# *HURLER'S SYNDROME: BIOSYNTHESIS OF ACID MUCOPOLYSACCHARIDES IN TISSUE CULTURE**

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Hurler's syndrome is an inborn error of the metabolism of acid mucopolysaccharides (AMPS) characterized by deposition in tissues and excretion in urine of chondroitin sulfate B (CS-B) and heparitin sulfate (HS).[1-6] Previous studies[6, 7] have demonstrated that CS-B and HS, obtained from tissues and urine of patients with Hurler's syndrome, are readily extracted with water. Such preparations contain less than one serine residue per polysaccharide chain and few other amino acid residues. In contrast, CS-B of normal skin prepared by proteolytic digestion contains larger amounts of amino acids.

The studies to be reported were undertaken to determine whether the metabolic defect in this disease can be demonstrated in fibroblasts in tissue culture. While these studies were in progress, Danes and Bearn[8] reported that fibroblasts cultured from individuals with Hurler's syndrome, as well as from heterozygotes, exhibited metachromatic granules. This finding was readily confirmed. The results indicate that fibroblasts cultured from patients with Hurler's syndrome contain 5–10 times as much AMPS as are present in cells cultured from normal individuals. A marked difference between normal cells and Hurler cells was found with respect to the incorporation of radioactive precursors into mucopolysaccharides.

*Materials and Methods.*—Carrier-free $H_2S^{35}O_4$ was obtained from the New England Nuclear Corp. Sodium acetate-$H^3$ (206 mc/mole) was obtained from Nuclear-Chicago. L-Serine-U-$C^{14}$ (110 mc/mole) was obtained from the International Chemical and Nuclear Corp. Puromycin hydrochloride was obtained from General Biochemicals, Inc. Chondroitin sulfate-A (CS-A) was a gift from Dr. J. A. Cifonelli.

*Tissue culture:* Fibroblast cultures were established from patients with Hurler's syndrome and normal children, using skin biopsies. The cells were grown at 38° in monolayers on 100-mm plastic Petri dishes in a 15% $CO_2$-85% air mixture in Eagle's medium containing 4.2 gm/liter of $NaHCO_3$ supplemented with 20% fetal calf serum and 10% calf serum. The cells used for this study, fifth to the seventh transfers, were grown for 2–3 weeks before study. The number of cells at plating time was $2.5 \times 10^6$ per plate.

*Isolation of AMPS:* AMPS were isolated from the media and cells following digestion with crystalline papain, dialysis, and precipitation with cetylpyridinium chloride (CPC), as previously described.[9]

Fractionation of AMPS was carried out on Dowex 1-X2 $Cl^-$ (200–400 mesh), with stepwise elution by NaCl (0.5 *M*, 1.0 *M*, 1.3 *M*, 1.7 *M*, and 2.0 *M*). The CS-B was recovered in the 1.7 *M* fraction,[10] and further purification was performed using $CuSO_4$.[11] Some fractions of AMPS were subjected to hyaluronidase digestion, and the undigested material was recovered by precipitation with $CuSO_4$. The polysaccharide obtained was passed over Dowex 50-X8 $H^+$ (200–400 mesh) for the removal of the copper ions.

*Analytical methods:* Uronic acid was determined by the carbazole method of Dische,[12] as well as by the orcinol reaction.[13] The hexosamine was determined by the Boas modification[14] of the Elson-Morgan method omitting the resin treatment. N-sulfated hexosamine, which is characteristic of heparin and HS, was determined by the nitrous acid reaction as described by Lagunoff and Warren.[15] Protein was precipitated with hot trichloroacetic acid (TCA),[16, 17] and determined quantitatively by the method of Lowry *et al.*[18]

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer as previously described.[9]

Digestion of AMPS with testicular hyaluronidase was carried out as previously described.[19] Purified hyaluronidase (19,000 IU per mg) was a gift from Dr. L. Rodén.

The intrinsic viscosity of purified CS-B was kindly determined by Dr. M. B. Mathews, by a method previously described.[20]

Paper chromatography of uronic acids was carried out, with Whatman no. 1 paper, using tertiary-amyl alcohol, isopropyl alcohol, and water (8:2:3) as solvent. The uronic acid spots were visualized with silver nitrate stain. Samples were prepared for chromatography by hydrolysis of CS-B with 1 *M* HCl for 90 min at 100°, followed by treatment with Dowex 3 $CO_3^-$, and passage over Dowex 50-X8 $H^+$.

The amino sugars of certain AMPS fractions were determined by the Technicon amino acid analyzer following hydrolysis with 4.0 *N* HCl for 6 hr at 100°.

Electrophoresis was carried out in a 0.1 *M* pyridine-formic acid buffer, pH 3.0, for 1 hr with a voltage gradient of 17 v/cm, using cellulose polyacetate strips (Sepraphore III). The strips were stained with a 1% solution of acridine orange.[21, 22]

*Preparation of subcellular fractions:* Fibroblasts were washed with cold isotonic sucrose solution and then removed from the tissue culture plates by rubber policemen. Following suspension in isotonic sucrose, the cells were disrupted at 4° in a Kontes-Duall homogenizer with 40 up and down strokes. The homogenates were centrifuged at 800 × *g* for 10 min at 4° to remove the nuclear fraction. The supernatant solution was centrifuged for 20 min at 15,000 × *g*. The 15,000 × *g* supernatant solution was recentrifuged in a Spinco model L centrifuge for 1 hr at 100,000 × *g*. The pellet of each fraction was dissolved in 0.1 *N* NaOH, dialyzed in the cold against 0.3 *M* $Na_2SO_4$, and then against distilled $H_2O$.

*Labeling experiments:* Cells were incubated for 24 and 48 hr with 10 ml of medium containing 20 μc of $S^{35}O_4^-$ or acetate-$H^3$. L-Serine-U-$C^{14}$ was used in a concentration of 10 μc per 10 ml of medium. In the experiment utilizing puromycin for 2 hr, 3 times as much radioactive precursors were used. After incubation, the medium was removed, the cells were treated with 0.25% trypsin, and counted in a Neubauer hemocytometer. AMPS were isolated from the cells and medium with 2 mg of carrier CS-A for each sample. In the experiments with L-serine-U-$C^{14}$, the media and cells were divided equally, one half for the isolation of AMPS, and the other half for the precipitation of proteins with hot TCA.

Puromycin hydrochloride was added simultaneously with the radioactive precursors, in a concentration of 25 μg/ml.

In pulse-labeling experiments, $S^{35}O_4^-$ and acetate-$H^3$ were in contact with cells for a 24-hr period, following which the cells were washed and incubated with nonradioactive medium for an additional 24 hr. AMPS were isolated from the labeling media, "chase" media, and from the cells. Cells used for preparation of subcellular fractions were pulse-labeled with $S^{35}O_4^-$ only.

*Results.*—When the growth medium was used for the isolation of AMPS, no striking difference in quantity was observed between cultures of Hurler and normal cells. The amounts of AMPS recovered were too small for complete identification. Electrophoresis of the crude AMPS showed a principal spot which migrated as does HA or chondroitin. In contrast, when AMPS were isolated from cells, 5–10 times as much AMPS were found in Hurler cells as in normal cells. Table 1 compares the quantity obtained from cells of two different patients, both of whom are afflicted with autosomal recessive forms of the disease, with the quantity of AMPS ob-

TABLE 1

ACID MUCOPOLYSACCHARIDES ISOLATED FROM NORMAL AND HURLER FIBROBLASTS

| Cell type | Uronic acid (μg per plate) | Hexosamine (μg per plate) |
|---|---|---|
| Normal | 12.4 | 11.4 |
| Normal | 7.9 | 8.9 |
| Hurler KF | 66.4 | 61.8 |
| Hurler BH | 67.5 | 74.2 |

Results are expressed per 100-mm plates. The number of cells per plate was roughly comparable, although normals tend to contain slightly larger numbers.

tained from cells of two normal children. Patient KF excretes HS while patient BH excretes CS-B.

In the case of patient BH, 6.6 mg (based on hexosamine) of AMPS were isolated from 30 plates. Electrophoresis of this preparation showed two major spots, which migrated like HA and like CS-B, with an additional, fainter spot, with a mobility similar to that of CS-A. When the crude AMPS were subjected to testicular hyaluronidase, a 40 per cent decrease in turbidity was found. The undigested polysaccharide was recovered by $CuSO_4$ precipitation, and on electrophoresis, only one discrete spot, identical in mobility with CS-B, was found. L-Iduronic acid was identified by paper chromatography in a hydrolysate of the undigested AMPS and galactosamine was found to be the only amino sugar present in this material. No N-sulfate groups were detected with the nitrous acid method, ruling out the presence of HS.

A portion of the AMPS was chromatographed on Dowex 1-X2 $Cl^-$ (200–400 mesh). AMPS were recovered in the 0.5 *M*, 1.0 *M*, and 1.7 *M* NaCl eluates. The 1.3 *M* NaCl and the 2.0 *M* NaCl fractions contained no AMPS. The analyses of the three major fractions are summarized in Table 2.

The 0.5 *M* NaCl eluate contained the bulk of material with an electrophoretic mobility identical with that of standard HA. This fraction was completely digested by testicular hyaluronidase, showed an optical rotation of $[\alpha]_D^{25} = -70$, and contained glucosamine as the sole amino sugar.

The 1.0 *M* eluate contained components that migrated on electrophoresis as did standard CS-B. However, it also contained a faint spot corresponding to HA.

After the 1.7 *M* fraction was further purified by precipitation with CPC in 0.5 *M* NaCl and reprecipitated with $CuSO_4$, it was free of HA as demonstrated by electrophoresis, and moved as did standard CS-B. The optical rotation was $[\alpha]_D^{25} = -73°$. The intrinsic viscosity was $[\eta] = 0.52$, corresponding to a molecular weight of approximately 22,400, using the equation $[\eta] = 3.0 \times 10^{-4} \times M^{0.74}$.[23] Analyses of the copper-precipitated material gave a carbazole:orcinol ratio of 0.37 and a carbazole:hexosamine ratio of 0.46. The amino sugar of this fraction was exclusively galactosamine.

Equivalent amounts of normal cells yielded approximately 10 per cent of the amount of AMPS isolated from Hurler cells. Electrophoresis showed spots corresponding in mobility to HA, CS-A, and CS-B. Hyaluronidase digestion resulted in an 80 per cent decrease in turbidity. No appreciable amounts of N-sulfate-containing AMPS were found in this material. The quantity was too small for further fractionation.

Since Hurler cells clearly contain larger amounts of AMPS than do normal cells, it was of importance to determine whether this difference is also reflected in the rate of biosynthesis. Experiments were carried out with acetate-$H^3$, $S^{35}O_4^=$, and L-serine-U-$C^{14}$. The AMPS were isolated from cells and medium after 24- and 48-hr incubation periods.

TABLE 2

ANALYSES OF AMPS ELUTED FROM DOWEX 1-X2 $Cl^-$

| Fraction | Uronic acid (μg/ml) | Hexosamine (μg/ml) | $[\alpha]_D^{25}$ | Per cent of total* |
|---|---|---|---|---|
| 0.5 *M* | 165 | 130 | −70° | 25 |
| 1.0 *M* | 95 | 70 | — | 13 |
| 1.7 *M* | 280 | 318 | −60° | 62 |

An aliquot of crude AMPS isolated from fibroblasts of patient B. H. was chromatographed on Dowex 1-X2 $Cl^-$ with stepwise elution by NaCl.
* Based on hexosamine content.

TABLE 3

INCORPORATION OF ACETATE-$H^3$ INTO ACID MUCOPOLYSACCHARIDES BY NORMAL AND HURLER FIBROBLASTS

| Incubation period (hr) | Cells | | Medium | |
|---|---|---|---|---|
| | Normal | Hurler | Normal | Hurler |
| | Specific Activity | | | |
| 24 | 4,800 | 4,900 | 10,000 | 16,500 |
| 48 | 2,000 | 4,100 | 15,400 | 23,800 |

Specific activity = cpm/mg uronic acid/cell × $10^6$.

TABLE 4

INCORPORATION OF SULFATE-$S^{35}$ INTO ACID MUCOPOLYSACCHARIDES IN NORMAL AND HURLER FIBROBLASTS

| Incubation period (hr) | Cells | | Medium | |
|---|---|---|---|---|
| | Normal | Hurler | Normal | Hurler |
| | Specific Activity | | | |
| 24 | 3,900 | 13,000 | 4,500 | 8,300 |
| 48 | 3,600 | 25,000 | 5,700 | 17,000 |

Specific activity = cpm/mg uronic acid/cell × $10^6$.

Table 3 summarizes the results of an experiment using acetate-$H^3$, and Table 4 the results with $S^{35}O_4^=$. In the case of acetate-$H^3$ incorporation into intracellular polysaccharide, there was little difference between Hurler and normal cells at 24 hr, but at 48 hr incorporation of radioactivity by the Hurler cells was distinctly higher than by normal cells. In the medium there was a somewhat higher incorporation in the Hurler cultures both at 24 and 48 hr. Much larger differences were observed with $S^{35}O_4^=$ than with acetate-$H^3$, particularly in the cells at 48 hr.

In view of the increasing accumulation of sulfated AMPS within the Hurler fibroblasts with time, pulse-labeling experiments were carried out. Table 5 illustrates the results of such an experiment. The Hurler cells show a 3–8 times increase in content of radioactive polysaccharide.

Cell fractionation was carried out in order to determine whether the newly synthesized sulfated AMPS are confined to cytoplasmic particles. Cells, pulse-labeled with $S^{35}O_4^=$, were homogenized and fractionated. The data in Table 6 indicate that the 15,000 × $g$ pellet contains radioactivity. Fractions derived from Hurler cells contain more than 6 times as much radioactivity as those derived from normal cells. A similar difference between the 100,000 × $g$ supernatant solutions is evident. The 100,000 × $g$ pellet, the ribosomal fraction, contained little radioactivity.

Since earlier studies had shown a deficiency of protein binding in AMPS obtained from Hurler tissues, the effect of puromycin on Hurler cells was examined. Normal and Hurler cells were incubated with acetate-$H^3$, $S^{35}O_4^=$, and L-serine-U-$C^{14}$ for 2 and 24 hr, in the presence of 25 μg/ml of puromycin. AMPS were isolated, purified, and radioactivity was determined on each fraction. The results of these experiments are summarized in Tables 7 and 8. In the experiments in which L-serine-U-$C^{14}$ was used, the incorporation of radioactivity into the hot TCA precipitate was also determined. The results indicate that puromycin inhibits polysaccharide synthesis in both normal and Hurler fibroblasts; the inhibition of the

TABLE 5

INCORPORATION OF ACETATE-$H^3$ AND SULFATE-$S^{35}$ INTO ACID MUCOPOLYSACCHARIDE BY NORMAL AND HURLER FIBROBLASTS IN 24-HR PULSE-LABELED EXPERIMENT

| Precursor | Cells | | Pulse Medium | | Chase Medium | |
|---|---|---|---|---|---|---|
| | Normal | Hurler | Normal | Hurler | Normal | Hurler |
| | | | Specific Activity | | | |
| Acetate-$H^3$ | 42,800 | 123,100 | 435,600 | 775,400 | 71,500 | 81,000 |
| Sulfate-$S^{35}$ | 16,100 | 113,900 | 69,300 | 89,900 | 12,200 | 16,200 |

Specific activity = cpm/mg uronic acid/cell × $10^6$.

TABLE 6

DISTRIBUTION OF INCORPORATED SULFATE-$S^{35}$ IN SUBCELLULAR FRACTIONS OF NORMAL AND HURLER FIBROBLASTS

| Fraction | Normal (cpm/mg protein) | Hurler (cpm/mg protein) |
|---|---|---|
| 15,000 × *g* pellet | 1,500 | 9,500 |
| 100,000 × *g* pellet | 136* | 242* |
| Supernatant | 535 | 3,600 |

* Counts expressed as total cpm because of extremely low protein.

sulfate incorporation is higher than that of acetate-$H^3$. After 2 hr (Table 8), the inhibition of acetate-$H^3$ incorporation is even less striking than that of $S^{35}O_4^=$. It should be pointed out that acetate is a precursor of HA as well as chondroitin sulfates. It is not yet known whether normal HA synthesis like chondroitin sulfate synthesis is dependent on protein synthesis. The incorporation of L-serine-U-$C^{14}$ into AMPS was somewhat lower in Hurler cells than normal cells, although in both cases inhibition by puromycin was striking. Inhibition of the incorporation of L-serine-U-$C^{14}$ into the hot TCA precipitate (protein) was almost complete in both Hurler and normal cells.

*Discussion.*—Although it has been known for some years that AMPS are excreted in urine and deposited in tissues in Hurler's syndrome, the biochemical defect has not been defined. Certain facts now seem established: (1) Both CS-B and HS are deposited in tissues and excreted in urine. The relative amounts of the two polysaccharides vary in different patients. (2) There are at least two genetically distinct types of Hurler's syndrome. Some authors have suggested that five separate diseases comprise this syndrome.[24] (3) Mucopolysaccharides isolated from Hurler tissues by methods not expected to rupture peptide or glycosidic bonds, show an abnormally low amino acid content. In the case of CS-B, predominantly serine is found and in a concentration of less than one serine residue per polysaccharide chain.[6] HS is found to be of low molecular weight and heterogeneous; one fraction contains almost all of the serine (the predominant amino acid present), while the others con-

TABLE 7

EFFECT OF 24 HR PUROMYCIN TREATMENT ON THE INCORPORATION OF RADIOACTIVITY IN ACID MUCOPOLYSACCHARIDES IN NORMAL AND HURLER FIBROBLASTS

| | Normal | | | Hurler | | |
|---|---|---|---|---|---|---|
| Precursor | Control (Sp. act.*) | Puromycin (Sp. act.*) | Inhibition (%) | Control (Sp. act.*) | Puromycin (Sp. act.*) | Inhibition (%) |
| Acetate-$H^3$ | 3,200 | 12 | 99.6 | 9,800 | 2,500 | 74.8 |
| Sulfate-$S^{35}$ | 2,400 | 44 | 98.2 | 5,100 | 212 | 95.8 |
| Serine-$C^{14}$† | 431,000 | 29,000 | 93.3 | 275,000 | 44,000 | 84.0 |
| Serine-$C^{14}$‡ | 1,437,000§ | 24,000§ | 98.4 | 1,227,000§ | 22,000§ | 98.3 |

* Cpm/mg uronic acid/cells × $10^6$.
† Serine in AMPS fraction.
‡ Serine in total cell protein precipitated by hot TCA.
§ Cpm/mg protein.

TABLE 8

EFFECT OF 2 HR PUROMYCIN TREATMENT ON THE INCORPORATION OF RADIOACTIVITY IN ACID MUCOPOLYSACCHARIDES IN NORMAL AND HURLER FIBROBLASTS

| | Normal | | | Hurler | | |
|---|---|---|---|---|---|---|
| Precursor | Control (Sp. act.*) | Puromycin (Sp. act.*) | Inhibition (%) | Control (Sp. act.*) | Puromycin (Sp. act.*) | Inhibition (%) |
| Acetate-$H^3$ | 52,600 | 42,000 | 20 | 48,300 | 25,500 | 47 |
| Sulfate-$S^{35}$ | 803,600 | 127,200 | 85 | 672,900 | 94,400 | 86 |
| Serine-$C^{14}$† | 1,165,000 | 70,900 | 94 | 486,400 | 45,000 | 91 |
| Serine-$C^{14}$‡ | 1,406,000§ | 70,500§ | 95 | 1,426,000§ | 66,100§ | 96 |

* Cpm/mg uronic acid/mg protein.
† Serine in AMPS fraction.
‡ Serine in total cell protein precipitated by hot TCA.
§ Cpm/mg protein.

tain only small amounts of amino acids.[7] Xylose and galactose, known to be present in the linkage region between AMPS and protein,[25] are present only in the fraction containing serine.[26] As in glycopeptides isolated from chondroitin sulfate-A protein complex[27] and heparin,[28] one xylose and two galactose residues are present per residue of serine. These findings indicate that the AMPS extracted from Hurler tissues do not exist as the native AMPS complex. (4) The data presented in this paper show that fibroblasts derived from patients with Hurler's syndrome contain an excess of intracellular AMPS. Isotope studies indicate that the rate of synthesis, as well as the storage within cells, is markedly augmented. Particularly striking is the increase not only in CS-B, but of hyaluronic acid and probably of CS-A (C), AMPS not previously implicated in Hurler's disease. (5) The fact that AMPS isolated from Hurler tissues have a low amino acid content may be due to an unusual biosynthetic mechanism which does not form a normal protein-polysaccharide complex; or if such a complex is formed, it is subsequently partially degraded.

Previous studies by Telser, Robinson, and Dorfman[29] have shown puromycin inhibition of chondroitin sulfate synthesis in embryonic chick chondrocytes. These findings were considered to result from an interference with synthesis of protein acceptor, necessary for the formation of AMPS chains. The experiments reported here indicate an identical effect of puromycin on AMPS in Hurler fibroblasts, indicating that a protein acceptor is necessary for synthesis of sulfated polysaccharides in Hurler fibroblasts as it is in normal chick chondrocytes. These data rule out the possibility that in Hurler fibroblasts, sulfated mucopolysaccharide chains are synthesized independently of protein acceptor. The situation with respect to hyaluronic acid in either normal or Hurler cells is less clear; it is possible that synthesis of hyaluronic acid does not require protein acceptor.

Any explanation of the biochemical defect in Hurler's disease must account for these facts. Since Hurler fibroblasts appear to synthesize AMPS at a rate considerably greater than normal, the previous suggestion by Van Hoof and Hers[30] of a deficiency of degradative enzymes does not seem valid. Although adequate information is not yet available to construct a detailed picture of the biochemical defect of this disease, the following working hypothesis may be formulated to account for the known facts. It is suggested that the fundamental genetic defect results in excess production of acid mucopolysaccharides. This may be due to: (*a*) a regulator gene mutation, although the presence of inclusion bodies in cells derived from heterozygotes is not consistent with the usual recessive nature of regulator gene mutations. The possibility that regulator gene mutations can express dominance, under certain conditions, exists as has been shown in bacteria,[31] (*b*) a structural gene defect resulting in a product that does not act as a feedback inhibitor, or (*c*) a defect in assembly or excretion from the cell. Little is yet known regarding the mechanisms responsible for assembly of complex macromolecules such as AMPS within cell and the manner by which they are excreted. It has been suggested that after completion at the endoplasmic reticulum, AMPS-protein complexes are accumulated in the Golgi apparatus and "packaged" prior to excretion from the cell.[32] Whether a defect in this complex process might result in "storage" of such molecules within cells is unknown.

Since it has been demonstrated that mucopolysaccharides accumulate in lysosome-like particles and that lysosomes contain proteolytic enzymes as well as a hyal-

uronidase,[33, 34] it is possible that the combined action of these enzymes may result in partially degraded CS-B and HS complexes. Recent studies in this laboratory by Fransson and Rodén[35] indicate that CS-B of pig skin is partially degraded by hyaluronidase with removal of the protein linkage region. A similar mechanism may obtain for the degradation of HS. The failure to accumulate CS-A (C) and hyaluronic acid in Hurler's syndrome may be due to the fact that these polysaccharides are more thoroughly degraded by lysosomal hyaluronidase.

This formulation is necessarily incomplete and leaves many interesting questions unanswered, but may serve as a useful extension of previous hypothesis for future experimentation.

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