# Fine Structural and Cytochemical Identification of Microperoxisomes in Developing Human Erythrocytic Cells

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**An alkaline diaminobenzidine (DAB) medium has been used to identify peroxidase activity in small granules (0.09 to 0.2 μ in diameter) present in all forms of maturing erythrocytic cells with the exception of erythrocytes. These granules, which were more frequent in proerythroblasts (from two to seven by thin section), were distinct from pleomorphic granules present in the close proximity to the Golgi apparatus. They were also distinct from ferritin molecules which were seen as aggregates in siderosomes of polychromatophilic erythroblasts. They often appeared in close association with the smooth membrane of the nuclear envelope. Optimal conditions for the visualization of these granules by incubation in alkaline DAB were obtained when the peroxidase activity of hemoglobin was reduced by addition of low concentrations of potassium cyanide. Lack of hydrogen peroxide in the incubation media completely inhibited the staining reaction of hemoglobin, while the positive reaction persisted in the granules. Aminotriazole in the incubation media prevented the staining of these organelles. These findings suggest that small granules seen in maturing erythroblasts contain catalase and that they correspond to microperoxisomes described in other tissues. The mechanism of their disappearance during reticulocyte maturation is unknown. The relationship between particulate catalase of erythroblasts and soluble erythrocytic catalase has not been elucidated. (Am J Pathol 79:523–536, 1975)**

THE TERM PEROXISOME is a biochemical concept formulated by de Duve *et al.*[1] Thus, in a number of cell types, catalase and hydrogen-peroxide-generating oxidases have been found associated with sedimentable particles. The peroxisomal catalase has been visualized cytochemically [2,3] by modifications of the 3,3′-diaminobenzidine (DAB) technic of Graham and Karnovsky [4] in small organelles with a nucleoid (microbodies) from a wide variety of tissues. Incubation of fixed cells in DAB medium, at a pH higher than that used for staining peroxisomes, facilitates identification of other smaller peroxisome-like particles which stain positively for catalase.[5–9] Their small size, lack of a nucleoid, and close association with smooth endoplasmic reticulum (SER) have led Novikoff and Novikoff to introduce the term "microperoxisome." [5,10] Recent studies suggest a more general distribution of microperoxisomes [7,8] than previously realized.

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The cytochemical reaction with alkaline DAB which has been employed by many investigators, however, is not specific for catalase. Other hemoproteins also react positively. Thus, the diffuse staining reaction produced in the cytoplasm of erythroblasts [11,12] has generally been attributed to the presence of hemoglobin. Red blood cells do, however, contain a catalase whose presence has been demonstrated by conventional biochemical technics.[13] In guinea pig, rabbit,[12] and human erythroblasts [11] the use of the alkaline DAB medium of Novikoff and Goldfisher [3] has not demonstrated the presence of particulate catalase under conditions in which the pseudoperoxidase activity of heme imparted a high cytoplasmic density.

The present studies were initiated to determine whether microperoxisomes are present in erythrocytic series from normal human bone marrow under conditions in which the peroxidase activity of heme is reduced. We have used two different methods to diminish the peroxidase activity of heme without inhibition of the peroxidatic activity of catalase. In addition, we have established optimum conditions for cytochemical demonstration of microperoxisomes in other bone marrow cells.

## Materials and Methods

### Collection and Fixation of Bone Marrow

Four bone marrow samples from normal subjects and two from patients with hereditary leukocyte myeloperoxidase deficiency were obtained by sternal aspiration. The marrow particles were immediately immersed in 1.25% distilled glutaraldehyde (TAAB Laboratories) in 0.1 M phosphate buffer (pH 7.2) at 4 C for 30 to 120 minutes. After fixation, the marrow blocks were washed three times in 0.1 M phosphate buffer for 30 minutes and once overnight at 4 C. In one experiment, 40-μ-thick sections were cut on a Smith-Farquhar TC-2 tissue sectioner [14] after 60 minutes of fixation and washing. The cut sections were fixed for an additional 60 minutes and then washed and maintained as marrow particles in cold buffer until incubation in DAB medium.

### Diaminobenzidine Reaction and Inhibitors

Five media were employed: a) The alkaline DAB medium of Novikoff and Goldfischer [3]; b) the modified medium of Novikoff *et al*,[5,6] at pH 9.7; c) a medium at pH 8 with low concentration of hydrogen peroxide and addition of manganese ions [15]; d) a medium high in hydrogen peroxide [15]; and e) a medium at pH 7.6 with 0.2% DAB, 0.02% hydrogen peroxide, and preincubation at 4 C for 12 to 14 hours in order to improve the penetration of DAB.[16]

Incubations were carried out at 37 C for 2 or 3 hours. Different methods which do not reduce the staining of catalase but do diminish the peroxidase activity of heme were utilized. Novikoff *et al* [6] have shown that the pH 8–low hydrogen peroxide medium (final concentration of hydrogen peroxide, 0.001%) and potassium cyanide at a concentration of $1 \times 10^{-2}$ M had no effect on microperoxisome staining.[6] Under these conditions, the peroxidatic activity of developing erythrocytes was either

greatly diminished or completely inhibited.[11,12] Forty-micron-thick sections and marrow blocks were preincubated for 10 minutes at 37 C in buffer (at pH 7.6, 8, 9, or 9.7) containing $1 \times 10^{-2}$ M potassium cyanide. After this treatment, specimens were incubated in medium supplemented with the same concentration of potassium cyanide. For inhibition of catalase with aminotriazole (AMT), the specimens were maintained in buffer containing $2 \times 10^{-2}$ M 3-amino-1,2,4-triazole for 30 minutes at 4 C prior to incubation and were subsequently incubated in the appropriate media, to which $2 \times 10^{-2}$ M of AMT had been added. For controls, specimens were incubated in the respective media without DAB or without hydrogen peroxide.

#### Postfixation and Embedding

After incubation, the blocks or 40-μ-thick sections were rinsed in phosphate buffer and postfixed in 1% osmium tetroxide in the same buffer at 4 C for 30 minutes, before being dehydrated in ethanol and embedded in Epon; 0.5- to 1-μ-thick sections were cut with glass knives and examined in the light microscope with oil immersion.

Thin sections were cut on a LKB Ultrotome III with diamond knives and examined unstained in a Philips 300 electron microscope at 60 kV with objective aperture of 25 μ.

## Results

#### Morphology

In proerythroblasts, slight granulations in close proximity to the Golgi apparatus were common.[17-20] In addition, some spherical or ovoid particles were seen dispersed throughout the cytoplasm.

Careful examination of polychromatophilic erythroblasts also revealed the presence of a few small granules. Their identification in bone marrow which had not been incubated in DAB medium was difficult because of their small size, their low number (usually one per cell section), and their low electron density (Figure 1). A close spatial relationship between granules and the nuclear envelope was frequently observed (Figure 1). At this stage of maturation, the nuclear envelope was devoid of ribosomes. In addition, similar elongated or spherical bodies were occasionally seen in the cytoplasm where their position paralleled that of short cisternae of the SER.

#### Cytochemistry

##### Technical Considerations

Attempts to identify small bodies in erythroblasts by DAB staining have not been successful at pH 7.6, even after prolonged incubation of fragments or 40-μ-thick sections. With the alkaline medium of Novikoff *et al*,[5,6] a strongly diffuse positive reaction occurred in late erythroblasts and reticulocytes; this reaction obscured the cytoplasm with the exception of nonreactive organelles. Under these conditions, it was difficult or impossible to identify reactive organelles. In polychromatophilic erythro-

blasts, the peroxidatic activity of hemoglobin did not mask the staining of microperoxisomes (Figure 2).

Following incubation of fragments of bone marrow, a peripheral zone at the surface of the blocks was reactive. This contrasted with the regular staining of incubated sections of bone marrow.

In the pH 8.0 medium containing a low hydrogen peroxide concentration or in the pH 9.7 medium containing potassium cyanide, the peroxidatic activity of hemoglobin was greatly diminished. After incubation in the pH 9.7 media in the absence of hydrogen peroxide, this activity was completely abolished. Under these conditions, the staining of microperoxisomes was not affected, and therefore, their identification in mature erythroblasts was facilitated (Figures 3 and 4). At low magnification, it was sometimes possible to confuse the DAB staining of peroxisomes with a siderosome, but enlargment clearly revealed the structure of clusters of ferritin molecules. The mitochondrial oxidation of DAB (demonstrated after incubation in the pH 8 medium containing a low hydrogen peroxide concentration) was inhibited by the addition of $10^{-2}$ M potassium cyanide; the presence of potassium cyanide had no effect on the staining of microperoxisomes. Optimum staining resulted from fixation of bone marrow for two hours in glutaraldehyde with subsequent incubation in the pH 9.7 medium containing $10^{-2}$ M potassium cyanide. The addition of $2 \times 10^{-2}$ M AMT to the incubation media prevented the staining of microperoxisomes, but had no effect on mitochondrial staining (Figure 5).

In all media tested, AMT was found to inhibit the reaction in microperoxisomes and, therefore, made their detection very difficult.

### Distribution in Relation to Maturation Stages

In the four samples of normal bone marrows that were examined, the shape, number, and localization of microperoxisomes were similar. In bone marrow samples from 2 patients with myeloperoxidase deficiency, the distribution of microperoxisomes in the erythrocytic series clearly resembled that seen in samples from normal subjects.

Light microscope examination of thick sections of material which had been incubated in the DAB medium, pH 9.7, containing potassium cyanide, clearly revealed the presence of small brown granules in erythroblasts and reticulocytes. Their number and localization varied with the stage of maturation. In proerythroblasts, these granules were numerous and were dispersed throughout the cytoplasm, while in polychromatophilic erythroblasts, their number was diminished and they were preferentially localized at the surface of the nucleus.

Observations made using the light microscope were confirmed in the electron microscope from counts of microperoxisomes in 35 micrographs of early and late erythroblasts. Proerythroblasts had about five DAB-staining granules in thin sections (from two to seven), whereas polychromatophilic erythroblasts showed about 2.5 microperoxisomes (from zero to four) in thin section.

The classification of immature cells in the red cell lineage was dependant on the identification of ferritin on the cell surface and also within rhopheocytic vesicles. Intimate contact between the proerythroblast and thin pseudopods of the macrophage of erythroblastic islands was always seen.[17]

In proerythroblasts, microperoxisomes ranged in size from 0.14 to 0.29 μ, and they were generally round. They were present in all areas of the cytoplasm (Figures 6 and 9). The DAB reaction product was deposited uniformly over the matrix of the microperoxisomes, obscuring their membrane. Small granules concentrated near the Golgi complex, which resembled microperoxisomes in size and density, were distinguished from microperoxisomes by a constant negative reaction with alkaline DAB media (Figure 7) and by the presence of some ferritin molecules in their matrix. In general, microperoxisomes (except those localized on the surface of the nucleus) were not seen in close contact with segments of endoplasmic reticulum (Figure 8). The close relationship between the nuclear envelope and microperoxisomes was more apparent in polychromatophilic erythroblasts than in proerythroblasts (Figures 2, 3, 5, 9, and 10).

Some microperoxisomes were embraced by two SER cisternae which paralleled their limiting membranes (Figure 11). Many microperoxisomes were elongated (Figure 4), and in some cases they exhibited a central constriction (Figure 10).

The length of the elongate forms ranged from 0.21 to 0.45 μ and the diameters, from 0.09 to 0.17 μ.

In reticulocytes, some microperoxisomes were included in vacuoles (Figure 12). These densely stained bodies were distinct from siderosomes which had a cluster of ferritin molecules; in the absence of DAB or the presence of AMT in the incubation medium, the staining of microperoxisomes was abolished, while an identical contrast of ferritin persisted. Red cells lacked all organelles, including microperoxisomes.

### Microperoxisomes in Other Bone Marrow Cells

In normal subjects, the use of alkaline medium containing potassium cyanide inhibited the staining of endoplasmic reticulum in both

promyelocytes and promonocytes, but the staining of myeloperoxidase in azurophil granules was not significantly inhibited. Therefore, it was difficult to determine whether the granulocytic series and the monocytic series contained microperoxisomes. In contrast, in patients with a hereditary deficiency of myeloperoxidase, use of DAB media at pH 7.6 or 9.7 did not reveal any staining reaction in azurophil granules. However, following incubation at pH 9.7, smaller granules were identified within the neutrophils from these patients. These granules were strongly stained and exhibited a similar size and shape to those seen in proerythroblasts.

In all samples of bone marrow cells, a few round blastic cells which lacked differentiating characters also contained some microperoxisomes.

It should be noted that macrophages also possessed a few small granules which could be stained by DAB at pH 9.7. In megakaryocytes and platelets, this medium gave a positive reaction in granules whose size and morphology differentiated them from α granules.[21]

## Discussion

Small granules, which were surrounded by a single membrane and stained with alkaline DAB method, have been localized in all developmental forms of erythrocytes with the exception of mature erythrocytes.

Alkaline DAB media were used for the visualization of catalase present in the small granules, which have recently been described in many tissues.[5–10,22] The staining of peroxisomes and microperoxisomes by alkaline DAB was a result of the peroxidatic activity of the catalase which they contain.[2,3,5] Fixation of tissue samples with glutaraldehyde enhanced the peroxidatic activity of catalase [23,24] but inhibited its catalytic activity. Addition of AMT to the incubation medium was found to prevent the staining of microperoxisomes by inhibition of their catalase while potassium cyanide had little or no effect on it.[5–7,9,25]

Another essential morphologic feature of microperoxisomes is its close spatial relationship to the SER.[5,6,8–10,25]

The morphology, size, relationship to the SER, DAB reactivity, and effects of inhibitors suggest that the small granules which we have identified in erythroblasts resemble the microperoxisomes of other tissues.

One interesting observation concerning pleomorphic granules in proerythroblasts was provided by DAB staining. Granules which were associated in small groups in the region of the Golgi apparatus and which contained acid phosphatase activity,[18] did not react positively in alkaline DAB media. Routine electron microscopy did not permit

recognition of the heterogeneity of these granules since their size and density were similar. At this stage in maturation, the infrequent visualization of connections between endoplasmic reticulum and microperoxisomes may be due to their mode of attachment. These relationships were easily discerned when microperoxisomes were attached to the nuclear envelope.

Our present data indicate that the production of microperoxisomes in erythroblasts occurs in the most immature cells, since their number decreases as maturation progresses, possibly as a result of mitosis.

It remains to be determined if these organelles are present during the very early stages of maturation in stem cells activated by erythropoietin.[26]

In polychromatophilic erythroblasts, it is surprising that this cellular component was not detected in preparations examined by routine electron microscopy. Some investigators have described a few lysosome-like granules,[20] in the late erythroblast, but these granules were concentrated near the Golgi complex and did not correspond to the localization of DAB-staining particles. The failure to recognize their existence probably resulted from several factors, firstly their small size and low number, and secondly their confusion with lysosomes. Previous cytochemical studies of erythroblasts [11,12] have been directed toward the detection of the peroxidatic activity of hemoglobin. This reaction tends to mask the peroxidase reaction in granules. In the present study the partial inhibition of the peroxidatic activity of heme by potassium cyanide or media lacking hydrogen peroxide facilitated their identification.

The difficulties encountered in a attempt to localize erythrocyte catalase by fluorescent antibody technics in frozen section were ascribed to fixation of cells that contain the enzyme in soluble form. Intracorpuscular catalase could be revealed [27] only after use of surface-active agents. Under the present experimental conditions, microperoxisomes were never detected in red cells. The mechanism of the disappearance of microperoxisomes during reticulocyte maturation and the relationship between catalase in microperoxisomes and soluble erythrocytic catalase remain to be determined.

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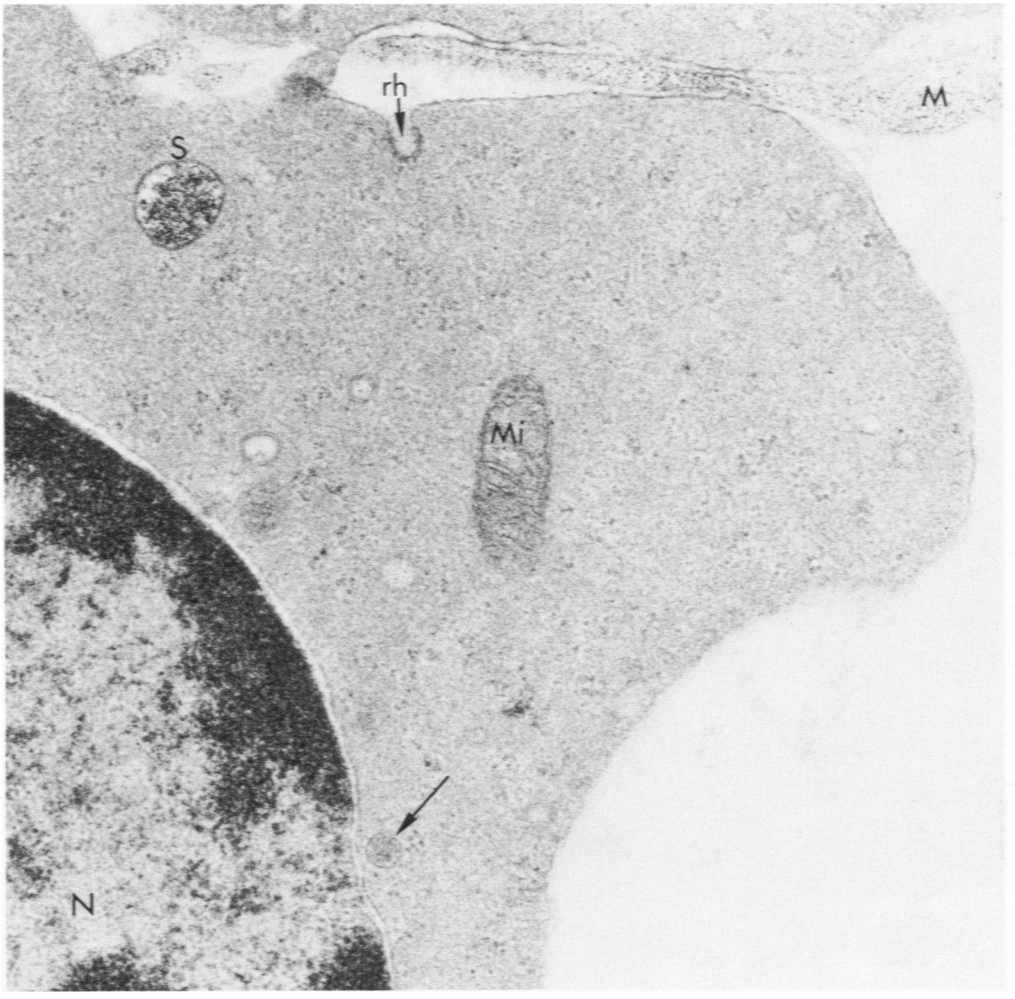
*[Illustrations follow]*

## Legends for Figures

**Fig 1**—Portion of a polychromatophilic erythroblast which was not incubated in DAB medium. One peroxisome (*arrow*) with moderate density may be seen adjacent to the smooth endoplasmic reticulum surrounding the nucleus (*N*) Note the presence of the characteristics of the maturing erythroblast: a siderosome (*S*) containing ferritin molecules, a rhopheocytotic (*rh*) depression in the membrane, and intimate association between the erythroblast and a macrophage (*M*) containing ferritin molecules. *Mi* = mitochondria. (Uranyl acetate and lead citrate, × 39,200)

**Fig 2**—Portion of a polychromatophilic erythroblast incubated in DAB medium at pH 9.7. Two microperoxisomes, each containing dense reaction product, may be seen situated at the surface of the perinuclear cisternae. At this maturation stage, hemoglobin imparts a cytoplasmic density which does not mask the strong staining of microperoxisomes (*arrows*). (× 56,800)

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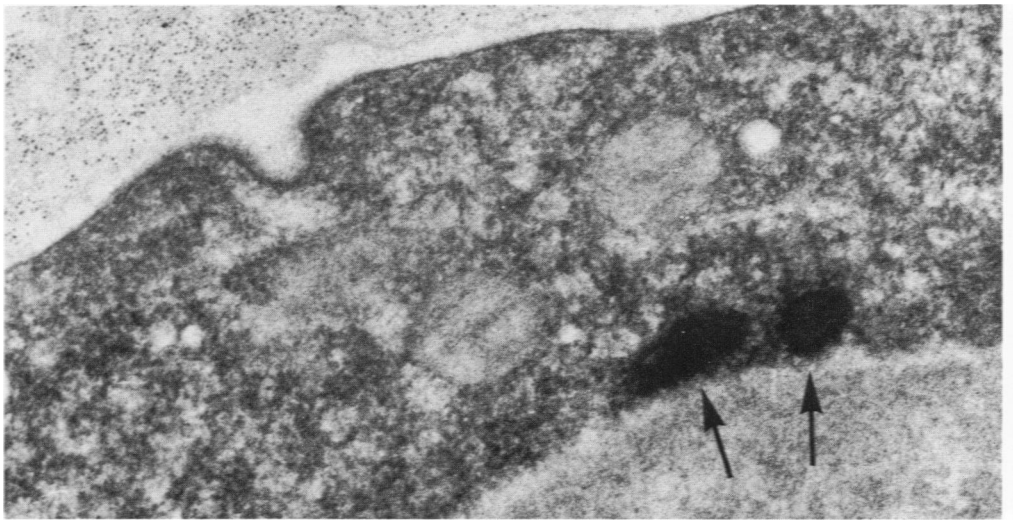

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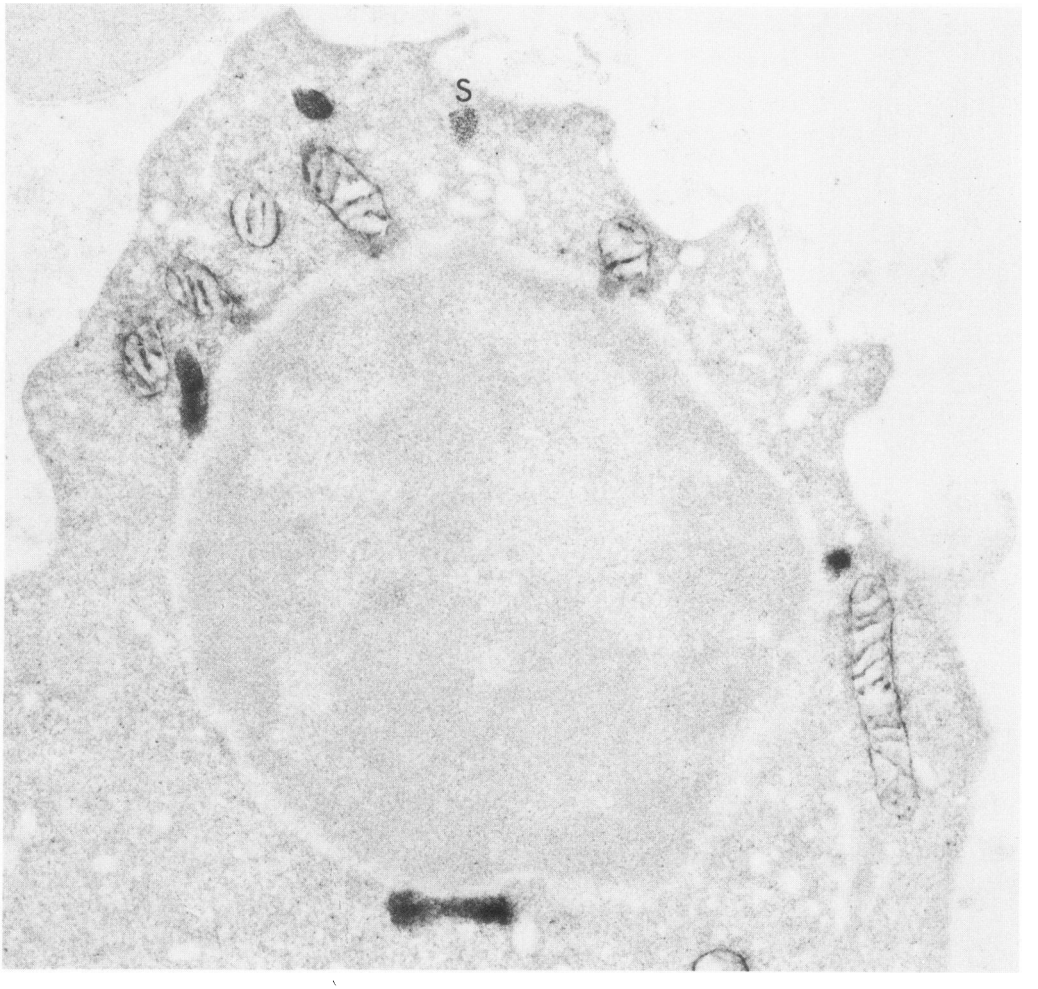


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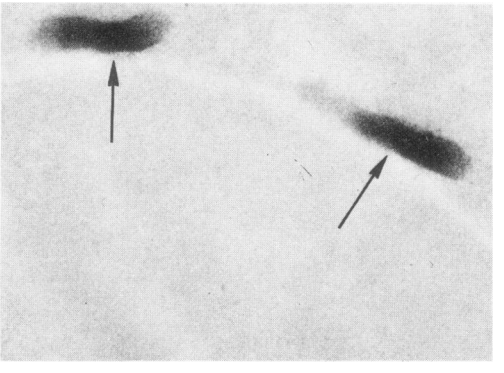

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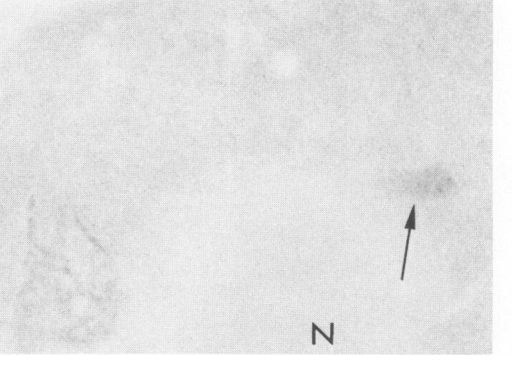


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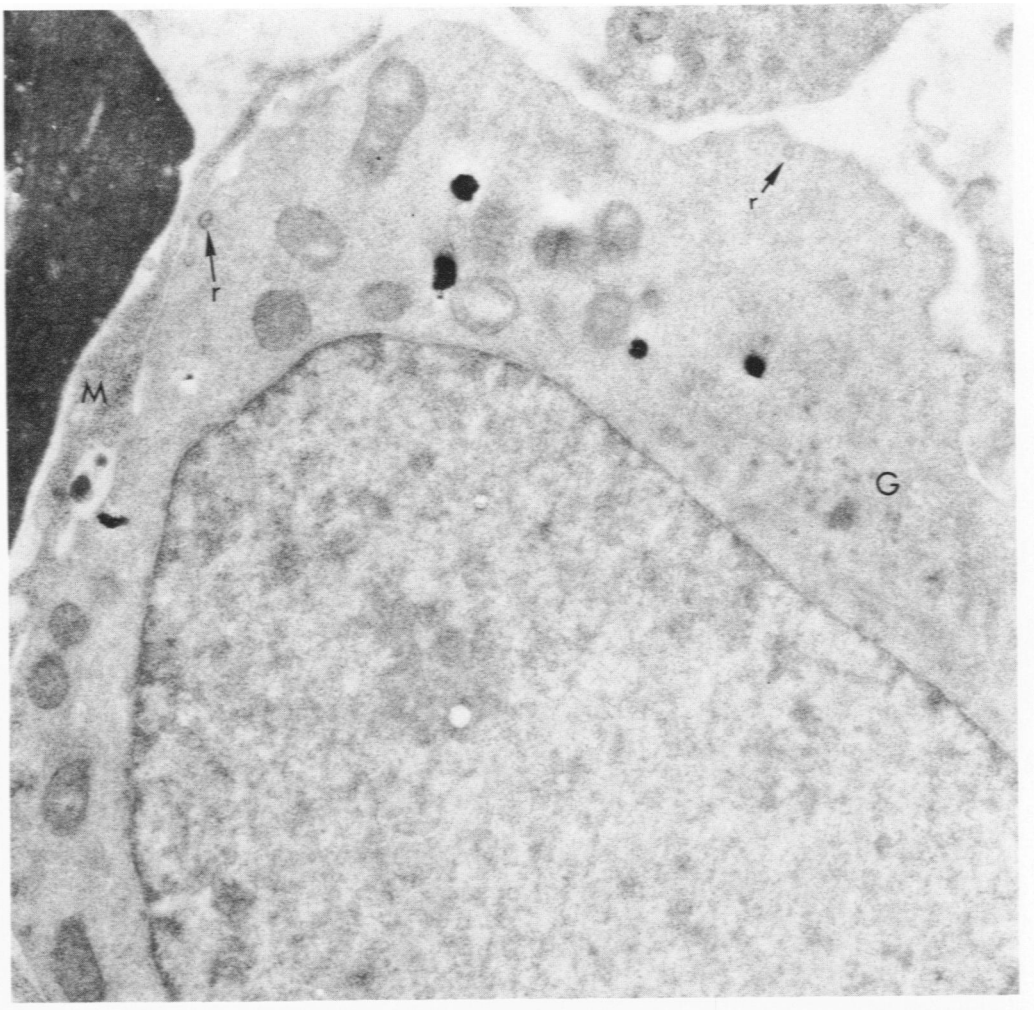
**Fig 3**—A later erythroblast which was incubated in DAB medium at pH 8.0 containing 0.001% hydrogen peroxide. Peroxidase activity of hemoglobin is greatly reduced, while staining of microperoxisomes persists. Three of the densely staining bodies may be seen close to the nuclear envelope. Note their elongated or ovoid shape. Mitochondria also contain reaction product. A cluster of ferritin molecules are responsible for the spontaneous density in a siderosome (*S*). (× 27,900) **Fig 4**—Part of a polychromatophilic erythroblast which was incubated in DAB medium at pH 8.0 containing both 0.001% hydrogen peroxide and potassium cyanide. Two elongated microperoxisomes (*arrow*) located at the surface of the nuclear cisternae remain reactive. (× 45,000) **Fig 5**—A portion of a polychromatophilic erythroblast which had been preincubated with aminotriazole and subsequently incubated in complete medium (containing the same inhibitor) at pH 8.0. Mitochondria contain reaction product, while a microperoxisome near the nucleus (*N*) is unreactive (*arrow*). (× 36,000)

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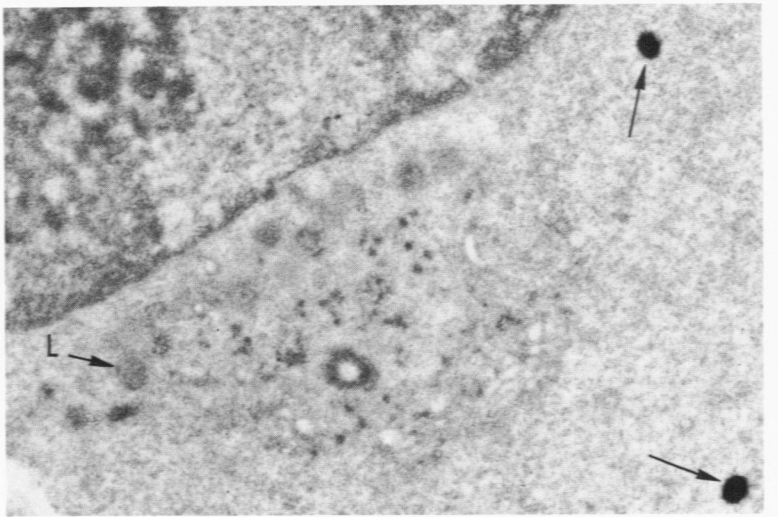


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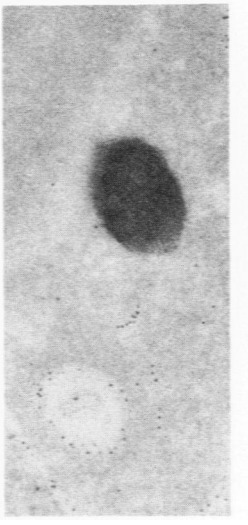

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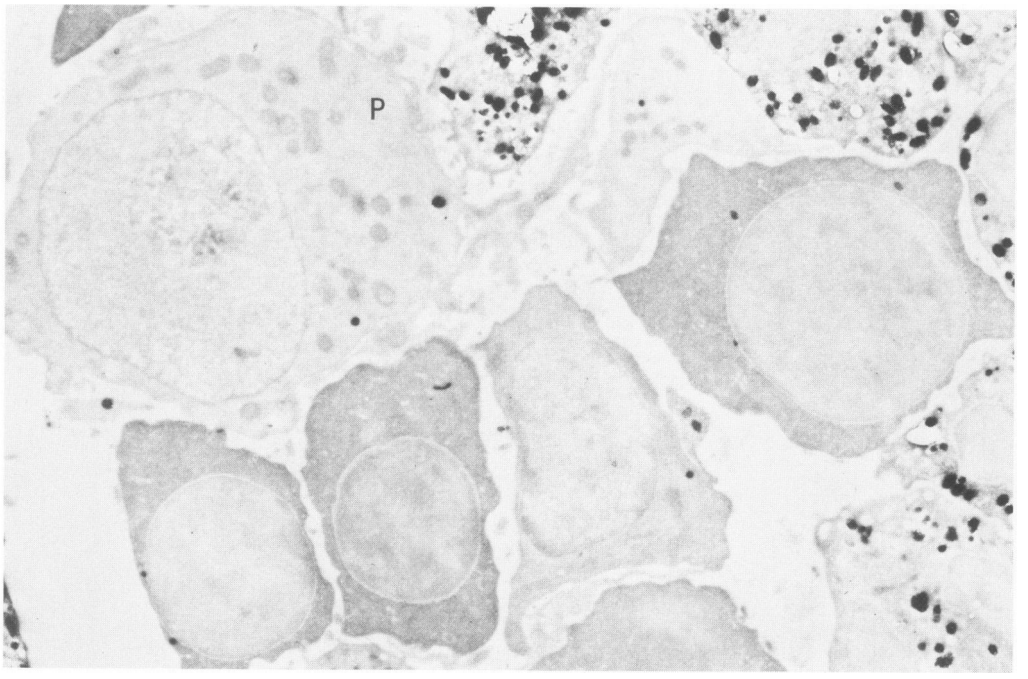
**Figs 6–8**—A proerythroblast which was incubated in DAB medium at pH 9.7. **6**—Five small DAB-positive particles are seen in the cytoplasm. A thin pseudopod of a macrophage (*M*) running along the cell, together with rhopheocytotic vesicles (*r*), characterize this early red cell precursor. *G* = Golgi complex. (× 14,200) **7**—A centriole is located in the middle of a Golgi apparatus. At the periphery of this organelle, several small unreactive granules, probably lysosomes (*L*), contrast with two strongly reactive microperoxisomes (*arrow*). (× 16,600) **8**—Ferritin molecules are dispersed throughout the cytoplasm and are also grouped in a rhopheocytotic vacuole but are not localized on a reactive microperoxisome which is adjacent to the perinuclear space. (× 64,300)

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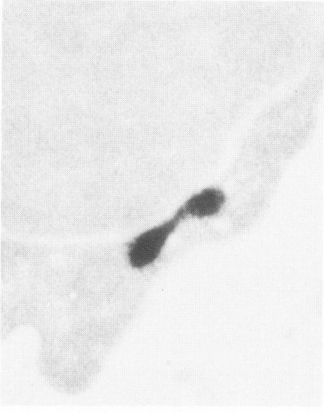

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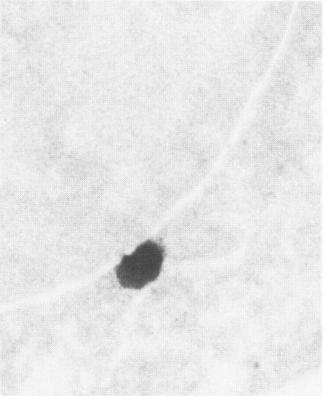

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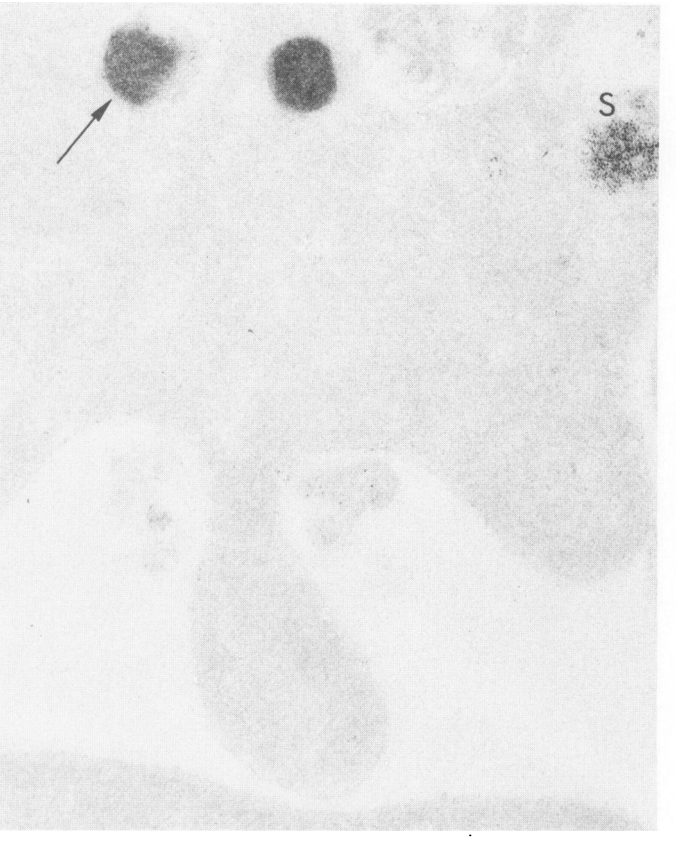


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**Fig 9**—A low-power view of an erythroblastic of islet in bone marrow which had been incubated in DAB medium (pH 9.7) in the presence of potassium cyanide. Five erythroblasts, one of which is a proerythroblast (*P*), may be seen to contain one to three microperoxisomes with varying shape and size. Note that the diffuse staining of the cytoplasm is not completely abolished by potassium cyanide. The intensity of reaction in microperoxisomes is similar to that seen in the azurophilic granules containing myeloperoxidase. (× 4700)
**Figs 10 and 11**—Part of mature erythroblast which had been incubated in DAB medium at pH 9.7 containing potassium cyanide. Microperoxisomes attached to the perinuclear space and a short saccule of endoplasmic reticulum are strongly stained. The cytoplasm shows only a slightly positive reaction (Figure 2). (× 22,500)
**Fig 12**—A portion of a reticulocyte following incubation in DAB medium (pH 8) containing both 0.001% hydrogen peroxide and potassium cyanide. The peroxidase activity of hemoglobin is abolished. Two microperoxisomes are identified by their DAB staining; one of these (*arrow*) seems to be included in a vacuole. At the right, a siderosome may be recognized. (× 59,400)