ON THE SITE OF SULFATION IN THE CHONDROCYTE

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ABSTRACT

As observed autoradiographically in the cartilage of embryonic rats, radiosulfate is bound and concentrated only in vesicles of the juxtanuclear Golgi apparatus of secreting chondrocytes within 3 minutes of its presentation. From this area, vacuoles migrate peripherally and lodge in the subcortex; their sulfated contents are thence discharged *via* stomata to the extracellular matrix. The label, apparently often associated with microvesicles at 10 and 20 minutes, is subsequently localized in the dense contents of the larger vacuoles. Bound radiosulfate is not detectable in other organelles. It is concluded that the vesicular component of the Golgi apparatus is the actual site of sulfation. Intracellular hyaluronidase-sensitive metachromatic granules are found chiefly at the cell periphery or mantle, rarely juxtanuclear in the main Golgi zone.

INTRODUCTION

The uptake of radioactive sulfur into cartilage from S^{35}-sulfate, either *in vivo* or *in vitro*, is, in general, a measure of the synthesis of its principal sulfomucopolysaccharides, chondroitin sulfates A and C (1–6). The radiosulfate is first concentrated in the chondrocytes (7–13) in which it is present chiefly as chondroitin sulfate (14, 15). This product is then apparently secreted into the extracellular matrix (7, 10, 15) presumably as the sulfomucopolysaccharide-protein complex (15, 17). An accepted view is that polysaccharide synthesis *via* uridine nucleotide intermediaries (see 17–19), as well as sulfate esterification by enzymatic transfer of sulfate from 3-phosphoadenosine-5 phosphosulfate to polysaccharide or oligosaccharide acceptors, occurs intracellularly. Neither the exact nature of the final sulfate acceptor nor the organelles of sulfate binding and sulfation is yet known. It is the purpose of this report to describe the intracellular sites of radiosulfate accumulation, and presumably of sulfation, as localized autoradiographically.

MATERIALS AND METHODS

Pregnant Sherman rats in the 15th to 17th day of gestation were injected intraperitoneally with $Na_2S^{35}O_4$, made up in balanced salt solution. The radioactive dose per rat ranged from 2 μc/gram to 20 μc/gram in successive experiments. Sulfate readily traverses the placenta and is concentrated in embryonal tissue (20). Embryos were extracted under ether anesthesia at ½, 1, 3, 10, and 15 hours after the injection. The tibiofemoral epiphyseal cartilages were split, divided into fragments not exceeding 1 mm^3, rinsed quickly in a balanced salt solution containing 100 times the concentration (w/v) of sodium sulfate employed in the radioactive solution, and fixed for 60 minutes in 1 per cent osmium tetroxide buffered at pH 7.3 with phosphates (21).

For study of shorter time-intervals, tibiofemoral epiphyseal cartilages dissected from newborn rats and cut into cubes of about 1 mm were incubated in a medium consisting of Tyrode's balanced salt solution and 2 per cent calf serum containing 0.5 or 2.0 mc/ml of S^{35} as sodium sulfate. At 1½, 3, 6, and 9 minutes, fragments were transferred to fresh medium ("chaser") containing one-hundredfold more "cold"

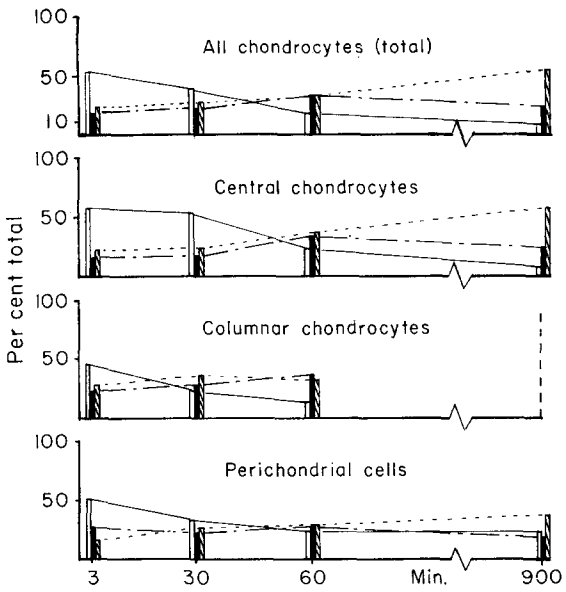


FIGURE 1 Bar graph representing the percentage of the total number of grains counted for each kind of chondrocyte over three areas of the cell at each time interval after $S^{35}O_4$ labeling. The juxtanuclear or main Golgi area is shown as the blank bar, the peripheral cytoplasm as the solid inked bar and the capsular matrix as the cross-hatched bar. The data are interpreted to show movement of isotopic material from the central juxtanuclear Golgi region to the cell periphery and subsequent discharge into the matrix.

sodium sulfate than that in the radioactive medium. Some fragments were fixed after a very brief rinse; others were allowed to remain in "chaser" for 1½, 5, and 15 minutes before fixation (pulse label). All fragments were fixed in osmium tetroxide as noted, washed, dehydrated, embedded in methacrylate, and sectioned at 1 μ for light microscopy and at about 60 to 80 mμ for electron microscopy.

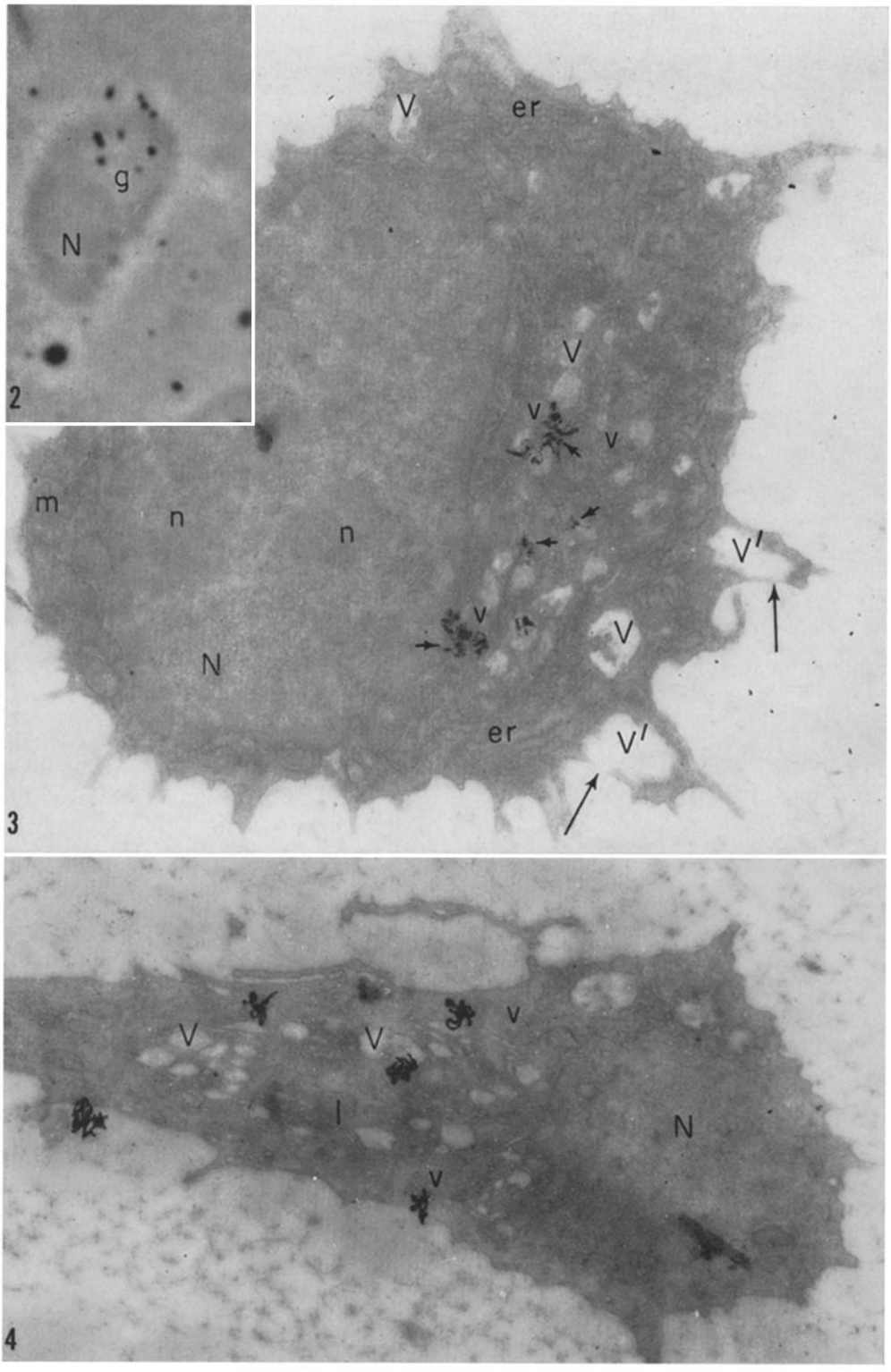
For phase microscopy, 1-μ sections mounted on gelatin-subbed slides were coated with melted Kodak NTB_2 emulsion by dipping after removal of the methacrylate with amyl acetate. They were then treated in a humidifier at 27°C, as prescribed by Kopriwa and Leblond (22), and after exposure for periods of from 2 to 6 weeks, were developed in Kodak D19 at 20°C for 2 minutes. For making grain counts, only those cells (with uptake) were included in which nucleus and perikaryon were fairly centrally sectioned.

Sections on carbonized grids were coated with a film of Ilford L4 emulsion and processed, according to the recommendations of Caro (23), after exposure times of from 4 to 8 weeks. Some grids were stained with the Karnovsky lead stain (24) or with lead tartrate (25) or citrate (26) to remove gelatin and augment contrast in the section, but to verify the accu-

FIGURE 2 Autoradiogram of a 1-μ section of a central chondrocyte fixed 3 minutes after presentation of radiosulfate *in vitro*, showing concentration of isotope over the juxtanuclear Golgi region *g* (pale area). *N* is the nucleus. Phase contrast, × 2000.

FIGURE 3 Electron micrograph of an autoradiogram illustrating the location of the label over the juxtanuclear Golgi zone of a central chondrocyte 10 minutes after presentation of radiosulfate *in vitro* (5 minutes in label, 5 minutes in chaser). Most of the grains indicated by small arrows overlie the small (15 to 40 mμ) vesicle component (*v*) of the large Golgi apparatus; the larger vacuoles of the Golgi zone marked *V* have moderately dense contents. Those vacuoles which have migrated to the periphery to lodge in the subcortex are marked V^1. After making contact with the cell membrane at one point, they establish continuity with the exterior through formation of stomata (large arrows). The nucleus is shown at *N*, nucleolus at *n*, rough-surfaced endoplasmic reticulum at *er*, and a mitochondrion at *m*. × 24,000.

FIGURE 4 Electron micrograph of an autoradiogram showing distribution of grains over the Golgi region 20 minutes after labeling (5 minutes in radiosulfate, 15 minutes in chaser). Most of the grains lie eccentric to the large vacuoles *V*, but actually over areas made up of clusters of small vesicles *v*, poorly delineated. Lamellar elements of the Golgi apparatus are at *l*. × 21,000.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racy of localization, unstained specimens were always examined in the electron microscope (an RCA EMU2E) with the emulsion in place.

Controls were prepared by fixing fragments of cartilage, labeled either *in vivo* or *in vitro* with radiosulfate, in the following: (*a*) 10 per cent formalin saturated with lead subacetate (to about 4 per cent); (*b*) 10 per cent formalin containing 4 per cent lead subacetate and 2 per cent acetic acid; (*c*) 10 per cent formalin containing 1 per cent calcium chloride; (*d*) 10 per cent formalin saturated with barium hydroxide (10, 14, 27); (*e*) 10 per cent formalin alone. These tissues were subsequently dehydrated, embedded in paraffin and sectioned at 5 μ. In mounting, ribbons were floated on 70 per cent alcohol containing a trace of cetylpyridinium chloride rather than on water. Some sections on labeled tissue (60 minutes), fixed in formalin, were digested with testicular hyaluronidase or pneumococcal hyaluronidase (courtesy of Dr. Karl Meyer), 15 TRU/ml made up in 0.12 M NaCl, pH 5.9, at 37°C for 6 and 18 hours. Autoradiograms were subsequently prepared by dipping in liquid Kodak NTB_2 emulsion.

To study the distribution of stainable acid mucopolysaccharide, the tibiofemoral, tarsal, and humeral epiphyses from 17-day rat embryos were fixed for 20 hours at 4°C in the formalin solutions listed above and in 0.5 per cent cetylpyridinium chloride in 10 per cent formalin (27, 28). In addition, fragments were quenched and substituted (29) in methanol, methanol saturated either with lead acetate, or hexammonium cobalt (30), or with acetone, at −50°C. Three μ sections of these tissues, doubly embedded in celloidin and paraffin, were stained in (*a*) 0.2 per cent toluidine blue in distilled water, pH 5.5; (*b*) 0.2 per cent toluidine blue in HCl-citrate buffer of ionic strength 0.01, pH 5.0; (*c*) 0.5 per cent toludine blue in phthalate-tartrate buffer, pH 4.4 (31); (*d*) 0.02 per cent azure B bromide at pH 4.0. Some sections were examined without dehydration in Apathy's mountant or glycerogel; others were dehydrated in tertiary butyl alcohol before clearing and mounting. Some sections were pretreated with hyaluronidase, as described above, or were subjected to acetylation in absolute acetic anhydride at 60°C for 2 hours before staining.

OBSERVATIONS

Ultrastructure of the Secreting Chondrocyte

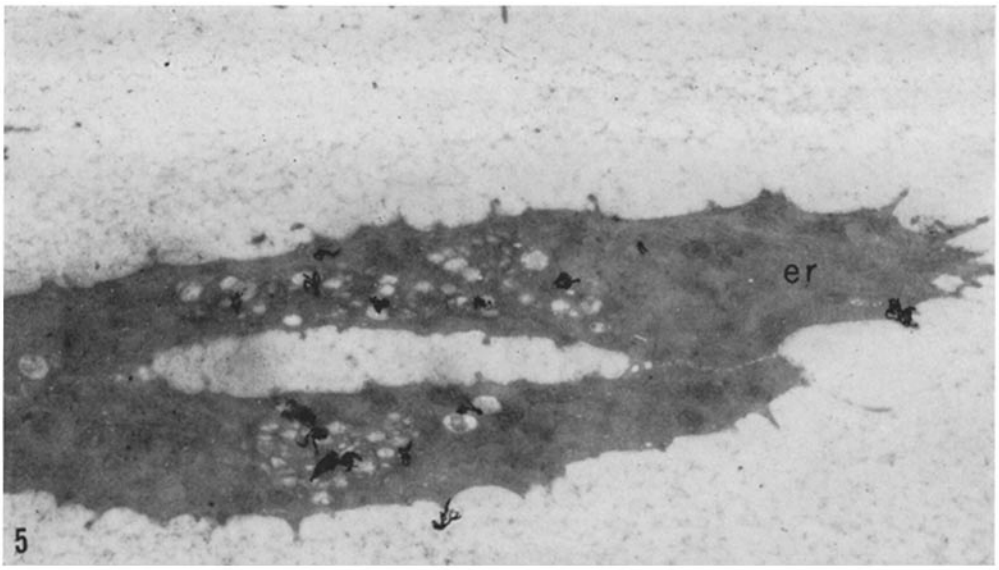
Especially prominent, once matrix formation begins, is the large and extensive Golgi apparatus of the chondroblast which has, in addition to lamellar elements, very numerous small vesicles and larger vacuoles with dense contents (Figs. 3 to 5, 11). These are gathered in a relatively large circumscribed area next to the nucleus, where the hypertrophied Golgi apparatus, long observed by light microscopists (32–34), constitutes a relatively chromophobic center of reduced density recognizable in the phase microscope (Figs. 2, 6, 7, 14). Vacuoles of the same kind are found all about the cell periphery, where, on attaining the surface, they burst and release their contents (Fig. 10), giving the cell outline a scalloped, bayed, and indented appearance (Figs. 3, 10). Smaller vesicles with less dense contents are also discharged at the cell surface. The basophilic parts of the condensed cytoplasm are occupied by tight arrays of rough-surfaced endoplasmic reticulum, often with dilated

FIGURE 5 Electron micrograph of an autoradiogram of an isogenous pair of columnar ("proliferating") chondrocytes, 20 minutes after presentation of radiosulfate (5 minutes in label, 15 minutes in chaser). The concentration of grains over the main Golgi area is evident. Compare with Fig. 6. The peripheral zone of ribosome-studded endoplasmic reticulum (*er*) is always unlabeled. × 17,000.

FIGURE 6 Autoradiogram of 1-μ section of columnar chondrocytes like those in Fig. 5, illustrating concentration of grains over the pale juxtanuclear Golgi region *g*. *N* is the nucleus; the vacuole *c* may be a cystic vacuole (see reference 36). Phase contrast, × 2500.

FIGURE 7 Autoradiogram of 1-μ section of central chondrocytes like those in Figs. 8 and 9, at 3½ hours after injection of radiosulfate *in vivo*. Concentration of grains over the juxtanuclear Golgi zone *g* is evident. Arrow points to grains over a sector of the cell periphery (mantle), from subcortical source of radiation. Increased numbers of grains are also seen in the matrix. Nuclei are at *N*; *c* is a large cystic vacuole or focus of cytoplasmic sequestration. Phase contrast, × 2000.

FIGURE 8 Electron micrograph of autoradiogram of central chondrocyte 20 minutes after radiosulfate administration, showing accumulation of grains over the Golgi area. More of these grains overlie the larger vacuoles in this cell and many are seen at or near the cell periphery. A mitochondrion is at *m*. × 24,000.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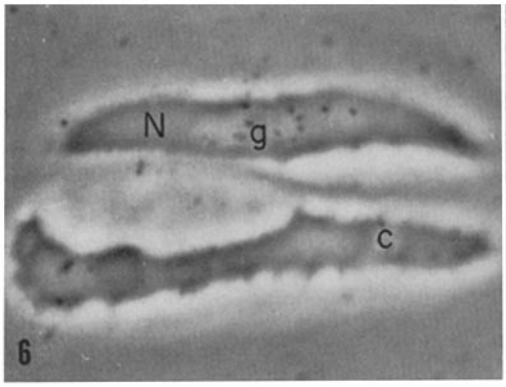
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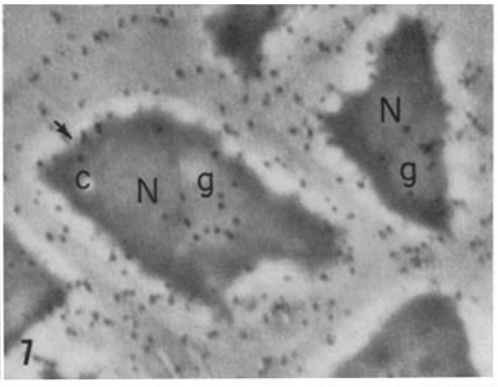
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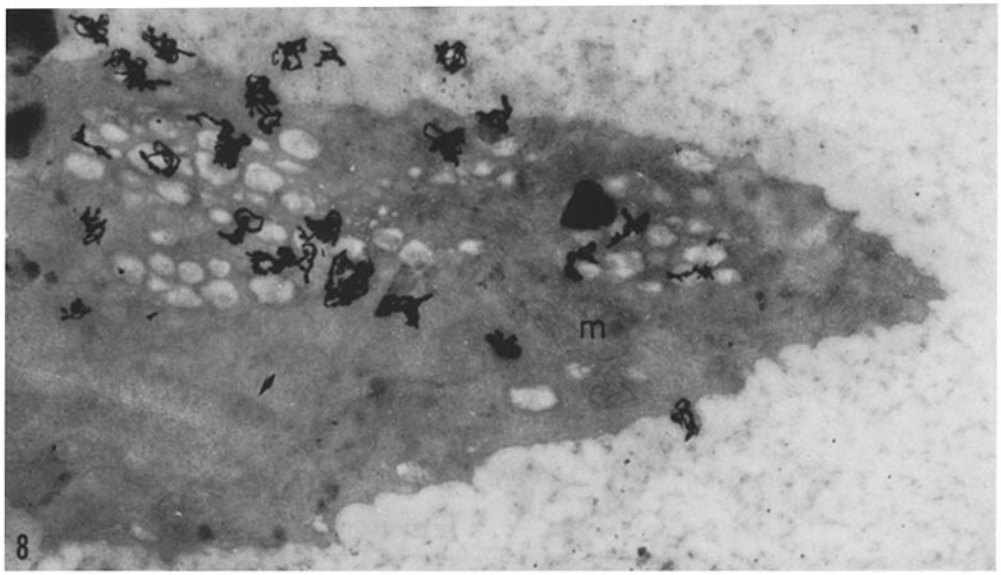
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TABLE I

Relative (Percentile) Distribution of Grains over Labeled Chondrocytes of Rat Epiphysial Cartilage in Autoradiograms of 1-μ Sections, at Successive Intervals after Administration of $S^{35}O_4$

Route of S^{35} administration	Exposure to $S^{35}O_4$	Exposure to chaser	Total elapsed time	Region	No. of Grains	Golgi area	Basophilic cytoplasm	Periphery (mantle)	Capsular matrix	Per cent of total
In vitro	3 min.	—	3 min.	Perichondrial	18	50.0	5.5	27.8	16.7	4.6
				Central	227	58.5	5.7	15.4	20.2	58.0
				Columnar	146	46.5	4.0	20.5	28.0	37.4
				Total	*391*	*53.7*	*5.3*	*17.9*	*23.0*	*100*
	9 min.	—	9 min.	*Total*	*214*	*60.7*	*8.8*	*15.8*	*17.2*	*100*
	5 min.	5 min.	10 min.	Central	*520*	*50.9*	*11.5*	*16.5*	*21.7*	*100*
	5 min.	15 min.	20 min.	Central	297	59.2	12.1	12.8	15.8	54.5
				Columnar	251	48.6	6.7	17.9	26.7	45.5
				Total	*548*	*54.4*	*9.7*	*15.1*	*20.8*	*100*
In vivo		—	30 min.	Perichondrial	83	33.7	19.2	21.6	25.3	13.6
				Central	298	52.6	8.3	16.7	22.1	49.1
				Columnar	227	24.2	13.2	28.1	34.3	37.3
				Total	*608*	*39.4*	*11.6*	*21.7*	*27.2*	*100*
		—	60 min.	Perichondrial	53	22.6	22.6	26.4	28.3	10.6
				Central	239	22.1	6.8	33.8	37.2	47.8
				Columnar	207	14.0	20.2	35.2	30.4	41.4
				Total	*499*	*18.0*	*14.0*	*33.6*	*33.6*	*100*
		—	15 hrs.	*Total*	*234*	*8.9*	*11.9*	*22.2*	*56.8*	*100*

cisternae. Cytoplasmic glycogen deposits are especially prominent in proliferating cells of the central (precolumnar) area, and again in hypertrophic chondrocytes of the epiphysial plate. In the cytoplasm of secreting chondroblasts there are often cyst-like bodies (actually, foci of sequestered cytoplasm), delimited by a doubled membrane, whose contents are also evidently disgorged to the exterior (35, 36).

Localization of Radiosulfate

HISTOLOGICAL: In all of the specimens taken within 3½ hours after radiosulfate administration the histologic aspect of the radioautograms was similar in that, as previously reported (1, 7, 37, 38), the perichondrium and superficial proliferating chondroblasts had few overlying grains; radioactivity increased progressively toward the epiphysial line, with intense activity of the precolumnar, columnar, and proximal hypertrophic cells. The proliferating chondrocytes in the center of the epiphysis, just proximal to the zone in which the chondrocytes become stacked into columns (therefore precolumnar), gave apparently higher grain counts than the columnar and hypertrophic cells (Table I). Degenerating chondrocytes or the late hypertrophic cells examined 1 hour after radiosulfate administration incorporated relatively little sulfate, but were more active than cells of the perichondrium.

At any one time point after 10 minutes, there was some variation in distribution of grains over the chondrocytes of a given category (*e.g.* precolumnar, columnar, etc.). Some cells were more "advanced," their label being dispersed in a manner more characteristic of later times, while others were "retarded," with grains mostly concentrated over the central Golgi areas. This suggests an asynchrony of function among chondrocytes of the same class.

CYTOLOGICAL: As early as 3 minutes after exhibition of radiosulfate *in vitro*, whether after a 90-second pulse or after 3 minutes in medium containing $S^{35}O_4$, isotope was fixed in the chondrocytes (Fig. 2). About 80 per cent of grains were counted over the cells as compared with adjacent capsular matrix (*i.e.* the moat or the pericellular area, and its margins) (Table I). 50 to 60 per cent of the grains were already localizable over the pale juxtanuclear zone identifiable in the phase microscope (Fig. 2) and in the electron microscope (Fig. 3) as the enlarged Golgi zone. This was

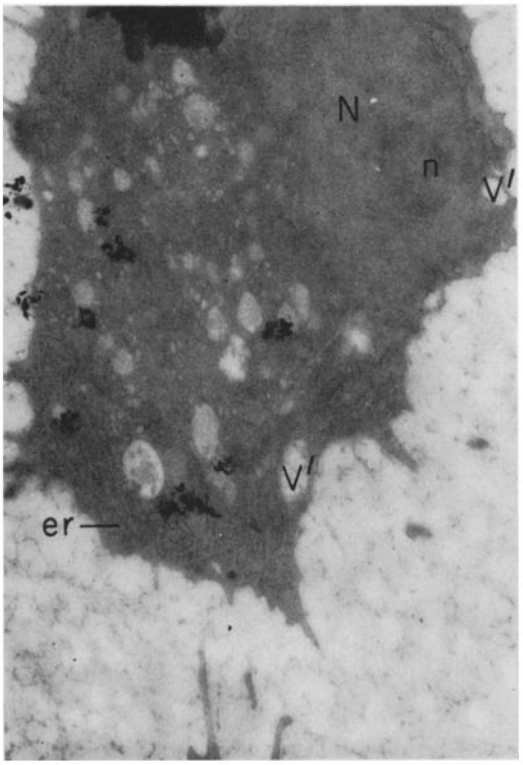

FIGURE 9 Electron micrograph of autoradiogram of a central chondrocyte 10 minutes (5 minutes in label, 5 minutes in chaser) after radiosulfate. At this time most of the grains in the cytoplasm overlie the area beside and between the larger vacuoles, in which clusters of microvesicles, poorly shown here, are present. Marginated subcortical vacuoles are at V^1; N is the nucleus, *er* an area of granular endoplasmic reticulum. Compare with Fig. 10. × 17,000.

the site of high radioactivity which progressively and very slowly began to diminish after 20 to 30 minutes and over the whole period of observation (Table I).

Most of these grains were associated with the vesicular component of the Golgi apparatus. At the earlier intervals (10 minutes) (Fig. 3), the grains were related not only to the large vesicles, but especially to the small (15 to 40 mμ) vesicles between them. In radioautograms of specimens taken within an hour of labeling, most of the grains were ecentrically placed in relation to the center of the larger vacuole near which they lay (Figs. 3 to 5, 8, 9), and an impression is sustained that the radioactive source was at or near the periphery of the vacuole rather than within. The microvesi-

cles lie closely related to the outer membranes of the larger vacuoles in the central Golgi zone. At later intervals, grains were usually found directly overlying the centers or the dense contents of the vacuoles (Figs. 10 and 11).

The silver deposits designated as "cytoplasmic" in the phase microscope were, in the electron microscope, localized over vacuoles detached and separated from the main juxtanuclear clusters (Figs. 4, 8, 9).

In the 1- and 3½-hour time intervals following the single injection of radiosulfate, grains overlying the cell margin or periphery became especially prominent, constituting about 50 per cent of the grains counted over the chondrocyte itself, which they appeared to ring (Figs. 7, 14). These, too, originated from vacuoles lying at the extreme cell periphery at or just adjacent to the cortex (Figs. 3, 9 to 11), which vacuoles, as depicted by Godman and Porter (35) and Godman (36), may form a discontinuous mantle zone around the chondrocyte. These, and the detached vacuoles with similar dense contents, are derived from the main juxtanuclear Golgi cluster from which they move out, apparently centrifugally.

The grains overlying the matrix appeared to have no preferred orientation or relation to the fibrils.

The apparent absence of radioactivity in the rough-surfaced endoplasmic reticulum and the contents of its cisterns, in the mitochondria, and in the large cystic vacuoles (sequestrae) bounded by double membranes is noteworthy.

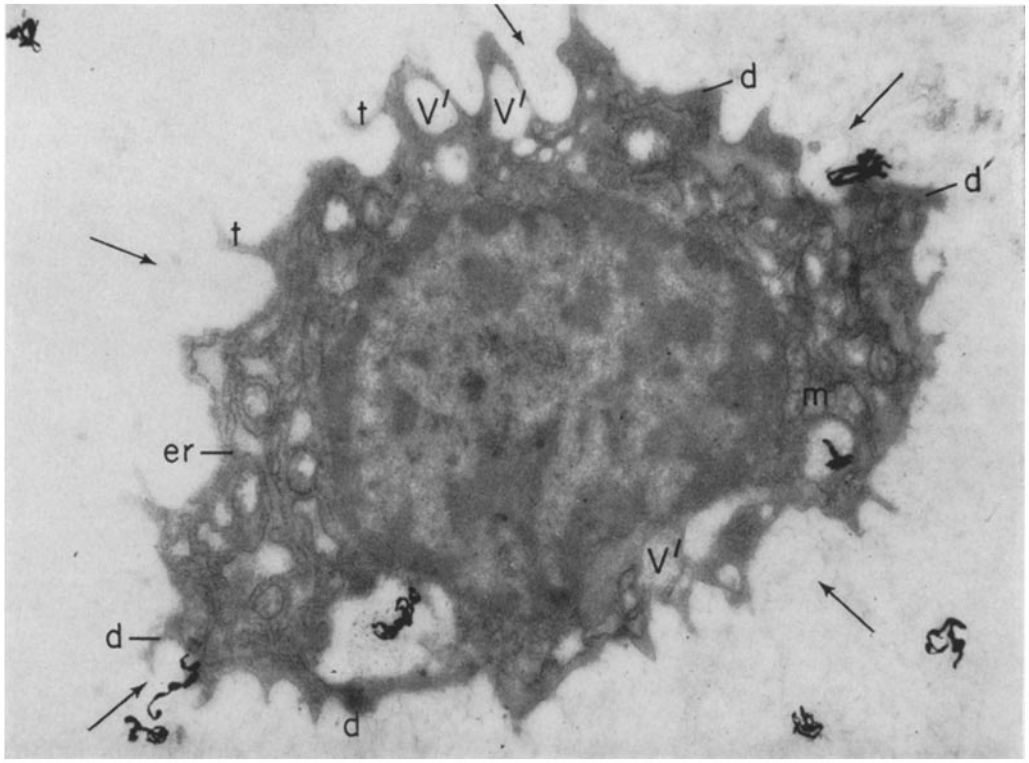

FIGURE 10 Electron micrograph of autoradiogram of a chondrocyte 19 hours after injection of radiosulfate. At this time, most of the grains in the cell overlie directly the dense stippled contents of the large vacuoles at the cell periphery. Those of the marginated vacuoles V^1 in process of discharging into the matrix are indicated by large arrows. The bursting of these vacuoles lifts off flaps or tabs of cortical cytoplasm, *t*. Cortical densities, *d*, believed to play some part in fibrillogenesis, are shown at *d* (see reference 36). The matrix is very radioactive at this time. × 20,000.

Transit of the Label

The relative concentrations of radiosulfate over the Golgi area, basophilic cytoplasm, cell periphery, and immediately surrounding matrix

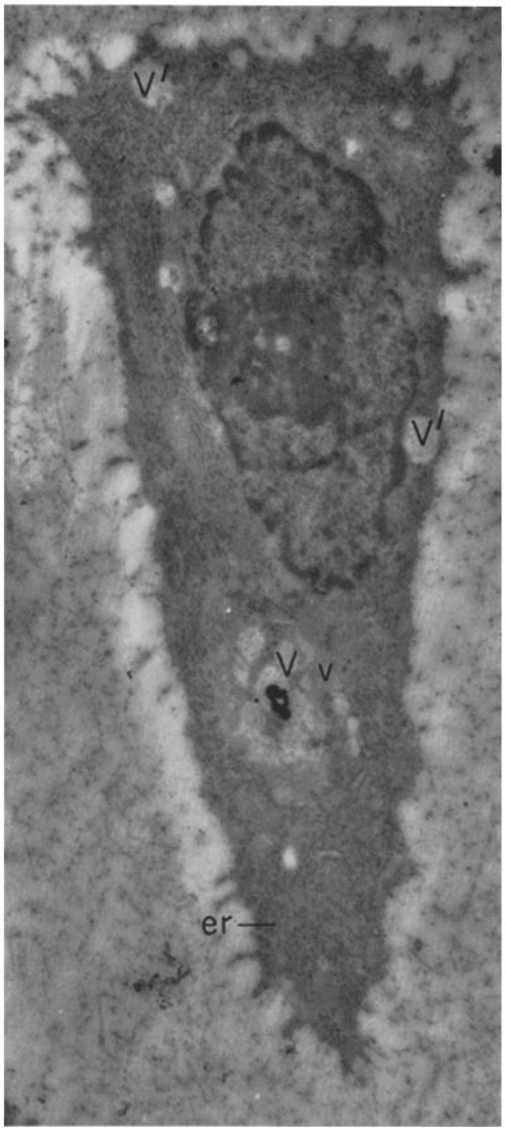

FIGURE 11 Electron micrograph of autoradiogram of a chondrocyte fixed 3 hours after radiosulfate administration. Its Golgi area contains a nest of large vacuoles *V* flanked by a pale zone *v* in which are numerous microvesicles (but poorly contrasted here). The grain overlies directly the dense stippled contents of a large vacuole. Subcortical vacuoles with similar contents are at V^1. An area of granular endoplasmic reticulum is at *er*. × 24,000. Micrograph taken by Dr. Lucien Caro.

at successive time intervals are tabulated in Table I and graphed in Fig. 1. Sulfate was trapped, transported, and accumulated in the central Golgi zone in some bound form within 3 minutes. Peripheral structures which might be involved in these processes, other than the vesicles, were not discerned with these methods or in these intervals. Some radiosulfur, about 20 per cent of the grains counted, was held in some form in the capsular or territorial matrix around the active chondrocytes even at 3 minutes after exposure to $S^{35}O_4$.

By 30 minutes and to a much greater degree by 60 minutes, there was a significant increase in the number of grains counted over the cell periphery (mantle) and in the surrounding matrix, and a comparable diminution in the concentration of grains overlying the juxtanuclear Golgi apparatus (Table I). At the hour interval, the grains over the matrix constituted a third of those counted, and by 15 hours over 50 per cent of the grains were extracellular. These data may be interpreted as showing a movement of sulfate into the cell, its early fixation and accumulation in vesicles of the Golgi apparatus, and the movement of sulfated materials thence to the cell periphery from which they are discharged into the extracellular space.

The Sulfated Materials

Fixation at 4°C for 20 to 24 hours in formalin saturated with barium hydroxide extracted almost all of the radioactive source from tissue which had been labeled either *in vitro* or *in vivo*, except for a very few residual grains.

Pretreatment with testicular hyaluronidase, as described, extracted all of the radioactivity from the matrix and most of the sulfated material from the cells in cartilage which had been labeled *in vivo* (60 minutes). The number of grains overlying the cells was reduced by 85 to 90 per cent.

Intracellular Metachromasia

Intracellular and pericellular sites of gamma metachromasia after staining with toluidine blue were sought in relation to the observed sites of concentration of radiosulfate. Metachromatic material was best preserved in frozen-substituted specimens, and in those fixed in lead subacetate formalin and cetavlon formalin. The violaceous metachromasia given by the abundant ribonucleoproteins in the cytoplasm of these cells was distinguishable from the reddish metachromasia of the sulfomucopolysaccharide. Intracellular or cell-associated gamma (red) metachromasia was only occasionally observed, after any method of toluidine blue staining, in any cell except the distal

hypertrophic and the degenerating chondrocytes, in which cells many such granules were seen. Fewer of the chondroblasts and central (precolumnar) chondrocytes contained metachromatic globules than did the columnar chondrocytes. Most frequently, this metachromatic material was distributed as a series of reddish granules (of about 0.5 to 3 μ) at the cell margin or periphery (Fig. 13), reminiscent of the corona of grains overlying the mantle of the chondrocytes in radioautograms of specimens taken 1 hour or more after radiosulfate administration (Figs. 7, 14). More rarely, a few reddish granules of 0.5 to 3 μ were found in the pale juxtanuclear regions (Golgi area). Some of these granules were surrounded by clear halos (Fig. 12). The contents of the large spherical vacuoles, most of which represent the cystic sequestrae in the cytoplasm, were usually unstained, and in no instance metachromatic. The metachromatic materials were not extracted by pretreatment of sections for 2 hours in 0.12 M sodium chloride or boiled testicular hyaluronidase for 18 hours, but disappeared after testicular hyaluronidase digestion; pneumococcal hyaluronidase diminished but did not extinguish the color intensity of both basophilic and metachromatic materials in the cartilage which later appeared pinkish. Acetylation, under the conditions employed, did not enhance metachromasia or reveal new sites, but intensified cytoplasmic basophilia generally. These observations suggest that the metachromatic component of the intracellular granules of the chondrocytes is chondroitin sulfate A and/or C.

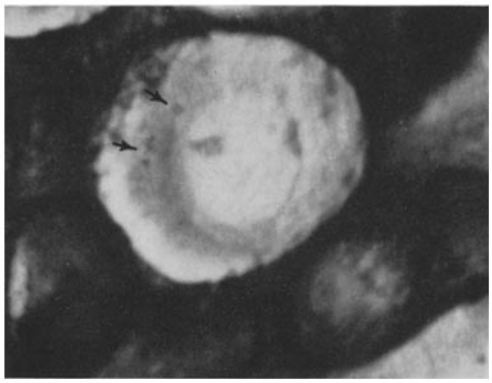

FIGURE 12 Section of chondrocyte and matrix showing metachromatic granules (arrows) in juxtanuclear pale area (Golgi region). Compare with Fig. 11. Metachromatic granules in this location are seldom seen. Prepared by freezing-substitution; toluidine blue stain; × 2000.

DISCUSSION

Fixation of Sulfate

Cartilage matrix is remarkably permeable to the sulfate ion (10), which is as rapidly fixed by and accumulated in the chondrocytes. The selective permeability of these cells and other matrix-secreting cells of the connective tissues, of goblet and

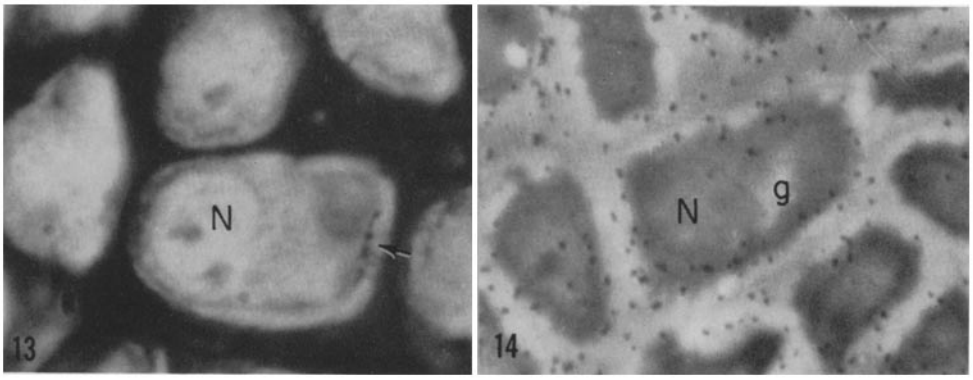

FIGURE 13 Chondrocyte and matrix showing series of metachromatic granules (arrow) at cell periphery (corresponding with rows of subcortical vacuoles V^1 as in Figs. 3 and 10). Such intracellular granules are more frequently encountered. Compare with Figs. 7 and 14. Prepared by freezing-substitution; toluidine blue stain; × 2000.

FIGURE 14 Autoradiogram of a 1-μ section of a chondrocyte 3½ hours after labeling with radiosulfate, showing peripheral (mantle) distribution of grains around cell (presumably corresponding to subcortical vacuoles V^1 as in Figs. 3 and 10, and, in some part, to metachromatic granules as in Fig. 13). Phase contrast, × 2000.

some mucous cells, and of certain neuroepithelial cells to inorganic sulfate, and their mechanism of trapping and retaining sulfate remain obscure. A transport process at the cell membrane requiring ATP has been postulated (39), but this analogy to the better-known cation pumps has not yet been elaborated experimentally. Presumably, there are special receptors at the surfaces of sulfate-permeable cells. The small pools of extracellular (10) and intracellular (39) inorganic sulfate, or the labile organic sulfate of small molecules, would hardly be retained as such, in appreciable concentration, after the washing and aqueous fixation employed to make the autoradiograms of this study. For this reason, most of the radioactivity observed in the cartilage matrix at 3 minutes is unlikely to be unbound inorganic sulfate; it may be protein-bound and possibly, in some part, newly esterified polysaccharide product.

Synthesis of Sulfomucopolysaccharide

Synthesis of sulfomucopolysaccharide (actually, sulfation) evidently proceeds almost immediately upon presentation of sulfate, for radioactive chondroitin sulfate has been detected in chondrocytes as early as within 5 minutes (39) and 10 minutes (15) of incubation in medium containing $S^{35}O_4$. This intracellular chondroitin sulfate fraction is chemically similar to that of the matrix, and is "undersulfated" (15).

Especially in longer term experiments, sulfate incorporation has been regarded as a general index of sulfomucopolysaccharide synthesis (1–6). However, the process of sulfation may proceed independently of the biosynthesis of the polysaccharide (19, 39–43). Sulfation, the transfer of active sulfate in adenosine 3′-phosphate 5′-phosphosulfate to an acceptor, is a reaction catalyzed by a phosphosulfokinase (44–46). Mucopolysaccharides (*e.g.* chondroitin sulfates, chondroitin, heparitin (45, 47–50), and oligo- and monosaccharides (47, 48) can serve as acceptors in cell-free systems, and it is not certain whether sulfation actually takes place in the cell by esterification of mono- or oligosaccharide units and their subsequent polymerization; or by esterification of such units as they are added to the sulfomucopolysaccharide chain; or by esterification of the preformed polysaccharide polymer. The available evidence favors the latter two possibilities (15, 19, 41–43, 49, 51), but not definitively.

It is now certain that most (perhaps 80 to 90 per cent) of the cellular radioactivity detected in autoradiograms, prepared as in this study, emanates from chondroitin sulfate (14, 15) even within 5 to 10 minutes after labeling (15, 39). But it is not known whether *all* of the bound sulfate is in this form. Although most observers report virtual abolition of radioactivity by digestion with testicular hyaluronidase (7, 11, 17, *inter alia*), our observation of a very small part of labeled material remaining after hyaluronidase, and the observations of Curran and Kennedy (52), would suggest that there may be a small enzyme-resistant residue of bound sulfate in the cells.

Localization of Bound Sulfate

However, sulfate is captured, activated, accumulated, and some of it probably already esterified as chondroitin sulfate within 3 minutes of its presentation. Since the only organelle in which it is bound in this time or later is the vesicular component of Golgi apparatus, it is probable that chondroitin sulfate is present only in the contents of the vesicles and vacuoles, and it is reasonable to postulate that at least the last step of sulfation occurs here. This process would appear to begin in the microvesicles, judging by the association of grains with clusters of them in the early (3- to 20-minute) specimens. Subsequent coalescence of microvesicles would produce the larger vacuoles. Absence of detectable radioactivity from mitochondria or endoplasmic reticulum, if, indeed, these organelles take up any sulfate for activation or transfer, would mean that (*a*) sulfate is present in them only in some soluble form, or that (*b*) its turnover rate therein is rapid and, therefore, their sulfate concentration is very low.

Resolution of 0.2 μ (and possibly better) can be achieved in autoradiograms with S^{35}, although the radiation is of relatively high energy ($E_{max.}$ = 0.167 Mev) (53). This might be sufficient to establish whether the membranes themselves of the large Golgi vacuoles are more radioactive at earlier times and the contents at later times, as the present observations suggest. It can be hypothesized, pending their isolation, identification, and analysis, that the Golgi membranes of chondrocytes have at least sulfokinase activity. If this were, indeed, the case, then the microvesicles, having a higher surface to volume ratio, might be expected to exhibit a higher turnover rate for sulfate than the larger vacuoles. Newly esterified chondroitin sulfate is secreted from isolated chondrocytes into the medium in about 10 minutes after radiosulfate

administration (15); by 30 minutes, *in vivo* (Table I), there has been an appreciable secretion of radioactive product.

Relative grain counts showing the apparently changing location of bound sulfate in the chondrocytes with the passage of time (Table I, Fig. 1) are consistent with the interpretation that sulfation takes place chiefly in the central (juxtanuclear) Golgi zone, and that vacuoles containing partly sulfated chondroitin sulfate are continuously detached from the juxtanuclear region, migrate peripherally through the non-glycogenic areas of the cytoplasm, and accumulate in the cortex (mantle) where they lodge for relatively long periods. These peripheral vacuoles (Figs. 3, 8 to 10), when fused, correspond to the confluent subcortical "rarefactions" depicted by Godman and Porter (35, 36). Their contents are then discharged to the matrix through the formation of stomata to the exterior (Figs. 3, 10), a process which also appears to detach sectors or tabs of the cell cortex (35, 36). With constant access to inorganic sulfate, it is probable that sulfate incorporation proceeds continuously in both large and small Golgi vacuoles (Figs. 4, 8), but most likely at different rates. Not until it becomes possible to measure the specific activity of the sulfated product in central and peripheral regions of the cell at various times after a sulfate "pulse," as Warshawsky *et al.* (54) have been able to do for secreted proteins of the pancreas, can turnover times and rates of transit in each zone be calculated.

Histologic estimation of the apparent avidity of the different zones of epiphysial cartilage for sulfate may encounter the hazard that, owing to closer concentration of the cells in columns, this area will appear to have concentrated more radiosulfate. However, grains per columnar chondrocyte are consistently somewhat fewer than grains counted per central (precolumnar, glycogen-containing) chondrocyte. Because in sagittal section the flattened lenticular columnar chondrocytes present only their thin edges (hence less surface per cell) to the emulsion, while the large central precolumnar cells expose a greater surface area, even this difference is more apparent than real. Both the large central and the columnar chondrocytes probably have similar amounts of intracellular sulfate at any time, but turnover or transit of sulfate through the latter appears to be always more rapid (Table I).

Stainability of Intracellular Sulfomucopolysaccharide

The chondroitin sulfates (A and C) in the chondrocyte are similar in composition and proportion to the extracellular products formed *in vitro* (15) They are not present as such in matrix or intracellularly, but in covalent linkage with non-collagen protein as sulfomucopolysaccharide-protein (chondromucoprotein) (16, 17, 19, 55, 56). Because turnover rates of $S^{35}O_4$ and C^{14}-lysine in cartilage are similar (55) and because of the parallel incorporation of $S^{35}O_4$ and radioactive amino acids in chondrocytes at 4 hours (16), it has been supposed that polysaccharide and protein moieties are synthesized simultaneously. These data, and the reported facilitation of sulfate uptake by amino acids (57), would suggest a dependency of sulfopolysaccharide synthesis or sulfation upon the presence of the protein moiety. The Golgi vesicles presumably contain the mucopolysaccharide-protein. If the protein moiety is synthesized in the endoplasmic reticulum (see Fig. 4 and reference 58), the sulfopolysaccharide-protein must finally be compounded in the Golgi vesicles, where the sulfation evidently occurs.

Detection of the intracellular sulfochondromucoprotein as a chromotrope by metachromasia with a thiazine dye would depend upon: (*a*) its amount and concentration (*i.e.* the density of anionic charges) (59–61), and (*b*) the availability of charged groups to the cationic dye (*i.e.* degree of electrostatic steric, or other interference by associated protein (62)). Intracellular gamma-metachromasia in secreting chondroblasts or fibroblasts has but rarely been reported. In active secretory chondroblasts, hyaluronidase-sensitive metachromatic granules, when found, are mostly at the cell periphery, very rarely juxtanuclear (Figs. 12, 13). The difficulty of demonstrating the sulfated product in the central Golgi zone must mean that: (*a*) it is more difficult to preserve through fixation and preparation; or that (*b*) it is somehow more protected from competitive interaction with dye by its associated protein; or (*c*) that it is present in too small amount or concentration; or (*d*) that its negative charge-density (*i.e.* its number or spacing of sulfate groups) is not yet sufficient to confer chromotropy. The last-named explanation would seem the more probable, because in aggregate the contents of the central Golgi zone appear sufficient in amount for visualization in the light micro-

scope, and are as adielectronic as the peripheral contents, and because acetylation of protein effected no change in stainability. Sulfation is probably a continuing process in the cell, and it may be that the new, undersulfated chondroitin sulfates in the juxtanuclear Golgi vacuoles continue to be further esterified as the vacuoles migrate peripherally where, as we have seen, they lodge for a time in the subcortex. In this peripheral location, they may achieve a charge density, through having acquired more sulfate and/or through concentration (inspissation) sufficient to confer the same chromotropic properties as those of the sulfomucopolysaccharide in the matrix and in the distal hypertrophic chondrocytes.

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