THE DEFECT IN HURLER AND HUNTER SYNDROMES, II. DEFICIENCY OF SPECIFIC FACTORS INVOLVED IN MUCOPOLYSACCHARIDE DEGRADATION

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Abstract.—Cultured fibroblasts, derived from patients with the Hurler and Hunter syndromes, show defective degradation of sulfated mucopolysaccharide. The aberrant metabolism of Hurler cells can be corrected by secretions of fibroblasts of genotype other than Hurler, and similarly, the defect of Hunter cells can be corrected by secretions of fibroblasts of genotype other than Hunter. The active factors in these secretions, which are heat labile and associated with macromolecules, accelerate the degradation of mucopolysaccharide.

The Hurler and Hunter syndromes are human genetic disorders of sulfated mucopolysaccharide (MPS) metabolism.[1] Affected individuals excrete in their urine and accumulate in their tissues excessive quantities of two MPS, chondroitin sulfate B and heparitin sulfate.[2] This biochemical derangement gives rise to severe clinical manifestations: gross skeletal deformities, thickening of blood vessel walls, enlargement of liver and spleen, retarded growth and mentation and early death. The two diseases are genetically distinct: the Hurler syndrome is transmitted as an autosomal recessive, while the Hunter syndrome, also recessive, is X-linked. The basic molecular lesion must therefore differ, though the laboratory and clinical manifestations are very similar.

Studies of these disorders have been stimulated by the discovery that fibroblasts cultured from the skin of affected individuals also accumulate excessive quantities of MPS, especially chondroitin sulfate B.[3, 4] We have previously shown that the kinetics of this accumulation exclude a defect in biosynthesis or secretion, but are compatible with a defect in the degradative mechanism.[5] We subsequently reported that when fibroblasts derived from Hurler and Hunter individuals are mixed with each other, or with normal fibroblasts, the kinetic pattern of MPS turnover was restored to normal. The correction did not require cell to cell contact, but was mediated by substances released into the medium.[6]

This phenomenon has now been studied further.[7] Data presented in this paper show that the corrective factors, which are heat labile and associated with macromolecules, accelerate the degradation of intracellular MPS in recipient cells. The factor which corrects the defect in Hurler cells is produced by all genotypes tested other than Hurler, and likewise, the factor which corrects the defect in Hunter cells is produced by all genotypes tested other than Hunter. Therefore, the deranged MPS metabolism in Hurler and Hunter fibroblasts is caused by the lack of factors specific for each defect.

Materials and Methods.—Fibroblast cultures were derived from skin biopsies and maintained in 100-mm Falcon plastic Petri plates in an atmosphere of 95% air and 5% CO_2.

Eagle's Minimum Essential medium with Earle salts and 10% fetal calf serum was modified by the substitution of $MgCl_2$ for $MgSO_4$, by the addition of nonessential amino acids and the following antibiotics: penicillin, 100 units/ml, streptomycin, 100 units/ml, and nystatin, 60 units/ml, and by reduction of the $NaHCO_3$ concentration to 1.6 g/L. The inorganic sulfate concentration was 0.4 mM. When protein-free medium was required, the formulation of Gorham and Waymouth[8] was used, with the following modifications: thymidine, serine, and alanine were reduced to one tenth the concentration specified; $MgSO_4$ was replaced by $MgCl_2$; phenol red, 5 μg/ml, additional glutamine, 292 μg/ml, and antibiotics as above, were added.

Procedures used to study MPS metabolism have been described in detail.[5] In brief, Petri plates containing approximately 1 mg of cell protein are incubated in the presence of 5–10 μC of $^{35}SO_4$ per ml of medium and samples analyzed periodically for radioactive MPS (MPS^{35}) content. Alternatively, cells are prelabeled by incubation for two days in medium containing $^{35}SO_4$; intracellular MPS^{35} is measured at intervals after replacement of radioactive with unlabeled medium. Advantage is taken of the insolubility of mucopolysaccharides in boiling 80% ethanol in performing these analyses. All incubations were carried out in the modified Eagle's medium, unless otherwise specified.

For preparation of corrective factors, we used medium which had been in contact with cells for 3 to 4 days. One to two heavily grown plates, visually estimated to be equivalent to 2 to 4 mg of cell protein, were used to prepare corrective factor for one test plate. For large quantities, the use of Bellco bottles (1410 cm^2) in a Bellco roller apparatus proved more convenient than the equivalent number of Petri plates. When the medium contained serum, factor activity was concentrated by precipitation with 70% ammonium sulfate; the precipitate was dissolved in water (1 ml for material from one plate) and dialyzed for 18 hr against four changes of 0.9% NaCl. Concentration by ultrafiltration gave better results when the medium contained no serum; the device obtained from the Amicon Corporation was used with either of two filters with exclusion limits of molecular weight 1,000 or 10,000 and 80 to 90 psi positive pressure under nitrogen. Medium from one roller bottle (150 ml) was reduced in volume to 5–10 ml, dialyzed against several changes of 0.9% NaCl for 4 to 18 hr. The preparations could be frozen for two weeks. They were passed through a bacteriological filter (Millipore Swinnex 13) before administration to cells. Where not otherwise specified, the corrective factors were prepared from the modified Eagle's medium with fetal calf serum.

Incubation of factor with hydrolytic enzymes was carried out under the following conditions. Each milliliter of incubation mixture contained serum-free factor preparation from a normal culture (approximately 0.2 mg of protein, 6 μg of nucleic acid), enzyme (trypsin, Calbiochem, 1.25 mg; pancreatic RNase, Worthington, 0.5 mg; or pancreatic DNase, Worthington, 0.5 mg) in 5 mM Tris buffer, pH 7.5, which contained 2.5 mM $CaCl_2$ for the trypsin experiment and 0.5 mM $MgCl_2$ for the DNase experiment. After 3 hr at 37°, 1 ml of the mixture plus 5 ml of fresh medium was applied per plate of prelabeled Hurler or Hunter cells and intracellular MPS^{35} was measured a day later.

Results.—Effect of corrective factors on the turnover of the intracellular pool: It had been shown previously that mixing cells of the Hurler and Hunter genotypes changes their characteristic pattern of radioactive MPS accumulation and decay to one which approaches the normal.[6] Typical of the latter is that the pool is smaller and turns over more rapidly than in the abnormal cells.

The same change in kinetic pattern is obtained when the appropriate factor preparations are used. As shown in Figure 1, Hurler cells, in the presence of factor from their own genotype, accumulate radioactive MPS at a nearly linear rate for four days (which is similar to that without added factor). In the presence of factor prepared from Hunter medium the curve approaches a plateau in two days, similar to the pattern of normal cells. A converse relationship holds

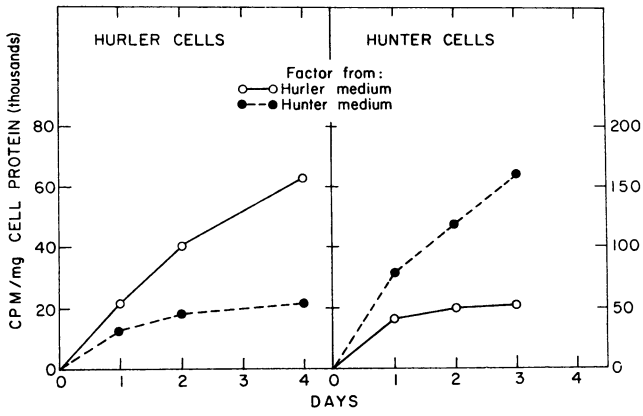

FIG. 1.—Accumulation of labeled MPS by Hurler and Hunter fibroblasts in the presence of factor preparations.

for Hunter cells, which are corrected by a preparation from Hurler culture medium.

Figure 2 confirms this finding using a pulse-chase method, wherein the cells are prelabeled for two days, radioactive medium is replaced with unlabeled medium and the disappearance of the radioactive MPS followed. Factor preparation from a different genotype accelerates the decay of the MPS^{35} pool. Changes in the half-life of that pool, calculated from semilog plots of the data in Figure 2, are from three days to 22 hours for the Hurler cells, and from two days to 13 hours for the Hunter cells. The usual half-life of the pool in normal cells is 8 hours.

The increased rate of turnover of the intracellular MPS in the presence of the corrective factors is due to increased degradation, as shown for Hunter fibroblasts in Figure 3. The radioactive MPS which disappears from the cells during

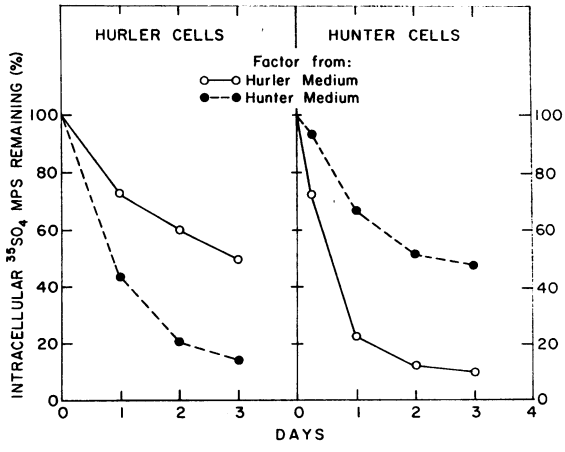

FIG. 2.—Chase of labeled MPS by Hurler and Hunter fibroblasts in the presence of factor preparations.

incubation with unlabeled medium is quantitatively recovered in the medium; in the control (*i.e.*, in the presence of factor prepared from a Hunter culture), 12 per cent is released in macromolecular form, while 29 per cent is dialyzable—that is, degraded. In the presence of the factor preparation from a normal culture, essentially all the MPS (82%) has been degraded. Likewise, in Hurler cells, the counts lost from intracellular MPS upon addition of the appropriate factor appear in the medium in dialyzable form.

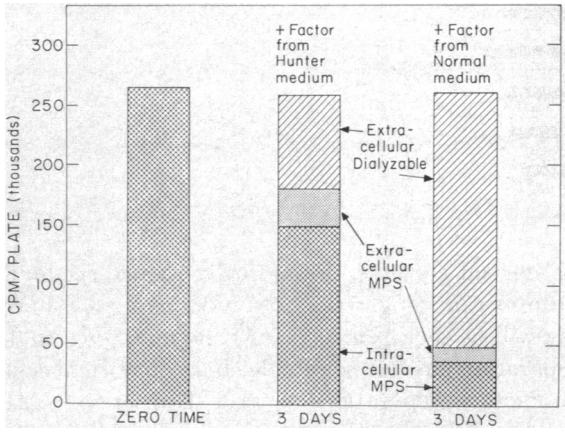

FIG. 3.—Effect of factor preparations on the fate of labeled MPS in Hunter fibroblasts. Cells were prelabeled for two days, trypsinized, and replated in unlabeled medium with factors from normal or Hunter cultures.

Specificity of corrective factors: The ability of preparations to exert a corrective effect depends on the genotype from which they were obtained. In the experiment shown in Figure 4, ammonium sulfate concentrates were prepared from culture medium of fibroblasts derived from patients with a variety of genetic diseases. The corrective activity of these preparations was compared with that of a control obtained by precipitating unused Eagle's medium.[9]

Of all those tested, only preparations from Hurler medium failed to lower MPS accumulation in Hurler cells, and conversely, only preparations from Hunter medium failed to affect Hunter cells. None of the preparations used in that experiment affected normal fibroblasts. It is interesting to note that a preparation from a Hunter heterozygote corrects Hunter cells. Since there was no attempt to quantitate production of factor activity per cell, we do not know whether the amount of factor produced is proportional to the gene dose.

Effect of serum protein on correction: Fibroblasts are routinely cultured in medium containing added serum. Since this would interfere with procedures designed to characterize the factors, we attempted to prepare serum-free material. Fibroblasts grown in Eagle's medium with fetal calf serum and subsequently fed Gorham-Waymouth synthetic medium remain viable and attached to glass and plastic surfaces for several weeks. Ultrafiltered concentrates of this medium,

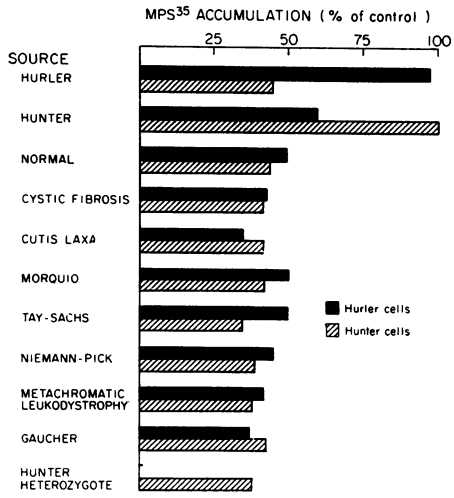

FIG. 4.—Effect of factor preparations from cultures of various genotypes on accumulation of labeled MPS in Hurler and Hunter fibroblasts.

preincubated with normal cultures, dramatically reduce accumulation of radioactive MPS by Hunter cells (Fig. 5). Activity is expressed in terms of protein content, though as will be seen below, there is no assurance that the factor is a protein. The preparation has but neglibible effect on normal cells.

The serum-free factor preparations correct Hunter cell MPS metabolism equally well when administered to the recipient cells in the presence or absence of serum. Ability to correct Hunter cells is therefore independent of serum proteins. The corrective factor for Hurler cells, however, became elusive when prepared in the absence of serum. It was totally absent from preparations dialyzed overnight, and present, though of low activity, in preparations dialyzed less than 7 hours. It is not yet clear whether serum is required for Hurler factor production, stabilization, or both.

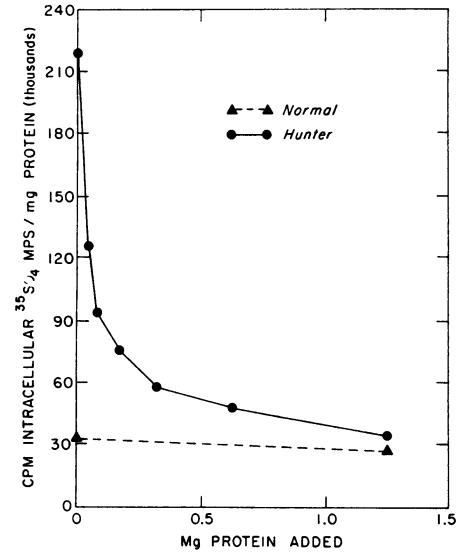

FIG. 5.—Dose response of normal and Hunter fibroblasts to a serum-free preparation from normal cells.

Preliminary experiments show both factors present in the 100,000 × g supernatant fraction of sonicated normal cells.

Characteristics of the corrective factors: (1) The factors are associated with macromolecules, as already evident from the method of preparation. In the presence of serum proteins, they are excluded from Sephadex G-100 and retarded by Sephadex G-200; however, their apparent size may be affected by binding to serum proteins. In the absence of serum, Hunter factor (i.e., the factor which corrects Hunter cells) moves as a broad peak toward the front of G-200 (equivalent to a protein molecular weight of 200,000 to 300,000). Because of the difficulties mentioned above in obtaining serum-free Hurler factor, we have not attempted to determine its size. (2) The factors are heat labile. Both in the presence and absence of serum proteins, activity is lost upon heating ten minutes at 100°. (3) The factors are not sulfated MPS, since the latter are excluded from Sephadex G-200. (4) The factors are probably not nucleic acids. Absorbance at 260 and 280 shows that a serum-free preparation such as was used in Figure 5 contains no more than 0.03 times as much nucleic acid as protein. Treatment of such a preparation with DNase or RNase did not affect Hurler or Hunter factor activity. (5) Are the factors protein? From the information above, one might speculate that they are, yet direct experimental evidence is lacking. Treatment with a high concentration of trypsin did not destroy the activity of either factor; however, this result is ambiguous as the biological activity of proteins is sometimes resistant to trypsin action.[10] Attempts to use pronase were unsuccessful, since pronase itself affects both the MPS metabolism and viability of the cells. (6) The factors are probably not β-galactosidases. The suggestion that they might be was recently made[11] because of the repeated observation of partial β-galactosidase deficiency in some tissues of Hurler and Hunter patients.[12] Corrective factor preparations from serum-free medium have essentially no β-galactosidase activity, as measured by hydrolysis of o-nitrophenyl galactoside.

Discussion.—Previous studies[5] indicated that fibroblasts secrete part of the newly synthesized sulfated MPS into the external medium, while the remainder is retained intracellularly and degraded. Each of the three processes—synthesis, secretion and degradation—must be associated with a finite intracellular pool of sulfated macromolecules. But because the synthetic and secretory pools are relatively small and rapidly saturated, they do not contribute significantly to the measured intracellular MPS[35]; the latter consists, therefore, almost entirely of material stored for degradation. Preliminary experiments suggest that this degradative pool is lysosomal.[13]

In fibroblasts derived from Hurler and Hunter patients, the amounts of MPS synthesized and secreted are similar to those in normal cells. However, the intracellular MPS pool is larger and turns over more slowly in the diseased cells; thus, half-lives of two to six days have been determined from pulse-chase experiments on Hurler and Hunter cells, as compared with eight hours for the normal (absolute rates cannot be given since the specific activity of the intracellular pool is not known). When accumulation of radioactive MPS is measured, this difference is manifested by normal cells reaching a steady state between 24 and 48

hours, while the abnormal ones continue to accumulate for many days. The slower turnover of the intracellular MPS has been attributed to a defect in the degradative pathway—presumably a different defect in each of the two genetic disorders.[5, 6] Further definition of the defects has been hampered by lack of information of the normal mechanisms by which the relevant MPS, chondroitin sulfate B and heparitin sulfate, are degraded.[14]

In the presence of factor preparations from fibroblasts of the appropriate genotype, the kinetic pattern of sulfated MPS metabolism in Hurler and Hunter cells is changed towards the normal. Degradation is increased; the intracellular MPS pool is reduced in size and its turnover accelerated. The mechanism by which this occurs is unknown, though it appears reasonable to speculate that the corrective factors replace their defective counterparts in the abnormal cells. The likelihood of such a mechanism is not diminished by the fact that it may require the entrance of macromolecules into the cells, for fibroblasts are known to take up macromolecules by pinocytosis.[15]

It is clear that the corrective factors are products of the alleles of those genes that are mutant in the Hurler and Hunter cells, since they can be found in all other genotypes tested. Present data, however, do not allow us to say that they are the primary products—i.e., the proteins whose structures are encoded in those alleles. The absence of the appropriate factor is responsible for the observed accumulation of MPS, a finding consistent with the recessive nature of the disorders. We conclude, therefore, that in the Hurler and Hunter syndromes, the lack of factor required for mucopolysaccharide degradation is the most specific defect thus far reported.

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[2] Synonyms: Chondroitin sulfate B = dermatan sulfate; heparitin sulfate = heparan monosulfate.

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[7] A preliminary report of this work has been presented at the meeting of the American Society for Clinical Investigation, Atlantic City, May 4, 1969; *J. Clin. Invest.*, **48**, 26a (1969).

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[9] Preparations from unused Eagle's medium, which contains fetal calf serum, lowered the accumulation of MPS^{35} to a variable degree (from 0 to 20%) compared to saline controls. There are evidently substances in the calf serum which affect fibroblast MPS metabolism. Human serum is known to affect it significantly (Hors-Cayla, M. C., P. Maroteaux, and J. de Grouchy, *Ann. Genetique*, **11**, 265 (1968)).

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