Differences in Glycosaminoglycans Synthesized by Fibroblast-like Cells from Chick Cornea, Heart, and Skin*

(Received for publication, January 3, 1977, and in revised form, April 13, 1977)

GARY W. CONRAD, CONNIE HAMILTON, AND ELAINE HAYNES
From the Division of Biology, Kansas State University, Manhattan, Kansas 66506

Glycosaminoglycan biosynthesis was studied in primary cultures in vitro to determine if fibroblast-like cells from different individual tissues synthesize the same or different patterns of glycosaminoglycans. Corneas, heart ventricles, and back skins from embryonic chicks were dissociated into cell suspensions. Corneal fibroblast-like cells were obtained by simple dissection, whereas heart and skin fibroblast-like cells were separated from nonfibroblastic cell types by their rapid rate of adhesion to plastic substrata. These populations were grown in vitro for 8 days and then were incubated in the continuous presence of 6-[3H]glucosamine and H235SO4 for another 2 weeks. All radioactive nutrient medium was collected, as was each cell layer at the end of the experiments. The amount of radioactivity incorporated into each glycosaminoglycan was determined.

The patterns of glycosaminoglycans synthesized by the three different fibroblast-like cell populations are clearly distinguishable. Corneal populations incorporate twice as high a percentage of radioactivity in heparan sulfate of media as in heparan sulfate of cell layers as in heparan sulfate of media. Heart populations, on the other hand, incorporate about the same proportion of radioactivity in cell layer heparan sulfate as in that from the media, whereas skin populations show a slightly higher percentage of radioactivity incorporated in cell layer heparan sulfate than in that from media. The proportions of radioactivity incorporated in hyaluronic acid are highest in corneal samples, next highest in skin samples, and lowest in heart samples. All three populations release a 2 to 3-fold higher proportion of radioactive hyaluronic acid into the media than they retain in their cell layers. Both heart and skin cultures incorporate almost twice as high a percentage of radioactivity in chondroitin 6-sulfate as do corneal cultures, whereas the percentage of radioactivity incorporated in chondroitin 4-sulfate is approximately the same in all three populations. Skin cultures incorporate a 3-fold higher percentage of radioactivity in hyaluronic acid released into the media as do heart cultures, yet these skin populations incorporate only half as much radioactive activity in cell layer dermatan sulfate as do heart cultures. Analysis of replicate samples indicates that the differences are all highly significant statistically.

Fibroblast-like cells isolated from whole embryos and from several specific tissues are being used widely for in vitro studies as presumably homogeneous populations. Such cells are used for important and divergent types of investigations, including diagnosis of genetic diseases (1), as well as studies of plasma membrane properties (2) and viral transformation (3, 4). However, if the fibroblast-like cell populations derived from different tissues of a single organism are not alike when grown in vitro, their differences obviously could have significant effects on the results of analyses which made use of such cells. Our data suggest that this is, indeed, the case: fibroblast-like cells derived from three tissues of one age of embryonic chick can be consistently distinguished from one another by their patterns of glycosaminoglycan biosynthesis. Fibroblast-like cells derived from human skin presently are used extensively in the diagnosis of genetic diseases (1, 5, 6). Some populations of such cells have even been shown to display metabolic patterns sufficiently distinct from normal to allow detection of the heterozygotic, as well as the homozygotic, states of the syndromes (5-7, but see Ref. 8). Studies of the mucopolysaccharidoses (1, 7), cystic fibrosis (6, 9), myotonic muscular dystrophy (10), and other syndromes (11-13) have examined the glycosaminoglycans synthesized by fibroblast-like cells grown in vitro from skin biopsy or foreskin samples. Although in Hurler's syndrome cell-types throughout the body, including both skin and corneal fibroblasts, express the mutant phenotype (14), in other syndromes the fibroblasts of only certain tissues express the defect. For example, in the Hunter syndrome, the authentic fibroblasts of cornea in vitro appear normal in ultrastructure, whereas those of the skin do not (15). Conversely, in muscular dystrophy (Groenouw's type II) the fibroblasts of skin appear normal, whereas those of the cornea do not (16). These data raise the possibility that each tissue contains a population of fibroblasts distinct from those of other tissues, at least distinct in their response to specific mutations. If so, such cells might express some of their tissue-specific properties even in vitro in the absence of nonfibroblastic cells, i.e. their differentiated states might be stable to some degree. Alternatively, a single labile population of fibroblastic cells dispersed throughout the body might be directed into different patterns of glycosaminoglycan biosynthesis by the different microenvironments that the various nonfibroblastic

* This work was supported in part by the National Institutes of Health under Research Grant EY00952 to G. W. Conrad. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.
Glycosaminoglycans from Fibroblast-like Cells

Tissues were dissected under sterile conditions from Day 14 White Leghorn embryonic chicks. Corneas were removed, trimmed free of scleral ossicles, and rinsed three times in calcium-, magnesium-free saline G, pH 7.2 (CMF), containing 10% chick serum (v/v) (CMF + CS). Heart ventricles were separated from auricles and major blood vessels, minced to small pieces, and rinsed three times in CMF + CS. Skin were obtained by inoculating the cell suspensions in culture medium, centrifuged at 400 x g, 10 min, room temperature, and then fed) and three times per week thereafter. Dishes were incubated at 37° (17). Heart cells, however, were mixtures of fibroblasts, endothelial cells, and cardiac muscle cells, and skin cells were mixtures of fibroblasts and epithelial cells. Fibroblast-like cell populations were grown as matched sets.

Isolation of Glycosaminoglycans

The nutrient medium used was Ham’s F-12 containing (final concentrations) fetal calf serum (10%, v/v), sodium bicarbonate (14 mM), sodium ascorbate (0.54 mM), potassium salt of penicillin G (48 units/ml), and streptomycin sulfate (0.033 mM, 48 µg/ml). The final concentration of inorganic sulfate in the complete medium, excluding that contributed by the fetal calf serum, was 0.038 mM. All sera and media were obtained from Grand Island Biological Co. (Grand Island, N.Y.); all antibiotics were from Sigma Chemical Co. (St. Louis, Mo).

Labeling of Cells

After 8 days of growth in vitro, cultures were labeled with both H3SO4 (5 µCi/ml; carrier-free; Schwarz/Mann, Orangeburg, N.Y.) and 16H125Iglycosaminoglycans (5 µCi/ml; 7.3 to 10.1 Ci/mmol; New England Nuclear, Boston, Mass.) simultaneously. Cultures were confluent monolayers at the time labeling began. Isotope was added in appropriate radioactive medium at the start of the labeling. H3 and 125I were added at three times per week thereafter at the times of routine feedings. At the time of each feeding, the radioactive nutrient medium was collected from each labeled culture and replaced by fresh labeling medium. Collected radioactive medium then was centrifuged immediately (10,000 x g, 10 min, 2-4°) and frozen. No discernible pellets resulted from such centrifugation, but this step was always performed to remove floating cells and cellular debris from the media.

Cultures were maintained as above in the continuous presence of radioactive medium for 14 days. At the end of the labeling period, the last batch of radioactive medium was collected, as described; the cell layers were rinsed twice over a 20-min period with 3 ml of fresh unlabeled nutrient medium at room temperature (all such rinses were collected, centrifuged, pooled with the previously collected radioactive medium, and frozen). The cell layers were then collected and resuspended in 2 ml of 0.15 n NaCl added to each culture dish, and frozen.

At the beginning of labeling in each experiment, all the dishes of each fibroblast-like population were separated into groups of five dishes each. All the labeled media collected from a given set of five dishes was pooled into a single media sample (v/v) (19). For labeling, the five cell layers within each group were pooled to make one cell layer sample. For each fibroblast-like population, a number of replicate media samples and cell layer samples were collected in this manner (cornea; 7; heart; 5; skin; 5). They were derived from four totally separate experiments, within each of which all three fibroblast-like populations were grown as matched sets.

Isolation and Analysis of Glycosaminoglycans

Total Glycosaminoglycans—Samples, consisting of collected radioactive medium or of isolated cell layers suspended in 0.15 n NaCl, were diluted with a concentrated solution of Tris/HCl to achieve a final concentration of 0.2 M (pH 8.0). Samples were immersed in a boiling water bath for 3 min, cooled, and then incubated for 60 min with predigested pronase (0.18 mg/ml, grade B; Calbiochem; San Diego, Calif.) in the presence of ethanol and CaCl2 (18). Enzyme was added after the first 24 h. Enzyme and other residual proteins were removed by treatment with cold trichloroacetic acid (5%, w/v, final concentration), blending on a Vortex mixer, incubation on ice for 30 min, and centrifugation at 12,000 x g for 15 min at 2-4°. Precipitates contained insignificant incorporated radioactivity and were discarded. Supernatants were extracted with chloroform/methanol (2:1, v/v) (19). Chloroform layers contained insignificant incorporated radioactivity and were discarded. Supernatants, together with material at the interface, were dialyzed in the presence of chloroform against cold running tap water and 0.05 n NaSO4 until no more radioactivity was detected in the bath, and then lyophilized. The sample and eluent were discarded. Non-dialyzable material was lyophilized to dryness in 20% ammonium acetate in 20% ethanol (v/v; chromatography solvent), and applied to columns containing Sephadex G-50 fine (Pharmacia, Uppsala, Sweden) (1 x 200 cm) equilibrated with the same solution. The void volume (Vo) of the column was 72 ml. Fractions of approximately 3 ml were collected, and aliquots were analyzed for radioactivity using a Packard Tri-Carb model 3320 liquid scintillation spectrometer and an ethanol/toluene mixture (20). Counting efficiency for H was 12% and for S was 44% (double label). The Sephadex columns were standardized by the elution of blue dextran (Mr = 5 x 106; Sigma). St. Louis Mo.) and by ultraviolet standards of glycosaminoglycans. Blue dextran and the glycosaminoglycans emerged in the excluded volume in Fractions 20 to 30.

Material eluted in the void volume fractions (total glycosaminoglycans) was pooled and lyophilized to dryness, as was material eluted in the retarded fractions (glycopeptides) (Fig. 1) (Note: Figs. 1 to 6 display data, from selected individual samples from media and cell layers, representative of each step during glycosaminoglycan analysis. In all cases, comparable data from the other fibroblast populations differ from the patterns shown only in relative peak heights, but not in the elution positions of the peaks shown).

Treatment with Nitrous Acid—The lyophilized samples of total glycosaminoglycans were treated with HNO3 to selectively degrade N-sulfated glycosaminoglycans, such as heparin and heparan sulfates (21). Samples in 2 volumes of glass-distilled water were mixed with 1 volume of 1 n HCl and 1 volume of freshly prepared 20% n-
butyl nitrite (v/v) (Kodak; Rochester, N.Y.) in absolute ethanol and the solution with 1 original volume of 1/2 h at room temperature. The reaction was stopped by neutralizing specific pattern of peaks (221 (Fig. 2). Test samples of heparin (2 mg; sulfate emerged as degraded products in the retarded fractions in a tides eluted in a broad zone after the excluded peak. A, heart glycosaminoglycans; it was pooled and lyophilized to dryness.

Material in Fractions 20 to 30 (arrow) was combined and constituted the total glycosaminoglycan pool. Glycopeptides eluted in a broad zone after the excluded peak. A, heart medium; B, heart cell layer. *--o, 3S; O--0, 3H.

Material eluted in the void volume fractions (HNO2-resistant glycosaminoglycans) was combined and constituted the HNO2-resistant glycosaminoglycan pool. Degradation products emerged in a characteristic set of peaks in the retarded fractions. A, corneal medium; B, corneal cell layer. *--o, 3S; O--0, 3H.

**Low Salt Fractions**—After dialysis and lyophilization, the low salt fractions from the Dowex 1-Cl- columns were dissolved in glass-distilled water. The proportion of radioactivity in hyaluronic acid, compared with that in chondroitin, was assayed in aliquots of these solutions in three ways: amino sugar analysis, sensitivity to Streptomyces hyaluronidase as determined by alcohol precipitation, and sensitivity to the Streptomyces enzyme as determined by paper chromatography.

For amino sugar analysis, aliquots of the low salt fractions above were air-dried, dissolved in 8 N HCl, and incubated in sealed tubes at 110° for 18 h under an atmosphere of N2. One milligram of glucosamine and 1 mg of galactosamine were added to each hydrolyzed sample as carrier. The samples were air-dried, redissolved in 0.3 N HCl, and applied to columns containing Dowex 50-H* (aggregate 50-W-X12, 200 to 400 mesh, hydrogen form; Bio-Rad) (0.8 x 6 cm) which were equilibrated and eluted with 0.3 N HCl (26). Fractions of 1.2 ml were collected and neutralized with NaOH. Glucosamine was eluted in Fractions 50 to 70, galactosamine in Fractions 75 to 95. Aliquots of 0.5 ml were assayed for radioactivity and for amino sugar by colorimetric assay (26). The proportion of radioactivity detected in the glucosamine peak is taken as a measure of the amount of hyaluronic acid synthesized, whereas the proportion of radioactivity in the galactosamine peak is taken as a measure of the amount of chondroitin synthesized.

**Low Salt Fractions**—After dialysis and lyophilization, the low salt fractions from the Dowex 1-Cl- columns were dissolved in glass-distilled water. The proportion of radioactivity in hyaluronic acid, compared with that in chondroitin, was assayed in aliquots of these solutions in three ways: amino sugar analysis, sensitivity to Streptomyces hyaluronidase as determined by alcohol precipitation, and sensitivity to the Streptomyces enzyme as determined by paper chromatography.

For amino sugar analysis, aliquots of the low salt fractions above were air-dried, dissolved in 8 N HCl, and incubated in sealed tubes at 110° for 18 h under an atmosphere of N2. One milligram of glucosamine and 1 mg of galactosamine were added to each hydrolyzed sample as carrier. The samples were air-dried, redissolved in 0.3 N HCl, and applied to columns containing Dowex 50-H* (aggregate 50-W-X12, 200 to 400 mesh, hydrogen form; Bio-Rad) (0.8 x 6 cm) which were equilibrated and eluted with 0.3 N HCl (26). Fractions of 1.2 ml were collected and neutralized with NaOH. Glucosamine was eluted in Fractions 50 to 70, galactosamine in Fractions 75 to 95. Aliquots of 0.5 ml were assayed for radioactivity and for amino sugar by colorimetric assay (26). The proportion of radioactivity detected in the glucosamine peak is taken as a measure of the amount of hyaluronic acid synthesized, whereas the proportion of radioactivity in the galactosamine peak is taken as a measure of the amount of chondroitin synthesized.
Sensitivity of labeled material in the low salt fractions to degradation by Streptomyces hyaluronidase was assayed in two ways.

**Assay 1:** Aliquots of the low salt fractions above were diluted with saline G, pH 7.2 (Sigma), 6 µCi/ml tritiated sulfate to give a final concentration of 0.02 µCi/ml. To half the tubes (experimental), 4 turbidity reducing units of Streptomyces hyaluronidase (ENZYME) or with buffer alone (CONTROL) as described in Assay 2 under “Experimental Procedures.” Aliquots of reaction solutions were subjected to descending paper chromatography. Dried chromatograms were cut into 1-cm strips which were placed in liquid scintillation vials for measurement of radioactivity. Hyaluronic acid is degraded by the enzyme to small products which migrate away from the origin as two peaks, whereas chondroitin is not degraded and remains at the origin. The proportion of radioactivity that moved from the origin is taken as a measure of the amount of hyaluronic acid synthesized; whereas the proportion of radioactivity in the precipitates is taken as a measure of the amount of chondroitin synthesized. The trace amounts of 35SO4 detected in the low salt fractions were ascribed to chondroitin, consistent with the original description of this glycosaminoglycan as a poorly sulfated (rather than a nonsulfated) polymer (27) and also consistent with the possibility that some of the chondroitin detected may be a metabolic precursor of chondroitin sulfates (27, 28).

**Assay 2:** Aliquots of the low salt fractions above were treated with Streptomyces hyaluronidase or with buffer, as in Assay 1. Aliquots of the reaction solutions were subjected to descending paper chromatography (27, S & S 300 Green Ribbon C paper; isobutyric acid-NH4OH, 5.3 v/v; 16 h). Chromatograms were air-dried, cut into 1-cm strips, placed in liquid scintillation vials, and assayed for radioactivity (Fig. 4). The proportion of radioactivity that moved from the origin is taken as a measure of the amount of hyaluronic acid synthesized, whereas the proportion of radioactivity remaining at the origin is taken as a measure of the amount of chondroitin synthesized.

In the experiments with hyaluronidase, control samples were always included containing 1 mg of hyaluronic acid (Sigma), with or without enzyme. The reaction was monitored at the end of incubation by addition of cetylpyridinium chloride solution to a final concentration of 1%: a white precipitate formed in buffer-treated tubes, but no precipitates formed in enzyme-treated tubes.

**Medium Salt Fractions**—After dialysis and lyophilization, the medium salt fractions from the Dowex 1-Cl columns were dissolved in 2.5 ml of 0.1 M Tris/HCl solution, pH 7.2, (to which solutions of 0.5 units of chondroitinase AC (4.2.2.5; Miles Laboratories, Elkhart, Ind.) in 0.1 M of the same buffer were added. Samples were incubated at 37° for 48 h, with fresh enzyme added after the first 24 h. At the end of incubation, 1 ml of absolute ethanol was added to each tube and samples were air-dried. They were then redissolved in 0.5 ml of glass-distilled water, mixed with 5 ml of absolute ethanol saturated with sodium acetate, and left at 2-4° overnight to allow polysaccharide to precipitate. No carrier polysaccharides were added; precipitates formed upon the ethanol addition in samples derived from cell layers, as well as in those from media. Samples were centrifuged (750 × g, 15 min, room temperature), supernatants (first supernatants) were stored, and precipitates were treated with chondroitinase AC a second time, exactly as described above. Alcohol precipitation then generated a second supernatant, as well as a pellet. The first and second supernatants were pooled; they contained the products of enzymic degradation of chondroitin 4- and 6-sulfates. These products, mostly unsaturated disaccharides, ΔDi-4S and ΔDi-6S, were separated from one another and from the unsulfated disaccharide, ΔDi-6S, by descending paper chromatography, as described above. All chromatograms were scanned with a Packard model 7201 radiochromatogram scanner and were cut up in 1-cm segments for liquid scintillation counting (Fig. 5). The proportion of radioactivity in the supernatant solutions which co-chromatographed with standard ΔDi-4S (monosulfate with ultraviolet lights) is taken as a relative measure of the amount of chondroitin 4-sulfate synthesized; correspondingly for ΔDi-6S and chondroitin 6-sulfate.

The precipitate that formed after the second alcohol precipitation above, mostly dermatan sulfate and presumptive poorly sulfated keratan sulfate, was dissolved in 0.8 ml of 0.01 M Tris/HCl solution (pH 8.0) to which was added 0.4 unit of chondroitinase ABC (4.2.2.4; Miles Laboratories) in 0.2 ml of the same buffer. Samples were incubated at room temperature for 24 h. At the end of the incubation, 0.5 ml of absolute ethanol was added to each tube and samples were air-dried. They were then redissolved in 0.5 ml of glass-distilled water and treated with ethanol saturated with sodium acetate to precipitate polysaccharides, as above. Supernatants contained mostly the product of enzymic degradation of dermatan sulfate, i.e., ΔDi-4S, which was identified by descending paper chromatography, as described above (Fig. 6). The final precipitates represented presumptive poorly sulfated keratan sulfates and any other material resistant to degradation by chondroitinases AC and ABC. They were assayed for radioactivity by liquid scintillation counting.

In the experiments with both chondroitinases, control samples were always included containing 2 mg of chondroitin sulfate (mixed isomers, mostly chondroitin 6-sulfate; Sigma), with or without en-
RESULTS

Recovery of total glycosaminoglycans and glycopeptides from media and cell layers is shown in Table I. The total amounts of radioactivity in the samples from any one population of fibroblast-like cells differ from those of the other two (e.g., glycosaminoglycans: heart > skin > cornea). This may arise because the cell densities which the populations attain at saturation differ in the same order (cell number/60-mm dish: heart, 4.55 \times 10^6 > skin, 3.13 \times 10^6 > cornea, 1.85 \times 10^6) (29). Accurate cell numbers could not be determined in the present experiments because after 3 weeks in vitro, the cell layers were sufficiently intermeshed with extracellular material to preclude their complete dissociation.

Differences between the cell types are also apparent in the proportions of radioactivity incorporated. Corneal populations, for example, differ from those of heart and skin in incorporating a much lower proportion of \(^\text{3H}\) than of \(^{\text{35S}}\). Such correction applied to data from corneal media (Table I) thereby raises the proportion of \(^\text{3H}\) incorporated in hyaluronic acid. The values of \(^\text{3H}\) incorporation in each class of sulfated polysaccharide are lower than the values of \(^{\text{35S}}\) incorporation because of the synthesis of the nonsulfated polysaccharide, hyaluronic acid. In order to compare the \(^\text{3H}\) incorporation levels directly with those of \(^{\text{35S}}\) for each sulfated polysaccharide, one must multiply the \(^\text{3H}\) incorporation by a factor (\(^{\text{3H}}\) correction factors: 100/100 - % of \(^\text{3H}\) in hyaluronic acid: cornea, 1.93; heart, 1.11; skin, 1.51. Such correction applied to data from corneal media (Table I) thereby raises the proportion of \(^\text{3H}\) incorporated in heparan sulfate to 24%, a value very similar to the level of incorporated \(^{\text{35S}}\) (22%). Moreover, such correction of the heparan sulfate data accentuates the difference of the corneal population from the other two and essentially obliterates the

\begin{table}[h]
\centering
\caption{Average incorporated radioactivity in each sample}
\begin{tabular}{lccc}
\hline
\textbf{Media} & \textbf{Cornea} & \textbf{Heart} & \textbf{Skin} \\
\hline
\textbf{Total} & \textbf{Glycosaminoglycans}\(^*\) & \textbf{Glycosaminoglycans}\(^*\) & \textbf{Glycosaminoglycans}\(^*\) \\
\hline
\(^{\text{3H}}\) & 1,950,000 & 3,900,000 & 3,330,000 \\
\(^{\text{35S}}\) & 8,460,000 & 19,200,000 & 10,700,000 \\
\hline
\textbf{Glycopeptides}\(^*\) & \textbf{Glycopeptides}\(^*\) & \textbf{Glycopeptides}\(^*\) & \\
\hline
\(^{\text{3H}}\) & 613,000 & 1,070,000 & 1,330,000 \\
\(^{\text{35S}}\) & 1,300,000 & 821,000 & 734,000 \\
\hline
\textbf{Cell layers} & \textbf{Cell layers} & \textbf{Cell layers} & \\
\hline
\textbf{Total} & \textbf{Glycosaminoglycans}\(^*\) & \textbf{Glycosaminoglycans}\(^*\) & \textbf{Glycosaminoglycans}\(^*\) \\
\hline
\(^{\text{3H}}\) & 102,000 & 730,000 & 303,000 \\
\(^{\text{35S}}\) & 854,000 & 3,540,000 & 1,500,000 \\
\hline
\textbf{Glycopeptides}\(^*\) & \textbf{Glycopeptides}\(^*\) & \textbf{Glycopeptides}\(^*\) & \\
\hline
\(^{\text{3H}}\) & 12,900 & 105,000 & 73,800 \\
\(^{\text{35S}}\) & 71,000 & 112,000 & 80,800 \\
\hline
\textbf{Number of samples analyzed} & \textbf{Number of samples analyzed} & \textbf{Number of samples analyzed} & \\
\hline
\textit{n = 7} & \textit{n = 5} & \textit{n = 5} & \\
\hline
\end{tabular}
\end{table}
The total glycosaminoglycan pools from 17 individual samples of media were isolated by chromatography on Sephadex G-50, as described under "Experimental Procedures." Table I, and Fig. 1. Each such sample then was subjected to a sequence of degradative and chromatographic steps which allowed the amount of radioactivity in each type of glycosaminoglycan to be measured. These amounts are expressed as a percentage of the radioactivity present in each original total glycosaminoglycan pool. The averages of those percentages are shown (X), as well as their standard deviations (S) and 95% confidence intervals. The averages from all pairs of cell types were compared by Student's t test and the probability values of the differences arising by chance are shown (P); values of 0.05 or greater were not considered significant (N.S.). Incorporation in hyaluronic acid and chondroitin was determined by degree of sensitivity to Streptomyces hyaluronidase, followed by alcohol precipitation (Assay 1). See text for details.

### Table II

Percentages of radioactivity in glycosaminoglycans from media

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage of radioactivity in glycosaminoglycans</th>
<th>95% Confidence Interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPARAN SULFATE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cornea</td>
<td>3_1</td>
<td>12</td>
<td>3 9-15 N.S.</td>
</tr>
<tr>
<td>heart</td>
<td>3_1</td>
<td>14</td>
<td>1 12-16 0.050</td>
</tr>
<tr>
<td>skin</td>
<td>3_1</td>
<td>15</td>
<td>3 7-16 0.001</td>
</tr>
<tr>
<td>HYALURONIC ACID</td>
<td></td>
<td>10</td>
<td>3 6-14 0.001</td>
</tr>
<tr>
<td>cornea</td>
<td>3_1</td>
<td>16</td>
<td>4 5-6 N.S.</td>
</tr>
<tr>
<td>heart</td>
<td>3_1</td>
<td>17</td>
<td>4 5-6 N.S.</td>
</tr>
<tr>
<td>skin</td>
<td>3_1</td>
<td>18</td>
<td>4 5-6 N.S.</td>
</tr>
<tr>
<td>CHONDROITIN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cornea</td>
<td>3_1</td>
<td>19</td>
<td>5 7-8 N.S.</td>
</tr>
<tr>
<td>heart</td>
<td>3_1</td>
<td>20</td>
<td>5 7-8 N.S.</td>
</tr>
<tr>
<td>skin</td>
<td>3_1</td>
<td>21</td>
<td>5 7-8 N.S.</td>
</tr>
<tr>
<td>DERMATAN SULFATE</td>
<td></td>
<td>22</td>
<td>6 8-10 N.S.</td>
</tr>
<tr>
<td>cornea</td>
<td>3_1</td>
<td>23</td>
<td>6 8-10 N.S.</td>
</tr>
<tr>
<td>heart</td>
<td>3_1</td>
<td>24</td>
<td>6 8-10 N.S.</td>
</tr>
<tr>
<td>skin</td>
<td>3_1</td>
<td>25</td>
<td>6 8-10 N.S.</td>
</tr>
<tr>
<td>UNIDENTIFIED</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cornea</td>
<td>3_1</td>
<td>26</td>
<td>6 8-10 N.S.</td>
</tr>
<tr>
<td>heart</td>
<td>3_1</td>
<td>27</td>
<td>6 8-10 N.S.</td>
</tr>
<tr>
<td>skin</td>
<td>3_1</td>
<td>28</td>
<td>6 8-10 N.S.</td>
</tr>
</tbody>
</table>

The proportion of $^3$H incorporated in hyaluronic acid is very different for each population, cornea showing the highest level and heart showing the lowest (Table II). The differences between the three populations, with regard to hyaluronic acid in the medium, are all very highly significant.

All three populations make both chondroitin 4- and 6-sulfates (Table II), with quite similar proportions of both $^{35}$S and $^3$H (after correction) being incorporated into chondroitin 4-sulfate by all cell types. Incorporation into chondroitin 6-sulfate, however, is more than twice as high in heart and skin populations as in those of cornea.

Incorporation of radioactivity into the remaining polysaccharide classes (chondroitin, dermatan sulfate, and presumptive keratan sulfates) was 10% or less, except for $^{35}$S incorporation in dermatan sulfate by the corneal population (17%). In spite of the generally low levels of radioactivity incorporated in these classes, corneal populations still showed several instances of significant differences ($p \leq 0.001$) from heart and skin populations, whereas many fewer differences existed between heart and skin populations.

Table III summarizes the average amounts of $^3$H and $^{35}$S radioactivity found in each type of polysaccharide from cell layers, as well as the statistical significance of pairwise comparisons of each population with comparable values from each of the other two populations. As was the case with samples from media, corneal cell layers are distinguished by the high proportions of $^{35}$S and $^3$H incorporated in heparan sulfate and of $^3$H incorporated in hyaluronic acid. The $^3$H correction factors for cell layer samples are: cornea, 1.23; heart, 1.06; skin, 1.10. Corneal populations incorporate approximately twice as much radioactivity in heparan sulfate as do heart and skin populations. Moreover, there is a significant difference between heart and skin cells in the amount of $^{35}$S incorporated into heparan sulfate.
As with samples from media, corneal cell layers show a much higher proportion of radioactivity incorporated in hyaluronic acid than do either heart or skin cells (Table III). Again, skin cells incorporated almost twice as much radioactivity in hyaluronic acid as did heart cells.

Radioactivity was incorporated into chondroitin 4- and 6-sulfates by all cell types, but as with media samples, incorporation into chondroitin 4-sulfate was about the same for all three populations (Table III). Incorporation into chondroitin 6-sulfate was much higher in heart and skin cells than in corneal cells.

Incorporation of radioactivity into the remaining polysaccharide classes (chondroitin, dermatan sulfate, and presumptive keratan sulfates) represented less than 10% of that in the total glycosaminoglycan pool. Unlike the case with media samples, in which corneal populations showed the highest incorporation of 35S in dermatan sulfate (17%) and heart and skin were very similar, cell layers from heart populations showed the highest 35S incorporation in dermatan sulfate (10%), whereas cornea and skin were very similar. Insufficient amounts of radioactivity were present in the samples of presumptive keratan sulfate from both media and cell layer samples to allow further characterization (e.g., assay of sensitivity to keratan sulfate β-endogalactosidase (31)).

The data from Tables II and III show that the amounts of radioactivity incorporated in hyaluronic acid in samples from both media and cell layers constitute probably the single most important parameter for distinguishing between the three cell populations. It was therefore important to verify, with other methods, the levels of radioactivity incorporated in this polysaccharide. As described under "Experimental Procedures," three methods were used to determine the proportion of radioactivity present in hyaluronic acid in the fractions eluted from the Dowex-1 columns with NaCl solutions of low molarity. The data presented in Tables II and III were derived from treating samples with *Streptomyces* hyaluronidase and then precipitating undegraded polysaccharide (e.g., chondroitin) with ethanol (i.e., Assay 1). Those data are presented again for comparison, in Table IV, as well as data derived from separating the degraded and undegraded products by paper chromatography (i.e., Assay 2). Amino sugar analysis constituted the third type of assay. In samples from media, the three types of analyses detected very similar amounts of radioactivity from any one sample. Thus, for example, the high proportions of radioactiv-
The total glycosaminoglycan pools from 17 individual samples of media and 17 individual samples of cell layers were isolated and the HNO_3-resistant glycosaminoglycans were fractionated by chromatography on Dowex 1-C^+`, as in Fig. 3. Amount of \(^3H\) radioactivity incorporated in hyaluronic acid and chondroitin of the low salt fractions was determined by amino sugar analysis on Dowex 50-H^+ and by sensitivity to \textit{Streptomyces} hyaluronidase, as assayed by alcohol precipitation (Assay 1) and by descending paper chromatography (Assay 2). See "Experimental Procedures" for details. Amounts of incorporation are expressed as a percentage of the radioactivity present in each original total glycosaminoglycan pool. Hyaluronic acid is sensitive to degradation by the enzyme and contains glucosamine; chondroitin is resistant to degradation by the enzyme and contains galactosamine.

### DISCUSSION

The data presented show that normal, primary fibroblast-like cell populations in \textit{vitro} isolated from embryonic chick cornea, heart, and skin make arrays of glycosaminoglycans which reproducibly differ from one another quantitatively. Thus, by the methods used in this study, the fibroblast-like cell populations derived from different tissues of the same organism are unique.

The question of whether different tissues contain different kinds of fibroblasts was first approached by Parker (33, 34). From nine different tissues he could isolate nine populations of fibroblast-like cells which differed from one another by several criteria. His results did not answer the question, however, for no precautions were taken to try to exclude those nonfibroblastic cell types which \textit{in vitro} can assume fibroblastic morphology. Although the term, fibroblast, has a rather restricted meaning for cell types \textit{in vitro}, frequently it also is used to describe populations of cells \textit{in vitro} having fibroblastic morphology regardless of whether the progenitors of such cells existed in \textit{vivo} as authentic fibroblasts (35). We choose to reserve the term, fibroblast, to designate only authentic \textit{in vivo} fibroblasts or their proven descendants \textit{in vitro} and to use the term fibroblast-like for cell populations \textit{in vitro} which display fibroblastic morphology, but whose progenitor cell types or their states of differentiation (e.g. fibroblast \textit{versus} fibrocyte) have not yet been established rigorously. In the present study, we have taken steps to try to exclude nonfibroblastic cell types. The purest population is that from cornea, an avascular tissue in which the stromal fibroblast population can be separated cleanly by dissection from the two nonfibroblastic populations (corneal epithelium and endothelium) and inoculated \textit{in vitro} in its entirety without the use of selective procedures. Although a previous study (17) demonstrated that the corneal cells cultured came only from the stroma, present information still does not reveal whether the stromal population of the Day 14 chick cornea \textit{in vivo} consists of fibroblasts, fibrocytes (i.e. "differentiated" fibroblasts), or a

### TABLE IV

Average percentage of \(^3H\) radioactivity incorporated in hyaluronic acid and chondroitin: determination by three assay methods

<table>
<thead>
<tr>
<th></th>
<th>Cornea</th>
<th>Heart</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hyaluronidase</strong></td>
<td>Assay 1</td>
<td>Assay 2</td>
<td>Assay 1</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>48</td>
<td>47</td>
<td>51</td>
</tr>
<tr>
<td>Chondroitin</td>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td><strong>Amino sugar</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>19</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>Chondroitin</td>
<td>8</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

The relative distribution of individual polysaccharides between cell layers and media can be determined by comparison of data between Tables II and III. For example, corneal cell layers contain a much higher proportion of radioactivity in heparan sulfate than do corneal media. This suggests that corneal cell layers are preferentially enriched in heparan sulfate whereas those from amino sugar analysis were always the highest, whereas those from Assay 2 were always the lowest. Insufficient amounts of radioactivity were recovered in the low salt fraction from cell layers to allow analyses by all three techniques. Nevertheless, the data obtained from the two diverse methods used were reasonably similar; within each method the amounts of radioactivity incorporated in hyaluronic acid, as in media samples, were cornea > skin > heart. By all methods, for both media and cell layers, the amounts of radioactivity in chondroitin were less than 10%.

The question of whether different tissues contain different kinds of fibroblasts was first approached by Parker (33, 34). From nine different tissues he could isolate nine populations of fibroblast-like cells which differed from one another by several criteria. His results did not answer the question, however, for no precautions were taken to try to exclude those nonfibroblastic cell types which \textit{in vitro} can assume fibroblastic morphology. Although the term, fibroblast, has a rather restricted meaning for cell types \textit{in vitro}, frequently it also is used to describe populations of cells \textit{in vitro} having fibroblastic morphology regardless of whether the progenitors of such cells existed in \textit{vivo} as authentic fibroblasts (35). We choose to reserve the term, fibroblast, to designate only authentic \textit{in vivo} fibroblasts or their proven descendants \textit{in vitro} and to use the term fibroblast-like for cell populations \textit{in vitro} which display fibroblastic morphology, but whose progenitor cell types or their states of differentiation (e.g. fibroblast \textit{versus} fibrocyte) have not yet been established rigorously. In the present study, we have taken steps to try to exclude nonfibroblastic cell types. The purest population is that from cornea, an avascular tissue in which the stromal fibroblast population can be separated cleanly by dissection from the two nonfibroblastic populations (corneal epithelium and endothelium) and inoculated \textit{in vitro} in its entirety without the use of selective procedures. Although a previous study (17) demonstrated that the corneal cells cultured came only from the stroma, present information still does not reveal whether the stromal population of the Day 14 chick cornea \textit{in vivo} consists of fibroblasts, fibrocytes (i.e. "differentiated" fibroblasts), or a

---

Glycosaminoglycans from Fibroblast-like Cells

by guest on August 15, 2017 http://www.jbc.org/ Downloaded from
mixture of the two. For this reason, we will continue to designate the in vitro corneal population as fibroblast-like even though we believe that originally nonfibroblastic cells were excluded. On the other hand, in tissues such as heart and skin the authentic fibroblasts are sufficiently intermixed with other cell types in vivo as to preclude their separation by simple dissection. We therefore relied upon the rapid adhesion of fibroblast-like cells to accomplish their separation from nonfibroblast-like cells. Thus, for these two tissues, unlike cornea, a selective procedure had to be employed to obtain the fibroblast-like cell populations. This means that, although the heart cells and skin cells obtained had uniformly fibroblast-like morphologies, we cannot yet prove that these cells existed in vivo only as authentic fibroblasts or that we obtained a representative sample of the in vivo fibroblast population. Thus, it may be inappropriate in a rigorous sense to compare corneal populations with those from heart and skin. By the same criteria, however, it is appropriate to compare heart populations with those from skin. Our results suggest that even these two populations differ from one another with respect to the glycosaminoglycans they synthesize. Our data are not compatible with theories suggesting that a single, generalized fibroblast-type is found in all tissues. However, the results are compatible with the possible existence of stably differentiated fibroblast populations in different tissues, although for the reasons discussed above, they do not prove it.

To what extent do the patterns of glycosaminoglycans made by the three populations in vitro resemble those that the same populations made in vivo, i.e., how stable is the differentiated state of each population? In the case of cornea, previous studies have shown that the ability to make keratan sulfate I, a glycosaminoglycan synthesized only in the cornea and only by the stromal fibroblasts (31, 36), is lost when corneal fibroblasts alone or even whole corneas are incubated in vitro (36). This phenomenon also has been seen recently in fibroblast-like populations from rabbit and bovine corneas (37–39). The ability to make other glycosaminoglycans, such as chondroitin 4-sulfate and heparan sulfate, is not lost, however, and the ability to synthesize chondroitin 6-sulfate is acquired (36, 39). Thus, substantial changes occur in the pattern of glycosaminoglycans synthesized when corneal fibroblast-like cells are grown in vitro.

Several studies have examined the glycosaminoglycans synthesized in the embryonic and adult heart and skin. Such data must be used carefully here, for not all the glycosaminoglycans of an intact tissue are synthesized by the resident fibroblasts. We therefore do not know for certain what glycosaminoglycans the authentic heart and skin tissue fibroblasts were synthesizing in vivo when they were removed from the Day 14 embryo. Embryonic chick hearts older than Day 3 of incubation contain cardiac myocytes, endocardium, epicardium, vascular cell types, valve tissue, and fibroblasts; such heart tissue contains hyaluronic acid, chondroitin sulfates, heparan sulfate, and dermatan sulfate (40), the same types of glycosaminoglycans as synthesized by heart fibroblast-like cells alone in vitro. Vertebrate skin contains at least epithelium, blood vessels, and dermal fibroblasts; unlike heart tissue, however, hyaluronic acid and dermatan sulfate constitute the two major glycosaminoglycans of skin, with heparan sulfate and chondroitin 4- and 6-sulfates as minor components (41, 42). During Day 14 of chick development, the concentration of hyaluronic acid (50%) in skin is declining, whereas that of dermatan sulfate (26%) is steadily rising; concentrations of chondroitin sulfates and heparan sulfate each remain at about 12% (43). The skin fibroblast-like cells in vitro studied here appear to synthesize far less dermatan sulfate and far more chondroitin sulfate than would be expected from the concentrations of glycosaminoglycans present in intact embryonic chick skin. Nevertheless, they appear to synthesize high concentrations of hyaluronic acid and significant amounts of heparan sulfate, in correspondence with whole skin. Thus, although insufficient data are available to reveal the extent to which the patterns of glycosaminoglycan biosynthesis of heart fibroblast-like cells are altered when they are removed from the ventricle, skin fibroblast-like cells may almost cease synthesis of dermatan sulfate and may approximately double their synthesis of chondroitin sulfate. Despite the fact that such changes in glycosaminoglycan biosynthesis probably occur in each of the three cell populations studied here, each population must change in a different way, for once adapted to in vitro conditions, the three populations are still distinguishable, albeit perhaps not by the same criteria which would be used to distinguish them were we able to identify and quantitate the products they synthesize in vivo.

It is of interest to compare the glycosaminoglycans synthesized by the three populations and their distributions between cell layers and media (Tables II and III). The proportion of radioactivity incorporated in heparan sulfate by corneal fibroblast-like cell populations was higher in cell layers than in media. This suggests that heparan sulfate to some extent remained associated preferentially with cell layers, as originally demonstrated by Kraemer (32) in several established lines of cultured cells. On the other hand, skin fibroblast-like populations showed only slight enrichment of the cell layer with heparan sulfate and those of heart showed no enrichment. Similar distributions were seen previously (22). Recent studies of human skin fibroblast-like cells (44) have suggested that, although some of the heparan sulfate bound in a cell layer appears to be intracellular and some may be on cell surfaces per se (trypsin-sensitive), the vast majority can be released from cell layers simply by treatment with collagenase (free of trypsin activity). The exact relationships of heparan sulfate proteoglycan to plasma membranes and collagen molecules remain to be elucidated.

Corneal and skin cultures incorporated substantial proportions of radioactivity in hyaluronic acid, whereas heart cultures did not. Using data from either media (Table II) or cell layers (Table III), one can distinguish each population from the other two. Indeed, aside from the total average amounts of radioactivity incorporated (Table I), levels of incorporation in hyaluronic acid constitute the most prominent distinction between heart and skin populations. These distinctions were confirmed by three methods of assay (Table IV). Irrespective of the exact amounts synthesized, in each of the three populations the media contained a higher proportion of the radioactive hyaluronic acid. This is consistent with the high solubility of hyaluronic acid in aqueous solutions and its apparent lack of covalent association with a protein core. Several previous studies of fibroblast-like cells in vitro have shown that, although hyaluronic acid biosynthesis is most active during logarithmic growth (cells used here were at confluency when labeling began), addition of fresh serum to cultures in stationary phase causes transient stimulation of hyaluronic acid biosynthesis; such stimulation occurs by a mechanism unrelated to that involved in serum stimulation of DNA synthesis (45, 46). In the present experiments we have not determined whether each of the three populations responds identically to serum stimulation. As noted under "Results," we have shown...
in other experiments that the cell densities at which saturation occurs in these cultures of fibroblast-like cells differ from one another (heart > skin > cornea) (29). Incorporation of radioactivity into hyaluronic acid by 3T3 fibroblast-like cells in vitro is progressively reduced at higher cell densities (47). In the present experiments, the levels of incorporation into hyaluronic acid were: heart < skin < cornea. It remains to be directly demonstrated if saturation density is the primary regulator of hyaluronic acid biosynthesis in these cells.

Data presented here demonstrate a heterogeneity in ability of embryonic fibroblast-like cell populations to synthesize glycosaminoglycans. They lead to the prediction that populations of fibroblast-like cells obtained by dissociating whole embryos, for example, will behave as though composed of mixtures of cell types. Heterogeneity in ability of such chick populations to produce virus has already been observed (48). Moreover, heterogeneity of fibroblast-like cells from human skin has been seen with respect to testosterone metabolism (49) and levels of lysosomal enzymes (50). Present data therefore suggest that heterogeneity with respect to other properties can be expected as well.

Acknowledgments — We thank Drs. J. A. Cifonelli and M. B. Mathews for highly purified reference standards of glycosaminoglycans and Dr. A. H. Conrad for criticism of the manuscript.

REFERENCES
33. Parker, R. C. (1939) Science 76, 219-220
34. Parker, R. C. (1933) J. Exp. Med. 58, 401-414
Differences in glycosaminoglycans synthesized by fibroblast-like cells from chick cornea, heart, and skin.

G W Conrad, C Hamilton and E Haynes