Sanfilippo Disease Type B: Enzyme Replacement and Metabolic Correction in Cultured Fibroblasts

Abstract. *α-Acetylglucosaminidase, purified from human placenta, corrected the defect in mucopolysaccharide degradation when added to cultured fibroblasts from patients with Sanfilippo disease type B. A small cellular concentration of enzyme gave a large corrective effect. The half-life of disappearance of enzyme activity was 4 to 7 days.*

Sanfilippo disease is an inborn error of mucopolysaccharide metabolism in which there is a massive storage of heparan sulfate and which is transmitted as an autosomal recessive trait. Two types of Sanfilippo disease are known: type A in which there is a deficiency of heparan sulfate sulfatase (*1*) and type B in which there is a deficiency of α-acetylglucosaminidase (*2*). Both enzymes appear to participate in heparan sulfate degradation at different steps, accounting for the mucopolysaccharide storage. Kresse *et al.* (*3*) demonstrated an impairment in mucopolysaccharide degradation in cultured fibroblasts from patients with Sanfilippo disease and showed that the defect in degradation could be normalized by adding corrective factors to media in which Sanfilippo cells were growing. Von Figura and Kresse (*4*) have shown that the Sanfilippo type B corrective factor is identical to α-acetylglucosaminidase.

We sought to determine whether partially purified preparations of α-acetylglucosaminidase are taken up by cultured skin fibroblasts, whether externally supplied enzyme corrects the metabolic defect, what concentrations of intracellular enzyme are required for correction, and whether intracellular enzyme degradation occurs in these cells, and, if so, at what rate.

Skin biopsies from patients with Sanfilippo disease and from control subjects were used to establish cultured fibroblast strains. All cell strains were grown in media described by Fratantoni *et al.* (*5*). Patients were typed as Sanfilippo type A or B by enzyme assays for α-acetylglucosaminidase (*2*) or by experiments on cross-correction of their cells (*3*). Disappearance of sulfate was determined as described (*5*). The cells were subcultured in sulfate-free media for 3 to 4 days, [^{35}S]sulfate was added, and the cells were grown for an additional 44 hours. One portion of cells was taken to determine the ^{35}S (in counts per minute) in the mucopolysaccharide (*5*); another portion, after being washed once in isotonic saline, was taken to determine α-fucosidase and α-acetylglucosaminidase activities with the use of the corresponding *p*-nitrophenyl substrates (*2*) (Koch-Light, Ltd.).

The enzyme was purified as follows. Buffer 1 contained phosphate (*p*H 6.0, 50 m*M*); buffer 2 contained phosphate (*p*H 6.0, 50 m*M*) and glycerol (20 percent by volume); and buffer 3 contained phosphate (*p*H 7.0, 10 m*M*) and glycerol (20 percent by volume). All enzyme purification steps were carried out at 4°C.

α-Acetylglucosaminidase was isolated from fresh frozen human placentas obtained at the delivery of full-term normal newborns. After the amniotic membrane was removed, the placental tissue was washed in buffer 1 and then homogenized in 1.5 volumes of buffer 2 in a Waring blender. The homogenate was centrifuged for 30 minutes at 14,600*g*, and the supernatant was dialyzed for 24 hours against buffer 2 and centrifuged again at 14,600*g* for 30 minutes to remove insoluble material. The supernatant was then adjusted to a protein concentration of 25 mg/ml and ammonium sulfate was added to 40 percent saturation. After 1 hour, the solution was centrifuged at 14,600*g*, the precipitate containing the enzyme was redissolved in buffer 2, and the resulting solution was dialyzed against the same buffer for 24 hours and centrifuged again for 30 minutes at 24,000g to remove the insoluble material. The protein concentration of the enzyme solution was adjusted to 13 mg/ml with buffer 2. Ammonium sulfate was added to 25 percent saturation. After 1 hour, the resulting suspension was centrifuged at 24,000*g* for 30 minutes. The precipitate was discarded, and the ammonium sulfate concentration of the supernatant was raised to 35 percent; after overnight equilibration, the suspension was centrifuged at 24,000*g* for 30 minutes. The sediment containing the enzyme was dissolved in buffer 2; the resulting solution was dialyzed for 24 hours against buffer 3, and then centrifuged at 24,000*g* for 30 minutes. The enzyme, now in the supernatant, had undergone an 80-fold purification.

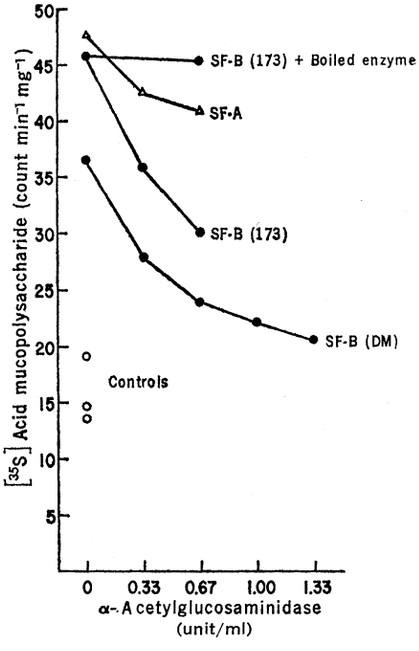

Fig. 1. Effect of α-acetylglucosaminidase on accumulation of acid mucopolysaccharide. Enzyme was added to skin fibroblasts, and cells were harvested 44 hours later; *SF-B* (Sanfilippo type B), patients 173 and DM; *SF-A* (Sanfilippo type A); controls were three normal subjects.

The enzyme solution was added to a column of diethylaminoethyl cellulose (Whatman DE-52), which had been degassed and washed with buffer 3. Buffer 3 was then added to elute α-acetylglucosaminidase, which did not adhere to the column. The enzyme was concentrated by ultrafiltration (Diaflo, model 65) with the use of a UM-10 membrane, and the concentrate was dialyzed against buffer 3. α-Acetylglucosaminidase prepared in this manner was purified 200-fold.

Both preparations of α-acetylglucosaminidase were used in the enzyme replacement experiments. α-Acetylglucosaminidase was dissolved in tissue culture media and added to the cells when the [^{35}S]sulfate was added.

When α-acetylglucosaminidase was added over a 20-fold range of concentration to fibroblasts from three patients with Sanfilippo disease type B (Table 1), activity in the sedimented cells was between 0.5 to 0.7 percent of that added in the media. Assays of α-acetylglucosaminidase activity in the cell-free media demonstrated that less than 5 percent of the enzyme was inactivated over a 72-hour period. α-Fucosidase activity in the sedimented cells remained constant over the same period. The enzyme-treated cells appeared morphologically identical to

Table 1. Addition of α-acetylglucosaminidase to Sanfilippo type B fibroblasts. Cells were harvested 44 hours after they were exposed to the enzyme. Assays of the media showed no significant losses of enzyme over this time period. Units given are nanomoles of substrate cleaved per hour. Correction values were calculated by determining the number of counts per minute in the ^{35}S-labeled mucopolysaccharide as a percentage of the controls. The enzyme preparation used here was purified 200-fold. Column A represents the amount of enzyme found in the sedimented cell pellet (units per milligram of protein). Column B represents the number of units in a volume of medium equal to the volume of the sedimented cell pellet (it is the amount expected on the basis of diffusion alone).

Enzyme in media (unit/ml)	Enzyme taken up (%)	Cell activity (% of normal mean)	^{35}S correction (%)	A (unit/mg)	B (unit/mg)	A : B
			Patient DM			
0				Less than 0.07		
1	0.61	2.0	42	0.110	0.217	51 : 100
2	0.49	3.2	61	0.173	0.370	46 : 100
3	0.47	4.7	70	0.253	0.540	46 : 100
4	0.52	6.9	78	0.375	0.712	53 : 100
20	0.56	24.6	92	1.330	2.720	49 : 100
			Patient RO			
20	0.74	33.2		1.792	4.840	37 : 100
			Patient 173			
20	0.62	27.6		1.493	3.620	41 : 100
			Controls (17 in all)			
0				5.4 ± 3.5 (2.1 to 12.6)		

controls. At the highest concentration of added enzyme, the cellular activity of α-acetylglucosaminidase in three Sanfilippo strains was 1.3 to 1.8 units or 24 to 33 percent of the mean activity of control fibroblasts (Table 1, column A).

From the volume of the cell pellet and the concentration of α-acetylglucosaminidase in an equivalent volume of media, a calculation was made to determine whether the Sanfilippo cells concentrated the enzyme against a gradient (Table 1, column B). The cells contained between 37 and 53 percent of the activity in an equivalent volume of media, an indication that selective concentration did not occur at any concentration of added enzyme.

Externally supplied α-acetylglucosaminidase corrected the defect in mucopolysaccharide metabolism in Sanfilippo B fibroblasts (Fig. 1). The degree of correction increased with increasing added enzyme, but the correlation was not linear. Twenty times more enzyme was required for 92 percent correction than for 42 percent correction. A small degree of correction occurred when α-acetylglucosaminidase was added to Sanfilippo type A cells, an indication that the preparation was contaminated with heparan sulfate sulfatase. A boiled enzyme preparation of α-acetylglucosaminidase had no corrective effect.

Both the 200-fold and the 80-fold purified α-acetylglucosaminidase preparations corrected the defect in mucopolysaccharide degradation, and the former preparation was somewhat more effective than the latter (Fig. 2). The reason for the difference between the two preparations is not known. When cellular activity of α-acetylglucosaminidase in the Sanfilippo type B cells was brought to 0.25 unit (5 percent of the normal mean), approximately 70 percent correction occurred; at 1.33 units (25 percent of the normal mean) 92 percent correction occurred (Table 1).

The rate of inactivation of the enzyme was determined as follows. Cells were exposed to α-acetylglucosaminidase (20 units per milliliter) for 64 hours, the medium was replaced, and cells were harvested at intervals for the next 72 hours. Intracellular enzyme activity was corrected for dilution caused by new cell growth, as determined from the protein content of the flasks. The rate of inactivation of the enzyme was similar in two cell lines (Fig. 3); the loss of activity in strain DM averaged 0.28 unit per day while that in strain RO averaged 0.33. The half-life of disappearance of the enzyme in these two strains was 4 and 7 days, respectively.

The results demonstrate that partially purified placental α-acetylglucosaminidase preparations correct the defect in mucopolysaccharide metabolism in cultured fibroblasts from patients with Sanfilippo disease type B. Bach *et al.* have demonstrated a similar effect by adding L-α-iduronidase prepared from normal human urine to cells from patients with Hurler's disease; the uptake of α-iduronidase from the medium was remarkably high, approaching 40 percent of that added (*6*). The uptake found by Bach *et al.* was 66 times higher than that we found for the uptake of α-acetylglucosaminidase over

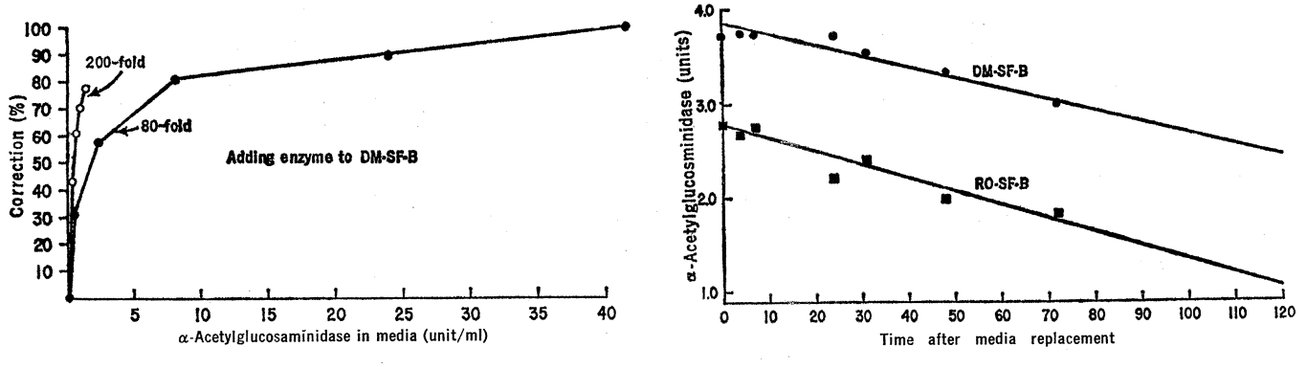

Fig. 2 (left). Effect of two different enzyme preparations on accumulation of acid mucopolysaccharide. Enzyme was added to fibroblasts from Sanifilippo type B patient DM, and the cells were harvested 44 hours later. Insufficient enzyme was available from the 200-fold purified preparation to carry out studies at all concentrations. Fig. 3 (right). Inactivation of α-acetylglucosaminidase (nanomoles per milligram of protein per hour) with time (in hours) after two Sanfilippo type B skin fibroblast strains (RO and DM) were treated with enzyme. Activities were corrected for dilution.

the same time interval. The reasons for the higher uptake of α-iduronidase as compared to α-acetylglucosaminidase are not apparent. Since we used the same media for culturing fibroblasts as Bach *et al.* (*6*), this would not seem to account for the difference. Similar uptake and correction studies in which arylsulfatase A preparations were added to cultured fibroblasts from patients with metachromatic leukodystrophy were carried out by Porter *et al.* (*7*) and by Wiessman *et al.* (*8*). Unfortunately, neither group reported values for the cellular uptake of this enzyme.

The degree of metabolic correction is large at very low cellular concentrations of enzyme; 42 to 70 percent correction occurred at concentrations that were 2 to 5 percent of the normal average. This fact is encouraging when considering enzyme replacement therapy in patients with Sanfilippo disease type B.

It is assumed that the cellular uptake of α-acetylglucosaminidase is accomplished by pinocytosis because uptake of other proteins by cultured cells appears to proceed via this mechanism (*9*). Our studies with α-acetylglucosaminidase indicate that the chief limiting factor in the correction of the metabolic defect by enzyme replacement is cellular uptake of the enzyme. Pinocytosis stimulants, such as basic polypeptides, may be useful adjuncts in enzyme replacement (*9*) because they enhance (up to ten times) the uptake of added proteins by cultured cells.

It is not surprising that trials of intravenous plasma infusions (100 to 200 ml of fresh normal plasma) have been ineffective in patients with Sanfilippo disease (*10*). Although α-acetylglucosaminidase activity in human plasma averages 20 units per milliliter (*11*), the amount of enzyme which would be supplied by administering 100 to 200 ml of plasma is approximately 250 times lower than should be metabolically effective if one assumes that enzyme uptake, metabolic correction, and enzyme degradation in the patient's tissues are similar to those in cultured fibroblasts. Enzyme replacement therapy with α-acetylglucosaminidase in higher quantities appears to be warranted in Sanfilippo disease type B.

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Osmolar Control of Prolactin Secretion in Man

Abstract. *To study the effect of changing serum osmolality on serum prolactin concentration 11 volunteers were given oral and intravenous hypotonic and hypertonic fluids. Mean serum prolactin fell to 10.5 percent of baseline after oral water loading and to 15 percent of baseline after intravenous hypotonic saline infusion. Conversely, mean prolactin rose to 417 percent of baseline after intravenous hypertonic saline administration. The correlation coefficient of simultaneously determined serum prolactin and osmolality was highly significant* ($P < .001$). *Isoosmolar changes in extracellular fluid volume did not consistently affect the concentration of prolactin in the serum. Thus, prolactin may be involved in the physiologic regulation of osmolar balance and the kidney may be an important target organ for prolactin.*

The adenohypophysial hormone prolactin exists in many vertebrate species (*1*). Among the many functions of prolactin, a role in osmoregulation has been demonstrated in teleost fish (*2*). After hypophysectomy certain euryhaline fish species are unable to maintain blood osmolality when placed in fresh water. Osmoregulation is restored by prolactin administration (*2*). Furthermore, in the intact fish the prolactin-producing eta cells appear to be functionally more active in fresh water than in salt water (*3*), an indication that prolactin secretion is increased in fresh water enabling the fish to maintain blood osmolality.

The role of prolactin in osmoregulation in mammals has not been studied systematically. There is, however, evidence that the kidney may be a target organ for prolactin. Specific binding activity for prolactin has been described in kidney homogenates (*4*). In some patients with renal failure concentrations of prolactin in the serum are elevated (*5, 6*). Rats (*7*), heart-lung-kidney preparations from cats (*8*), and humans (*9*) respond to prolactin administration with marked renal retention of sodium, potassium, and water. These lines of evidence suggest that prolactin may play a role in osmoregulation in mammals.

Table 1. Prolactin concentrations during administration of hypotonic and hypertonic fluid.

Subject	Sex	Prolactin concentrations (ng/ml) at								
		0′	10′	30′	50′	60′	70′	90′	120′	180′
					Oral water					
J.R.	F	11		7		8		4	6	9
J.M.	F	6		3		<1		<1	4	3
A.R.	M	4		3		5		5	1	2
M.W.	F	4		1		<1			<1	<1
M.B.	F	5		<1		<1			<1	
G.P.	M	3		9		2			<1	<1
					Hypotonic saline infusion					
S.M.	F	21		9	17		17			
C.L.	F	17	9	3	10		8			
J.F.	M	5	2	<1	<1		<1			
B.M.	M	4	2	<1	<1		<1			
					Hypertonic saline infusion					
S.M.	F	17	17	15	27		19			
C.L.	F	2	25	20	14		5			
J.F.	M	2	3	4	<1		<1			
J.H.	F	5	5	12	5		10			