

CYTOPLASMIC GRANULE FORMATION IN MYELOCYTES

An Electron Microscope Radioautographic Study on the Mechanism of Formation of Cytoplasmic Granules in Rabbit Heterophilic Myelocytes

MARTHA E. FEDORKO and JAMES G. HIRSCH

With the technical assistance of Carol Dwyer

From The Rockefeller University

ABSTRACT

The intracellular flow of tritiated lysine as revealed by electron microscope radioautography was studied in heterophilic myelocytes of rabbit marrow. Label over the Golgi complex rose to a maximum of 37% of total cytoplasmic grains 30 min after initial exposure to the tracer and fell to 11% after 3 to 4 hr of incubation. Coincident with decrease in label over the Golgi complex, grain counts over granules rose to 32% after 3 to 4 hr. The time sequence of incorporation and flow of tritiated lysine and the per cent distribution of label was similar in bone marrow myelocytes under *in vivo* and *in vitro* conditions. The results demonstrate a function of the Golgi complex in incorporating or packaging certain basic amino acids or proteins into cytoplasmic granules of heterophilic myelocytes.

INTRODUCTION

The cytoplasmic granules of mature polymorphonuclear leucocytes exemplify primary lysosomes, i.e., they are membrane-bounded organelles containing hydrolytic enzymes which apparently have not yet been involved in digestion. Immature white cells in the bone marrow which are actively forming cytoplasmic granules are thus suitable for a study of primary lysosome formation. Recent morphologic observations at the ultrastructural level suggest that the Golgi complex has a definite role in the formation of cytoplasmic granules found in these cells (1, 2). Our study of granule genesis in the rabbit myelocyte was patterned after the study of zymogen granule formation in pancreatic acinar cells by Caro and Palade (3). The purpose of this paper is to report on the mechanism of formation of cytoplasmic granules in rabbit heterophilic myelocytes as reflected by the intracellular flow of an incorporated basic

amino acid observed by electron microscope radioautography.

MATERIALS AND METHODS

Tissue Preparation

IN VITRO STUDIES

Rabbits of either sex, 4 to 5 wk of age and 500 to 600 g in weight were killed by a blow on the head. Femoral red bone marrow was rapidly taken out, dissected, and placed in a "basic" medium of Hanks' solution with bicarbonate containing a final concentration of 10% newborn calf serum (Microbiological Associates, Inc., Bethesda, Maryland). Penicillin was added to this medium to give a final concentration of 3300 units per ml; no other antibiotic was used. Blocks of bone marrow approximately 1 mm³ were placed at the bottom of 25-ml flat-bottomed tubes (2 cm diameter) which contained 2 ml of

medium; pH was adjusted with a mixture of 5% CO₂ and air.

DL-lysine-4,5-H³ (New England Nuclear Corp., Boston, Massachusetts) with a specific activity of 5.0 c/mmole was used as a tracer. Blocks were incubated for 10 min at 38° C in medium containing the tritiated lysine at a concentration of 100 μc/ml. They were then washed on a stainless steel screen by repeated gentle flooding with basic medium containing a chaser of "cold" DL-lysine at a concentration of 100 μg/ml. The blocks were transferred to culture tubes containing basic medium and incubated for various time periods. During the first hour of incubation, bone marrow specimens were removed and fixed at 10-min intervals. Specimens were then fixed at hourly intervals up to 4 hr.

IN VIVO STUDIES

A 5 week-old, 550 g rabbit was anesthetized with ether and intravenously given 10 mc of DL-lysine-4,5-H³ (New England Nuclear Corp.) specific activity, 4.13 c/mmole. A chaser containing 100 mg of cold DL-lysine in 1 ml of isotonic saline was administered intravenously 4 min later. Bone marrow samples were obtained by aspiration with a University of Illinois sternal marrow needle from the distal end of the femur or proximal end of the tibia at 9, 25, 30, and 240 min after the initial injection. The marrow obtained was placed directly into fixative. Ether anesthesia was administered prior to each marrow aspiration.

Fixation of Specimens

The bone marrow was fixed for 1 hr at 4° C in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, made by diluting 50% glutaraldehyde (Fisher Scientific Company, Pittsburgh, Pennsylvania) 1:20 in 0.1 M phosphate buffer. The specimens were then washed twice in cold isotonic saline for 30 min and then postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hr in the cold. Blocks were then washed twice in cold isotonic saline before being dehydrated in graded alcohols and embedding in Epon (4).

Preparation of Specimens for Electron Microscope Radioautography

Sections 0.06 to 0.1 μ thick were cut with a Porter-Blum microtome and placed on Formvar-carbon coated grids. Ilford L-4 Nuclear Research Emulsion was applied according to the method of Caro and van Tubergen, as modified by Dales (5, 6). After a period of 3 to 6 wk' storage at room temperature, the grids were processed in Microdol X (Eastman Kodak Co., Rochester, New York) and Kodak rapid fixer and then stained for 7 min in an aqueous solution of

1% uranyl acetate followed by 2 min in lead citrate (7). Some grids were also floated briefly on a drop of 0.05 M NaOH before being stained with uranyl acetate (8). When dilute NaOH was used before staining, there was no evidence of grain loss or displacement. The background level of grains over grid squares not containing tissue sections was low, averaging less than 5% of the counts over a similar area of a cellular marrow section. Grains located over the extracellular portion of tissue sections accounted for 9% of the total grains in the 9- and 10-min samples, but were less than 5% at later time periods.

Method of Grain Counting

The cells selected for counting were limited to large myelocytes that had suitable areas of the following structures present in the section: Golgi complex, granules, rough endoplasmic reticulum, cytoplasmic matrix, and nucleus. Progranulocytes and metamyelocytes were excluded from the study. Grains located over the nuclear membrane were included with those over the endoplasmic reticulum. The few grains occurring over mitochondria were recorded separately. Frequently, grains were found at the junction of the Golgi complex and the surrounding rough endoplasmic reticulum; since accurate assignment of these grains was not possible, they were allotted equally between the adjacent components in a predetermined alternating pattern. Those grains which were partially over granules were included in the granule count if more than one-half of the grain was located over the granule; otherwise, the grain was counted as label of the cytoplasmic matrix. Counts over the cytoplasmic matrix were combined with those over the rough endoplasmic reticulum, since these two components could not reliably be separated in the cells studied. The per cent of nuclear label, determined for each time interval, was found to remain constant; this was approximately 30% of the total grain count. Analysis was therefore limited to the distribution of grain counts over cytoplasmic structures.

Grain counts were made directly from the image seen on the fluorescent screen of the Siemens Elmiskop I at × 10,000. The grids to be examined were numbered by code, and the count was made by two investigators who had no knowledge of the code. These counts, thus made in "blind" and independent fashion, agreed remarkably well. From bone marrow specimens, 230 to 400 total grain counts were obtained for each time period studied. Three experiments performed under in vitro conditions gave essentially the same results. The dose of label given and the processing techniques employed resulted usually in a range of 2 to 6 grains per labeled cell. This level did not differ detectably at various time periods.

RESULTS

Morphology

The cell type selected for electron microscope radioautographic study was the large heterophilic myelocyte, an example of which is shown in Fig.

1. Typically, the nucleus was large, oval, and eccentrically located. The Golgi complex of these cells was composed of lamellae, clear vesicles, and vacuoles partially filled with electron-opaque material ("nascent granules"). Rough-surfaced endoplasmic reticulum was prominent and widely

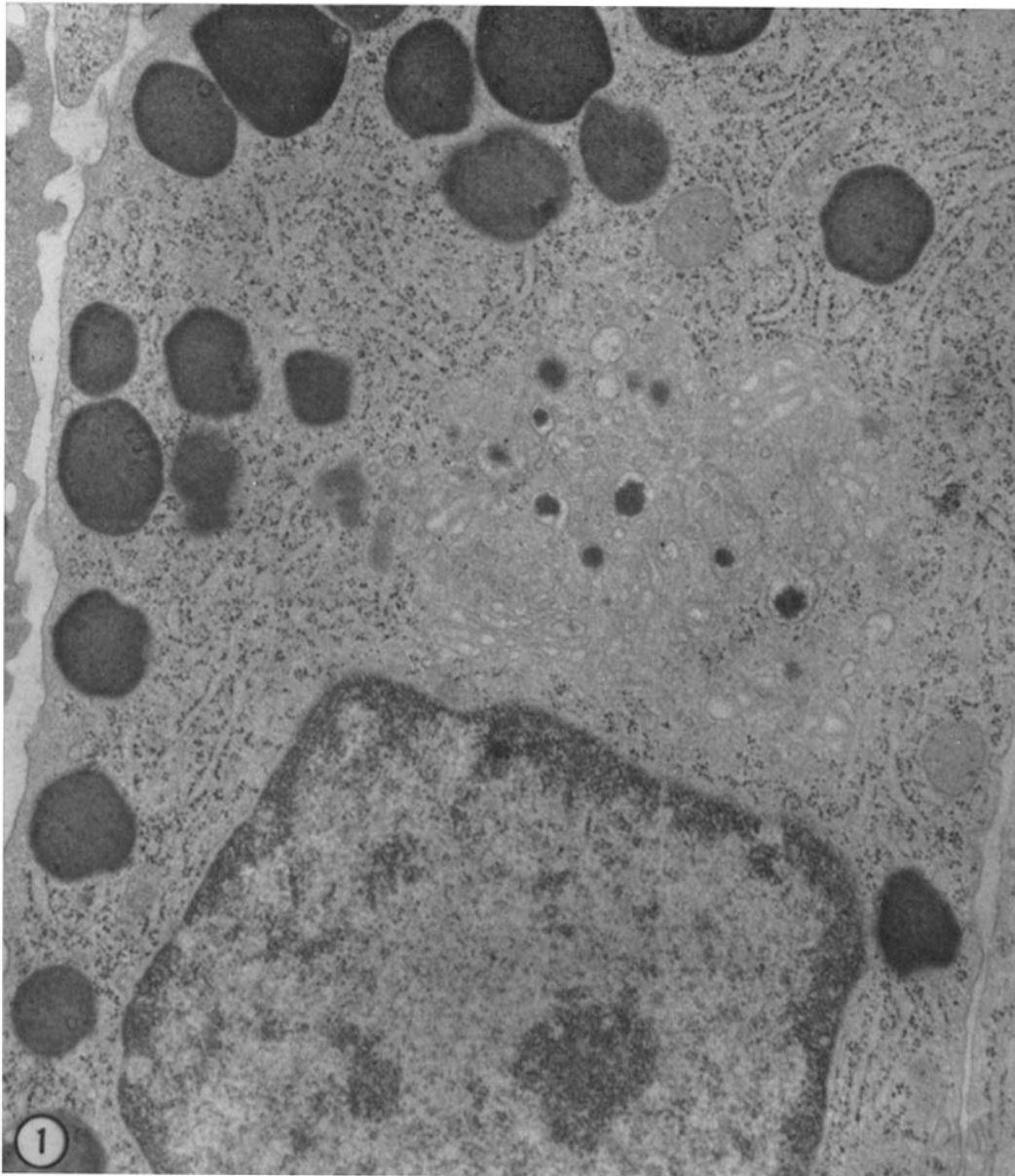


FIGURE 1 Thin section of heterophilic myelocyte found in rabbit bone marrow. Golgi complex, granules, rough endoplasmic reticulum, and nucleus are evident. Nascent granules are seen within the Golgi complex. $\times 26,500$.

distributed in the cytoplasm. Cytoplasmic granules appeared as large dense bodies, homogenous, or, in some instances, composed of a dark core surrounded by a less dense layer.

In Vitro Studies

Cell preservation appeared to be good in specimens of marrow blocks up to approximately 4 to 5 hr of incubation under in vitro conditions. Marrow blocks exposed to pulse label incorporated 25% of the total content of tritiated lysine into the cold 10% trichloroacetic acid-insoluble fraction within 10 min and 60% after 3 hr. Table I shows per cent distribution of cytoplasmic grain counts at time intervals after exposure to tritiated lysine. Seven time periods were used to determine the pattern of flow of label.

There was flow of approximately 30 to 40% of the label incorporated into cytoplasmic structures. Label over the Golgi complex reached a peak of 37% 30 min after exposure to tritiated lysine and fell to 11% within 3 hr. Quantity of label over cytoplasmic granules rose steadily until a value of 32% was reached at 3 hr of incubation. Label over rough endoplasmic reticulum and cytoplasmic matrix was 71% at 10 min, fell to 45 to 55% within 30 min, and was maintained at 54% throughout the remainder of the experiment. The extent of label over the nucleus remained constant at approximately 30% of total grain counts for each time period studied.

In Vivo Studies

There was a possibility that cell metabolism and extent of amino acid incorporation into granules would be somewhat compromised in the later stages of the in vitro experiment. Therefore, the intracellular flow of label was studied within the first 4 hr after a rabbit received an intravenous pulse of tritiated lysine. The technique of bone marrow aspiration in the rabbit was adopted and perfected so that specimens could be obtained from the same rabbit at successive time intervals. The distribution of grain counts over cytoplasmic structures under in vivo conditions is shown in Table II. Four time periods were used to determine the pattern of flow of tritiated lysine.

Under conditions of the experiment, 9 min after intravenous injection of tritiated lysine, the amount of label over the Golgi complex was considerable (25%); Golgi labeling was 31% at 30 min and subsequently fell to 14% at 4 hr. Label

TABLE I
Labeling of Cytoplasmic Structures in Rabbit Myelocytes Incubated in Vitro in Medium Containing Tritiated Lysine

Cytoplasmic structures	Per cent distribution of grain counts Minutes after initial exposure to tritiated lysine						
	10	20	30	40	50	120	180
Golgi complex	11	22	37	26	17	16	11
Granules	17	18	18	21	27	29	32
Cytoplasmic matrix + RER*	71	58	45	53	54	54	56
Mitochondria	1	2			2	1	1

* RER, rough-surfaced endoplasmic reticulum.

TABLE II
Labeling of Cytoplasmic Structures in Rabbit Myelocytes after Intravenous Tritiated Lysine

Cytoplasmic structures	Per cent distribution of grain counts Minutes after tritiated lysine injection			
	9	25	30	240
Golgi complex	25	29	31	14
Granules	5	13	14	30
Cytoplasmic matrix + RER*	69	58	55	55
Mitochondria		1		1

* RER, rough-surfaced endoplasmic reticulum.

over granules rose progressively to 30% at 4 hr. The extent of label over rough endoplasmic reticulum and cytoplasmic matrix fell from the initial value of 69% at 9 min to 55% by 30 min, and was maintained at this level until the termination of the experiment. Counts at 25 and 30 min were essentially the same, which indicated the reliability of the sampling and counting techniques.

Figs. 2, 3, and 4 illustrate radioautographic labeling of various subcellular structures in heterophilic myelocytes at 10, 30, and 240 min after exposure to label.

Comparison of in Vitro and in Vivo Studies

Data obtained under in vivo and in vitro conditions were similar in the following respects: there was flow of 30 to 40% of cytoplasmic label;

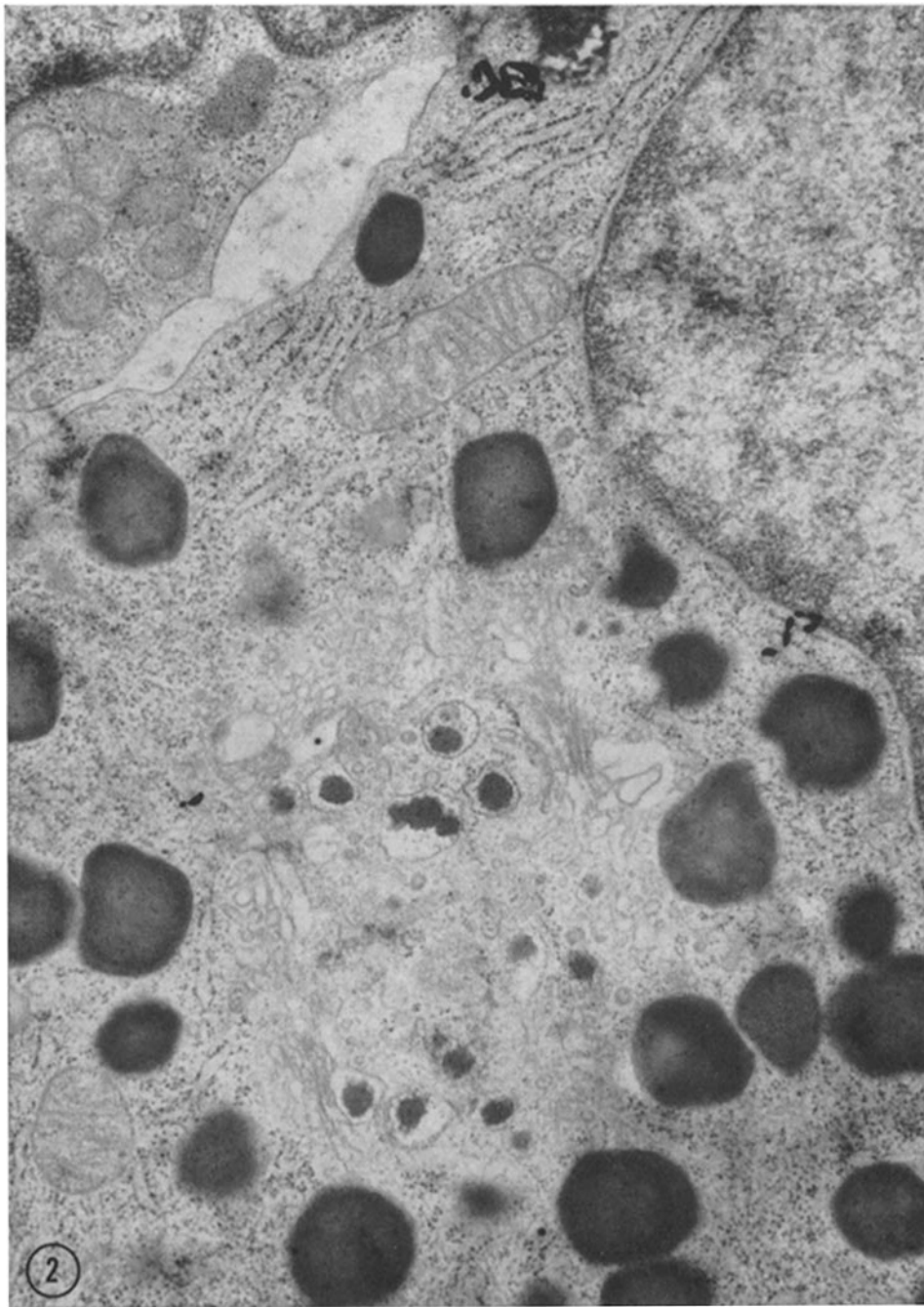


FIGURE 2 Electron microscope radioautograph of a rabbit myelocyte 10 min after exposure to tritiated lysine. Grains are present over the rough endoplasmic reticulum, nuclear membrane, and at the edge of the Golgi zone. $\times 30,000$.

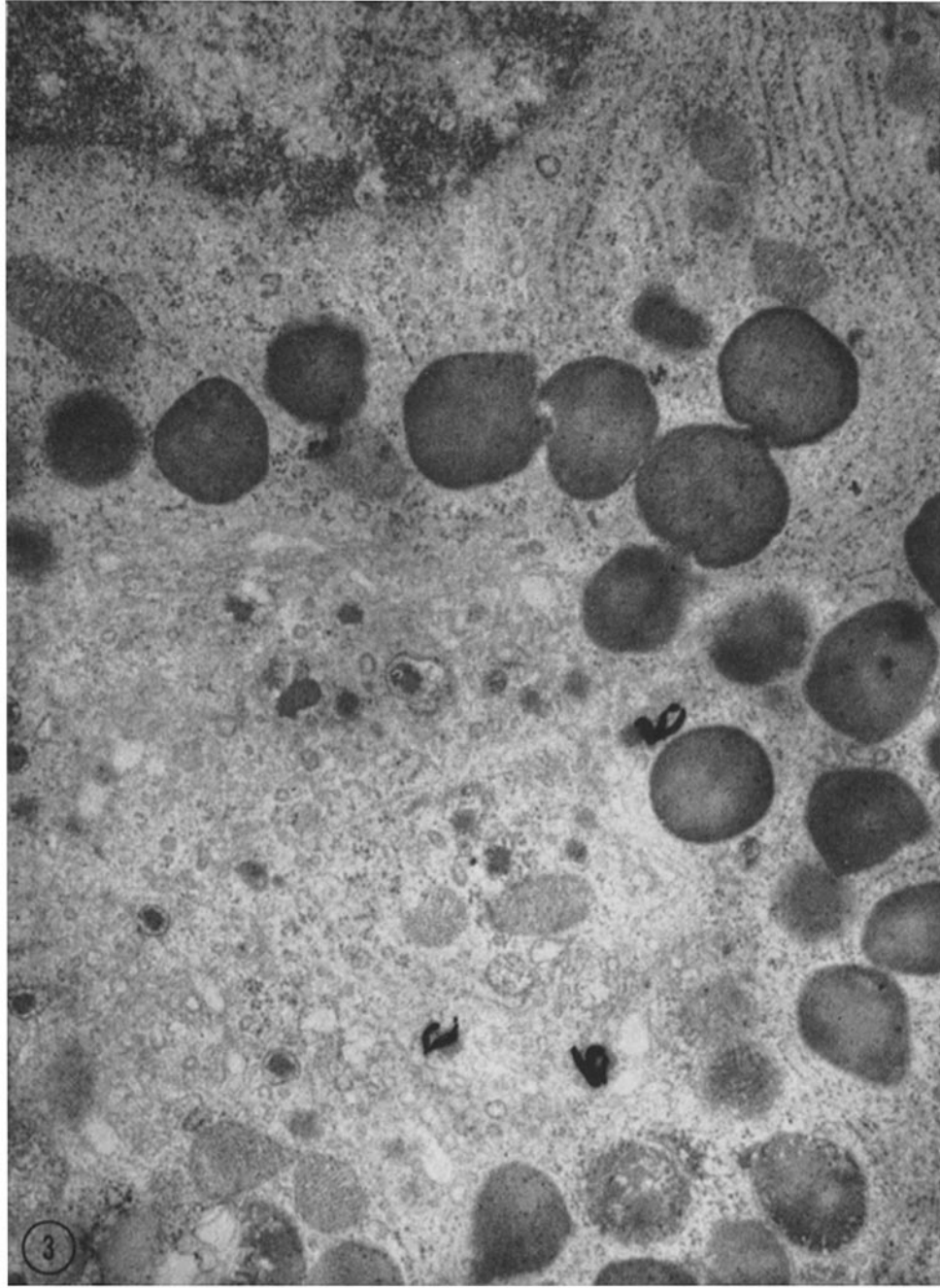


FIGURE 3 Electron microscope radioautograph of a rabbit myelocyte 30 min after exposure to tritiated lysine. Three grains are evident at the periphery of the Golgi complex; each appears to be associated with a nascent granule. $\times 30,000$.

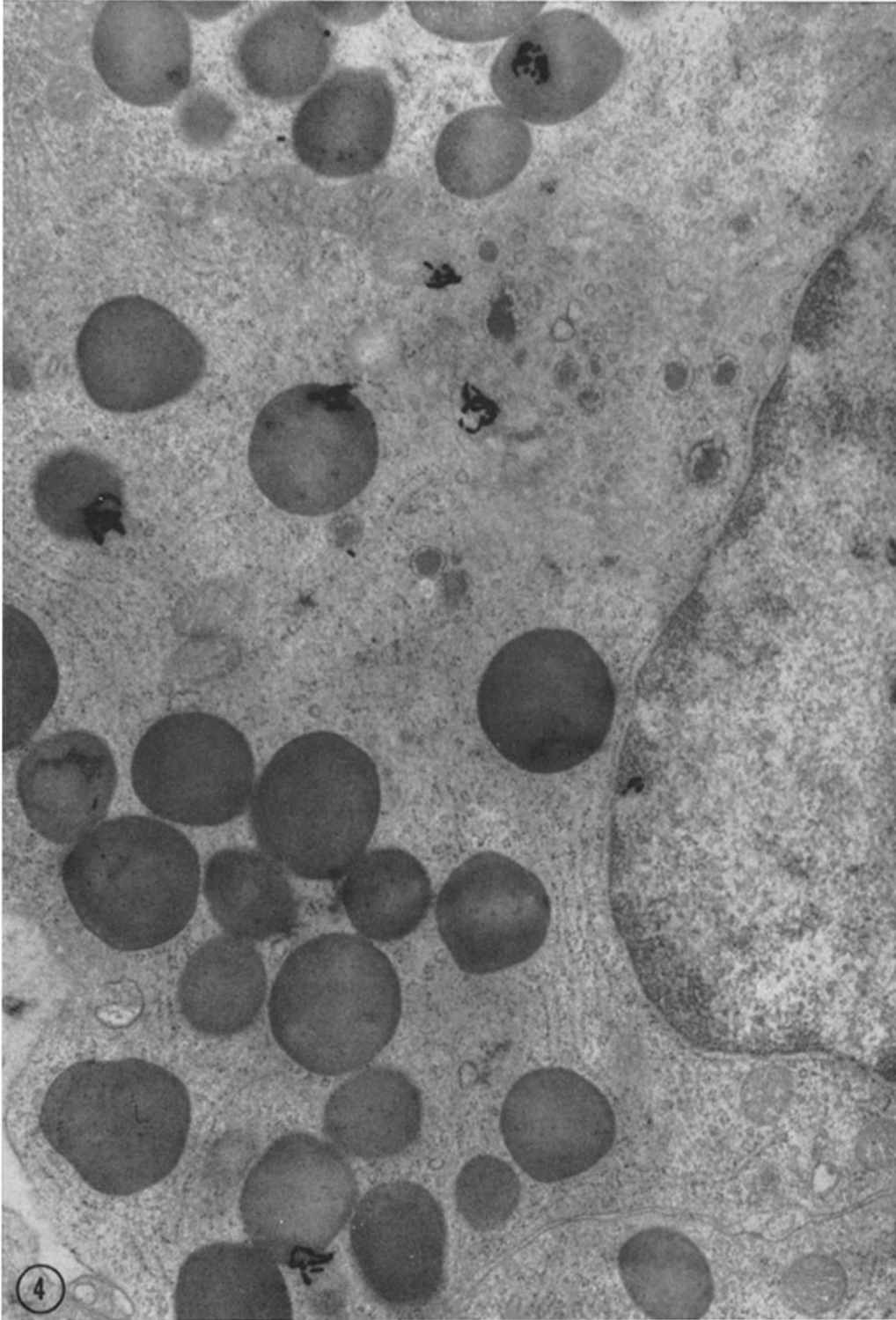


FIGURE 4 Electron microscope radioautograph of a rabbit myelocyte 240 min after exposure to tritiated lysine. Four granules are labeled, and, in addition, grains are seen over the Golgi complex, a mitochondrion, and the nucleus. $\times 28,000$.

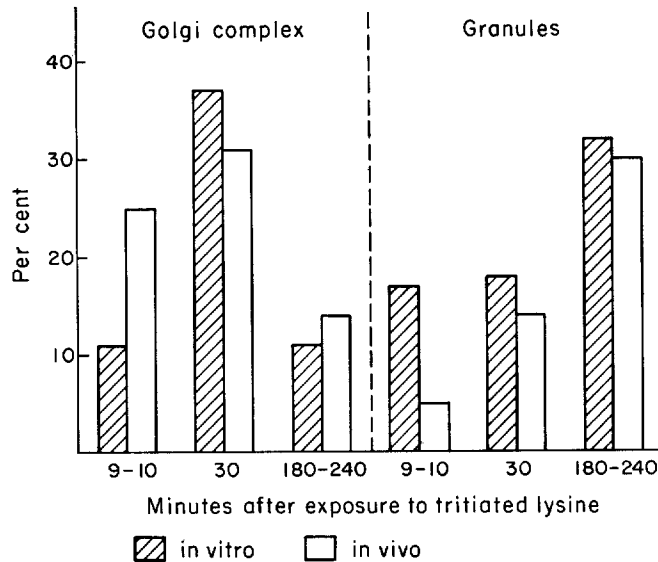


FIGURE 5 Per cent of label over Golgi complex and granules in rabbit myelocytes under in vitro and in vivo conditions.

the Golgi complex had a high quantity of label at 30 min; and approximately 3 hr were required for 30 to 35% of the label to be evacuated from the Golgi complex and to be incorporated into cytoplasmic granules. Fig. 5 summarizes the results in a histogram which illustrates apparent flow of label through the Golgi complex into granules under both conditions and at the three critical time periods. The only significant differences between results obtained in vivo and in vitro were seen at the earliest sampling time, when a higher value was obtained for the granules in vitro than in vivo, and a higher value was obtained for the Golgi complex in vivo than in vitro.

DISCUSSION AND INTERPRETATION

Little is known about the mechanism of formation of cytoplasmic granules in rabbit heterophilic myelocytes. The studies reported here demonstrate that, after a pulse label, there is flow of cytoplasmic tritiated lysine through the Golgi complex before incorporation into the cytoplasmic granules of the myelocyte.

Rabbit myelocytes served quite well as subjects for an electron microscope radioautographic study of cytoplasmic granule formation. Content and structure of the large myelocytes was such that, for purposes of grain counting, the cell could be divided into 4 major compartments: Golgi complex, granules, cytoplasmic matrix including rough endoplasmic reticulum, and nucleus. Each

of these compartments occupied roughly an equal area in selected thin sections of myelocytes, in contrast with other stages of heterophil maturation. Progranulocytes have a larger nucleus, small Golgi complex, and fewer granules, whereas metamyelocytes have a lobulated nucleus, small Golgi complex, and few scattered strands of rough endoplasmic reticulum.

The myelocyte represents a stage in the maturation from stem cell to mature polymorphonuclear leukocyte. Thus, the possibility existed that some of the cells counted at later time periods had derived from cells labeled in the promyelocyte stage, or that some of the labeled myelocytes differentiated during the incubation period into metamyelocyte forms not included in the counts. Studies on the rate of maturation of mammalian myelocytes indicate, however, that the compartment time for this stage is sufficiently long so that a great majority of the cells included in the counts were, in fact, in the same phase of development during the relatively short 3 to 4 hr time period of our experiments (9). Furthermore, only a small proportion of the myelocytes would be expected to have undergone mitosis in 4 hr (10). During later time periods of our experiments, there was no evidence of dilution of intensity of label.

Recently perfected methods for electron microscope radioautography made this study feasible. There are certain inaccuracies inherent in these methods when they are applied to these cells.

Counts over the cytoplasmic matrix and the rough endoplasmic reticulum had to be combined, because the rough endoplasmic reticulum was distributed so extensively in the cytoplasm, and resolution was inadequate to distinguish between the reticulum and adjacent matrix. It was also impossible to determine the exact localization of label over the transitional zone between the Golgi complex and the rough endoplasmic reticulum. For this reason, counts over this transitional zone were divided equally between the two adjacent structures. Transitional zone labeling was particularly high during peak Golgi complex labeling and was probably associated with the Golgi complex or perhaps indicated an avenue of entry into the Golgi complex. To clarify this point, grain counts were performed on samples of marrow obtained 25 and 30 min after exposure to a pulse label *in vivo*; in both instances, transitional zone grains accounted for 30% of the grains found over the combined Golgi and transitional zones.

Grain count distributions for bone marrow specimens, aspirated 25 and 30 min after administering tracer intravenously, were very similar. Furthermore, there was agreement in the results obtained for three experiments in which marrow was incubated under standardized *in vitro* conditions. These data thus demonstrate reproducibility of techniques used in marrow sampling, incubation of marrow, tissue processing, and counting of label.

Borsook and others have demonstrated that amino acids, including lysine, are rapidly removed from the blood stream within 15 min after intravenous administration and are incorporated into tissue proteins (11). It has been shown that granules of polymorphonuclear leukocytes contain basic protein (12). Tritiated lysine was therefore selected as an appropriate tracer for studying the origin of cytoplasmic granules of the myelocyte.

Under *in vitro* conditions, approximately 25% of the tritiated lysine incorporated into cytoplasmic structures was transported from the rough endoplasmic reticulum and cytoplasmic matrix to the Golgi complex within the first 30 min after a pulse label. The transported amino acid might have been absorbed on protein or might have represented transport of protein synthesized in the rough endoplasmic reticulum. Current views on protein synthesis would tend to favor the latter alternative (13). After 3 hr of incubation, tritiated lysine incorporation into the trichloroacetic acid-

insoluble proteins of the bone marrow block rose to 60% of the total tissue content of tracer. Trichloroacetic acid fractionation of the incubated marrow block and liquid scintillation counting of the fractions was, however, of limited value in relation to the radioautographic results, since the population of myelocytes formed only a small proportion of the cells present.

The quantity of label over the rough endoplasmic reticulum and cytoplasmic matrix fell to 45 to 55% within 30 min; this level was maintained throughout the remainder of the experiment. Persistently high labeling of the rough endoplasmic reticulum and cytoplasmic matrix at later time intervals in these myelocytes differs from the progressive fall in label over the rough endoplasmic reticulum in pancreatic acinar cells (3). The peak label in the Golgi region and the disappearance of extracellular label eliminates the possibility of attributing persistence in cytoplasmic label to continued uptake of the labeled amino acid precursor. The remaining cytoplasmic label in myelocytes could be incorporated into granules at a later stage in maturation. On the other hand, myelocytes, in contrast to pancreatic acinar cells, are undergoing cell division; thus, synthesis of new cytoplasmic structural protein may well explain the high level of cytoplasmic labeling.

Previous studies have demonstrated a role for the Golgi complex in the formation of secretory granules or proteins in the pancreas, mammary gland, thyroid, and fibroblast (3, 14-16). In our investigation, it appeared that grains in the Golgi complex at the 30 min time period were commonly located at the periphery, and that they frequently were associated with vacuolar structures containing dense material. We termed such structures "nascent granules" (see Fig. 3). Although these findings differ in some respects from those of Bainton and Farquhar, they do support the general concept that there may be specialization of function within the Golgi complex (1). In any event, the function of at least part of the Golgi complex in the formation of leukocyte granules seems clear.

The incorporation of basic amino acids into the cytoplasmic granules of polymorphonuclear leukocytes may not, of course, be restricted solely to the myelocyte stage we studied. The role of the Golgi complex in other stages of heterophil maturation and in other cell types of the bone marrow still has to be clarified.

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