THE SANFILIPPO B CORRECTIVE FACTOR : A N-ACETYL-α-D-GLUCOSAMINIDASE

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Received June 6, 1972

SUMMARY : Fibroblasts from patients with Sanfilippo's disease fall into two groups - A and B, each deficient in a specific protein factor which is required for normal degradation of sulfated mucopolysaccharide. From normal human urine the factor deficient in the B subgroup was copurified with N-acetyl-α-D-glucosaminidase by ammonium sulfate precipitation and successive chromatography on Sephadex G-200 and carboxymethyl cellulose. Both the factor and the enzyme had the same relative mobility in three different polyacrylamide gel electrophoresis systems at several gel concentrations. Since Sanfilippo B fibroblasts were found to be strikingly deficient in N-acetyl-α-D-glucosaminidase activity, this inactivity is proposed to be the basic defect in Sanfilippo B disease.

Sanfilippo's disease (mucopolysaccharidosis III) is an inherited disorder of mucopolysaccharide metabolism, characterized by an increased intralysosomal storage and an excessive urinary excretion of heparan sulfate (1). Fibroblasts cultured from the skin of Sanfilippo patients perpetuate the basic defect - an inadequate degradation of heparan sulfate, which is demonstrable by its abnormally long turnover time and the inefficiency in degradation of pinocytosed heparan sulfate containing proteoglycans (2,3). As in Hurler and Hunter fibroblasts the metabolic defect in Sanfilippo fibroblasts can be remedied by a specific protein "factor" which is found in fibroblast secretions and in urinary concentrates derived from individuals of other genotype. However, Sanfilippo fibroblasts can be subdivided into two groups, each deficient in a different corrective factor and therefore showing cross-correction. These two groups were arbitrarily designated A and B, such that Sanfilippo A fibroblasts lack the Sanfilippo A factor and Sanfilippo B fibroblasts the B factor for proper mucopolysaccharide metabolism (2).

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It has been shown previously that a highly purified Sanfilippo A factor preparation from human urine was active towards $^{35}SO_4$-labelled heparan sulfate releasing a product which behaved as inorganic sulfate (4). This paper provides evidence that the Sanfilippo B factor is a N-acetyl-α-D-glucosaminidase.

EXPERIMENTAL: Procedures for the culture of fibroblasts and for the assay of corrective factor activity were as previously described (4,5). Reference cell lines for both Sanfilippo subgroups were those used in Dr. Neufeld's laboratory (National Institutes of Health, Bethesda, Md., USA).

For the preparation of partially purified Sanfilippo B factor from normal human urine ammonium sulfate precipitation and gel filtration on Sephadex G-200 were employed as described for the purification of Sanfilippo A factor, except that the pH of the eluent was raised to 6.5 for Sephadex G-200 chromatography (4).

For the measurement of enzyme activities fibroblasts grown to confluency in a 75 cm^2 flask (10 - 20 days after subcultivating, about 8 x 10^6 cells, 4 mg cell protein) were harvested by scraping and extracted with 1.0 ml 0.15 M NaCl by 10 cycles of freezing and thawing. After centrifugation 0.6 - 0.8 mg of protein and more than 75 % of the enzyme activities tested were found in the supernatant.

The conditions for the assay of N-acetyl-α-D-glucosaminidase were as follows: 0.005 M UDP-N-acetyl-α-D-glucosamine, 0.05 M citrate buffer, pH 4.4, 0.02 % NaN_3, up to 100 µl enzyme preparation, final volume 200 µl. The incubation period was 2 - 18 hours at 37 °C. The liberated N-acetyl-glucosamine was then determined by the Morgan-Elson reaction (6). The assay followed zero order kinetics. Using phenyl-N-acetyl-α-D-glucosaminide as a substrate (7) the same specific activities were determined, but the enzyme blank was more pronounced. N-acetyl-ß-D-hexosaminidase was assayed according to Werries et al. (7), other glycoside hydrolases and sulfatases as previously described (4).

The methodology of polyacrylamide gel electrophoresis is that described by Rodbard and Chrambach (8) using two multiphasic buffer systems developed by Jovin et al. (9, system 2123.3 and system 2243.3) and one described by Maurer (10). The operative pH of the separation gel at 0 °C is 8.0 for system 2123.3, 8.5 for system 2243.3, and 9.0 for the third system. 200 µg of the urinary factor preparation were applied to each gel in 50 µl upper buffer containing 0.02 M NaCl and 20 % sucrose. At the end of the run the gel was frozen, cut transversely in 1.2 mm wide slices; the slices were homogenized in 1.0 ml 0.01 M Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl and 0.1 % bovine serum albumin. After centrifugation the supernatant solution was assayed for factor and N-acetyl-α-D-glucosaminidase activity.

RESULTS : Deficiency of N-acetyl-α-D-glucosaminidase - Skin fibroblasts from Sanfilippo B patients exhibited normal activity for N-acetyl-ß-D-hexosaminidase, N-acetyl-α-D-galactosaminidase, ß-D-glucuronidase, ß-D-galactosidase, ß-D-glucosidase, α-D-glucosidase, and arylsulfatase A, but are strikingly deficient in N-acetyl-α-D-glucosaminidase activity (Table 1). Thus, cells from three different unrelated Sanfilippo B patients had, at the most, 6 % of the activity found in fibroblasts of other genotypes, inclusive Sanfilippo A fibroblasts. The profound deficiency of N-acetyl-α-D-glucosaminidase is not due to the presence of endogenous inhibitors since mixing of cell extracts of Sanfilippo B patients with extracts of cells of other genotype yielded the expected intermediary activity.

Co-purification of Sanfilippo B factor and N-acetyl-α-D-glucosaminidase - In the course of purification of urinary Sanfilippo B factor by successive chromatography on Sephadex G-200 and carboxymethyl cellulose no separation of factor and N-acetyl-α-D-glucosaminidase activity could be achieved (Fig. 1 and 2). Arylsulfatases A and B and all glycoside hydrolases listed above differed in their elution maxima on Sephadex G-200 and did not adsorb to carboxymethyl cellulose. Whereas in all purification steps enzyme

Table 1

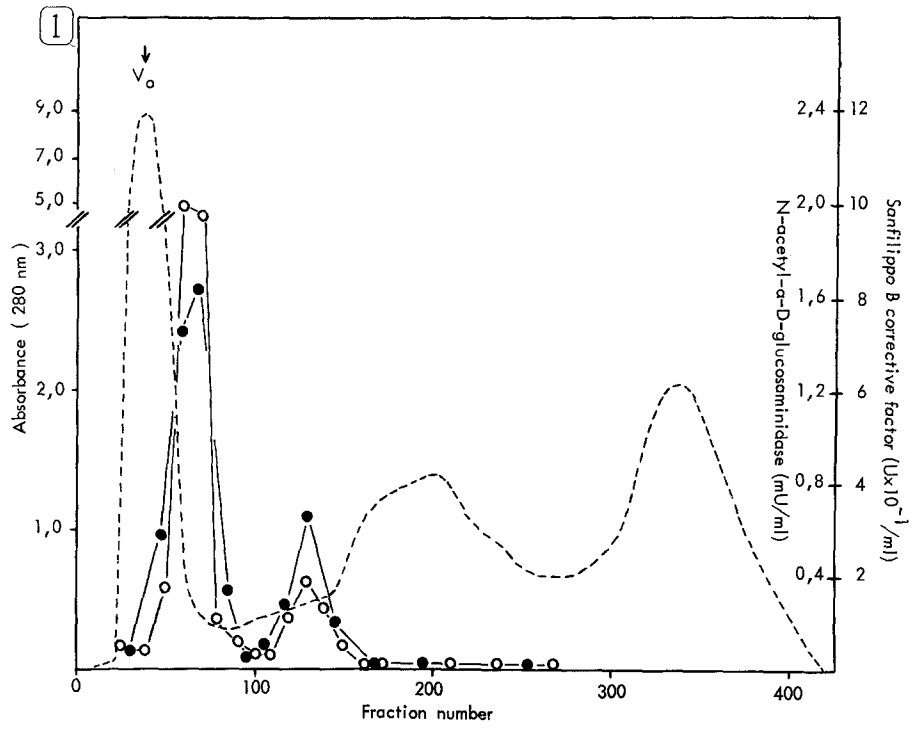
Activity of N-acetyl-α-D-glucosaminidase in cultured skin fibroblasts

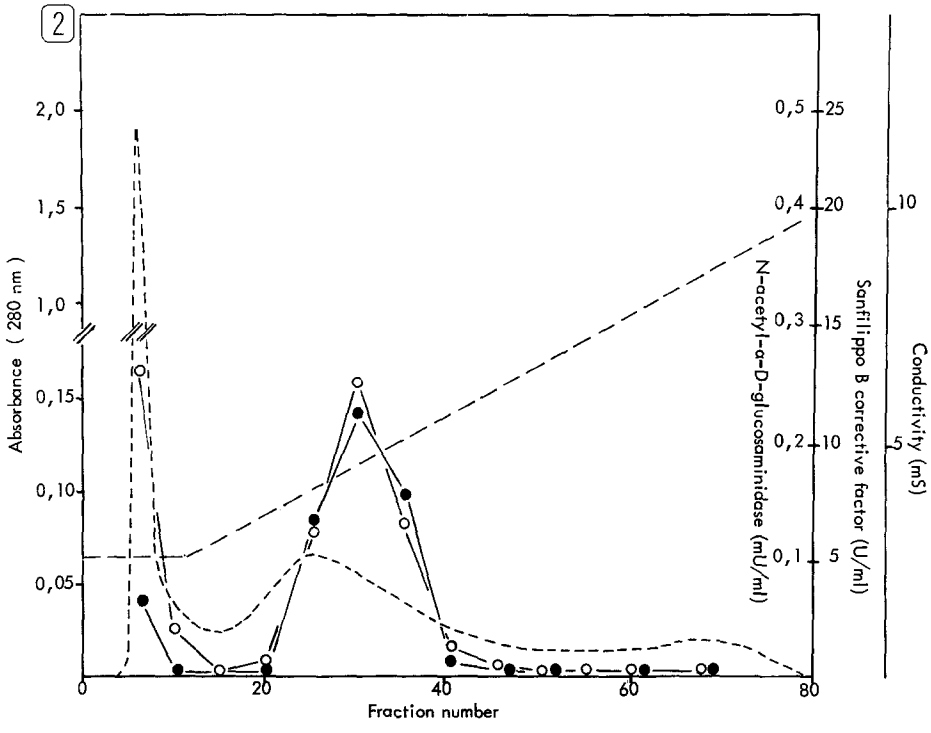
Genotype	N-acetyl-α-D-glucosaminidase mU / mg protein
Normal	0,248
Sanfilippo B : B.M.	0,006
V.K.	0,012
S.	0,012
Sanfilippo A : S.D.	0,360
F.D.	0,190
Hurler	0,202
Hunter	0,348

and factor activity showed a fairly constant ratio of 1 factor unit to 0.02 - 0.04 mU of enzymatic activity, that ratio was 1 : 0.08 for the material not adsorbed to carboxymethyl cellulose. This may be explained by an alteration of the enzyme protein not affecting the catalytic activity in vitro, but impairing the rate of pinocytosis, which results in a decrease of the biological activity.

Mobility of corrective factor and N-acetyl-α-D-glucosaminidase in polyacrylamide gel electrophoresis - In three different anodic polyacrylamide gel electrophoresis systems the factor and N-acetyl-α-D-glucosaminidase activity showed the same relative mobility at three different gel concentrations (Fig. 3). The plot of log R_f of factor or N-acetyl-α-D-glucosaminidase activity against gel concentration ("Ferguson" plot) gives rise to straight lines. Their slopes or retardation coefficients (K_R) are 0.13 at pH 8.0, 0.12 at pH 8.5 , and 0.11 at pH 9.0.

DISCUSSION : In Sanfilippo B fibroblasts the accumulated mucopolysaccharide is predominantly heparan sulfate (11). Since its accumulation is caused by an impaired degradation one could assume that the basic defect in Sanfilippo B fibroblasts is the lack


1
V_o
Absorbance (280 nm)
9,0
7,0
5,0
3,0
2,0
1,0
0
100
200
300
400
Fraction number
N-acetyl-α-D-glucosaminidase (mU/ml)
2,4
2,0
1,6
1,2
0,8
0,4
Sanfilippo B corrective factor (Ux10^{-1}/ml)
12
10
8
6
4
2


2
Absorbance (280 nm)
2,0
1,5
1,0
0,15
0,10
0,05
0
20
40
60
80
Fraction number
N-acetyl-α-D-glucosaminidase (mU/ml)
0,5
0,4
0,3
0,2
0,1
Sanfilippo B corrective factor (U/ml)
25
20
15
10
5
Conductivity (mS)
10
5

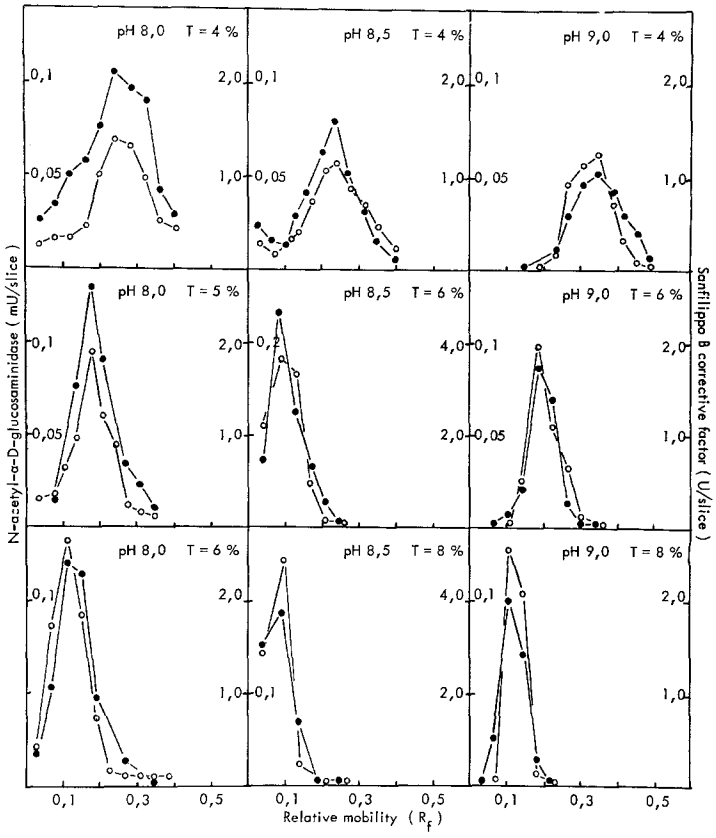


Fig. 3. Relative mobility of Sanfilippo B factor (●——●) and N-acetyl-α-D-glucosaminidase (o——o) in analytical polyacrylamide gel electrophoresis at pH 8.0 (left panel), pH 8.5 (central panel), and pH 9.0 (right panel). T = total gel concentration.

Fig. 1. Elution profile of Sanfilippo B factor (●——●) and N-acetyl-α-D-glucosaminidase (o——o) after chromatography on Sephadex G-200. The ammonium sulfate precipitate of human urine was suspended in 40 ml 0.15 M NaCl and dialyzed against 0.5 M NaCl in 0.01 M sodium phosphate, pH 6.5 . 50 ml of the dialyzed solution (absorbance 72.0 at 280 nm) were applied to a 7.5 x 130 cm column of Sephadex G-200, and eluted with the same buffer in fractions of 15 ml, at 75 ml / hour.

Fig. 2. Elution profile of Sanfilippo B factor (●——●) and N-acetyl-α-D-glucosaminidase (o——o) after chromatography on carboxymethyl cellulose. After chromatography on Sephadex G-200 fractions 61 - 80 were pooled and made 70 % saturated with ammonium sulfate. The precipitate was dissolved in 4.3 ml 0.05 M NaCl in 0.1 M sodium acetate, pH 4.2, and dialyzed against the same buffer. 4.2 ml of the dialyzed solution (absorbance 16.0 at 280 nm) were loaded onto a carboxymethyl cellulose column, 1.5 x 50 cm, equilibrated with 0.05 M NaCl in 0.1 M sodium acetate, pH 4.2 . A linear gradient was applied with 700 ml of the same buffer and 700 ml 0.4 M NaCl in 0.1 M sodium acetate, pH 4.2 . Fractions of 14 ml were collected at 60 ml / hour.

of an enzyme involved in the degradation of heparan sulfate. In view of the occurence of α-glucosaminidic bonds in heparan sulfate, the profound deficiency of N-acetyl-α-D-glucosaminidase in Sanfilippo B fibroblasts strongly suggests, that this might be the cause of Sanfilippo B disease. While this work was in progress, O'Brien came to the same conclusion by the demonstration of a deficiency of N-acetyl-α-D-glucosaminidase in fibroblasts and organs of Sanfilippo B homozygotes, but also of a markedly reduced activity in fibroblasts of heterozygotes (12).

A corrective protein factor which normalizes the mucopolysaccharide metabolism mirrors directly the basic defect of a mucopolysaccharidosis. The identity of an enzyme with such a protein factor would give final evidence for the nature of the disease. The Sanfilippo B corrective factor and the N-acetyl-α-D-glucosaminidase could not be separated from each other by Sephadex G-200 and carboxymethyl cellulose chromatography. Furthermore, factor and enzyme activity migrated with the same velocity in three different polyacrylamide gel electrophoresis systems. It seems most unlikely that two different proteins behave identical under such conditions. Therefore, we propose the absence of N-acetyl-α-D-glucosaminidase to be the basic defect of Sanfilippo B disease.

ACKNOWLEDGEMENTS : We are indebted to Drs. E.F. Neufeld and C. Hooft for supplying us with cultures of fibroblasts, to Drs. E. Werries and G. Mersmann for a gift of phenyl-N-acetyl-α-D-glucosaminide and phenyl-N-acetyl-α-D-galactosaminide, and to Miss D. Bolte and Miss H. Völkert for technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

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