A Novel Assay for the Biosynthesis of Sulphated Polysaccharide and its Application to Studies on the Effects of Somatomedin on Cultured Cells

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1. A simple method is described for the determination of small amounts of [^{35}S]sulphated polysaccharide with 95–100% recovery in the range from 0.3 to 150 μg of polysaccharide. 2. The method is based on precipitation with cetylpyridinium chloride of polysaccharide samples applied to filter paper. It is not significantly disturbed by the presence in the sample of a large excess of inorganic $^{35}\text{SO}_4^{2-}$. 3. Sulphated glucosamino- and galactosaminoglycans may be determined separately by treatment of the sample with chondroitinase ABC. 4. The method is applicable to the assay of [^{35}S]sulphated polysaccharide biosynthesis in cell cultures. A stimulation of sulphate incorporation obeying a linear dose-response curve, was demonstrated in somatomedin-incubated fibroblast and glia cell cultures. 5. The described system provides a new assay method for somatomedin. 6. The stimulatory effect of somatomedin on the synthesis of [^{35}S]sulphated polysaccharide appeared to be general, rather than specific, for a particular type of polysaccharide.

The incorporation of radioactive sulphate into acid mucopolysaccharides (glycosaminoglycans) (Boström, 1952) has become important in the study of the metabolism of these compounds. The determination of sulphate uptake, either in terms of total or specific radioactivity, should be based on a complete recovery of the labelled product. Methods for quantitative isolation of milligram quantities of glycosaminoglycans from tissues are well established (Scott, 1960). However, the precipitation of polysaccharide from dilute solutions is often incomplete and irreproducible, and, generally, microgram quantities of polysaccharide are obtained in acceptable yields only by isolation along with an excess of exogenous carrier. These difficulties have hampered the study of polysaccharide metabolism in situations where small absolute quantities of polysaccharide are present, e.g. in most cell culture systems. In this paper a novel method is introduced for the assay of small amounts of ^{35}S -labelled polysaccharide. It is based on the precipitation of the polysaccharide on filter paper with cetylpyridinium chloride.

Experimental

Materials

Chemicals and enzymes. Carrier-free inorganic $^{35}\text{SO}_4^{2-}$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. It was dissolved in 0.15M-NaCl to a concentration of 10 $\mu\text{Ci}/50\mu\text{l}$.

Pronase was obtained from Calbiochem, Los Angeles, Calif., U.S.A. It was used as a 2% (w/v) solution in water. Papain, obtained in crude form from Sigma Chemical Co., St. Louis, Mo., U.S.A.,

was purified by the procedure of Kimmel & Smith (1954) and was used as a suspension (0.1M-sodium acetate buffer, pH4.5) containing approx. 40mg of protein/ml. Chondroitinase ABC and AC were purchased from Miles Lab. Inc., Kankakee, Ill., U.S.A.

Cetylpyridinium chloride was obtained from AB Recip, Stockholm, Sweden. Triton X-100 was supplied by Sigma Chemical Co.

Carrier chondroitin sulphate was isolated from bovine nasal septum as described previously (Wasteson, 1971). Chondroitin [^{35}S]sulphate was isolated from epiphyseal cartilage of 13-day-old chick embryos, after incubation *in vitro* with inorganic $^{35}\text{SO}_4^{2-}$. The incubation medium was Eagle's (1959) minimal essential medium supplemented with 10% (v/v) baby calf serum and serine (0.35mM). The incubation was performed at 37°C for 5h. The isolation of chondroitin [^{35}S]sulphate included digestion with papain and precipitation of the liberated polysaccharide with cetylpyridinium chloride (Scott, 1960). The details of the procedure were as described previously (Wasteson *et al.*, 1972). The specific radioactivity of the preparation was approx. 15000 c.p.m./ μg of uronic acid.

^{35}S -labelled heparin was generously supplied by Dr. S. Ögren of this Institute. The preparation was isolated from mouse mastocytoma after labelling *in vivo* with $^{35}\text{SO}_4^{2-}$ (S. Ögren, unpublished work). The specific radioactivity of the preparation was approx. 5000 c.p.m./ μg of uronic acid.

Somatomedin (Daughaday *et al.*, 1972) was prepared from pooled human plasma as described by Uthne (1973). The preparation contained approx. 80 units of somatomedin/mg dry wt., as determined

by its stimulatory activity on sulphate incorporation in embryonic chick cartilage (Hall, 1970). The insulin content of the preparation was approx. 100 μ units/mg dry wt. (Hales & Randle, 1963). Before use the material was dissolved in 0.1 M-acetic acid.

Reference serum was pooled from 800 healthy human donors. It was diluted to the appropriate concentration with phosphate-buffered saline (3.4 mM-KCl - 170 mM-NaCl - 10 mM-sodium phosphate, pH 7.3).

Cells. A cell line of human foetal lung fibroblasts (460Hel) was initiated from trypsin-treated lung tissue of a foetus after 4 months of gestation, as described by Hayflick (1965). A cell line of human glia (622CG) was cultivated from a brain biopsy specimen as described previously (Pontén & Macintyre, 1968). The cells were grown as monolayer cultures on 50 mm Petri dishes (Falcon Plastics, Los Angeles, Calif., U.S.A.) in 5 ml of Eagle's (1959) minimum essential medium containing baby calf serum (10%, v/v), penicillin (100 units/ml), streptomycin (50 μ g/ml) and amphotericin B (1.25 μ g/ml).

Methods

Liquid-scintillation counting. This was performed in a Beckman model LS250 instrument with 1 ml samples and with 10 ml of Insta-Gel (Packard Instruments, Warrenville, Ill., U.S.A.) as the scintillation medium. Background value was approx. 70 c.p.m.

Incubation of cells with [35 S]sulphate. Incorporation experiments were performed when the fibroblast and glia cell cultures had reached a density of approx. 2×10^6 and 0.5×10^6 cells per dish respectively. The cells were then washed briefly with 5 ml of phosphate-buffered saline, and 5 ml of Ham's (1965) F-12 medium without serum was added. A second change of medium was made 48 h later with 2 ml of Ham's F-12 medium. After another 1 h, 10 μ Ci of $^{35}\text{SO}_4^{2-}$ was added to the cultures. In some experiments the cultures were preincubated with somatomedin or reference serum for 1 h before the addition of $^{35}\text{SO}_4^{2-}$. The incorporation of the isotope was allowed to proceed for various periods of time (4–48 h) and was terminated by the addition of 200 μ l of Triton X-100 (1%, v/v) and freezing to -90°C . Controls were established by adding the radioactive precursor only at the time of termination of the cultures, i.e. along with the detergent.

Determination of ^{35}S -labelled polysaccharide. After thawing of the samples, 2 mg of Pronase was added. Proteolysis was continued for 5 h at 37°C , yielding a clear, homogeneous solution with no cell fragments visible. Portions (100 μ l) of this solution were applied in duplicate to strips (4 cm $\times$ 26 cm) of Whatman 3MM filter paper at intervals of 4 cm. In some experiments, portions of the same digests were further treated with chondroitinase ABC or AC (Yamagata

et al., 1968) (after heat-inactivation of the proteolytic enzyme) before application to the filter-paper strips. All the strips were mounted vertically in steel racks carrying eight strips each (LKB, Stockholm, Sweden). The dried strips were washed for 30 min in each of five changes of 1000 ml of 1% (w/v) cetylpyridinium chloride in 0.3 M-NaCl and dried again. Squares (4 cm $\times$ 4 cm) corresponding to the applied samples were cut into small pieces and transferred to scintillation vials. After the addition of 1 ml of water and 10 ml of Insta-Gel, the samples were left to equilibrate for 1 h and were then subjected to liquid-scintillation counting.

The recovery of polysaccharide-bound ^{35}S radioactivity by the cetylpyridinium chloride filter-paper technique was studied separately. A comparison with established cetylpyridinium chloride precipitation methods (Scott, 1960) was effected by the following experiment. Portions (0.5 ml) of the heat-inactivated Pronase digests were mixed with 150 μ l of 1.0 M-sodium acetate buffer, pH 5.5, 0.05 M-EDTA, 0.05 M-cysteine-HCl, 2 mg of papain and 3 mg of carrier chondroitin sulphate. The mixture was incubated at 60°C for 20 h. The polysaccharide was then precipitated from the solution by the addition of 0.5 ml of 1% (w/v) cetylpyridinium chloride. The cetylpyridinium-polysaccharide complex was dissolved in 0.5 ml of 2 M-KCl and reprecipitated by the addition of 2.5 ml of 0.1% (w/v) cetylpyridinium chloride. This last step was repeated once and the precipitate was dissolved in 0.5 ml of 2 M-NaCl-10% (v/v) ethanol. The polysaccharide was finally precipitated with 3 vol. of ethanol, dissolved in 0.5 ml of water and subjected to liquid-scintillation counting.

Results

Recovery of ^{35}S -labelled polysaccharide

Experiments with known amounts of $^{35}\text{SO}_4^{2-}$ and chondroitin [^{35}S]sulphate showed complete removal of the inorganic sulphate with no appreciable loss of the labelled polysaccharide (Table 1). No detectable ^{35}S radioactivity was eluted in the washing solution after five changes with cetylpyridinium chloride. Analysis of chondroitin [^{35}S]sulphate at various concentrations (Fig. 1) yielded a linear standard curve in the tested range (0.3–150 μ g of polysaccharide). Further, comparison with controls, not subjected to washing in cetylpyridinium chloride, indicated recoveries of 95–100%.

Like chondroitin sulphate, heparin was recovered quantitatively by the present method (Table 2). Treatment with chondroitinase of mixtures of [^{35}S]heparin and chondroitin [^{35}S]sulphate decreased the recovered radioactivity in proportion to the contribution of chondroitin [^{35}S]sulphate to the total radioactivity.

Table 1. Recovery of ³⁵S radioactivity from fibroblast cultures after the addition of exogenous ³⁵S-labelled polysaccharide and/or inorganic ³⁵SO₄²⁻

Chondroitin [³⁵S]sulphate (approx. 70000 c.p.m.) was added at a stage in the assay procedure corresponding to the termination of isotope incorporation (see the Experimental section). Inorganic ³⁵SO₄²⁻ (10 μCi) was added either at the same time, or immediately after proteolysis. Portions of the proteolysates (100 μl) were analysed by the cetylpyridinium chloride (CPC) filter-paper technique; the values represent the mean of two determinations with duplicate samples.

Addition of chondroitin [³⁵ S]sulphate	Addition of ³⁵ SO ₄ ²⁻		Washing with CPC		Radioactivity (c.p.m.)
	Before proteolysis	After proteolysis			
			×5	×10	
+	+	—	+	—	2586
+	—	+	+	—	2485
+	+	—	—	+	2550
+	—	—	+	—	2479
—	+	—	+	—	97
—	—	+	+	—	77
+	—	—	—	—	2611

Fig. 1. Recovery of ³⁵S radioactivity from ³⁵S-labelled polysaccharide by the cetylpyridinium chloride filter-paper technique

Samples of chondroitin [³⁵S]sulphate in serial dilution (1:2) were applied to the filter-paper strips. The radioactivity of each spot was determined by liquid-scintillation counting, either with (●—●) or without (○—○) previous washing of the strips in cetylpyridinium chloride. The recorded values represent duplicate determinations.

Incorporation of sulphate

Incorporation of ³⁵SO₄²⁻ in cell cultures. The time-course of ³⁵SO₄²⁻ incorporation into fibroblast poly-

saccharide is shown in Fig. 2. The incorporation was almost linear during the first 20 h; during the last 24 h of incubation the rate of incorporation seemed to decrease. Determination of incorporated radioactivity by conventional precipitation with cetylpyridinium chloride in the presence of carrier polysaccharide gave results nearly identical with those obtained by the filter-paper technique (Fig. 2). The proportion of chondroitinase-resistant polysaccharide was approx. 40% of the total radioactivity and apparently did not change with incubation time (Fig. 2).

Stimulation of incorporation by somatomedin. The effect of somatomedin or serum on the incorporation of ³⁵SO₄²⁻ into fibroblast or glia cell polysaccharide was studied by use of the cetylpyridinium chloride filter-paper technique. The incorporation of ³⁵SO₄²⁻ was significantly stimulated by the presence in the culture medium of 0.5 unit of somatomedin or 1% (v/v) of reference serum. After 24 h of incubation the stimulated fibroblast cultures had accumulated 50% more polysaccharide-bound radioactivity than the controls (Fig. 3). The glia cell cultures were stimulated to a slightly lower extent (not shown). The relative amounts of chondroitinase-resistant polysaccharide were approximately the same in stimulated and in non-stimulated cultures, indicating that somatomedin and serum affected the different classes of polysaccharide to the same extent (Table 3).

The stimulation of sulphate incorporation in fibroblast polysaccharide at various dosages of somatomedin is shown in Fig. 4. The dose-response curve was almost linear on a log/lin plot in the concentration range from 0.03 to 0.5 unit/ml. At higher concentrations sulphate uptake remained constant or, occasionally, decreased.

Table 2. Recovery of ^{35}S -labelled polysaccharides by the cetylpyridinium chloride filter-paper technique

A solution of chondroitin[^{35}S]sulphate or [^{35}S]heparin or a 1:1 (v/v) mixture of both was analysed. The ratio of chondroitin[^{35}S]sulphate to [^{35}S]heparin was 3:2 with respect to radioactivity. The values represent two determinations on duplicate samples.

^{35}S -labelled polysaccharide applied	Treatment with chondroitinase	Radioactivity (c.p.m.)
Chondroitin sulphate	—	31000
Heparin	—	19200
Chondroitin sulphate+heparin	—	25200
Chondroitin sulphate+heparin	+	9400

Fig. 2. Incorporation of $^{35}\text{SO}_4^{-2}$ into fibroblast polysaccharide after various times of incubation

$^{35}\text{SO}_4^{-2}$ was added after 1 h of preincubation in the presence of 1% (v/v) human serum (see the Experimental section). The incorporation was estimated either by the present cetylpyridinium chloride filter-paper technique (●—●) or by conventional methods (○—○), including papain digestion and precipitation with cetylpyridinium chloride in the presence of carrier chondroitin sulphate (see the Experimental section). The incorporation of $^{35}\text{SO}_4^{-2}$ into polysaccharide, resistant to treatment with chondroitinase ABC, as measured by the cetylpyridinium chloride filter-paper method, is also recorded (■—■). Radioactivities refer to 100 μl of culture proteolysate; each value represents two determinations with duplicate samples.

Discussion

It is well known that a variety of connective tissue cells, such as fibroblasts, chondrocytes, osteocytes or synovial cells, have the ability to produce glycosaminoglycans (see e.g. Schubert & Hamerman, 1968). Glycosaminoglycan synthesis has also been demonstrated in mast cells (Selye, 1965; Helting *et al.*, 1972),

Fig. 3. Incorporation of $^{35}\text{SO}_4^{-2}$ into fibroblast polysaccharide under various culture conditions

The conditions were: non-supplemented culture medium (○—○); 1% of normal human serum present (●—●); somatomedin present (0.5 unit/ml) (■—■). The accumulated incorporation was estimated by using 100 μl portions of culture proteolysate with the cetylpyridinium chloride filter-paper technique. The recorded values represent two determinations with duplicate samples.

Fig. 4. Stimulatory effect of different doses of somatomedin on the incorporation of $^{35}\text{SO}_4^{-2}$ into fibroblast polysaccharide

The relative increase in sulphate incorporation at various concentrations of somatomedin, expressed as the percentage of a non-stimulated control, has been recorded on a log/lin scale. Each value represents two determinations with duplicate cultures.

Table 3. *Differential incorporation of $^{35}\text{SO}_4^{2-}$ into total and chondroitinase-resistant polysaccharide in somatomedin-, serum- or non-stimulated fibroblast cultures*

The cultures had been incubated with the radioactive isotope for 24h. Duplicate samples from duplicate cultures were analysed for ^{35}S -labelled polysaccharide before and after treatment with chondroitinase ABC.

Culture conditions	^{35}S -labelled polysaccharide (c.p.m.)		Chondroitinase-resistant ^{35}S -labelled polysaccharide (% of total)
	Before chondroitinase	After chondroitinase	
Somatomedin (0.5 unit/ml)	1698	627	37
Serum (1%)	1557	593	38
Non-stimulated	830	280	34

human circulating leucocytes (Olsson *et al.*, 1968), enterocytes (Carlstedt & Fransson, 1972) or brain cells (Bachawat *et al.*, 1972). The idea that cells not derived from fibroblasts may be able to produce acid mucopolysaccharides has received an increasing amount of support from studies on cultured cells, showing polysaccharide synthesis in e.g. HeLa cells (Suzuki *et al.*, 1970), ascites carcinoma cells (Saito & Uzman, 1971), glia or glioma cells (Dorfman & Ho, 1970; Å. Wasteson & B. Westermark, unpublished work) or lymphoid cells (Saito & Uzman, 1971). An overt or repressed capacity to produce these kinds of substances may be retained in most, if not all, cells.

Present knowledge of polysaccharide metabolism in cells is incomplete both with respect to the nature and regulation of the biosynthetic or degradative processes and the function of the polysaccharides. Difficulties in assaying small amounts of polysaccharide have hampered studies in this field. The present method seems to offer a relatively simple and sensitive system as a complement to previously described methods for the recovery of polysaccharides in general (Scott, 1960) or, more specifically, for the assay of small amounts of radioactively labelled polysaccharide in cell cultures (Fratantoni *et al.*, 1968; Saito & Uzman, 1971). The technique is based on the specific precipitation of glycosaminoglycans by aliphatic ammonium salts, e.g. cetylpyridinium chloride, as described by Scott (1960).

Analysis of known amounts of polysaccharide, from 0.3 to 150 μg , indicated that the assay of ^{35}S -labelled polysaccharide was quantitative (Fig. 1) and not influenced by the presence of a large excess of non-polysaccharide-bound radioactivity (Table 1). The minor losses of chondroitin[^{35}S]sulphate during the wash in cetylpyridinium chloride (see the Experimental section and Fig. 1) may be due to the presence in the chondroitin sulphate preparation of low-sulphated or low-molecular-weight fragments having a significant solubility in cetylpyridinium chloride–0.3M-NaCl. However, 0.3M-NaCl was preferred to

water as a solvent for cetylpyridinium chloride because with water alone, precipitation of non-polysaccharide radioactivity occasionally occurred.

Most polysaccharide-synthesizing cells in culture seem to produce more than one type of polysaccharide (Saito & Uzman, 1971). The differential assay of individual classes of polysaccharides in the same culture is readily applicable in the present system by use of specific chondroitinases (Yamagata *et al.*, 1968). Thus it was found that the human embryonic fibroblast cell line produced a sulphated polysaccharide, two-thirds of which was susceptible to chondroitinase ABC. Alternatively, discrimination between polysaccharides, differing with respect to charge or molecular size, may be achieved by varying the ionic strength of the cetylpyridinium chloride solution.

By applying the present method to the study of incorporation of sulphate in response to somatomedin it was shown that this substance afforded a maximal increase in the production of ^{35}S -labelled polysaccharide by approx. 50–100%, which was demonstrable within less than 10h. The stimulation apparently obeyed a linear dose-response curve; on maximal stimulation somatomedin and human serum seemed to produce approximately equivalent effects. The possibility that the insulin activity of the preparation was responsible for the observed effects on sulphate incorporation seems to be ruled out by the fact that the actual insulin concentration (1 $\mu\text{unit/ml}$) were far below the range (1 munit/ml) in which such effects have been demonstrated (Salmon *et al.*, 1968).

An analysis of the ^{35}S -labelled polysaccharide produced under the stimulation of somatomedin or serum showed that the ratio of chondroitinase-susceptible to chondroitinase-resistant polysaccharide was mainly unchanged compared with the non-stimulated control, thus indicating a general rather than a type-specific effect on the polysaccharide synthesis. This finding is compatible with the view that the 'sulphation factor' activity of somatomedin may

be exerted via an effect on the protein synthesis (see Daughaday, 1971).

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