Synthesis of Acid Mucopolysaccharides by Glial Tumor Cells in Tissue Culture*

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Communicated by Leon O. Jacobson, March 23, 1970

Abstract. A clonal strain of rat glial tumor cells was shown to produce the acid mucopolysaccharides, hyaluronic acid, chondroitin 4-SO₄, and heparan sulfate in tissue culture. The mucopolysaccharides were isolated both from the growth medium and the cells.

The presence of acid mucopolysaccharides in the nervous system was first suggested by the histochemical studies of Abood and Abul-Haj¹ and has more recently been corroborated by isolation of polysaccharides from brain,²⁻¹⁰ spinal cord, and peripheral nerve.¹¹ Hyaluronic acid and chondroitin 4- and 6-sulfate are the major mucopolysaccharide constituents of the nervous system. Heparan sulfate^{3,6,9-11} and possibly small amounts of dermatan sulfate⁵ are also present.

Although the functional significance of the mucopolysaccharides in the nervous system has not yet been clearly defined, several observations indicate that they may play an important role in the physiology of the nervous system.¹²⁻¹⁴ In view of the long-standing uncertainty as to the nature and function of the intercellular matrix of the central nervous system, it seemed important to examine the origin of mucopolysaccharides in brain since these substances are characteristic components of the extracellular spaces in other tissues. Although the glial cells of the brain sometimes have been regarded as the counterpart of the connective tissue cells of other tissues, their role in the physiology of the brain has not been adequately elucidated, and it is, therefore, of particular interest to establish whether these cells are capable of producing mucopolysaccharides. The possible production of hyaluronic acid in tissue culture of avian brain has been previously reported by Grossfeld.¹⁵ Recently, Benda *et al.*¹⁶ have isolated a clone of glial cells from a rat glial tumor, induced by injection of *N*-nitrosomethylurea. Cells from this clone were kindly supplied by Dr. Gordon Sato. In the present work it is shown that these cells in culture synthesize hyaluronic acid, chondroitin 4-sulfate and heparan sulfate.

Materials and Methods. Sodium acetate-³H (270 mCi/mmole) was purchased from Nuclear-Chicago. Carrier-free H₂³⁵SO₄ was purchased from New England Nuclear Corp. Streptococcal hyaluronidase was obtained from Lederle Laboratories and bovine testes hyaluronidase (activity 20,000 International Units/mg) was obtained from Leo, Helsingborg, Sweden. Chondroitinase was prepared by Dr. H. C. Robinson in this laboratory.¹⁷ Twice crystallized papain was prepared by Dr. Lennart Rodén in this

laboratory.¹⁸ Cetylpyridinium chloride was purchased from K and K Lab. Eagle's medium supplemented with 10% fetal calf serum (Hyland Laboratories) as modified by Matalon and Dorfman¹⁹ was used.

Cell growth: Glial cells were cultured in 100 mm Falcon plastic tissue culture dishes. Trypsinized suspensions were plated at 1×10^6 cells per plate and incubated in 10% CO₂. Cells were fed three times during a 10-day culture period. An average of 3.6×10^7 cells were obtained per plate. During the last 24 hr of incubation, 10 ml of medium containing 20 μ Ci of ³⁵SO₄ and 20 μ Ci of Na acetate-³H was placed on each of 10 plates. The growth medium from all of the plates was pooled. The cells were removed by a rubber policeman after several rinses with buffered saline.

Isolation of acid mucopolysaccharides: Crude acid mucopolysaccharides were precipitated by the addition of 10% cetylpyridinium chloride to the growth medium after dialysis and fivefold concentration. The precipitate was suspended in 2.0 M NaCl. Four vol of ethanol were added to the suspension and the precipitate that formed was washed twice with 80% ethanol and dried with ether. The dry powder was suspended in 20 vol of 0.1 M acetate buffer, pH 5.5, containing 0.005 M cysteine and 0.005 M EDTA. After addition of 40 mg of papain, the mixture was incubated for 36 hr at 65°C; an additional 20 mg of papain was added after 24 hr. The digest was boiled for 2 min and made 5% with respect to trichloroacetic acid. Following removal of the precipitate by centrifugation and extensive dialysis of the solution, acid mucopolysaccharides were precipitated by cetylpyridinium chloride in the presence of 0.03 M NaCl. The cetylpyridinium chloride-polysaccharide complex was dissolved in 2.0 M NaCl, and the acid mucopolysaccharides were precipitated in 80% ethanol and dried with ether. The cetylpyridinium chloride precipitation was repeated. The acid mucopolysaccharides were then fractionated by the differential salt solubility method of Schiller, Slover, and Dorfman.²⁰

For isolation of acid mucopolysaccharides from cells, the cell mass was digested with papain and the method described above was used on the digest.

Enzyme digestions: Streptococcal hyaluronidase (0.5 mg/ml) digestion was performed in 0.1 M PO₄-0.15 M NaCl buffer, pH 5.2, for 17 hr at 37°C. Testicular hyaluronidase (2500 International Units/ml) digestions were performed in 0.1 M acetate-0.15 M NaCl buffer, pH 5.0, for 17 hr at 37°C. Chondroitinase (0.2 unit/ml) digestion was performed in 0.01 M Tris acetate buffer, pH 8.0, at room temperature for 17 hr.

The enzyme digests were separated on a 200 $\times$ 1.0 cm Sephadex G-25 superfine column.

Descending paper chromatography of the chondroitinase digests was performed in isobutyric acid:2 N ammonia (5:3) for 20 hr on Schleicher and Schuell, 589 green ribbon paper. Electrophoresis was performed in formic acid-pyridine buffer, pH 3.0, using cellulose acetate strips.

Analytical methods: Hexosamine was determined by the Boas method omitting the Dowex treatment.²¹ Uronic acid was determined by the method of Dische²² and N-sulfate by the Lagunoff and Warren method.²³ Glucosamine and galactosamine were determined on the Technicon amino acid analyzer.

Determination of radioactivity was performed in the toluene scintillation mixture with Bio-Solv BBS-3 (Beckman) in a Packard tricarb scintillation counter.

Results. At the end of 10 days 91 plates yielded 9 ml of packed cells representing approximately 3.3×10^9 cells. A total of 10.0 mg of polysaccharide-bound uronic acid (approx. 25 mg of mucopolysaccharides) was isolated from the growth medium (2500 ml), and this material was separated by the method of Schiller, Slover, and Dorfman.²⁰ The results of this separation are shown in Table 1. Seventy-five per cent of the uronic acid appeared in the 0.04 M NaCl fraction, which is expected to contain hyaluronic acid or chondroitin. The remainder was extracted with 1.24 M NaCl, and no significant amount of uronic acid was found in the 2.1 M fraction which ordinarily contains heparin. The 0.40 M fraction contained little radioactive sulfate, whereas the bulk of the

TABLE 1. *Acid mucopolysaccharides produced by glial cells.*

Fraction	Uronic Acid		³⁵ SO ₄		³ H	
	mg	%	Total cpm	%	Total cpm	%
Original	10.0	100	4.3×10^5	100	3.4×10^5	100
0.40 <i>M</i>	7.6	75	0.2×10^5	5	2.7×10^5	77
1.24 <i>M</i>	2.3	22	3.2×10^5	75	0.3×10^5	10

labeled acetate was found in this fraction. In contrast, the 1.24 *M* fraction contained 75% of the total sulfate radioactivity but only 10% of the acetate label. The polysaccharide in the 0.04 *M* fraction was further characterized by the following procedures. Quantitative analyses showed a molar ratio of uronic acid to hexosamine of 1.06, and the $[\alpha]_D^{25}$ was -71°C . On digestion with streptococcal hyaluronidase, followed by Sephadex chromatography, 98% of the radioactive acetate emerged in a retarded position corresponding to the effluent volume of a disaccharide. Paper chromatography of this material showed radioactivity which migrated as an unsaturated, nonsulfated disaccharide. The material eluted with the void volume on gel filtration contained all the radioactive sulfate of the 0.4 *M* fraction, and was presumably also contaminated with small amounts of glycoproteins, since it contained both glucosamine and galactosamine as well as large amounts of amino acids. On paper electrophoresis and staining with acridine orange, the 0.40 *M* fraction yielded a single spot with a mobility similar to that of hyaluronic acid.

The 1.24 *M* fraction showed two spots on electrophoresis with mobilities corresponding to those of authentic samples of chondroitin 4-sulfate and heparan sulfate. After prior treatment of the 1.24 *M* fraction with testicular hyaluronidase electrophoresis showed only one spot corresponding to heparan sulfate. Accordingly, the entire fraction was treated with hyaluronidase and then chromatographed on Sephadex G-25. The chondroitinase digest of the retarded material demonstrated on paper chromatography a radioactive spot which corresponded in mobility with a standard sample of 3-O- β - Δ -4, 5 glucuronosyl-*N*-acetyl-D-galactosamine 4-O-SO₄.

The hyaluronidase-resistant material which represented 38% of the 1.24 *M* fraction showed a uronic:hexosamine ratio of 1.2 and an $[\alpha]_D^{25}$ of $+55^\circ\text{C}$. The amino sugar was found to be entirely glucosamine, 31.3% of which was *N*-sulfated. Clearly this fraction consists of heparan sulfate.

The washed glial cells were digested with papain and acid mucopolysaccharides were isolated as indicated above. From 3.3×10^9 cells, 6.8 mg of uronic acid (approx. 15 mg of mucopolysaccharides) was isolated. On separation by the method of Schiller, Slover, and Dorfman,²⁰ 63% was recovered in the 0.40 *M* fraction and 37% in the 1.24 *M* fraction. Electrophoresis showed the same pattern as observed in the acid mucopolysaccharide fractions isolated from the growth medium.

Previous experience in this laboratory has indicated that fetal calf serum contains acid mucopolysaccharides. It was, therefore, essential to determine what proportion of the material isolated from the growth medium was derived from this source. By the procedure employed, a total of 0.9 mg of uronic acid (approx. 2.4 mg of mucopolysaccharides) was found per liter of medium. This

corresponds to approximately 25% of the total polysaccharide recovered from the medium of the cultured cells. On fractionation of the polysaccharide from one liter of fresh medium 0.2 mg of uronic acid was recovered in the 0.40 *M* fraction and 0.1 mg in the 1.24 *M* fraction. On electrophoresis, the 0.40 *M* fraction migrated slightly faster than did standard hyaluronic acid, whereas the 1.24 *M* fraction had the same mobility as chondroitin 4-sulfate. Since these fractions were completely digested by testicular hyaluronidase, the heparan sulfate recovered from the growth medium was derived entirely from the glial cells.

Discussion. The results reported indicate that a clonal glial cell strain is capable of synthesizing three different acid mucopolysaccharides: hyaluronic acid, chondroitin 4-sulfate, and heparan sulfate. That the cell strain is indeed of glial origin is supported by the fact that it also makes the S-100 brain protein.¹⁶ The formation of these acid mucopolysaccharides by glial cells is of importance with reference to the suggestions that acid mucopolysaccharides are essential for the development of myelin of normal structure² and also for the regulation of Na⁺ and K⁺ in nerve excitation.¹ Intracisternal injection of testicular hyaluronidase in cats has been found to cause lethargy, rigidity, and myoclonic convulsions.^{13,14} The formation of heparan sulfate by glial cells is of particular interest since it has been suggested by Kaplan²⁴ that excretion of this compound in mucopolysaccharidoses is associated with mental retardation. Thus, the glial cells may represent a source of heparan sulfate in the forms of mucopolysaccharidoses, especially Sanfilippo's syndrome, in which mental retardation is prominent and in which heparan sulfate is the predominant polysaccharide excreted. It is worthy of note that skin fibroblasts in tissue culture do not produce heparan sulfate even when derived from patients with Sanfilippo's syndrome. The only previous report of the synthesis of this compound in tissue culture is that of Kraemer²⁵ utilizing an established cell line of Chinese hamster fibroblasts.

The authors are grateful to Drs. J. A. Cifonelli and Allen Stoolmiller for their help in these studies and to Dr. Lennart Rodén for many valuable suggestions.

* These studies were supported by USPHS grants AM-05996 and 1-PO1 HD-04583.

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