# LOCALIZATION OF HYALURONIC ACID IN SYNOVIAL CELLS BY RADIOAUTOGRAPHY

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ABSTRACT

Cultured human synovial cells secrete hyaluronic acid (HA) into the culture medium. Glucosamine-6-$^3$H was shown to be a direct and relatively specific precursor of HA-$^3$H by the following observations: the susceptibility of nondialyzable radioactivity in the medium to hyaluronidase, its migration with hexuronic acid on zone electrophoresis in polyvinyl chloride, its exclusion from Sephadex G-200, and the localization of radioactivity to glucosamine after hydrolysis of the labeled polysaccharide. The presence of intracellular HA-$^3$H was established by sequential extraction of labeled cells and by radioautography of synovial cell cultures digested with hyaluronidase *in situ*. When cells were exposed to medium lacking glucose, glucosamine-$^3$H-uptake was enhanced; and this made possible electron microscopic radioautographic studies. These studies demonstrate the early and continued presence of HA-$^3$H within the Golgi apparatus.

Hyaluronic acid (HA) is found in the matrix of many connective tissues where it appears to be synthesized by the local mesenchymal cells (1). This high molecular weight, anionic polysaccharide is also synthesized by cultures of cells derived from connective tissues (2–6). However, the intracellular organelles involved in the formation and secretion of HA are not known.

This report describes our observations on the ultrastructural sites of HA synthesis and storage. Electron microscopic radioautography has been performed on cultures of human synovial cells that incorporate glucosamine-$^3$H and synthesize and secrete HA-$^3$H. Parallel biochemical studies demonstrate that glucosamine is a relatively specific precursor of the HA which is produced by these cells and that the majority of the intracellular radioactivity visualized by radioautography is present as labeled HA. Possible artifacts created during the fixation and preparation of the cells for electron microscopy were excluded by pertinent additional experiments.

Synovial cells cultured in conventional medium incorporate only small amounts of glucosamine. In order to obtain adequate numbers of radioautographic grains from ultrathin sections, it was necessary to increase greatly the uptake of glucosamine-6-$^3$H by these cells. This was accomplished by using a medium deficient in glucose and containing pyruvate as an energy source.

## MATERIALS AND METHODS

### *Tissue Culture*

Samples of synovial membrane from human joints were obtained at arthrotomy and explanted to plastic Petri dishes within 4 hr. Biopsy tissues were "floated" in medium in sterile Petri dishes, which permitted small samples of the superficial lining cells to be removed to another culture dish. In this way it was possible to obtain a culture consisting principally of lining cells and a few subsynovial fibroblasts, inflammatory cells, fat cells, and capillary endothelial cells. The explants were nourished initially in medium consisting of Dulbecco-Vogt modified Eagle's medium

containing 25% calf serum in an atmosphere of 10% $CO_2$ in room air. The concentration of calf serum was reduced to 10% when a confluent monolayer of cells had developed. Subcultures were made as required from confluent cultures at saturation density. All experiments were performed on early confluent cultures.

### *Identification of Tritiated HA in Culture Medium*

Cultures of synovial cells were incubated for 30 min to 96 hr in medium containing 5–10 μc of glucosamine-6-$^3$H.[1] Known volumes of medium were dialyzed in 1 liter of acetate buffer, pH 4.8 (0.1 M NaCl, 0.05 M potassium acetate, and acetic acid). A small aliquot was taken for counting. Then the percentage of counts in the dialyzed medium susceptible to digestion with testicular hyaluronidase and thereby rendered dialyzable or filterable was determined. Two aliquots of dialyzed medium were taken; testicular hyaluronidase[2] was added to one, and the other served as a control. Both samples were incubated overnight at 37°C and then filtered or dialyzed.

COUNTS FILTERED: Millipore filters (27 mm, 10 mμ) in Pyrex holders were set up and washed with pH 4.8 buffer. The control and hyaluronidase-digested samples were added to separate filters, and the filtrates were collected quantitatively and analyzed for radioactivity. Negligible counts were detected in the control filtrate. The percentage of counts rendered filterable by hyaluronidase was then calculated.

COUNTS DIALYZABLE: The samples of control and hyaluronidase-digested medium were dialyzed exhaustively in potassium acetate (0.02 M). After dialysis the volumes of medium were measured, and an aliquot was taken for counting. The percentage of counts rendered dialyzable by hyaluronidase was determined.

Radioactivity was measured in a Packard Tri-Carb (Packard Instrument Co., Downers Grove, Ill.) liquid scintillation spectrometer with Bray's solution or a toluene-Cab-O-Sil (Cabot Corporation, Boston, Mass.) solution as counting fluid. Quenching was monitored by the use of an external standard or by the addition of an internal standard.

### *Zone Electrophoresis*

About 40 ml of medium containing HA-$^3$H were dialyzed in potassium acetate (0.02 M) and lyophilized. The powder was dissolved in 3 ml of distilled water and applied to a block (40 × 8 cm) of polyvinyl chloride.[3] Electrophoresis was carried out in a cold room for 16 hr at 350 v, 60 mamp. The block was cut into segments approximately 1 cm wide, and the contents were eluted with water and analyzed for counts, hexuronic acid (7), and protein (8).

### *Chromatography*

SEPHADEX: A 41 × 1.3 cm column containing G-200 Sephadex[4] was prepared with potassium acetate (0.15 M) as the solvent.

PAPER CHROMATOGRAPHY: Samples were hydrolyzed, in sealed tubes, in 4 N HCl for 5 hr at 100°C. The acid was removed under vacuum in the presence of NaOH. Ascending chromatography was carried out on Whatman No. 1 paper (8 × 8 inches) in butanol, pyridine, water (3:2:1.5, three ascents) or in ethyl acetate, pyridine, water (8.6:1:1.15, upper phase four ascents). Sugar spots were detected with silver nitrate and alcoholic NaOH.

For scanning chromatograms for radioactivity the paper was cut into 3-cm strips and counted in a Vanguard Autoscanner 880 (Vanguard Instrument Corp., Roosevelt, N.Y.).

### *Cell Extractions*

Cell cultures were incubated for 1 hr in a medium containing 10 μc of glucosamine-6-$^3$H per milliliter, and for another hour in fresh, unlabeled medium. The medium was aspirated, and the cultures were washed three times with cold phosphate-buffered saline, pH 7.2. The cells were policed into suspension with a rubber policeman, collected in a test tube, and centrifuged (1500 rpm for 5 min); the supernatant was discarded. The cells were then extracted with 60% ethanol at 100°C for 1 hr. The suspension was centrifuged, and the sediment was dried with acetone on a Buchner funnel. The residue was digested with 0.1 N $H_2SO_4$ for 60 min at 80°C. After centrifugation the residue was hydrolyzed in 2 N HCl for 4 hr at 100°C. Appropriate aliquots were analyzed for radioactivity (as above).

### *Uptake of Glucosamine-6-$^3$H*

Replicate cultures of synovial cells were incubated for 1–4 hr in whole medium or in Krebs-Ringer phosphate buffer (0.12 M NaCl, 0.013 M KCl, 0.01 M $KH_2PO_4$, 1 mM $CaCl_2$, and 0.65 mM $MgSO_4$, and NaOH to pH 7.4) containing 20 mM pyruvate, 10 μc glucosamine-6-$^3$H per milliliter and glucose in concentrations varying from 0.0 to 30 mM. After incubation the labeled medium was aspirated, and the cells

[1] Purchased from New England Nuclear Corp., Boston, Mass. Specific activity 238 mc/mmole.

[2] Generously supplied by Wyeth Laboratories, Philadelphia, Pa.

[3] B. F. Goodrich Chemical Co., Cleveland, Ohio. PVC #427.

[4] Pharmacia Fine Chemicals Inc., New Market, N.J.

were washed five times with unlabeled Krebs-Ringer buffer. The cells were policed into suspension and filtered onto 0.45 $\mu$ Millipore filters which were placed in counting vials, dried, digested with 1 ml of ammonium hydroxide, and counted in Bray's solution.

### *Radioautography*

Monolayer subcultures of synovial cells were incubated at 37°C for 5, 15, and 30 min in Krebs-Ringer phosphate buffer, pH 7.4, containing 20 mM pyruvate and 125 $\mu$c/ml of glucosamine-6-$^3$H. Additional cultures were incubated in this medium for 30 min and then in fresh whole medium for an additional 30, 90, and 150 min. Following these incubations the cultures were washed with fresh Krebs-Ringer phosphate, fixed in cold 3% glutaraldehyde in Krebs-Ringer phosphate, pH 7.2, for 60 min, and postfixed in cold 1% $OsO_4$ in Krebs-Ringer buffer for 30 min. The cells were then policed into 50% ethanol and centrifuged into a pellet at 2500 rpm for 5 min. The pellets were cut into blocks which were dehydrated and embedded in Epon 812.

1 $\mu$ sections were mounted on glass slides for light microscope radioautography. The slides were coated with a 1:1 dilution of K-5 emulsion (Ilford Ltd., Ilford, Essex, England), by dipping. The slides were developed 3–14 days later in VHD developer (Brandywine Photo Chemical Co., Avondale, Pa.) for 2 min and fixed in Kodak Rapid Fixer. The sections were mounted in dilute glycerol and examined with a phase microscope.

The method of preparing radioautographs for electron microscopy was adapted from the methods of Salpeter and Bachmann (9) and Granboulan (10). Thin sections were mounted on slides coated with 1% nitrocellulose. They were coated with emulsion by dipping in a 1:4 dilution of L-4 emulsion (Ilford Ltd.) and exposed at room temperature in Bakelite (Union Carbide Corporation, New York) boxes containing desiccant. Slides were developed after 4–10 wk in Microdol-X (Eastman Kodak Co., Rochester, N.Y.) for 5 min, fixed in 15% thiosulfate, and washed briefly. After they had been dried, the films were floated onto water, and the sections were covered with 200-mesh copper grids. The films were then picked up on a clean slide and air dried. The sections were stained with lead citrate and examined in a Siemens Elmiskop I electron microscope, at 60 or 80 kv, with a 30 $\mu$ objective aperture. Grain counts were made from low-power micrographs by listing the principal organelle underlying the smallest circle encompassing each grain.

Possible artifacts of extraction or adsorption of radioactivity (11) resulting from fixation and dehydration were investigated. Monolayer cultures of synovial cells grown on cover slips were incubated for 30 min in medium labeled with glucosamine-6-$^3$H. Individual cover slips were then fixed at room temperature in 3% glutaraldehyde in Krebs-Ringer phosphate buffer (pH 7.2) for 10 min, 10% formalin in 0.1 M phosphate buffer (pH 7.4) for 15 min, 1% osmium tetroxide in Krebs-Ringer phosphate buffer (pH 7.2) for 15 min, and absolute methanol for 10 min. The cover slips were then dehydrated through ethanol, air dried, and coated with K-5 emulsion. After exposure for 1 wk they were developed in VHD developer, mounted on glass slides, and examined with a phase microscope.

As a determination of whether HA-$^3$H had been extracted from the cells during their fixation and embedding in epoxy resins, aliquots of glutaraldehyde, osmium tetroxide, and ethanols were dialyzed against water and then assayed for radioactivity in a liquid scintillation spectrometer, as above.

The susceptibility of intracellular radioactivity to hyaluronidase was investigated by radioautographic methods. Monolayers of synovial cells grown on cover slips were incubated in Krebs-Ringer phosphate buffer (pH 7.4) containing 20 mM pyruvate and 50 $\mu$c/ml of glucosamine-6-$^3$H for 15 and 45 min. The cells were then washed rapidly five times in unlabeled Krebs-Ringer phosphate with pyruvate. They were then fixed in cold 1.5% glutaraldehyde in Krebs-Ringer buffer (pH 7.2) for 10 min, followed by washing (continuous stirring) for 48 hr in three changes of 0.1 M phosphate (pH 7.4) containing 0.2 M sucrose. Cover slips were then digested overnight with testicular hyaluronidase in 0.1 M acetate buffer (pH 4.7) at 37°C. Control cover slips were incubated in acetate buffer alone under the same conditions. Both sets of cover slips were then washed, air dried, and coated with a 1:1 dilution of K-5 emulsion. After exposure for 2–10 days, the radioautographs were developed in VHD developer which contained 0.025% benzotriazole for reduction of fog. The cover slips were then mounted on glass slides, and grain counts were made by direct observation with a phase microscope.

## RESULTS

### *Identification of HA-$^3$H in Cell Culture Medium*

Tritiated hyaluronic acid (HA-$^3$H) was identified in the medium of synovial cell cultures by dialysis followed by digestion of the dialyzed medium with testicular hyaluronidase and by filtration of the digest through 10-m$\mu$ Millipore filters or redialysis as described in Materials and Methods. After incubation of synovial cells with glucosamine-6-$^3$H, 75–80% of the nondialyzable radioactivity in the medium was susceptible to hyalu-

TABLE I

*Susceptibility of Nondialyzable Radioactivity in Culture Medium to Testicular Hyaluronidase*

| Medium* | dpm added | Filtrate‡ | | Nonfilterable | |
|---|---|---|---|---|---|
| | | dpm | % | dpm | % |
| Testicular hyaluronidase, 24 hr | 30,800 | 24,300 | 78 | 3,600 | 11.9 |
| Buffer, *pH* 4.5, 24 hr | 7,220 | 1,080 | 14 | 2,900 | 41.5 |

* Medium harvested from synovial cell cultures after 5 hr incubation with glucosamine-6-$^3$H, 10 $\mu$c/ml.

‡ After filtration through a 10 m$\mu$ Millipore filter.

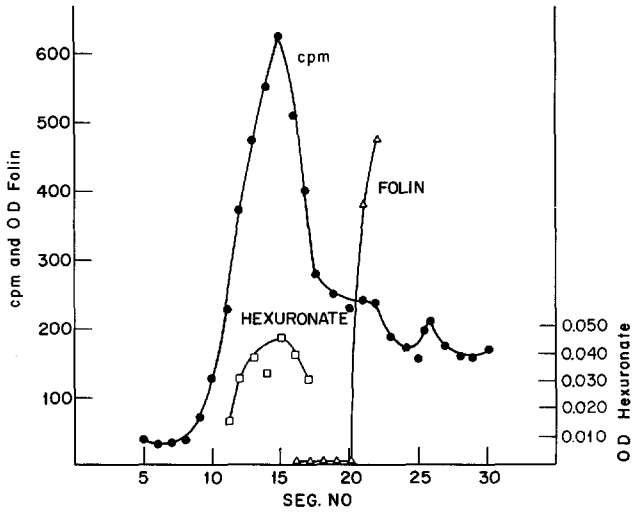



FIGURE 1 Zone electrophoresis (pH 7.4) of $^3$H medium. Synovial cell cultures incubated for 48 hr in medium containing 10 $\mu$c glucosamine-6-$^3$H per milliliter. Medium dialyzed and then subjected to zone electrophoresis (see text). Segments were eluted with water and analyzed for radioactivity (●—●), hexuronic acid (□—□), and protein (△—△).

ronidase (Table I). In comparison, when tritiated glucose was used in place of glucosamine, only 30% of the counts in the dialyzed medium were rendered filterable by hyaluronidase. HA-$^3$H was detectable as early as 30 min after exposure to glucosamine-6-$^3$H, and additional HA-$^3$H was secreted into the medium 12 hr after the removal of the glucosamine-6-$^3$H and addition of cold medium. Similar results were obtained whether the glucosamine-6-$^3$H was added to whole culture medium or to Krebs-Ringer phosphate buffer containing 20 mM pyruvate.

A number of steps were carried out for rigorous identification of the HA-$^3$H. First, medium was subjected to zone electrophoresis as described in Materials and Methods. The major portion of the radioactivity migrated in segments which contained hexuronic acid and ahead of the proteins in the medium (Fig. 1). These segments were pooled, concentrated, and applied to a column of G-200 Sephadex. Most of the radioactivity and all the hexuronic acid emerged in the void volume; this indicated exclusion of the radioactive macromolecule from the column. Part of the labeled eluate in the void volume was digested with streptococcal hyaluronidase[5] and added again to the same Sephadex column. Now all the radioactivity was markedly retarded on the column, and it appeared in a late eluate corresponding in location to the disaccharide produced by streptococcal hyaluronidase digestion of known HA (Fig. 2). The remainder of the labeled eluate in the void volume from the Sephadex column was dialyzed in distilled water, hydrolyzed in acid, and studied by paper chromatography. Radioactivity was confined to a single spot corresponding in location to known glucosamine identified by a silver stain (Fig. 3).

[5] Wyeth Laboratories, Philadelphia, Penn.

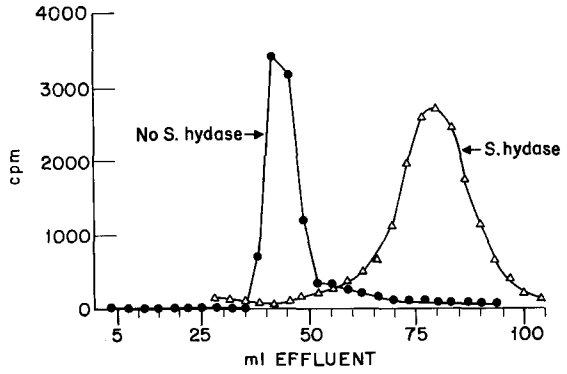



FIGURE 2 Hyaluronate-$^3$H on Sephadex G-200. The radioactive polysaccharide obtained from zone electrophoresis of dialyzed medium was chromatographed on Sephadex G-200, and counts per minute were measured in 3.5-ml fractions (●—●). All the radioactivity was recovered in the void volume, and these fractions were pooled and digested with streptococcal hyaluronidase. The digest was then rechromatographed on Sephadex G-200 and the effluent was assayed for radioactivity (△—△).

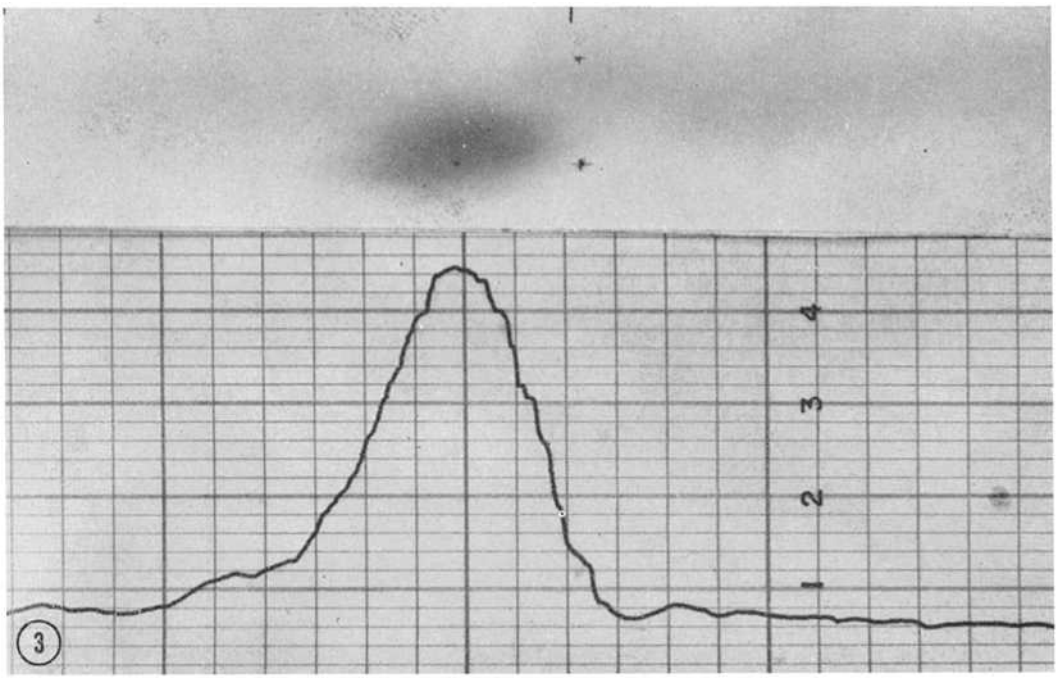



FIGURE 3 Radioactive scan of a paper chromatogram of an hydrolysate of hyaluronate-$^3$H to which cold carrier glucosamine was added. Upper strip is a silver nitrate stain of the chromatogram.

Thus the isolated polysaccharide contained hexuronic acid and radioactive glucosamine and was susceptible to streptococcal hyaluronidase digestion. These findings prove that glucosamine is incorporated directly into HA. This incorporation is detectable in the medium as early as 30 min after exposure to glucosamine-$^3$H, and it continues for a number of hours after withdrawal from the tritiated precursor. These findings also indicate the existence of a cellular pool of HA or a specific precursor with a slow turnover.

### *Identification of HA-$^3$H in Cells*

Serial extractions of synovial cells after their incubation with glucosamine-6-$^3$H revealed that 74% of the original radioactivity was extracted after hydrolysis with 2 N HCl (Table II). Further hydrolysis of this extract in 4 N HCl, neutralization, desalting, and paper chromatography revealed a radioactive spot in the location of known glucosamine. Another radioactive spot was also present but has not yet been identified. These

TABLE II

*Extractions of Radioactivity from Synovial Cells Incubated with Glucosamine-6-$^3H$*

| Extraction procedure | extracted cpm | Probable source |
|---|---|---|
| | % | |
| 60% ethanol, 100°C | 19 | UDP-sugar |
| 0.1 N $H_2SO_4$, 80°C, 60 min | 7 | Terminal glycosidic sialic acid |
| 2 N HCl, 100°C, 120 min | 74 | Glycosidic sugars |

TABLE III

*The Effect of Testicular Hyaluronidase Digestion on Radioautographs of Synovial Cells Incubated with Glucosamine-6-$^3H$*

| Incubation time with glucosamine-6-$^3$H | Hyaluronidase | No. cells counted | No. grains counted | Grains/cell |
|---|---|---|---|---|
| *min* | | | | |
| 15 | + | 50 | 292 | 5.84 |
| 15 | 0 | 50 | 1358 | 27.16 |
| 45 | + | 25 | 454 | 18. |
| 45 | 0 | 20 | 982 | 49. |

TABLE IV

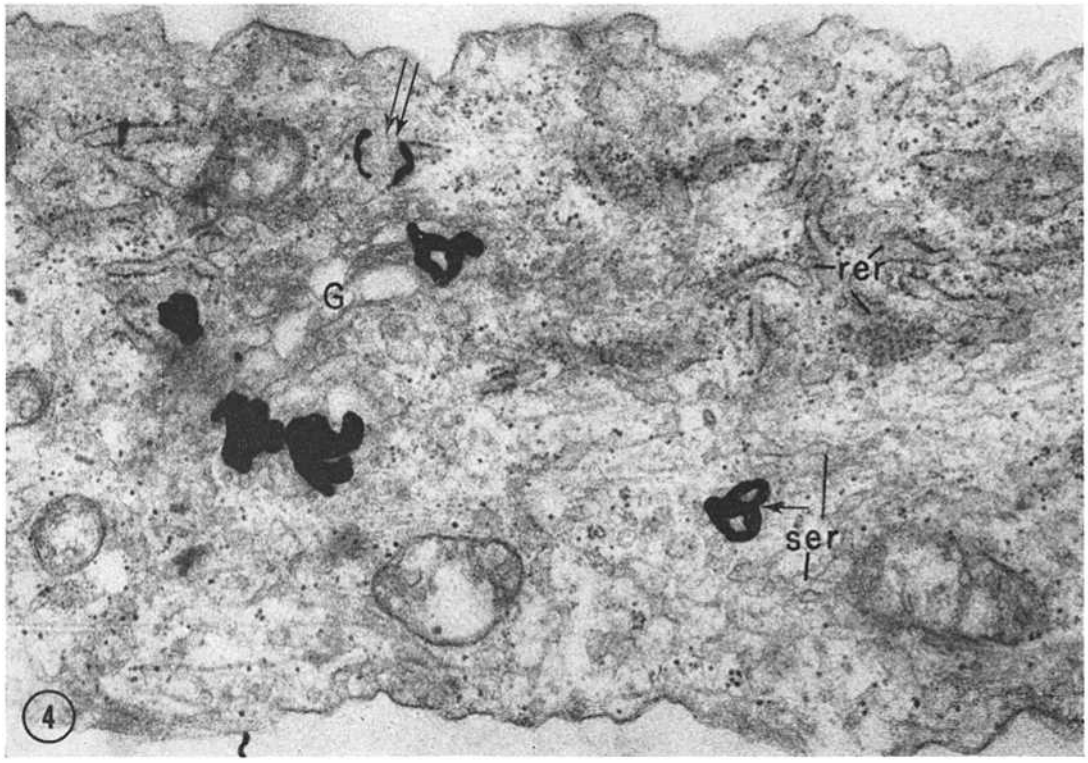
*Uptake of Glucosamine-6-$^3H$ by Replicate Cultures of Synovial Cells*

| Incubating conditions | Time | cpm/plate |
|---|---|---|
| | *min* | |
| Krebs-Ringer phosphate + 10 μc glucosamine-6-$^3$H + 20 mM pyruvate, no glucose | 60 | 937 |
| Krebs-Ringer phosphate + 10 μc glucosamine-6-$^3$H + 20 mM pyruvate + 2 mM glucose | 60 | 411 |
| Krebs-Ringer phosphate + 10 μc glucosamine-6-$^3$H, no pyruvate, no glucose | 60 | 540 |
| Whole culture medium + 10 μc glucosamine-6-$^3$H | 60 | 121 |

results indicate that the majority of the intracellular radioactivity was in the form of glycosidically bound glucosamine. Only 19% of the total intracellular radioactivity was extracted with 60% ethanol. These counts are presumed to originate from free glucosamine, phosphorylated glucosamine, and nucleotide sugar. However, this extract was not identified further since it was likely that almost all of this radioactivity would be extracted during the fixation and dehydration of these cells prior to their being sectioned and therefore it would not be a source of radioautographic grains. Only 7% of the intracellular radioactivity was extracted with 0.1 N $H_2SO_4$. These counts presumably correspond to terminal glycosidic neuraminic acid residues (12). They were not identified further since they represented such a small percentage of the intracellular radioactivity.

Further proof of intracellular radioactivity in HA was obtained by radioautography. Monolayers of synovial cells grown on Petri dishes were exposed to glucosamine-6-$^3$H for 15 and 45 min and then fixed; they were digested with hyaluronidase or pH 4.8 buffer alone and prepared for light microscope radioautography as described under Materials and Methods. Digestion with hyaluronidase resulted in an 80% reduction in the number of grains per cell at 15 min and a 63% loss at 45 min (Table III). Since the cells were not extracted with ethanol in these experiments, some of the radioactivity detected in the radioautographs of hyaluronidase-digested preparations probably represents material, such as nucleotide sugars, which would be extracted during embedding in epoxy resins for electron microscopic radioautography.

Initial attempts at electron microscopic radioautography were unsatisfactory because of insufficient intracellular radioactivity, except after prolonged exposure to glucosamine-6-$^3$H for 1 hr or more. The effect of glucose in the incubating medium on the uptake of glucosamine therefore was investigated. It was reasoned that the sugars might compete for transport into the cell and for incorporation into HA. Table IV shows that glucosamine uptake was markedly enhanced when Krebs-Ringer phosphate buffer containing 20 mM pyruvate as an energy source was substituted for whole medium containing glucose. It was found that glucosamine uptake from the buffer solution could be inhibited by the addition of




Figs. 4–11 are electron micrographs processed for radioautography, as described in the text, and stained with lead citrate.

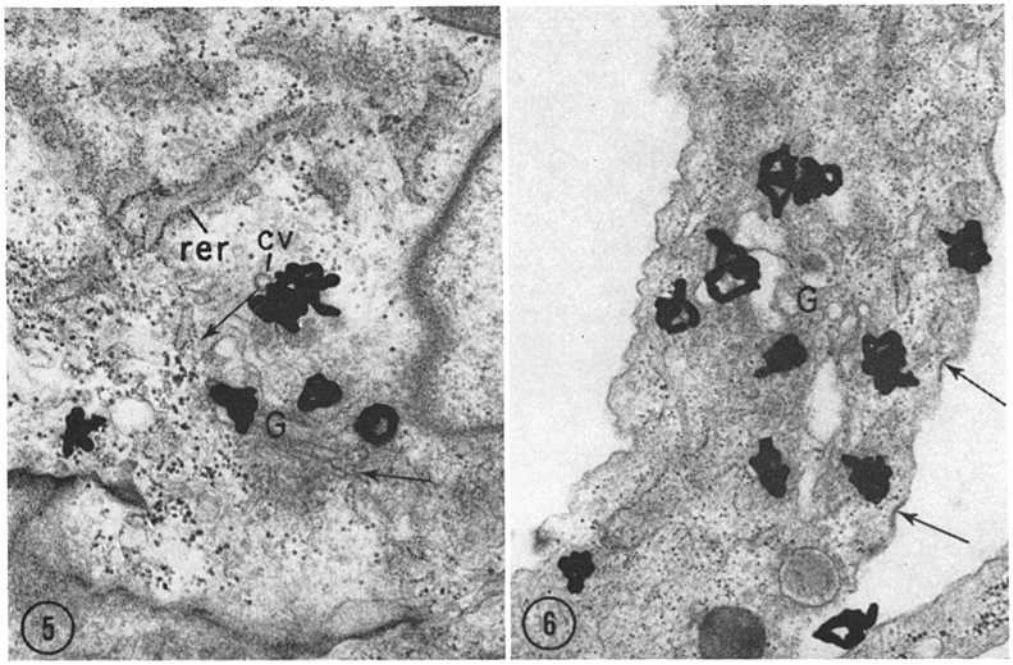
FIGURE 4 A synovial cell incubated in medium with glucosamine-6-$^3$H for 5 min. Four of the five silver grains seen are located over the Golgi apparatus (*G*). The remaining grain (arrow) is situated over a region of smooth endoplasmic reticulum (*ser*). Grains are absent over the area of rough endoplasmic reticulum (*rer*) and the mitochondria depicted. Partially developed grains (double arrow) were not uncommon but were not included in the grain counts. × 31,000.

glucose. Therefore we employed Krebs-Ringer buffer with pyruvate and 25–125 μc of glucosamine-6-$^3$H per milliliter as the incubating medium for our electron microscopic radioautographic studies. The increased uptake of isotope resulted in a satisfactory number of developed silver grains after only 5 min of incubation.

### *Fine Structure*

The cytoplasm of these cultured synovial cells presents certain characteristic features when it is examined with the electron microscope. The rough endoplasmic reticulum (RER) occupies a major portion of the cytoplasm and extends from the perinuclear area into the distal cytoplasmic processes. It consists of flattened cisternae filled with a finely granular material of moderate electron opacity.

Transitions of RER to agranular membrane systems appear to occur in two forms. In one form, the cisternae dilate and the membranes lose their attached ribosomes and become infolded. In addition, the cisternal contents become less dense (Fig. 7). Similar clear vacuolar structures with infolded, limiting membranes are often seen in the cytoplasm near the plasma membrane, and they have no attachment to the RER. However, their continuity with the RER may occur in another plane of section. Occasionally these vacuoles appear to be in direct continuity with the extracellular space. A second form of transition of RER is in the region of the Golgi apparatus where small vesicles devoid of ribosomes appear to arise from narrow cisternae of RER and merge with the small vesicles commonly seen in the Golgi zone (Figs. 5, 7).




FIGURES 5 and 6 Synovial cells incubated for 5 min in medium with glucosamine-6-$^3$H. In both figures the clustering of grains over the Golgi apparatus (*G*) is clearly illustrated. In Fig. 5, the rough endoplasmic reticulum (*rer*), though closely connected with the Golgi apparatus (arrows at possible points of transition), appears to be unlabeled. A coated vesicle (*cv*) is seen in the Golgi zone. × 48,000. In Fig. 6, coated dense "pits" are seen along the membrane surface (arrows). × 22,000.

A well-developed Golgi apparatus surrounds the nucleus and frequently extends peripherally between the cisternae of RER. It consists of four or five flattened saccules. The ends of the saccules are frequently dilated to form clear vacuoles enclosed by a smooth limiting membrane. Similar vacuoles are also seen along the concave surface of the Golgi saccules. These vacuoles often contain some finely granular material along their inner surface (Figs. 9, 10). Vacuoles, resembling these Golgi vacuoles, are sometimes seen near the cell membrane, but a clear differentiation between these and the vacuoles which arise directly from the RER is usually not possible. In addition, two types of small vesicles are present in the Golgi apparatus. One type is coated on its external surface by a clearly demarcated zone of condensed cytoplasm containing radiate striations, while the other is usually smaller and has no external coating (Figs. 5, 7, 9, 10). Both kinds of vesicles are more prominent along the convex aspect of the Golgi saccules.

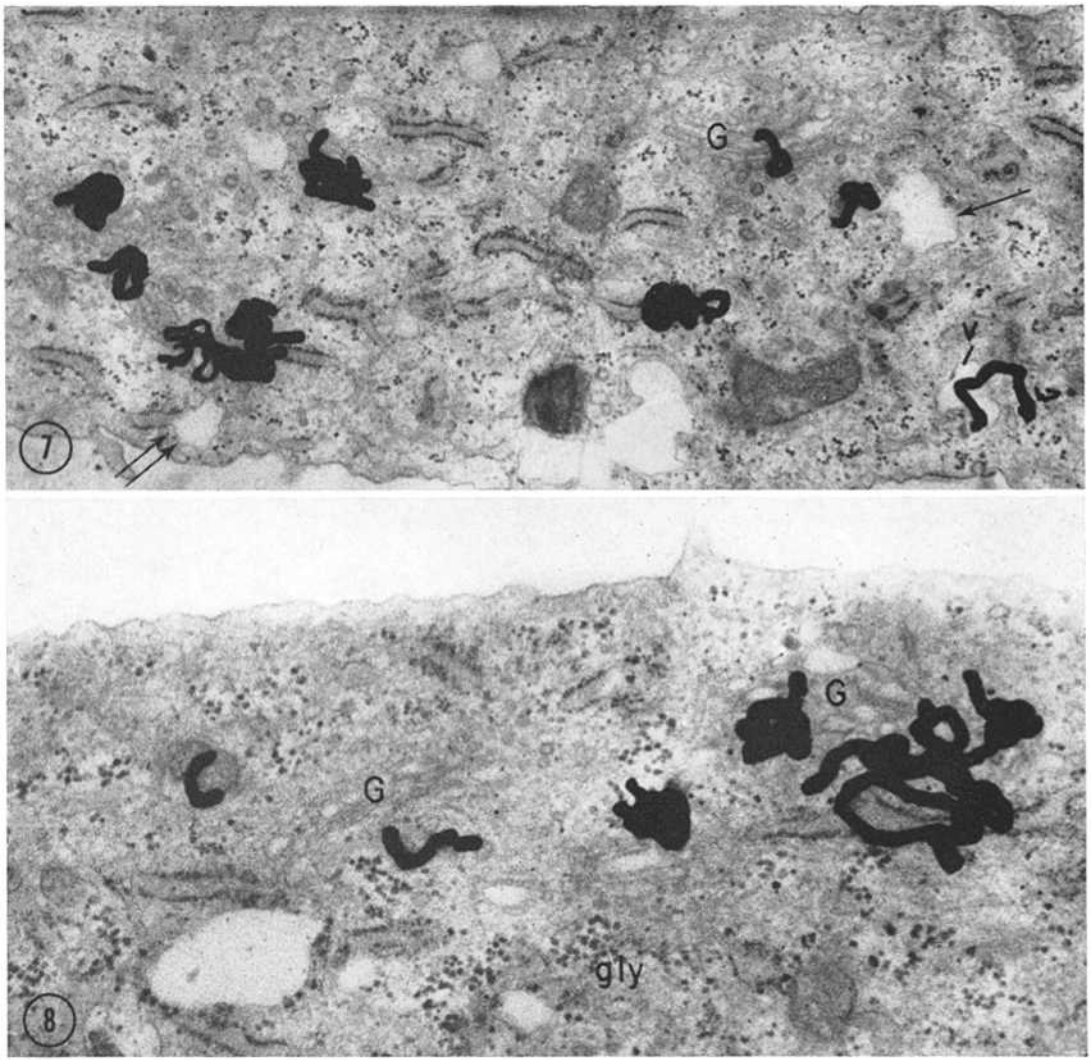
The perinuclear cytoplasm also shows frequent dense granules containing membranous lamellae, occasional multivesicular bodies, and autophagic vacuoles inside of which endoplasmic reticulum is seen (Fig. 9). Small clumps of dark granules, resembling glycogen deposits of other cells, are scattered throughout the ground cytoplasm.

Segments of the plasma membrane appear indented and darker and are lined by a layer of condensed cytoplasm (Fig. 6) resembling the "pits" described in other cells. Numerous small pinocytotic vesicles are seen in continuity with the plasma membrane (Fig. 9). Occasional finger-like projections of the cytoplasm extend into the extracellular space. Peripherally the cytoplasm takes the form of narrow processes which contain, in addition to the RER, lipid droplets and bundles of filamentous material.

No differences in fine structure were noted between cells exposed to and those not exposed to a medium containing glucosamine-6-$^3$H.

### *Radioautography*

After 5 min of incubation with glucosamine-6-$^3$H numerous radioautographic grains were seen overlying the cytoplasm of the synovial cells. The




FIGURES 7 and 8 Synovial cells incubated in medium with glucosamine-6-$^3$H for 15 min. The Golgi apparatus (*G*) in both cells is labeled by numerous grains.

FIGURE 7 Most of the grains overlie portions of the Golgi apparatus (*G*). However, one grain is situated over a cytoplasmic vacuole (*v*). The relation of such vacuoles to vacuoles in the Golgi apparatus (arrow) and to dilatations of the RER (double arrow) has not been resolved. × 28,000.

FIGURE 8 Small granules occurring in clusters within the cytoplasm are presumed to be glycogen (*gly*). These areas are not appreciably labeled compared with the Golgi apparatus (*G*) within which the radioactivity appears at this time. × 35,000.

grains were especially frequent over the concave aspect of the Golgi apparatus which consists chiefly of clear vacuoles. However, all parts of this organelle including the saccules and vesicles were associated with some of the silver grains (Figs. 4–6). As is shown in Table V, 47% of all the grains counted after 5 min of labeling were situated over the Golgi apparatus. The remaining grains were evenly distributed over the rest of the cytoplasmic organelles in approximate proportion to the areas occupied by these structures in the cell cytoplasm. Many of these grains lay within the perinuclear Golgi zone but were assigned to the Golgi apparatus only if the grain overlaid some recognizable element of this organelle.

When incubation with glucosamine-6-$^3$H was

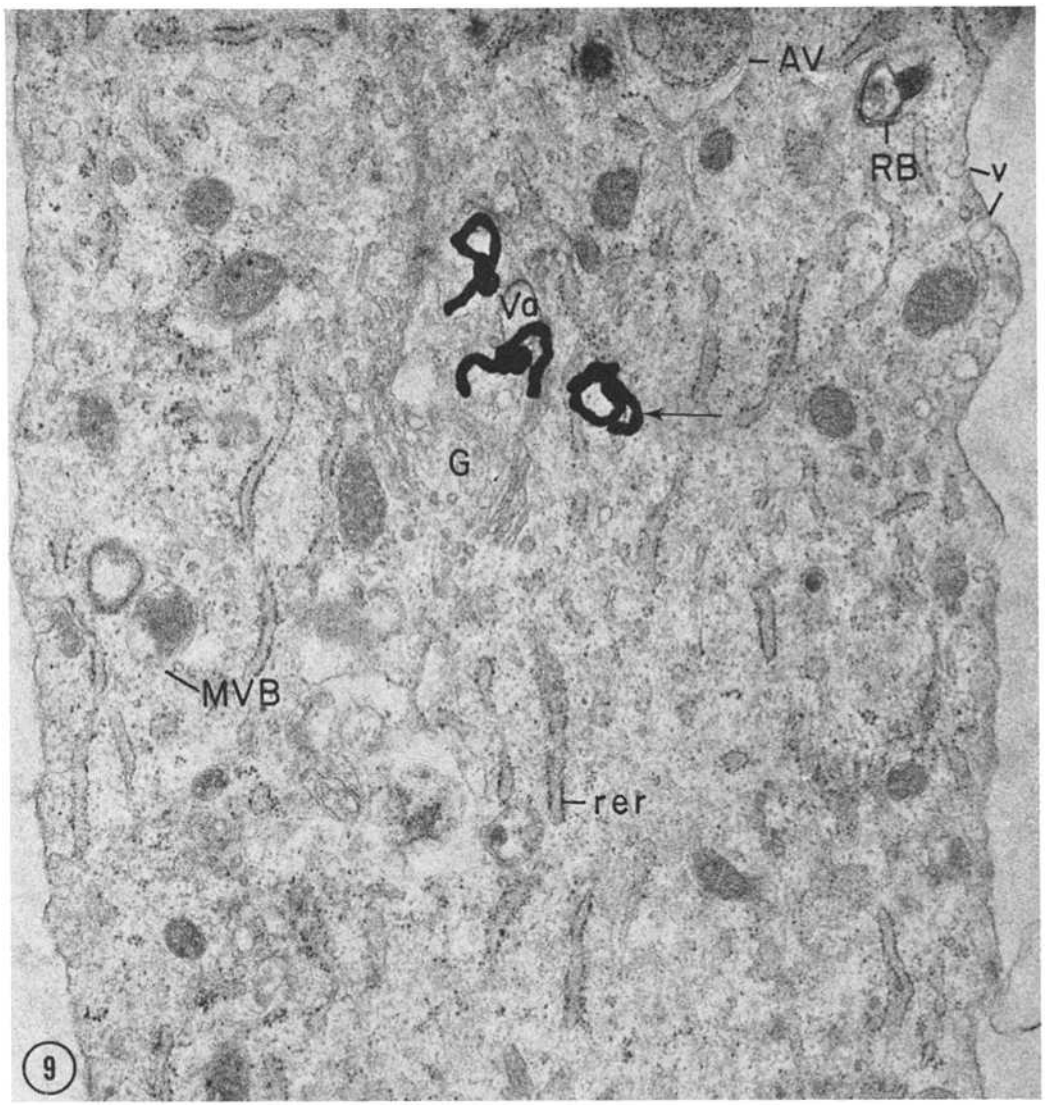



FIGURE 9 Synovial cell incubated for 30 min in medium with glucosamine-6-$^3$H. Two grains lie over the concave aspect of the Golgi apparatus (*G*) where a typical Golgi vacuole is seen (*Va*). The third grain overlies a cisterna of the abundant rough endoplasmic reticulum (*rer*) immediately adjacent to the Golgi apparatus (arrow). Other characteristic features of the perinuclear cytoplasm of these cells are a multivesicular body (*MVB*), a residual body (*RB*), and an autophagic vacuole (*AV*). Pinocytotic vesicles (*v*) are in continuity with the plasma membrane. × 31,000.

continued for 15 and 30 min, frequent radioautographic grains were again present over the synovial cell cytoplasm. At these times the Golgi apparatus continued to be the predominantly labeled organelle, and no other organelle appeared to become more consistently radioactive (Table V, Figs. 7–9).

Synovial cells, incubated for 30 min with glucosamine-6-$^3$H, then washed, and reincubated with unlabeled medium, showed a progressive decrease in the number of radioautographic grains developed per cell as compared with cultures which had been fixed immediately after 30 min incubation in the same concentration of iso-

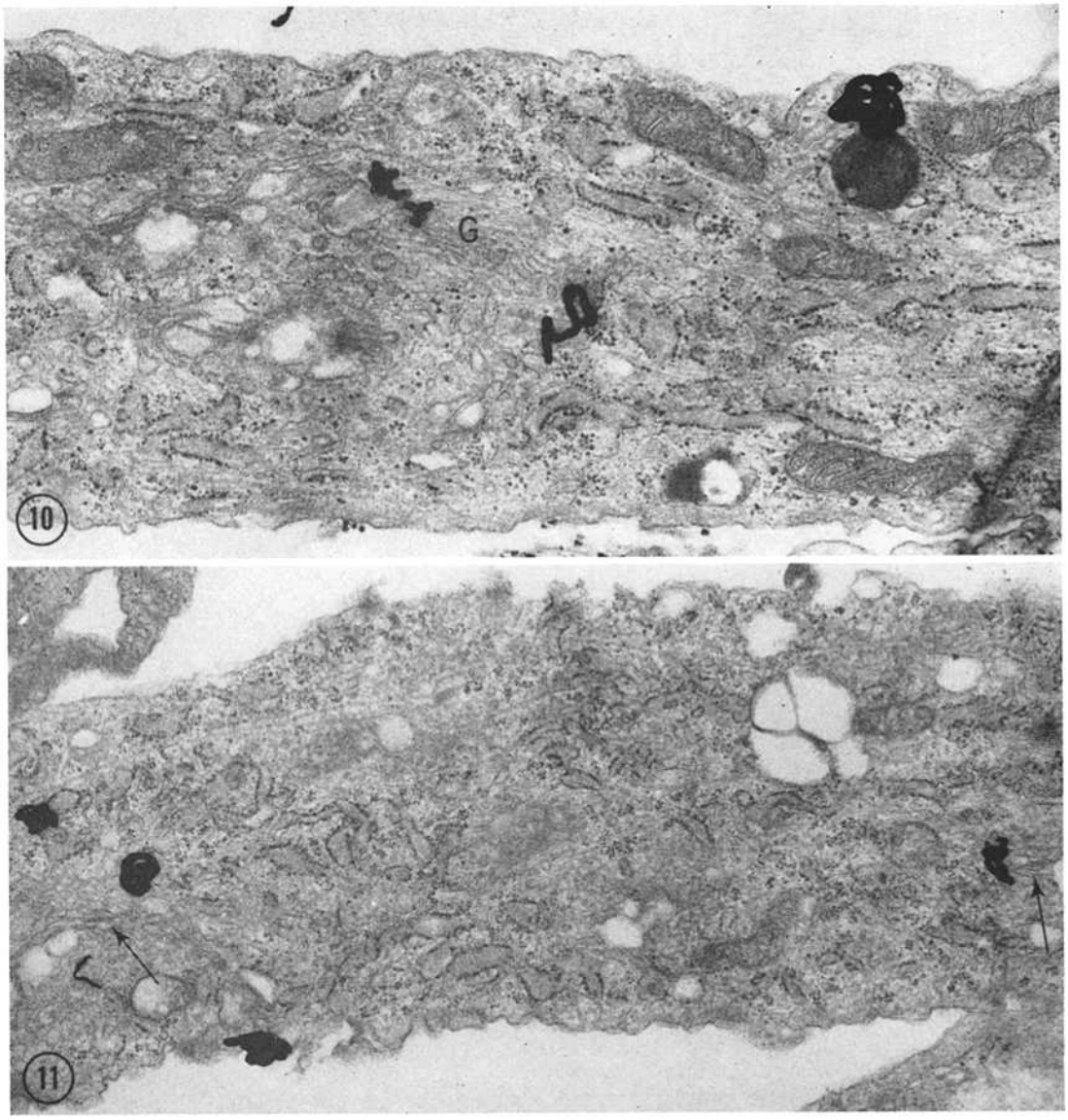



FIGURE 10 Synovial cell incubated for 30 min in medium with glucosamine-6-$^3$H and for an additional 90 min in unlabeled medium. Though this section was exposed to L-4 emulsion for 3 months, very few grains were developed. Grains remain associated with the Golgi apparatus (*G*). × 27,000.

FIGURE 11 Synovial cell incubated for 30 min in medium with glucosamine-6-$^3$H and then for 150 min in unlabeled medium. Exposure for 3 months, as in Fig. 10. Scattered grains are still associated with the Golgi apparatus (arrows). × 21,000.

tope and exposed to the radioautographic emulsion for the same period of time. This decline in the amount of intracellular HA-$^3$H was especially prominent after 90 and 150 min in unlabeled medium and was accompanied by a decrease in the percentage of grains overlying the Golgi apparatus (Table V). The increase in the percentage of grains associated with the ground cytoplasm and cell membrane represents an actual decrease in absolute radioactivity within these structures because of the over-all reduction in radioactivity within the cells which is reflected

TABLE V

*The Distribution of Radioautographic Grains Within Cultured Synovial Cells after Incubation with Glucosamine-6-$^3H$*

| Incubation Time | | | Distribution | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Glm-$^3$H | Cold medium | Grains counted | Golgi | RER* | Cell memb. | Vacuo‡ | Grd cyto§ | SER‖ | Mito¶ | Nucl** | Lytic bodies‡‡ |
| *min* | *min* | | % | % | % | % | % | % | % | % | % |
| 5 | 0 | 294 | 47 | 7.5 | 13.6 | 2.4 | 14.2 | 0 | 11 | 3 | 1.5 |
| 15 | 0 | 392 | 33 | 18 | 12.5 | 9 | 13.5 | 2 | 2 | 9.5 | 0 |
| 30 | 0 | 125 | 39 | 12 | 5.5 | 4 | 21 | 10 | 2 | 7 | 0 |
| 30 | 30 | 255 | 36 | 17 | 14 | 12 | 7 | 5 | 5 | 3 | 1 |
| 30 | 90 | 270 | 28 | 11 | 22 | 3 | 23 | 3 | 2.5 | 4 | 3.5 |
| 30 | 150 | 207 | 16 | 5.8 | 29 | 5 | 33 | 0 | 1.6 | 7 | 2.2 |

* Rough endoplasmic reticulum
‡ Large clear cytoplasmic vacuoles
§ Ground cytoplasm
‖ Smooth endoplasmic reticulum
¶ Mitochondria
** Nucleus
‡‡ Autophagic vacuoles and residual bodies as identified by morphological appearance.

in many fewer grains developed over each cell (Figs. 10, 11).

In the radioautographs of cover slip preparations fixed with a variety of agents there were no significant differences noted in the distribution or the number of grains developed per cell. In addition, no appreciable nondialyzable radioactivity was detected in the fixatives and alcohols used in the epoxy embedding of labeled cells for radioautography.

## DISCUSSION

This report appears to be the first demonstration of the use of glucosamine as a relatively specific precursor of connective-tissue anionic polysaccharides, although a number of workers have reported its incorporation into glycoproteins by liver cells (13–15). This conclusion is established on the basis of our studies of the culture medium. Most of the nondialyzable radioactivity secreted into the medium by cells incubated with glucosamine-6-$^3$H is susceptible to digestion with testicular hyaluronidase and streptococcal hyaluronidase. On zone electrophoresis the counts migrate with a hexuronic acid-containing component in a zone corresponding to known carrier HA. After hydrolysis of the isolated polysaccharide, all the radioactivity chromatographs with carrier glucosamine. Tritiated glucose and galactose have been used as precursors of chondroitin-sulfate synthesis in chondrocytes (14). However, in our synovial cell cultures, glucose-$^3$H was much less specific than glucosamine-$^3$H as a precursor of HA-$^3$H. Only 30% of the radioactivity in the medium of the cells exposed to labeled glucose was susceptible to hyaluronidase digestion, as compared to 75–80% in the case of glucosamine-$^3$H. On theoretical grounds as well, glucosamine offers advantages over other sugars used in earlier studies since glucose and galactose enter a wide variety of metabolic pathways (16) and are not components of the repeating disaccharide of either HA or chondroitin sulfate.

One key to success in these studies was the use of culture medium without glucose and with pyruvate as an energy source. Only in this way did synovial cells take up sufficient glucosamine-$^3$H to permit visualization of intracellular HA-$^3$H. The mechanism for the augmented uptake of glucosamine in the absence of glucose by synovial cells in culture is not presently understood. Competitive inhibition between sugars for transport across cell membranes has been well documented (17, 18) and may be the mechanism here. Enhanced glucosamine uptake in low glucose medium has also been demonstrated for ascites tumor cells (19).

Both radioautographic evidence and biochemi-

cal evidence are presented to show that after incubation with glucosamine-$^3$H the principal intracellular source of radioactivity is HA-$^3$H. After 15 and 45 min the majority of the radioactivity in synovial cells is susceptible to hyaluronidase digestion, as judged by radioautography. Serial extractions of cells previously incubated with glucosamine-$^3$H for 1 hr and incubated in unlabeled medium for an additional hour also reveal that approximately 75% of the intracellular radioactivity is in glycosidic glucosamine-$^3$H exclusive of terminal neuraminic acid residues.

Electron microscopic radioautography shows HA-$^3$H to be localized to the Golgi apparatus. The distribution of grains over this organelle suggests that the Golgi vacuoles contain most of the HA-$^3$H, but the resolution of this technique does not permit a more precise localization. Circumstantial evidence indicates that the Golgi apparatus is the site of HA-$^3$H biosynthesis since it attains increasing radioactivity with shorter incubation times. In addition, no other cell organelle or region becomes increasingly labeled during the shorter incubations. Such an increase would be expected if HA were transported to the Golgi apparatus from an earlier site of synthesis. In analogous studies of protein synthesis in rat pancreatic acinar cells with tritiated leucine (20), the RER was the predominant site of radioactivity after 5 min. In our studies at 5 min, however, the Golgi apparatus attained its highest radioactivity; this suggests that HA-$^3$H is indeed synthesized in this organelle. However, we cannot exclude the possibility that HA-$^3$H is very rapidly synthesized in some other organelle. The Golgi apparatus is also believed to be the site of synthesis of chondroitin sulfates in other cells. Godman and Lane (21) combined radioautography and electron microscopy to study the site of incorporation of $S^{35}O_4$ into chondroitin sulfate in embryonic rat chondroblasts. The label was first localized to the vesicles of the Golgi apparatus and later to large cytoplasmic vacuoles which appeared to fuse with the cell membrane. Neutra and Leblond (16) used glucose-6-$^3$H and galactose-1-$^3$H as precursors in a light microscope radioautographic study of chondroitin-sulfate synthesis in chondroblasts. They found early localization of radioactivity in a perinuclear zone interpreted to be the Golgi apparatus. This radioactivity was partially susceptible to testicular hyaluronidase digestion. Revel (22) used colloidal thorium as an electron-opaque stain for anionic chondroitin sulfate in cartilage cells of the salamander, mouse, and rabbit. The thorotrast was localized to the Golgi vacuoles in these cells.

The Golgi apparatus appears also to serve as a site of intracellular storage of HA. It remains the predominantly labeled organelle up to 30 min after incubation with glucosamine-$^3$H. In addition, no cytoplasmic structure, such as the large cytoplasmic vacuoles, becomes more radioactive as the radioactivity in the Golgi apparatus diminishes.

The greater percentage of grains over the ground cytoplasm and cell membrane at later times is most likely a reflection of the small numbers of grains over each cell at these times. Under these conditions nonspecific background grains (fog) become increasingly important and tend to overlie those areas of the cell occupying the greatest areas which, in these large attenuated cells, are the ground cytoplasm and the cell membrane. In addition, because of the geometry of these cells in thin section, any grain developed at some angular distance from its source in the section is likely to overlie the ground cytoplasm. The manner in which HA is transported to the extracellular space is not resolved by these studies, but the time of secretion is apparently a short phase of the intracellular life-span of this molecule.

In another approach to the biosynthesis of HA, Stoolmiller and Dorfman (23) have recently reported that HA synthesis in streptococci is not dependent upon new protein synthesis. In our laboratory, cycloheximide or puromycin did inhibit HA synthesis by synovial cells, but only partially.[6] It is interesting that HA synthesis may be separated morphologically as well as biochemically from protein synthesis by polyribosomes in the RER.

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[6] Smith, C., and D. Hamerman. Unpublished results.

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