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The Hurler Syndrome: Treatment of Cultured Hurler Fibroblasts with Normal Human Serum[1] (39001)

J. KENNETH HERD,[2] BARBARA HAYHOME, AND JANICE TSCHIDA
(Introduced by Hugh J. Phillips)

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

Cultured skin fibroblasts from patients with the Hurler Syndrome accumulate increased quantities of acid mucopolysaccharides, mostly dermatan sulfate, due to a failure of the degradative process in their lysosomes (1–3), more specifically, a deficiency of the acid hydrolase, α-L-iduronidase (4, 5). This enzyme is secreted by normal fibroblasts into their culture medium, is found in normal human urine (6, 7) and its activity can be measured with a synthetic substrate (8).

It had been reported that enrichment of the synthetic culture medium with normal human serum in place of the usual fetal calf serum resulted in decreased intracellular mucopolysaccharides in Hurler cells and increased mucopolysaccharides in the culture medium (9). That report prompted our initial studies (10) which indicated the presence of a corrective factor activity in normal human serum when assayed by a 25–48 hr chase assay. The experiments reported here were designed to reexamine the corrective effect of normal human serum on the acid mucopolysaccharide content of Hurler cells following prolonged treatment and to investigate its mode of action during chase assays.

Materials and methods. Materials and methods of cell culture and isolation of mucopolysaccharides have been described (11). Eagles minimum essential medium in Earle's salts (MEM) was obtained from the Grand Island Biological Company. Purified testicular hyaluronidase, Lot M151, 335 turbidity reduction units per mg., was supplied by Dr. George Warren, the Wyeth Laboratories, Marietta, Pa. Enzyme grade ammonium sulfate was purchased from the Schwarz/Mann Company and bead polymerized G-75 Sephadex, superfine, from the Pharmacia Company. Chondroitin-4,6-sulfate (CS) was prepared from bovine nasal septa (12). Dermatan sulfate, Fraction III, was prepared from pigskin (13).

Blood was drawn from five healthy adult male donors following an overnight fast and clotted 2 hr at room temperature. Serum was removed aseptically and added to MEM to treat Hurler fibroblasts on the day the blood was drawn.

Passage 10 Hurler fibroblasts from a common cell suspension were plated out in 100 petri dishes in MEM enriched with 20% fetal calf serum (FCS). After 3 days, medium was aspirated and the dishes divided into experimental and control groups of 50 dishes each. Experimental dishes received 10 ml of MEM enriched with 20% fresh normal human serum (NHS) on days 0, 4 and 7. Control dishes were treated identically with MEM enriched with 20% heat-inactivated fetal calf serum (HIFCS). On day 11 treatment medium was removed and the cells rinsed with 2.0 ml. of 0.15 *M* NaCl which was added to the medium.

Cells were harvested by scraping, and homogenized in a Teflon-glass tissue homogenizer in ice with two strokes of a Tri-R motor set at 4. Aliquots were taken for determination of DNA (0.3 ml) and protein (0.1 ml) (11). Homogenates were brought to pH 6.2 in 0.1 *M* acetate-EDTA buffer and twice digested with papain (1 mg/g protein) at 63° for 24 hr. Acid mucopolysaccharides (AMPS) were isolated by precipitation with

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cetyl pyridinium chloride (CPC) (1) and analyzed for uronic acid with carbazole (14) and orcinol (15), hexosamine (16), and protein (17). Specimens were electrophoresed before and after treatment with testicular hyaluronidase (18). AMPS were isolated from pooled media collected at each feeding by previously described methods (1) following concentration by flash evaporation and dialysis 24 hr in tap H_2O and 24 hr in distilled H_2O.

In a repeat experiment, nine dishes of passage 13 Hurler fibroblasts were continuously labeled with 20 μC $^{35}SO_4$ per dish and divided into three equal groups treated with medium enriched as follows: Group 1, 20% NHS; Group 2, 20% FCS; and Group 3, 20% HIFCS. NHS contained 7.30 g. protein per 100 ml and was diluted in sterile 0.15 *M* saline to equal the protein concentration of FCS, 4.20 g/100 ml before addition to MEM. All media contained 2 μcuries of sterile-filtered $^{35}SO_4$ per ml. Cells were harvested and mucopolysaccharides isolated as before except for the addition of 2 mg of non-radioactive CS to each homogenate just prior to precipitation. Radioactivity was measured by liquid scintillation as described in Method 1 (11). Identification of $^{35}SO_4$-AMPS by scanning the electrophoretograms for radioactivity before and after treatment with testicular hyaluronidase has been described (19).

Corrective factor preparations of serum proteins were precipitated at 0° by the slow addition of 9.3 ml of saturated aqueous ammonium sulfate to 4.0 ml of fresh serum with constant stirring at pH 7.5–7.9. After standing 1 hr in ice, the precipitated proteins were collected by centrifugation at 27,000 *g*, redissolved in 1.5 ml of water, dialyzed overnight in five separate 1-liter vol of 0.15 *M* NaCl at 4°, and adjusted to 6.0 ml with 0.15 *M* NaCl. Reconstituted precipitates were sterile-filtered and protein content determined (17). Corrective factor activity of reconstituted ammonium sulfate precipitates was determined by addition of measured aliquots of equal protein content to 8.0 ml of chase medium in the corrective factor assay as described (11, Method 2). Duplicate determinations made on cells from 2 petri dishes were averaged in all corrective factor assays.

Chase media from selected experiments was examined for the size of $^{35}SO_4$-labeled fragments as follows: 20 ml was concentrated to 5 ml by flash evaporation, made 0.05 *M* with respect to acetic acid, applied to a 76 × 1.2 cm column of G-75 Sephadex, superfine, previously equilibrated with 0.05 *M* acetic acid (pH 3.2), and eluted at 22°, 10 ml/hr, with 0.05 *M* acetic acid to prevent protein binding by the matrix. Two-milliliter fractions were collected, o.d. at $\lambda = 280$ nm recorded, and radioactivity determined (11). Macromolecules in the eluate corresponding to Blue dextran 2000, were pooled, dialyzed 5 days in three 4-liter changes of distilled H_2O at 4°, and concentrated to 4.0 ml; aliquots were taken for determination of protein (17) and radioactivity. All fractions after the macromolecules were similarly pooled, dialyzed, and concentrated, and radioactivity was determined.

Results. The results of the first experiment are shown in Table I. Hurler fibroblasts treated with medium enriched with fresh normal human serum contained more macromolecular AMPS than control Hurler fibroblasts treated with heat-inactivated fetal calf serum. The AMPS from both experimental and control cells gave only one spot on electrophoresis which had the same mobility as reference DS and was resistant to digestion with testicular hyaluronidase. The AMPS from both sets of cells had a carbazole: orcinol ratio approaching that of DS. When the data were expressed for quantity of cell protein, human serum-treated cells still contained two to three times more macromolecular AMPS than control cells. The medium also contained more mucopolysaccharides when the cells were grown in the presence of normal human serum. The predominant mucopolysaccharide was hyaluronic acid. Although the total micrograms of dermatan sulfate in the medium was the same with either treatment, dermatan sulfate represented a smaller percent of the total mucopolysaccharide in the medium during treatment with normal human serum than during treatment with fetal calf serum.

These results were supported by the $^{35}SO_4$

TABLE I. EFFECT OF NORMAL HUMAN SERUM ON THE MACROMOLECULAR AMPS OF HURLER FIBROBLASTS.[a]

Material	Analysis	Treatment	
		NHS[b]	HIFCS[c]
Cells			
Cell homogenate	DNA (mg)	1.719	0.874
	Protein (mg)	357	233
Total AMPS	Uronic Acid		
	carbazole (μg)	499	119
	orcinol (μg)	1178	336
	carbazole/orcinol	0.42	0.35
	Hexosamine (μg)	601	113
Adjusted AMPS	AMPS/Protein		
	carbazole (μg/g)	1398	511
	hexosamine (μg/g)	1683	571
	AMPS/DNA		
	carbazole (μg/mg)	290	136
	hexosamine (μg/mg)	350	152
Medium			
Total AMPS	Carbazole (μg)[d]	6080	3100
	Orcinol (μg)[d]	4080	1840
	Hyaluronic Acid[e]	75%	81%
	Dermatan sulfate[f]	3%	8%

[a] Hurler cells were exposed to medium enriched with either 20% heat-inactivated fetal calf serum (HIFCS) or 20% normal human serum (NHS), 50 dishes each, from the fourth to the fifteenth day after plating. Used medium was replaced with fresh medium every 3–4 days. AMPS were extracted from cells and pooled medium after the fifteenth day and analyzed as described under Methods.

[b] NHS—normal human serum (9.8 g protein/100 ml).

[c] HIFCS—fetal calf serum, heated 30 minutes at 56°, (4.3 g protein/100 ml).

[d] Totals eluted from Dowex, 1X 2 Cl^-, at all molarities NaCl.

[e] Eluted from Dowex with 0.5 *M* NaCl; contained only glucosamine; electrophoresed with mobility of reference HA.

[f] Eluted from Dowex with 1.3 *M* NaCl; electrophoresed with mobility of reference DS.

TABLE II. CONTINUOUS $^{35}SO_4$-LABELING OF MACROMOLECULAR MUCOPOLYSACCHARIDES BY NORMAL HUMAN SERUM-TREATED CULTURED HURLER FIBROBLASTS.

Sample	Analysis	NHS[b]	FCS[c]	HIFCS[d]
Cell homogenate	Protein (mg)	10.0	5.0	4.6
	DNA (mg)	0.065	0.057	0.046
Total AMPS (cells and carrier)	Uronic acid (μg)	350	250	320
	Radioactivity (cpm)[e]	394,478	99,000	86,258
	Specific activity[f]	112,708	79,000	58,599

[a] Hurler cells were treated as in Table I with the following changes: three dishes of cells were used for each treatment group; the protein concentration of the sera were made equal before enrichment of the medium; each dish received 20 μCi $^{35}SO_4$ with each medium change; AMPS were extracted from cells in the presence of nonradioactive carrier CS.

[b] NHS—normal human serum (8.1 g protein/100 ml) was diluted with 0.15 *M* NaCl to 4.4 g protein/100 ml.

[c] FCS—fetal calf serum, 4.4 g protein/100 ml.

[d] HIFCS—fetal calf serum—heated 2 hr at 56° (4.6 g protein/100 ml).

[e] Radioactivity is total counts per minute (cpm) less background cpm.

[f] Specific activity = (cpm/mg uronic acid)/(mg protein).

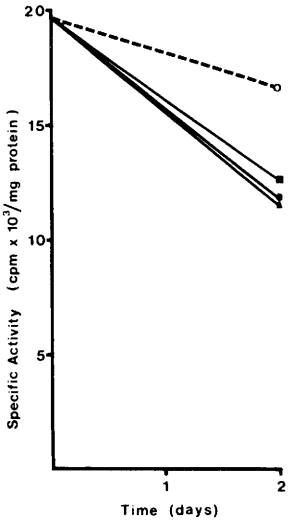

FIG. 1. Loss of $^{35}SO_4$-AMPS from Hurler cells during treatment with whole serum. $^{35}SO_4$-labeled Hurler cells were chased with medium enriched to 20% with heat-inactivated fetal calf serum (- - ○), fetal calf serum ■, normal human serum ▲, or Hurler serum ● (from patient whose cells were being tested). The two human sera (78 and 72 mg protein/ml) were diluted to the protein concentration of fetal calf serum (30 mg/ml) before addition to chase medium.

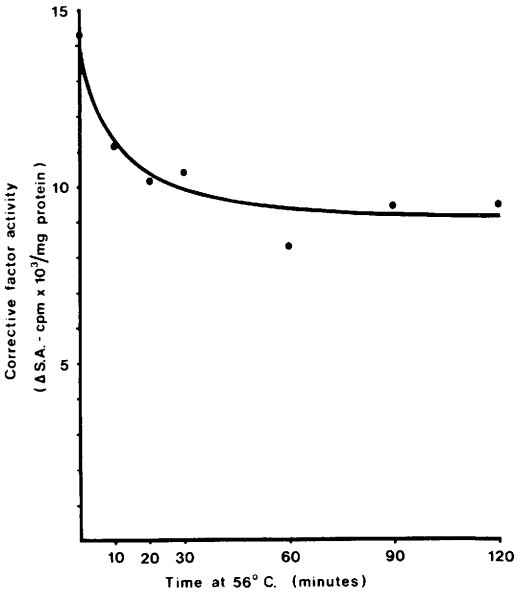

FIG. 2. Heat inactivation of corrective activity of normal human serum. Fresh pooled normal human serum (NHS), heated at 56° for times indicated on the abscissa, replaced fetal calf serum (FCS) during 24-hr chase of $^{35}SO_4$-labeled Hurler cells. The decrease in specific activity of the labeled Hurler cells is indicated in positive numbers on the ordinate.

accumulation data shown in Table II. Accumulation of radioactive macromolecular AMPS by Hurler cells treated with NHS was considerably greater than accumulation by Hurler cells treated with either FCS or HiFCS. Recovery of AMPS was comparable in all three groups. Radioactivity of AMPS from all cells had the same electrophoretic mobility as reference DS and was resistant to digestion with testicular hyaluronidase, while carrier CS was no longer detected after hyaluronidase treatment. The growth-stimulating effect of NHS noted in the first experiment was still apparent, although both NHS and FCS were used at the same protein concentration. Heat-inactivation of FCS caused some loss of growth-supporting properties, but previous experiments had shown that HIFCS supported sufficient $^{35}SO_4$ labeling of AMPS by Hurler fibroblasts for corrective factor assay (11).

Fresh NHS was compared to fresh Hurler serum (HS) and FCS (not heat-inactivated) for corrective factor activity as measured by the chase of $^{35}SO_4$-AMPS from Hurler cells (11). The results are shown in Fig. 1. Uncorrected Hurler cells spontaneously lose little specific activity in 48 hr. Corrective factor activity is here defined as the difference between the specific activity of the uncorrected Hurler cells and the specific activity of the Hurler cells treated with the three different sera. There was no difference between the corrective factor activity in all three sera, and the effect is therefore not genotype specific.

Heating normal human serum for use in the corrective factor assay gave the results shown in Fig. 2. Inactivation of corrective factor activity of normal human serum was maximal at 2 hr as had been shown for fetal calf serum (11). However, normal human serum which had been maximally heat-inactivated for 2 hr, still retained considerable corrective activity.

Aliquots of reconstituted ammonium sulfate precipitates of NHS were tested for corrective factor activity on Hurler and normal cells, and were compared to reconstituted precipitates of FCS on Hurler cells. The results are shown in Fig. 3. Whereas 60 mg.

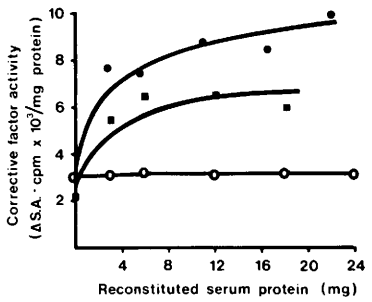

FIG. 3. Corrective activity of reconstituted ammonium sulfate precipitates of serum. Ammonium sulfate precipitates were prepared from NHS (■ and ○) and FCS (●) and tested on Hurler cells (solid symbols) and Normal cells (open circles) by 24-hr corrective factor assay as described in the text.

protein as whole FCS in the standard chase medium accounted for the loss of 2500 cpm specific activity from Hurler cells, the addition of 6 mg protein as reconstituted ammonium sulfate precipitate of NHS accounted for the loss of an additional 2850 cpm specific activity. Each dish contained equal cell protein after treatment excluding a false positive effect. In this experiment, correction by precipitated proteins from FCS was greater than that by precipitated proteins from NHS. Reconstituted ammonium sulfate precipitates of NHS had a barely perceptible corrective effect on normal cells.

The relative size of the $^{35}SO_4$-labelled particles secreted into the medium by Hurler cells during various treatments was determined by chromatography on G-75 Sephadex. The results when Hurler cells were treated with conditioned medium are shown in Fig. 4A. There was essentially no change in the quantity of macromolecular $^{35}SO_4$, but a moderate decrease in the oligosaccharide $^{35}SO_4$-labeled fragments secreted by Hurler cells treated with medium conditioned by normal cells when compared to the result when Hurler cells were tested with medium conditioned by other Hurler cells. There was a small increase in the secretion of $^{35}SO_4$-labeled fragments corresponding to ionic sulfate. The ratio of protein to polysaccharide (o.d.:cpm) was relatively constant in all fractions.

The results when Hurler cells were treated with medium enriched with normal human serum or fetal calf serum are seen in Fig. 4B. There was a threefold increase in the macromolecular $^{35}SO_4$, a 20 to 100-fold increase in the largest oligosaccharide fragments, and a similar though smaller increase in the smaller oligosaccharides secreted by Hurler cells treated with NHS compared to Hurler cells treated with FCS. Treatment with NHS doubled the secretion of $^{35}SO_4$-labeled fragments corresponding to ionic sulfate. Fifty percent of the pooled radioactivity excluded by Sephadex, but less than 0.1% of the pooled radioactivity retarded by Sephadex, were retained after extensive dialysis. The ratio of protein to polysaccharide (o.d.:cpm) in the macromolecular components excluded by Sephadex was the same when Hurler cells were treated with medium enriched with either serum, but the ratio of protein to polysaccharide in the largest of the intermediate-sized fragments slightly retarded by Sephadex was greatly decreased when Hurler cells were treated with medium enriched with normal human serum.

Discussion. The results of the first two experiments show that cultured Hurler cells accumulate two to three times more acid mucopolysaccharide when the culture medium is enriched with fresh whole normal human serum than when the medium is enriched with the usual fetal calf serum. This difference is not the result of the higher protein content of normal human serum because it persisted when the two sera were used at equal protein concentrations. The increased mucopolysaccharide had the characteristics of dermatan sulfate, the predominant mucopolysaccharide stored by cultured Hurler cells (1).

This result is at variance with the original report of Hors-Cayla *et al.* (9). Explanation of this difference from their results is difficult due to the lack of experimental detail in their report. Most notable is the brief exposure of their cells to human serum, 4 days in their longest experiment compared to 11 days in our study. It was surprising in view of our earlier finding that normal human serum showed corrective activity capable of increasing the loss of isotopically labeled mucopolysaccharides from Hurler cells (10). The cell kinetics of mucopolysaccharide metabolism that could account for this finding are: increased synthesis of DS, increased pinocytosis of preformed DS from the

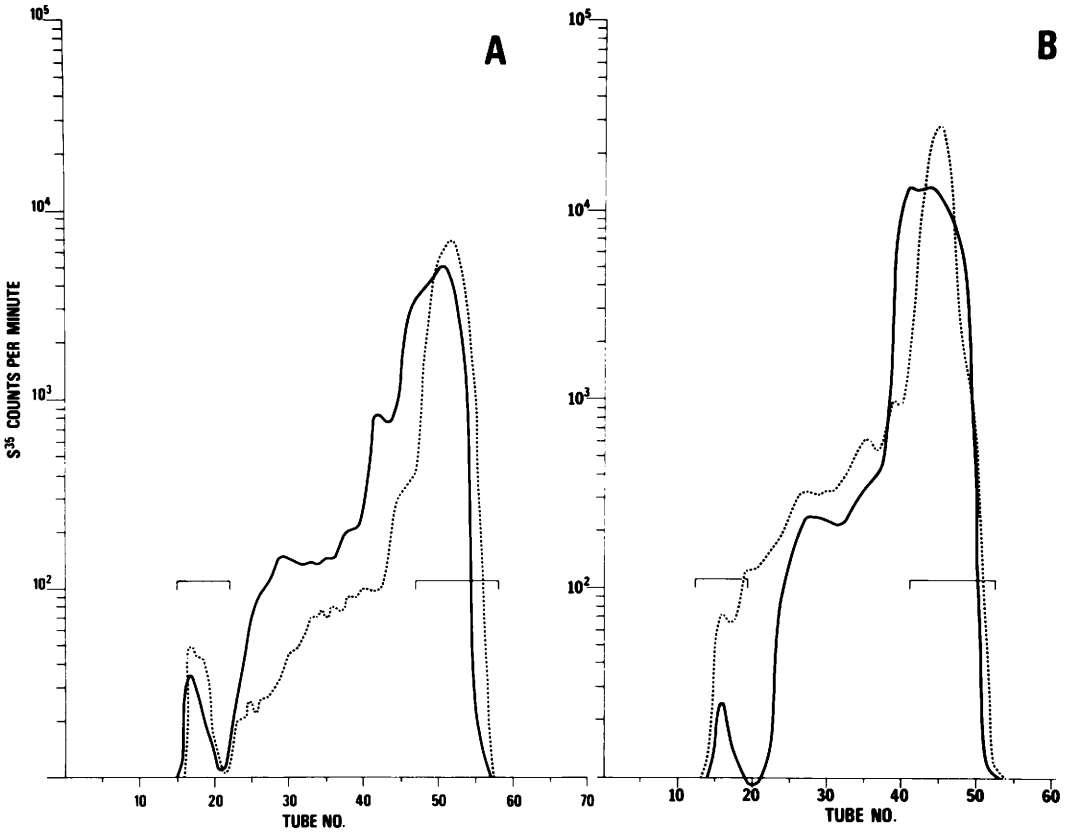

FIG. 4. Secretion of $^{35}SO_4$-AMPS by Hurler cells. Medium from $^{35}SO_4$-labeled Hurler cells during 48 hour chase treatment was chromatographed on G-75 Sephadex as described in the text. A) Hurler cells treated with medium conditioned by Hurler cells (——) and by normal cells (-----). B) Hurler cells treated with unused medium enriched 20% with FCS (——) or NHS (-----). Elution volumes for blue dextran 2000 and $SO_4^=$ are indicated by inverted brackets.

medium, decreased degradation of DS, decreased secretion of DS, or any combination of part or all of the above. We have examined some of these mechanisms.

Our earlier work indicated that normal human serum briefly augmented the secretion–degradation mechanism (11) as detected by corrective factor assay of $^{35}SO_4$-labeled Hurler cells. Corrective factor activity for Hurler cells as used here is defined as any activity that augments the chase of macromolecular $^{35}SO_4$ from Hurler cells, as opposed to the concept of genotype specific Hurler corrective factor (7, 8), α-L-iduronidase, which augments the chase of $^{35}SO_4$-AMPS only from Hurler and Scheie cells (20). We have shown that whole human serum and whole fetal calf serum possess equal corrective activity for Hurler cells (Fig. 1). This corrective activity augmented the loss of $^{35}SO_4$-AMPS from Hurler cells and was heat labile (Fig. 2). The retention of some corrective activity by NHS under conditions that completely inactivated corrective activity of FCS may be due to partial denaturation of the activity of FCS during prolonged frozen storage. We have not tested human serum after prolonged frozen storage. The recognition of equal corrective factor activity in whole NHS, FCS and Hurler serum indicates a nonspecific effect for whole serum when assayed in this system.

Corrective factor activity of NHS could be recovered from ammonium sulfate precipitates as had been done in the preliminary isolation of genotype-specific corrective factors from conditioned media (6). Corrective factor activity for Hurler cells was

similarly recovered from FCS and from Hurler serum (data not shown) by ammonium sulfate precipitation. Saturation of the corrective effect of these reconstituted precipitates as shown in the dose-response curves for Hurler cells (Fig. 3) implied a specific cell mechanism was involved but provided no evidence of its nature. The lack of a significant corrective effect of these precipitates for normal cells is inadequate evidence to confirm Hurler genotype specificity. They must be similarly tested for corrective effect on other types of mucopolysaccharide-accumulating fibroblasts. Such studies have not been done.

We next examined the processes of secretion and degradation in Hurler cells as influenced by treatment with whole normal human serum. This was accomplished by comparison of the size of the $^{35}SO_4$-labeled particles released into the medium by Hurler cells during corrective factor assays by means of G-75 Sephadex chromatography. The molecular weight of intracellular DS in cultured Hurler cells after papain digestion is 40,000 (21). Without papain digestion the mucopolysaccharides retained in the intracellular pool are moderately larger (3).

Comparing the result when Hurler cells were chased with medium conditioned by normal cells (known to contain α-L-iduronidase) to the result when Hurler cells were chased with medium conditioned by other Hurler cells (deficient in α-L-iduronidase), the effect was a decrease in the secretion of oligosaccharides (Fig. 4A). This effect is not the result of increased retention by the cells as shown by the decreased specific activity of the cells in the chase assay. It therefore represents increased degradation occurring in the cell. This is further supported by the elevated inorganic sulfate peak. When the corrective activity of fresh normal human serum was compared to that of fetal calf serum by this method (Fig. 4B), the effect on polysaccharide secretion was reversed. Secretion of macromolecular and large oligosaccharide fragments were both increased by normal human serum. Secretion of inorganic sulfate was again increased. Thus, the corrective effect of normal human serum treatment of Hurler cells appears to be due to a markedly increased secretion of large oligosaccharides, and a moderately increased secretion of macromolecular polysaccharides and inorganic sulfate. A similar effect has been reported from one study *in vivo* which showed increased urinary excretion of medium-sized oligosaccharides of DS by a Hunter patient treated with plasma infusion therapy (22).

In the presence of nonspecific corrective factor activity in normal human serum, we must then account for the increased dermatan sulfate accumulated in our first experiments when Hurler cells were treated with normal human serum. The corrective factor effect of whole serum appears to be of brief duration, only 24 hr in the studies with fetal calf serum (11). It may be operative only during the initial mitotic burst that accompanies the first addition of serum (23). This may account for the effect on intracellular mucopolysaccharides in the shorter, single treatment experiments of Hors-Cayla (9). The prolonged exposure to NHS in our first studies here allows other mechanisms to overwhelm this initial corrective effect. Such mechanisms could be increased pinocytosis of dermatan sulfate from the medium or increased synthesis under more favorable nutritional conditions.

Seventy-five percent of the mucopolysaccharide available for pinocytosis from the medium was hyaluronic acid. If pinocytosis was increased, one would expect the cells should also have contained increased hyaluronic acid, but this was not found. It is possible that increased hyaluronic acid was pinocytosed, but was degraded and metabolized by normally operative metabolic mechanisms. It is also possible that increased dermatan sulfate was pinocytosed selectively from the medium despite the large excess of hyaluronic acid. Although it seems unlikely that a cell would selectively pinocytose a minor polysaccharide which it cannot degrade, it is possible. The fact that the total dermatan sulfate in either treatment media is less than one-half the total dermatan sulfate content of the control cells and only one-tenth of that in normal human serum treated cells speaks against this possibility, but does not exclude it. We know of no evidence supporting selective pinocytosis of mucopolysaccharide by Hurler fibroblasts.

The possibility of increased synthesis of dermatan sulfate in human serum treated Hurler cells remains to be considered.

Examination of overall true rates of polysaccharide synthesis in intact cells has not been accomplished due to the simultaneous occurrence of degradation and secretion, and due to the large number of individual enzymatic steps involved in the synthetic process (24). However, the increased protein and DNA in our human serum-treated Hurler cells suggests increased synthesis of those materials and agrees with the reported favorable effects of fresh serum on cultured fibroblasts (23, 25, 26). A single protein capable of stimulating DNA synthesis in cultured cells has been isolated from human serum (27). It is possible that synthesis of dermatan sulfate was similarly increased throughout prolonged treatment of Hurler cells with fresh normal human serum.

Conclusions. Prolonged replacement of fetal calf serum by normal human serum for the enrichment of medium during tissue culture of Hurler fibroblasts resulted in increased acid mucopolysaccharides in the cells and in the medium. The predominant intracellular mucopolysaccharide had the characteristics of dermatan sulfate when Hurler cells were treated with either serum.

Normal human serum contains a nonspecific corrective factor capable of augmenting the loss of $^{35}SO_4$-AMPS from Hurler cells, but not from normal cells. Fetal calf serum and Hurler serum have similar corrective factor activity for labeled Hurler cells. The corrective factor activity of all three sera was recovered from reconstituted dialyzed ammonium sulfate precipitates. The corrective factor of normal human serum did not increase degradation of mucopolysaccharide, but increased secretion of macromolecular and large oligosaccharide components.

Failure of the corrective factor of normal human serum to effectively decrease the dermatan sulfate content of Hurler cells during prolonged exposure may be a quantitative phenomenon due partly to the brief duration of corrective factor activity and partly to increased synthesis of mucopolysaccharide.

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