THE USE OF ^{55}FE IN HIGH-RESOLUTION RADIOAUTOGRAPHY OF DEVELOPING RED CELLS

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INTRODUCTION

The radioisotope ^{59}Fe is used extensively for biochemical investigations of hemoglobin synthesis in maturing red cells. Its usefulness for radioautography is more limited, largely because high-energy beta particles emitted by ^{59}Fe exhibit a long path and, therefore, produce scattered patterns of reduced silver grains in photographic emulsion overlying labeled cells.

Another isotope, however, low-energy emitting ^{55}Fe, would appear to be more favorable for radioautographic work on the basis of theoretical considerations (Forberg et al., 1964) and early results from light microscopic radioautography (Fowler et al., 1966). The former paper indicates that ^{55}Fe disintegrations produce three physical classes of emissions which include röntgen radiation, mainly X-rays, with an energy of 6 kev (0.19/d[1]) and Auger electrons of either 5–6.5 kev (0.5/d) or 0.5–0.6 kev (1.2/d).

The present paper demonstrates that particles originating in ^{55}Fe foci are sufficiently localized to permit high-resolution radioautographic analysis of red cells viewed in thin sections. This localization is shown first with the use of remnants of hemolyzed reticulocytes as structurally defined sources of ^{55}Fe label and, in a second aspect of the work, by the examination of immature erythroid cells in splenic red pulp.

[1] d = disintegration.

MATERIALS AND METHODS

Labeled RBC Ghosts

An adult male rabbit was injected subcutaneously with 25 mg phenylhydrazine hydrochloride in 2.5 ml saline daily for 8 days. This treatment produced marked reticulocytosis (Borsook et al., 1952). 12 hr after the last injection, 2–3 ml of blood were collected in a heparinized tube, and 1.0 ml distilled water was added. Some cells hemolyzed to form ghosts. The blood was then centrifuged three times in saline at 1000 *g* for 5 min, and the supernatant was discarded.

1 ml saline containing 15 μg rabbit transferrin (lot No. R2598, Mann Research Laboratories, Inc., New York) was incubated in a shaker at 37°C for 30 min with 50 μl ^{55}Fe (Chloride, carrier-free, <0.1 mg/ml, 2.4 mc/ml; New England Nuclear Corp., Boston, Mass.). A volume of 0.2 ml packed red cells was added to the transferrin-^{55}Fe solution, and the cells were resuspended and incubated for 2 min. Then 0.3 ml of this cell suspension was fixed in 10 ml of 5% paraformaldehyde-glutaraldehyde (Karnovsky, 1965) for 30 min, rinsed in 0.2 M cacodylate buffer, and postfixed for 30 min in 1% OsO_4. For each change the cells were pelleted at 2000 *g* for 5 min. They were then run through alcohol and propylene oxide solutions and embedded in Epon.

Labeled Spleen

Adult Swiss mice were sacrificed, and their blood was collected in a heparinized beaker. 1 ml of packed red cells, spun three times in saline at 1000 *g* for 5 min, was then administered intraperitoneally to an adult mouse, and the procedure was repeated the following day. This treatment elevated the hematocrit value and suppressed erythropoiesis. 4 days later the animal was given an intraperitoneal injection of 6 units of step III sheep plasma erythropoietin[2] Armour Pharmaceutical Co., Kankakee, Illinois, lot k147137; preparation supplied by the National Heart Institute, United States Public Health Service) to induce a wave of red cell maturation. 30 hr later the mouse was injected with 55 μc ^{55}Fe (CEA,[3] Citrate, Lot No. Fe-55-5-2, specific activity 2.33 mc/mg). After an 18-hr period of iron incorporation, the spleen was fixed in 3% glutaraldehyde for 1 hr, rinsed in phosphate buffer, and postfixed in 1% OsO_4.

Thick sections mounted on glass slides were dipped in Ilford K5 emulsion diluted in 8 volumes distilled water, and thin sections of both reticulocytes and spleen were coated with Ilford L4 emulsion (Caro, 1961). All were exposed for 3–6 wk.

RESULTS AND DISCUSSION

Reticulocyte ghosts (Figs. 1–4) consist of the cell membrane and a small amount of adhering cytoplasm but are devoid of other organelles. The cytoplasmic remnants probably correspond to that component which remains in place along the membrane in centrifuged intact reticulocytes instead of becoming "stratified" with the ribosomes and mitochondria (Beams and Kessel, 1966). In addition to the cell membrane (hereafter the traces of cytoplasm will be considered as part of the membrane), aggregates of dense material are frequently encountered within the otherwise empty ghosts. This material is most likely the remains of Heinz bodies which are known to form in red cells after treatment with phenylhydrazine (Jandl and Allen, 1960; Rifkind and Danon, 1965), and it may include portions of the membrane.

Although some areas of each ghost are sectioned obliquely, in most regions the plane of section is perpendicular to the membrane which together with the adhering cytoplasm is generally less than 0.1 μ thick, this is an important feature for our consideration of ^{55}Fe radioautographic resolution. The mean diameter of silver halide crystals in Ilford L4 emulsion is 0.14 μ, hence a given crystal might span the entire width of the underlying structure. To demonstrate high resolution with transferrin-^{55}Fe, we hoped to find at least a portion of each reduced silver grain lying directly over the membrane. Nearly all grains fulfilled this requirement.

We photographed and counted a total of 289 grains over 20 labeled red cell ghosts (Table I) and found 244 or 84% of the grains over the membrane. This result indicates a resolution of the order of 0.1 μ. The same degree of localization was obtained in a second count when 100 labeled cells were viewed in the microscope. Of 1138 grains located within 2–3 μ of the ghosts, 1012 or 89% were found over the membrane. Since radioactive ^{55}Fe is essentially a monoenergetic beta emitter, the 0.5–0.6 kev particles probably do not reach the photographic emulsion, these highly resolved grains would seem to be the result of interacting Auger electrons of 5–6.5 kev. This resolution agrees well with the 0.1 μ resolution obtained with tritium (Caro, 1962), a beta emitter which yields particles having a mean energy of 5.5 kev.

The remaining 45 grains or 16% occurred in the area between the membrane and at a distance of 0.5 μ, and of these grains 32 were located within 0.2 μ. It is highly probable that many or all of the latter were reduced by beta particles originating at transferrin-^{55}Fe receptor sites on the cell membrane. Such a view is tenable if we assume that an interacting particle entering a silver halide crystal can produce a latent image in any portion of the crystal and that during development the silver grain can grow away from the radioactive source. These factors, as indicated in an earlier study (Bachmann and Salpeter, 1965), contribute to the technical problems that result in poorly resolved grains in electron microscopic radioautography. That is to say, an electron traveling a path perpendicular to the emulsion can reduce a silver halide crystal directly over the membrane and still suffer a loss in resolution as high as 0.14 μ if, as in the present study, the portion of the grain nearest the labeled membrane is considered the site of latent image formation. Alternatively, poorly resolved grains can be produced by emissions that do not travel a path perpendicular to the emulsion, and hence they penetrate and produce latent images in crystals adjacent to those directly overlying the labeled site (Caro, 1962), in this instance red cell membranes.

[2] Kindly provided by Dr. Albert S. Gordon.

[3] Obtained from the Commissariat á L'Énergie Atomique, France.

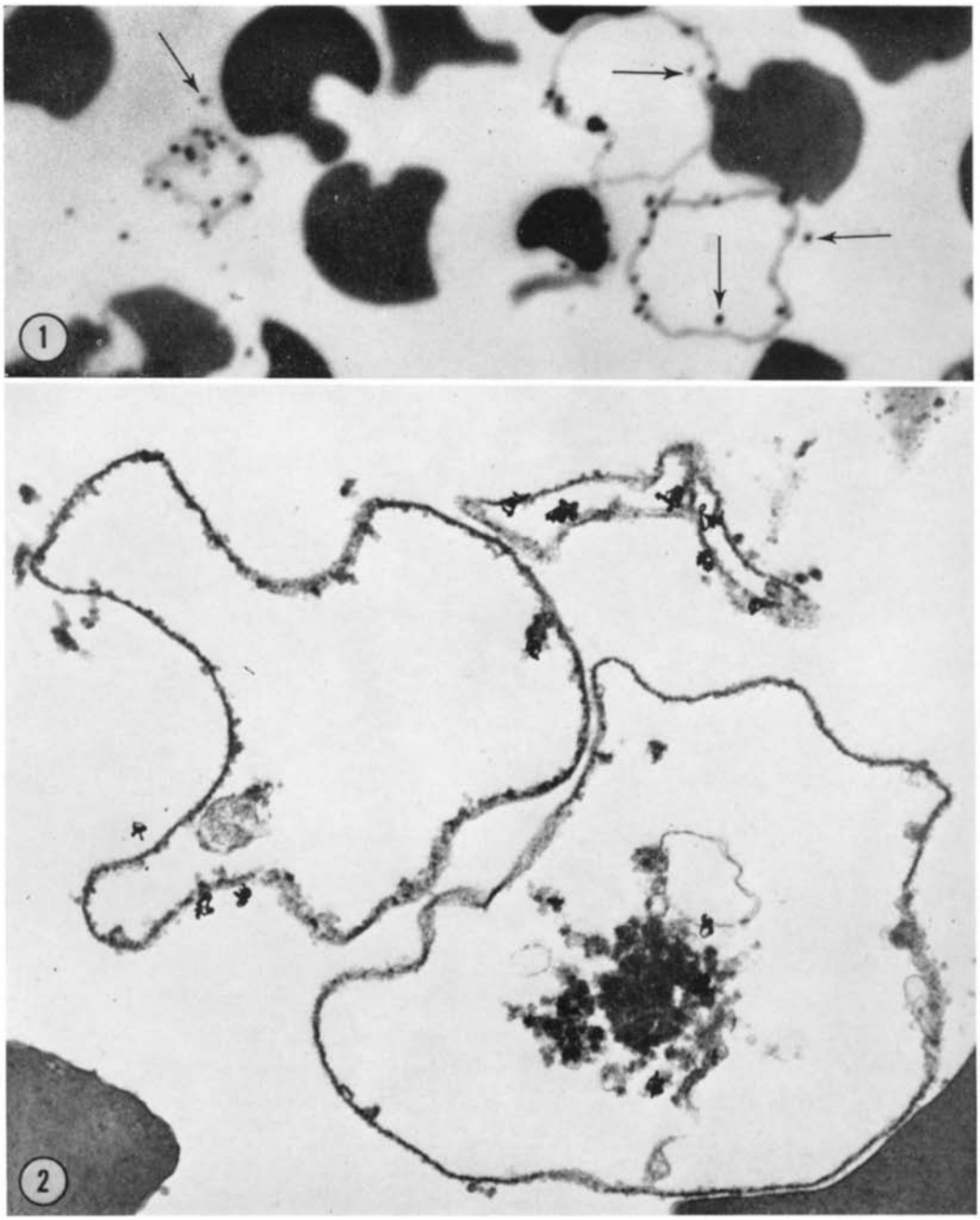
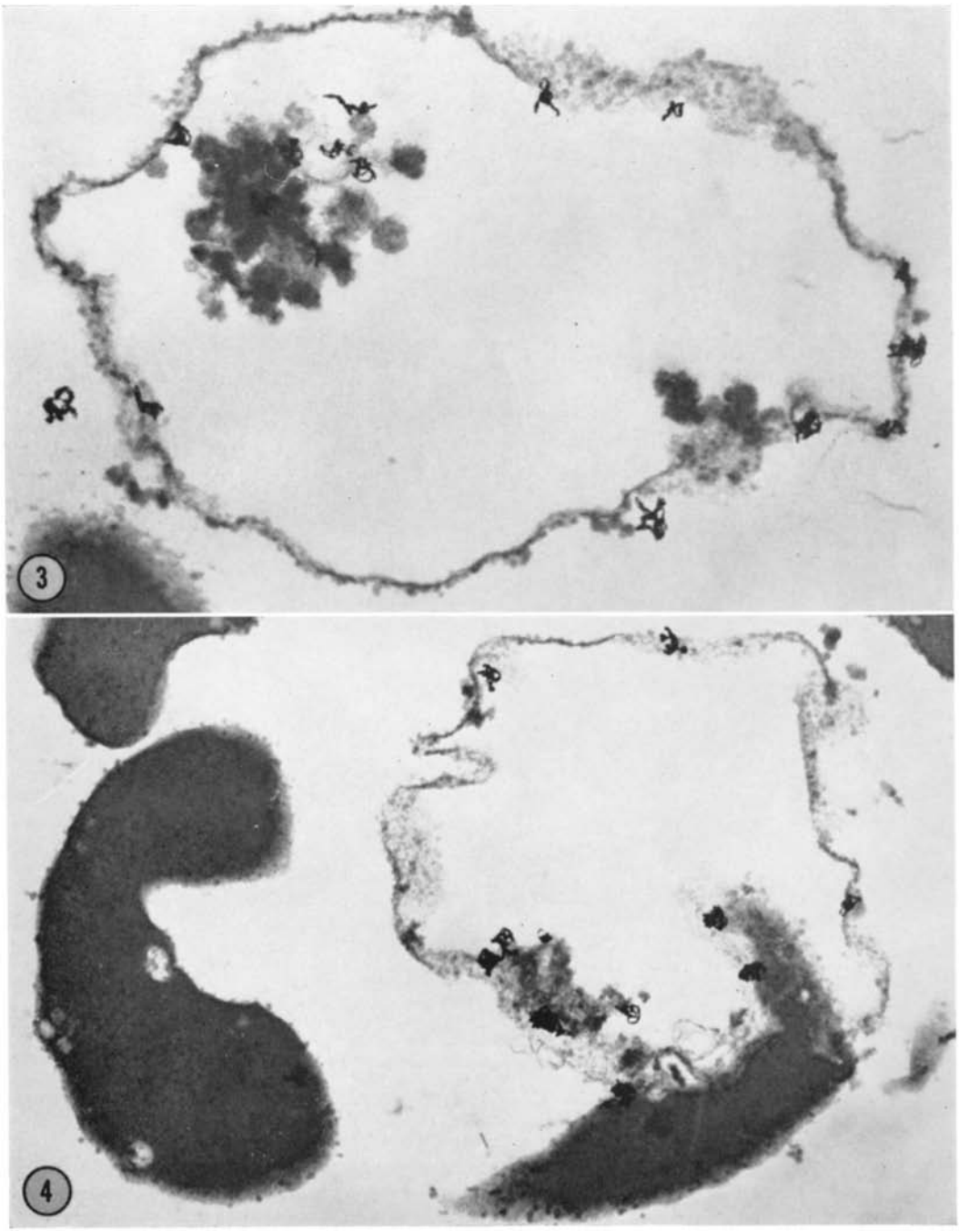


FIGURE 1 Light microscope radioautograph of three rabbit red cell ghosts after incubation with transferrin-^{55}Fe for 2 min. Nearly all silver grains lie directly over the ghosts. Arrows indicate several poorly resolved grains. Those grains seen in the left margin of the micrograph are probably due to nonspecific background reduction. × 2,300.

FIGURE 2 Electron micrograph of three rabbit red cell ghosts. Two ghosts are clearly labeled, but the third shows grains only over the dense inner material thought to be Heinz bodies combined with portions of the cell membrane. The latter cell may be the remains of an erythrocyte no longer capable of binding large numbers of transferrin-^{55}Fe molecules. × 13,400.



FIGURES 3 and 4 Rabbit reticulocyte ghosts labeled with ^{55}Fe. All except one grain (left margin of Fig. 3) fall over the membrane. Fig. 3, × 17,800; and Fig. 4, × 14,400.

TABLE I

Distribution of Silver Grains over the Membrane and Surrounding Areas of ^{55}Fe-Labeled Red Cell Ghosts Seen in Electron Microscope Radioautographs

Grains located over the membrane	Grains located at a distance from the membrane						
	No.	*0.05 μ*	*0.1 μ*	*0.2 μ*	*0.3 μ*	*0.4 μ*	*0.5 μ*
	9						
	19			1	2		
	6		2	1			
	5		2				
	3		1				
	26		1	1			
	11			1	1		
	6						
	3		1				
	12			2			
	4			1			
	27			2	1		
	7	1					
	7			1			
	24		2	1	1	2	
	9	1			1		1
	8		1	3			
	26	2	2	1		2	
	3		1				
	29				2		
Totals	244	4	13	15	8	4	1
Per cent	84.43%	1.38%	4.50%	5.19%	2.77%	1.38%	0.35%

A total of 20 labeled red cell ghosts were photographed and examined for grain distribution. 84% of all grains were localized over at least a portion of the cell membrane and its adhering cytoplasm. For the remaining 16%, localization was determined on the basis of the distance from the ghost to the nearest part of the silver grain. In addition to the above totals, we counted 100 labeled red cell ghosts in the electron microscope, without photographing them, and found 1138 grains located within 2–3 μ of the ghosts. Of these, 1012 or 89% were seen directly over the membrane.

Finally, although we cannot conclude with certainty that grains located between 0.3 and 0.5 μ from the ghost are not the result of Auger electron interactions, these grains are more likely due to nonspecific background reduction or ^{55}Fe emitted X-rays. Most X-rays of course would not be expected to interact with the silver halide crystals in relatively nonsensitive Ilford L4 emulsion, but the possibility that some are effective can not be categorically dismissed since X-ray backscatter might lead to the formation of latent images at distances farther than 0.2 μ from the ghost.

According to the above considerations, transferrin binding apparently can occur at membrane receptors independently of cellular integrity or demand for iron, but it should be noted that adsorption of "free" ^{55}Fe or transferrin-^{55}Fe might occur under these experimental conditions. The fact that some ghosts are not labeled, however, argues against the latter possibility and supports the view that binding is selectively occurring at transferrin receptors. These unlabeled ghosts are thought to be the remains of mature erythrocytes which are known to retain only a small fraction of the transferrin binding capacity of reticulocytes (Jandl and Katz, 1963), a fraction perhaps too small to provide the quantities of label needed for radioautography.

Spleens of adult mice are organs of considerable erythropoietic activity. Experimentally induced

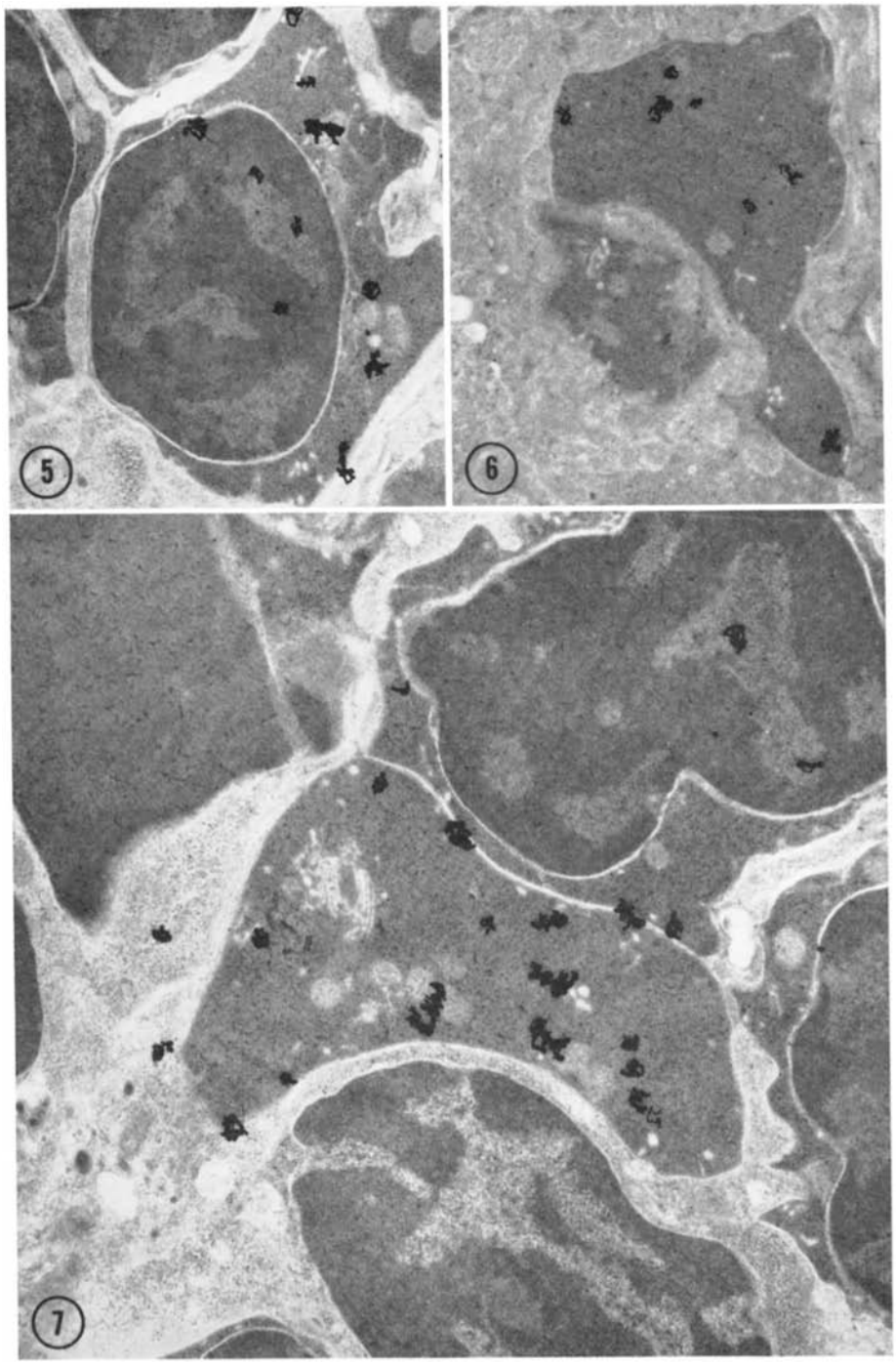


FIGURE 5 An erythroblast in mouse spleen with ^{55}Fe label in both its cytoplasm and nucleus. The nuclear grains lie over chromatin masses as well as over interchromatin material. This maturing cell, like those in Figs. 6 and 7, was presented with ^{55}Fe for 18 hr before fixation. × 9,000.

FIGURE 6 A late erythroblast or reticulocyte in mouse spleen showing several foci of ^{55}Fe. × 8,000.

FIGURE 7 Erythroid elements at different stages of maturation are seen in this area. The late erythroblast or reticulocyte in the center is labeled with ^{55}Fe, as is one of the erythroblasts (two silver grains are seen in the large interchromatin region of its highly condensed nucleus). The mature erythrocyte in the upper left corner is not labeled. × 11,500.

polycythemia, however, suppresses this function, so that one can initiate a well-defined wave of red cell maturation with a single injection of exogenous erythropoietin. The activated stem cells give rise during the first day to proerythroblasts which are replaced by erythroblasts and reticulocytes at 48 hr; and finally 24 hr later most of the population consists of reticulocytes (Gurney et al., 1961; Orlic et al., 1965). Iron is generally considered to be incorporated throughout maturation but most avidly by late erythroblasts.

^{55}Iron was injected at a time corresponding to the middle portion of the wave of experimentally-induced red cell maturation, 30 hr after erythropoietin administration, and was found in maturing red cells 18 hr later (Figs. 5–7). Both cytoplasm and nuclei become labeled, and the nuclear grains appear over chromatin masses as well as interchromatin regions (Fig. 5). Since these dense chromatin masses are large in late erythroblasts, it is likely that overlying silver grains are due to ^{55}Fe disintegrations within these masses, and that labeled hemoglobin is dispersed throughout the entire nucleus. The presence of nuclear label agrees with an earlier ultrastructural report which indicates that hemoglobin occurs within this organelle in nucleated erythrocytes (Davies, 1961) and also supports data that suggest the presence of hemoglobin within condensed chromatin (Tooze and Davies, 1963).

Some late erythroid elements derived from stem cells differentiated by the action of erythropoietin appear to be more heavily labeled than others (Fig. 7). This apparent difference could be the result of a more avid ^{55}Fe incorporation in a fraction of the maturing red cells at the time of isotope administration, or may be due to a higher rate of accumulation during the 18-hr period after ^{55}Fe administration. It should be noted, however, that the number of grains per cell can be meaningful only if related to total cytoplasmic area included in the section. On this basis, a cell displaying large areas of cytoplasm such as the one seen in the center of Fig. 7, a late erythroblast or reticulocyte, would be expected to show higher grain counts than erythroblasts sectioned through the nuclear region.

No labeled erythrocytes, identified as cells devoid of all cytoplasmic organelles, were found in these preparations through splenic red pulp. This finding suggests that developing erythroid cells incorporating ^{55}Fe made available 30–48 hr after the initiation of a wave of erythropoietin-induced red cell formation do not reach full maturation as erythrocytes within this period of time.

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Note Added in Proof:

Since the present paper was submitted, there appeared an article in which it was proposed that the radioisotope ^{59}Fe could be used for electron microscope radioautography. This article "Localization of iron and leghaemoglobin in the legume root nodule by electron microscope autoradiography" by M. J. Dilworth and D. K. Kidby appeared in *Expt. Cell Res.* **49**:148–159 (1968).

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