PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 642-649 (1976)

In Vitro Correction of Hurler Fibroblasts with Bovine Testicular Hyaluronidase (39277)

J. K. HERD, B. A. HAYHOME, J. TSCHIDA, AND T. FORREST
(Sponsored by Dr. I. Wells)

Department of Pediatrics, Creighton University School of Medicine, Omaha, Nebraska 68131

Preliminary isolation from normal human serum of corrective factor activity for cultured Hurler fibroblasts revealed the presence of both hyaluronidase and phenyl-α-L-iduronidase activities (1). Because hyaluronidase is commercially available and α-L-iduronidase is not, we have investigated the corrective effect of various hyaluronidase preparations on cultured Hurler fibroblasts. Preliminary results with crude and refined preparations indicated that bovine testicular hyaluronidase, which effectively degraded hyaluronic acid and the chondroitin sulfates, was a potent corrective factor for Hurler cells, and bacterial hyaluronidase, which effectively degraded only hyaluronic acid, was not a corrective factor for Hurler cells (2). Bovine testicular hyaluronidase had negligible corrective activity on normal cells. Several other enzymes including β-glucuronidase, aryl sulfatase, trypsin, papain, and pronase, when used at the same protein concentration as hyaluronidase in the culture medium, had no corrective activity on Hurler cells (2). More recently we have shown that the corrective effect of bovine testicular hyaluronidase on Hurler fibroblasts is associated with increased degradation of some of the intracellular mucopolysaccharides, increased secretion of oligosaccharides from the cells into the medium, and that α-L-iduronidase is present as a contaminant in all samples of bovine testicular hyaluronidase tested (3). The purpose of this report is to present the detailed results of those experiments.

Materials and methods. Samples of bovine testicular hyaluronidase of varying purity and activity were obtained as follows: purified unsterile Wyeth hyaluronidase (Lot M-151) and commercial grade Wyeth hyaluronidase (Wydase, 4734091) from Dr. J. Gold, Wyeth Laboratories, Philadelphia, Pa.; purified HSEP Worthington hyaluronidase, (Lot 54J589) from Dr. H. Bungay, Worthington Biochemical Corp., Freehold, N.J.; highly purified Leo hyaluronidase (Batch No. S-110) from the A. B. Leo Co., Helsingborg, Sweden; purified Apsen hyaluronidase (Lot 102) of the Apsen Laboratories from Dr. C. Dietrich of Sao Paulo, Brazil. Papain, 38 units/mg, was obtained as a suspension from Worthington (PAP) and activated with cysteine in EDTA buffer before use. Trypsin was obtained frozen, 2.5%, from Gibco and diluted in 0.15 *M* NaCl before use. Pronase, B. grade, 45,000 PUK units/g, was obtained from Calbiochemical Co. as a dry powder and diluted in 0.15 *M* NaCl before use. Bead polymerized, G-200 Sephadex was purchased from the Pharmacia Co., Piscataway, N.J. Carrier-free $H_2{}^{35}SO_4$, 43 Ci/mg, was purchased from New England Nuclear. Phenyl-α-L-iduronide, synthesized by Dr. B. Weissmann, was obtained from Dr. E. Neufeld, NIAMD, Bethesda, Md. *Para*-nitrophenyl phosphate and *para*-nitrophenyl-β-D-galactopyranoside were obtained from the Sigma Chemical Co., St. Louis, Mo. One Hunter, one Hurler, and all normal fibroblast lines were grown from primary explants from local patients. An additional Hunter fibroblast line was obtained from Dr. A. Greene, The Mutant Cell Repository, Camden, N.J.

Methods of cell culture and radioactive chase assay of corrective factor activity have been described (4). Examination of $^{35}SO_4$-labeled cells and medium for the size of $^{35}SO_4$-labeled mucopolysaccharides following hyaluronidase treatment was accomplished by gel filtration in columns of Sephadex G-200. After a 48-hr chase with nonradioactive treatment medium, cells of duplicate petri dish cultures were rinsed with 5 ml of cold 0.15 *M* NaCl, harvested with a rubber policeman, pooled in 2.5 ml of cold saline, disrupted by four cycles of fast freez-

ing (−53°) and thawing (+30°) and centrifuged at 2100*g* for 20 min. The supernatant containing the mucopolysaccharides was saved, and the pellet was washed in 0.5 ml of H_2O and recentrifuged. The supernatants and washings were combined and dialyzed for 24 hr against cold running tap H_2O and 24 hr against distilled H_2O at 4°. After the dialyzed solution was concentrated to 2.0 ml by flash evaporation, it was applied to the top of a column (85 × 1.7 cm) of G-200 Sephadex which had been equilibrated with 1.0 *M* NaCl. Elution with 1.0 *M* NaCl at a flow rate of 30 ml/hr was by gravity under 15 cm H_2O pressure, and the eluate was collected in 2.0-ml fractions. The media from duplicate petri dishes were combined with the saline wash of the cells, clarified by centrifugation, dialyzed, concentrated, and chromatographed by identical methods to those used for the cells.

Cell homogenates were prepared for enzyme assays according to the method of Hall and Neufeld (5). α-L-Iduronidase, β-galactosidase, and acid and alkaline phosphatase activities of cell homogenates were determined by published methods (5, 6). Hyaluronidase activity was assayed by the Worthington modification (7) of the Tolksdorf method (8) using a full 30-min incubation and was expressed in turbidity reduction units (TRU).

Results. Hurler and normal fibroblasts were plated from common suspensions of each cell type into two sets of 14 petri dishes each, and grown 5 days in standard medium +20% fetal calf serum (FCS). The cells were labeled with 20 μCi of $^{35}SO_4$ per dish for 2 days, rinsed, and two dishes of each line were harvested for determination of specific activity of the pulsed cells by methods previously described (4). The remaining 12 dishes of each cell line were divided equally into control and experimental groups. Each dish of control cells received 8 ml of medium enriched with 20% heat-inactivated fetal calf serum (HIFCS). Each dish of experimental cells received 8 ml of medium enriched with 20% HIFCS and 32 μg of Worthington hyaluronidase. Two control and two experimental dishes of each cell line were harvested after 1, 2, and 3 days and the specific activities of the cells were determined. Radioactivities were adjusted to account for natural decay of the isotope in all samples counted after the pulse cells. The results are shown in Fig. 1.

Hyaluronidase treatment caused a sevenfold increase in the turnover (sp act pulse minus sp act chase) of $^{35}SO_4$-labeled mucopolysaccharides in Hurler cells on the first day, a threefold increase on the second day, and a twofold increase on the third day compared to the turnover in control Hurler cells not treated with hyaluronidase. Hyaluronidase treatment of Hurler cells increased the turnover of $^{35}SO_4$-mucopolysaccharides to more than twice that of normal cells in the first day. Hyaluronidase had a similar, though very small, effect on normal cells.

Nonspecificity was examined by determining the corrective activity of two different hyaluronidase preparations simultaneously in Hurler, Hunter, and normal cells. The experiment was set up as before except that treatment was for 24 hr in all cells. Control dishes of each cell line were given 8 ml of the same medium containing 16 μg of either Wyeth (M-151) hyaluronidase (5.4 TRU) or Worthington hyaluronidase (42.8 TRU). Treated cells were harvested after 24 hr and their specific activities were deter-

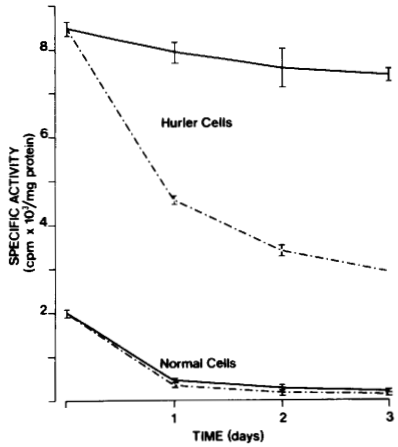

FIG. 1. Loss of $^{35}SO_4$ mucopolysaccharides from 48-hr $^{35}SO_4$-labeled Hurler and normal fibroblasts treated with control medium enriched with 20% HIFCS (——) or control medium (20% HIFCS) + 32 μg Worthington hyaluronidase (-·-·-) per petri dish. Vertical lines represent the range of duplicate determinations of specific activity of two petri dishes for each point.

mined. The results are shown in Table I. Hyaluronidase treatment evoked nearly as great an increase in mucopolysaccharide turnover in Hunter cells as it did in Hurler cells and again had a nearly negligible effect in normal cells. The magnitude of the corrective effect of hyaluronidase was the same for each sample tested, although one preparation had eight times more hyaluronidase activity.

Corrective activity of Wyeth and Worthington enzymes were further compared in 24-hr dose-response chase assays. Strong corrective activity was seen with less than 1 μg of enzyme per petri dish. Worthington hyaluronidase (0.4 μg) effected the same decrease (3674 cpm/mg protein) in specific activity of Hurler cells as did 1.6 μg of Wyeth hyaluronidase. In this experiment these amounts of each enzyme equaled 0.48 TRU of hyaluronidase measured with bovine nasal septal chondroitin 4-sulfate as a substrate.

The effect of hyaluronidase treatment of Hurler cells on the size distribution of the $^{35}SO_4$-labeled mucopolysaccharides was examined by Sephadex G-200 chromatography. Hurler cells were labeled for 3 days with 50 μCi of $^{35}SO_4$ per dish, rinsed, and treated for 2 days with 10 ml of medium enriched with 10% FCS (control) or with 10 ml of medium enriched with 10% FCS and 20 μg of Wyeth (M-151) hyaluronidase (6.75 TRU). Cells and treatment (chase) media were harvested after 2 days and chromatographed on G-200 Sephadex as described in Methods. Radioactivity was determined by liquid scintillation analysis of 0.6-ml aliquots from each fraction. The results are shown in Figs. 2A and B. Radioactivity from the pulsed cells (Fig. 2A) was distributed in three rather well-defined peaks, labeled I, II, and III. Peak I is rather sharp and corresponds to the void volume for blue dextran 2000. Peak III is rather broad and terminates just ahead of the effluent volume for ionic sulfate. Peak II is a broad peak occupying the effluent volume between I and III. In Hurler cells treated with control medium there was loss of mucopolysaccharide from peaks I and II, while peak III was unchanged. In Hurler cells treated with hyaluronidase there was a further loss of mucopolysaccharide from peak I and II and an increase in mucopolysaccharide in peak III. Mucopolysaccharides secreted (Fig. 2B) by Hurler cells treated with control medium consisted mostly of macromolecular material and small amounts of large oligosaccharides corresponding to peaks I and II in the cells, plus a very small amount of material toward the last part of peak III. Hyaluronidase-treated Hurler cells secreted about one-half as much macromolecular mucopolysaccharide, but 5–10 times as much oligosaccharide, the increase being greatest in the smallest oligosaccharides.

Because of the many impurities known in commercial testicular hyaluronidase it was important to consider whether corrective ef-

TABLE I. CORRECTIVE EFFECT OF HYALURONIDASE ON HURLER, HUNTER, AND NORMAL FIBROBLASTS.[a]

Treatment	Cell Lines			
	Hurler[b]	Hunter 1[b]	Hunter 2[b]	Normal
Pulse	16,919[c]	15,269	31,315	9,522
Chase				
Control – 20% HIFCS	15,725	9,945	27,123	1,390
Hyaluronidase				
20% HIFCS + 16 μg Wy[d]	10,271	6,471	20,265	1,003
20% HIFCS + 16 μg Wo[e]	10,430	6,430	18,388	974

[a] Fibroblast cell lines were pulse labeled with 20 μCi of $^{35}SO_4$ per petri dish from Days 5–7 after plating, then exposed to the three different treatment media indicated for 24 hr. The specific activity of cells harvested after treatment (chase) may be compared with the specific activity of cells harvested after labeling (pulse).

[b] These Hurler cells are from a different patient than those in Fig. 1. Hunter 1 is a local patient; Hunter 2 is cell line GM-39 from Dr. A. Greene, Mutant Cell Repository, Camden, N.J.

[c] Numbers are specific activities (cpm/mg protein) and represent average of duplicate determinations of two petri dishes.

[d] Wy = Wyeth hyaluronidae (M-151), 5.4 TRU/petri dish.

[e] Wo = Worthington hyaluronidase (HSEP), 42.8 TRU/petri dish.

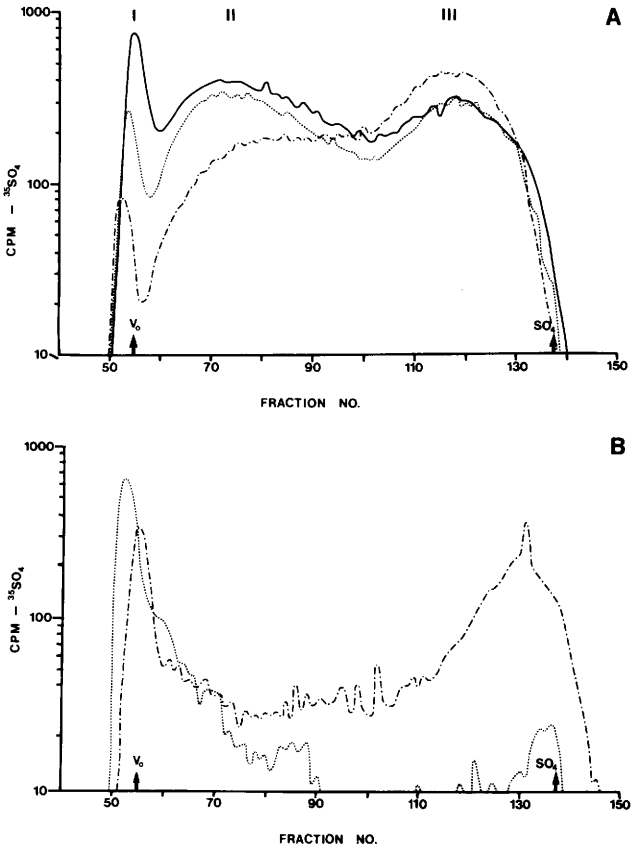

FIG. 2. G-200 sephadex chromatography elution profiles of $^{35}SO_4$-mucopolysaccharides from (A) Hurler cells and (B) Hurler medium. Hurler cells labeled 3 days with 50 μCi of $^{35}SO_4$/dish (pulse, ——) were treated 2 days with standard medium (control, ·····) or standard medium plus 20 μg of Wyeth (M-151) hyaluronidase (experimental, -·-·-).

fect could have been imparted by nonhyaluronidase enzymes such as protease, α-L-iduronidase, and sulfatase. We tested large amounts of three proteolytic enzymes for corrective activity by a 24-hr chase assay on $^{35}SO_4$-labeled Hurler cells and a 2-hr chase assay on $^{35}SO_4$-labeled normal cells. The results are shown in Table II. None of these proteolytic enzymes showed significant correction. Trypsin may even have been inhibitory to correction. Also, no decrease in cell protein has been observed in hyaluronidase-treated cells during a corrective factor assay. Sulfatase activity was unlikely to have caused this effect as is discussed later.

We then examined one hyaluronidase sample directly for phenyl-α-L-iduronidase activity. The result, shown in Fig. 3, revealed definite iduronidase activity, but of low magnitude. All the hyaluronidase preparations available to us were then examined for both phenyl-α-L-iduronidase activity and hyaluronidase activity (Table III). Those preparations with greatest hyaluronidase activity also had the greatest iduronidase activity. The Wyeth hyaluronidase (M-151) had the least iduronidase activity.

Since we did not yet have pure hyaluronidase we repeated treatment of $^{35}SO_4$-labeled Hurler cells with Wyeth hyaluronidase for 24 hr as in the first experiment and measured the iduronidase content of the cells before and after treatment. The results are shown in Table IV. Treatment of Hurler cells with 5.4 TR units of hyaluronidase containing only 0.11 units of phenyl-α-L-iduronidase effected a large increase in the turnover of $^{35}SO_4$-mucopolysaccharides, but

TABLE II. CORRECTIVE EFFECT OF PROTEOLYTIC ENZYMES ON HURLER AND NORMAL FIBROBLASTS.[a]

Treatment	Cell Lines	
	Hurler	Normal
Pulse	19,988[b]	2,200
20% FCS (control)	16,116	1,282
20% FCS + Papain[c]	16,460	1,185
20% FCS + Trypsin[c]	17,343	1,318
20% FCS + Pronase[c]	15,591	1,246
20% FCS + Test. Hyase[c]	13,295	1,045

[a] Hurler and normal fibroblasts were labeled with 20 μCi of $^{35}SO_4$ for 48 hr and treated with 8 ml of control medium (MEM + 20% FCS) or 8 ml of control medium + 40 μg of the indicated enzyme. Specific activity was determined for cells harvested after labeling (pulse) and after treatment for 24 hr (Hurler cells) or 2 hr (normal cells).

[b] Numbers are specific activities (cpm ^{35}S/mg protein) and represent averages of duplicate determinations of two petri dishes.

[c] Forty micrograms of each enzyme per petri dish (5 μg/ml medium).

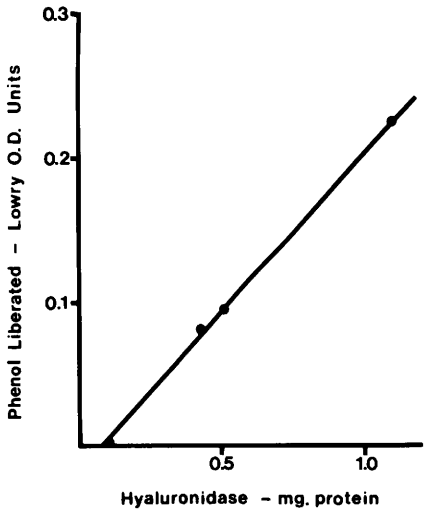

FIG. 3. Phenyl-α-L-iduronidase activity of Wyeth (M-151) hyaluronidase.

caused no recognizable increase in iduronidase content measured at the end of the treatment.

Discussion. The first experiment was performed under nearly identical conditions to a previously reported study on the corrective factor activity of fetal calf serum (4) and the results in Fig. 1 were drawn to the same scale as Fig. 2 in that report. Although the magnitude of the corrective effect of hyaluronidase appears to be two to three times that of fetal calf serum, true comparison of corrective activity can only be made by examination of the maximal corrective effect of each material. This has not been done. The effect of hyaluronidase in Fig. 1 was still evident during the third day, as seen by the greater turnover of the hyaluronidase-treated Hurler cells compared to that of the control Hurler cells. However, the corrective effect of fetal calf serum was not evident beyond 24 hr. Since the specific activity of the hyaluronidase-treated normal cells had no overlapping values with specific activity of the control normal cells, we presume that there was a small corrective effect of hyaluronidase on the normal cells similar to the very small corrective effect of fetal calf serum on normal cells. Nonspecificity of the corrective effect of hyaluronidase, as suggested by this small effect on normal cells, was confirmed by demonstration of a large corrective effect on two different Hunter cell lines, as shown in Table I. The observation that 43 TRU of hyaluronidase in the Worthington preparation produced no greater correction in the Hurler cells or in one of the Hunter cell lines than 5.4 TRU of hyaluronidase in the Wyeth preparation, suggested that 5.4 TRU of hyaluronidase effected maximal correction. Further tests

TABLE III. PHENYL-α-L-IDURONIDASE ACTIVITY IN HYALURONIDASE ENZYME PREPARATIONS.[a]

Preparation	Iduronidase[b] (U/mg pro.)	Hyaluronidase[c] (U/mg pro.)	Iduronidase/hyaluronidase
Wyeth (M-151)	7	335	0.020
Wydase (4734091)	14	286	0.048
Worthington, HSEP (54J589)	2501	2677	0.934
A.B. Leo (S-110)	262	2500	0.105
Apsen (L-102)	156	55	2.830

[a] Iduronidase and hyaluronidase activities were determined for each of several hyaluronidase preparations at two or more different protein concentrations.

[b] One unit of iduronidase activity releases 1 nmole of phenol from phenyl-α-L-iduronide per 18 hr at 22°.

[c] One turbidity reduction unit (TRU) reduces the acid albumin turbidity of 0.2 mg of hyaluronic acid to that of 0.1 mg of hyaluronic acid in 30 min at 30°.

TABLE IV. EFFECT OF HYALURONIDASE TREATMENT ON HURLER CELL ENZYMES.[a]

Treatment	Specific activity[c]	Total enzyme activity units per petri dish[b]			
		α-L-iduronidase	β-Galactosidase	Phosphatase	
				Acid	Alkaline
Pulse	14,536	0	6	7.2	0.4
Chase					
MEM + 20% HIFCS	11,925	0.08	19	9.4	0.6
MEM + 20% HIFCS + 16 μg Hyaluronidase[d]	7,946	0	20	7.2	0.5

[a] Hurler fibroblasts were labelled with 20 μCi of $^{35}SO_4$ from Day 5 to Day 7 and then treated with control medium or control medium plus hyaluronidase for 24 hr. Specific activity was determined for cells harvested after labeling (pulse) and after treatment (chase). Hurler cells, plated from the same suspension as those for the pulse-chase analysis, were treated identically but for the omission of $^{35}SO_4$ and were harvested, homogenized, and analyzed for enzyme activities as described in Methods.

[b] Activity units are: iduronidase – nanomoles phenol released/18 hr; galactosidase and both phosphatases – nanomoles paranitrophenol released/30 min.

[c] Counts per minute of ^{35}S per milligram of protein (average of four petri dishes).

[d] Sixteen micrograms of Wyeth hyaluronidase added per petri dish contained 5.4 TRU hyaluronidase activity and 0.11 units iduronidase activity.

showed that a very large correction could be achieved with as little as 0.48 TRU of testicular hyaluronidase. They further showed that corrective activity at this low-dose level was directly proportional to the hyaluronidase activity when two preparations with different hyaluronidase activities were compared.

Since hyaluronidase is primarily a degradative glycosidase enzyme, we sought evidence for mucopolysaccharide degradation in the Hurler cells by examination of the size of the particles bearing the $^{35}SO_4$ label. Hyaluronidase treatment caused a decrease in the macromolecular and large oligosaccharides and an increase in the small oligosaccharides intracellularly, accompanied by a large increase in the secretion of oligosaccharides into the medium, as seen in Fig. 2. Thus, the corrective effect of hyaluronidase seems due to glycolytic degradation of macromolecular mucopolysaccharides to oligosaccharides and enhanced secretion of oligosaccharides from the cell. However, the marker for recognition of the polysaccharide was the labeled sulfate group, so it was important to consider whether the effect could have been due to sulfatase activity. This was most unlikely for two reasons. First, sulfatases with substrate specificity restricted to acid mucopolysaccharide sulfate radicals is ineffective on large polymers or monosaccharides. They require disaccharides as substrates (9, 10), although a recent report indicates limited activity of one sulfatase on polymeric chondroitin-6-sulfate (11). Second, all specimens were exhaustively dialyzed free of ionic sulfate so the increase in small oligosaccharides of peak III intracellularly and the increase in small oligosaccharides in the medium following hyaluronidase treatment must represent oligosaccharide chains with covalently bound sulfate.

Results of similar chromatographic examination of intracellular $^{35}SO_4$ mucopolysaccharides from Hunter cells by Bach and Neufeld (12) indicated that mucopolysaccharides eluted just after the void volume were largely dermatan sulfate, while those eluted later were largely heparan sulfate. Resolution into three somewhat discrete peaks by our chromatographic procedure using G-200 (Fig. 2A) may be attributed to the use of a slightly longer column. Examination by electrophoresis (13) of pooled eluates from peaks II and III of pulsed cells failed to yield the identity since neither material migrated sufficiently from the origin. However, since dermatan sulfate has hyaluronidase-susceptible internal glycoside bonds and heparan sulfate does not, it seems likely that peaks I and II contain largely dermatan sulfate since only they were decreased following hyaluronidase treatment, and peak III contains largely heparan sulfate, since it was not decreased by hyaluronidase treatment.

That proteolytic activity contaminating the hyaluronidases either could have freed up intracellular mucopolysaccharides from their core protein or created holes in the cell or lysosomal membranes allowing greater loss of mucopolysaccharides is unlikely for several reasons. Treatment of Hurler cells with large doses of papain, trypsin, or pronase caused no loss of metachromatic staining (2) and showed no corrective activity (Table II). Furthermore, thousands of units of hyaluronidase were required to detect contaminating protease (14), whereas less than 1 unit of hyaluronidase has remarkable corrective activity on Hurler cells. Also, if the main corrective effect of hyaluronidase was freeing up intact polysaccharide chains by proteolysis of their core protein allowing their increased secretion, then increased macromolecular polysaccharides should have appeared in the hyaluronidase-treated Hurler cell medium. However, secretion of macromolecular polysaccharide by hyaluronidase-treated Hurler cells was reduced by 50% (Fig. 2B).

Since the specific metabolic block in Hurler cells is known to be a deficiency of α-L-iduronidase, and since all commercial samples of testicular hyaluronidase preparations were contaminated with other enzymes, it was important to examine our hyaluronidase preparations for the presence of α-L-iduronidase activity. Rabbit epididymus was reported to be a rich source of iduronidase activity (15), so it was not surprising to find that all hyaluronidase preparations examined contained iduronidase activity (Table III). To determine if hyaluronidase, free of iduronidase activity, has corrective activity will require purification of hyaluronidase to a single protein free of other enzyme activities. That work is in progress. A preliminary answer was attempted by examining the iduronidase content of Hurler cells before and after treatment with Wyeth hyaluronidase M-151 containing the least iduronidase. Wyeth hyaluronidase supplied 5.4 TR units of hyaluronidase activity and 0.11 units of iduronidase activity to the medium of each effectively corrected dish of Hurler cells. Hurler cells in this experiment (Table IV) had no measurable iduronidase activity before or after treatment. Using nearly the same value for units of iduronidase activity, Bach and Neufeld (16) have shown that the addition of 175 units of unpurified urinary iduronidase to the medium of one petri dish of cells was required to achieve 90% maximal correction of Hurler cells with the subsequent recovery of 65 units of iduronidase from the cells. Although these authors point out that the efficiency of iduronidase uptake by Hurler cells and subsequent metabolic correction improves with the use of smaller amounts of iduronidase, it is unlikely that the addition of as little at 0.11 units of iduronidase could have accounted for near maximal correction in this experiment. Furthermore, it should be pointed out that normal human urine contains hyaluronidase with an acid pH optimum (17) similar to that reported for lysosomal hyaluronidase (18–20). We have found hyaluronidase, active only at pH 3.5, in unpurified 70% saturated ammonium sulfate precipitates of normal human urine prepared according to Bach and Neufeld (21). Despite the large corrective effect earlier shown by 0.48 TRU of hyaluronidase, it is not possible to conclude that hyaluronidase is a more potent corrective factor than iduronidase.

Summary. Bovine testicular hyaluronidase (endo-β-*N*-acetyl hexosaminidase) has a significant corrective effect on cultured Hurler fibroblasts. Nonspecificity of this effect is indicated by its equally strong corrective effect on Hunter fibroblasts. Although all specimens of hyaluronidase also possessed iduronidase activity, a separate corrective effect could be attributed to the endo-*N*-acetyl hexosaminidase activity of at least one hyaluronidase (Wyeth M-151) for four reasons: (i) its very low content of iduronidase activity; (ii) a decrease in intracellular macromolecular mucopolysaccharides (believed to be largely dermatan sulfate) with a corresponding increase in intracellular and extracellular oligosaccharides; (iii) no measurable increase in iduronidase activity of hyaluronidase-treated cells despite near maximal correction; (iv) direct correlation between Hurler cell correction and hyaluronidase activity when enzymes of different strength were used at less than maximal correction.

This work was supported by Grant AM 17020 of the NIAMD and in part by Grant 6-74-63 from The Na-

tional Foundation-March of Dimes and in part by institutional GRS Grant No. 5S01 RR05390-14. We thank Betty Chao, Lyle Motycka, and Claudia Hastings for technical assistance.

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Received September 2, 1975. P.S.E.B.M., 1976, Vol. 151.