Cytochemical Localization of Peroxidase Activity in the Developing Erythrocyte

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Peroxidase activity, demonstrated with diaminobenzidine as the electron donor according to the method of Graham and Karnovsky, was used as a cytochemical marker in a study of developing erythrocytes in guinea pig and rabbit bone marrow. Peroxidase activity was deposited diffusely in the cytoplasm and nuclear matrix of developing cells and was thought to represent hemoglobin, which others have shown by independent criteria to have a similar distribution. Diffuse localization was first observed in erythroblasts and at all subsequent stages of development. Another finding was the significant particulate localization of peroxidase activity apparently associated with cytoplasmic ribosomes and nuclear particles of immature erythrocytes. This activity differentiated the most primitive erythroid precursors from hemocytoblasts of other marrow cell lines, a distinction impossible by strictly morphologic criteria. Particulate peroxidase localization was identified in erythroid hemocytoblasts, erythroblasts, normoblasts and reticulocytes but not in mature erythrocytes. The nature of the particle-associated peroxidase activity was not determined with certainty. However, it could not be differentiated from the diffuse activity, thought to reflect hemoglobin, by several inhibitors and could not be attributed to erythrocyte catalase. The possibility is therefore raised that this activity represents hemoglobin, newly assembled either on or immediately adjacent to nuclear particles and cytoplasmic ribosomes (Am J Pathol 67:303–326, 1972).

A VARIETY OF ENDOGENOUS PEROXIDASE ACTIVITIES exists in plant and animal tissues and may be detected by both biochemical and histochemical methods.[1]

Several of the enzymes responsible for this activity have been well characterized and all have been found to be heme proteins. These include the peroxidases of milk, white blood cells, thyroid microsomes and the horseradish plant.[2]

Mature erythrocytes of many species exhibit peroxidase activity. This activity has generally been attributed to the presence of the heme protein, hemoglobin, which catalyzes the oxidation of a variety of

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substrates by hydrogen peroxide and which is often referred to as a "pseudoperoxidase."[1] Red blood cells also contain a catalase which might contribute activity under certain circumstances. The peroxidase activity of erythrocytes is stable during fixation and tissue processing, can produce an insoluble reaction product which is readily visualized in the electron microscope and might, therefore, be expected to afford a useful cytochemical marker in developing cells.

We have used peroxidase activity, as demonstrated with diaminobenzidine as the electron donor, to study the development of erythrocytes in guinea pig and rabbit bone marrow. We have been able to identify red blood cell precursors at earlier stages of differentiation than has been possible by morphologic criteria alone. An unexpected finding was the presence of significant peroxidase activity associated with ribosomes and nuclear particles of immature erythrocytes, an observation which may have relevance to biochemical theories of hemoglobin synthesis.

Materials and Methods

Observations were made on 14 normal adult English short hair guinea pigs and 2 adult New Zealand rabbits whose bone marrow had been stimulated by treatment with phenylhydrazine.[3] Animals were sacrificed and small blocks of femoral bone marrow were immediately fixed by immersion for 2 hours at room temperature in a mixture[4] containing 1% paraformaldehyde, 1.25% glutaraldehyde and 1.5 × 10^{-3}M $CaCl_2$ in 0.1M sodium cacodylate buffer, pH 7.4. Tissues were then washed overnight in 0.1M sodium cacodylate buffer, pH 7.4.

Normal Morphology

Tissue was postfixed in 1.5% collidine-buffered osmium tetroxide for 2 hours at 4°C before being dehydrated in a graded series of alcohols and embedded in Epon 812. For purely morphologic observation, sections were stained lightly with lead citrate.[5] However, for comparison with tissues reacted for peroxidase activity, sections were routinely examined unstained, and only photographs of unstained sections are illustrated in this paper.

Cytochemistry

After the overnight wash in 0.1M sodium cacodylate buffer, 40 μ sections were cut with a Smith-Farquhar tissue chopper and incubated for 1 hour at room temperature in Graham and Karnovsky's medium[6] containing 5 mg of 3-3′ diaminobenzidine tetrachloride (DAB-Sigma Chemical Co, St Louis, Mo) and 0.01% hydrogen peroxide in 10 ml of 0.05M Tris-HCl buffer at pH 7.6 or pH 6.0. Sections were also incubated according to the method of Novikoff and Goldfischer.[7] A variety of controls was employed. In some instances either DAB or H_2O_2 was omitted from the reaction mixture. To exclude nonspecific binding of reduced DAB to tissue components, the sections were incubated for 1 hour in 0.05M Tris-HCl buffer containing 5 mg of DAB, were washed three times in buffer and were then incubated for 15 minutes in 0.05M Tris-HCl buffer containing 10^{-3}M potassium ferricyanide.[6] To exclude nonspecific binding of oxidized

DAB to tissue components, 5 mg of DAB in 0.05M Tris-HCl buffer was oxidized by reaction for 15 minutes with 10^{-3}M potassium ferricyanide. Bone marrow sections were then added to the mixture, incubated for 1 hour at room temperature and then washed three times in buffer.

Following appropriate incubation, the sections of bone marrow were washed overnight in 0.05M Tris-HCl buffer, pH 7.6, postfixed, dehydrated, and embedded as described above. Sections were cut with an LKB II ultrotome and were studied in an RCA 3F or Philips 200 electron microscope. To avoid confusion of peroxidase reaction product with deposits of heavy metal stain, only unstained sections were examined.

Inhibitors. The inhibitory effects of several chemicals and heat on the cytochemical reaction were evaluated. Forty μ chopped sections were preincubated for 30 minutes at room temperature in 0.05M Tris-HCl buffer, pH 7.6 containing either 10^{-1}M or 10^{-2}M potassium cyanide, 10^{-1}M sodium azide, or 2×10^{-2}M 3-amino- 1,2,4- triazole or were boiled for 10 minutes in buffer. After this treatment, sections were incubated for 1 hour in complete Graham and Karnovsky's medium supplemented with the same concentration of the appropriate inhibitor. After incubation, sections were processed as above and unstained sections were examined in the electron microscope.

Peroxidatic Activities of Hemoglobin and Hematin. The peroxidatic activities of hemoglobin (bovine, twice crystallized, Sigma Chemical Company, St Louis, Mo) and hematin were measured by following spectrophotometrically the change in absorbance due to the oxidation of O-dianisidine in the presence of H_2O_2 [8,9] and were compared to the values obtained using horseradish peroxidase (Type II, Sigma Chemical Co). According to the manufacturer, the preparation of the hemoglobin studied contained up to 75% by weight methemoglobin, the remainder being largely oxyhemoglobin. Bovine hemin (Mann Research Laboratories, NY, NY) was solubilized and quantitatively converted to hematin by dissolution in water with NaOH. The reaction mixture contained 0.003% H_2O_2 and 0.008% O-dianisidine in 0.01M phosphate buffer, pH 7.3. One-tenth milliliter aliquots of the various compounds tested were added to 3 ml of reaction mixture, and the change in absorbance at 460 mμ was measured at 15-second intervals at room temperature. Activity was expressed as the change in absorbancy per minute per micromole as measured in a Zeiss spectrophotometer.

Results

Normal Morphology of Developing Red Blood Cells

The fine structure of developing red blood cells has been described by several authors [10–13] and our results agree with their findings. In brief, the most primitive marrow cell, the hemocytoblast, was a large cell with a large nucleus and prominent nucleoli. The pale interchromatinic substance (nuclear matrix) contained small particles many of which resembled ribosomes in size and shape. Some, however, were larger and more irregular than ribosomes. The cytoplasm contained many free ribosomes, often grouped as polysomes, varying numbers of mitochondria, and a Golgi area. Phenylhydrazine-treated rabbits had large numbers of hemocytoblasts, presumably of the erythroid series, in their marrow. Until differentiation took place (for example, granule

production in the myeloid series) one could not reliably determine the developmental potential of a given marrow hemocytoblast by morphologic criteria alone.

Differentiation in the erythroid series involved progressive reduction in nuclear and cytoplasmic size and condensation of nuclear chromatin into large dense angular masses. The nuclear interchromatinic space with its complement of particles also became markedly reduced in size. Prominent nuclear pores were seen, some of which were bridged by a thin septum.

The cytoplasm of the erythroblast contained numerous free ribosomes, often grouped as polysomes, a few mitochondria, and a small Golgi zone. Ferritin particles were adherent to the fuzzy surface coat at cup-shaped depressions of the cell membrane and were apparently taken into the cell as these membrane depressions invaginated to form vesicles. With further maturation, fewer ribosomes were visualized and the cytoplasm and nuclear matrix contained homogeneous, light staining material identified as hemoglobin.[11] The normoblast had a greatly condensed nucleus with little interchromatinic space. With nuclear extrusion, the normoblast became a reticulocyte, a cell retaining moderate numbers of ribosomes. Finally, the mature erythrocyte was a dense, anucleate cell, smaller than the reticulocyte, and lacking ribosomes.

Cytochemical Localization of Reaction Product in Differentiating Erythrocytes

The various stages of erythrocyte development in guinea pig and rabbit bone marrow, as illustrated in micrographs of sections incubated for peroxidase activity, appear in Figure 1, 2, 5–8, and 10–13. Figure 3, 4 and 9 represent micrographs of sections not reacted for peroxidase. None of the sections illustrated was exposed to heavy metal stains. The results obtained in the two species were similar and are considered together. Cytochemical incubation of guinea pig marrow at pH 7.6, 6.0 and 9.0 gave comparable results.

Hemocytoblasts. Primitive erythrocyte precursors, indistinguishable from other blast cells by morphologic criteria in routine sections, were readily identified by the presence of peroxidase reaction product on their cytoplasmic ribosomes (Figure 2 and 8). The interchromatinic area of the nucleus of these cells (nuclear matrix) also contained positively reacting particles some of which had the size and shape of ribosomes (Figure 2). Mitochondria, perinuclear cisternae, and small Golgi vesicles and saccules were uniformly negative. The density of the nucleolus of these cells was not increased after incubation in cytochemical reaction mixture and was therefore also considered negative. The ribo-

somes, positive for reaction product, were aggregated into polysomes generally containing 4–6 ribosomal units (Figure 10). The cytoplasm of erythrocyte hemocytoblasts lacked diffuse positive cytoplasmic material (Figure 10) corresponding to the hemoglobin of more mature cells.

The ribosomes of other hemocytoblasts in the bone marrow, presumably the precursors of nonerythrocyte cell lines, did not show peroxidase activity (Figure 1). Myeloblasts had numerous ribosomes, usually associated with endoplasmic reticulum, but these invariably lacked reaction product. Focal staining of eosinophil myelocyte ribosomes was noted occasionally but usually in an area adjacent to intense reaction product associated with the strong peroxidase reaction of the mature granule. It could not be decided whether this erratic staining represented diffusion of enzyme or reaction product or in fact reflected ribosomal synthesis of eosinophil peroxidase.

Erythroblasts. Erythroblasts were somewhat smaller cells with a smaller nucleus and less ample cytoplasm (Figure 1, 4, 5, 7 and 8). The nucleus contained larger aggregates of chromatin. The nuclear matrix, in contrast to that of nonerythroid cells, contained peroxidase-positive material (compare Figure 1, 5 and 8, peroxidase reacted, with Figure 4). Nuclear particulate staining was less obvious in older erythroblasts (Figure 7) which had fewer nuclear particles. The cytoplasmic ribosomes of erythroblasts, for the most part arranged as polysomes, were intensely positive (Figure 5 and 11). In addition, reaction product was sometimes deposited focally in the cytoplasm between ribosomes and unassociated with any cell organelles (Figure 11). In agreement with earlier studies [11] this localization was thought to represent newly synthesized hemoglobin.

Normoblasts. Normoblasts were smaller cells with a small nucleus containing dense aggregates of chromatin in osmium-collidine postfixed tissues. After incubation in peroxidase reaction medium, the interchromatinic areas of the nucleus of these cells contained peroxidase-positive material, not associated with particles, and thought to represent hemoglobin. Fewer ribosomes were present, although those remaining were intensely positive for reaction product and stained more densely than the diffuse deposits of cytoplasmic hemoglobin (Figure 6 and 12). Ferritin was also recognized within cytoplasmic vesicles of these cells and was attached to cup-shaped depressions of the surface membrane. Ferritin was readily distinguished from cytochemical reaction product at all stages of cell development.

Reticulocytes. Reticulocytes lacked nuclei and had relatively fewer ribosomes than normoblasts. Residual ribosomes stained more intensely

for peroxidase activity than did the surrounding cytoplasm which was intermediate in density between that of normoblasts and mature erythrocytes (Figure 7).

Mature red blood cells

Mature erythrocytes lacked ribosomes and the cytoplasm was diffusely and densely positive for reaction product (Figure 8).

Controls

No cytochemical reaction. Material postfixed in osmium-collidine and unstained with heavy metals was examined to determine the relative densities of structures in developing red cells in the absence of a cytochemical reaction. In this material, it was noted that ribosomes were slightly more dense than the surrounding cytoplasm, and the interchromatinic area of the nucleus had minimal density (Figure 3, 4 and 9). The relative densities of cellular structures were thus similar to those observed in unstained sections of nonerythroid bone marrow cell lines. Following incubation for peroxidase activity, however, the striking changes in density described above, cytoplasmic and nuclear, particulate and diffuse, became obvious (compare Figure 2 and 8, incubated, with Figure 3, nonincubated; likewise, compare Figure 1, 5, 7 and 8 with Figure 4; and Figure 11 with Figure 9). Similar dense particulate and diffuse reaction product did not develop in nonerythroid cells following incubation for peroxidase activity except for rare staining of ribosomes in eosinophils adjacent to strongly positive specific granules.

Absence of H_2O_2 or DAB from reaction media. Omitting either H_2O_2 or DAB resulted in a negative cytochemical reaction in developing and mature red blood cells, analogous to that observed in Figure 3, 4 and 9.

Control for nonspecific absorption of reduced DAB. When tissue was preincubated for 1 hour in the presence of DAB but without H_2O_2 and subsequently incubated for 15 minutes in 0.05M Tris-HCl containing 10^{-3} potassium ferricyanide, developing and mature red blood cells lacked densities of the type observed in cells incubated in complete medium. This is convincingly illustrated when Figure 9, a high magnification micrograph of an erythroblast studied for nonspecific absorption of DAB, is compared with Figure 11, a micrograph of a similar cell reacted for peroxidase and photographed at the same magnification.

Control for nonspecific absorption of oxidized DAB. When sections of bone marrow were incubated in DAB which had been oxidized with potassium ferricyanide, developing and mature erythrocytes were again

negative for reaction product, indicating that nonspecific binding of oxidized DAB to the tissue was not responsible for the results observed.

Inhibitors

The peroxidase activity observed on ribosomes and nuclear particles, as well as the diffuse nuclear and cytoplasmic activity of mature and developing erythrocytes, were completely inhibited by 10^{-1}M but not 10^{-2}M KCN. Sodium azide, 10^{-1}M, caused a moderate loss of activity in mature red cells and a greater loss of reactivity, both particle associated and diffuse, in immature erythrocytes. Aminotriazole, in a concentration of 2×10^{-2}M, produced no detectable inhibition of peroxidase activity in either the mature or developing red blood cell (Figure 8 and 13). Lack of inhibition of the peroxidatic activity of mature red blood cells with this concentration of aminotriazole has been previously described.[14,15] Heating caused a patchy and irregular loss of peroxidase activity in red cells with sparing of activity in intervening areas.

The peroxidase activity of mature eosinophil granules was unaffected by 10^{-1}M KCN, 10^{-1}M sodium azide, and 2×10^{-2}M aminotriazole, confirming earlier work.[16,17]

Peroxidatic activities of hemoglobin and hematin. Since the cytochemical peroxidase activity observed in erythrocytes could be attributed either to free hematin or to hemoglobin, it was of interest to measure the relative peroxidatic activities of these compounds biochemically with reference to the true peroxidase derived from the horseradish plant. The results of this comparison, using O-dianisidine as the electron acceptor are summarized in Table 1. The relative peroxidase activities of bovine hematin, bovine hemoglobin, and horseradish peroxidase, expressed in molar terms, were found to be 1, 11 and 7852 respectively. Comparable relative activities of hematin and hemoglobin were obtained by Bancroft and Elliott.[18]

Table 1—Peroxidatic Activities of Bovine Hematin and Hemoglobin Relative to Horseradish Peroxidase

	Molecular weight	Units*/μ mole	Relative molar activities
Hematin	633	2.7	1
Hemoglobin	68,000	29.7	11
Horseradish peroxidase	40,000	21,200	7852

* Change in absorbancy at 460 mμ/min at room temperature and at pH 7.3

Discussion

We have used the well known peroxidase activity of erythrocytes as a cytochemical marker in studies of red blood cell differentiation in the bone marrows of guinea pigs and phenylhydrazine-treated rabbits. Mature erythrocytes, reticulocytes, normoblasts and late erythroblasts of both species contained diffuse deposits of peroxidase-positive material, unassociated with ribosomes, in their cytoplasm corresponding to the distribution of hemoglobin identified by others in strictly morphologic studies. Similar deposits were observed in the nuclei of erythroblasts and of older cells in the erythroid series. These cytochemical data, therefore, considered in the light of earlier studies of erythrocyte ultrastructure,[11,13] absorption microspectroscopy [19] and fluorescence microscopy,[20] suggest that the diffuse nuclear and cytoplasmic peroxidase activity observed may be attributed to hemoglobin. Although small amounts of free heme may be present in the mature erythrocyte, quantitative considerations make it likely that the great bulk of cytochemical activity reflects the presence of hemoglobin.[21] Peroxidase staining was not observed in mitochondria, the site of heme synthesis, and in a biochemical assay system bovine hematin was found to possess only one-eleventh of the peroxidatic activity of hemoglobin.

Unexpected was the demonstration of peroxidase activity associated with ribosomes and nuclear particles of developing erythrocytes. Particle-associated peroxidase activity, both nuclear and cytoplasmic, appeared at an earlier stage of development than diffuse activity (presumably hemoglobin) and was well developed in erythroid hemocytoblasts and erythroblasts but was not seen in nonerythroid hemocytoblasts, myeloblasts or lymphoblasts. With differentiation, additional peroxidase activity appeared diffusely both in the nucleus and in the cytoplasm (Figure 6, 11 and 12), unassociated with cell organelles, as described above. As the nucleus condensed, the interchromatinic space with its particulate and diffuse peroxidase activities was reduced in size and little nuclear activity remained in the mature normoblasts. Ribosome associated activity persisted in the cytoplasm through the reticulocyte stage (Figure 7) but mature erythrocytes, lacking ribosomes, had only diffuse staining (Figure 8).

The particle associated reactivity observed in the cytoplasm was localized to ribosomes. Breton-Gorius,[22] in a study of developing human bone marrow erythrocytes, also suggested the possibility of ribosomal peroxidase activity. However, her observations did not exclude the possibility of diffusion from the adjacent, hemoglobin-rich, cytoplasm. In our study the cytochemically reactive particles were commonly

arranged in configurations with the appearance and dimensions of polysomes (Figure 10–13), and in the youngest erythroid cells, lacking diffuse peroxidase activity, unstained ribosomes could not be visualized between peroxidase positive cytoplasmic particles. Finally, the particulate cytoplasmic reactivity and the number of cytoplasmic ribosomes declined in parallel as erythrocytes matured such that both were considerably reduced in reticulocytes and absent in mature erythrocytes.

The nuclear particles observed by us in erythrocyte precursors would seem to be analogous to those which have been previously noted in the nuclei of other cells.[23,24] These particles have some morphologic similarities to ribosomes but have less tendency to form polysome-like structures. The finding that erythrocyte nuclear particles have peroxidase activity similar to that of cytoplasmic ribosomes is of interest since Hammell and Bessman have demonstrated hemoglobin synthesis in nuclear preparations of avian erythrocytes.[25] Similarly, Orlic [26] has demonstrated ^{55}Fe incorporation in the nuclei of mouse spleen erythroblasts using electron microscopic autoradiography. Some authors have felt that nuclear hemoglobin resulted from the flow of this material from the cytoplasm, where it was synthesized, to the nucleus by means of the nuclear pores.[13,19] The most primitive erythroid cells we observed contained nuclear particles as well as cytoplasmic, polysome-associated peroxidase activity. Primitive cells containing only cytoplasmic or nuclear activity were not identified. We therefore have no morphologic evidence for a flow of particles or of hemoglobin between nucleus and cytoplasm. Indeed, the nuclear particles and hemoglobin observed could have resulted from the generalized mixing of nuclear and cytoplasmic elements at the time of cell division.

The nature of the particle associated peroxidase activity in developing erythrocytes was not established with certainty. Ribosome or microsome-bound peroxidases have been described in plant and animal tissues.[27,28] Glutathione peroxidase does occur in erythrocytes but this enzyme has been shown to be devoid of activity at pH 6.0 [29] and therefore probably did not contribute to our results. Neither particulate nor diffuse activity was inhibited by aminotriazole at any stage of erythrocyte development. Since this compound is a known inhibitor of the catalases isolated from several organs and species, including the human erythrocyte, it seems unlikely that either activity observed in our studies could be attributed to catalase.[30] A variety of other enzyme inhibitors were likewise unable to distinguish between diffuse and particle associated peroxidase activity, possibly suggesting that both reflect the activity of the same protein; namely, hemoglobin. However,

hemoglobin is not known to be bound to ribosomes in significant quantities.[31,32] Current biochemical opinion [33] holds that the α chains of globin are released from polysomes as soon as they are synthesized and combine with newly completed β chains still attached to ribosomes. Subsequently, it is thought, αβ subunits are freed into the cytoplasm and only then combine with heme which is synthesized independently by cytoplasmic and mitochondrial enzymes. Finally, the αβ subunits with attached heme join to form the tetrad of $\alpha_2\beta_2$ hemoglobin. Only a small amount of ^{59}Fe becomes associated with rabbit reticulocyte polysomes *in vitro* and this is not released by puromycin whereas nascent chains of globin are completely released. Moreover, well washed rabbit reticulocyte ribosomes that have been passed through a sucrose density gradient do not possess peroxidase activity in our hands.[34] Therefore, whatever the nature of the particle associated peroxidase activity, it would seem to be loosely bound to ribosomes. Possibly this activity represents newly synthesized hemoglobin whose assembly was completed either on or immediately adjacent to nuclear particles and cytoplasmic ribosomes. Further work will be required to settle this question.

Endogenous peroxidase activity in ribosomes has also been reported in salivary gland acinar cells [35] and in epithelial cells of the large intestine.[36] In salivary gland cells which synthesize a peroxidase for secretion, Strum and Karnovsky noted that focal clusters of ribosomes were peroxidase positive. While this staining was thought to reflect newly synthesized enzyme, the possibility was also considered that enzyme was released from adjacent peroxidase-containing organelles and became nonspecifically bound to ribosomes. The irregular and focal ribosomal staining noted in eosinophils in our material is likewise subject to either interpretation. However, differentiating erythrocytes lack peroxidase containing organelles other than ribosomes and nuclear particles. Further, the particle staining in these cells probably cannot be attributed to diffusion from the sites of hemoglobin reaction in the nucleus or cytoplasm since particle reactivity appeared in young cells before diffuse nuclear or cytoplasmic enzyme activity could be identified (Figure 10 and 13). It remains possible that diffuse hemoglobin is present at activities below the sensitivity of our methods and during fixation and processing becomes bound to ribosomes in concentrations sufficient to be detected. This, however, seems unlikely due to the uniformity of ribosomal staining observed.

Peroxidase staining of ribosomes and nuclear particles was observed in guinea pig hemocytoblasts situated in islands of erythropoiesis, and

we conclude that these cells are committed to develop in the erythroid series—*ie*, that hemocytoblasts exhibiting particulate peroxidase activity are in fact erythroid hemocytoblasts. Support for this inference was obtained from study of phenylhydrazine-treated rabbits whose marrow showed striking erythroid hyperplasia with numerous immature forms. Here the great majority of hemocytoblasts present could safely be assumed to be erythroid precursors and were found in fact to possess peroxidase-positive particulate material both in their nuclei and cytoplasm. Thus, the presence of particulate peroxidase activity allowed identification of erythroid hemocytoblasts at a stage of development when they could not otherwise be distinguished from the hemocytoblast precursors of other marrow cell lines. This finding suggests that primitive cells, undifferentiated and indistinguishable morphologically from the blast cells of the marrow, spleen, or lymph nodes, may in fact already be committed to differentiate in a particular cell line. Independent evidence for this conclusion is found in the cytochemical work of Avrameas and Leduc [37] who localized antibody to horseradish peroxidase on the ribosomes of primitive lymphoid cells from lymph nodes, thereby demonstrating that another type of blast cell is already committed to the synthesis of a specific protein. Taken together, these data indicate that not all hemocytoblasts are totipotential but do not exclude the possibility that some are or that the blasts described by us and by Avrameas and Leduc arose from some unrecognized common totipotential precursor.

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[*Illustrations follow*]

All figures represent electron photomicrographs of guinea pig and rabbit bone marrow sections which were not exposed to heavy metal stains.

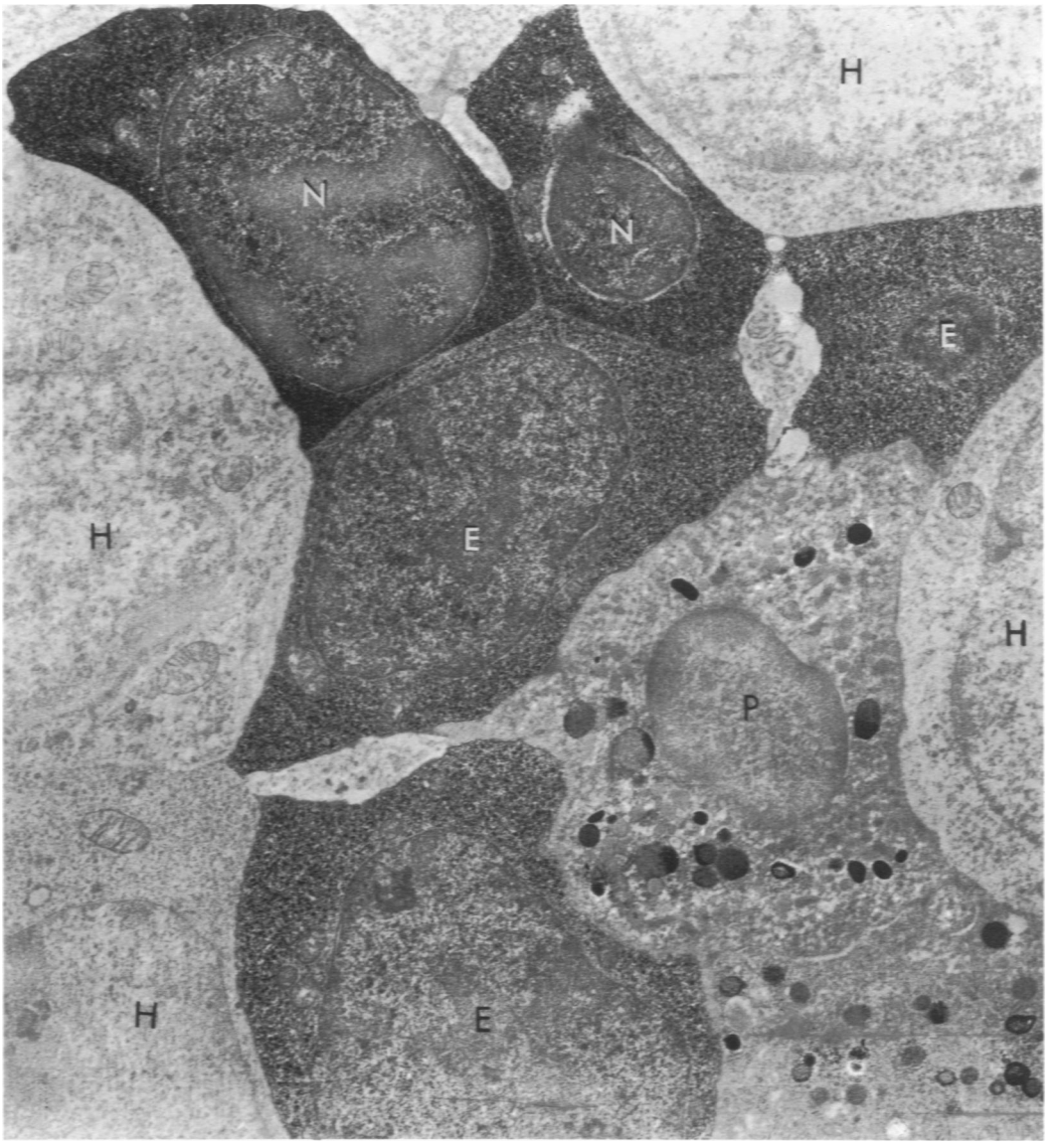


Fig 1—Specimen of guinea pig bone marrow incubated for peroxidase activity. This low magnification picture shows negative hemocytoblasts (*H*). These cells, presumably not of the erythroid series, show cytoplasmic ribosomes and nuclear interchromatinic particles that are negative for peroxidase activity. Also present are a polymorphonuclear leukocyte (*P*) with positive granules, three positive erythroblasts (*E*), and two positive normoblasts (*N*) (× 9000).

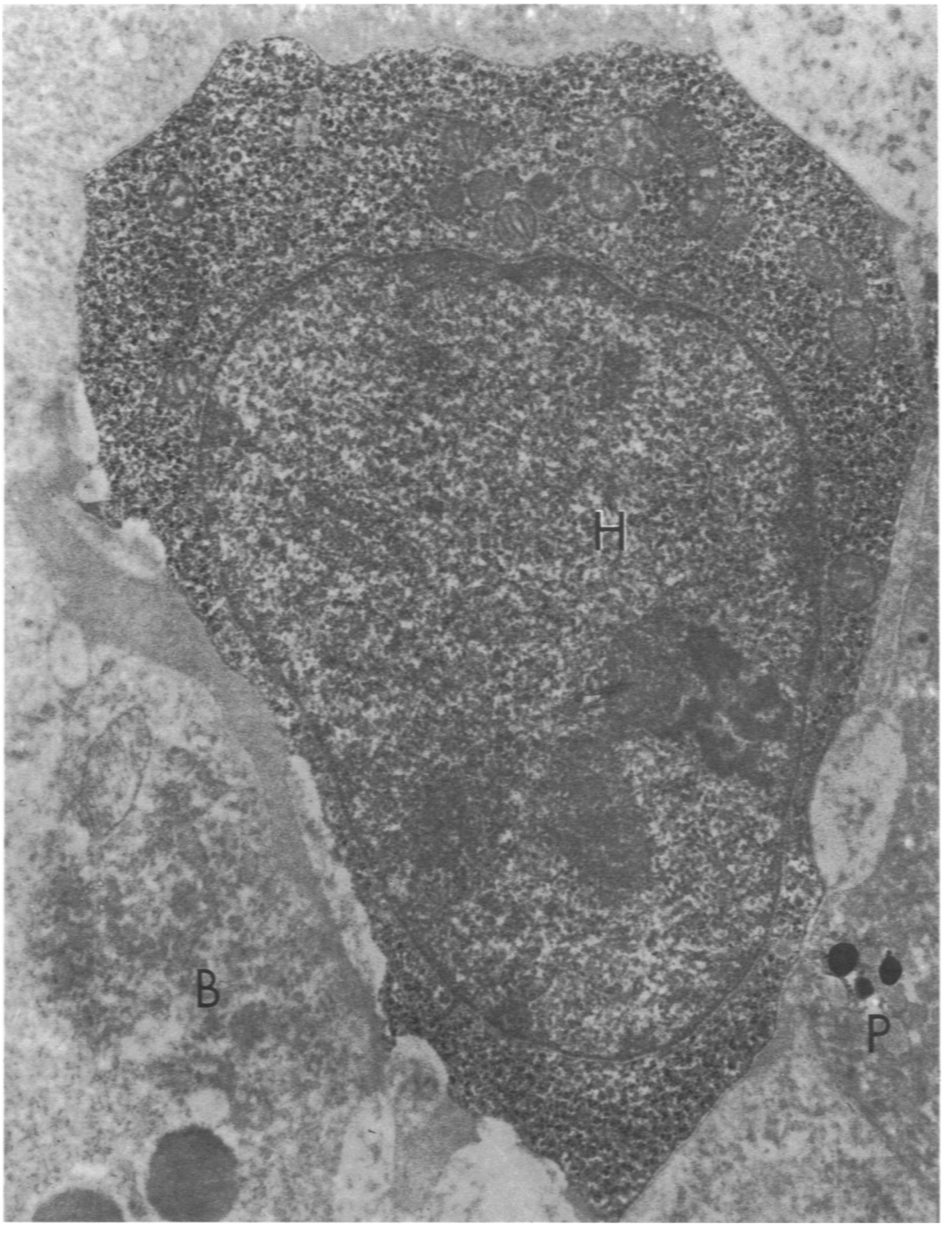
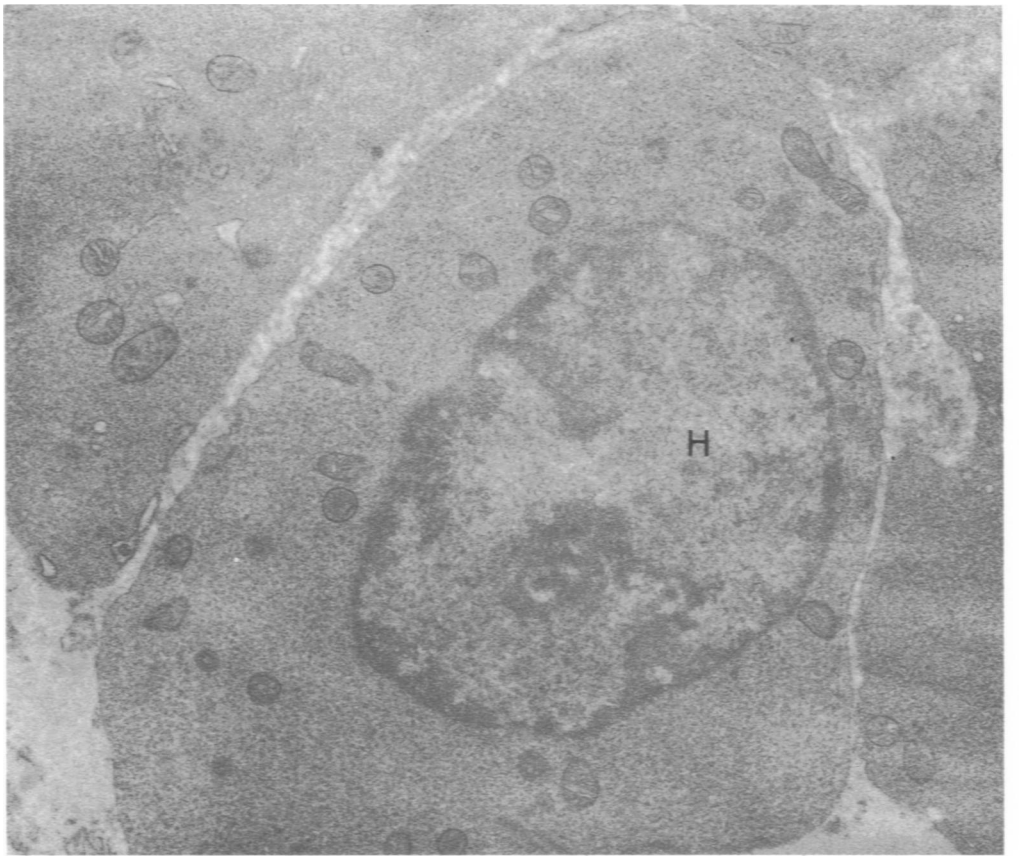


Fig 2—Specimen of guinea pig bone marrow incubated for peroxidase activity. The large erythroid hemocytoblast (*H*) has peroxidase-positive polysomes throughout the cytoplasm. The nuclear matrix is filled with positive particles. The adjacent polymorphonuclear leukocyte (*P*) has several positive cytoplasmic granules. The basophilic leukocyte (*B*) has negative granules (× 16,000).



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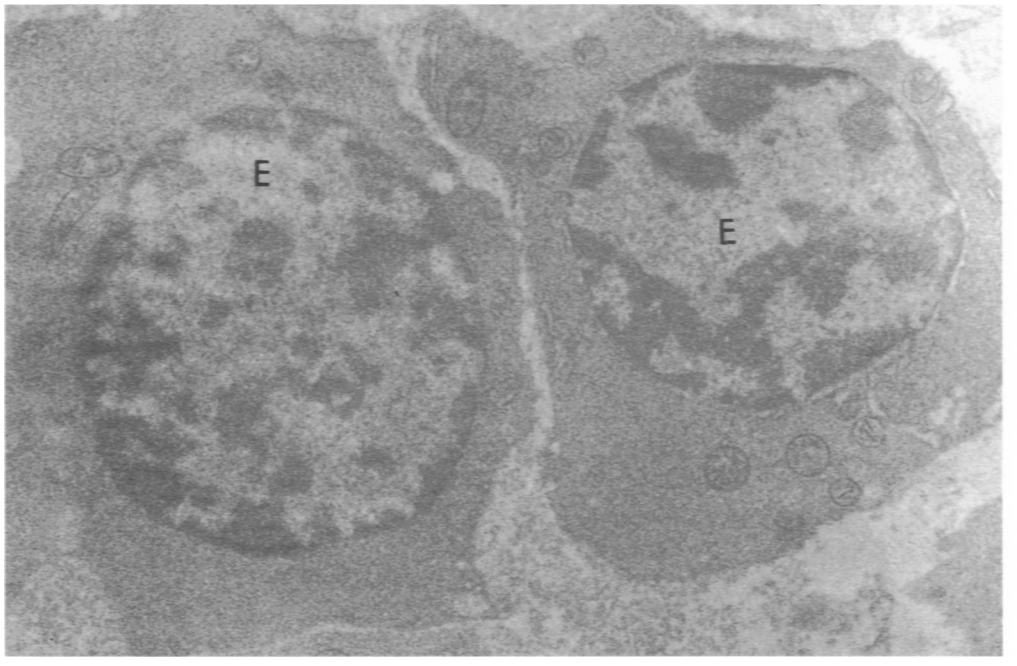

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Fig 3—Specimen of guinea pig bone marrow not incubated for peroxidase activity. The nuclear matrix of the hemocytoblast (*H*) has minimal density. In addition, the cytoplasmic ribosomes are visible but are of low density when compared with the ribosomes in erythroid hemocytoblasts incubated in the medium for peroxidase activity, as seen in Figure 2 (× 14,000). **Fig 4**—Specimen of guinea pig bone marrow not incubated for peroxidase activity. The erythroblasts (*E*) are smaller than hemocytoblasts and have more densely condensed nuclear chromatin. Nuclear matrix is minimally dense. Ribosomes have low density compared with ribosomes in erythroblasts incubated in medium for peroxidase activity, as seen in Figure 5 (× 14,500).

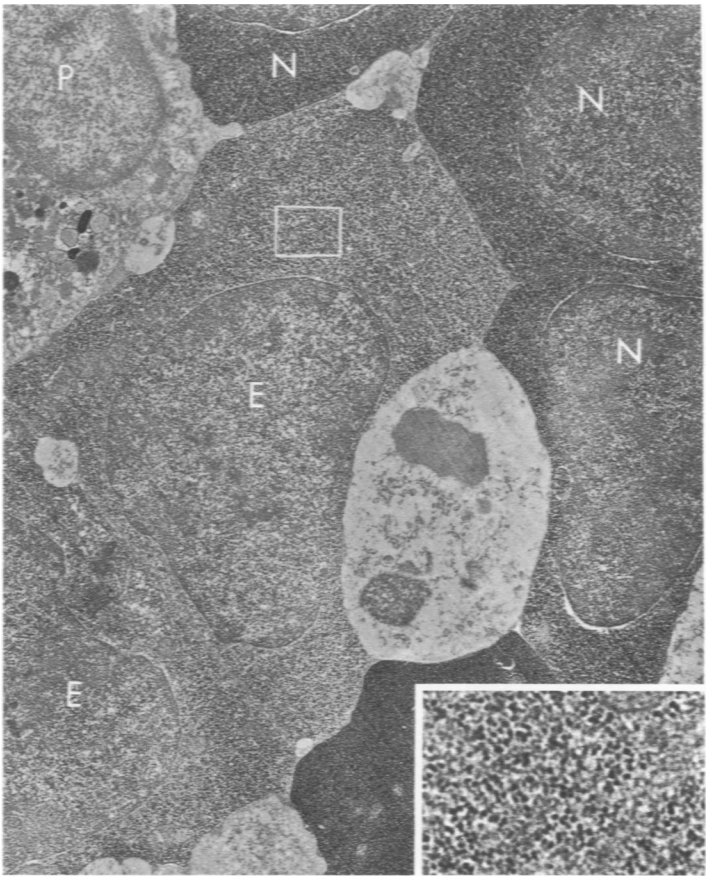


Fig 5—Specimen of guinea pig bone marrow incubated for peroxidase activity. In contrast to Figure 4, erythroblasts (*E*) have intensely positive particles filling nuclear matrix and positive ribosomes in cytoplasm. Polymorphonuclear leukocyte (*P*) with positive granules and several positive normoblasts (*N*) also present (× 9000). Inset is a higher magnification of positive cytoplasmic ribosomes and polysomes in erythroblast cytoplasm (× 29,000).

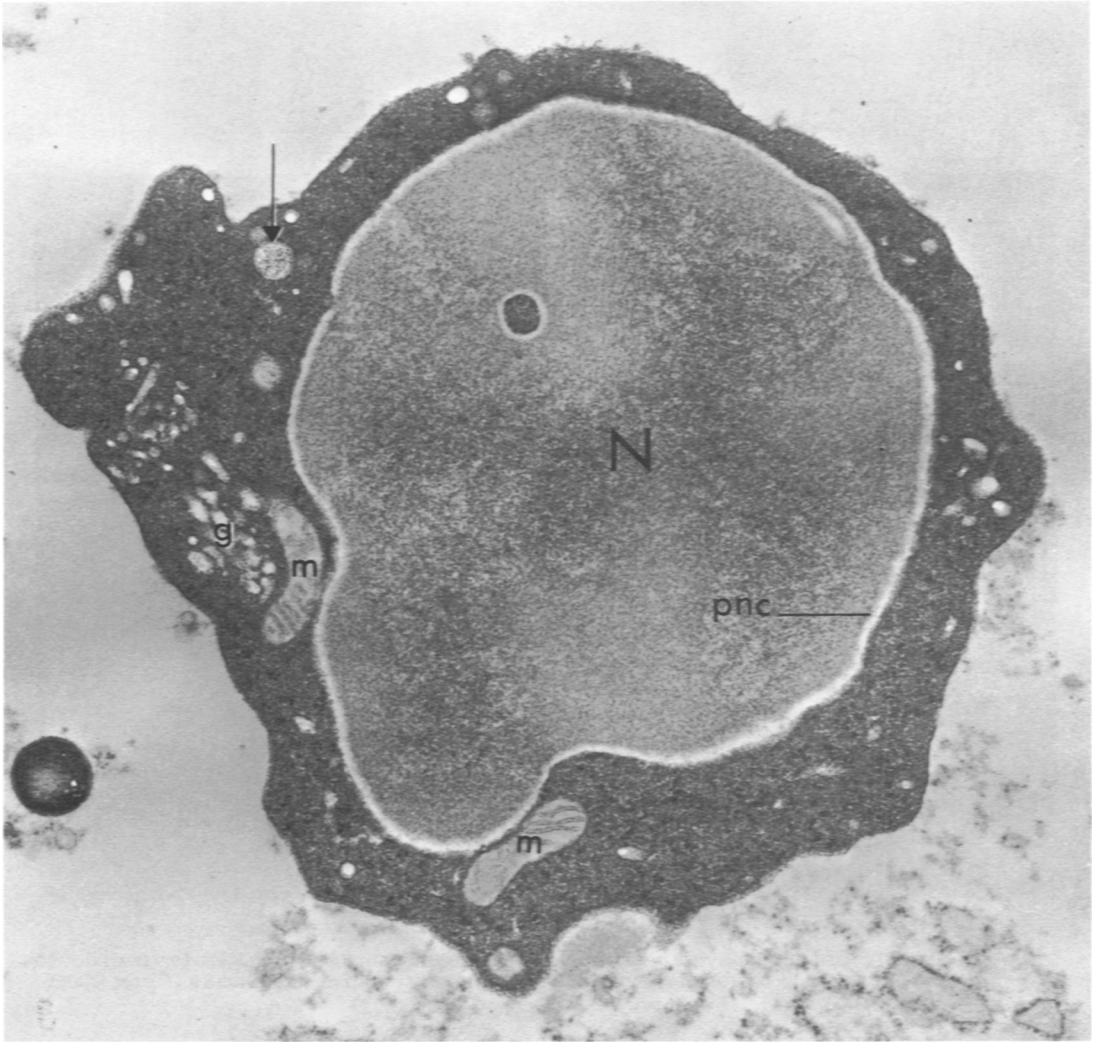


Fig 6—Specimen of guinea pig bone marrow incubated for peroxidase activity. This higher magnification of normoblast (*N*) demonstrates diffuse positive staining in the nuclear matrix and cytoplasm. Perinuclear cisternae (*PNC*), Golgi saccules (*G*) and mitochondria (*m*) are negative. Cytoplasmic vesicle filled with particles of ferritin also present (*arrow*) (× 22,000).

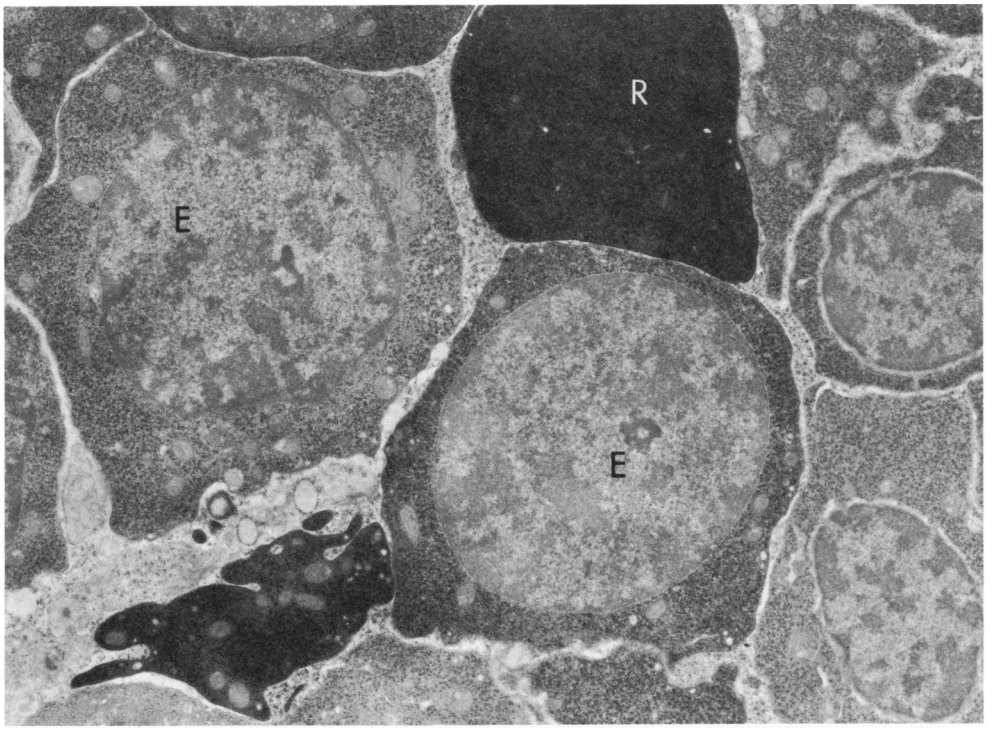


Fig 7—Specimen of phenylhydrazine-treated rabbit bone marrow incubated for peroxidase activity showing several positive erythroblasts (*E*), and one positive reticulocyte (*R*) (× 8000).

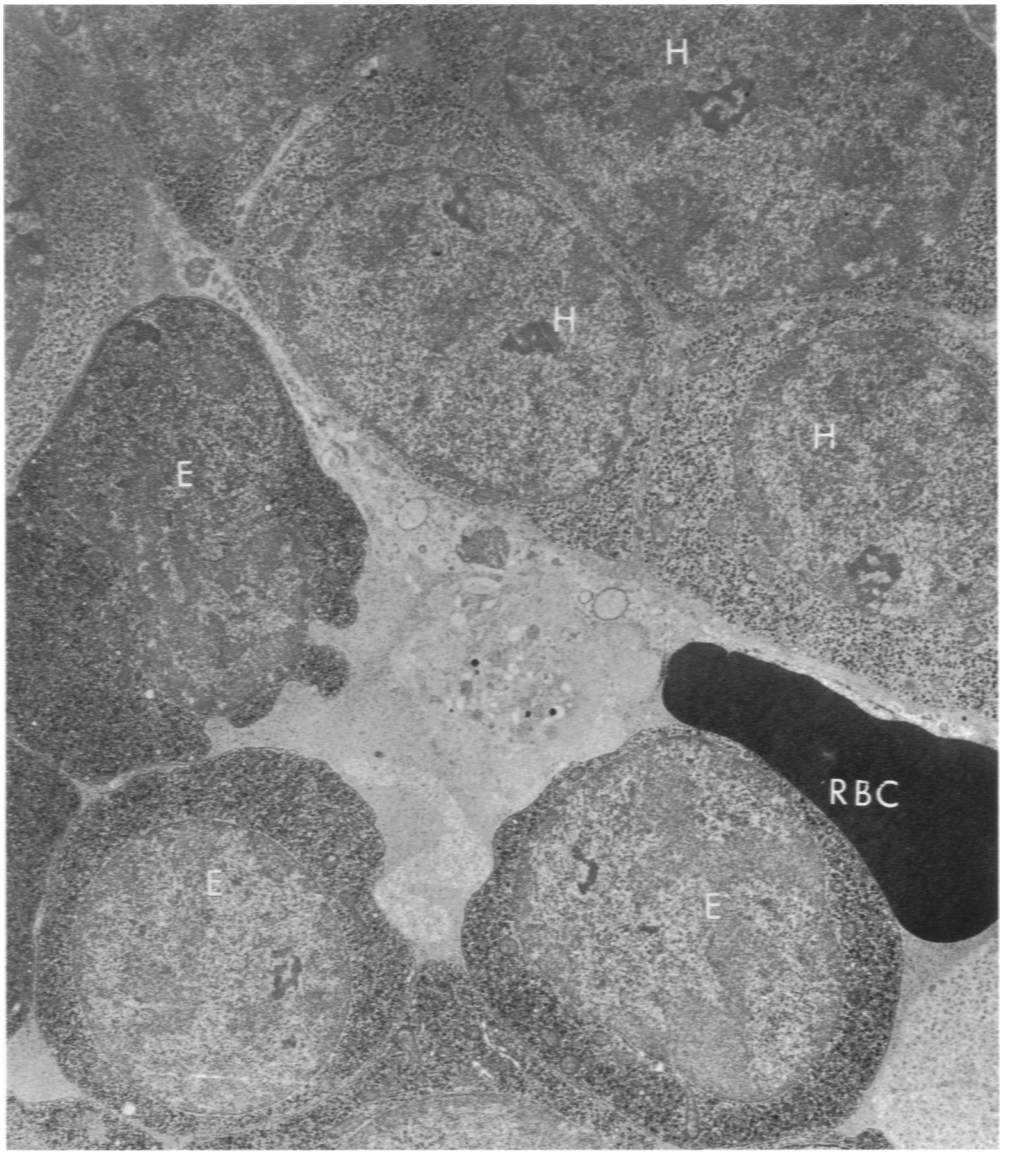


Fig 8—Specimen of phenylhydrazine-treated rabbit bone marrow, preincubated with aminotriazole followed by incubation in complete medium for peroxidase, also contained aminotriazole. Numerous positive erythroid hemocytoblasts (*H*) and erythroblasts (*E*) are seen. A mature red blood cell (*RBC*) also is positive. This lack of inhibition of reaction product formation indicates positive material is not red blood cell catalase (× 8000).

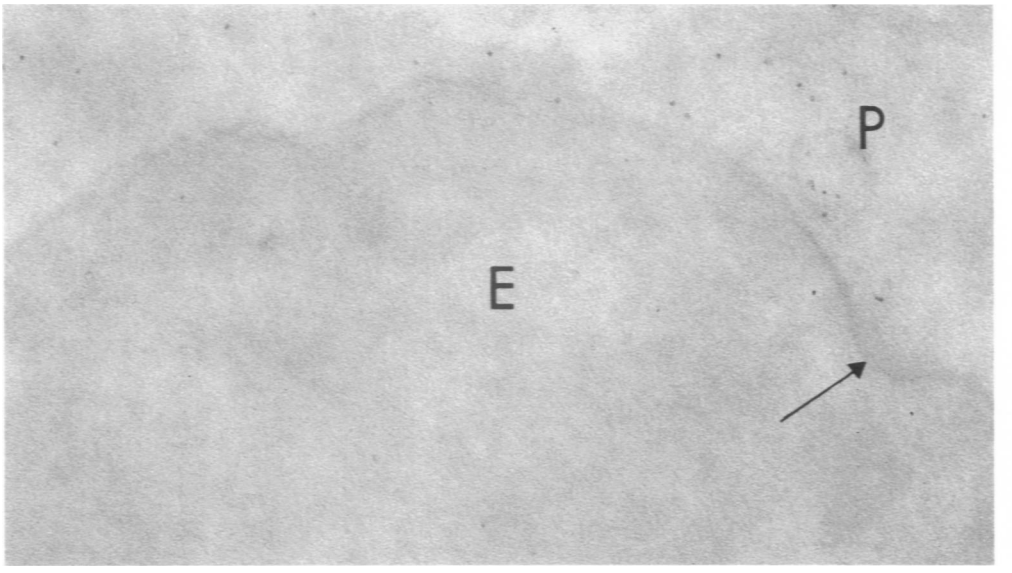
Fig 9—Specimen of phenylhydrazine-treated rabbit bone marrow, incubated in DAB alone followed by 10^{-2}M potassium ferricyanide, was a control for nonspecific absorption of reduced DAB. This high magnification photograph of an erythroblast (*E*) shows cytoplasm of low electron density. Ribosomes are not visible. Cytoplasmic density is similar to that of adjacent plasma (*P*) which contains ferritin particles. Cup-shaped depression (*arrow*) of cell membrane is site to which ferritin particles adhere before being ingested. Compare with Figure 11, a similar magnification photograph of an erythroblast incubated for peroxidase activity (× 120,000).

Fig 10—Specimen of phenylhydrazine-treated rabbit bone marrow incubated for peroxidase activity, illustrating a portion of the cytoplasm of an erythroid hemocytoblast. The interribosomal cytoplasm is minimally dense similar to negative control cells shown in Figure 9. However, numerous positive ribosomes are present; *arrow* points to a positive polysome (× 120,000).

Fig 11—Specimen of phenylhydrazine-treated rabbit bone marrow incubated for peroxidase activity, illustrating a portion of the cytoplasm of an erythroblast. The cytoplasm contains diffuse, amorphous, dense deposits which presumably represent hemoglobin. Compare with the cytoplasm of the negative control erythroblast in Figure 9. The arrow points to a positive polysome. Many other positive ribosomes are present (× 120,000).

Fig 12—Specimen of phenylhydrazine-treated rabbit bone marrow incubated for peroxidase activity. This high magnification photograph illustrates the cytoplasm of a late normoblast. The amount of amorphous electron-dense cytoplasmic material is greater in this more mature cell than in the erythroblast of Figure 11. The arrow points to a positive polysome. Other positive ribosomes are present (× 120,000).

Fig 13—Specimen of phenylhydrazine-treated rabbit bone marrow inhibited with aminotriazole and incubated for peroxidase activity. This high magnification micrograph of the cytoplasm of an erythroid hemocytoblast shows a negative interribosomal cytoplasm as in hemocytoblasts that were not treated with aminotriazole (see Figure 10). The arrow points to a peroxidase-positive polysome indicating that the activity observed is not due to erythrocyte catalase which aminotriazole inhibits (× 120,000).



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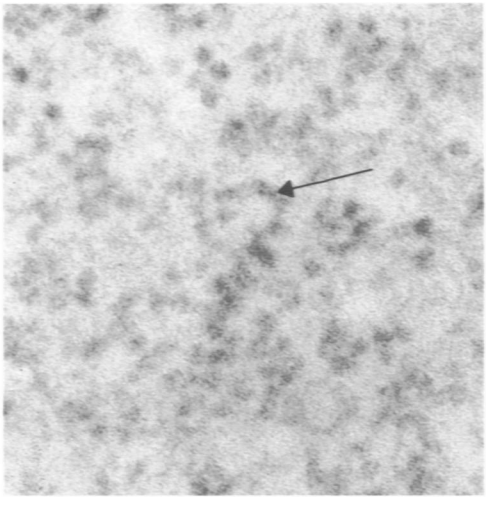

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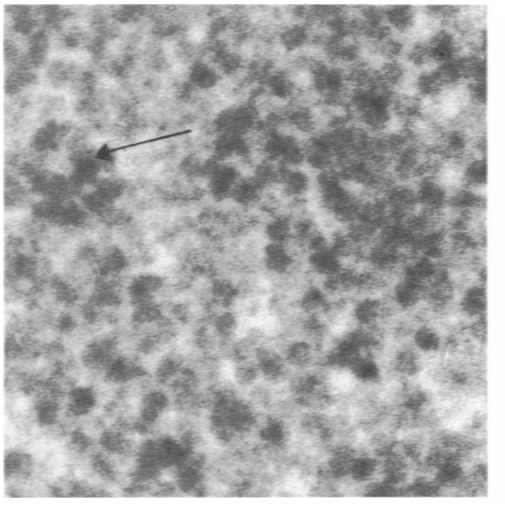

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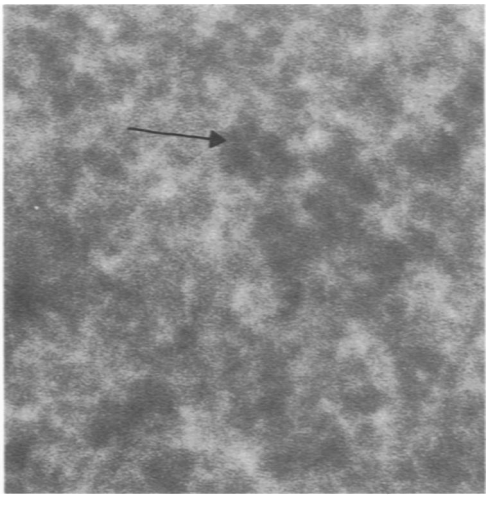

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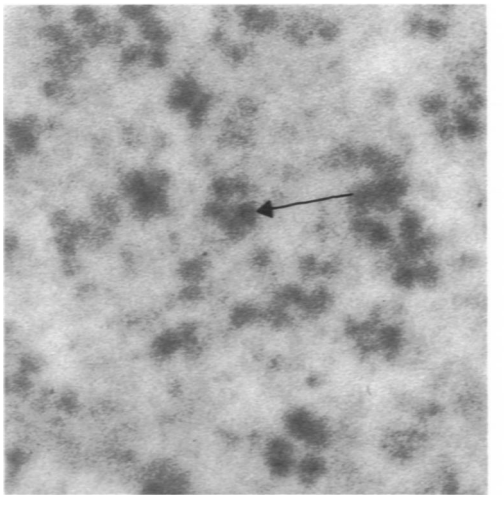

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