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PAYNE, DALE NATHANIEL
AN AUTORADIOGRAPHIC STUDY OF THE UPTAKE AND
LOCALIZATION OF SODIUM(35) SULFATE,
D-GLUCOSE-6-TRITIUM, AND L-LYSINE 4,5-TRITIUM
BY DEVELOPING HUMAN NEUTROPHILS OF THE BONE
MARROW.

THE OHIO STATE UNIVERSITY, PH.D., 1978
AN AUTORADIOGRAPHIC STUDY OF THE UPTAKE AND LOCALIZATION OF
\(^{35}\)SODIUM SULFATE, D-GLUCOSE-6-H\(^3\), AND L-LYSINE 4.5-H\(^3\)
BY DEVELOPING HUMAN NEUTROPHILS OF THE BONE MARROW

DISTRIBUTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Dale Nathaniel Payne, B.S., M.S.

* * * * *

The Ohio State University
1978

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DEDICATION

Dedicated to my parents, Mr. and Mrs. William N. Payne. Without their moral support and strength, this work would not have been possible.
ACKNOWLEDGMENT

The author wishes to express his most sincere gratitude to his adviser and friend, Dr. G. Adolph Ackerman, for his innumerable contributions to this work and to the author's education.
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RESEARCH GRANTS AND FELLOWSHIPS:
1. General Research Support Grant from The Ohio State University, 1974-1975 - "Relationship of Intracellular Metabolism and Cell Surface Characteristics of Developing Granulocytes on Normal Bone Marrow. An Autoradiographic Study."


ORGANIZATIONAL AFFILIATIONS:
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PRESENTATIONS:

PUBLICATIONS:


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GENERAL INTRODUCTION

The human neutrophilic leukocyte develops in the bone marrow through a series of morphologically and histochemically distinct stages, viz., myeloblast, promyelocyte, myelocyte, metamyelocyte, band neutrophil and segmented neutrophil. Although much is known about the morphology and histochemistry of these cell types, little is known about the intracellular metabolic changes that occur in the neutrophil during development, because of the inadequacies of present day cellular separation techniques. Autoradiography, however, is a method whereby individual cells may be analyzed for uptake and distribution of radioactively labeled precursor substances. Therefore, in order to study the changes that occur in the uptake and metabolism of carbohydrates, amino acids, and inorganic sulfate during neutrophil maturation, radioactively labeled glucose, lysine and sulfate were incubated with aspirates of human bone marrow and high resolution electron microscopic autoradiography was utilized to trace those substances intracellularly. Part I of this dissertation deals specifically with $^{35}$S labeled sodium sulfate uptake by developing human neutrophils and Part II describes uptake and localization of glucose-6-$H^3$ and lysine 4,5-$H^3$ by these cell types.
PART I

ULTRASTRUCTURAL AUTORADIOGRAPHIC STUDY OF
THE UPTAKE AND INTRACELLULAR LOCALIZATION
OF $^{35}$S-SULFATE BY DEVELOPING HUMAN NEUTROPHILS

INTRODUCTION

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 (1-10). 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 (3, 4, 11, 12). 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 (12-27).
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 (12, 21, 22, 24, 28, 29). Biochemically, the principal variety of sulfated glycosaminoglycan present in leukocytes and bone marrow cells is chondroitin-4-sulfate (30-38). Olsson (34-36) 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 promyelocyte stage (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 (11, 12, 39).

In a preliminary report (11) it was demonstrated that $^{35}$S-sulfate uptake by developing neutrophils from human bone marrow occurs 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 (12, 21, 23, 26, 27, 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 (21, 22, 25).

It is the purpose of this section (Part I) to report 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.
MATERIALS AND METHODS

Preparation of Tissue Samples

Human bone marrow specimens (1-2ml) 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 Rosewell Park Memorial Institute tissue media 1640 (RPMI 1640) [Gibco, Grand Island, N.Y.] and resuspended in 5 ml of either PBSP or RPMI containing 200 microcuries/ml of carrier-free $^{35}$S-sulfate-labeled sodium sulfate ($\text{Na}_2^{35}\text{SO}_4$) with specific activity 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) [41]. Slides were stored
in light-tight black boxes containing anhydrous calcium sulfate for 56 days. Autoradiographs were then developed in gold Elonal ascorbic acid developer (42) 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 microscope.

Analysis of Autoradiographs

All cells employed in detailed autoradiographic analysis were sectioned through the midplane and included the Golgi complex and portions of the nucleus. Photographs of these cells were subjected to point hit analysis (43) using a grid calibrated to points per 0.25 square micrometer 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 areas (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 subcellular organelles, standard deviations of grain counts, grain density (total grains counted over an organelle divided by total grains counted x 100), and relative grain density (percentage of total grains of an organelle divided by the percent of total area occupied by that organelle) [44]. Relative grain density values above 1 indicated a level of labeling above purely random [44].

Probability circle analysis (44, 45) was also used on a number of autoradiographs. A 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 $^{14}$C), drawn around a developed silver grain represented the area within which there would be a 50% probability of locating the $^{35}$S radiation locus [46]. Thus, circles with 336 nm were drawn around silver grains on many autoradiographs to approximate more closely the subcellular location of $^{35}$S-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 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 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 through 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 (square micrometer) 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 Figures 1 through 4.

Figure 5 is representative of the $^{35}$S-sulfate uptake by the neutrophilic myelocytes 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 $^{35}$S-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 $^{35}$S-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. $^{35}$S-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 not commonly 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 often 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 $^{35}$S-sulfate label concentrated over segments of the Golgi complex, condensing saccule, and over a few of the small pleomorphic granules in the proximal cytoplasm at the 10 min interval (Figs. 8 through 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 $^{35}$S-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 pro-
myelocytes. With successive chase times, there was a marked
decrease in Golgi labeling with a concomitant increase in label-
ing of the granule 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/square micrometer of organelle)
alanalysis of the developing neutrophils (Fig. 13) emphasized the
changing pattern of $^{35}$S-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 den-
sities 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 re-
tained 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 den-
sity 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 den-
sity 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; seg-
mented neutrophils had membrane-labeling values just above random
only at the 110 min interval.
DISCUSSION

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 labelled sulfate was directly related to the extent of azurophilic (primary) granule formation and is consistent with the studies of Horn and Spicer (3), Wetzel (12), and Young (39) 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, 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 (29, 34-36). 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 (12-26). 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. 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 formation. A definitive answer is not possible because of the larger 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 also 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. Such independent lines of research, including this 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 in the cell membrane and is consistent with the identification of a sulfated glycosaminoglycan component in the plasma membrane noted in other cell forms (48).
Table 1

Distribution of Grains over Neutrophilic Cells following 10 minute Pulse Label with $^{35}$S Sodium Sulfate

<table>
<thead>
<tr>
<th>Medium (Minutes)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Mean Grains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>±</td>
<td>Mean</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>PBSP</td>
<td>1846 (82%)</td>
<td>62 (3%)</td>
<td>129 (62%)</td>
<td>208 (9%)</td>
<td>123.1 ± 49.8</td>
</tr>
<tr>
<td>RPMI</td>
<td>159 (58%)</td>
<td>19 (7%)</td>
<td>39 (14%)</td>
<td>58 (21%)</td>
<td>10.6 ± 5.5</td>
</tr>
<tr>
<td>RPMI</td>
<td>120 (52%)</td>
<td>11 (5%)</td>
<td>45 (19%)</td>
<td>55 (24%)</td>
<td>8.0 ± 5.0</td>
</tr>
<tr>
<td>RPMI</td>
<td>168 (53%)</td>
<td>14 (5%)</td>
<td>57 (18%)</td>
<td>74 (24%)</td>
<td>11.1 ± 6.6</td>
</tr>
<tr>
<td>RPMI</td>
<td>224 (59%)</td>
<td>17 (5%)</td>
<td>59 (16%)</td>
<td>77 (20%)</td>
<td>14.9 ± 8.4</td>
</tr>
</tbody>
</table>

A = Promyelocyte; B = Myelocyte; C = Metamyelocyte; D = Segmented Neutrophil
## Table 2

**Area of Cell Organelles and Distribution of Silver Grains over Developing Neutrophils Pulse**

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Mean Area (um^2) and (% of Total Area per Cell Stage)</th>
<th>Mean Grains and (% 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></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>20.2(12.1)</td>
<td>19.6(10.6)</td>
<td>20.9(11.3)</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>Mitochondria</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>23.0(36.6)</td>
<td>16.2(25.2)</td>
<td>10.4(75.5)</td>
</tr>
<tr>
<td>Total</td>
<td>62.9(100)</td>
<td>45.9(100)</td>
<td>39.4(100)</td>
</tr>
</tbody>
</table>

A = Promyelocyte; B = Myelocyte; C = Metamyelocyte; D = Segmented Neutrophil

* Grain Density = Grains counted/area (um^2) of organelle

** Relative Grain Density = % grains counted over organelle/ % area occupied by organelle
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 \textsuperscript{35}S-sulfate in PBSP. No counterstain. X 15,000

Fig. 2  Higher magnification of early promyelocyte (Fig. 1) showing silver grains over two immature azurophilic granules and near RER cisternae. X 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 \textsuperscript{35}S-sulfate in PBSP. No counterstain. X 23,000
Fig. 4  Silver grains overlying segments of Golgi cisternae (G) and concentrated over condensing saccules 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. X 44,000

Fig. 5  Neutrophilic myelocyte with both azurophilic granules (A) and specific granules in various stages of development (S). Condensing saccules (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. X 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 PBSP. Counterstained with lead citrate and uranyl acetate. X 24,000

Fig. 7  Higher magnification of Golgi (G) region of metamyelocyte shown in Figure 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
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. X 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. X 41,000
Fig. 10  Cisternae on concave face of Golgi (G) of a segmented neutrophil showing modest electron density similar to adjacent oblong saccules (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. X 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. X 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. X 45,000
Fig. 13  Distribution of silver grains over Golgi complex, cytoplasmic granules, and cell membrane in neutrophilic cells after 10 min pulse label with sodium $^{35}$S-sulfate in RPMI and chase periods of 50, 110, and 230 min shown as grain density (grains/square micrometer). (A) Promyelocyte; (B) Myelocyte; (C) Metamyelocyte; (D) Neutrophil.
Figure 13
PART II
AN ULTRASTRUCTURAL AUTORADIOGRAPHIC
STUDY OF THE UPTAKE OF
D-GLUCOSE-\(^3\)H BY DEVELOPING HUMAN NEUTROPHILS

INTRODUCTION

Stages in the sequential development of the neutrophilic leukocyte in the bone marrow have been based primarily on morphological criteria derived from light and electron microscopy. Changes in certain intracellular components in this cell line correlate developmentally with specific intracellular events, e.g., the elaboration of three distinct populations of cytoplasmic granules each characterized by their content of selected enzymes, protein and glycoprotein components (12, 14, 15, 19, 22, 23, 47, 49, 50). Chemical differences in cell surfaces have been demonstrated between various hemic cells of the bone marrow (51-56) and changes in neutrophil surface characteristics can be correlated with the extent of neutrophil maturation (51, 52, 55, 56). Histochemically, an increase in cell surface labeling occurs during the final stages of neutrophil development in the
bone marrow, viz., metamyelocytes to segmented neutrophils, when concanavalin A (51, 52) and cationized ferritin (55) are employed as selective markers for glucosyl and mannosyl residues and for surface anionic binding sites, respectively. Net surface charge of developmental forms of neutrophils as measured by electrophoretic mobilities has been found to decrease during neutrophil development; this change has been correlated with an increase in neutrophil deformability and motility (56).

Intracellular metabolism also is modified during neutrophil development and mature neutrophils have been found to depend primarily on anaerobic rather than aerobic glycolysis (50, 57-60). Increased glycogen stores become evident during the late phases of neutrophil development and large accumulation of the carbohydrate complex are present in the segmented or mature neutrophils (49, 59, 61, 62). Biochemical evidence involving the uptake and utilization of glucose by neutrophils in the blood and in inflammatory sites indicates that mature neutrophils demonstrate a high level of anaerobic glycolysis for energy production utilizing either exogenous sugars and/or endogenous glycogen stores (50, 57-61). A marked increase in glucose oxidation via the hexose monophosphate shunt occurs in neutrophils during phagocytosis with stored glycogen providing an important energy reserve (50, 59, 63-67). Considering the importance of glucose in cellular
metabolism and activity, it is surprising that so little is known regarding the uptake and utilization of glucose during the development and maturation of the neutrophilic cells in the bone marrow.

In order to further define the dynamics of neutrophil maturation and glucose utilization an ultrastructural autoradiographic investigation utilizing D-glucose-6-$^3$H was initiated. It is the purpose of this section (Part II) to detail observations regarding the uptake and localization of D-glucose-6-$^3$H within developing neutrophilic leukocytes from normal human bone marrow. D-glucose-6-$^3$H incorporation will be related to distinctive maturational stages of the neutrophil and the cellular uptake of this isotope will be compared with the glycogen content of these cells. L-lysine 4,5-$^3$H was also used in similar experiments as an index of protein synthetic capacity and transport viability of the bone marrow cells.
MATERIALS AND METHODS

Preparation of Tissue Samples

Human bone marrow specimens (1-2 ml) were obtained from posterior iliac crest aspiration of healthy volunteers who gave appropriate informed consent. Marrow was anticoagulated by adding the aspirate to heparin (1 mg/ml) in Sorenson's phosphate buffer, pH 7.4, containing 4% polyvinylpyrrolidone and 1% sucrose (PBSP) at room temperature. Siliconized glassware was used throughout, minimizing surface adherence of cells. Buffy coats obtained by low speed centrifugation (1200 rpm for 15 mins) were rinsed once in cold (4°C) PBSP and centrifuged. Cells were suspended in PBSP at room temperature and adjusted to 10^8 cells/ml. Five ml of this cell suspension was added to 2 ml of the patient's marrow plasma followed by the addition of 250 micro-curies of D-glucose-6-H^3 (New England Nuclear, Boston, Mass.). This cell suspension was incubated for 30 minutes in a 37°C water bath. Cells exposed to glucose-H^3 were washed three times within a 10 min period in cold (4°C) PBSP; a Clay-Adams Serofuge was used for centrifugation at 3400 rpm for 1 min between washes.
The cells were then fixed in suspension in 2.5% glutaraldehyde at room temperature in PBSP for 10 min and centrifuged into a pellet at 3000 rpm for 20 min. They were then fixed in 1% chrome osmium in PBSP for 1 hour at 4°C, washed, dehydrated in graded ethanol, and embedded in Araldite. In addition to the above, other studies were performed in which bone marrow buffy coats were incubated at 37°C in 5 ml PBSP containing 2 ml of bone marrow plasma and 250 microcuries L-lysine 4,5-H³ (New England Nuclear, Boston, Mass.) for 30 min. These cells were washed and fixed with glutaraldehyde as above, post-fixed in osmium tetroxide, washed and embedded in Araldite. In some of the L-lysine experiments, cell suspensions were subjected to the Graham-Karnovsky 3,3' diaminobenzidine reaction (68) after glutaraldehyde fixation.

Preparation of Autoradiographs

Silver-gold sections were cut and placed on 1% collodion coated slides and dipped in Ilford L-4 nuclear photographic emulsion using a semiautomatic coating device to obtain a monolayer of emulsion (purple interference color) [41]. Slides were stored in light-tight black boxes containing anhydrous calcium sulfate for 60 days. Autoradiographs were developed in gold Elon-ascorbic acid developer (42) for 7 min at room temperature, rinsed for 1 min in water, fixed in 24% sodium thiosulfate for 2 min and then thoroughly washed and air 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 iso-amylacetate for 1 min. Sections were stained with aqueous uranyl acetate and lead citrate and viewed in a Philips 300 electron microscope.

**Analysis of Autoradiographs**

All cells which contained large portions of a nucleus and cytoplasm were randomly photographed and subjected to point hit analysis (43) using a grid calibrated for points per 0.25 square micrometer for determination of cytoplasmic and nuclear areas. Area data were collected for neutrophilic maturational stages, viz., myeloblasts, promyelocytes, myelocytes, metamyelocytes, band neutrophils, and segmented neutrophils.

All randomly photographed cells were separated into their morphologically designated stages of maturation. Except for the myeloblast stage, for which only 12 cells were found, at least 50 cells per stage were utilized. Silver grains were counted over each autoradiograph and assigned either to the nucleus or the cytoplasm according to the direct proximity of the grain. Statistical analysis consisted of mean grain counts per cytoplasm, nucleus, and total cell, and grain density (grains counted over a structure divided by the total area occupied by that structure) of these respective categories (42, 44). Probability circle analysis was inappropriate for this experiment (44, 45).
Histochemical Localization of Glycogen

The periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP) reaction was used for the intracellular localization of glycogen (69, 70). Bone marrow buffy coats were fixed and embedded in the routine manner as described above. Free-floating sections were floated on the surface of a 1% aqueous solution of periodic acid for 30 min, thoroughly washed with several changes of distilled water and subsequently floated on 1% thiosemicarbazide (Matheson, Coleman, and Bell, Norwood, Ohio) in 10% acetic acid with a final rinse of distilled water. Sections were then floated on 1% aqueous silver proteinate (Roboz Surgical Instrument Co., Inc., Washington, D.C.) for 30 min in the dark, washed thoroughly in distilled water and mounted on copper grids for ultrastructural examination. Controls included exposure of thin sections to 0.5% alpha amylase (Diastase of malt; Merck and Co., Rathway, N.J.) in 0.1M phosphate buffer (pH 7.4) at 37°C for 1 hr prior to exposure to the PA-TSC-SP reaction sequence.
RESULTS

Silver grain distribution was evaluated on autoradiographs of 410 cells of the neutrophilic series from normal human bone marrow pulse labeled in vitro with 250 microcuries D-glucose-6-H\(^3\) for 30 min at 37°C in PBSP containing human plasma. Nearly all cells (greater than 90%) in each morphological category exhibited D-glucose uptake as revealed by silver grain localization (Figs. 14, 15). Background labeling was virtually zero. The number of cells counted, cell areas, and grain densities (grains/square micrometer) for each cell category are shown in Table 3; cytoplasmic and nuclear grain densities are also indicated for each cell group. Mean grain densities for each cell group are displayed in Figure 16. These data indicate that low but significant levels of D-glucose uptake occurred in cells from the myeloblast through the metamyelocyte stages and the mean ranged from 0.06 to 0.12 grains/square micrometer. The extent of D-glucose incorporation for each of these cell groups was quite similar and in most instances the silver grains were randomly distributed over nucleus and cytoplasm.
Visual inspection did not indicate any preferential labeling of Golgi, rough endoplasmic reticulum, mitochondria, or cytoplasmic granules (azurophilic or specific); glycogen was not readily detected in these cell forms in routinely stained preparations until the late metamyelocyte stage of development. Although not reflected in the data (Table 3), late forms of metamyelocytes commonly exhibited higher grain counts than the younger metamyelocytes (Figs. 15, 17, 18).

In contrast to the earlier developmental neutrophils, band and segmented neutrophils (2-4 lobes) exhibited extensive labeling (Figs. 19, 20), viz., 0.89 and 1.44 grains/square micrometer for band and segmented neutrophils, respectively (Table 3). Older multi-segmented neutrophils in some instances were found to incorporate less glucose than band and younger (2-3 lobes) forms of segmented neutrophils. Cytoplasmic labeling was greater than nuclear labeling in band and segmented neutrophils with cytoplasmic silver grain localization primarily found over non-granular regions containing glycogen clusters (Figs. 21, 22). Glycogen was present in large quantities in both band and segmented neutrophils. Silver grains were also found over cytoplasmic granules (azurophilic, specific and tertiary) present in large numbers in these mature cells. Definitive point analyses to determine specifically which of these structures were labeled were not possible in autoradiographs because of the close
proximity of the granules and glycogen particles to each other. Preferential labeling of the Golgi zone and of formative tertiary granules was not evident in the segmented and band neutrophils or in the late form of metamyelocytes when tertiary granule formation is quite active. Occasionally small cytoplasmic vesicles or short cisternae were found in close approximation to small clusters of glycogen in band and segmented neutrophils as well as late metamyelocytes; silver grains were commonly found over such areas (Fig. 22).

Eosinophils and basophils and their developmental forms were less heavily labeled than comparable cells in the neutrophil series as judged by visual estimation; segmented forms showed greater labeling than promyelocytes and myelocytes in these series. Erythrocytes were rarely labeled while a small number of grains were located over the cytoplasm of nucleated erythroidic cells in the late stages of development. Monocytes and most lymphocytes in the bone marrow exhibited limited uptake of D-glucose although a few lymphocytes were modestly labeled with grains overlying both nucleus and cytoplasm. The few megakaryocytes encountered in this study were moderately labeled.

Morphologic and autoradiographic evidence suggested a close correlation with apparent glycogen content of the developing neutrophilic cells. In order to more precisely identify
glycogen, the PA-TSC-SP reaction was evaluated in the neutrophilic series (Fig. 23). It is noteworthy that modest amounts of diastase-labile glycogen were present throughout early neutrophil development in PA-TSC-SP preparations. The amount of glycogen increased slowly between the myeloblast and metamyelocyte stages and then rose sharply in the metamyelocyte, band and younger (2-3 lobes) segmented neutrophil stages. Glycogen appeared as individual particles rather than clusters in the early neutrophilic cells with scattered glycogen clusters first appearing in the myelocyte. Glycogen rosettes increased in size and number during the final stages of development; both rosettes and monodispersed particles were present from the myelocyte to the segmented neutrophil. In routinely stained sections (uranyl acetate and lead citrate) similarities in size and density of glycogen particles and free ribosomes, present in younger cells, precluded the morphological distinction of these two cell components.

In complimentary autoradiographic studies, 3H-lysine was found to be heavily incorporated into all granulocytes in the bone marrow, particularly those involved in the elaboration of cytoplasmic granules. Localization occurred over both nucleus (chromatin) and cytoplasm. Figures 24 to 26 are representative of the lysine-H3 incorporation of promyelocytes, myelocytes and late metamyelocytes. In each instance, granulogenesis (azurophilic, specific and tertiary) is active, and labeled lysine is
associated with the Golgi complex and formative granules; the
cisternae of the rough endoplasmic reticulum is labeled in
promyelocytes (Fig. 24). The cytoplasmic matrix also may be
labeled in cells from the myelocyte to segmented neutrophil
stages. The pattern of lysine-H\(^3\) uptake and intracellular
localization was distinctive from that observed with D-glucose-
H\(^3\).
DISCUSSION

This autoradiographic study has shown that the rate of glucose incorporation by developing neutrophilic cells from normal human bone marrow is directly related to the extent of neutrophil maturation. Developing neutrophils from the myeloblast through the early metamyelocyte stage incorporate limited amounts of glucose. However, in concurrence with increased quantities of intracellular glycogen, commencing in the late metamyelocyte stage, neutrophilic cells show a dramatic increase in their capacity to concentrate this sugar. The ability to incorporate large quantities of glucose further increases in the band and segmented neutrophil stages. The amount of glucose taken up by the younger neutrophilic cells is 10-30% of that incorporated by band and segmented neutrophils. Similar patterns of glucose incorporation also occur in the eosinophilic and basophilic leukocyte series although the level of uptake is not generally as pronounced as found in the neutrophilic cells.

Glucose metabolism has been extensively studied in peripheral leukocytes (50, 57-60, 63-67, 71, 72), however, little is known concerning the membrane transport kinetics of glucose or
the fate of glucose in developing neutrophils in the bone marrow. Glucose is metabolized in leukocytes via glycolytic and Krebs cycle pathways involved in cellular respiration and energy production (50, 58-60, 73). Human leukocytes use glucose only to a limited extent as a substrate for cell respiration (59). In the bone marrow, myeloid elements exhibit both aerobic and anaerobic glycolysis while anaerobic glycolysis predominates in peripheral neutrophils (58, 59). Changes occurring in glycolysis and glucose metabolism at different phases of leukocyte maturation in the marrow have been inadequately studied because cell separatory methodologies to date remain unsatisfactory. Glucose metabolism, however, has been examined in leukemias, and the myeloid elements in both acute and chronic myelocytic leukemia (CML) utilize glucose less than normal neutrophils (50, 59, 60). These leukemic cells have a high rate of aerobic glycolysis but a lower rate of anaerobic glycolysis than normal peripheral leukocytes (50, 59, 60); in vitro glycogen production is unimpaired in CML (59, 60). In this short-term labeling study the low glucose uptake noted in the majority of developing marrow cells from normal individuals suggests that the rate of glucose utilization (in the presence of exogenous glucose) is relatively low as compared to segmented leukocytes.

Evidence from many cell systems has shown that intracellular glucose can be transformed into nucleotide sugars used for
the synthesis of glycogen, glycolipids, glycoproteins and glycosaminoglycans (73-76). In autoradiographic studies of cells involved in the synthesis of glycoprotein and/or glycosaminoglycans, i.e., goblet and thyroid follicular cells, glucose-$^3$H labeling occurred rapidly over the Golgi and subsequently localized in membrane-bound secretory vesicles and granules (74-76). Developing neutrophils are known to elaborate sulfated glycosaminoglycans during two separate phases of their maturation, e.g., in the promyelocyte stage with the formation of azurophilic granules and in metamyelocytes, band and young segmented neutrophils with the formation of tertiary granules (3, 12, 22, 77). Earlier studies indicated that $^{35}$S-sulfate is rapidly and preferentially incorporated into the Golgi and formative granules during both azurophilic and tertiary granulogenesis (12, 77); myelocytes involved in the synthesis of specific granules did not incorporate $^{35}$S-sulfate. In contrast, L-lysine 4,5-$^3$H uptake was heavy throughout neutrophil maturation and the intracellular localization of this amino acid was in both nuclear and cytoplasmic areas. In promyelocytes, the cytoplasmic lysine label was preferentially distributed in the rough endoplasmic reticulum, Golgi and developing azurophilic granules while in subsequent maturational stages, with sparse endoplasmic reticulum, cytoplasmic localization was associated with the Golgi and formative granules (both specific and tertiary). D-glucose-6-$^3$H did not localize in these cell components. Significantly, glucose uptake was quite limited
in the younger developmental neutrophils and intracellular localization was random while in later maturational stages glucose uptake was heavy and localization was primarily associated with particulate glycogen. Such autoradiographic data suggests that of the glucose taken into developing neutrophils only a limited portion may be directed into the pathway for the synthesis of glycosaminoglycan component(s) of these cells while a major portion appears to be directed toward the glycogen pathway.

Glycogen formed in blood leukocytes is derived principally from glucose rather than from products of intermediary metabolism (58). Leukocyte glycogen regeneration is rapid; neither anaerobic nor aerobic conditions influence the content or metabolic turnover of leukocyte glycogen provided glucose is present in the medium (58, 64, 71, 78). Peripheral glycosyl residues of glycogen molecules turn over rapidly while those of the central core are slowly renewed (71, 79). Seitz (59) found the glycogen content of nucleated marrow cells was nearly half that of normal blood leukocytes although $^{14}$C-glucose turnover in glycogen in marrow cells was higher than in peripheral leukocytes. In cells actively synthesizing glycogen, e.g., hepatic and skeletal muscle cells, glucose-$H^3$ has been localized autoradiographically in areas of particulate glycogen and in areas having smooth endoplasmic reticulum in proximity to forming glycogen clusters (80, 81).
Similarly, correlated morphological and biochemical studies
(62) of rabbit neutrophils and eosinophils from peritoneal exu-
dates showed glycogen to occur in two forms, single and grouped.
Membrane cisternae were found to be associated with but not
attached to the glycogen particles in both the intact leukocytes
and the isolated glycogen fraction (62). The glycogen-rich
microsomal fraction of these exudate cells exhibited high
phosphorylase activity in relationship to glycogen content (62).
Phosphorylase and phosphorylase kinase activity have been found
directly associated with the sarcoplasmic reticulum of skeletal
muscle (81). Activation of this complex with calcium appears to
be related to glycogenolysis occurring during muscular contrac-
tion (81). In developing neutrophils the appearance of histo-
chemically demonstrable phosphorylase corresponds closely with
the appearance and relative amount of glycogen visualized at
the light microscopic level (49, 82). During glycogen synthesis,
glucose combines with uridine diphosphate and is transferred to
an acceptor polysaccharide chain (usually glycogen) by glycogen
synthetase (73) which is bound to particulate glycogen (81, 83);
an activator of this enzyme has been reported in the microsomal
fraction of hepatocytes (83). In the present study, 'D-glucose-
H3 was found in intimate association with glycogen in late develop-
mental neutrophilic cells which show heavy glucose incorporation.
In a number of instances, in these cells, silver grains were
closely approximated with short cytoplasmic cisternae or vesicles located near small glycogen clusters. A similar glycogen-cisternal relationship was not apparent in autoradiographs of younger developmental neutrophils where glucose uptake was low and glycogen not readily detected.

Light microscopic histochemistry has indicated the initial appearance of glycogen in the myelocyte stage (56, 61, 82); this has been supported by electron microscopic studies (12, 15, 19, 47, 50). In the present study, using the PA-TSC-SP reaction for diastase-labile glycogen, it has been shown that glycogen is present in all stages of neutrophil development. While sparse in myeloblasts and early promyelocytes, glycogen gradually increases in amount until the metamyelocyte stage. It then undergoes a sharp rise extending into the multi-segmented stage. Ultrastructurally, in routinely stained (uranyl acetate and lead citrate) preparations, glycogen is quite difficult to recognize until the late myelocyte or early metamyelocyte stages. In the more immature cells, glycogen occurs in a monodispersed form rather than clustered and individual glycogen particles have a size and electron density very similar to free ribosomes which are abundant in the younger cells.

This data has shown that glucose incorporation by granulocytes (neutrophils, eosinophils and basophils) in human bone marrow is a function of cell maturity and is related to the amount of
intracellular glycogen. Functional maturity in glucose uptake may reflect a modification or differentiation of the cell membrane, its components, receptors and/or transport proteins. Unfortunately, very little is known about the cell surface, membrane composition and transport kinetics in developmental forms of granulocytes. Recent ultrastructural histochemical studies demonstrate that differentiative changes do occur on the cell surface as the neutrophil matures (51, 52, 55), particularly in the late developmental stages, e.g., a differentiative increase in surface anionic binding sites (55) and in the number of mannosyl and glycosyl residues (51, 52). Proteins involved in glucose transport across the cell membrane also may vary with cell maturation and an increase in these proteins and/or Na-K-ATPase activity could enable the more mature granulocytes to incorporate larger quantities of glucose than earlier forms. Changes in number or access of selected hormone receptors, i.e., insulin receptors on the surface of differentiating leukocytes also may reflect or help govern the rate of glucose incorporation and its utilization in differentiating and mature granulocytes. No information is presently available regarding insulin binding sites on differentiative granulocytes although it is known that peripheral granulocytes do have limited capacity for insulin binding (84, 85). In addition, glucose incorporation and utilization may be dependent upon possible changes in the content and/or activity of
intracellular enzymes directly involved in glucose metabolism as has been suggested for leukocytes in patients with CML. In this regard, phosphorylase activity also has been found to vary with the stage of granulocyte differentiation. Lastly, glucose incorporation may be functionally dependent upon the quantity of intracellular glycogen. Since each glycogen particle represents a carbohydrate-enzyme complex, glucose is known to be metabolized in greater quantities and more rapidly as the number of glycogen particles increase (79). The mechanism governing the rapid accumulation of intracellular glycogen in more differentiated neutrophilic cells is unknown. This study suggests that glucose is directed in large measure along the glycogen pathway in granulocytes during their late stages of differentiation.
<table>
<thead>
<tr>
<th>Stage of Development</th>
<th>Total Cells</th>
<th>Total Grains</th>
<th>Total Area (μm²)</th>
<th>Total Mean Grain Density (S.D.)</th>
<th>Cytoplasmic Mean Grain Density (S.D.)</th>
<th>Nuclear Mean Grain Density (S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloblast</td>
<td>12</td>
<td>53</td>
<td>497</td>
<td>0.11 (0.10)</td>
<td>0.08 (0.06)</td>
<td>0.15 (0.11)</td>
</tr>
<tr>
<td>Promyelocyte</td>
<td>50</td>
<td>174</td>
<td>2444</td>
<td>0.07 (0.05)</td>
<td>0.07 (0.06)</td>
<td>0.09 (0.09)</td>
</tr>
<tr>
<td>Myelocyte</td>
<td>126</td>
<td>341</td>
<td>5414</td>
<td>0.06 (0.06)</td>
<td>0.06 (0.06)</td>
<td>0.06 (0.06)</td>
</tr>
<tr>
<td>Metamyelocyte</td>
<td>50</td>
<td>246</td>
<td>2053</td>
<td>0.12 (0.20)</td>
<td>0.12 (0.22)</td>
<td>0.10 (0.16)</td>
</tr>
<tr>
<td>Band Neutrophil</td>
<td>63</td>
<td>1813</td>
<td>2037</td>
<td>0.89 (0.53)</td>
<td>1.07 (0.66)</td>
<td>0.34 (0.32)</td>
</tr>
<tr>
<td>Segmented Neutrophil</td>
<td>109</td>
<td>5005</td>
<td>3483</td>
<td>1.44 (0.81)</td>
<td>1.77 (0.92)</td>
<td>0.49 (0.45)</td>
</tr>
</tbody>
</table>
Fig. 14  Silver grain distribution representing typical glucose-H\(^3\) uptake by developing human neutrophils. (P) - promyelocyte. (M) - early metamyelocyte. (S) - segmented neutrophil. Counterstained with lead citrate and uranyl acetate. X 14,000
Fig. 15  Glucose-\(H^3\) incorporation by early (E) and late (L) metamyelocytes. Counterstained with lead citrate and uranyl acetate.  X 11,500
Fig. 16 Distribution of silver grains over the total cell, cytoplasm, and nucleus after a 30 min pulse label with D-glucose-6-H$^3$ in PBSP, shown as grain density (grains/square micrometer). (A) myeloblast. (B) promyelocyte. (C) myelocyte. (D) metamyelocyte. (E) band neutrophil. (F) segmented neutrophil.
Fig. 17  Early neutrophilic metamyelocyte labeled with D-glucose-H$^3$. Only low levels of glucose incorporation are evident. Particulate glycogen (g) occurs in small quantities among cytoplasmic granules. Counterstained with lead citrate and uranyl acetate. X 26,000

Fig. 18  A late neutrophilic metamyelocyte with more glycogen (g) than shown in Figure 17 and demonstrating increased glucose incorporation. Counterstained with lead citrate and uranyl acetate. X 26,000

Fig. 19  Band neutrophil with increased uptake of glucose. Silver grains are in most instances located over clusters of particulate glycogen. Counterstained with lead citrate and uranyl acetate. X 13,000
Fig. 20  Glucose-H\(^3\) labeling of typical segmented neutrophil. Silver grains are localized primarily over the cytoplasmic portions of the cell usually within non-granular areas. Counterstained with lead citrate and uranyl acetate. X 28,000

Fig. 21  Higher magnification of cytoplasmic area of segmented neutrophil from Figure 20 labeled with glucose-H\(^3\). Silver grain localization is mainly over non-granular regions containing glycogen clusters. Counterstained with lead citrate and uranyl acetate. X 70,000

Fig. 22  Segmented neutrophil demonstrating localization of silver grains over small cytoplasmic vesicles and cisternae (arrows) situated adjacent to small glycogen clusters. Counterstained with lead citrate and uranyl acetate. X 60,000
Fig. 23 Glycogen content of developing human neutrophils as revealed by the periodic acid-thiosemicarbazide-silver protein reaction (PA-TSC-SP) reaction. (P) promyelocyte. (M) myelocyte. (S) segmented neutrophil.

X 13,000
Fig. 24 Neutrophilic promyelocyte labeled with L-lysine-H\(^3\) followed by peroxidase reaction. Lysine is concentrated in the Golgi region (G) and associated with formative azurophilic granules; cisternae of rough endoplasmic reticulum, and azurophilic granules are peroxidase-positive. Counterstained with lead citrate and uranyl acetate. X 16,000

Fig. 25 L-lysine-H\(^3\) incorporation by neutrophilic myelocyte involved in active secondary granule formation. Peroxidase reaction, counterstained with lead citrate and uranyl acetate. X 16,000

Fig. 26 Late neutrophilic metamyelocyte labeled with L-lysine. Lysine is actively incorporated into the Golgi (G) which in this cell is producing tertiary granules. Peroxidase reaction; counterstained with lead citrate and uranyl acetate. X 16,000
APPENDIX A

Electron Microscopic Autoradiographic Coating Technique

Specimen Preparation: Microscope slides with frosted ends are cleaned by wiping them with Kimwipes and then with lens paper. It is important that the cleaned slides remain dust free. The slides are then dipped, individually, into freshly prepared 0.8% collodion in isoamylacetate. So that the collodion coat will not separate from the slide prematurely during processing, it must extend a few millimeters into the frosted end of the slide. The slides are dried vertically overnight in a dust-free chamber.

Flattened, silver-gold sections of labeled material are transferred to the central portion of the glass slides (frosted side up) with a 3 mm diameter platinum wire loop. Three to four individual groups of sections are placed on each slide. The location of each group of sections can be marked on the back of the slides by a small circle of white tape or by an "x" placed by a diamond pencil. Excess water transferred to the slide by the platinum loop must be removed immediately with a wedge of filter paper.

Coating sections with photographic emulsion: Ilfo-d L-4 photographic emulsion (Ilford, Essex, England) is stored in light proof containers refrigerated at 4°C. For use the emulsion is diluted with double or triple distilled, deionized water on a
volume basis in a light proof darkroom using a safelight (Kodak, Wratten Series No. 2) for illumination. The dilution is accomplished by marking the required amount of distilled water needed for a particular dilution on a beaker with a grease pencil. The final level which the diluted emulsion will have after the water and emulsion are mixed is also marked on the beaker. In the darkroom the photographic emulsion is added to the proper amount of distilled water until the marked level is reached. Before stirring, the beaker with the emulsion and water is covered and placed in a preheated waterbath at 40°C for 20-30 min with the safelight turned off. The emulsion and water are then thoroughly mixed with a porcelain spoon being very careful not to introduce many bubbles into the mixture. For Ilford L-4 photographic emulsion the dilution is 1:2.5 to 1:3 (emulsion and distilled water). The diluted mixture is then transferred to the waterbath of the semiautomatic coating apparatus where, in order to evaporate any air bubbles introduced into the emulsion, it is kept covered for 20 min at the preset temperature of 32°C with the safelight off.

The collodion coated slides containing sections of labeled material are placed in the slide holders of the semiautomatic coating apparatus. It is advisable not to coat more than 2-3 slides at a time. The slides are lowered into the emulsion using the handle on the right side of the coating apparatus, and they are withdrawn from it mechanically by flipping the electric motor
switch on the left side of the coating apparatus. The slides should be withdrawn from the emulsion at a constant, slow speed of 85 mm per minute. This is obtained by placing the pulley belt on the left side of the coating apparatus into the middle grooves of the pulleys. The emulsion coating should completely cover the areas of sections and extend at least 1 cm beyond the sections. It is important to remember that the speed of removal of the slides and the dilution and temperature of the emulsion determines the thickness of the emulsion coat. A closely packed monolayer of emulsion is necessary for accurate reproducible autoradiographs. This is obtained by checking a test slide. A monolayer of Ilford L-4 emulsion has a purple interference color when looked at from an angle in normal room lighting. After dipping the slides, they are dried in a vertical position on plastic racks at room temperature.

Exposure of Autoradiographs: After drying, the slides are placed in black plastic boxes containing 20 gms indicating Drierite wrapped in a Kimwipe. The boxes are sealed with black plastic tape, and are placed in a refrigerator at 4°C. The boxes are stored so that the slides remain in a horizontal position with the sections facing up and the Drierite bag on the bottom of the box. The duration of exposure is 30-60 days depending on results obtained from development of test slides. If the exposure is prolonged over 30 days, the Drierite bag should be removed.
Developing of Autoradiographs: The slides are removed from the black plastic boxes and placed one or two at a time in slide holders. The processing fluids for development must cover the sections but must not extend above the top of the collodion coating. The autoradiographs are developed in either Kodak D-19 diluted 1:5 or 1:10 (developer:distilled water) for 1 min for large grain development; for fine grain development slides are developed in gold-Elon-ascorbic acid for 5-7 min (See Appendix B for details). After development slides are placed in distilled water for 30 min, fixed for 2 min in 24% sodium thiosulfate and washed five times in distilled water. For the final wash, slides may be placed in a waterbath with continuously running distilled water. The slides are dried thoroughly and a circle is gently cut through the collodion coat around the tissue sections with a needle which has been wet with distilled water. The slide is carefully fogged by breathing on it and the collodion emulsion complex containing the tissue sections is floated off the glass onto the surface of distilled water. In many cases the collodion will not separate from the glass. If this occurs, it is helpful to drop 1% hydrofluoric acid on the edge of the cut collodion. This will effectively lift the collodion from the glass and it can now be floated onto distilled water. While the films are floating, 200-300 mesh Athen type EM grids are carefully placed with forceps onto the tissue sections and the complex of collodion, tissue
sections, emulsion and grid are removed from the water by placing the end of a clean index card next to the grid and pushing it down and into the water causing the autoradiograph complex to adhere to the index card. The card is removed from the water, dried thoroughly, and the grids containing the autoradiographs are removed with forceps. The collodion coat which is now on the top of the grid covering the tissue section and emulsion may be removed by floating the grid face down on isoamylacetate for 30 seconds and washing under slowly running distilled water. The sections are then stained by usual procedures by submerging in aqueous uranyl acetate for 1 min and floating face down on Reynold's lead citrate for 5-10 min.
APPENDIX B

Preparation of Gold-Elon Ascorbic Acid Developer and Development Procedure.

Gold latensification: (Use ultraclean glassware and prepare just prior to use). 27% gold chloride (Fisher Chemical) stock solution is made by adding 15 grains of gold chloride to 48 ml distilled water. 0.5 ml of this 27% stock solution is added to 10 ml of distilled water. Next add 0.125 g potassium thiocyanate (Fisher Chemical, p. 317). This will cause the solution to turn brown, however, with continued stirring the solution will completely clear. If the solution does not clear, discard and begin again with cleaner glassware. After the above solution is clear, add 0.15 g potassium bromide (Fisher Chemical, p. 205) and dilute to 250 ml with distilled water. For use in autoradiographic development this solution is diluted again 1:10 (gold:water) prior to use.

Elon-Ascorbic Acid Developer: (prepare the day before developing autoradiographs; use at 18-24 hours of age).

Elon (metal) 0.225 g (Kodak E90)
Ascorbic acid 1.500 g
Sodium borate (Borax) 2.500 g
Potassium bromide 0.500 g
Water to make 500 ml
Developmental steps of autoradiograph:

(a) 1 min in distilled water at 20°C.
(b) 1 min in gold solution at 20°C.
(c) 30 sec rinse in distilled water at 20°C.
(d) 5-7 min in developer at 24°C.*
(e) 30 sec in distilled water for stop bath at 20°C.
(f) 2 min in 24% sodium thiosulfate for fix at 20°C.
(g) wash in distilled water x5 for 1 min each or in running distilled water x3-4 min.
(h) air dry

*5 min for small grain development; 7 min for large grain development.
APPENDIX C

Quantitation of Silver Grain in Autoradiography

Cell Areas - (30): A transparent grid containing points (intersections) per 0.25 micron is placed over each electron micrograph. Points are counted and assigned to specific cell organelles, i.e., nucleus, Golgi, granules, etc. depending on the exact location of the point. Total points are added and divided by four to determine areas in square microns for each organelle.

Grain Analysis - (29, 30, 31, 32): Silver grains are counted and assigned to specific organelles depending on their exact location on the electron micrograph. Total counts per organelle were tabulated and statistical results consisted of:

(a) total grain count per cell and subcellular organelle.

(b) mean grain count per cell and subcellular organelle.

\[
\text{grain density} = \frac{\text{total grains per structure}}{\text{total number of structures counted}}
\]

(c) grain density

\[
= \frac{\text{total grains per structure}}{\text{total area occupied by structure}}
\]
(d) relative grain density

\[ \text{relative grain density} = \frac{\text{percentage of total grains of organelle}}{\text{percentage of total area occupied by that organelle}} \]

(Relative grain density values above 1 indicate a level of labeling above purely random [31]).
BIBLIOGRAPHY


