Ultrastructural Autoradiographic Study of the Uptake and Localization of D-Glucose-\(^3\)H

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The uptake and localization of D-glucose-6-\(^3\)H was studied in developing neutrophils from normal human bone marrow by means of ultrastructural autoradiography. Marrow cells were pulse labeled for 30 min in vitro at 37°C with 250 \(\mu\)Ci D-glucose-6-\(^3\)H in phosphate buffer (pH 7.4) containing bone marrow plasma. Autoradiographic data indicated that glucose incorporation in developing neutrophils is a function of maturity and is related to the amount of intracellular glycogen. Developing neutrophils from the myeloblast stage through the early metamyelocyte stage incorporate limited amounts of glucose; however, in concurrence with increased quantities of intracellular glycogen, commencing with the late metamyelocyte stage, neutrophils 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. D-Glucose-\(^3\)H concentrated in areas of glycogen clustering and occasionally in areas containing small cytoplasmic vesicles that were in close approximation to small clusters of glycogen in late metamyelocytes and band and segmented neutrophils. Specific cellular components, e.g., rough endoplasmic reticulum and Golgi and both formative and definitive primary (azurophil) and secondary (specific) granules, were not preferentially labeled in the promyelocyte and myelocyte stages. Selective glucose-\(^3\)H labeling also was not observed in the Golgi and formative tertiary granules in the metamyelocyte, band, and young segmented neutrophil stages; primary, secondary, and tertiary granules in these cells were occasionally labeled, but labeled granules were invariably found to be closely associated with glycogen clusters.

Although glucose metabolism has been extensively studied in peripheral leukocytes, \(^1\) \(^1\) little is known regarding the uptake and utilization of glucose by developing neutrophils in the bone marrow. Glucose provides a major energy source for leukocytes and can be stored in the form of glycogen or metabolized via glycolytic and Krebs cycle pathways for energy production and respiration, \(^1\) \(^3\) \(^10\) \(^11\) although peripheral leukocytes use glucose only to a limited extent as a substrate for respiration. \(^10\) In bone marrow, myeloid elements show both aerobic and anaerobic glycolysis, \(^1\) \(^10\) while anaerobic glycolysis predominates in peripheral leukocytes and those in inflammatory sites \(^1\) \(^11\) with the utilization of either exogenous sugars and/or endogenous glycogen stores. Glucose also may be incorporated into glycosaminoglycan and glycoprotein components elaborated by the cell, e.g., certain cytoplasmic granules and carbohydrate complexes of the cell surface. At the present time, the only morphologic-histochemical evidence suggestive of a differential uptake and utilization of glucose by developing neutrophils is the appearance of appreciable amounts of glycogen during the final stages of neutrophil maturation, with large accumulations of this carbohydrate complex in mature neutrophils \(^10\) \(^12\) \(^18\) and perhaps the elaboration of glycosaminoglycan-
containing cytoplasmic granules during specific stages of neutrophil development.\textsuperscript{12,19,22}

This report describes the uptake and localization of D-glucose-6-\textsuperscript{3}H within the developing neutrophilic leukocytes from normal human bone marrow. D-Glucose-6-\textsuperscript{3}H incorporation will be related to distinctive maturational stages of the neutrophil and to the granulogenic activity and glycogen content of these cell forms.

\section*{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. Marrow was anticoagulated by adding the aspirate to heparin (1 mg/ml) in Sorenson 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 min) were rinsed once in cold (4\(^\circ\)C) PBSP and centrifuged. Cells were suspended in PBSP at room temperature and adjusted to 10\(^6\) cells/ml. Then 5 ml of this cell suspension was added to 2 ml of patient marrow plasma, followed by the addition of 250 \(\mu\)Ci D-glucose-6-\textsuperscript{3}H (New England Nuclear, Boston, Mass.). This cell suspension was incubated for 30 min in a 37\(^\circ\)C water bath. Cells exposed to glucose-\textsuperscript{3}H were washed three times within a 10-min period in cold (4\(^\circ\)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 hr at 4\(^\circ\)C, washed, dehydrated in graded ethanol, and embedded in Araldite.

\textit{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).\textsuperscript{22} Slides were stored for 60 days in light-tight boxes containing anhydrous calcium sulfate. Autoradiographs were developed in gold Elon-ascorbic acid developer\textsuperscript{24} for 7 min at room temperature, rinsed in 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 isomylacetate for 1 min. Sections were stained with aqueous uranyl acetate and lead citrate and viewed in a Phillips 300 electron microscope.

\textit{Analysis of autoradiographs.} All cells containing large portions of a nucleus and cytoplasm were randomly photographed and subjected to point hit analysis\textsuperscript{25} using a grid calibrated for points per 0.25 \(\mu\)m\textsuperscript{2} for determination of cytoplasmic and nuclear areas. Area data were collected for neutrophilic maturational stages—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 grains' direct proximity. 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.\textsuperscript{24,26,27} The level of significance between morphologic groups of cells was determined by Student's \(t\) test with \(p < 0.01\) reported as significant.

\textit{Histochemical localization of glycogen.} The periodic acid–thiosemicarbazide–silver proteinate reaction (PA-TSC-SP) was used for the intracellular localization of glycogen.\textsuperscript{28,29} Bone marrow buffy coats were fixed and embedded in the routine manner as described above. Free-floating tissue 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, & Bell, Norwood, Ohio) in 10\% acetic acid for 18–24 hr. Sections were rinsed with three changes of 10\% acetic acid and then with 5\% and 1\% acetic acid, with a final rinse of distilled water. Sections were then floated on 1\% aqueous silver proteinate (Roboz Surgical Instruments, 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, Rahway, N.J.) in 0.1 M phosphate buffer (pH 7.4) at 37°C for 1 hr prior to exposure to the PA-TSC-SP reaction sequence.

RESULTS

Evaluation of silver grain distribution was done on autoradiographs of 410 cells of the neutrophilic series from normal human bone marrow pulse labelled in vitro with 250 μCi D-glucose-6-3H for 30 min at 37°C in PBSP containing human plasma. Nearly all cells (greater than 90%) in each morphologic category had D-glucose uptake as shown by silver grain localization (Figs. 1 and 2). Background labeling was virtually zero. The number of cells counted, cell areas, and grain densities (grains/μm²) for each cell category are shown in Table 1; cytoplasmic and nuclear densities are also indicated for each cell group. These data indicate that low

![Fig. 1. Silver grain distribution representing typical glucose-3H uptake by developing human neutrophils. P, promyelocyte; M, early metamyelocyte; S, segmented neutrophil. Counterstained with lead citrate and uranyl acetate. × 14,000.](image-url)
levels of d-glucose uptake occurred in cells from the myeloblast through the metamyelocyte stages; the mean ranged from 0.06 to 0.12 grains/μm². 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. Differences in total, cytoplasmic, and nuclear mean grain densities between myeloblast, promyelocyte, and myelocyte stages were not significant \( p > 0.02 \). A significant level \(< 0.005\) was noted in total and cytoplasmic mean grain densities between the myelocyte and metamyelocyte stages. Although not reflected in the data (Table 1), late forms of metamyelocyte commonly had higher grain counts than the younger metamyelocytes (Figs. 2–4). Visual inspection did not indicate preferential labeling of specific cellular components, e.g., Golgi, rough endoplasmic reticulum, mitochondria, and cytoplasmic granules (azurophilic or specific); no evidence of selective labeling of formative cytoplasmic granules was noted. Glycogen was not readily detected in these cell forms in routinely stained preparations until the metamyelocyte stage of development.

**Table 1. Cell Area and Distribution of Silver Grains Over Developing Neutrophils Pulse-Labeled for 30 min in PBSP Containing d-Glucose-6-\(^{3}H\)**

<table>
<thead>
<tr>
<th>Stage of Development</th>
<th>Total Cells</th>
<th>Total Grains</th>
<th>Total Area (μm²)</th>
<th>Total Grain Density (Mean ± SD)</th>
<th>Cytoplasmic Grain Density (Mean ± SD)</th>
<th>Nuclear Grain Density (Mean ± SD)</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. 3. Early neutrophilic metamyelocyte labeled with D-glucose-\(^3\)H. 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. \(\times\) 26,000.

Fig. 4. Late neutrophilic metamyelocyte with more glycogen (g) than shown in Fig. 3 and showing increased glucose incorporation. Counterstained with lead citrate and uranyl acetate. \(\times\) 26,000.

Fig. 5. 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. \(\times\) 13,000.
In contrast to the earlier developmental neutrophils, band and segmented neutrophils (two to four lobes) had extensive labeling (Figs. 5 and 6), 0.89 and 1.44 grains/μm² for band and segmented neutrophils, respectively. Significant differences were found in total, cytoplasmic, and nuclear mean grain densities of the band-segmented neutrophil group and the more immature cell group (p < 0.001), although the nuclear grain density was uniformly much less than cytoplasmic density in the band-segmented neutrophil group. Significant differences (p < 0.001) also were noted in the total and cytoplasmic mean grain densities between the band and segmented neutrophils and in cytoplasmic and nuclear grain densities between the metamyelocytes and band neutrophils. Older, multisegmented neutrophils, in some instances, were found to incorporate less glucose than band and younger (two or three lobes) segmented neutrophils.

Ultrastructurally, cytoplasmic labeling localized primarily over or in close approximation to particulate glycogen (Figs. 7 and 8). Large quantities of glycogen were present in these cells, and many of the glycogen clusters were labeled. In addition, silver grains were found to overlie a number of the cytoplasmic granules (primary, secondary, and tertiary) in these cells; labeled granules were in close apposition to glycogen clusters. Definitive point analyses to determine specifically which of these cell components were labeled were not possible in autoradiographs because of the close proximity of the granules and glycogen particles to each other. However, areas containing glycogen but free of cytoplasmic granules were commonly found to be labeled. Occasionally small cytoplasmic vesicles or short cisternae were found associated with small clusters of glycogen in band and segmented neutrophils as well as in late metamyelocytes; silver grains were commonly found over such areas (Fig. 8). Preferential labeling of the Golgi zone, of formative tertiary granules, and of the plasma membrane was not observed either in the segmented and band neutrophils or in the late form of metamyelocytes, where tertiary granule formation is quite active.

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 was located over the cytoplasm of nucleated erythrocytic cells in the late stages of development. Monocytes and most lymphocytes in the bone marrow had 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. 9). 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 (two or three 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 stage. Glycogen clusters increased in size and number during the final stages of development; both
Fig. 6. Glucose-\textsuperscript{3}H labeling typical segmented neutrophil. Silver grains are localized primarily over the cytoplasmic portions of the cell usually within nongranular areas. Counterstained with lead citrate and uranyl acetate. × 28,000.

Fig. 7. Higher magnification of cytoplasmic area of segmented neutrophil from Fig. 6 labeled with
Fig. 9. Glycogen content of developing human neutrophils as shown by the periodic acid–thiosemicarbazide–silver proteinate (PA-TSC-SP) reaction. Compare the glycogen content of these cells with the glucose uptake by similar cells shown in Fig. 1. P, promyelocyte; M, myelocyte; S, segmented neutrophil. × 13,000.

Glycogen–3H. Silver grains localize over both nongranular regions containing glycogen clusters and over cytoplasmic granules, which are in close proximity to glycogen particles. Counterstained with lead citrate and uranyl acetate. × 70,000.

Fig. 8. Segmented neutrophil demonstrating localization of silver grains over small cytoplasmic vesicles and cisternae (arrows) situated adjacent to small glycogen clusters. Note that in most instances label is some distance from the cytoplasmic granules. Counterstained with lead citrate and uranyl acetate. × 60,000.
clusters and monodispersed particles were present from the myelocyte stage 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 morphologic distinction of these two cell components.

**DISCUSSION**

Our autoradiographic study showed that the amount 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 the 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.

Intracellular localization of glucose-$^3$H in the younger developmental neutrophils, including the promyelocyte and myelocyte, is random with no evidence of preferential labeling of any of the cytoplasmic organelles or granule populations. In contrast, glucose uptake in late maturational stages is heavy and intracellular localization is primarily cytoplasmic and associated with particulate glycogen. D-Glucose-$^3$H did not localize preferentially over either the Golgi region or formative tertiary granules, which are elaborated in the metamyelocyte through early segmented neutrophil stages of development. Only in these late stages, when both cytoplasmic granules and glycogen are abundant, was the glucose label found occasionally over mature forms of granules (primary, secondary, and tertiary). In these instances, particulate glycogen was always found in close association with the labeled granule. It would seem unlikely that fully developed granules in these late neutrophilic cells would have appreciable synthetic capabilities and be able to actively incorporate glucose.

Evidence from many cell systems has shown that intracellular glucose can be utilized not only for the synthesis of glycogen but also in the synthesis of glycolipids, glycoproteins, and glycosaminoglycans.$^{30-34}$ 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 in the Golgi granules and subsequently localized in membrane-bound secretory vesicles and granules.$^{31-34}$ As indicated, this pattern of localization was not observed in developing neutrophils with D-glucose-$^3$H although sulfated glycosaminoglycans are known to be elaborated during two separate phases of neutrophil maturation, e.g., in the promyelocyte stage with the formation of primary granules and in metamyelocyte, band, and young segmented neutrophils with the formation of tertiary granules.$^{19-22}$ Autoradiography, using $^{35}$S-sulfate, has indicated a rapid and preferential incorporation of this label into the Golgi and formative granules during
both primary and tertiary granulogenesis;" myelocytes involved in the synthesis of secondary granules do not incorporate $^{35}$S-sulfate. In a corollary study, we found l-lysine-3H (250 $\mu$Ci l-lysine-4,5-3H in an incubation medium identical to that used for glucose labeling for 30 min) to be heavily incorporated into all developmental forms of granulocytes in the marrow; most cellular components were heavily labeled. The localization of this amino acid within the Golgi and in formative cytoplasmic granules (primary, secondary, and tertiary) in the neutrophilic series shows the viability and active synthetic capacity of the cell system used in our studies. Furthermore, the pattern of uptake and localization obtained with lysine-3H, as well as $^{35}$S-sulfate, is distinctive from that noted with d-glucose-3H. It is also noteworthy that no labeling of the cell surface was noted with glucose in this investigation, although the incubation period was relatively short. We feel our autoradiographic data are consistent with the view that of the glucose taken into developing neutrophils only a limited portion is directed into the pathway for the synthesis of glycosaminoglycan component(s) of these cells while a major portion of the glucose present in the cytoplasm is directed along the glycogen synthetic pathway.

Leukocyte glycogen is derived principally from glucose rather than from products of intermediary metabolism. Glycogen regeneration is rapid; neither anaerobic nor aerobic conditions influence the content or metabolic turnover of leukocyte glycogen provided that glucose is present in the medium. Glucose incorporation is functionally dependent upon the quantity of intracellular glycogen, with glucose being metabolized in greater quantities and more rapidly as the number of glycogen particles increase. Peripheral glycosyl residues of glycogen molecules turn over more rapidly than those of the central core. During glycogen synthesis, glucose combines with uridine disphosphate and is transferred to an acceptor polysaccharide chain (usually glycogen) by glycogen synthetase, which is bound to particulate glycogen; an activator of this enzyme is present in microsomal fractions of hepatocytes. In cells actively synthesizing glycogen, e.g., hepatic and skeletal muscle cells, glucose-3H has been localized to particulate glycogen and to areas of smooth endoplasmic reticulum in proximity to forming glycogen clusters. Similarly, correlated morphologic and biochemical studies of rabbit neutrophils and eosinophils from peritoneal exudates have been found to have short cisternae commonly associated with but not attached to glycogen particles. In the present study, glucose-3H labeling was occasionally found over or near cytoplasmic vesicles or cisternae located near small glycogen clusters in the late developmental neutrophilic cells, which collaborates the relationship of the smooth-surfaced cisternae to particulate glycogen and glycogen turnover in these cells.

From a histochemical standpoint, light microscopy has indicated the initial appearance of glycogen in developing neutrophilic cells to occur in the myelocyte stage; this finding has been supported by routine electron microscopy. In contrast, we have shown ultrastructurally, using the PA-TSC-SP reaction for diastase-labile glycogen, 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 multisegmented stage. Glycogen, in the more
immature cells, is quite difficult to distinguish in routine electron micrographs because particulate glycogen occurs in a monodispersed form rather than as the clusters noted in late maturational stages and because individual glycogen particles have a size and electron density similar to free ribosomes, which are abundant in the immature cells.

Our data showed that glucose incorporation by granulocytes (neutrophils, eosinophils, and basophils) in normal human bone marrow is a function of cell maturity and is closely related to the amount of intracellular glycogen. The glucose label appears to be predominantly associated with cytoplasmic glycogen on the cytoplasmic matrix immediately surrounding particulate glycogen; little, if any glucose is localized either in the granule populations or in association with the cell surface after short-term incubation.

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