Biochemical Studies on the Sulphated Glycosaminoglycan Fraction of Skin Fibroblasts Cultured from a Patient with the Hurler Syndrome

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1. The metabolism of the sulphated glycosaminoglycan fraction in cultured skin fibroblasts derived from a patient with the Hurler syndrome and from a normal subject was studied. Two labelled precursors, $\text{Na}_2^{35}\text{SO}_4$ and D-[2- ^3H]glucose, were used and their intracellular fates during uptake and 'chase' periods were assessed after separation of sulphated glycosaminoglycans from hyaluronic acid. After 4 or 8 h of exposure to culture medium containing both labels, [^{35}S]sulphate incorporation into the sulphated glycosaminoglycan fraction was twofold greater in Hurler-syndrome cells than in normal cells. At the same time, the rate of incorporation of [^3H]glucose into the sulphated glycosaminoglycan fraction was approximately the same for both cell types. Consequently, an increased $^{35}\text{S}/^3\text{H}$ ratio (nmol of [^{35}S]sulphate incorporated/nmol of [^3H]glucose incorporated) was observed for Hurler-syndrome cells compared with normal cells. 2. The results of 'chase' experiments revealed that although the expected loss and relative retention of labelled sulphate occurred in the sulphated glycosaminoglycan fraction of normal and Hurler-syndrome cells, both cell types retained all of their radioactivity derived from [^3H]glucose. 3. After 34 h exposure to a 'corrective-factor' preparation from urine, the sulphated glycosaminoglycan content (as hexosamine and [^{35}S]sulphate) of the Hurler-syndrome cells approached normal values. At the same time, there was an increase in specific radioactivity of 'corrected' Hurler-syndrome cells.

The Hurler syndrome is an inborn error of glycosaminoglycan metabolism determined by an autosomal recessive mutation. Accumulation of extensively degraded derivatives of dermatan sulphate and heparan sulphate in various tissues is a major pathogenetic element of the disease. Preservation of the basic biochemical phenotype in serially subcultured skin fibroblast cell strains (Schafer *et al.*, 1966; Danes & Bearn, 1966; Matalon & Dorfman, 1966) has led to their use as a source of glycosaminoglycans for structural and metabolic studies. Faulty degradation of dermatan sulphate and heparan sulphate owing to a deficit of α -L-iduronidase (Matalon *et al.*, 1971; Matalon & Dorfman, 1972) appears to be the primary biochemical defect in the Hurler syndrome. Nevertheless, multiple consequences of this enzyme deficit remain to be clarified. These include: (1) the explanation for storage in liver and spleen of readily soluble, highly degraded glycosaminoglycans (Dorfman, 1964; Knecht *et al.*, 1967; Dean *et al.*, 1971); (2) the presence of hypersulphated dermatan sulphate in the spleen of a Hurler-syndrome patient (Calatroni & DiFerrante, 1968); (3) the reason for hyaluronic acid accumulation in cultured skin fibroblasts (Matalon & Dorfman, 1966; Schafer *et al.*, 1968).

The present paper describes a study of sulphated glycosaminoglycan metabolism in Hurler-syndrome and normal cultured skin fibroblasts by using a double-labelling technique. The concomitant incorporation of [^3H]glucose and [^{35}S]sulphate into the sulphated glycosaminoglycans of Hurler-syndrome and normal cells permitted two metabolically separate parts of this fraction to be examined. Previous studies of this type (Matalon & Dorfman, 1966; Danes & Bearn, 1966) are not comparable with the present one, since, in the earlier studies, separation of intracellular glycosaminoglycans into sulphated and hyaluronic acid fractions was not performed. The double-label method has also been applied here to Hurler-syndrome and normal fibroblasts exposed to a crude preparation of corrective factor (Neufeld & Cantz, 1971).

The disturbance of hyaluronic acid metabolism present in the Hurler-syndrome cells is described in the accompanying paper (Germinario *et al.*, 1973).

Materials and Methods

Cell culture

The strain of Hurler-syndrome fibroblasts was obtained from Dr. Elizabeth F. Neufeld (National

Institutes of Health, Bethesda, Md. 20014, U.S.A.). It was derived from a patient with the typical clinical syndrome and has been characterized *in vitro* by its inability to produce Hurler-syndrome corrective factor (E. F. Neufeld, personal communication). Both cell types were cultured in roller bottles (Bellco; 670 cm²) (Bellco, Vineland, N.J., U.S.A.) in Eagle's minimal essential medium (Eagle, 1959) supplemented with 1 mM-pyruvate and 12% (v/v) calf serum (a 1:1 mixture of foetal and newborn calf serum; Flow Laboratories Inc., Rockville, Md., U.S.A., mycoplasma-screened). Repeated samples of these cultures were sent to the laboratory of Dr. L. Hayflick (Stanford University) for mycoplasma screening with negative results. The cells were grown in an atmosphere of CO₂ + air (5:95), the medium being changed every 3 days. The cells were harvested from the roller bottles after incubation for 10 min with 0.02% (w/v) EDTA and 5 min with 0.25% (w/v) trypsin (Difco Labs., Detroit, Mich., U.S.A.) (Martin, 1964).

Isolation of glycosaminoglycans

The glycosaminoglycans were isolated from cells after digestion with papain (Schwarz/Mann, Orangeburg, N.Y., U.S.A.) and precipitation with 1% (w/v) cetylpyridinium chloride (KABI, Stockholm, Sweden). Cetylpyridinium chloride-saturated cellulose micro columns were prepared as described by Antonopoulos *et al.* (1961), and separation into hyaluronic acid and sulphated fractions was done by a modification (H. Triller & N. Kalant, personal communication) of the procedure originally described by Thunell *et al.* (1967). The modification concerned only the separation of the sulphated glycosaminoglycans. After elution of the hyaluronic acid with 0.3 M-NaCl in 0.05% (w/v) cetylpyridinium chloride (Thunell *et al.*, 1967), the sulphated glycosaminoglycans were eluted in one step with 1.5 M-MgCl₂ in 0.05% (w/v) cetylpyridinium chloride. The fractionation was done at 30–34°C.

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951) with a bovine serum albumin standard (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and DNA by the method of Burton (1956). Hexosamines were determined by a modified Elson-Morgan reaction (Antonopoulos *et al.*, 1964). Sulphate was determined by a modification of the benzidine precipitation method (Antonopoulos, 1962).

Double-labelling method

D-[2-³H]Glucose and carrier-free Na₂³⁵SO₄ were obtained from New England Nuclear Corp. (Boston,

Mass., U.S.A.). All samples were counted for radioactivity in 10 ml of a scintillation solution prepared as previously described (Kahlenberg, 1969). Double-radioisotope counting was done in a liquid-scintillation spectrometer (Packard Tri-Carb model 3320), automatic external standardization being used to correct for overlap (26–27%) of ³⁵S into the ³H channel. Counting efficiencies were approx. 23 and 37% for ³H and ³⁵S respectively. A Wang 700A/701 programmable calculator was used for the processing of liquid-scintillation data.

Labelling experiment protocol

Sulphate-free minimal essential medium (Grand Island Biological Co., Grand Island, N.Y., U.S.A.) was employed in labelling experiments. It contained 0.25 mM-sulphate after addition of streptomycin sulphate (Unik Medical Laboratories, Montreal, P.Q., Canada). At the time of their use in these experiments, both normal and mutant cultures were visually confluent and had approximately the same population density. Each experiment included a 2 h preincubation in unlabelled medium and an 8 h incubation in double-labelled medium. For 'chase' experiments, there followed a 12 h incubation in unlabelled medium. The specific radioactivities of D-glucose (6 × 10⁵ d.p.m./nmol) and sulphate (12.5 × 10⁶ d.p.m./nmol) in the minimal essential medium were determined to allow quantitative measurement of the amount (nmol) of substrate incorporated into the glycosaminoglycan fractions. Incorporation is defined as the net difference between the rate of synthesis of labelled glycosaminoglycans minus the rate of removal of labelled glycosaminoglycans from the cell by secretion plus degradation.

Preparation of urine corrective factor

A crude preparation of urine corrective factors was prepared as described by Neufeld & Cantz (1971). This preparation is now known to contain α-L-iduronidase, the specific Hurler-syndrome corrective factor (Bach *et al.*, 1972). Freshly collected human urine was concentrated tenfold in an Amicon pressure chamber (model 2000) fitted with a Diaflo XM-50 membrane filter. The concentrated urine was dialysed at 5°C against several changes of 0.15 M-NaCl and two changes of 0.15 M-NaCl–0.01 M-sodium phosphate buffer, pH 6, for 6 h.

Correction experiment protocol

The experimental protocol included a 12 h incubation period of normal and Hurler-syndrome cells in minimal essential medium (1 mM-sulphate) with the corrective-factor preparation. This was followed by labelling and 'chase' periods during which the corrective-factor preparation was present in the medium.

The amount of urine protein used in a typical experiment was 0.1 mg/ml of medium.

Results

The inability of cultured Hurler-syndrome cells to lose [^{35}S]sulphated glycosaminoglycans when 'chased' with unlabelled medium is shown in Fig. 1. Such results, together with normal initial uptake and secretion patterns of sulphate, caused Neufeld and her colleagues (Fratantoni *et al.*, 1968, 1969) to conclude that defective degradation was the proximate cause of sulphated glycosaminoglycan storage in Hurler-syndrome cells. This disability was overcome in Hurler-syndrome cells exposed to corrective factor (Fig. 1), thereby demonstrating characteristic behaviour of the cell strain used in these experiments.

To supplement the valuable results of Neufeld's group, simultaneous measurements of incorporation of glucose and sulphate into the isolated sulphated glycosaminoglycan fraction of Hurler-syndrome and normal cells were obtained (Fig. 2). The results described in Fig. 2(a) show that the amounts (nmol) of [^{35}S]sulphate recovered from the sulphated glycos-

aminoglycan fraction after 4 and 8 h exposure to the label were twofold higher in the Hurler-syndrome cells than in the normal cells, but the amounts (nmol) of [^3H]glucose concomitantly incorporated into the sulphated glycosaminoglycan fraction of normal and Hurler-syndrome cells were the same (Fig. 2b). These results yielded an increased $^{35}\text{S}/^3\text{H}$ ratio (nmol of [^{35}S]sulphate incorporated/nmol of [^3H]glucose incorporated) in the Hurler-syndrome cells than in the normal cells. This difference in the $^{35}\text{S}/^3\text{H}$ ratio was observed in each of eight separate experiments done under the same conditions (Fig. 3). The variation among experiments could not be related to the age of the cell strains *in vitro* at the time these experiments were performed. Increasing the routine sulphate concentration in the medium eightfold (to 2 mM) did not affect the amount of [^{35}S]sulphate incorporated into an ethanol precipitate of the cell pellet.

Having established the uptake patterns of the two labels, i.e. increased amounts of [^{35}S]sulphate incorporated into the sulphated glycosaminoglycans of the Hurler-syndrome cells with equal amounts of [^3H]glucose incorporated into the same fraction of

Fig. 1. Changes in the contents of [^{35}S]sulphated glycosaminoglycans of cultured skin fibroblasts in 'pulse-chase' experiments

The results show the intracellular [^{35}S]sulphated glycosaminoglycans remaining in ethanol precipitates of the cell pellets, in normal cells (○), Hurler-syndrome cells (●), normal cells exposed to the corrective-factor preparation (□) and Hurler-syndrome cells exposed to the corrective-factor preparation (■). Procedures were as described by Fratantoni *et al.* (1968, 1969). The cells were exposed to the crude corrective-factor preparation (0.1 mg of urine protein/ml of medium) and [^{35}S]sulphate for 48 h before 'chasing' with unlabelled medium.

Fig. 2. Incorporation of $[^{35}\text{S}]$ sulphate and ^3H from $[^3\text{H}]$ glucose into the sulphated glycosaminoglycan fraction

The incorporation of $[^{35}\text{S}]$ sulphate (a) and of ^3H from $[^3\text{H}]$ glucose (b) into the sulphated glycosaminoglycan fraction of normal (○) and Hurler-syndrome (●) cells after 4 and 8 h exposure to double-labelled medium is shown. All experiments included a 2 h preincubation in unlabelled medium before exposure to double-labelled medium.

Fig. 3. $^{35}\text{S}/^3\text{H}$ ratios observed in normal and Hurler-syndrome cells after 4 h exposure to double-labelled medium

The results show the $^{35}\text{S}/^3\text{H}$ ratios obtained in eight separate experiments (RG-1–RG-8) performed under the same conditions for normal (□) and Hurler-syndrome (■) cells. The labelling protocol was as in Fig. 2.

both cell types, a series of 'chase' experiments was undertaken to determine the catabolic fate of both labels in Hurler-syndrome and normal cells. After

8 h exposure to double-labelled medium, the cells were 'chased' with unlabelled medium (Figs. 4a and 4b). The normal cells rapidly lost $[^{35}\text{S}]$ sulphate from

Fig. 4. Loss of radioactivity from the sulphated glycosaminoglycan fraction during the 'chase' with unlabelled medium

(a) Retention of radioactivity derived from $[^3\text{H}]$ glucose and (b) the retention of $[^{35}\text{S}]$ sulphate in the sulphated glycosaminoglycan fraction of normal (○) and Hurler-syndrome cells (●). The labelling protocol was as in Fig. 2. The 'chase' portion of the experiment was performed by using unlabelled medium.

the sulphated glycosaminoglycan fraction (Fig. 4b) but retained the radioactivity derived from $[^3\text{H}]$ glucose (Fig. 4a). The Hurler-syndrome cells retained radioactivity derived from $[^3\text{H}]$ glucose (Fig. 4a) but did not lose $[^{35}\text{S}]$ sulphate from the sulphated glycosaminoglycans as efficiently as did normal cells (Fig. 4b).

Effects of the corrective-factor preparation

The 'correction' experiment in Fig. 1 was done by the method of Fratantoni *et al.* (1969) (i.e. Hurler-syndrome and normal cells were exposed to corrective factor and $[^{35}\text{S}]$ sulphate for 48 h before being 'chased' with unlabelled medium). In subsequent experiments performed by the method described here, 'correction' of the Hurler-syndrome cells from the viewpoint of $[^{35}\text{S}]$ sulphate retention was not seen clearly until 12 h after the 'chase' (i.e. 34 h exposure to the corrective-factor preparation; Fig. 5a), at which time the content of sulphated glycosaminoglycans in the 'corrected' cells approached normal values (Fig. 5b). Additionally, Fig. 5(c) shows that there was a simultaneous increase in the specific radioactivity (nmol of $[^3\text{H}]$ glucose/nmol of hexosamine) of the sulphated glycosaminoglycan fraction of the 'corrected' Hurler-syndrome cells. This indicated that corrective factor induced a preferential loss of unlabelled sulphated glycosaminoglycans stored in Hurler-syndrome cells. As noted above, from the viewpoint of $[^3\text{H}]$ glucose incorporation, the Hurler-syndrome and normal cells expressed little difference in their sulphated glycosaminoglycan fractions after

8 h exposure to double-labelled medium (Fig. 6a). During the 'chase' period, however, striking changes occurred in normal cells exposed to corrective factor (Fig. 6b), whereas unexposed Hurler-syndrome, normal and exposed Hurler-syndrome cells were all essentially similar (Fig. 6b).

Discussion

The Hurler-syndrome cell strain used in these experiments was first characterized with regard to sulphate retention and responsiveness to a corrective-factor preparation (Fig. 1) with methods described by Fratantoni *et al.* (1968, 1969). Then experiments on the isolated sulphated glycosaminoglycan fraction of the cells were done.

Increased incorporation of $[^{35}\text{S}]$ sulphate into the sulphated glycosaminoglycan fraction of Hurler-syndrome cells compared with normal cells was apparent after 4 h of exposure to the label.

Despite accelerated $[^{35}\text{S}]$ sulphate incorporation, the Hurler-syndrome cells did not incorporate radioactivity from $[^3\text{H}]$ glucose at a faster rate than normal. These results caused an increased $^{35}\text{S}/^3\text{H}$ ratio in the sulphated glycosaminoglycan fraction of Hurler-syndrome cells than in normal cells.

Incorporation of $[^{35}\text{S}]$ sulphate in excess of ^3H from $[^3\text{H}]$ glucose prompted reconsideration of the idea that the Hurler syndrome resulted from a biosynthetic defect expressed primarily as oversulphation of certain species of glycosaminoglycans, and secondarily by defective degradation. Results of previous studies were compatible with this hypothesis

Fig. 5. Effects of a crude corrective-factor preparation on the sulphated glycosaminoglycan fractions of normal and Hurler-syndrome cells during the 'chase' with unlabelled medium

(a) Retention of $[^{35}\text{S}]$ sulphate in the sulphated glycosaminoglycan fraction by normal (○), Hurler-syndrome (●) and corrective-factor-exposed Hurler-syndrome (■) cells. (b) Sulphated glycosaminoglycan content (as hexosamine) in normal (□), Hurler-syndrome (■) and corrective-factor-exposed Hurler-syndrome (▨) cells. (c) Specific radioactivity in corrective-factor-exposed (■) and unexposed (●) Hurler-syndrome cells. The Hurler-syndrome and normal cells were exposed to the crude preparation of corrective factor (0.1 mg of urine protein/ml of medium) for 12 h before the labelling and 'chase' protocols were done as described in the legends to Figs. 2 and 4 respectively.

(Matalon & Dorfman, 1966; Calatroni & DiFerrante, 1968; Schafer *et al.*, 1968; Dean *et al.*, 1971; DiFerrante *et al.*, 1971; Germinario *et al.*, 1971). However, the interpretation of 'chase' experiments reported here, and evidence bearing on the properties of Hurler-syndrome corrective factor (Neufeld & Cantz, 1971; Barton & Neufeld, 1971) make this extremely unlikely.

Several mechanisms could explain the increased $^{35}\text{S}/^3\text{H}$ ratio in the sulphated glycosaminoglycan fraction of the Hurler-syndrome cells: they are not necessarily exclusive, and certain combinations could be complementary. They include: (1) exchange of ^{32}S for ^{35}S on preformed molecules of dermatan sulphate; (2) regional oversulphation of disaccharide units containing iduronic acid within native molecules of dermatan sulphate; (3) preferential desulphation during the course of normal dermatan sulphate degradation; and (4) preferential utilization of ^3H -labelled degradation products during resynthesis of new sulphated glycosaminoglycan molecules.

If 'exchange' were predominantly responsible, rapid loss of $[^{35}\text{S}]$ sulphate from Hurler-syndrome cells would have been expected during the 'chase' period, a result not observed (Figs. 1, 4b and 5a). The capacity of dermatan sulphate for hypersulphation is exemplified by the demonstration of sulphate-acceptor activity of dermatan sulphate of chick embryo cartilage (Meezan & Davidson, 1967), and is supported by the finding that oligosaccharide fragments from dermatan sulphate rich in glucuronic acid are not oversulphated (Fransson & Rodén, 1967b), whereas those resistant to hyaluronidase (i.e. rich in iduronic acid) are (Fransson & Rodén, 1967a). Further, oversulphated dermatan sulphate has been extracted from Hurler-syndrome spleen (Calatroni & DiFerrante, 1968). Deficient α -L-iduronidase activity might enhance the capacity of dermatan sulphate molecules for oversulphation merely by increasing their intracellular half-life.

Evidence for preferential desulphation during normal catabolism of sulphated glycosaminoglycans is available, but meagre. DiFerrante *et al.* (1971) suggested that this process might explain the higher ^{35}S specific radioactivity of intracellular 'bound' versus extracellular 'bound' sulphated glycosaminoglycans in normal fibroblast cultures. Similarly, Dorfman & Matalon (1969) found significant amounts of free sulphate, without concomitant recovery of oligosaccharide, after incubation of $[^{35}\text{S}]$ sulphate-labelled dermatan sulphate with normal or Hurler-syndrome cultured fibroblasts. The equality of normal and mutant fibroblasts in this system suggests that α -L-iduronidase activity is not a prerequisite for such desulphation, but definitive studies of this type are needed. If the sulphatase activity described by Dorfman & Matalon (1969, 1972) operates on glycosaminoglycan molecules of intermediate size, as does

Fig. 6. Additional effects of corrective factor on normal and Hurler-syndrome cells

(a) Incorporation of ^3H from ^3H glucose into the sulphated glycosaminoglycan fraction and (b) the retention of radioactivity derived from ^3H glucose during 'chase' with unlabelled medium of normal (○), Hurler-syndrome (●), corrective-factor-exposed normal (□) and corrective-factor-exposed Hurler-syndrome (■) cells. The correction, labelling and 'chase' protocols were as in the legends to Figs. 2, 4 and 5 respectively.

liver sulphatase (Tudball & Davidson, 1969), then the absence of α -L-iduronidase in Hurler-syndrome cells would result in the storage of molecules of dermatan sulphate in the Hurler-syndrome cells containing proportionately more ^{35}S sulphate than hydrolysed dermatan sulphate molecules in normal cells. Conversely, these partially hydrolysed normal fragments would have proportionately less sulphate and may still be precipitable by cetylpyridinium chloride thus generating a lower $^{35}\text{S}/^3\text{H}$ ratio in the normal cells.

The similarity in the fates of ^3H radioactivity in the sulphated glycosaminoglycan fraction of normal and mutant cells during the 'chase' period (Fig. 4a) may be largely superficial. The retention of radioactivity derived from ^3H glucose by the Hurler-syndrome cells largely reflects decreased degradation (Fratantoni *et al.*, 1968; Matalon *et al.*, 1971; Matalon & Dorfman, 1972); that of the normal cells could reflect predominantly reutilization of breakdown products during turnover of the sulphated glycosaminoglycan fraction. Metabolic salvaging of glucuronide during glycosaminoglycan turnover has been suggested previously (Neufeld & Fratantoni, 1970).

Whatever the origin(s) of the greater $^{35}\text{S}/^3\text{H}$ ratio in the sulphated glycosaminoglycan fraction of the Hurler-syndrome cells than in normal cells, the difference is readily interpretable as a consequence of

a primary defect in the degradation of iduronic acid-containing glycosaminoglycans.

When the preparation of corrective factor from urine was added to the medium, the sulphated glycosaminoglycan fraction of the Hurler-syndrome cells did not respond until 12h after the 'chase' period began (34h exposure to 'factor'). The response was reflected simultaneously by a decrease in the amount (Fig. 5b) and in the total ^{35}S sulphate radioactivity (Fig. 5a) of the sulphated glycosaminoglycan fraction. A striking concomitant of the response to 'correction' was the dramatic increase in ^3H specific radioactivity of the sulphated glycosaminoglycan fraction (Fig. 5c). This would suggest that unlabelled sulphated glycosaminoglycan molecules were preferentially lost during this period of 'correction', and it implies the existence of pools of 'old' and 'new' molecules of sulphated glycosaminoglycans destined for degradation and/or secretion. The clear effect of the corrective-factor preparation on normal cells at the earliest point of observation in the 'chase' period (26h exposure to 'factor') is readily interpretable in the same terms; namely, accelerated turnover of the sulphated glycosaminoglycan fraction with reutilization of ^3H -labelled degradation products from sulphated glycosaminoglycans and other labelled cell components acted on by the preparation of corrective factor. Indeed, the

behaviour of normal cells exposed to corrective factor supports the idea that retention of ^3H radioactivity by the sulphated glycosaminoglycan fraction of normal cells not exposed to the factor (Fig. 4a) is due partly to resynthesis of sulphated glycosaminoglycan molecules with ^3H -labelled degradation products. It is important to note that the 'correction' response of the Hurler-syndrome cells was apparent in their hyaluronic acid fraction after only 18h exposure to the 'factor' (Germinario *et al.*, 1973), i.e. some 16h before it was observable in their sulphated glycosaminoglycan fraction.

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