

THE DEFECT IN HURLER'S AND HUNTER'S SYNDROMES: FAULTY DEGRADATION OF MUCOPOLYSACCHARIDE

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Hurler's syndrome is a genetically transmitted disorder of mucopolysaccharide metabolism, distinguished chemically by excessive intracellular accumulation and urinary excretion of chondroitin sulfate B and heparitin monosulfate, and clinically, by mental retardation, skeletal deformities, corneal clouding, and early death.¹ While Hurler's syndrome follows autosomal recessive inheritance, there also exists a sex-linked variant, Hunter's syndrome, which is chemically similar, but clinically less severe. The study of these disorders has been greatly facilitated by the discovery that fibroblasts cultured from the skin of affected individuals also manifest the disease, in that they store an abnormal amount of mucopolysaccharide as determined by histological observation, chemical analysis, and measurement of isotope incorporation.^{2, 3}

We have undertaken a study of the kinetics of this accumulation in fibroblasts to determine whether it is a result of excessive synthesis, insufficient degradation, or defective secretion of mucopolysaccharide. Although accumulation of mucopolysaccharide has been attributed to overproduction,³ our results suggest that the lesion(s) in both Hurler's and Hunter's syndromes is faulty degradation.⁴

Materials and Methods.—Culture of fibroblasts: Fibroblasts were grown from skin biopsies or from infant foreskin obtained at circumcision. Tissue was obtained from two normal infants, a normal adult, a Hurler infant, and a Hunter adult, and cultured for 2–8 months prior to use. Cultures were maintained as monolayers at 37° in 100-mm Falcon plastic Petri dishes in an atmosphere of 5% CO₂–95% air. They were fed three times per week with Eagle's minimal essential medium (in which MgCl₂ was substituted for MgSO₄) supplemented with nonessential amino acids, 10% fetal calf serum, penicillin, streptomycin, and, in some cases, Mycostatin. Inorganic sulfate, derived primarily from the streptomycin, was about 0.4 mM.

When used, the Hurler and Hunter cells displayed strong metachromasia in toluidine blue (a histological marker for mucopolysaccharide). They were stained essentially as described by Danes and Bearn,³ except that methanol fixation was omitted and the cover slips were immersed for 8 min in 0.1% toluidine blue in 60% ethanol. This modification was introduced because, in some cultures, mucopolysaccharide appeared to be leached out in the 30% methanolic solution of dye originally recommended.

Incorporation of S³⁵O₄ into mucopolysaccharide: A simple assay was devised to measure the incorporation of S³⁵O₄ into mucopolysaccharides, based on the fact that the latter are the only macromolecules of the fibroblast to become labeled with inorganic sulfate (mammalian cells in general do not introduce sulfate into cystine or methionine residues of proteins⁵).

Cells were incubated in the medium described above to which had been added 3–15 × 10⁶ cpm of carrier-free H₂S³⁵O₄ (New England Nuclear Corp.) per ml. Petri dishes routinely contained 1–3 million cells (0.5–1.5 mg protein). At specified intervals, the labeled medium was removed and dialyzed for 4 hr against 0.1 M (NH₄)₂SO₄, followed by running tap water for 20 hr. The cells were washed with isotonic saline, detached from the plate by treatment with 0.4% trypsin (Microbiological Associates) in saline at 37° for 15 min, centrifuged, and extracted four times with boiling 80% ethanol (1 min boiling

in 2 ml ethanol each time). The dialysis of the medium and the ethanol extraction of the cells removed $S^{35}O_4$ and low-molecular-weight compounds, leaving mucopolysaccharide in the residue. An aliquot of the dialyzed medium was counted directly, while the extracted cell residue was dissolved with careful heating in 10% NaOH, for determination of radioactivity and of protein.⁶

For decay experiments, cells were pre-labeled with medium containing $S^{35}O_4$ for 2 days. The radioactive medium was removed, the cells detached from the plate and from each other with trypsin, washed, replated, and incubated in unlabeled medium for specified periods of time.

Analytical methods: Uronic acid was measured by the Gregory carbazole or the Meibum orcinol procedures;⁷ protein by the method of Lowry *et al.*;⁸ sulfate by the benzidine procedure of Antonopoulos.⁹ Radioactivity was measured in a liquid scintillation spectrometer, using 0.5 ml of aqueous or ethanolic solution per 10 ml of scintillation fluid (0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis-2'(5-phenyloxazolyl)-benzene in toluene-methylcellosolve, 1:1, v/v).

For degradation of protein associated with mucopolysaccharide, 2 ml of solution or suspension containing labeled mucopolysaccharide was incubated at pH 6 with 0.020 ml of 0.5 M potassium ethylenediaminetetraacetate (EDTA), 0.020 ml of 0.5 M cysteine, 0.200 ml of 1 M sodium acetate buffer, pH 6, and 1.6 mg of crystalline papain (Worthington, 13 units per mg) at 60° for 24 hr. During that time, additional papain and cysteine were added once. Residual protein was removed by precipitation with trichloroacetic acid, and the supernatant was dialyzed for 20 hr against running water.

Electrophoresis of mucopolysaccharide was carried out on strips of Whatman 1 for 16–20 hr in 0.15 M $ZnSO_4$;¹⁰ mucopolysaccharide was stained with toluidine blue O after scanning for radioactivity. Chondroitin sulfate B from pig skin was generously supplied by Dr. Eugene Davidson; other reference standards were commercial preparations.

Gel filtration was performed on Sephadex G-200 in a column 1.5 × 43 cm; the eluent, (0.02 M $(NH_4)_2SO_4$ -0.02% NaN_3) was allowed to flow at 20 ml per hr.

Results.—Kinetics of intracellular mucopolysaccharide accumulation: The patterns of accumulation of radioactivity in intracellular mucopolysaccharide of fibroblasts from normal individuals and from Hurler and Hunter patients differ markedly, as seen in Figure 1. In normal cells, the amount of label incorporated into intracellular mucopolysaccharide increases linearly for about five hours, as labeled material replaces the unlabeled already present in the cells; after one day,

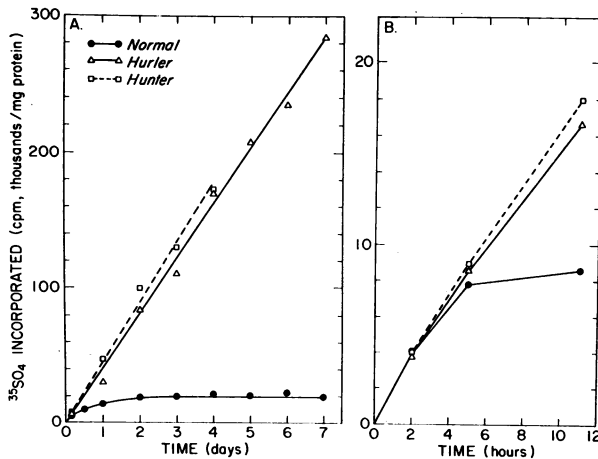


FIG. 1.—Incorporation of $S^{35}O_4$ into intracellular mucopolysaccharide by fibroblasts from a normal subject and from patients with the Hurler and Hunter syndromes. An expanded plot of the first 12 hr of the experiment is shown in B. The specific activity of the labeled sulfate was 20,000 cpm per nmole.

it reaches a steady state when the rate of exit from the intracellular pool is equal to the rate of entry ("exit" is the sum of degradation and secretion; "entry" is concerned, but is not synonymous, with synthesis. These points will be treated more fully in the *Discussion*). At that time, 1 nmole of SO_4 has been replaced per milligram of cell protein. Identical results were obtained with fibroblasts from infant and adult subjects.^{10a} In contrast, the accumulation of label by the diseased cells does not reach a steady state, but proceeds linearly for the duration of the experiment. However, *the early rates of accumulation by normal and abnormal fibroblasts are identical*, as seen in Figure 1, particularly in the enlarged plot of the early time points shown in *B*. This is a significant point, as will be discussed below.

Kinetics of removal of intracellular mucopolysaccharide (secretion and degradation): Mucopolysaccharide can leave the cell in two ways, one of which is by secretion into the medium. This route can be seen in Figure 2 to follow a similar

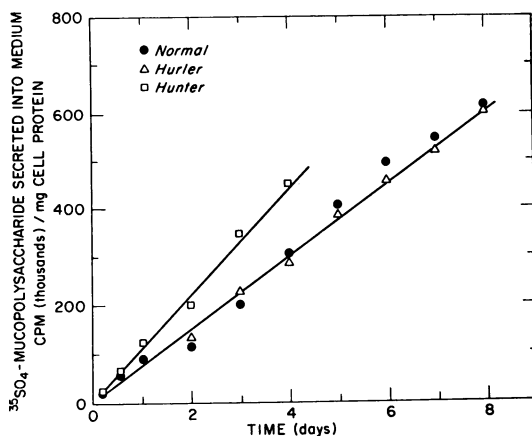


FIG. 2.—Secretion of S^{35}O_4 -labeled mucopolysaccharide into culture medium. The specific activity of the sulfate was 20,000 cpm per nmole. The medium was changed every 2 days; it is the cumulative secretion that is plotted.

time course for normal and Hurler cells, the Hunter culture showing a somewhat higher rate. It should also be noted that this rate is linear from the earliest points (2 hr in the experiments shown and about 30 min in a separate experiment).

Removal of intracellular mucopolysaccharide may also occur by degradation, which can be followed by the disappearance of prelabeled mucopolysaccharide (Fig. 3). The radioactive mucopolysaccharide content of normal cells declined at a rate which mirrors the rate of accumulation observed in Figure 1, only 32 per cent of the label remaining 11 hours after the cells had been transferred to unlabeled medium. On the other hand, labeled intracellular mucopolysaccharide of Hurler cells remained as high as 70 per cent of the initial value after three days in unlabeled medium, while that of Hunter cells declined slowly to 50 per cent in three days. (The experiment described in Fig. 3 was performed on cells prelabeled for 48 hr, but a similar difference in decay rates was observed if the cells were prelabeled for only 4 hr, at which time all three cultures had incorporated S^{35}O_4 to a similar extent.)

Most of the labeled mucopolysaccharide lost from the normal cell was found in

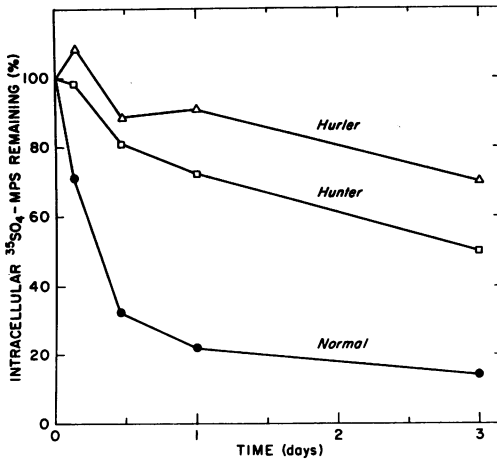


FIG. 3.—Loss of radioactivity from intracellular mucopolysaccharide (MPS) during chase with unlabeled medium.

the medium *not as macromolecule, but as dialyzable material* (Table 1). This shows the slow loss of intracellular mucopolysaccharide to be due to degradation into small fragments (oligosaccharides or inorganic sulfate), a process quite different from the secretion shown in Figure 2.

Characteristics of labeled intracellular and secreted mucopolysaccharide: The intracellular and extracellular $S^{35}O_4$ -labeled mucopolysaccharides of normal, Hurler, and Hunter cells all belong predominantly to the same molecular species, namely chondroitin sulfate B (dermatan sulfate), as determined by coelectrophoresis with authentic material in $ZnSO_4$, under conditions which separate chondroitin sulfate B from chondroitin sulfate A and C.^{10, 11} However, since these electrophoretic separations are of necessity carried out after extensive digestion with papain, they shed no light on how much protein the original macromolecules contained or how large they may have been.

The bulk of the intracellular mucopolysaccharide may be obtained in soluble form if cells are disrupted for two minutes in a Raytheon sonifier. (From 10 to 40% of the label remains bound to particles.) When subjected to filtration on Sephadex G-200 this material is retarded (Fig. 4), while the secreted mucopolysaccharide is excluded from the gel. After treatment with papain, mucopolysaccharides from the cells and medium were identical in size and both somewhat smaller and more polydisperse than the original intracellular material. These results were obtained with Hurler and Hunter cells. The intracellular material from normal cells, however, showed a more disperse macromolecular pattern,

TABLE 1. *Degradation of intracellular mucopolysaccharide (MPS) by normal cells.*

Time (hr)	Intracellular MPS (cpm)	Medium MPS (cpm)	MPS lost (calculated) (cpm)	Dialyzable in medium* (cpm)
0	36,500
2	30,500	2,800	3,200	4,700
9	14,200	3,800	18,500	17,200
24	6,100	3,300	27,100	33,200

* Corrected for 4,000 cpm zero-time value.

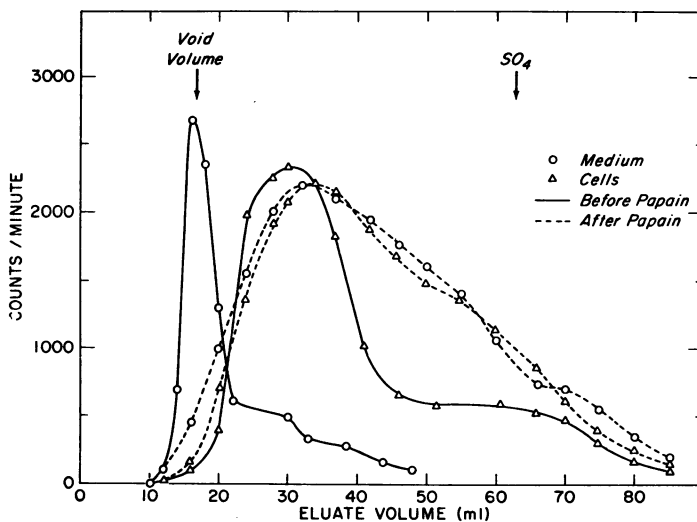


FIG. 4.—Gel filtration on Sephadex G-200 on intracellular and secreted mucopolysaccharides from Hunter fibroblast. Arrows indicate the peak of elution of dextran blue and inorganic sulfate.

plus a peak corresponding to inorganic sulfate. Control experiments showed that the apparent small size of intracellular material or the large size of the secreted mucopolysaccharide are not artifacts resulting from sonication or adsorption to proteins in the medium, respectively.

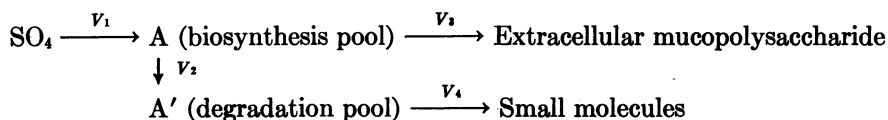
Discussion.—The observations made in this study must be examined in light of the existing knowledge of the metabolism of sulfated mucopolysaccharides. These compounds occur in normal tissues as complex structures, with polysaccharide covalently linked to protein.¹² The biosynthesis of protein-polysaccharides, such as chondroitin sulfate A or B or heparitin sulfate, is thought to take place in an organized fashion, beginning with the synthesis of the protein core on the ribosomes, and followed by the sequential addition of monosaccharide residues from appropriate sugar nucleotide precursors and by sulfation via phosphoadenosine 5'-phosphosulfate. The later events, glycosylation and sulfation, are presumed to occur in the membranes of the endoplasmic reticulum and the Golgi apparatus.¹³⁻¹⁵ Polysaccharide is neither formed nor sulfated if protein synthesis is inhibited by puromycin, indicating that the sequence cannot be reversed, i.e., polysaccharide chains cannot be formed first and bound to protein secondarily.¹⁶ The obligatory requirement of protein synthesis for sulfation is true of Hurler's as well as of normal fibroblasts.⁸

Relatively little is known of the catabolism of sulfated mucopolysaccharides. Chondroitin sulfate A and C are probably degraded in lysosomes by the combined action of proteases, sulfatases, hyaluronidase, β -glucuronidase, and β -*N*-acetylgalactosaminidase.^{17, 18} The fates of chondroitin sulfate B and of heparitin sulfate, both of which are essentially resistant to hyaluronidase, are obscure. The dearth of information has given rise to an implicit, though unjustified,

assumption in the literature that normally there is little turnover of these two mucopolysaccharides whose metabolism is disturbed in Hurler's syndrome.

The intracellular mucopolysaccharide in fibroblasts had *a priori* been thought to comprise a single pool, destined for secretion. Our data suggest, however, that there are two distinct intracellular pools, one of which is not secretory. First of all, the intracellular mucopolysaccharide, which is obtained in soluble form after brief sonication, is smaller in size than that secreted into the medium, and behaves as if it had less protein associated with it, as evidenced by a smaller change in size after papain treatment. Since puromycin experiments have shown that protein synthesis is a prerequisite for sulfate incorporation,³ we may not postulate that the intracellular mucopolysaccharide is awaiting "binding" to a protein before release from the cell and, instead, must presume that it is in the process of degradation. Secondly, the kinetics of secretion suggest that the bulk of the intracellular mucopolysaccharide is not the precursor of secreted material, for the cells continue to accumulate radioactivity long after the rate of secretion of macromolecule has become linear (at which time the secretory pool would have reached constant specific activity). Finally, chase experiments show that most of the pre-labeled intracellular mucopolysaccharide is not secreted as a macromolecule, but is broken down to dialyzable fragments. These data all substantiate the existence of two pools of mucopolysaccharide within the cell: a secretory pool, which is small, turns over rapidly and is probably associated with particles; and a separate pool, in which mucopolysaccharides are stored until degraded and which is relatively large, turns over slowly and is readily solubilized.

From these considerations of the metabolism of mucopolysaccharides, we can draw a schematic model to help interpret the experimental results:



At any time the rate of intracellular accumulation is the net difference between the rate of synthesis (V_1) and the rate of removal from the cell by secretion (V_3) or degradation (V_4). Immediately after introduction of isotopic sulfate, while pools A and A' are still predominantly unlabeled, the rate of isotope incorporation is a measure of the synthetic rate. Within 30 minutes the rate of secretion becomes linear, meaning that the specific activity of A must have reached that of the administered S^{35}O_4 . After that the rate of intracellular incorporation becomes $V_1 - V_3$. It is then primarily a reflection of V_2 , the rate of labeling of an intracellular pool which is turning over relatively slowly. When the specific activity of A' approaches that of the inorganic sulfate, and V_4 approaches V_2 , a steady state is reached. In both Hurler and Hunter cells, V_2 and V_3 appear normal, since the rate of mucopolysaccharide accumulation during the first six hours and the rate of mucopolysaccharide secretion are similar to those of normal fibroblasts. However, a steady state is not reached in the diseased cells since V_4 , the rate of degradation, is impaired. Instead, the intracellular pool increases indefinitely until the cytoplasm is engorged with mucopolysaccharide, as can be demonstrated histologically and chemically.^{2, 3}

Preliminary experiments have shown that part of the intracellular polysaccharide may be material that had been secreted and reingested (perhaps by pinocytosis, which is known to occur in cultured mammalian cells¹⁹). The quantitative significance of this method of accumulation has not yet been determined. However, the consequences of decreased exit from pool A' are the same, whether that pool is filled from external or internal material.

The rate of filling of the degradative pool, A', is almost half the rate of secretion. This can be seen in normal cells in the first few hours of the experiment, or in the Hurler and Hunter cells at any time. It seems surprising that the cell should divert one third of the newly synthesized mucopolysaccharide only to degrade it. Perhaps this is not a specific peculiarity of mucopolysaccharide metabolism, but one of cells secreting in tissue culture. Since both secretory products and lysosomes originate in the Golgi apparatus,²⁰ an especially vigorous synthesis of lysosomes by cultured cells might result in the entrapment of considerable material which, under other circumstances, would be secreted.^{20a}

That Hurler's syndrome might be a degradative disorder was originally proposed by van Hoof and Hers²¹ on the basis of electron micrographic studies which showed mucopolysaccharide stored in lysosome-like structures in Hurler liver cells. The hypothesis is particularly attractive since it readily explains the recessive nature of the disorder and renders it analogous to other storage diseases such as glycogenosis II²² and several sphingolipidoses.²³ Chondroitin sulfate B and heparitin sulfate excreted in Hurler urine or stored in Hurler liver and spleen are smaller than their normal counterparts found in skin and aorta, and are relatively free of protein.²⁴ It is as if Hurler lysosomes, unable to fully degrade these two mucopolysaccharides, cleaved as many bonds as possible with protease and hyaluronidase²⁵ before releasing the indigestible residue which is then excreted in the urine. The inefficiency of release of partly digested mucopolysaccharides would account for the observed accumulation in some tissues and for the pathological results. Since Hurler's syndrome is a disorder of degradation, we may deduce that the quantity of chondroitin sulfate B and heparitin sulfate excreted by affected patients (sometimes higher than 100 mg per day) is roughly equivalent to the amount catabolized by normal individuals.

The mechanism by which these mucopolysaccharides are broken down, and hence, the precise enzymatic lesion(s) in the autosomal and X-linked mucopolysaccharidoses are subjects currently under investigation.

Summary.—Kinetics of sulfated mucopolysaccharide turnover in fibroblasts derived from skin show that about two thirds of the newly synthesized mucopolysaccharide is secreted and one third diverted to an intracellular pool where it is degraded. Fibroblasts derived from the skin of patients with two genetic forms of mucopolysaccharidosis, the Hurler and Hunter syndromes, synthesize and secrete mucopolysaccharide normally but accumulate intracellular mucopolysaccharide because of inadequate degradation.

Note added in proof: The kinetics of accumulation and chase of intracellular mucopolysaccharide of Hurler and Hunter cells can be converted to a near-normal pattern if these cells are incubated with each other, or if either type is incubated with normal cells. Metachromasia is likewise lost after mixing of Hurler and Hunter cells. These preliminary data

suggest that the genetic lesion in each of the abnormal cell lines is the lack of a diffusible factor necessary for mucopolysaccharide degradation, which can be supplemented by cells of a different genotype.

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¹ McKusick, V. A., *Hereditary Disorders of Connective Tissue* (St. Louis: C. V. Mosby Co., 1966), 3rd ed.; Dorfman, A., in *The Metabolic Basis of Inherited Disease*, ed. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson (New York: McGraw-Hill, 1966), p. 963.

² Danes, B. S., and A. G. Bearn, *Science*, **149**, 987 (1965); Danes, B. S., and A. G. Bearn, *J. Exptl. Med.*, **123**, 1 (1966); *ibid.*, **124**, 1181 (1966).

³ Matalon, R., and A. Dorfman, these PROCEEDINGS, **56**, 1310 (1966).

⁴ A preliminary report of this work has been presented: Fratantoni, J. C., C. W. Hall, and E. F. Neufeld, *Federation Proc.*, **27**, 596 (1968).

⁵ Gregory, J. D., and P. W. Robbins, *Ann. Rev. Biochem.*, **29**, 347 (1960).

⁶ Results thus obtained agree well with values obtained by isolation of mucopolysaccharide as cetylpyridinium complexes, as shown in the following experiment. Carrier mucopolysaccharide (2 mg each of hyaluronic acid and chondroitin sulfate C) was added to the ethanol-extracted cell residue and to the dialyzed medium, and these were subjected to papain digestion, prolonged dialysis to remove amino acids and peptides, precipitation with cetylpyridinium chloride, dissociation of the complex with 1.5 M NaCl, precipitation in 66% ethanol, and drying. The specific activity (cpm per mg of carrier uronic acid) was tested before the dialysis, after dialysis, and at the end of the entire procedure, and was shown to remain essentially constant during all these manipulations: Hurler cell residue 149,700, 162,400, 156,700; normal cell residue 40,900, 41,200, 37,700; Hunter medium 121,400, 132,800, 135,100; normal medium 91,600, 91,900, 88,800.

⁷ Davidson, E. A., in *Methods in Enzymology*, ed. E. F. Neufeld and V. Ginsburg (New York: Academic Press, 1966), vol. 8, p. 53.

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⁹ Antonopoulos, C. A., *Acta Chem. Scand.*, **16**, 1521 (1962).

¹⁰ Haruki, F., and J. E. Kirk, *Biochim. Biophys. Acta*, **136**, 391 (1967).

^{10a} If normal plates are much heavier than recommended, the intracellular pool of labeled mucopolysaccharide does not reach a steady state but keeps increasing at a slow rate. The reason for this is unknown.

¹¹ This method does not separate heparitin sulfate from chondroitin sulfate B. However, Matalon and Dorfman³ found no heparitin sulfate in fibroblasts. Further identification of the mucopolysaccharides is in progress.

¹² Rodén, L., *Abstracts, 7th International Congress on Biochemistry, Tokyo, 1967*, (International Union of Biochemistry, 1967), vol. 1, p. 69.

¹³ Godman, G. C., and N. Lane, *J. Cell Biol.*, **21**, 353 (1964).

¹⁴ Fewer, D., J. Threadgold, and H. Sheldon, *J. Ultrastruc. Res.*, **11**, 166 (1964).

¹⁵ Suzuki, S., M. Okayama, K. Kimata, T. Yamagata, H. Saito, M. Hoshino, and I. Suzuki, *Abstracts, 7th International Congress on Biochemistry, Tokyo, 1967* (International Union of Biochemistry, 1967), vol. 4, p. 704; Horwitz, A., and A. Dorfman, *Federation Proc.*, **27**, 346 (1968).

¹⁶ Telser, A., H. C. Robinson, and A. Dorfman, these PROCEEDINGS, **54**, 912 (1965).

¹⁷ Hutterer, F., *Biochim. Biophys. Acta*, **115**, 312 (1966).

¹⁸ Tudball, N., N. Aronson, Jr., and E. A. Davidson, *Federation Proc.*, **26**, 856 (1967).

¹⁹ Ryser, H. J. P., *Science*, **159**, 390 (1968).

²⁰ DeDuve, C., and R. Wattiaux, *Ann. Rev. Physiol.*, **28**, 435 (1966).

^{20a} The amount of mucopolysaccharide secreted is somewhat underestimated, since there exists an intercellular pool, released by trypsin, which is not accounted for in the present data.

²¹ Van Hoof, F., and H. G. Hers, *Compt. Rend.*, **259**, 1281 (1964).

²² Hers, H. G., *Advan. Metab. Disorders*, **1**, 1 (1964).

²³ Brady, R. O., *New Engl. J. Med.*, **275**, 312 (1966).

²⁴ Knecht, J., J. A. Cifonelli, and A. Dorfman, *J. Biol. Chem.*, **242**, 4652 (1967).

²⁵ Fransson, L. A., and L. Rodén, *J. Biol. Chem.*, **242**, 4170 (1967).