# Production of Heparin Related Mucopolysaccharides by Mammalian Cells in Culture[1] (34920)

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(Introduced by L. B. Jaques)

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The uptake of $^{35}S$ heparin by four mammalian cell lines in culture has been recently reported (1). During this investigation, it was observed that control cultures grown in the presence of $^{35}S$ inorganic sulfate incorporated considerable amounts of radioactivity in compounds possessing chromatographic and electrophoretic mobilities similar to heparins obtained from some mammalian species as well as heparitin sulfate (2). The present paper describes the isolation and partial characterization of a group of *N*-sulfated mucopolysaccharides resembling heparin or heparitin sulfate in HeLa, L, mouse embryo, and rat embryo cells in culture.

The presence of mucopolysaccharides, *e.g.*, chondroitin sulfates, has been extensively demonstrated, in several lines of mammalian cells in culture (3). However, heparin-like compounds had never been found in established lines of cells in culture, except for the Chinese hamster fibroblast cells grown in suspension culture (4).

*Methods. Cell cultures.* HeLa, L, mouse embryo, and rat embryo cells were grown in stationary cultures as previously described (1). Mouse and rat embryo cell cultures were obtained from 12-day embryos according to the method described elsewhere (5). In all cases the cells were grown in 10 ml of medium containing 0.1 mCi of inorganic sulfate $^{35}S$, (15 mCi/mmole). In some instances heparin (200 $\mu$g/ml) was also added to the cultures. After growth for 72 hr the cells were centrifuged and washed repeatedly in the cold with Eagle's essential amino acid medium until no radioactivity was detected in the supernatant (usually four washes). After the last washing the precipitated cells were freeze dried and weighed. 10 mg of dry cells were suspended in 1 ml of water and agitated for 0.5 hr with 1 ml of 80% phenol. After centrifugation at 3000*g* for 30 min the water phase was separated and extracted with three 5-ml portions of ether to remove dissolved phenol. The ether layer was discarded and the water phase was freeze dried. The residue was dissolved in 0.2 ml of water and the mucopolysaccharides were precipitated with 5 vol of 95% ethanol. The white precipitate was dried, resuspended in 200 $\mu$l of water, and analyzed.

*Chemicals.* $^{35}S$ sodium sulfate (15.0 mCi/mmole) was purchased from Nuclear Chicago (Des Plaines, Illinois). *N*-$^{35}S$ heparin was purchased from Calbiochem (Los Angeles, Calif.). Heparin was obtained from Lederle Laboratories, Pearl River, N. Y. through the courtesy of Dr. E. H. Dearborn. Chondroitin sulfate A/C and B were gifts from the late Dr. A. Winterstein, Hoffman LaRoche AG Basel.

*Enzymes.* Chondroitinase ABC and chondroitinase AC were purchased from Miles Laboratories (Elkhart, Indiana). Enzymes from induced and noninduced *Flavobacterium heparinum* (heparinases and chondroitinases) were prepared as previously described (6, 7). Pronase and ribonuclease were purchased from Calbiochem (Los Angeles, Calif.).

*Analytical.* The radioactive mucopolysaccharides obtained from the mammalian cells were analyzed by chemical and enzymatic hydrolysis as previously described for hepar-

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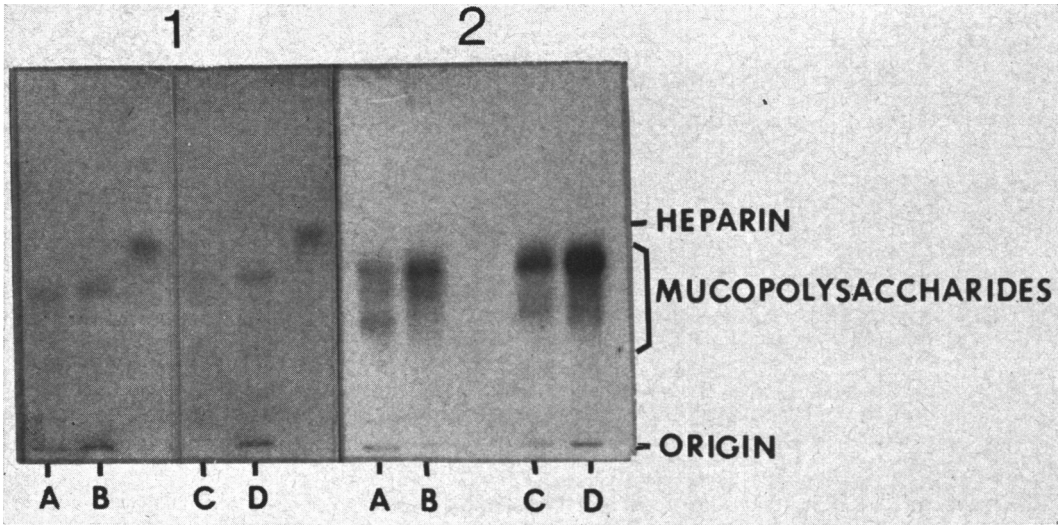


FIG. 1. Microelectrophoresis of the radioactive mucopolysaccharides from the mammalian cells: A, HeLa cells; B, L cells; C, mouse embryo cells; D, rat embryo cells; (1) toluidine blue stain; (2) radioautography of the stained microelectrophoresis slide.

in and other mucopolysaccharides (8–10). The radioactive products obtained from the enzymatic or chemical degradation were chromatographed in isobutyric acid–1 *M* $NH_4OH$ (5:3, v/v) in Whatman no. 1 paper, or subjected to electrophoresis in 0.3 *M* pyridine acetate buffer, pH 4.2, in Whatman 3MM paper.

The radioactive materials were localized by radioautography with Kodak Royal Blue X-ray film (Eastman Kodak Co.). The areas of the chromatograms containing the radioactive materials were then cut out and counted in 10 ml of a solution of 5 g of diphenyloxazole in 1 liter of toluine in a Beckman liquid scintillation spectrometer (LS-100). Agarose gel microelectrophoresis of the mucopolysaccharides and their quantitation were performed according to the method described by Jaques *et al.* (2).

*Results. Microelectrophoresis of the radioactive mucopolysaccharides obtained from the 4 cell lines.* Three to 4 radioactive bands were detected by radioautography of the microelectrophoresis slides after fixation with Cetavlon (cetyltrimethylammonium chloride) and staining with toluidine blue, as shown in Fig. 1. The fixation of the radioactive materials in the slides with Cetavlon indicates that they have a polyanionic structure, since Cetavlon is unable to precipitate sulfated materials with a molecular weight lower than 2000, such as sulfated oligosaccharides obtained from heparin after bacterial degradation (Dietrich, unpublished). Furthermore, the radioactive materials are not dialyzable and are excluded by gel filtration in Sephadex G-25. None of the radioactive mucopolysaccharides have the same electrophoretic mobilities of the standard commercial heparin used in this experiment. Nevertheless they have similar mobilities to heparins obtained from some species (dog, rat) as well as heparitin sulfate (2). No change in migration or amount of the radioactive bands in microelectrophoresis was observed after treatment of the extracts with pronase and ribonuclease (Fig. 2). These radioactive materials were completely degraded by the action of enzymes (heparinases plus chondroitinases) extracted from induced cells of *F. heparinum* (Fig. 2).

In this experiment, the amount of radioactive mucopolysaccharides was large enough to be detected by toluidine blue reagent. Part of this material had the typical metachromatic activity (purple staining) observed for heparin and some other mucopolysaccharides. In the microelectrophoresis slides of all extracts, several bands staining in blue with

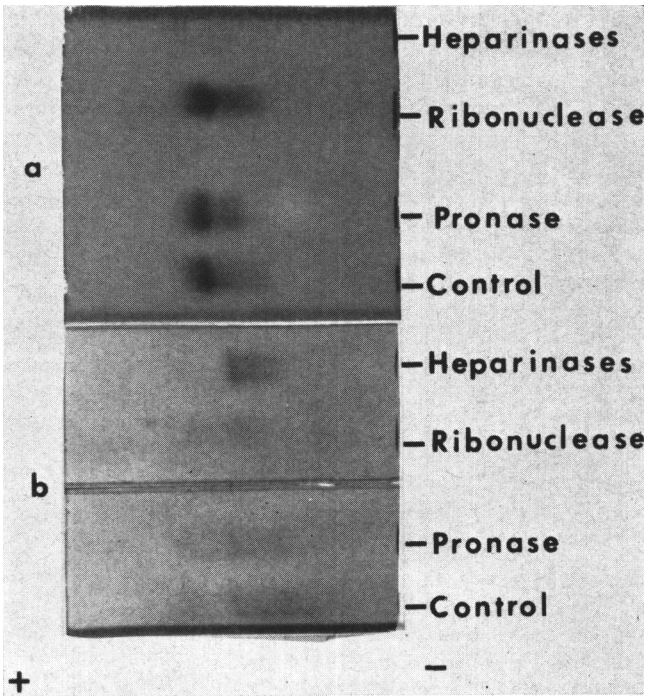


FIG. 2. Action of ribonuclease, pronase and heparinases + chondroitinases upon the radioactive mucopolysaccharides: 2000 cpm of a mixture of mucopolysaccharides from the 4 cell cultures were incubated with 10 $\mu$g of pronase or 10 $\mu$g of ribonuclease or 10 $\mu$g of heparinases + chondroitinases for 18 hr at 25° containing 0.04 *M* phosphate buffer, pH 7.0, and 5 m*M* $MgCl_2$ in a final volume of 20 $\mu$l; 2 $\mu$l were applied to microelectrophoresis; a, radioautography of the microelectrophoresis slide; b, toluidine blue stain.

toluidine blue reagent were also observed (Fig. 2). These bands, however, did not contain any detectable amount of radioactivity and were absent when the extracts were treated with ribonuclease prior to the microelectrophoresis, indicating that these bands are probably ribonucleic acid fractions. Thus, these preliminary data indicate that the radioactive compounds found in the extracts of the 4 cell lines are sulfated mucopolysaccharides with a molecular weight higher than 2000 and susceptible to the action of mucopolysaccharidases.

*Incorporation of $^{35}S$ inorganic sulfate into mucopolysaccharides.* The amounts of radioactive mucopolysaccharides extracted by the phenol method from the cells grown in the presence and absence of heparin are summarized in Table I. No appreciable difference in the incorporation of $^{35}S$ inorganic sulfate into these compounds was observed when heparin was used in the growth medium. Table I also shows that the mouse embryo and rat embryo cells synthesize higher amounts of sulfated mucopolysaccharides by weight than the HeLa and L cells.

*Mild acid hydrolysis of the radioactive mucopolysaccharides.* The radioactive mucopolysaccharides extracted from the mammalian cells were subjected to hydrolysis in 0.04 *N* HCl for 1 hr at 100°. Between 46 and 66% of the radioactivity was released as inorganic sulfate after the treatment (Table II). Among the known mucopolysaccharides, only heparin and heparin sulfate have *N*-linked sulfates which are labile under these conditions. Thus, the results indicate that the compounds isolated from the cells are at least partially *N*-sulfated heparin or heparitin sulfate.

*Action of* Flavobacterium heparinum *enzymes upon the radioactive mucopolysaccharides extracted from the mammalian cells in culture. F. heparinum* grown in the ab-

sence of heparin produces chondroitinases able to degrade chondroitin sulfates and hyaluronic acid. When the bacterium is grown in the presence of heparin, heparinases able to degrade heparin and heparitin sulfate are also formed (7).

TABLE I. Amount of Mucopolysaccharides Synthesized by Mammalian Cells in Culture in the Presence and Absence of Heparin.

| Cell line | Additions to the growth medium | $^{35}S$ mucopolysaccharides ($\mu$g/mg of dry cells)[a] |
|---|---|---|
| HeLa | None | 0.065 |
| L | None | 0.081 |
| Rat embryo | None | 0.175 |
| Mouse embryo | None | 0.200 |
| HeLa | Heparin | 0.060 |
| L | Heparin | 0.082 |
| Rat embryo | Heparin | 0.155 |
| Mouse embryo | Heparin | 0.182 |

[a] Extractable by the phenol method. After extraction of the $^{35}S$ mucopolysaccharides from the cells as outlined in "Methods" aliquots of the material were counted in the liquid scintillation spectrometer and also subjected to microelectrophoresis after treatment with ribonuclease as described in Fig. 2. The microelectrophoresis slides were then stained with toluidine blue and the amount of mucopolysaccharides present was measured by densitometry as previously described (2). From these data the specific activity of the mucopolysaccharides was then calculated.

TABLE II. Release of Radioactive Inorganic Sulfate from the Mucopolysaccharides After Mild Acid Hydrolysis.

| Mucopolysaccharide source | Inorganic sulfate formed after hydrolysis (%) |
|---|---|
| HeLa | 46.0 |
| L | 46.0 |
| Rat embryo | 57.5 |
| Mouse embryo | 66.5 |
| Heparin[a] | 92.5 |
| Chondroitin sulfate A/C[b] | 5.2 |

[a] N-$^{35}S$ heparin.

[b] Sulfate measured by a colorimetric procedure (11). About 1000 cpm of each mucopolysaccharide was hydrolyzed in 0.04 *N* HCl for 2 hr at 100°. After the hydrolysis the mixtures were applied in Whatman 3MM filter paper and submitted to electrophoresis. The inorganic sulfate was localized by autoradiography and counted in a liquid scintillation spectrometer.

Figure 3 shows the formation of degradation products from the radioactive mucopolysaccharides by the action of the heparinases and chondroitinases from *F. heparinum*. All the mucopolysaccharides were at least partially degraded by the action of these enzymes. When the substrates were incubated with the enzymes for long periods (18 hr) most of the radioactivity was found as glucosamine *N*-sulfate and inorganic sulfate characterized by their relative migrations in electrophoresis (8). Some of the radioactive products from all the cell lines had the same chromatographic migration as the degradation products of heparin incubated withe the same enzymes (Fig. 3). Among these products glucosamine 2,6-disulfate and glucosamine *N*-sulfate were the most characteristic. These two compounds are produced by degradation of heparin and heparitin sulfate only and are not

TABLE III. Susceptibility of $^{35}S$ Mucopolysaccharides from Mammalian Cells in Culture to the Action of Chondroitinases and Heparinases.[a]

| Cell line | Mucopolysaccharides (%) nondegraded by: Condroitinases | Chondroitinases + heparinases |
|---|---|---|
| HeLa | 41.0 | 12.0 |
| L | 52.5 | 12.4 |
| Rat embryo | 55.9 | 14.9 |
| Mouse embryo | 43.6 | 7.1 |

[a] About 2000 cpm of each mucopolysaccharide, was incubated for 2 hr at 25° with 10 $\mu$g of enzymes prepared from *F. heparinum* cells previously grown in glucose (chondroitinases) or heparin (chondroitinases + heparinases). The incubation mixtures also contained 0.04 *M* potassium phosphate buffer and 5 m*M* of $MgCl_2$ in a final volume of 20 $\mu$l. After incubation, the mixtures were spotted in Whatman no. 1 filter paper and chromatographed in isobutyric acid:1 *M* $NH_4OH$ (1:0.6; v/v). The chromatograms were then radioautographed and the nondegraded mucopolysaccharides (remaining at the origin of the chromatogram) were counted. The results were expressed in percentage of the total radioactivity present in the chromatogram.

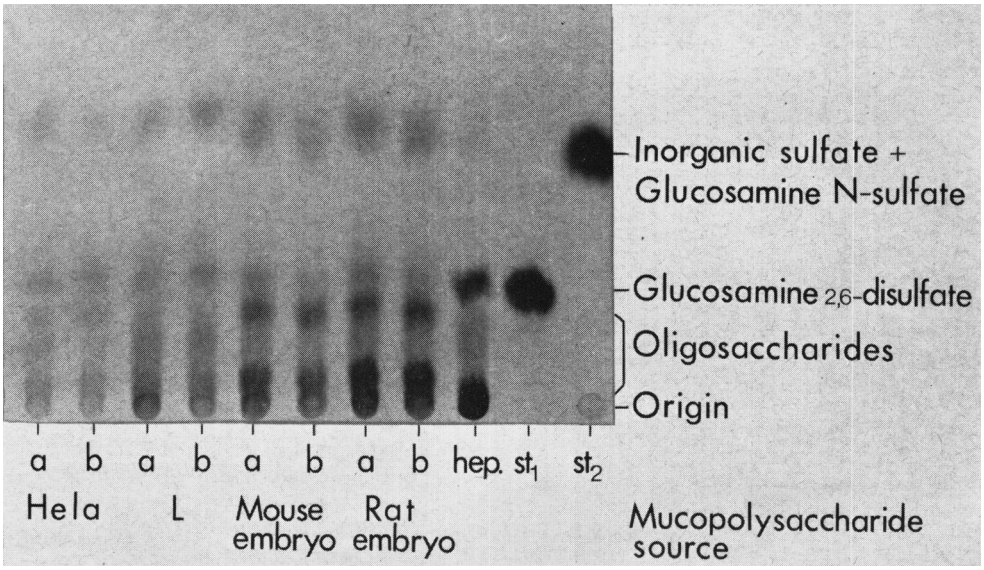


FIG. 3. Action of chondroitinases and heparinases upon the radioactive mucopolysaccharides from the 4 cell lines: The radioactive mucopolysaccharides from 1 mg of dry cells previously grown in the presence and absence of heparin were incubated with induced cells of *F. heparinum* as described in Table III. After chromatography in isobutyric acid:1 *M* $NH_4OH$ (5:3; v/v) the paper was radioautographed; a, mucopolysaccharides from mammalian cells grown in the presence of heparin; b, mucopolysaccharides from the mammalian cells grown in the absence of heparin. hep., 20,000 cpm of $N^{35}S$ heparin incubated with the same enzymes; $st_1$, glucosamine 2, 6-disulfate; $st_2$, inorganic sulfate. Undegraded mucopolysaccharides remain at the origin of the chromatogram (not shown).

found as degradation products of chondroitin sulfate A, B, or C under the same treatment. No appreciable difference was found in the degradation products of the radioactive mucopolysaccharides produced by the mammalian cells grown in the presence or absence of heparin.

Table III shows the amount of radioactivity remaining in the origin of the chromatograms after degradation of the mucopolysaccharides by the action of enzymes prepared from induced and noninduced *F. heparinum* cells. About half of the total incubated radioactivity remains in the origin after the action of enzymes from noninduced *F. heparinum,* while about 90% of the radioactive material moves from the origin when the incubation is performed using enzymes from induced bacteria. These results indicate that about 50% of the radioactive mucopolysaccharides produced by the mammalian cell lines in culture is chondroitin sulfate-like (degraded by chondroitinases) and the other 50% is heparin-like material (degraded by heparinases).

*Action of chondroitinase ABC and chondroitinase AC upon the radioactive mucopolysaccharides.* Table IV shows the amount of degradation products formed from the radioactive mucopolysaccharides by the action of excess purified chondroitinase ABC (from Proteus) and chondroitinase AC (from *F. heparinum*). Between 40 and 70% of the radioactive products were susceptible to the action of these enzymes. The amounts of degradation products were the same with both enzymes. The only radioactive product formed had the same chromatographic mobility of a disaccharide obtained by the degradation of chondroitin sulfate A/C by the action of the same enzymes. Compounds with the chromatographic mobility of glucosamine 2,6-disulfate or glucosamine *N*-sulfate could not be detected.

The resistance of the remaining 30–60% of the extracted radioactive mucopolysac-

TABLE IV. Action of Chondroitinases ABC and AC upon the Radioactive Mucopolysaccharides Obtained from the Mammalian Cells in Culture.[a]

| Cell line | Degradation products (%) formed with: Chondroitinase ABC | Chondroitinase AC |
|---|---|---|
| HeLa | 39.2 | 39.3 |
| L | 43.6 | 43.2 |
| Rat embryo | 69.2 | 69.2 |
| Mouse embryo | 64.5 | 64.1 |

[a] 0.2 units of chondroitinases ABC or AC were incubated with 2000 cpm of the radioactive mucopolysaccharides for 5 hr at 37°. The incubation mixtures also contained 0.05 *M* Tris–HCl buffer, pH 8.0, and 5 m*M* $MgCl_2$ in a final volume of 20 $\mu$l. For chondroitinase AC the Tris buffer was substituted for 0.04 *M* phosphate buffer, pH 7.0. The compounds were spotted in Whatman no. 1 paper and chromatographed in isobutyric acid ammonia solvent. The products formed by the action of these enzymes (sulfated disaccharides) were located by radioautography and counted. Controls using heated inactivated enzymes have shown that the undegraded mucopolysaccharides remained at the origin of the chromatography.

charides to the action of the chondroitinases is illustrated by the microelectrophoresis method (Fig. 4). Two to three radioactive bands are still present in the radioautograms of these microelectrophoresis slides performed after treatment of the mucopolysaccharides with the enzymes (compare with Fig. 1). Control incubations of chondroitin sulfates A/C and B with these enzymes show that they completely disappear from the microelectrophoresis slide while heparin is unaffected by the same enzymatic treatment.

The results again clearly indicate that from 40 to 70% of the mucopolysaccharides extracted by the phenol method from the cells grown in culture are chondroitin sulfate A and/or C.

*Identification of glucosamine N-sulfate and glucosamine 2,6-disulfate in the radioactive mucopolysaccharides isolated from the mammalian cells.* The compounds with the same chromatographic mobility of glucosamine 2,6-disulfate derived from the mucopolysaccharides from the 4 cell lines (Fig. 3) were eluted from the chromatography paper, hydrolyzed with 0.04 *N* HCl at 100° for 1 hr, and subjected to electrophoresis. The radioautogram of the electrophoresis paper revealed two radioactive components, one with the same migration as inorganic sulfate and another with the migration of glucosamine *O*-sulfate.

In another group of experiments the eluted compounds were subjected to paper chromatography in two different solvents (butanol: acetic acid:water 3:1:1 and ethyl acetate: pyridine:acetic acid:water 5:5:1:3). The compound produced by all the lines of cells studied had the same chromatographic mobilities in these solvents as the authentic glucosamine 2,6-disulfate.

The compounds with the same chromatographic mobility as glucosamine *N*-$SO_4$ or inorganic sulfate in the chromatograms shown in Fig. 3 were also eluted from the paper and subjected to electrophoresis. Two radioactive spots with the mobilities of inorganic sulfate (67%) and glucosamine *N*-$SO_4$ (33%) were detected by radioautography. Mild acid hydrolysis of the latter produced radioactive inorganic sulfate.

*Discussion and Conclusions.* The presence of heparin-like mucopolysaccharides in the four mammalian cells in culture is indicated by the following:

1. The $^{35}S$ compounds extracted by the present method have a polyanionic structure, molecular weight higher than 2000, and electrophoretic mobility in agarose gel microelectrophoresis similar to some heparins and/or heparitin sulfate;

2. Inorganic sulfate is formed from these sulfated mucopolysaccharides after mild acid hydrolysis;

3. The compounds are partially resistant to hydrolysis by chondroitinases from noninduced cells of *F. heparinum*, as well as by chondroitinases AC and ABC, but are almost completely hydrolyzed by heparinases from induced *F. heparinum*;

4. Glucosamine 2,6-disulfate and glucosamine *N*-sulfate are isolated from these mucopolysaccharides by the action of the heparinases.

These results do not exclude, however, the

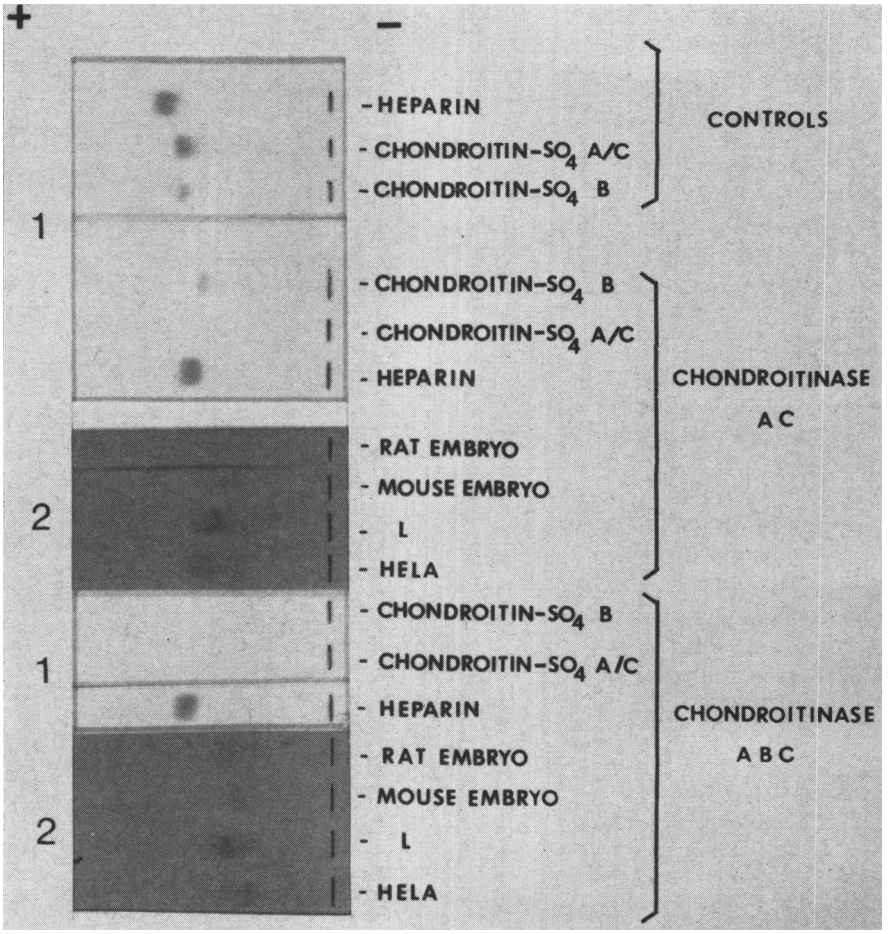


FIG. 4. Action of chondroitinases ABC and AC on the mucopolysaccharides from the 4 cell lines. This experiment was performed as described in Table IV. Nonradioactive chondroitin sulfate A/C, chondroitin sulfate B, and heparin (100 $\mu$g) were also incubated with the same enzymes. 2 $\mu$l of the incubation mixtures were applied to the microelectrophoresis; (1) toluidine blue stain; (2) radioautography.

possibility of one of these mucopolysaccharides being a new group of heparin-like compounds.

Chondroitin sulfates have already been isolated from many mammalian cells in culture (3), but no mention has been made of the existence of *N*-sulfated mucopolysaccharides. Probably, the *N*-sulfated mucopolysaccharides were unseen among the vastly more abundant chondroitin sulfates existent in these cells in a protein-bound form. We were able to exclude most of these compounds by using a method in which the unbound mucopolysaccharides were preferentially extracted into the aqueous phase. A similarly selective procedure was used by Kraemer (4) to extract *N*-sulfated mucopolysaccharides from the Chinese hamster fibroblasts.

All the mammalian cells in culture so far studied are able to synthesize *N*-sulfated mucopolysaccharides. The function of these compounds in the cells is not known, however. Since an increase in mitotic activity in the presence of heparin has been observed in the four mammalian cell lines in culture (1), it could be suggested that the mucopolysaccharides synthesized by the cells play a role in the regulation of mitosis.

Recently, Saito and coauthors (10) described a method to distinguish several chondroitin sulfates in microgram amounts by the use of specific chondroitinases. The heparinases used in the present work permit the extension of this procedure to heparin-related mucopolysaccharides.

*Summary.* Radioactive mucopolysaccharides were isolated from HeLa, L, mouse embryo, and rat embryo cells grown in culture medium in the presence of radioactive inorganic sulfate, for 72 hr. The isolated mucopolysaccharides were submitted to mild acid hydrolysis as well as to the action of several mucopolysaccharidases and the degradation products were characterized by their migration in several chromatographic systems and on electrophoresis. It was demonstrated that part of these mucopolysaccharides are chondroitin sulfate A/C and part *N*-sulfated-heparin-like compounds susceptible to hydrolysis by heparinases releasing glucosamine 2, 6-disulfate as the major amino sugar residue.

We thank Dr. S. M. C. Dietrich for help in the preparation of the manuscript.

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Received Jan. 6, 1970. P.S.E.B.M., 1970, Vol. 134.