The Tissue Culture Detection of Corrective Factor Activity for Hurler Fibroblasts (37340)

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Accumulation of abnormally large amounts of acid mucopolysaccharides which occurs in cultured skin fibroblasts from patients with the Hurler syndrome results from failure of intracellular degradation (1–3). Disappearance of isotopically labeled mucopolysaccharides from Hurler fibroblasts is slower than from normal fibroblasts. A deficiency of iduronidase that could account for these findings has been demonstrated in Hurler fibroblasts (4). A protein isolated from normal human urine has been found to have corrective activity for cultured Hurler cells and has also been shown to have α-L-iduronidase activity (5, 6).

Numerous studies have used the detection of decreased radioactivity of specifically labeled intracellular mucopolysaccharides to describe corrective factors for cultured Hurler fibroblasts (2, 3, 7–10). Although not as definitive as classical biochemical isolation of mucopolysaccharides (1), the isotope method is simpler, quicker and less expensive. Testing of therapeutic agents for efficacy in treating the Hurler syndrome relies on methods for the detection of correction in the cultured Hurler fibroblasts.

The experiments reported here were undertaken to confirm the findings of the isotope method using alternate techniques of mucopolysaccharide isolation, to establish a standard method of expressing the data as quantity of substrate degraded, and to establish the baseline decay curve for uncorrected Hurler and normal fibroblasts.

Materials and Methods. F-10 nutrient medium and Type II fetal calf serum (frozen) were purchased from the Grand Island Biological Co. and $H_2{}^{35}SO_4$, carrier free, (42 Ci/g) from the New England Nuclear Co. Chondroitin-4,6-sulfate was obtained from Dr. J. A. Cifonelli, the Pritzker School of Medicine. The scintillation mixture, Permablend (Packard Co.), was used for detection of radioactivity, 5.5 g/liter of toluene.

Biopsies of skin from the deltoid area were minced in Ham's F-10 medium enriched with 20% fetal calf serum, incubated in Falcon plastic tissue culture dishes at 37° in a moist atmosphere of 5% CO_2 in air. After 3–5 wk, monolayer cells were subcultured in 250 ml plastic bottles after exposure to 0.25% trypsin for 5 min. Cells subcultured on glass cover slips were stained with 0.5% aqueous toluidine blue on the fifth day after plating (11). Cells for isotope labeling experiments were subcultured in 100 mm culture dishes. Two methods were used for labeling and isolation of radioactive mucopolysaccharides from Hurler cells following treatment.

1. The first method for the isolation of acid mucopolysaccharides of relative purity has been described (12). Briefly, Hurler test cells plated in culture dishes were given 10 ml of fresh medium every 3–4 days for 10 days, then labeled with 20 μCi $^{35}SO_4$ in 10 ml of fresh medium/dish, for 48 hr. The radioactive medium was removed, the cells were rinsed with 2.5 ml of sterile medium and then given 10 ml of the medium to be tested for corrective activity. After 1–3 days, the test medium was removed, the cells were rinsed with 0.15 *M* NaCl, harvested with trypsin and the amount of sulfate-labeled acid mucopolysaccharide ($^{35}SO_4$-AMPS) was determined. Cells suspended in 0.25% trypsin

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were counted in a hemocytometer, 2.0-ml aliquots were taken for the determination of DNA (13, 14) and the remainder was used for the isolation of $^{35}SO_4$-AMPS following papain digestion and precipitation with cetyl pyridinium chloride (CPC) as described (1). Aliquots of the aqueous solution of acid mucopolysaccharides were taken for the determination of uronic acid with carbazole (15) and radioactivity by liquid scintillation in water–ethanol–toluene (0.6:6.0:8.4). Radioactivity was expressed as specific activity (sp act) which was calculated as total counts per minute over background (cpm) per total milligrams of uronic acid (UA) per million cells which may be expressed algebraically as:

$$\frac{\text{cpm/mg UA}}{\text{no. of cells}/10^6} = \frac{\text{cpm} \times 10^6}{\text{mg UA} \times \text{no. of cells}}$$

Specific activity calculated per milligram of DNA in place of cell numbers gave identical results.

2. In the second method (2, 3), Hurler fibroblasts were grown for 5 days in F-10 medium enriched with 20% fetal calf serum in plastic petri dishes. On the fifth day the cells were labeled with 10 ml fresh medium containing 20 μCi $^{35}SO_4$ for 48 hr. The radioactive medium was removed, the cells were rinsed with 2.5 ml of sterile medium and then given 8 ml of fresh medium to be tested for corrective activity. Following removal of the test medium, the cells were rinsed with 0.15 *M* NaCl, harvested by treating exactly 10 min with 0.25% trypsin, and promptly refrigerated at 4°. Suspended trypsinized cells were transferred to centrifuge tubes in ice and centrifuged at 1000 rpm for 3 min. The supernatant fluid was discarded, the walls of the tube were rinsed with NaCl and centrifuged as before. The saline was discarded, the cells were suspended in 2.0 ml of 80% ethanol and heated to bubbling in a near-boiling water bath for 2 min. The precipitate was collected by centrifugation at 3000 rpm for 3 min. The extraction in hot ethanol was repeated twice, the precipitate was dissolved in 2.0 ml 10% NaOH in a near-boiling water bath for 1–2 min, cooled to room temperature and duplicate aliquots were taken for the measurement of radioactivity (0.6 ml) and protein (0.2 ml). Radioactivity was determined by liquid scintillation as in the first method. Protein was measured by the Lowry *et al.* method (16) after neutralization by the addition of 0.3 ml of 2 *N* acetic acid. Specific activity in this method was calculated as total counts per minute over background (cpm) per milligram of protein or:

$$\frac{\text{cpm}}{\text{mg protein}}.$$

Fetal calf serum was heat-inactivated in a 56° water bath prior to its addition to the F-10 nutrient medium. No precipitates formed although some darkening was usually observed.

Results. The first experiments confirmed the report that cultured normal fibroblasts release into the culture medium factors which have a corrective effect on the slow loss of $^{35}SO_4$-AMPS from cultured Hurler fibroblasts. The control experiment measured the corrective effect of culture medium exposed to other unlabeled Hurler fibroblasts. Several dishes of 10-day-old Hurler fibroblasts were $^{35}SO_4$ labeled for 48 hr. The cells of 2 dishes were rinsed, the intracellular mucopolysaccharides were isolated by classical methods and the sp act was determined (method 1). The cells on the remaining dishes were then rinsed and exposed to nonradioactive medium which had previously been in contact with either Hurler or normal cells of the same age in culture. After 1 and 3 days their intracellular mucopolysaccharides were similarly isolated and the sp act was determined. The difference between the sp act of the mucopolysaccharides isolated from the labeled Hurler fibroblasts before and after treatment, *i.e.*, the decrease in sp act, represents loss of macromolecular $^{35}SO_4$-AMPS from Hurler cells either by degradation or excretion or both. It is a direct measure of the corrective activity of the medium used in the treatment. The data are shown in Table I. More $^{35}SO_4$-AMPS were lost from Hurler cells during treatment with medium previously exposed to normal cells (than during treatment with medium previously exposed to Hurler cells) as reported (7).

An experiment was designed to compare the two methods of acid mucopolysaccharide

TABLE I. The Decrease in Macromolecular $^{35}SO_4$-Acid Mucopolysaccharides of Hurler Cells During Treatment.[a]

Treatment with medium used by	Test no.	Decrease in sp act[b] 24 hr	72 hr
Hurler cells	1	2434	5064
	2	2740	3730
	Mean	3087	4397
Normal cells	1	5888	7676
	2	4380	6255
	Mean	5134	6965

[a] Hurler test fibroblasts were labeled with 20 μCi $^{35}SO_4$ for 48 hr, then exposed to nonradioactive medium previously used by either Hurler or normal cells. After 1- and 3-day intervals the intracellular mucopolysaccharides were isolated by classical methods and their specific activity (sp act) was determined by method 1. The numbers represent the difference between the sp act of the mucopolysaccharides of the test fibroblasts before and after treatment, and are a direct measure of the corrective activity of the medium.

[b] Sp act = (cpm $\times$ 10^3)/(mg UA $\times$ no. of cells).

isolation and analysis. Four dishes of 10-day-old Hurler fibroblasts were labeled as before. Two dishes were rinsed and the cells were harvested with trypsin as described. The remaining two dishes were rinsed and treated with unused fresh medium for 24 hr after which the cells were again rinsed and harvested with trypsin as before. At each harvest, the trypsinized cells were evenly suspended, a drop was taken for cell count and equal aliquots were removed for the simultaneous isolation of the mucopolysaccharides and determination of their sp act by each method. The data are shown in Table II. The greater sp act in method 2 is an artifact due to the method of calculation; the raw data showed 30% more counts per minute isolated by method 1. By either method of isolation, the same percentage of the original labeled mucopolysaccharide was recovered from the cells after 24 hr of treatment. Since method 2 was quicker, required less handling, and was equally reliable for corrective factor assay, it was used in subsequent tests.

For the accurate assay of corrective factor for Hurler fibroblasts, it was necessary to establish the baseline decay curve for uncorrected $^{35}SO_4$-labeled Hurler cells. This was accomplished by heat-inactivating the corrective factor presumed present in the fetal calf serum.

An experiment was designed to determine the time required for inactivation of all corrective activity when fetal calf serum was heated to 56°. Hurler fibroblasts were labeled with $^{35}SO_4$ for 48 hr. The cells of 2 dishes were rinsed, harvested and the sp act of the mucopolysaccharides was determined. The cells in the remaining dishes were rinsed and 2 were treated with medium enriched with 20% fetal calf serum for 48 hr. These cells were rinsed, harvested, and the sp act of the mucopolysaccharides was determined. The change (decrease) in sp act represented the maximal corrective activity of this batch of fetal calf serum plus the inherent ability of the Hurler fibroblasts to lose $^{35}SO_4$-AMPS spontaneously by whatever means. The re-

TABLE II. Specific Activity of $^{35}SO_4$-Acid Mucopolysaccharides in Hurler Fibroblasts Measured by Two Methods.[a]

After labeling (hr)	Method 1[b]	Method 2[c]
48	9120	13,370
	7720	12,580
	8420	12,970
24	5025	9930
	7460	8000
	6242 (74%)	8065 (70%)

[a] Four dishes of 10-day-old Hurler fibroblasts were labeled with $^{35}SO_4$ for 48 hr as described. The cells from 2 dishes were harvested with trypsin and the sp act of their acid mucopolysaccharides was determined. The cells of the remaining 2 dishes were rinsed twice with sterile unused medium, then treated with fresh unused medium for 24 hr. At the end of this time these cells were harvested with trypsin and the sp act of their acid mucopolysaccharides was determined. At each harvest the $^{35}SO_4$-AMPS were isolated from equal aliquots of the trypsin-suspended cells by each of two different methods and their sp act was determined as described in the text.

[b] Sp act = (cpm $\times$ 10^6)/(mg UA $\times$ no. of cells).

[c] Sp act = cpm/mg protein.

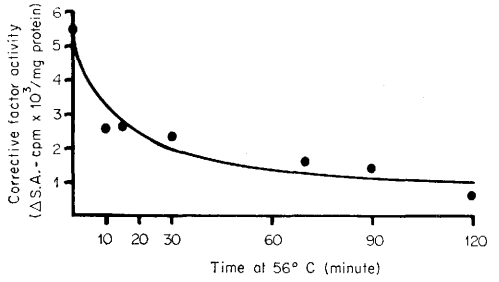

FIG. 1. Heat inactivation of corrective factor activity of fetal calf serum at 56°. Several dishes of Hurler fibroblasts were labeled with $^{35}SO_4$ as described and the sp act of the mucopolysaccharides of the cells in 2 dishes was determined. The radioactive medium was removed from the cells in the remaining dishes. These cells were rinsed with sterile medium containing no serum and then exposed to medium enriched with 20% fetal calf serum which had been heated at 56° for varying times. After 48 hr these cells were harvested and the sp act of their mucopolysaccharides also determined. The change in sp act following treatment is plotted against the time of heating the fetal calf serum at 56°.

maining dishes of cells were treated 48 hr with fetal calf serum which had been heated to 56° for increasing lengths of time. They were rinsed, harvested as the others, and the sp act of the mucopolysaccharides was determined. The decrease in the sp act of the mucopolysaccharides of these cells from the sp act of the mucopolysaccharides of the untreated cells represented the corrective activity remaining in the fetal calf serum after heating. The results are shown graphically in Fig. 1. The numbers on the ordinate represent the corrective activity remaining in the fetal calf serum (expressed as change in specific activity) after heating at 56° for the time shown in minutes on the abscissa. Most of the corrective factor actvity was destroyed in the first 30 min. None was left once the curve had plateaued at about 120 min. The small amount of correction (decrease of sp act) which still occurred represented the inherent ability of the Hurler fibroblasts to turn over $^{35}SO_4$-AMPS. Separate tests have been run which show that fetal calf serum heated at 56° for 2 hr supports cell metabolism adequately for $^{35}SO_4$ labeling of acid mucopolysaccharides.

The variability of the method was determined in a separate experiment using 5 dishes of cells at each time point. Measurement of the sp act of the intracellular acid mucopolysaccharides was made before and after a 12-hr treatment with Ham's F-10 enriched with 20% heat-inactivated fetal calf serum. The data are shown in Table III. Variability of the sp act had a standard deviation which was 2% of the mean for Hurler cells and 4% of the mean for normal cells.

Further evidence that heating fetal calf serum inactivated the corrective factor activity for Hurler cells was obtained by determining the baseline decay curve of the sp act of $^{35}SO_4$-labeled Hurler and normal cells using medium enriched with either 20% fetal calf serum or 20% heat-inactivated fetal calf serum. Approximately equal num-

TABLE III. Specific Activity[a] of $^{35}SO_4$-Acid Mucopolysaccharides Isolated by Method 2.[b]

	Sp act after	
	Labeling 44 hr	Treating 12 hr[c]
Hurler cells	11,045	9900
	10,613	9898
	10,235	9706
	10,474	10,046
	10,325	10,471
Mean ± 1 σ	10,538 ± 318	10,004 ± 218
Normal cells	1687	628
	1658	563
	1797	609
	1710	587
	1672	557
Mean ± 1 σ	1705 ± 55	589 ± 30

[a] Sp act = cpm/mg protein.

[b] Dishes of 5-day-old Hurler and normal fibroblasts were labeled with 20 μCi $^{35}SO_4$ for 44 hr. The radioactive medium was removed, the cells were rinsed, 5 dishes of each type were harvested and the sp act of the mucopolysaccharides was determined by method 2. The remaining 5 dishes of each type were exposed to fresh nonradioactive medium enriched with 20% heat-inactivated fetal calf serum for 12 hr. The cells were rinsed, harvested with trypsin and the sp act of the mucopolysaccharides was determined by method 2.

[c] Fresh medium enriched with 20% heat-inactivated (56°, 2 hr) fetal calf serum.

bers of Hurler and normal fibroblasts were grown to confluency in 28 separate dishes. All were labeled with $^{35}SO_4$ for 48 hr. Two dishes of each cell type were rinsed, the cells were harvested and the sp act of the mucopolysaccharides was determined by method 2. The remaining cells were rinsed and the dishes were divided into 2 groups. One group of 12 (6 of each type) was treated with medium enriched with 20% heat-inactivated fetal calf serum. The other group of 12 dishes (6 of each type) was treated with medium enriched with 20% fetal calf serum which had not been heat-inactivated. Two dishes of cells were taken from each group at 1-day intervals, the cells were rinsed, harvested and the sp act of the mucopolysaccharides was determined as before. The mean sp act of each pair is plotted on the graph in Fig. 2. Agreement of the duplicate data was within the variability of the method. Heating fetal calf serum inactivated the factor responsible for the correction of Hurler fibroblasts and had a similar, though smaller effect on the factor responsible for the loss of $^{35}SO_4$-AMPS from normal cells. It is seen that the decay curve for uncorrected Hurler fibroblasts decreased 1900 cpm/mg protein in the first 24 hr.

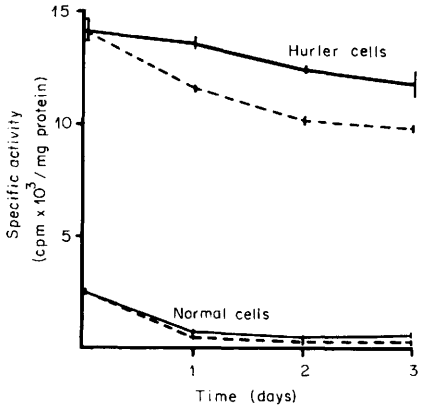

FIG. 2. The loss of $^{35}SO_4$-AMPS from uncorrected Hurler and normal fibroblasts. Fourteen dishes of Hurler and 14 dishes of normal fibroblasts were labeled with $^{35}SO_4$ as described and the sp act of the mucopolysaccharides of the cells in 2 dishes of each type was determined. The remaining cells were rinsed and treated with medium enriched with either 20% fetal calf serum (- - - - -) or 20% heat-inactivated fetal calf serum (———). At 1-, 2-, and 3-day intervals cells were harvested and the sp act of their mucopolysaccharides was determined. The sp act of the mucopolysaccharides (mean of 2 dishes) is plotted against the time of treatment (days).

Assurance that corrective factor from the fetal calf serum of the labeling medium had not lingered in the cells during treatment and contributed to the observed corrective activity was obtained by measuring the loss of $^{35}SO_4$-AMPS from Hurler cells using heat-inactivated fetal calf serum in the labeling medium as well as in the test medium. The results were identical to those in Fig. 2.

Discussion. Using an alternate extraction procedure relatively specific for acid mucopolysaccharides, our data confirms the findings of Neufeld and Fratantoni (17), that the slow loss of $^{35}SO_4$-AMPS from Hurler cells is augmented during treatment with medium from normal cells. Direct comparison of the classical acid mucopolysaccharide extraction method with the Neufeld and Fratantoni method during treatment of Hurler cells produced identical results. Although the second method recovers less of the labeled product by a less specific technique and consumes the sample in analysis, it is quicker, requires less handling, and gives equally reproducible results.

Both methods are susceptible to false interpretations. If a given treatment increases the cell numbers in method 1 or the cell protein in method 2 without changing the amount of macromolecular $^{35}SO_4$-AMPS in the cell, a false positive corrective effect will be noted. This possibility cannot be avoided by comparing the total labeled intracellular mucopolysaccharide in a dish before and after treatment. The radioactivity must be related to some parameter of the cell number, *e.g.*, DNA, protein. Furthermore, trypsinization of cells may result in small peptide fragments not precipitated by hot 80% ethanol and may release as much as 50% of $^{35}SO_4$-AMPS from Hurler cells. Using method 2, with carefully controlled trypsinization, no significant change has been observed in total cell protein during these treatments, and the false result has been avoided.

Neither method measures biochemical degradation and removal of $^{35}SO_4$-AMPS from the cell, since both methods fail to detect oligosaccharide fragments due to their

solubility in either CPC or hot 80% ethanol. The detection of such fragments may be accomplished by Sephadex chromatography (5), but this complicates the test beyond usefulness for assay purposes. The isotope method can be used effectively to screen materials for corrective factor activity with greater ease than biochemical analysis and with more specificity than metachromatic stains (18, 19).

This isotope assay for corrective factor activity measures differences in the loss of macromolecular $^{35}SO_4$-AMPS from Hurler cells rather than differences in mucopolysaccharide accumulation, and thus directly reflects degradation of intracellular mucopolysaccharide, the catabolic process which is at fault in these cells (2, 3). In this assay cells are not trypsinized and replated between labeling and treatment, because 50% of the $^{35}SO_4$-AMPS are lost during such treatment and the trypsinized mucopolysaccharides may be qualitatively different than those retained by the cells. False interpretations would then occur if the retained mucopolysaccharides were more or less susceptible to corrective factor action than the trypsinized mucopolysaccharides.

In searching for materials with corrective factor activity it is necessary to remove or inactivate corrective factors in the serum used to enrich the basic medium. Since cells in culture thrive best with serum enrichment, we retained the serum in the medium and heat inactivated its corrective factors. These experiments give convincing evidence of the presence of a heat-labile corrective factor for Hurler cells in fetal calf serum. Because of a similar, though smaller, effect on normal cells, the corrective factor activity is presumed nonspecific. The results do not otherwise contribute to our understanding the nature of the action of the corrective factor of fetal calf serum.

Heat-inactivated fetal calf serum-enriched medium was then used to study the inherent capacity of Hurler and normal fibroblasts to catabolize sulfated mucopolysaccharides. It was found that normal cells turn over about 2.5 times more substrate than Hurler cells in 24 hr. We have observed that normal cells treated with fetal calf serum-enriched medium turn over 10% of the substrate after 1 hr, 61% after 6 hr and 89% by 24 hr. If these data are plotted with time as the abscissa, during the first 6 hr the slope is steep and relatively straight, so the rate of turnover of substrate as measured in this assay is presumed constant. Actually, the true rate is constant for as long as the cells are healthy. However, when examining by isotope labeling, only observations made during the first 6–12 hr will accurately reflect cell metabolism in normal cells. The small amount of $^{35}SO_4$-AMPS eliminated by the Hurler cells probably represents the secretion of undegraded macromolecular mucopolysaccharides (3).

Summary. The isotope method of detection of corrective factor activity for cultured Hurler fibroblasts using radioactive sulfate ($^{35}SO_4$) has been confirmed and improved as a sensitive screening method. Its limitations have been identified and discussed. Using this method it has been shown that cultured Hurler fibroblasts turn over 2–3 times less macromolecular $^{35}SO_4$-AMPS in 12–24 hr than do cultured normal fibroblasts. The results also establish the presence of a heat-labile corrective factor in fetal calf serum, which, because of its similar, though smaller effect on normal cells, is presumed to be nonspecific.

This work was performed at the Children's Hospital, Buffalo, NY and was supported by a grant from the United Health Foundation of Western New York, Inc., by Grants RR-05493 and RR-05400 from the General Research Support Branch, Division of Research Resources, NIH, and by Project No. 417 of the Maternal and Child Health Service of HEW. The author thanks Betty Chao and Lyle Motycka for technical assistance, and Dr. A. J. Bollet, Medical College of Georgia for assistance in the preparation of this manuscript. Dr. Herd was a recipient of a Dr. Henry C. Buswell and Bertha H. Russell Research Fellowship.

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Received Sept. 1, 1972. P.S.E.B.M., 1973, Vol. 143.