Ultrastructural Autoradiographic Study of the Uptake and Intracellular Localization of $^{35}$S-Sulfate by Developing Human Neutrophils

By Dale N. Payne and G. Adolph Ackerman

The uptake and intracellular localization of $^{35}$S-sulfate was studied in developing neutrophils from normal human bone marrow by means of ultrastructural autoradiography in order to identify the pathway and sites of sulfate incorporation into glycosaminoglycans present in selected granule populations in these cell forms. Marrow cells were pulse labeled for 10 min in vitro at 37°C with 200 μCi/ml Na$_2^{35}$SO$_4$ in either phosphate buffer (pH 7.4) or RPMI 1640 and cold chased for intervals up to 4 hr. Autoradiographic data indicated that promyelocytes involved in primary (azurophilic) granule synthesis showed a striking and preferential uptake of labeled sulfate with localization of silver grains over the Golgi and formative granules, while $^{35}$S-sulfate uptake by myelocytes engaged in secondary (specific) granule formation was minimal. A second period of $^{35}$S-sulfate uptake occurred during the final stages of neutrophil maturation extending from the metamyelocyte into the segmented neutrophil stages. $^{35}$S-sulfate concentrated in the Golgi and in condensing saccules arising from the concave Golgi face and was subsequently localized in small round to oblong (tertiary) granules formed in these cells. Labeled sulfate uptake by metamyelocytes and segmented neutrophils was appreciably less than noted for neutrophilic promyelocytes. Thus, autoradiography using $^{35}$S-sulfate permitted the delineation of three distinct phases of neutrophil maturation. Two of these phases involved the uptake of labeled sulfate which became incorporated into distinct types of cytoplasmic granules reported to contain sulfated glycosaminoglycans, viz., primary granules in promyelocytes and tertiary granules formed during late maturational stages of the neutrophilic leukocyte.

The incorporation of radiolabeled sulfate by leukocytes and bone marrow cells in vivo and short-term autoradiographic studies in vitro have indicated that $^{35}$S-sulfate uptake occurs primarily in immature granulocytes (neutrophilic and eosinophilic series) and in megakaryocytes, while erythrocytes and their precursors fail to incorporate appreciable amounts of $^{35}$S-sulfate. The selective incorporation of $^{35}$S-sulfate by developing granulocytes and megakaryocytes in bone marrow has been correlated with the presence, number, and elaboration of azurophilic granules. In relation to neutrophil maturation, it is well recognized from ultrastructural evidence that azurophilic (primary) granule formation is restricted to the promyelocyte stage, while specific (secondary) granule formation characterizes the myelocyte stage of neutrophil development.

In addition to the distinctive enzymatic content of these two granule populations, both histochemical and biochemical evidence has indicated the presence of sulfated glycosaminoglycans in the azurophilic but not in specific granules. Biochemically, the principal variety of sulfated glycosaminoglycans...
glycan present in leukocytes and bone marrow cells is chondroitin-4-sulfate.\textsuperscript{30-38} Olsson\textsuperscript{36} has shown that \( ^{35}S \)-sulfate-labeled chondroitin-4-sulfate isolated from leukocytes and from bone marrow cells is localized primarily within the granule fraction. Autoradiographic studies designed to trace neutrophil maturation in the bone marrow and their emergence into the circulation have indicated a decrease in \( ^{35}S \)-sulfate labeling of the developing neutrophil after the pro-myelocyte stage.\textsuperscript{3,9} This decline in \( ^{35}S \) labeling correlates with the lack of formation of new glycosaminoglycan-containing azurophilic granules during the later half of neutrophil maturation and with the dilution of \( ^{35}S \)-labeled azurophilic granules resulting from mitotic activity during the maturation process. Ultrastructural autoradiographic studies have demonstrated the intracellular localization of \( ^{35}S \)-sulfate in the Golgi complex and formative azurophilic granules of neutrophilic promyelocytes.\textsuperscript{3,11,12,39}

In our preliminary report,\textsuperscript{31} we demonstrated that \( ^{35}S \)-sulfate uptake by developing neutrophils from human bone marrow seems to occur in two distinct phases: i.e., in the promyelocytes coincident with active azurophilic granule formation and again after the myelocyte stage during the final phases of neutrophil maturation (metamyelocytes and some segmented neutrophils). This second period of \( ^{35}S \)-sulfate uptake is considered to be directly related to the elaboration of so-called tertiary granules reported to occur during the late stages of neutrophil differentiation.\textsuperscript{12,21,23,25,40} Although the existence of a tertiary granule population in the neutrophil has not received general acceptance, recent histochemical and biochemical findings have indicated that pleomorphic granules in late neutrophilic cells not only contain certain hydrolytic enzymes but also possess sulfated glycosaminoglycans.\textsuperscript{21,22,25}

It is the purpose of this paper to report our definitive ultrastructural autoradiographic data regarding the uptake by and the localization of \( ^{35}S \)-sulfate within developing neutrophilic leukocytes from normal human bone marrow. The relative rate of \( ^{35}S \)-sulfate uptake by these marrow cells will be related to distinctive maturational stages, and the intracellular sites of localization to the pathway involved in active granulogenesis in these cell forms.

\textbf{MATERIALS AND METHODS}

\textit{Preparation of Tissue Samples}

Human bone marrow specimens (1-2 ml) were obtained from posterior iliac crest aspirations of healthy volunteers who gave appropriate informed consent. The immediate suspension of marrow in 18 ml of Sorenson’s phosphate buffer, pH 7.4, containing 4% polyvinylpyrrolidone and 1% sucrose (PBSP) at room temperature obviated the need for anticoagulant; cell suspension was achieved without clotting or clumping. Siliconized glassware was used throughout, minimizing surface adherence of cells. Buffy coats obtained by low-speed centrifugation (1200 rpm for 15 min) were rinsed once in room temperature PBSP or RPMI 1640 (Gibco, Grand Island, N.Y.) and resuspended in 5 ml of either PBSP or RPMI containing 200 \( \mu \)Ci/ml of carrier-free \( ^{35}S \)-sulfate labeled sodium sulfate (\( \text{Na}_2^{35}\text{SO}_4 \)) with specific activities of approximately 650 mCi/mM (New England Nuclear, Boston, Mass.).

Cells were incubated in a water bath at 37°C for 10 min. Cell concentration was adjusted to \( 10^8 \) cells/ml incubation medium. Cells exposed to \( ^{35}S \)-sulfate in PBSP were washed three times within a 5 7-min period in cold (4°C) PBSP; a Clay-Adams Serofuge was used for centrifugations at 3400 rpm for 1 min between washes. The cells were then fixed in suspension in 2.5% glutaraldehyde in PBSP for 10 min at room temperature. In other experiments, cells incubated in RPMI...
were rinsed in a similar manner but with cold RPMI. An aliquot was removed, the cells were
rinsed in PBSP prior to fixation in suspension with glutaraldehyde, and the remaining cells
were incubated at 37°C in RPMI containing unlabeled sodium sulfate as a cold chase. Aliquots were
withdrawn from the incubation media after periods of 50, 110, and 230 min. Suspensions were
centrifuged and the cells were washed in cold PBSP prior to fixation in glutaraldehyde.

Preparation of Tissue Blocks

After fixation in glutaraldehyde, cells were washed twice in PBSP and centrifuged into a pellet
at 3000 rpm for 20 min. They were then fixed in 1% chrome osmium tetroxide in PBSP for 1 hr at
4°C, washed, dehydrated in graded ethanol, and embedded in Araldite.

Preparation of Autoradiographs

Silver-gold sections were cut and placed on collodion-coated glass slides and dipped in Ilford
L-4 nuclear photographic emulsion using a semiautomatic coating device to obtain a monolayer of
emulsion (purple interference color). Slides were stored in light-tight black boxes containing
anhydrous calcium sulfate for 36 days. Autoradiographs were then developed in gold Elon–ascorbic
acid developer for 5 min at room temperature, rinsed for 1 min, fixed in 24% sodium thiosulfate
for 2 min, and then thoroughly washed and dried. Tissue sections were removed from the glass
slides by flotation onto distilled water and picked up on 200-mesh copper grids. Collodion was
removed from the sections by immersion of the grids in isoamylacetate for 1 min. Sections were
stained with aqueous uranyl acetate and lead citrate and viewed in a Philips 300 electron micro-
scope.

Analysis of Autoradiographs

All cells employed in detailed autoradiographic analysis were sectioned through the midplane and
and included the Golgi complex and portions of the nucleus. Photographs of these cells were subjected
to point hit analysis using a grid calibrated to points per 0.25 sq μm for the determination
of cell and subcellular compartment areas. Area data were obtained for neutrophilic cells at each
stage of maturation, viz., promyelocytes, myelocytes, metamyelocytes, and segmented neutrophils.
Areas, as well as percentage area (area of an organelle divided by the total area of cell analyzed
per time period), were also determined for the following subcellular structures: cytoplasmic
granules, endoplasmic reticulum, Golgi complex, mitochondria, nucleus, and cell membrane.
Fifteen cells from each stage of maturation were randomly selected from each incubation period
for grain analysis of autoradiographs. Silver grains were counted over each autoradiograph and
assigned to a subcellular compartment according to the grain’s direct proximity. Statistical
analysis for each time period consisted of the following: mean grain counts per cell and sub-
cellular organelle, standard deviations of grain counts, grain density (total grains counted over
an organelle divided by total area occupied by that organelle), percentage of total grains (total
grains counted over an organelle divided by total grains counted x 100), and relative grain den-
sity (percentage of total grains of an organelle divided by the percent of total area occupied by
that organelle). Relative grain density values above 1 indicated a level of labeling above
purely random.

Probability circle analysis was also used on a number of autoradiographs. The circle of
radius approximately 336 nm, i.e., 1.5–1.7 times the value of the line source (determined to be 210
nm based upon similar energy radiation with 14C), drawn around a developed silver grain rep
sented the area within which there would be a 50% probability of locating the 35S radiation
locus. Thus, circles with 336 nm were drawn around silver grains on many autoradiographs to
approximate more closely the subcellular location of 35S-sulfate. Unfortunately, this circle
proved to be much too large for determining the labeling characteristics of very small subcellular
structures, such as cytoplasmic granules, which were upon observation quite apparently labeled by
multiple silver grains.

RESULTS

Morphologic evaluation of the grain distribution was done on a large number
of autoradiographs of developing neutrophils from normal human bone
### Table 1. Distribution of Grains Over Neutrophilic Cells Following 10-min Pulse Label With Sodium $^{35}$S-Sulfate

<table>
<thead>
<tr>
<th>Medium</th>
<th>Chase (min)</th>
<th>Total (and Percent) Grains</th>
<th>Mean ($\pm$ SD) Grains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>PBS</td>
<td>0</td>
<td>1845 (82)</td>
<td>62 (3)</td>
</tr>
<tr>
<td>RPMI</td>
<td>0</td>
<td>159 (58)</td>
<td>19 (7)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>120 (52)</td>
<td>11 (5)</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>168 (53)</td>
<td>14 (5)</td>
</tr>
<tr>
<td></td>
<td>230</td>
<td>224 (59)</td>
<td>17 (5)</td>
</tr>
</tbody>
</table>

Abbreviations: A, promyelocyte; B, myelocyte; C, metamyelocyte; D, segmented neutrophil.

### Table 2. Area of Cell Organelles and Distribution of Silver Grains Over Developing Neutrophils Pulse Labeled for 10 min in PBS Containing Sodium $^{35}$S-Sulfate

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Mean Area in sq $\mu$m (and Percent of Total Area per Cell Stage)</th>
<th>Mean Grains (and Percent of Total Grains per Cell Stage)</th>
<th>Grain Density (and Relative Grain Density)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Membrane</td>
<td>2.0 (3.2)</td>
<td>1.7 (3.6)</td>
<td>1.5 (3.9)</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>20.2 (32.1)</td>
<td>20.5 (44.7)</td>
<td>19.6 (49.6)</td>
</tr>
<tr>
<td>R.E.R.</td>
<td>7.3 (11.6)</td>
<td>2.0 (4.3)</td>
<td>0.9 (2.2)</td>
</tr>
<tr>
<td>Mitochondr</td>
<td>1.4 (2.2)</td>
<td>0.4 (1.0)</td>
<td>0.3 (0.7)</td>
</tr>
<tr>
<td>Golgi</td>
<td>2.6 (4.1)</td>
<td>1.1 (2.4)</td>
<td>0.5 (1.3)</td>
</tr>
<tr>
<td>Granules</td>
<td>6.4 (10.2)</td>
<td>4.0 (8.8)</td>
<td>5.8 (14.8)</td>
</tr>
<tr>
<td>Nucleus</td>
<td>23.6 (36.6)</td>
<td>16.2 (35.2)</td>
<td>10.8 (27.5)</td>
</tr>
</tbody>
</table>

Total: 62.9 (100) 45.9 (100) 39.4 (100) 35.0 (100) 123.0 (100) 4.1 (100) 8.7 (100) 13.9 (100)

Abbreviations: A, promyelocyte; B, myelocyte; C, metamyelocyte; D, segmented neutrophil.

*Grain density = grains counted/area (sq $\mu$m) of organelle. Relative grain density = percent grains counted over organelle/percent area occupied by organelle.
marrow exposed to sodium $^{35}$S-sulfate pulse labeling for 10 min. Virtually every promyelocyte was extensively labeled and exhibited distinctive intracellular grain localization. Only a small number of myelocytes demonstrated $^{35}$S-sulfate uptake and the few silver grains observed were more randomly distributed over these cells. In contrast with the myelocyte group, most of the metamyelocytes and segmented neutrophils exhibited active $^{35}$S-sulfate uptake with selective intracellular localization. The extent of labeled sulfate uptake by metamyelocytes and segmented neutrophils was significantly less than that noted for the promyelocyte group.

Detailed grain analysis, as outlined above, was done on randomly selected cells from each incubation medium and selected time interval and included groups of 15 cells from each morphologic category of neutrophil development. The grain distribution and percentage of grains overlying the differentiating neutrophilic cells in autoradiographs following incubation of marrow cells in sodium $^{35}$S-sulfate in PBSP and in RPMI for 10 min are reported in Table 1. This table also shows these parameters for the RPMI medium following 50-, 110-, and 230 min chase containing unlabeled sodium sulfate. The data indicate that $^{35}$S-sulfate incorporation was much greater in cells exposed to $^{35}$S-sulfate in PBSP than in RPMI; however, the pattern of uptake for each cell category was similar. In addition, the uptake of $^{35}$S-sulfate by promyelocytes in the PBSP medium was proportionately greater than the uptake by those incubated in RPMI, while sulfate uptake by the metamyelocytes and segmented neutrophils was proportionately less in the PBSP medium than in RPMI. Myelocytes in both incubation media showed limited labeling values after a 10-min pulse label with sodium $^{35}$S-sulfate.

Figures 1–4 illustrate the $^{35}$S-sulfate labeling pattern for neutrophilic promyelocytes. The extent of labeling was related to the degree of active azurophilic (primary) granulogenesis. Promyelocytes having large Golgi zones, abundant condensing saccules, numerous immature azurophilic granules concentrated in the proximal cytoplasm, and extensive dilated rough endoplasmic reticulum (RER) were the most heavily labeled cells in the promyelocyte group. The more immature and older forms of promyelocytes showing more limited morphologic signs of azurophilic granule formation exhibited more limited $^{35}$S-sulfate uptake. The majority of silver grains associated with the promyelocytes localized over the Golgi, including condensing saccules, and to a lesser extent over some of the immature azurophilic granules at the 10-min period.

Table 2 details the grain counts and the grain distribution over individual cell compartments expressing these as grains per area (sq μm) occupied by a given organelle (grain density) and as a percentage of grains to percentage area occupied by the organelle (relative grain density). Data are also presented as mean grains per organelle as well as percentage of the total grains overlying an organelle. The grain density analyses suggest that, in addition to the Golgi complex and azurophilic granules becoming labeled, the cell membrane and perhaps RER and mitochondria exhibit low levels of $^{35}$S-sulfate incorporation in the promyelocyte group. The close association of silver grains with these organelles is illustrated in Figs. 1–4.

Figure 5 is representative of the $^{35}$S-sulfate uptake by the neutrophilic myelo-
Fig. 1. Early promyelocyte containing few azurophilic granules and abundant slender cisternae of RER. Silver grains concentrate near Golgi zone (G) and over three immature azurophilic granules; several grains lie near segments of RER (arrows). Incubated 10 min with sodium $^{35}$S-sulfate in PBSP. No counterstain. $\times$ 15,000.

Fig. 2. Higher magnification of early promyelocyte (Fig. 1) showing silver grains over two immature azurophilic granules and near RER cisternae. $\times$ 30,000.

Fig. 3. Typical neutrophilic promyelocyte with prominent Golgi complex, dilated cisternae of RER, and numerous azurophilic granules. Silver grains localize over the Golgi complex (G) and are also associated with several immature azurophilic granules (A) in the proximal cytoplasm. An occasional grain (arrows) lies near the RER adjacent to the Golgi zone. Incubated 10 min with sodium $^{35}$S-sulfate in PBSP. No counterstain. $\times$ 23,000.
**Fig. 4.** Silver grains overlying segments of Golgi cisternae (G) and concentrated over condensing sacules and small formative azurophilic granules (arrows) in this promyelocyte. Several grains lie near dilated RER (R) adjacent to Golgi complex. Incubated 10 min in sodium $^{35}$S-sulfate in PBSP. Counterstained with lead citrate and uranyl acetate. × 44,000.

**Fig. 5.** Neutrophilic myelocyte with both azurophilic granules (A) and specific granules in various stages of development (S). Condensing sacules (arrows) along convex face of Golgi indicate active specific granulogenesis. No silver grains are evident in this cell. Incubated 10 min in sodium $^{35}$S-sulfate in PBSP. Counterstained with lead citrate and uranyl acetate. × 28,000.
Fig. 6. Neutrophilic metamyelocyte with typical azurophilic (A) and specific (S) granules differing in size from smaller round to oblong granules (T). Silver grains overlie the Golgi and several of the small granules (arrows) in the proximal cytoplasm. Incubated 10 min in sodium $^{35}$S-sulfate in PBS. Counterstained with lead citrate and uranyl acetate. x 24,000.

Fig. 7. Higher magnification of Golgi (G) region of metamyelocyte shown in Fig. 6. Note electron density of outer Golgi cisternae (F) suggestive of granule formation and the localization of silver grains within this region. Small granules with overlying silver grains (arrows) can be compared to similar unlabeled granules (T) and to specific (S) and azurophilic (A) granules. Counterstained with lead citrate and uranyl acetate. x 36,000.
cytes at the 10-min period. In spite of morphologic evidence of active specific (secondary) granule formation in these cells, both visual observation and grain analysis (Table 2) showed that myelocytes exhibited low levels of 35S-sulfate uptake and no selective localization was apparent. The few silver grains analyzed in this cell group were distributed randomly in the cytoplasm and other organelles.

Figures 6 and 7 demonstrate 35S-sulfate localization in the neutrophilic metamyelocyte. Metamyelocytes had one or two Golgi stacks of moderate size and most of the cells showed small elongated condensing saccules containing a substance of moderate electron density; the condensing saccules generally were found to arise from the more concave Golgi face. In addition to azurophilic and specific granules in the metamyelocytes, a number of pleomorphic granules were evident. These granules differed from typical azurophilic and specific granules, being of smaller size and frequently having an oblong or dumbbell shape. 35S-sulfate labeling was observed to concentrate over areas of the Golgi, over the condensing saccules, and near or overlying some of the pleomorphic granules localized near the Golgi zone. Silver grains were uncommonly found overlying other types of granules. Grain analysis (Table 2) verified the concentration of grains over the Golgi (and condensing saccules) and over the granule population. No attempt was made to separate granule types in the data analysis since absolute identification of a given granule during grid analysis was not uncommonly open to question.

Approximately 80% of the segmented neutrophils from bone marrow revealed a selective incorporation of labeled sulfate. Examination of these cells revealed the presence of slender condensing saccules containing a matrix of modest electron density which arose from the more concave face of the Golgi; a number of small oblong granules of similar density were commonly found near the Golgi zone. Similar granules were quite numerous in the remainder of the cytoplasm. These granules differed from typical azurophilic and specific granules, and the number of these granules in segmented neutrophils was greater than that noted in metamyelocytes. The intracellular 35S-sulfate label concentrated over segments of the Golgi complex, condensing saccules, and over a few of the small pleomorphic granules in the proximal cytoplasm at the 10-min interval (Figs. 8–11); labeling of pleomorphic granules in the more peripheral cytoplasm was evident in chase experiments (Fig. 12). Grain density analysis (Table 2) confirmed this grain distribution for the segmented neutrophils; few grains were distributed over other cell components in this cell group.

Chase studies for periods greater than 30 min required the use of a medium other than PBSP, since after this time interval appreciable signs of cellular degeneration occurred. RPMI 1640 preserved morphologic integrity for periods up to 4 hr at 37°C. The uptake of 35S-sulfate by neutrophilic cells in the RPMI incubation medium was much less than with PBSP, but the relative uptake by these cells was similar after the 10-min pulse period at each time interval (Table 1). Grain density analysis of RPMI chase studies indicated that the initial concentration of label occurred in the Golgi and some azurophilic granules of the promyelocytes. With successive chase times, there was a marked decrease in Golgi labeling with a concomitant increase in labeling of the granule
Fig. 8. Segmented neutrophil with two small Golgi stacks (G). Silver grains overlie one of these units and near several cytoplasmic granules (arrow) adjacent to the Golgi zone. Incubated 10 min in sodium $^{35}$S-sulfate in PBSP. Counterstained with uranyl acetate. $\times$ 23,000.

Fig. 9. Segmented neutrophil with silver grains over several Golgi (G) cisternae, near small vesicles (V), and over a small oblong granule (arrow) of modest electron density. Azurophilic (A), specific (S), and tertiary (T) granules are indicated. Incubated 10 min in sodium $^{35}$S-sulfate in PBSP. Counterstained with uranyl acetate. $\times$ 41,000.
Fig. 10. Cisternae on concave face of Golgi (G) of a segmented neutrophil showing modest electron density similar to adjacent oblong sacules (arrow) considered to represent formative tertiary granules. Silver grains localize over these cell components. Incubated 10 min in sodium $^{35}$S-sulfate in PBSP. Counterstained with lead citrate and uranyl acetate. $\times$ 40,000.

Fig. 11. Silver grains overlying portions of Golgi (G) and near small cytoplasmic granules of modest density adjacent to Golgi in this segmented neutrophil. Smaller tertiary granules (T) can be compared to larger specific granules (S) in cytoplasm. Incubated 10 min in sodium $^{35}$S-sulfate in PBSP. Counterstained with lead citrate and uranyl acetate. $\times$ 35,000.

Fig. 12. Portion of cytoplasm of segmented neutrophil exposed to sodium $^{35}$S-sulfate in RPMI for 10 min, followed by 45-min chase, showing silver grains near several cytoplasmic granules. Typical specific granules (S) are indicated. Counterstained with lead citrate and uranyl acetate. $\times$ 45,000.
population. A similar pattern was obtained with the metamyelocytes and segmented neutrophils. These changes in the three cell types were significant at the 0.001 level.

Grain density (grains/sq µm of organelle) analysis of the developing neutrophils (Fig. 13) emphasized the changing pattern of 35S-sulfate localization in the Golgi, granule, and plasma membrane fractions of each cell group during the 4-hr interval. Only these cell compartments had relative grain densities above 1 at points along the 4-hr span and were considered to be above random. Grain density patterns suggested a more rapid passage of label from the Golgi to granules in the promyelocytes than in metamyelocytes and segmented neutrophils. Only low levels of Golgi labeling occurred in promyelocytes after 50 min, while the Golgi zones in metamyelocytes and segmented neutrophils retained appreciable but decreasing levels of label during the 4-hr period. Since during chase periods newly formed labeled granules migrated peripherally from the Golgi region, midplane sections as employed in analysis would miss many of these labeled granules, so that direct comparison of combined Golgi-granule labeling may appear as a decrease or loss of total label. Relative grain density values indicated the random labeling of the cell compartments in the myelocyte group incubated in the RPMI medium, except for the Golgi at 10 min and the cell membrane at 230 min, which had values slightly above 1. Grain density and relative grain density values also suggested the limited labeling of cell membranes of promyelocytes and metamyelocytes at 110 min, which decreased by 230 min with relative grain densities appreciably above 1; segmented neutro-

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**Fig. 13.** Distribution of silver grains over Golgi complex, cytoplasmic granules, and cell membrane in neutrophilic cells after 10-min pulse label with sodium 35S-sulfate in RPMI and chase periods of 50, 110, and 230 min, shown as grain density (grains/sq µm). (A) Promyelocyte. (B) Myelocyte. (C) Metamyelocyte. (D) Neutrophil.
DISCUSSION

Our autoradiographic data indicate that $^{35}$S-sulfate uptake occurs in two separate and distinct phases during neutrophil maturation, i.e., in the promyelocyte stage and during late stages of maturation (metamyelocyte and segmented neutrophil). These two phases of $^{35}$S-sulfate uptake were separated by a period of limited sulfate incorporation which corresponded developmentally to the myelocyte stage. During short-term incubation of normal human bone marrow in vitro the greatest uptake of $^{35}$S-sulfate was noted in neutrophilic promyelocytes and could be related to the extent of azurophilic (primary) granule formation. The high uptake of $^{35}$S-sulfate by promyelocytes and incorporation of labeled sulfate was directly related to the extent of azurophilic (primary) granule formation and is consistent with the studies of Horn and Spicer, Wetzel, and Young using rabbit, rat, and mouse bone marrow.

Neutrophilic myelocytes featured specific (secondary) granule formation rather than azurophil granulogenesis and revealed only limited $^{35}$S-sulfate uptake. In contrast, metamyelocytes and many of the segmented neutrophils showed appreciable $^{35}$S-sulfate uptake, although the extent of labeling of these cells was less than that noted for neutrophilic promyelocytes in the same preparations. Morphologically, cells incorporating $^{35}$S-sulfate during the late stages of neutrophil maturation were involved in the elaboration of small round to oblong cytoplasmic granules which differed from typical azurophilic and specific granules. The $^{35}$S-sulfate labeling pattern was consistent with the concept of selective uptake of sulfate by a small active Golgi and the transfer of sulfate to newly formed granules. The differential in $^{35}$S-sulfate uptake between promyelocytes and late developmental neutrophils suggests that the rate of sulfation of azurophilic granules in promyelocytes is greater than that of sulfate-containing granules formed in metamyelocytes and young segmented neutrophils. Alternatively, these differences may reflect the relative concentration of sulfated glycosaminoglycan incorporated into these two granule populations or may represent in part a differential in granule size.

Azurophilic granules of neutrophils possess a variety of enzymes (e.g., peroxidase and acid hydrolases) and are known to contain sulfated glycosaminoglycans, principally chondroitin-4-sulfate. The protein and enzymatic components incorporated into azurophilic granules in promyelocytes form within the RER, pass to the Golgi, and are transferred to condensing saccules derived from the concave and lateral sides of the Golgi. These saccules enlarge and fuse with adjacent saccules to form immature azurophilic granules, which undergo further maturation indicated by changes in size and electron density. Our data indicate that sulfation of the glycosaminoglycan component of these granules occurs primarily in the Golgi cisternae and condensing saccules. Limited sulfation within the RER also is suggested by the close approximation of silver grains near cisternae of RER in early promyelocytes and in dilated RER cisternae approaching the Golgi zone of typical promyelocytes exhibiting morphologic signs of extensive azurophilic granule for-
mation. A definitive answer is not possible because of the large size of the $^{35}$S probability circle around individual silver grains.

Multiple silver grains overlying immature azurophilic granules located at considerable distances from the Golgi as well as those located in the proximity of the Golgi zone after brief $^{35}$S-sulfate pulse labeling suggest that many immature azurophilic granules have or retain the capability of glycosaminoglycan sulfation. Such observations could also be considered consistent with the concept suggested by Scott and Horn$^{23}$ that some azurophilic granules may arise directly from the RER without involving the Golgi elements.

The concentration of $^{35}$S-sulfate in the Golgi and its packaging into small granules in neutrophilic metamyelocytes and segmented (2–3 lobes) neutrophils establishes not only that the active granulogenesis persists much later in neutrophil evolution than generally regarded, but that the chemical nature of these granules is distinct from specific granules. These observations provide strong confirmational data for the existence of a third or tertiary granule population in neutrophils as originally reported by Spicer and his colleagues.$^{12,20,23,26,27}$ Histochemically, tertiary granules have been shown to have acid phosphatase and aryl sulfatase activity and to contain sulfated glycosaminoglycans.$^{20,22,26,27}$ It is pertinent that leukocytes isolated from peripheral blood in man and several other species exhibit active uptake of $^{35}$S-sulfate and incorporate this label into sulfated glycosaminoglycans, principally chondroitin-4-sulfate separated from the granule fraction.$^{34,35}$ Such independent lines of research, including our autoradiographic study, provide substantial evidence that glycosaminoglycan synthesis, sulfation, and active granulogenesis occur during very late stages of neutrophil maturation, even extending into the segmented stage where neutrophils have been conventionally felt to be mature and not involved in granulogenic activity.

With the development of new concepts for the identification of granulocyte disorders which include alterations in number and composition of granule populations,$^{47}$ the recognition of tertiary granules as a separate and distinct population is essential for future evaluation of such disorders. It should be noted that although the majority of labeled sulfate utilized by developing granulocytes is directed toward the sulfation of glycosaminoglycan of primary and tertiary granules, at least a small portion of the labeled sulfate is incorporated into the cell membrane and is consistent with the identification of a sulfated glycosaminoglycan component in the plasma membrane noted in other cell forms.$^{48}$

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Ultrastructural autoradiographic study of the uptake and intracellular localization of 35S-sulfate by developing human neutrophils

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