BIOCHEMICAL HETEROGENEITY OF THE SANFILIPPO SYNDROME: PRELIMINARY CHARACTERIZATION OF TWO DEFICIENT FACTORS

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SUMMARY: Fibroblasts cultured from the skin of Sanfilippo patients show excessive accumulation and prolonged turnover time of sulfated mucopolysaccharide. This abnormality can be corrected by a macromolecular factor contained in the secretions of fibroblasts of differing genotype, as well as in normal human urine. By cross-correction tests, the Sanfilippo fibroblasts can be subdivided into two groups, each deficient in a different factor. Analytical polyacrylamide gel electrophoresis shows the two factors, which are probably proteins, to have a similar molecular weight (ca. 200,000) but to differ in charge at pH 8.5.

The Sanfilippo syndrome is an autosomal recessive disorder of mucopolysaccharide metabolism, characterized by severe mental retardation and excessive excretion of heparan sulfate in the urine (1). Though related clinically and biochemically to the Hurler and Hunter syndromes, it is genetically distinct.

Fibroblasts cultured from the skin of Sanfilippo patients accumulate elevated quantities of sulfated mucopolysaccharide, especially dermatan sulfate (2). As was demonstrated for fibroblasts from Hurler and Hunter patients (3, 4), the metabolic defect in Sanfilippo fibroblasts can be remedied by secretions+ of fibroblasts or by dialyzed concentrates of urine derived

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+The term "secretions" is used here to denote macromolecular substances produced by fibroblasts and released into the medium. Such release could presumably occur by exocytosis, by cell death or by the shedding of membrane components, as well as by true secretion.

from individuals of different genotypes. It was unexpectedly found that Sanfilippo fibroblasts fall into two subgroups showing mutual correction. Therefore, the syndrome can be attributed to the absence of either of two distinct factors necessary for normal mucopolysaccharide metabolism. These have been subjected to analytical polyacrylamide gel electrophoresis for an estimate of molecular size and charge, as had been previously done for that factor which is deficient in the Hunter syndrome (5).

EXPERIMENTAL: Procedures for the culture of fibroblasts, for the measurement of $^{35}SO_4$ incorporation into intracellular mucopolysaccharide, for the preparation of concentrates of preincubated medium, and for assay of corrective factor activity have been previously described (3, 5). For the preparation of urinary Sanfilippo factors, fresh morning urine was made 70% saturated with ammonium sulfate. The precipitate was dissolved in a small volume of 0.01 M sodium phosphate, pH 5.0, dialyzed against 0.01 M sodium acetate, pH 5.2, and applied to a DEAE-cellulose column equilibrated with the same buffer. The Sanfilippo factors passed through the column with a resultant 3-fold increase in specific activity.

The methodology of polyacrylamide gel electrophoresis is that described by Rodbard and Chrambach (6) using two multiphasic buffer systems developed by Jovin et al. (7). The operative pH of the separation phase at 0° C is 4.0 for system No. 35.7 and 8.5 for system No. 2243.3. Partially purified urinary factor preparation (400 μg protein) was applied to each gel in 100 μl of 0.01 M NaCl containing 20% sucrose and 0.001% methyl green or phenol red. Factor activity was localized by slicing the gels transversely and assaying an extract of the slices (5).

RESULTS: Metabolism of intracellular mucopolysaccharides - In the presence of $^{35}SO_4$, Sanfilippo fibroblasts accumulate more radioactive mucopolysaccharide than do normal fibroblasts, but less than Hurler and Hunter fibroblasts (Fig. 1, left panel). From the right panel, the rate of disappearance of radioactive mucopolysaccharide, when labeled medium is replaced by unlabeled, is likewise seen to be intermediate in Sanfilippo fibroblasts.

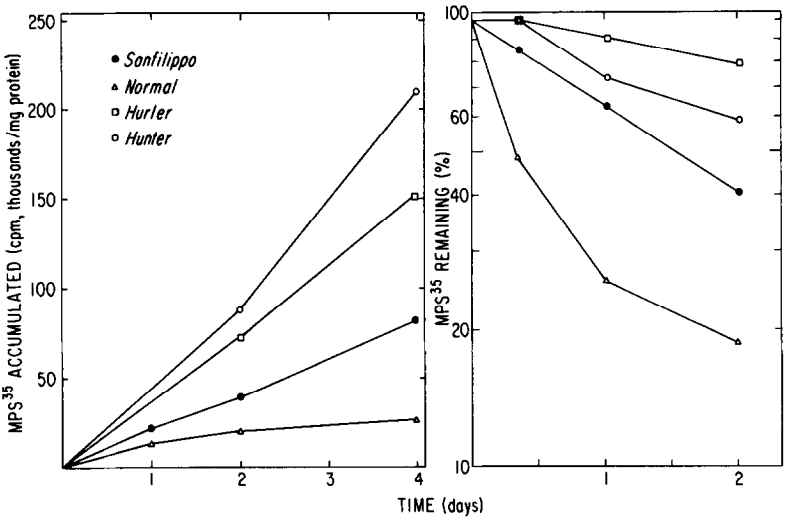

Fig. 1. Metabolism of sulfated mucopolysaccharide by fibroblasts from Sanfilippo, Hurler and Hunter patients, and from a normal subject. Left panel: accumulation of intracellular radioactive mucopolysaccharide, expressed on the basis of cell protein; right panel: loss of radioactive mucopolysaccharide during chase with unlabeled medium.

Correction by cell secretions - Concentrates of medium preincubated with cells of genotype other than Sanfilippo reduce the accumulation of radioactive mucopolysaccharide by Sanfilippo fibroblasts (Table 1). Similar corrective activity was found in medium preincubated with certain Sanfilippo fibroblast lines. As shown in Table 2, secretions of cells from patient A.B. reduce the accumulation in the cells of P.L. and M.L., but not in those of B.N.; the converse is true of the secretions of cells from K.M. By such cross-correction tests, Sanfilippo lines could be classified into two groups, arbitrarily designated A and B, such that secretions of A correct the defect in cells of B and vice-versa, but that there is no correction within each category. Of 17 Sanfilippo lines examined thus far, 11 were in the A and 6 in the B group. Both sexes are represented and there may be more than one affected child per family, indicating autosomal recessive inheritance for each group.

Correction by urinary factor - Macromolecular concentrates of normal human urine decrease the accumulation of radioactive mucopolysaccharide in

Table 1

Reduction of radioactive mucopolysaccharide accumulation in Sanfilippo fibroblasts by secretions from fibroblasts of other genotypes

Source of secretions	Mucopolysaccharide accumulation (cpm/mg cell protein)
Control*	34,100
Normal	11,300
Hurler	16,400
Hunter	19,100
Morquio	12,700
Scheie	16,500
Cystic Fibrosis	15,500

*Sanfilippo line used for assay also used as source of secretions.

Table 2

Cross-correction between Sanfilippo fibroblasts

Source of secretions	Mucopolysaccharide accumulation (cpm/mg cell protein) in fibroblasts from Sanfilippo patients		
	P.L.	M.L.	B.N.
Control*	14,700	12,900	22,000
Sanfilippo, A.B.	6,700	6,200	22,600
Sanfilippo, K.M.	14,700	11,800	12,200

*As in Table 1.

Hurler, Hunter and both subgroups of Sanfilippo fibroblasts, but have no effect on normal fibroblasts (4). Urine samples from two Sanfilippo patients were examined: one had Sanfilippo A factor (i.e., the factor required to reduce mucopolysaccharide accumulation in Sanfilippo fibroblasts A) but lacked the Sanfilippo B factor (correspondingly defined); the second urine had Sanfilippo B factor but not A. Both had normal amounts of Hurler and Hunter factors. The presence of urinary Sanfilippo factor therefore appears to be genotype-specific.

The Sanfilippo factors are heat-labile. The B factor activity is destroyed by heating 10 min at 70° in 0.9% NaCl, while the A factor activity, stable at 70°, is destroyed by 10 min at 100°.

Characterization by polyacrylamide gel electrophoresis - The two Sanfilippo factors migrated as cations in the pH 4.0 system and as anions in the pH 8.5 system. The latter was used for determination of molecular size and charge.

The relative electrophoretic mobility (R_f) of the two factors was determined at several gel concentrations. A plot of log R_f against gel concentration (Ferguson plot, Fig. 2) gives rise to straight lines; the slopes (retardation coefficients, K_R) were found to be 0.090 ± 0.004 (S.D.) and 0.086 ± 0.012 (S.D.) for the A and B factors, respectively. The K_R of a molecule is a function of its size; there is a linear relationship between $\sqrt{K_R}$ and the molecular radius, $\bar{R}$, assuming an unhydrated sphere (6, 8). K_R was determined experimentally for 7 proteins of known molecular weight (6) and a standard curve constructed (Fig. 3). By interpola-

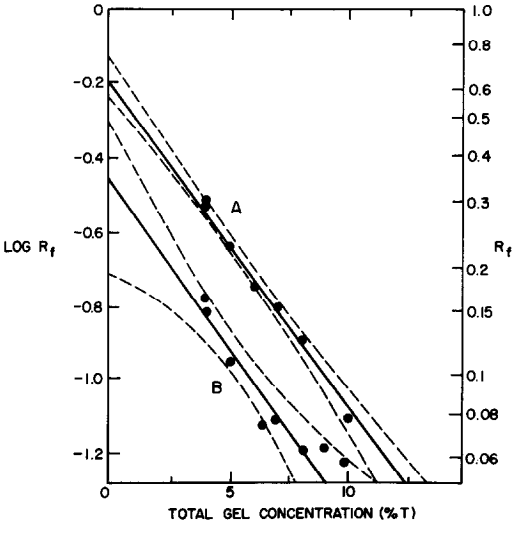

Fig. 2. Ferguson plot for the two Sanfilippo factors. Automated data processing was used to compute regressions and confidence limits (6). —— Weighted regression of log R_f on T; .... 95% confidence limits for the regression line.

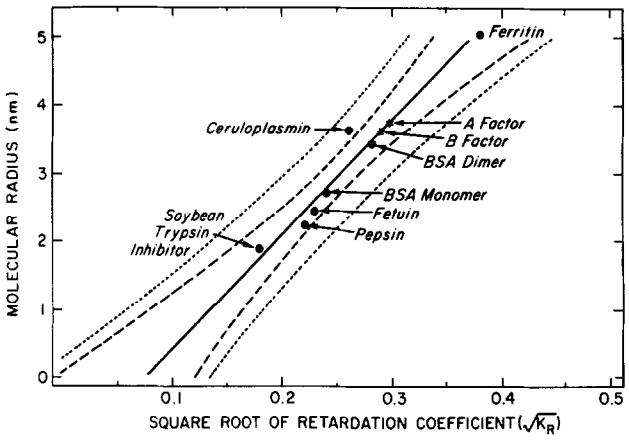

Fig. 3. Standard curve for the estimation of molecular size. —— Unweighted regression of $\bar{R}$ on K_R; ---- 95% confidence limits for the line; .... 95% confidence limits for a single observation around the line, which were used to compute the confidence limits for the radius of the factors.

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tion, $\bar{R}$ of the Sanfilippo A factor is found to be 3.79 nm, with 95% confidence limits 2.93-4.65 nm; this corresponds to a molecular weight of 186,000 with 95% confidence limits of 86,000-343,000. For the Sanfilippo B factor, $\bar{R}$ is 3.69 nm (2.84-4.54 nm) and the molecular weight 171,000 (78,000-319,000).

The free electrophoretic mobility (M_o) obtained by extrapolation of the Ferguson plot to zero gel concentration is used to calculate the net charge (8). At pH 8.5, the Sanfilippo A factor has a net charge of -6.6 protons, and the Sanfilippo B factor, -3.2.

DISCUSSION: Biochemical heterogeneity within the Sanfilippo syndrome is documented by several lines of evidence. Sanfilippo fibroblasts can be sorted into two groups, such that secretions from one group contain a macromolecular factor which normalizes mucopolysaccharide metabolism in cells of the other; the urine of Sanfilippo patients is deficient in only one of these two corrective factors; the two factors are separable by heat treatment and electrophoretic mobility. Though the clinical summaries available to us do not reveal any obvious phenotypic differences between the two biochemical subgroups of Sanfilippo patients, subtle differences may eventually be

uncovered with the aid of biochemical classification.

The properties of the Sanfilippo factors--heat lability, non-adsorption to DEAE-cellulose at pH 5.2, and migration as a cation at pH 4.0 and an anion at pH 8.5--are consistent with properties of proteins. This, in turn, allows comparison with known proteins for estimates of size and charge. It is our working hypothesis that the two factors are enzymes or enzyme activators, required for proper metabolism (degradation?) of dermatan sulfate and heparan sulfate, and that in Sanfilippo patients one factor has been rendered ineffective by mutation.

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