SPHINGOMYELINASE DEFECT IN NIEMANN-PICK DISEASE, TYPE C, FIBROBLASTS

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1. Introduction

Niemann-Pick disease is inherited in at least five different phenotypes [1] all of which exhibit degrees of sphingomyelin accumulation, particularly in the reticuloendothelial system. The diseases differ with respect to age of onset, degree of neurological involvement and clinical course of the disorder. In Niemann-Pick diseases types A and B, where the lipidosis is most severe, sphingomyelinase activity is profoundly deficient. However, in types C, D and E disease, sphingomyelinase activity is normal [1,2] or only partially reduced [2,3]; the biochemical basis for these conditions has yet to be firmly established. Recently, Callahan et al. [4] have indicated that a specific sphingomyelinase component, identifiable by isoelectric focusing, is deficient in Niemann-Pick type C (NPC) liver and brain [5] and in Niemann-Pick type E fibroblasts [6]. These authors did not study NPC fibroblasts but suggested that these might also be deficient in this sphingomyelinase component.

Recent work [7] has indicated that fibroblast sphingomyelinase probably exists in a bound state but that the various enzyme components may be separated by isoelectric focusing in the presence of Triton X-100. This technique has been applied to the separation of sphingomyelinases in NPC fibroblasts in order to identify any missing component. The resulting enzyme profile indicated that NPC cells were profoundly deficient in two cathodic forms of sphingomyelinase.

2. Materials and methods

[(*Me*-^{14}C)-choline]sphingomyelin was obtained from the Radiochemical Centre, Amersham (UK); Ampholine from LKB Produkter AB (Sweden); sodium cholate from Sigma Chemical Co. Ltd. (USA) and purified Triton X-100, bovine sphingomyelin and 4-methylumbelliferyl 2-acetamide-deoxy-β-D-glucopyranoside from Koch Light Labs. (UK).

2.1. *Cultured skin fibroblasts*

Niemann-Pick type A (NPA) fibroblasts were kindly provided by Dr A. D. Patrick (London) and Niemann-Pick type C fibroblasts (GM-110) by the Human Genetic Mutant Cell Repository (New Jersey, USA). Methods of cell culture and enzyme extraction, into glycine, for isoelectric focusing have been described elsewhere [7]. Sodium cholate (5 mg/ml) extracts of cell pellets were prepared by four cycles of freeze-thawing (in liquid N_2), leaving for 30 min at 4°C and the 1000 × *g* × 10 min supernatant (1–2 mg protein/ml) being used for assay.

2.2. *Enzyme assay*

Sphingomyelinase activity was determined as described [7,8]. The assay mixture contained enzyme extrac or column eluate (50 μl or 100 μl), 250 μg sodium cholate, 68 nmol [^{14}C]sphingomyelin (40 000 cpm) and 20 μmol acetate buffer, pH 5.0, in a final volume of 0.2 ml. Incubation (3 h at 37°C) was stopped with cold water (0.9 ml), 10% (w/v) bovine serum albumin (0.1 ml) and 50% (w/v) trichloroacetic acid (0.2 ml). Released [^{14}C]phosphorycholine was counted in Bray's scintillation mixture.

For K_m determination, a fixed sphingomyelin: sodium cholate ratio was maintained in the substrate mixture.

Activity of β-hexosaminidase was determined as described [9] and protein measured by the Lowry method [10].

2.3. Isoelectric focusing

Electrofocusing was performed in 10 ml J-tubes [7] in the presence of 0.1% (v/v) Triton X-100. On completion (18 h at 400 V and 4°C) fractions (8 drops, about 0.25 ml) were collected and analysed for pH (4°C) and enzyme activity.

3. Results and discussion

Sphingomyelinase activity of NPC fibroblasts was low (table 1) but well above that of NPA fibroblasts. The activity fell within the range of values (35–113 nmol/h/mg protein, mean 73 units) obtained from thirthy-six control fibroblasts strains. The low activity did not result from incomplete enzyme extraction, since 98% of total sphingomyelinase was extracted from NPC cells, as compared with 94–99% in the controls and 89% in the NPA cells.

The effect of pH on sphingomyelinase activity is shown in fig.1a. Optimum activity for control fibroblasts was at pH 5.0, whereas for NPC cells, maximum activity was at pH 5.5. The plot for NPC cells suggests considerable loss of enzyme activity at pH 5.0, due to isoelectric precipitation; in the absence of Triton most of the remaining sphingomyelinase activity of NPC cells would have a p*I* around 5.0 [7]. In the presence of Triton, however, both NPC and control fibroblasts exhibited broad pH optima, at pH 5.0 (fig.1b). At pH 5.0 and in the presence of Triton X-100, the apparent K_m of NPC fibroblasts was 76 μM (fig.2), less than half that (179 μM) of control cells. The control value agrees well with the previous results [11] obtained in the absence of Triton X-100, when an apparent K_m of 203 μM was recorded for control fibroblasts and 243 μM for NPA fibroblasts.

Earlier isoelectric focusing studies [7] on fibroblast sphingomyelinase had demonstrated at least five enzyme peaks. In the present study, where a larger

Table 1
Fibroblast sphingomyelinase activities nmoles/h/mg protein

NPC	47.4
NPA	0.61
Control	72.2
Control	98.3
Control	98.7

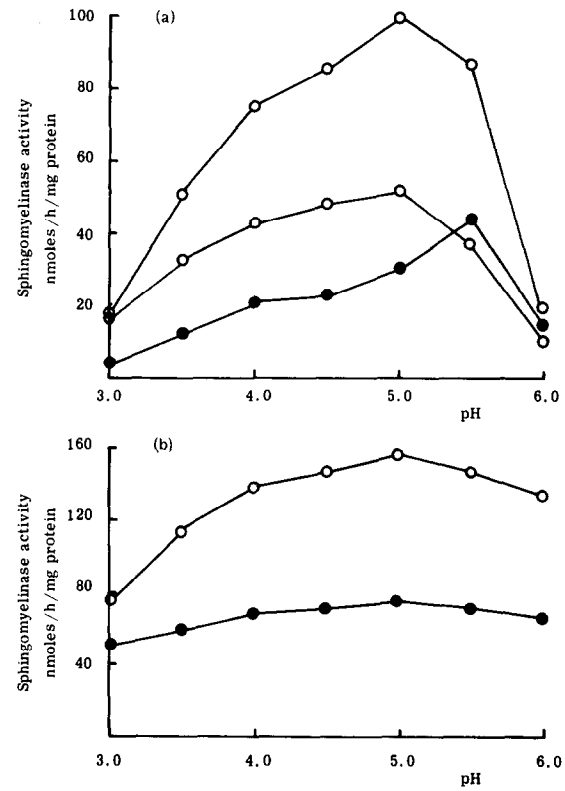

Fig.1. Effect of pH on fibroblast sphingomyelinase activity of cholate extracts (○ control; • NPC), in the absence (a), or presence (b) of 0.025% (v/v) Triton X-100.

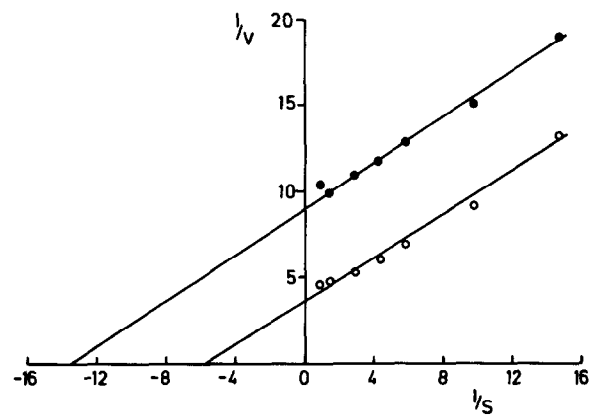

Fig.2. Lineweaver–Burk plot for sphingomyelinase activity of fibroblast extracts (○ control, 64 μg protein; • NPC, 78 μg protein). Assay for 2 h at pH 5.0 in the presence of 0.025% (v/v) Triton X-100. V = nmoles/h/mg protein × 10^{-3}, S = mM.

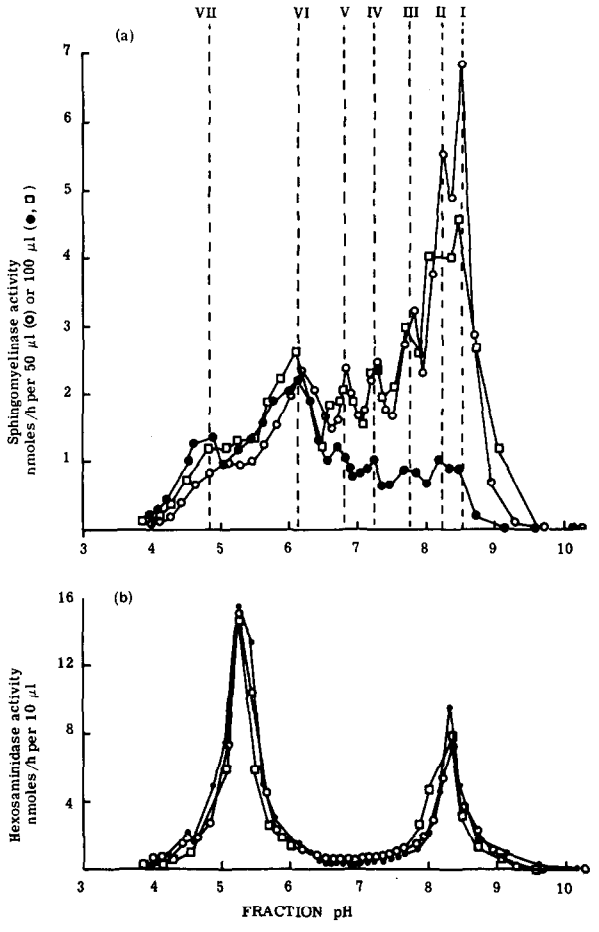

Fig.3. Isoelectric focusing profiles of fibroblast sphingomyelinase (a), and β-hexosaminidase activities (b).○ Control extract, 1.22 mg protein, sphingomyelinase activity 140 nmoles/h/mg protein (83% enzyme extraction); □ control extract, 1.06 mg protein, sphingomyelinase activity 99 nmol/h/mg protein, sphingomyelinase activity 69 nmol/h/mg protein (84% extraction).

number of fractions was collected, seven enzyme peaks were separated (fig.3a), with p*I* values of 8.50, 8.25, 7.75, 7.25, 6.80, 6.15 and 4.85. NPC fibroblast extracts contained all seven species but peaks I and II (pI 8.50 and 8.25) were considerably reduced in enzyme activity, with the result that peak VI now represented the major enzyme form in these cells. There was no marked increase in activity associated with any other sphingomyelinase peaks and the observed phenomenon would appear to result from an apparent enzyme defect rather than altered p*I* values. Shown in fig.3b are the focusing profiles of β-hexosaminidase activity, obtained for reference purpose for each cell extract; no defect was apparent in either the hexosaminidase A (p*I* 5.25) and hexosaminidase B (p*I* 8.37) peaks.

Properties of pooled peaks I and II were compared with those of pooled peaks V and VI, from control fibroblasts. Neither when assayed directly (in the presence of ampholytes, sucrose and Triton X-100) nor following dialysis against 0.1% (v/v) Triton X-100 was any major difference observed with respect to pH optimum K_m or heat stability. The pH optima of the two fractions were slightly lower (pH 4.0–5.0) than that obtained for cholate extracts but like these profiles, were considerably flattened. The K_m values were, however, much reduced being 26 μM and 19 μM before dialysis and 17 μM and 12 μM after dialysis, for components I plus II and components V plus VI respectively. At 50°C in the presence of 10 mM–acetate buffer, pH 6.0, and bovine serum albumin (0.1 mg/ml) both fractions lost about 65% activity after 1 h; similar results were obtained for both NPC and control fibroblast extracts.

If the metabolic lesion in NPC disease is, like other types of this disorder [12], expressed in cultured fibroblasts then sphingomyelin storage would be predicted, despite the persistence of sphingomyelinase components that are catalytically active in vitro. It may be that subtle changes underlie this disorder rather than any specific enzyme deficiency. The effect of detergents on enzyme activity [13]. loss of activity during enzyme purification [2,14] and different K_m values recorded for the enzyme would suggest that other factors might control catalytic activity. The physiological significance of a low mol. wt activator of sphingomyelinase [15] has yet to be assessed and, like β-glucocerebrosidase [16], the presence of certain effectors may be essential for full activation. A defect in the structure of interaction in vivo of any of these could underlie the defect in NPC disease.

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