechoslovak boy presented at 1 year with slight hepatosplenomegaly. There was developing and marked neurological involvement including ataxia and vertical gaze paralysis. He died aged 4 years and 8 months. Tissues were removed 5 h after death and stored at −40° C for approximately 3 years before study. Fibroblast sphingomyelinase activity was approximately 40% of normal with an abnormal isoelectric focusing pattern.

Case R.B., group C NPD: This Czechoslovak boy presented shortly after birth with a rapidly developing hepatosplenomegaly. This subsequently gave way to progressive and finally profound neurological involvement leading to death at 4 years. Tissues were removed 3 h after death and stored at −40° C for approximately 1 year before study. Fibroblast sphingomyelinase activity was approximately 40% of normal with an abnormal isoelectric focusing pattern.

Further details on these last two patients were reported by Elleder and colleagues (1984, 1985).

Other patients studied include a case of infantile neuropathic Gaucher disease (type 2), a Scottish infant (R.L.) who died aged 6 months; a case of non-neuropathic Gaucher disease (type 1), a Scottish man (D.C.) who died aged 68 years with acute myelomonocytic leukaemia (these two patients had approximately 2% of normal β-glucosidase activity in cultured fibroblasts); and a patient (D.B.) with GM_1-gangliosidosis (type 1), a Scottish boy aged 14 months at death.

Control tissues were obtained from subjects aged 3 months–6 years (juvenile controls) or 74–81 years (adult controls) whose causes of death were unrelated to the conditions discussed here. Tissues were removed approximately 6–24 h after death and had been stored at −40° C over similar periods to the NPD samples.

Lipid analysis

Samples of frozen tissue (approximately 100 mg wet wt.) were homogenised by hand in 20 vol. chloroform–methanol (2:1). The total extract was centrifuged at 1000 *g* for 10 min and the supernatant decanted and kept, the residue was re-extracted as before but in 10 vol. solvent. The combined supernatants were partitioned (Folch-Pi *et al.*, 1957) and the lower lipid-rich phase retained after further washing with approximately 15 vol. theoretical upper phase. The lipid phase

was dried under a stream of nitrogen and *in vacuo* over phosphorus pentoxide before reconstituting in chloroform–methanol (2:1) in a ratio of 0.5 mg original wet wt per μl solvent. Analytical chromatography was carried out on activated silica gel 60 HPTLC plates (Merck 5631) using 5 μl extract and developing in chloroform–methanol–water (70:30:5 by vol.). Glycolipids were located with orcinol spray and phospholipids with acid molybdate spray. Quantitative analysis of phospholipids was carried out basically as described by Rouser and colleagues (1970) following two-dimensional TLC. Briefly, 10 μl lipid extract was spotted on activated HPTLC plates, run in the first direction with chloroform–methanol–ammonia (65:35:5 by vol.) and second direction with chloroform–acetone–methanol–acetic acid–water (30:40:10:10:5 by vol.). Relevant areas, located by exposure to iodine vapour and with reference to known standards, were scraped into glass stoppered tubes, digested with 10 N sulphuric acid (0.25 ml) for 4–5 h at 155° C and after decolourisation with 30% hydrogen peroxide (50 μl) were analysed for phosphate content (Gomori, 1942).

Enzyme assays

Tissue samples were homogenised by hand in 20 vol. 1 g/L Triton X-100, followed by sonication and centrifugation at 8500 *g* for 2 min (Besley and Moss, 1983); both total and supernatant fractions were assayed.

The activity of acid sphingomyelinase was determined at pH 5.0 as described earlier (Besley and Moss, 1983) using [^{14}C]sphingomyelin as substrate. Neutral Mg^{2+}-dependent sphingomyelinase activity was determined at pH 7.4 as described (Wenger *et al.*, 1981) and the activities of β-glucosidase, β-galactosidase and *N*-acetyl-β-glucosaminidase were measured using 4-methylumbelliferone conjugates (Besley and Moss, 1983).

The effect of pH on brain sphingomyelinase activity was determined in the presence of the following buffers: 0.2 mol/L acetate, 0.1 mol/L cacodylate–HCl and 0.1 mol/L Tris–HCl in the range pH 3.0–9.0 and with substrate dispersed in Triton X-100 (Wenger *et al.*, 1981). The effect of substrate concentration on acid sphingomyelinase activity was measured at pH 5.0 in acetate buffer at sphingomyelin concentrations between 5 and 55 nmol per 100 μl assay and in the presence of a fixed Triton X-100 concentration of 100 μg.

Isoelectric focusing

Brain supernatant fractions prepared in 1 g/L Triton X-100 and containing 5–10 mg protein were subjected to sucrose gradient isoelectric focusing in the presence of 1 g/L Triton X-100 (Besley, 1977; Besley and Moss, 1983). Separated fractions were assayed for sphingomyelinase, β-glucosidase and *N*-acetyl-β-glucosaminidase activities.

RESULTS

Phospholipid levels in brain samples from Niemann–Pick patients and controls are given in Table 1. A marked increase in sphingomyelin concentration was measured

Table 1 Phospholipid concentrations in brain samples from patients with Niemann–Pick disease and controls

	Total phospholipid	*Sphingomyelin*	*Phosphatidylcholine*	*Phosphatidyl-ethanolamine*	*Phosphatidylserine and phosphatidylinositol*
NPD type A (M.Z.)	66.0	25.0	15.7	11.6	7.1
NPD type B (J.B.)	60.8	5.59	20.7	18.5	8.5
NPD group C (P.L.)	60.4	6.47	25.2	18.1	7.5
NPD group C (R.B.)	54.8	5.0	17.2	11.0	5.6
Control (6 years)	49.2	3.38	20.6	16.5	3.7
Control (81 years)	46.0	2.94	17.8	16.8	6.0

Values expressed as μmol per g wet wt

Table 2 Phospholipid concentrations in spleen samples from patients with Niemann–Pick disease and controls

	Total phospholipid	*Sphingomyelin*	*Phosphatidyl-choline*	*Phosphatidyl-ethanolamine*	*Phosphatidylserine and phosphatidylinositol*	*Bis(monoacyl-glycero)phosphate*
NPD type A (M.Z.)	111	99	22.3	5.1	1.64	5.3
NPD type B (J.B.)	60	41	8.9	3.1	1.40	3.7
NPD group C (P.L.)	55	24.4	14.9	4.0	1.84	3.8
NPD group C (R.B.)	51	24.7	14.3	5.2	1.79	5.2
Control (6 years)	24	3.9	11.0	5.2	1.13	0

Values expressed as μmol per g wet wt

in type A NPD, being increased about 8-fold over control levels and representing nearly 40% of the total phospholipid concentration. However, sphingomyelin levels were only marginally increased in brains from patients with type B NPD and group C NPD, representing 9–10% of total phospholipid concentration. Other phospholipid levels in NPD brain samples appeared normal and no bis(monoacylglycero)phosphate could be detected in any of these samples. In spleen, sphingomyelin levels were markedly raised in all three forms of NPD (Table 2). Concentrations were raised 25-fold in type A NPD, 10-fold in type B NPD and 6-fold in group C NPD. Bis(monoacylglycero)phosphate levels were also markedly raised in all NPD spleens examined, with values reaching approximately 5 μmol per g wet wt. None of this phospholipid was identified in control spleen. Increases also of cholesterol and glucosylceramide were evident on thin layer chromatography of spleen extracts from all NPD variants; however, levels of glucosylceramide were most striking in group C NPD spleens.

Lysosomal enzyme activities were measured in extracts of brain and spleen from these NPD patients and controls including Gaucher disease samples. Activities were determined in both total extracts and supernatant fractions to check for defects in enzyme extraction. In all samples studied and for all three enzymes, total activity was recovered in the supernatant fraction; there was no evidence for incomplete extraction in any enzyme deficient or abnormal tissue. The results of specific

Table 3 Enzyme activities in brain supernatant fractions

	Sphingomyelinase	β-*Glucosidase*	β-*Galactosidase*
NPD type A (M.Z.)	0.20 (1.3%)	66.8	33.9
NPD type B (J.B.)	2.89 (12.1%)	53.3	78.5
NPD group C (P.L.)	21.2	28.5	52.4
NPD group C (R.B.)	19.2	25	74.5
Gaucher type 1	39.2	8.3 (17%)	78.5
Gaucher type 2	17.3	0.88 (3%)	63.2
GM_1-gangliosidosis	29.9	139	7.2
Juvenile controls (n=5)			
mean	15.1	29.2	40.9
range	(12.0–19.5)	(25.8–34.9)	(33.4–50.7)
Adult controls (n=3)			
mean	23.8	49.1	55.5
range	(22.7–25.8)	(45.5–52.5)	(49.1–63.1)

Activities expressed as nmol/h per mg protein

activities in supernatant fractions only are given in Table 3 for brain enzymes and in Table 4 for spleen enzymes. Marked deficiencies of sphingomyelinase activity were observed in brains from patients with type A and type B NPD. However, residual activity in the type B variant was considerable, being 10 times higher than that in type A. Since magnesium-dependent neutral sphingomyelinase activity also extracted quantitatively into the supernatant, this might have contributed to residual acid sphingomyelinase activity in type B NPD brain. Activity of the neutral enzyme in NPD and control brain extracts was as follows: 56, 103, 89, 54, 67, 92 nmol/h

Table 4 Enzyme activities in spleen supernatant fractions

	Sphingomyelinase	β-*Glucosidase*	β-*Galactosidase*
NPD type A (M.Z.)	0.16	158	144
NPD type B (J.B.)	0.26	185	406
NPD group C (P.L.)	6.11	62.8	226
NPD group C (R.B.)	6.16	43.1	210
Gaucher type 1	3.61	1.93	375
Gaucher type 2	5.40	2.42	387
Control (3 years)	2.47	37.2	263
Control (6 years)	6.73	34.2	202

Activities expressed as nmol/h per mg protein

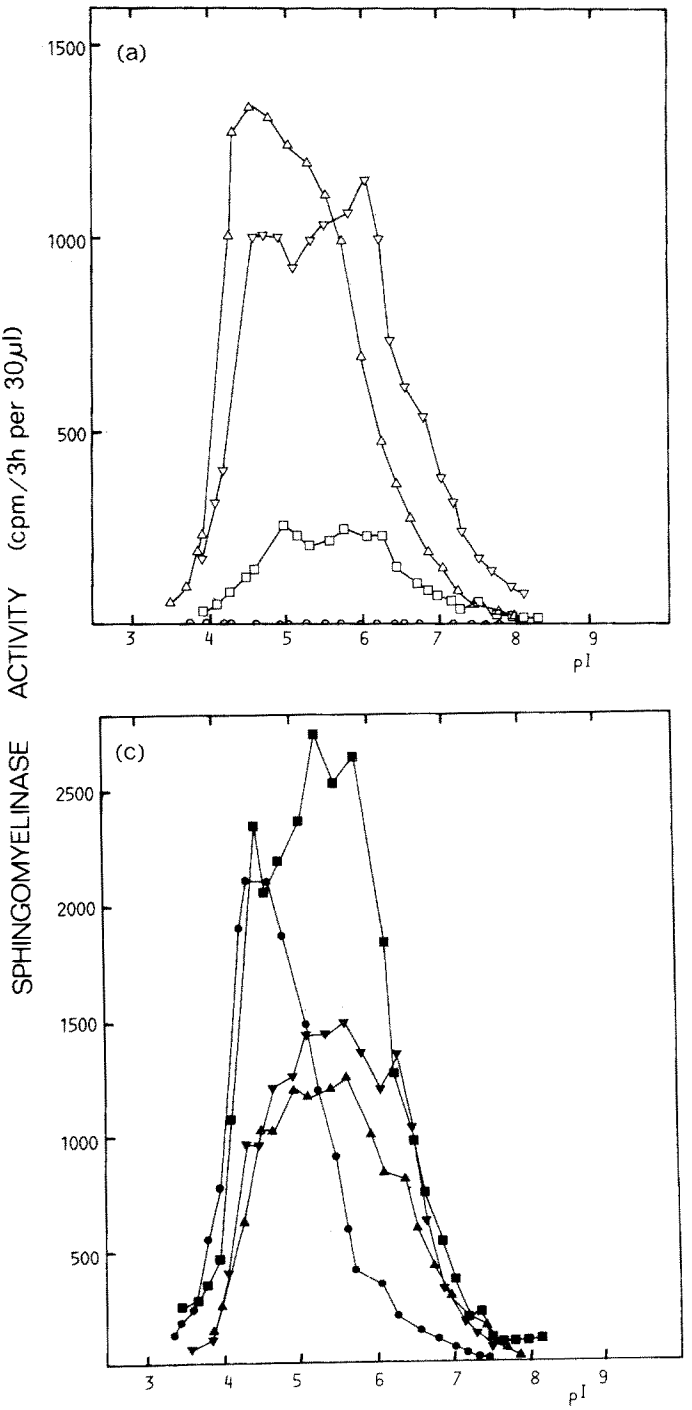
per mg protein for types A, B, C (P.L.), C (R. B.) and two juvenile controls. However, in the presence of 0.25 mmol/L EDTA, acid sphingomyelinase activity in type B NPD brain was reduced by only 1%. Sphingomyelinase and β-glucosidase activities appeared normal in group C NPD brains; however, deficiencies of β-glucosidase activity were measured in Gaucher brains. Again residual activity was considerably higher in the non-neuropathic (type 1) form.

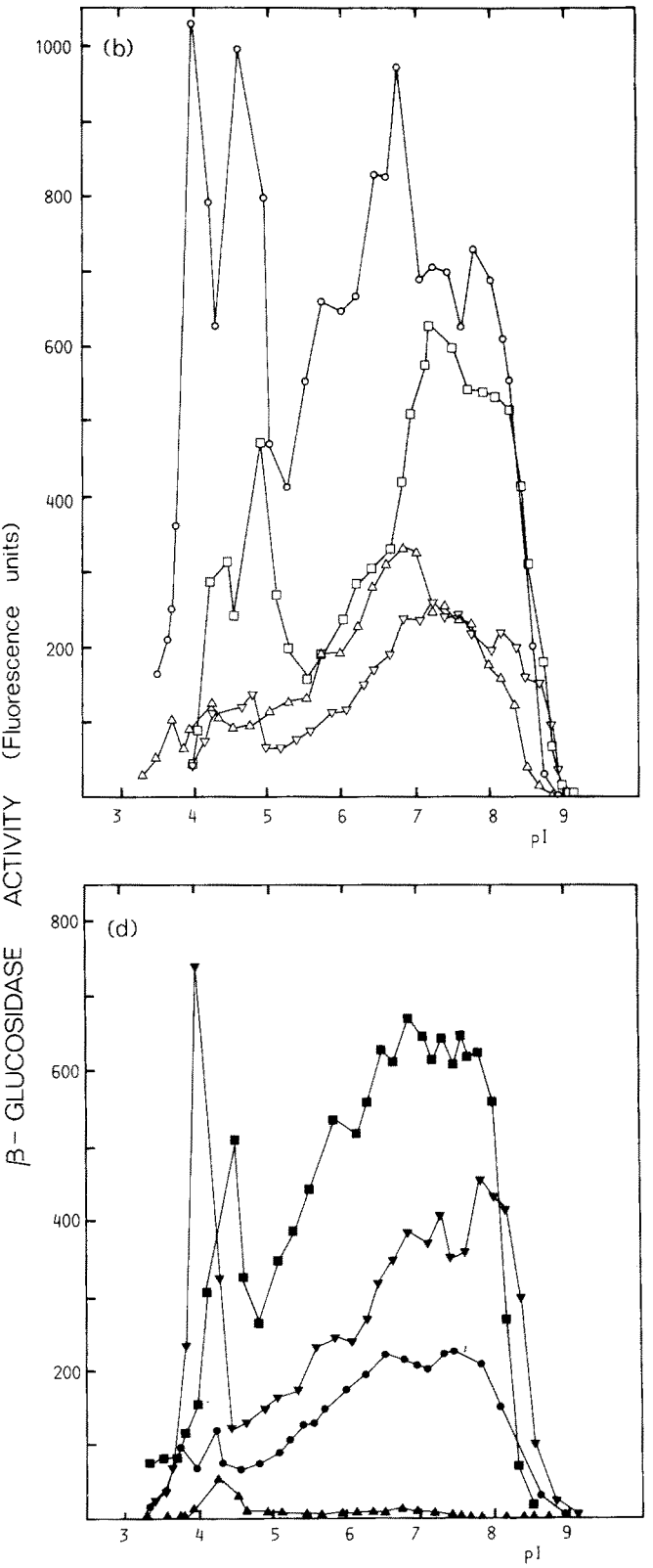
Similar degrees of sphingomyelinase deficiency were measured in type A and type B NPD spleen (Table 4). No reduction in activity was seen in group C NPD spleen for this enzyme or for β-glucosidase. Deficiencies of the latter enzyme, however, were recorded in Gaucher spleens and to a similar extent (about 6%) in type 1 and type 2 disease.

The effect of pH on brain sphingomyelinase activity indicated two peaks with optima at pH 5.0 and pH 7.0–7.5 corresponding to acid and neutral forms. Activity of the latter was generally 2–6-fold higher than that of the former. Significant residual acid sphingomyelinase activity was observed in type B NPD brain whereas negligible activity was present in type A; no abnormalities were observed with respect to pH profiles in group C NPD brain. Apparent K_m values were determined for acid sphingomyelinase activities in NPD and control brains, using Lineweaver–Burk plots based on computed best fit lines. The regression coefficient for type A NPD plot was $r = 0.990$ whereas for all others was $r > 0.997$. The apparent K_m values, expressed as mmol/L, were 1.32, 0.39, 0.35, 0.53, 0.34 and 0.40 for types A, B, C (P.L.), C (R.B.), infant control and adult control. Apparently normal values were therefore observed in type B and group C NPD brains whereas a higher value was found in type A NPD brain. However, this might be an underestimate since no corrections were made for endogenous sphingomyelin, which would be present in a different physiological state from the *in vitro* substrate dispersed in detergent.

Brain extracts were subjected to isoelectric focusing and the separated fractions assayed for acid and neutral sphingomyelinase activities. There was no loss of activity during this procedure and all activity applied was recovered in the separated fractions. Acid sphingomyelinase activities exhibited a degree of heterogeneity (Figure 1(a),(c)) with activity peaks between pI 4.5 and 6.5. Four control samples

Figure 1 Isoelectric focusing of brain sphingomyelinase and β-glucosidase activities. Separation was carried out at 4°C on a sucrose gradient in the presence of 1 g/L Triton X-100 and 10 g/L (w/v) Ampholine (LKB Ltd). Fractions (approximately 0.25 ml) were assayed for enzyme activity and pH after 18 h at 400 V. Activities of acid sphingomyelinase (panels a,c) and lysosomal β-glucosidase (panels b and d) are plotted against isoelectric point (pI). Samples were from patients with type A (○), type B (□), type C, P. L. (△) and type C, R. B. (▽) Niemann–Pick disease (top panels) and from patients with Gaucher disease type 2 (▲), GM_1-gangliosidosis (■) and control infants (●,▼) (lower panels).

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were analysed; one sample, having a single major peak (Figure 1(c)) at pI 4.5, was analysed on three occasions to check for reproducibility, and this was confirmed. Patient P.L., group C NPD, was analysed on two occasions to confirm results. The profiles shown in Figure 1 are representative of the findings made. No abnormality was observed in the two patients studied with group C NPD. In type B NPD brain, although activity was attenuated, all activity peaks remained and there was no evidence for loss of any specific component. In type A NPD brain no significant activity could be identified. Neutral sphingomyelinase activities were also measured in fractions from type B NPD, group C NPD (R.B.) and control brain; all activity fell between pI 4.2 and 5.0, either as a single peak, pI 4.5, or double (second peak pI 5.0 in type B NPD). Activity of this enzyme may have been associated with bands of precipitated protein on the column.

Similar profiles of β-glucosidase activity were observed in all brain samples studied (Figure 1(b), (d)) including all NPD variants, except that from Gaucher (type 2) brain where only a single minor peak was observed (Figure 1(d)) between pI 4.2 and 4.5. The major peak of brain β-glucosidase activity was between pI 7 and 8 with several minor bands on the anodic side. There was no evidence of any abnormality in group C NPD brains. The profile for *N*-acetyl-β-glucosaminidase activity was recorded in all samples for reference purposes and to exclude any inter-run variation. The results, not presented here, were similar to those reported earlier (Besley and Broadhead, 1976).

DISCUSSION

The phospholipid levels reported here for spleens agree well with a recent series by Vanier (1983) and, together with fibroblast enzyme activites and clinical presentation, establish the biochemical diagnosis of NPD in the patients studied. Marked storage of sphingomyelin and bis(monoacylglycero)phosphate was identified in spleens from all three variants. The most striking accumulation of sphingomyelin was associated with the most severe deficiency of sphingomyelinase activity in type A NPD. No overall deficiency of sphingomyelinase activity nor that of β-glucosidase was identified to explain the lipidosis observed in group C NPD. Similar results have been reported by Schneider and Kennedy (1967), Harzer and colleagues (1978) and Guibaud and colleagues (1979) although some reduction of spleen sphingomyelinase activity was reported by Gilbert and colleagues (1981) and a 'diminished extractability' of the enzyme was observed by Harzer and colleagues (1978). We found no defect in sphingomyelinase extraction from group C NPD tissues in this study nor from liver or fibroblasts in a previous study (Besley and Moss, 1983). It is, however, generally agreed (Callahan *et al.*, 1975; Vanier *et al.*, 1980; Besley and Moss, 1983) that normal levels of sphingomyelinase activity are found in group C NPD liver. For type A and type B NPD similar degrees of sphingomyelinase deficiency have been reported in liver (Vanier *et al.*, 1980; Lawlor *et al.*, 1981) despite the more severe lipidosis observed in type A variant. In the patient described here with type B NPD, sphingomyelinase activity in liver was approximately 10% of control value for both malignant and non-malignant

areas although pathological changes consistent with NPD were only minimal (Elleder, unpublished observation).

Lipid storage patterns and enzyme activities in brain samples from NPD variants have not been extensively documented although a number of early reports (Crocker, 1961; Norman *et al.*, 1967; Fredrickson and Sloane, 1972) point to sphingomyelin storage in patients with the classical disease but not in the group C or juvenile variant. A recent report by Elleder and colleagues (1985) confirms this observation with respect to group C NPD and provides further evidence for the storage of glucosyl- and lactosylceramides. A similar storage pattern has been found (Rao and Spence, 1977) in type D NPD brain. The question of brain involvement in type B NPD has not been satisfactorily answered, due primarily to the lack of appropriate tissue for study. In a study by Wenger and colleagues (1981) no acid sphingomyelinase activity was detected in the brain of a fetus presumed to have type B NPD, although neutral activity appeared normal. During the preparation of this manuscript, Vanier and Rousson (1984) also reported a marked deficiency of acid sphingomyelinase activity in a type B NPD brain and, like ourselves, failed to demonstrate any storage of sphingomyelin, but accumulation was present in the type A variant. In our study significant (12% of control) residual acid sphingomyelinase activity was measured in adult type B NPD brain and no reduction in activity was elicited by EDTA, known to inhibit neutral activity. A similar situation for neuropathic and non-neuropathic forms of disease was also observed (Table 3) for residual lysosomal β-glucosidase activity in Gaucher brain as well as being reported elsewhere (Wenger and Olsen, 1981; Daniels *et al.*, 1982). Whether the pattern observed here for type B NPD is represenative, or specific to our patient, must await further studies on other patients. In Gaucher disease specific mutations are reported (Ginns *et al.*, 1982) to differentially affect the processing or subunit composition of β-glucosidase in neuropathic and non-neuropathic types of the disease. To date no such evidence is available to explain the phenotypic expression in type A and type B NPD, although complementation studies have suggested these forms are allelic (Besley *et al.*, 1980).

Residual acid sphingomyelinase activity in type B NPD brain although low in activity appeared otherwise normal with respect to pH optimum, apparent K_m and isoelectric focusing profile. Whether or not this activity was sufficient *in vivo* to prevent sphingomyelin accumulation can only be speculated upon at this stage; however, it would seem likely in this instance.

In brain from group C NPD patients, no evidence was provided by these studies or others (Norman *et al.*, 1967; Muller and Harzer, 1980; Vanier and Rousson, 1984; Elleder *et al.*, 1985) for any significant accumulation of sphingomyelin or for any defect in sphingomyelinase activity. Furthermore, on isoelectric focusing no abnormal distribution of enzyme activity was observed in these cases. In particular, there was no evidence for loss in activity of certain cathodic components, as reported by Callahan and colleagues (1975). These results therefore agree with our earlier observations (Besley and Moss, 1983) for group C NPD liver but show an entirely different pattern of sphingomyelinase activity, as well as β-glucosidase activity, in the two tissues. Sphingomyelinase activity was more anodic in brain than in liver,

whereas β-glucosidase activity was more cathodic. These patterns also contrast with those observed in cultured fibroblasts (Besley, 1977; Besley and Moss, 1983) and lend support to the notion that for these enzymes, patterns of expression may be tissue-specific. Thus, for the group C NPD patients studied here, partial deficiencies of sphingomyelinase and β-glucosidase activities, and abnormal sphingomyelinase profiles, were confined to cultured fibroblasts. However, it has yet to be established how these observations in group C NPD fibroblasts (Besley and Moss, 1983; Vanier *et al.*, 1983) and those concerning fibroblast sphingomyelin metabolism (Maziere *et al.*, 1982; Kudoh *et al.*, 1983; Vanier and Rousson, 1984) relate to the primary defect in this elusive disorder.

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