

# Development of the Liver in the Chicken Embryo

## II. ERYTHROPOIETIC AND GRANULOPOIETIC CELLS

GENE K. WONG AND MICHAEL J. CAVEY

*Department of Biological Sciences, Division of Zoology, The University of Calgary,  
Calgary, Alberta, Canada*

**ABSTRACT** Hepatic hemopoiesis is apparent in the chicken embryo on day 7 of incubation (Hamburger and Hamilton Stage 30), and a peak in hemopoietic activity occurs on day 14 (Stage 40). During this period, the differentiation of hemopoietic cells was examined by light microscopy and by transmission and scanning electron microscopy. Glycol methacrylate sections were used in lieu of smears to study hemopoietic cells, thus minimizing the problems of cell shrinkage and rupture. The sections were superior to smears for close examination of nuclear and cytoplasmic morphologies and for precise localization of hemopoietic cells to intravascular and extravascular sites. The avian liver is involved directly with erythropoiesis and granulopoiesis only. Erythropoietic cells, occurring in intravascular and extravascular locations, appear throughout the time frame examined. Blood islands with granulopoietic cells were not observed until days 8-9 (Stage 35). Granulopoiesis in the liver produces only eosinophilic leukocytes. Individual granulopoietic cells appear first in the connective tissue sheaths of hepatic vessels, and these cells subsequently congregate into blood islands. Endothelial cells of the sinusoidal linings, through asymmetric divisions, frequently release daughter cells into the circulation, and Kupffer cells are actively engaged in phagocytosis of erythrocytes. From a comparative standpoint, the elements deemed critical to hemopoiesis in the mammalian liver—prehepatocyte population, hepatic vasculature, and compartments for stem cell differentiation—may not hold the same importance in the bird, owing to an inordinate reliance on intravascular hemopoiesis in this vertebrate class. © 1993 Wiley-Liss, Inc.

**Key words:** Capillaries, Chick embryo, Vascular endothelium, Hematopoiesis, Kupffer cells, Liver, Microscopy, Morphogenesis

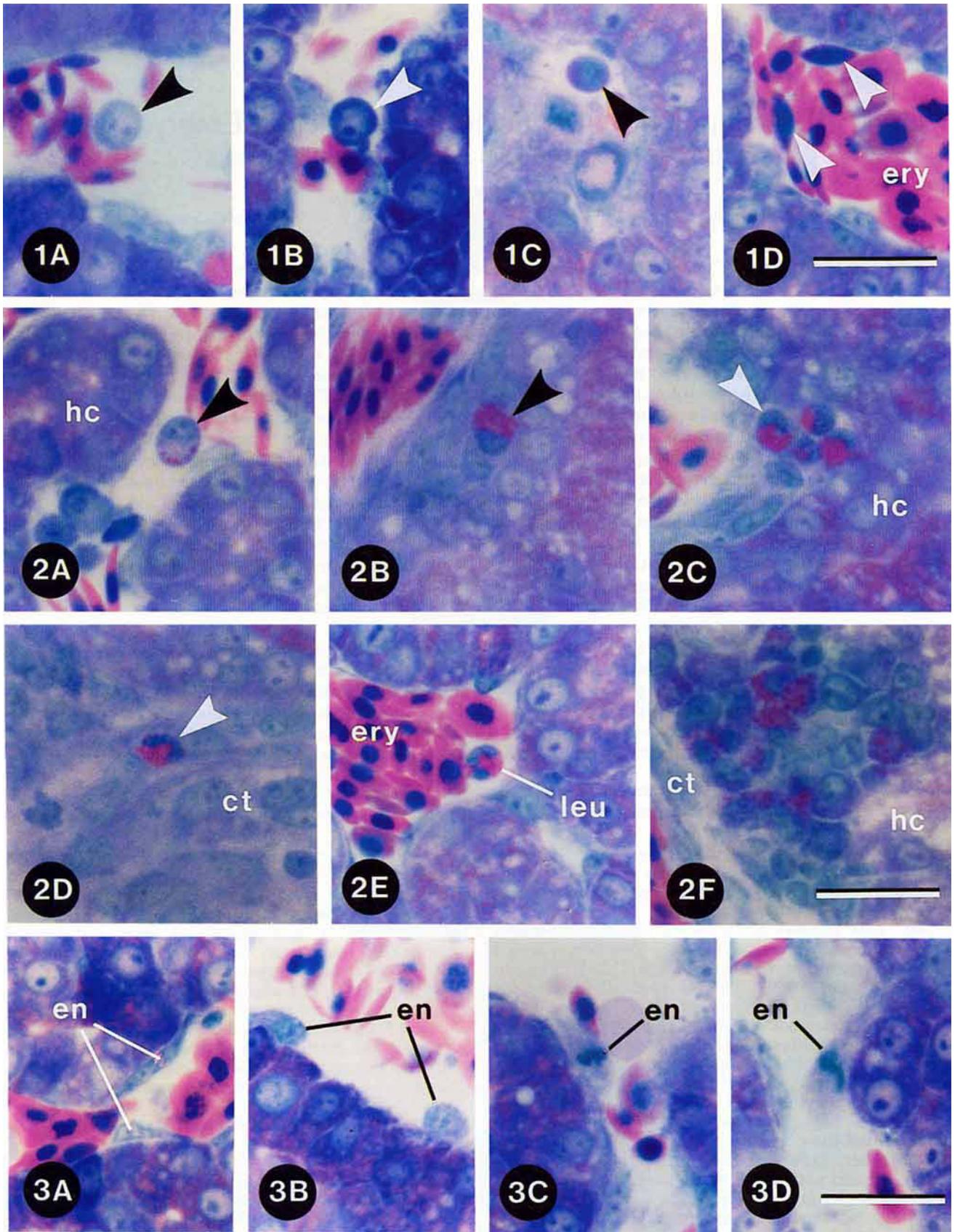
Erythropoietic cells are well characterized in the mammal on the basis of cytoplasmic staining and nuclear morphology. A stem cell (hemocytoblast) passes through five stages (proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, orthochromatic erythroblast or normoblast, and reticulocyte) before a mature erythrocyte enters the circulation (Rifkind et al., 1969). The earliest description of erythropoietic cells in the bird identified two stem cells: the megaloblast of a primitive erythrocyte line and the hemocytoblast of a definitive erythrocyte line (Romanoff, 1960). The megaloblast and hemocytoblast progress through 3 stages (proerythroblast, erythroblast, and proerythrocyte) before becoming mature erythrocytes. Later studies on the chicken embryo, which treated the primitive and definitive erythrocyte lines together, expanded the number of stages to be more consistent with those of the mammal. Intermediate stages between the stem cell and mature erythrocyte thus became the proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, and acidophilic erythroblast or reticulocyte (Ceresa-Castellani and Leone, 1969; Small and Davies, 1972).

Cytoplasmic staining characteristics and nuclear morphology are also used to describe granulopoietic cells. Staining of specific granules in the cytoplasm differentiates the neutrophilic, eosinophilic, and basophilic leukocytes in mammals. A stem cell for the mammalian leukocyte line (myeloblast) proceeds through 5 stages (promyelocyte, myelocyte, metamyelocyte, band cell, and segmented leukocyte) before appearing as a mature granular leukocyte (Huser, 1970). Granulopoietic cells of the bird resemble those of the mammal. The promyelocyte I, promyelocyte II, myelocyte, and metamyelocyte were originally proposed as intermediate stages between the myeloblast and ma-

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Mr. G.K. Wong is now at Department of Anatomy and Cell Biology, University of Alberta, Faculty of Medicine, Edmonton, Alberta, Canada T6G 2H7.

Address reprint requests to Dr. M.J. Cavey, Division of Zoology, Department of Biological Sciences, The University of Calgary, 2500 University Drive N. W., Calgary, Alberta, Canada T2N 1N4.



Figs. 1-3.

**TABLE 1. Stages of embryonic development with corresponding embryonic ages (after Hamburger and Hamilton, 1951) and hemopoietic landmarks**

| Stage | Incubational age (days) | Hemopoietic landmarks  |
|-------|-------------------------|--|
| 29    | 6.0                     | Macrophages in the process of phagocytosing primitive erythrocytes (present study) |
| 30    | 6.5                     |  |
| 31    | 7.0                     | Onset of hepatic erythropoiesis (Romanoff, 1960; Medda et al., 1977)               |
| 32    | 7.5                     |  |
| 33    | 7.5 to 8.0              |  |
| 34    | 8.0                     |  |
| 35    | 8.0 to 9.0              | Onset of hepatic granulopoiesis (present study)                                    |
| 36    | 10.0                    | Macrophages begin to phagocytose definitive erythrocytes (present study)           |
| 37    | 11.0                    | Intense granulopoietic activity commences (Romanoff, 1960)                         |
| 38    | 12.0                    |  |
| 39    | 13.0                    |  |
| 40    | 14.0                    | Peak of hepatic hemopoiesis (Medda et al., 1977)                                   |
| 41    | 15.0                    |  |
| 42    | 16.0                    |  |

ture granular leukocyte (Romanoff, 1960). Granular leukocytes of the bird were subclassified as eosinophilic, pseudoeosinophilic, or basophilic on the basis of the staining characteristics of their specific granules. A more recent scheme recognizes the eosinophilic and basophilic leukocytes but substitutes a heterophilic leukocyte for the pseudoeosinophilic cell. Intermediate stages of cell differentiation now include the progranulocyte, myelocyte (mesomyelocyte), metamyelocyte, and band cell (Campbell, 1988).

Extensive studies of the mammalian liver, a major site of intraembryonic hemopoiesis, have identified the prehepatocyte population, hepatic vasculature, and compartments for stem cell differentiation as elements critical to hemopoiesis. The "hemopoietic environment," a collective term for all three elements, can be subdivided into microenvironments for the erythropoi-

etic, granulopoietic, lymphopoietic, and thrombopoietic cell lines (Medlock and Haar, 1983). Hepatocytes within each microenvironment conceivably provide a cytotericulum which supports the hemopoietic cells during maturation (Asano et al., 1987).

The aim of the present study was to better characterize the elements of the hemopoietic environment in the liver of the chicken embryo and consider their impact on blood cell differentiation. Examination of the prehepatocyte population and preliminary coverage on the hepatic vasculature appear in a companion paper (Wong and Cavey, 1992). Erythropoietic cells of the definitive line and granulopoietic cells were identified by light- and electron-microscopic techniques for comparison to their mammalian counterparts. The involvement of sinusoidal cells in hemopoiesis is documented.

## MATERIALS AND METHODS

The embryonic material, specimen numbers, and preparative procedures used in this study are presented in a companion paper (Wong and Cavey, 1992). To better examine the hemopoietic cells in glycol methacrylate-embedded livers, a set of spaced serial sections was stained with a solution of 1 part Giemsa stain and 13 parts demineralized water at pH 4.8 (Du Pont Company, 1981).

Nuclear and cytoplasmic features were used to identify the erythropoietic and granulopoietic cells (Ceresa-Castellani and Leone, 1969; Campbell, 1988) in the histological and ultrastructural sections.

## RESULTS

### *Hepatic Hemopoiesis*

At Hamburger and Hamilton (1951) Stage 30 (Table 1), erythrocytes and granulocytes at various stages of maturation occur intravascularly and extravascularly in the liver. On occasion, lymphocytes and monocytes are also found extravascularly, but these cells tend to remain in the bloodstream. Thrombocytes appear exclusively in the circulation. Lymphopoiesis, monopoiesis, and thrombopoiesis were never observed in the em-

Fig. 1. Erythropoietic cells. **A:** Proerythroblast (arrowhead) from the general circulation. The nucleus contains two prominent nucleoli. **B:** Basophilic erythroblast (arrowhead) with a pale nucleus and prominent nucleolus. **C:** Polychromatophilic erythroblast (arrowhead) with a "checkerboard" nucleus and moderately acidophilic cytoplasm. **D:** Acidophilic erythroblasts (arrowheads) with shapes comparable to those of circulating erythrocytes (ery).  $\times 1,125$ . Bar, 20  $\mu$ m.

Fig. 2. Granulopoietic cells. **A:** Promyelocyte (arrowhead) beginning to accumulate specific (eosinophilic) granules. hc, hepatic cord. **B:** Eosinophilic myelocyte (arrowhead) in the connective tissue of a blood vessel. **C:** Metamyelocyte (arrowhead), together with other eosinophilic cells at various stages of maturation, in vascular connective tissue. hc, hepatic cord. **D:** Band cell (arrowhead) in the connective tissue (ct) of a blood vessel. **E:** Mature eosinophilic leukocyte (leu) in circulation with erythrocytes (ery). **F:** Differentiating leukocytes in blood islands within the vascular connective tissue (ct). hc, hepatic cord.  $\times 1,125$ . Bar, 20  $\mu$ m.

Fig. 3. Endothelial cells of the sinusoidal linings. **A:** Flattened endothelial cells (en) are the predominant cells (cf. Fig. 11A). **B:** Endothelial cells (en) often bulge into the lumina (cf. Fig. 11B). **C:** Both daughter cells of an endothelial cell (en) division may remain in the sinusoidal lining (cf. Fig. 11C). **D:** An endothelial cell (en) division may release one daughter cell to the bloodstream and retain the other in the sinusoidal lining (cf. Fig. 11D).  $\times 1,125$ . Bar, 20  $\mu$ m.

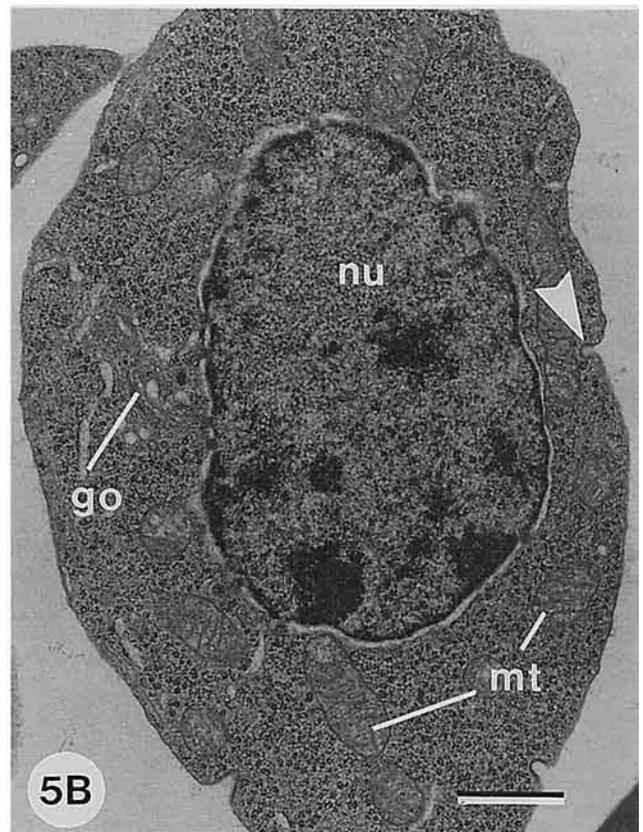
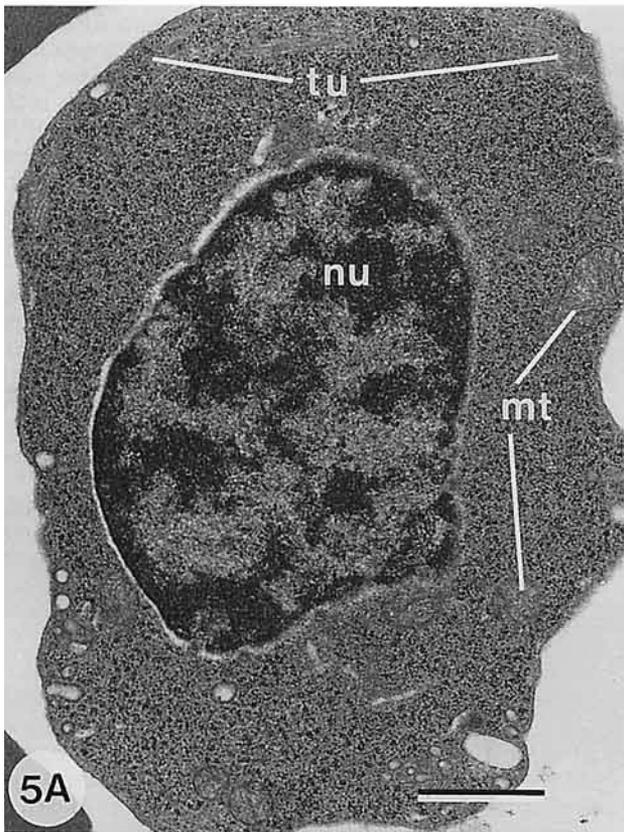
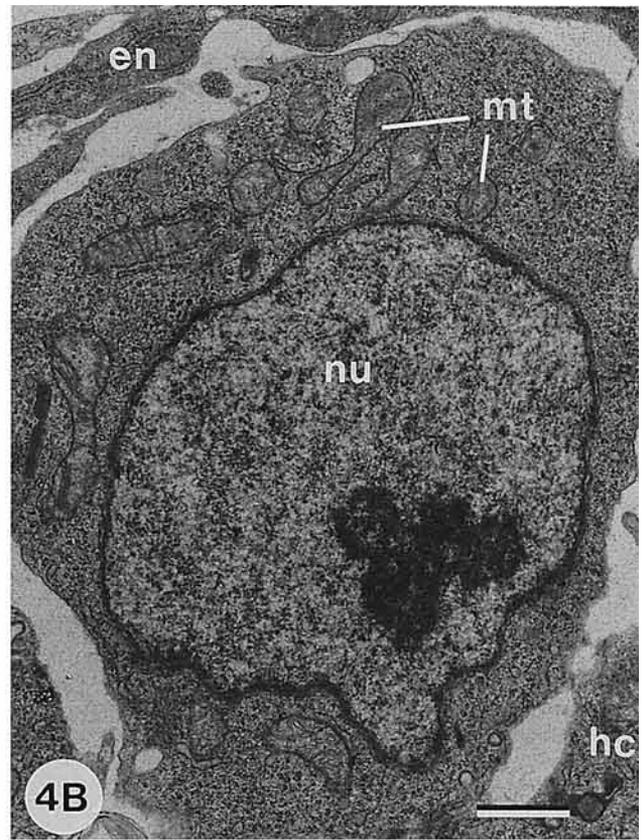
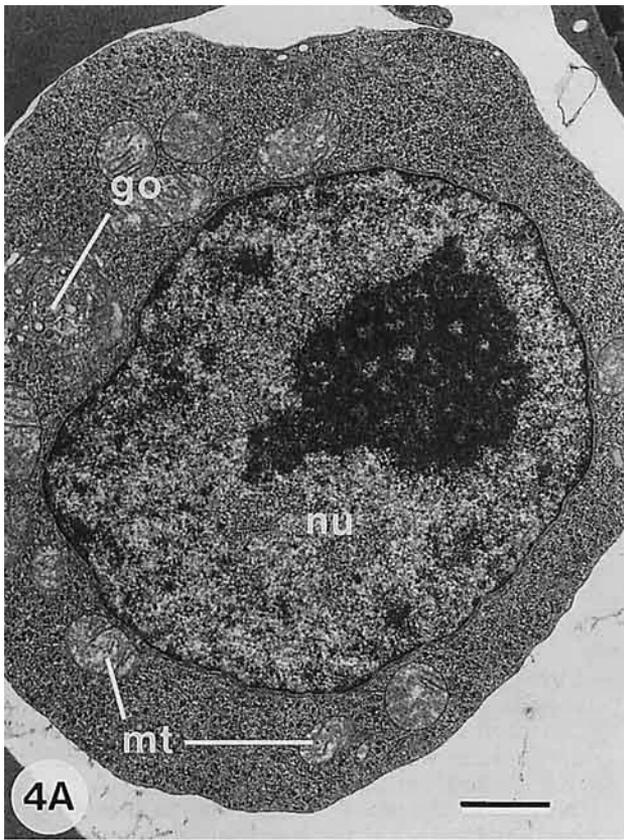


Fig. 4. Proerythroblasts. **A:** The nucleus (nu) of a proerythroblast (go, Golgi apparatus; mt, mitochondrion) from the general circulation displays a massive nucleolus. The cytoplasm is packed with ribosomes.  $\times 11,600$ . Bar,  $1 \mu\text{m}$ . **B:** A perisinusoidal cell situates between a proerythroblast (mt, mitochondrion; nu, nucleus) and an endothelial cell (en). hc, hepatic cord.  $\times 12,400$ . Bar,  $1 \mu\text{m}$ .

Fig. 5. Basophilic erythroblasts. **A:** Clumps of condensed chromatin are observed in the nucleus (nu) of a basophilic erythroblast (mt, mitochondrion; tu, microtubules).  $\times 16,500$ . Bar,  $1 \mu\text{m}$ . **B:** This basophilic erythroblast (go, Golgi apparatus; mt, mitochondrion; nu, nucleus) from the bloodstream shows evidence of plasmalemmal vesiculation (arrowhead).  $\times 14,000$ . Bar,  $1 \mu\text{m}$ .

bryonic stages studied, although Romanoff (1960) claims that lymphopoiesis does take place in the liver.

Improper blood-smearing techniques can create thick preparations and induce cell shrinkage and/or rupture (Campbell, 1988). Such problems can be overcome by embedding tissue in glycol methacrylate. Sectioned material is superior to smears for detailed observations of nuclear and cytoplasmic features and for accurate localization of hemopoietic cells to intravascular and extravascular sites. Common stains applied to blood smears can be adapted for use on embedded and sectioned cells.

#### *Erythropoiesis*

Erythropoietic cells of the avian embryo, identified by light microscopy, share many cytoplasmic and nuclear characteristics with their mammalian counterparts (Fig. 1). The notable difference in erythropoiesis between the two groups is the absence of an anucleate cell in the bird. Avian erythrocytes retain, rather than extrude, their pycnotic nuclei. The proerythroblast, basophilic erythroblast, and polychromatophilic erythroblast are spherical cells with spherical nuclei. The acidophilic erythroblast and mature erythrocyte are biconvex cells with spherical or ovoid nuclei.

The proerythroblast nucleus shows dispersed chromatin and possesses one or two substantial nucleoli (Fig. 1A). The cytoplasm of a proerythroblast contains a Golgi apparatus, mitochondria, and numerous ribosomes (Fig. 4A). Ribosomes account for the moderate basophilia of the cytoplasm. Neither granular nor agranular endoplasmic reticulum (ER) is prevalent, and small cytoplasmic vesicles likely derive from plasmalemmal invaginations. The random microtubules, observed throughout the cytoplasm of the proerythroblast by Small and Davies (1972), were never encountered in the present study.

Proerythroblasts in the liver exist both intravascularly and extravascularly. Extravascular cells either occur in the connective tissue sheaths of blood vessels or mingle with the connective tissue fibers in the perisinusoidal spaces (Fig. 4B). Both intravascular and extravascular proerythroblasts are capable of division. Extravascular blood islands, akin to those in the mammalian liver (Ackerman et al., 1961), were not observed.

The nucleus of a basophilic erythroblast retains one or two nucleoli in early phases of differentiation (Fig. 1B). Chromatin condensation begins beneath the nuclear envelope and proceeds toward the central nucleoplasm (Fig. 5A,B). Intense basophilia of the cytoplasm can be attributed to a growing concentration of free ribosomes. A Golgi apparatus is no longer evident in the cytoplasm, and mitochondria are few in number. Microtubules accumulate in the peripheral cytoplasm, immediately beneath the plasmalemma, as the cell prepares to change shape. Coated pits and vesicles are also found in a basophilic erythroblast. Plasmalemmal vesiculation is higher in basophilic erythroblasts than in proerythroblasts.

Hemoglobin formation in a polychromatophilic erythroblast makes the cytoplasm more acidophilic (Fig. 1C). Acidophilic and basophilic patches impart a grayish coloration to the cytoplasm in light-microscopic sections treated with Giemsa stain. Free ferritin

granules are observed in the cytoplasm shortly before the onset of hemoglobin production (Fig. 6A,B). Some small aggregations of granules can be found but, contrary to the findings of Ceresa-Castellani and Leone (1969), they are not enveloped by membranes to form siderosomes. Ferritin granules accumulate into larger and larger aggregations as a cell matures. With further chromatin condensation, the nucleoli disappear and the nucleoplasm assumes a "checkerboard" appearance. Organelles appear in various stages of degeneration. In later phases of its differentiation, the polychromatophilic erythroblast assumes a quasi-biconvex shape, similar to that of a mature erythrocyte. The density of microtubules beneath the plasmalemma is higher than elsewhere in the cytoplasm.

Acidophilic erythroblasts have a biconvex shape like mature erythrocytes. Cytoplasmic ferritin declines as more and more granules are mobilized for hemoglobin production (Fig. 7A,B). In a mature cell, the cytoplasmic matrix can be very electron-opaque owing to its hemoglobin content. Hemoglobin accounts for the increasing acidophilia of the cytoplasm. Mitochondria and other organelles continue to degenerate and are eventually eliminated from the cell. The nucleus must condense further to acquire its final form.

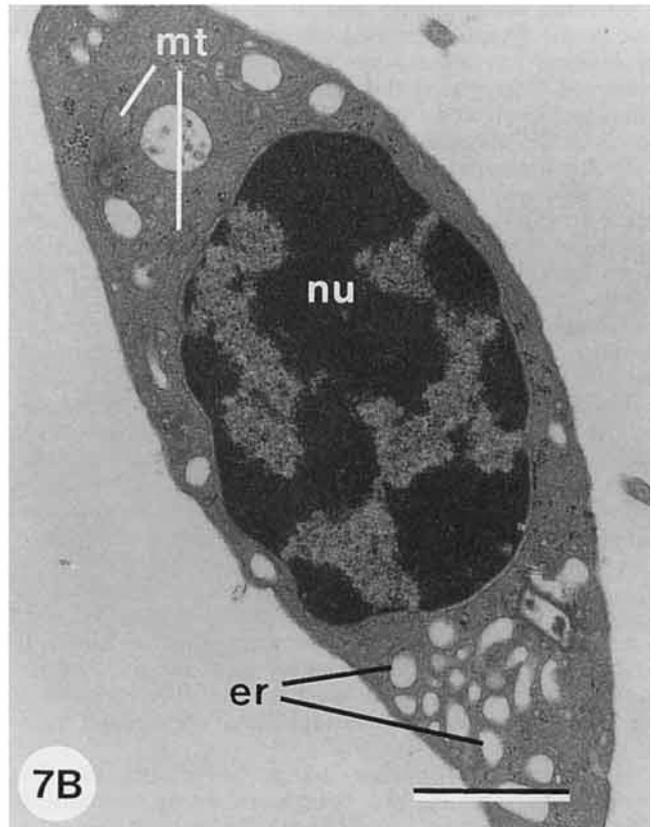
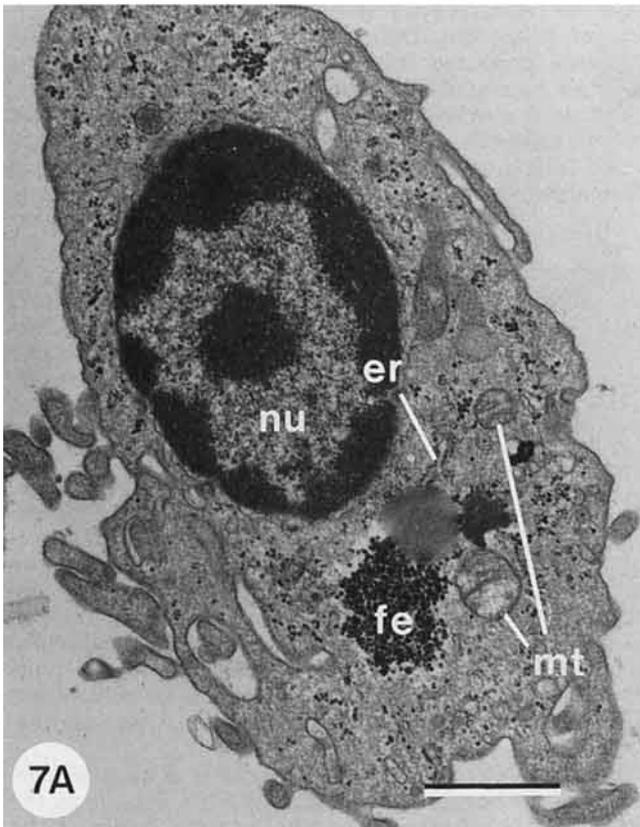
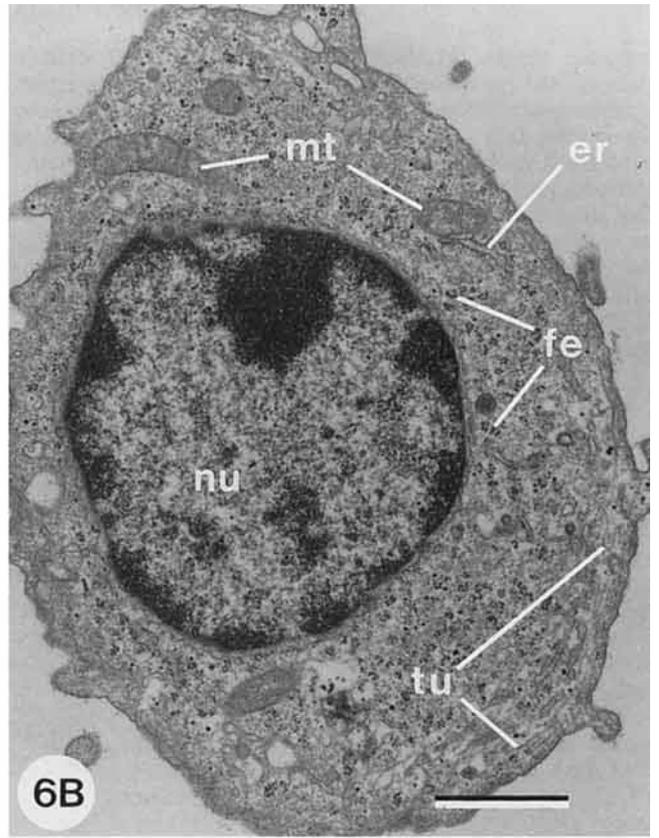
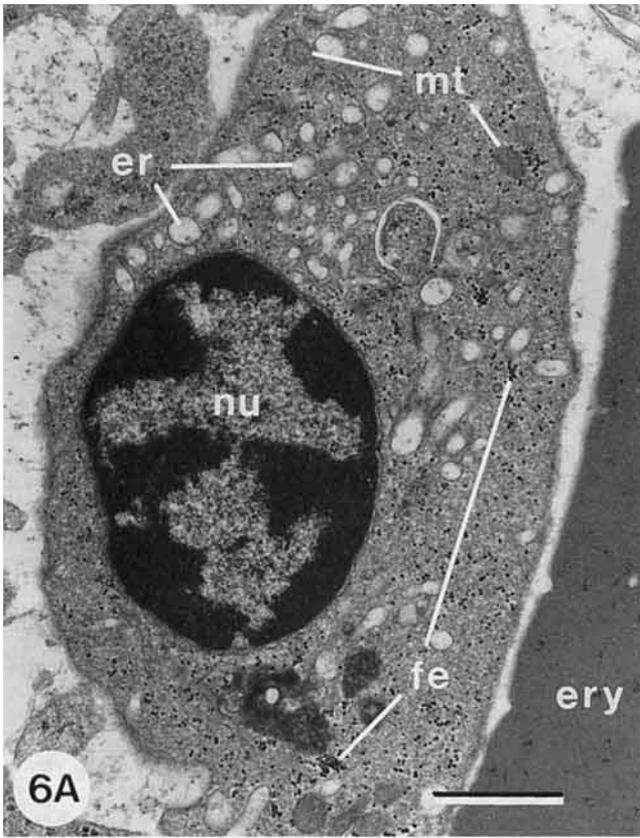
Mature erythrocytes of the primitive and definitive lines can be distinguished in photomicrographs. Both have condensed nuclei and acidophilic cytoplasm (Fig. 1D), but they are disparate in size (Romanoff, 1960). A primitive erythrocyte is distinctly larger than a definitive cell (Fig. 8A,B). A primitive erythrocyte may contain the remnants of organelles, dispersed among persistent ribosomes. Definitive erythrocytes, being the cell line produced intraembryonically, contain little else but hemoglobin in the cytoplasm. Plasmalemmal blebbing is a consistent feature of mature erythrocytes in both lines (Ceresa-Castellani and Leone, 1969). Flattened cells display more surface activity than rounded (less mature) erythrocytes (Fig. 9).

#### *Sinusoidal Cells*

Macrophages control the erythrocyte population in the embryo by phagocytosis. They are allied mainly with the sinusoidal linings, where they probably represent immature Kupffer cells. Free macrophages can also be found in the bloodstream. They occasionally leave the circulation, taking up residence in the perisinusoidal spaces or in the extracellular spaces between hepatocytes of the cords.

Ultrastructurally, phagocytotic vacuoles and residual bodies occupy the bulk of the cytoplasm of a macrophage (Fig. 10). The nucleus is displaced to the peripheral cytoplasm, along with most other organelles and inclusions. A close association with the sinusoidal lining is often evident, and in some cases, the plasmalemmata of the macrophage and endothelial cell come into contact. As the sinusoids become progressively more discontinuous, the immature Kupffer cells either fill the nascent gaps or displace the existing endothelial cells.

Fukuda (1976) noted the appearance of hepatic macrophages at Stage 34, coinciding with a decline in the phagocytotic activity of endothelial cells. In our study, however, macrophages were observed as early as Stage 29, a time when primitive erythrocytes are being re-



Figs. 6-7.

placed by definitive cells. Macrophages participate in the destruction of primitive erythrocytes and, as this population wanes, begin to phagocytose aging cells of the definitive line. Phagocytosis of definitive erythrocytes commences around Stage 36.

Endothelial cells of the hepatic sinusoids are either flattened (Fig. 3A) or rounded (Fig. 3B) in configuration, and both forms are capable of mitotic division (Fig. 3C,D). Most endothelial cells in the sinusoidal linings are flattened (Figs. 3A, 11A). The endothelial cell nucleus is anucleolate. Diminutive mitochondria and short cisternae of the granular endoplasmic reticulum are encountered near the nucleus. Many ribosomes and polysomes are distributed throughout the cell, and vacuoles become increasingly evident from Stage 34 onward.

Rounded endothelial cells bulge into the sinusoidal lumina (Figs. 3B, 11B). These cells have an ovoid nucleus which may display a single nucleolus. Organelles in a rounded cell are similar to those in a flattened cell, and plasmalemmal invaginations are prevalent. The rounded endothelial cells also maintain a close association with the perisinusoidal cells and hepatocytes. Flattened cells gradually supplant the bulging cells during hepatic development, and the endothelium assumes its definitive simple squamous classification.

Division of endothelial cells may yield one of two results. If the mitotic apparatus is centrally positioned, a cell appears to divide equally, and both daughter cells probably remain in the sinusoidal lining (Figs. 3C, 11C). If the mitotic apparatus is displaced toward the edge of a cell, one daughter cell may be released into the bloodstream, and the other stays in the sinusoidal lining (Figs. 3D, 11D). The rounding of endothelial cells and their subsequent projection into the lumen, observed as early as Stages 18 and 19, were once viewed as preparatory changes for cell division (Fukuda, 1976). Electron micrographs of flattened cells in the process of division (Fig. 11C) would indicate that such changes are not mandatory.

#### *Granulopoiesis*

Granulopoiesis has not been studied extensively in the avian liver. Kingsbury et al. (1956) noted a few eosinophilic leukocytes at intravascular and extravas-

cular sites at Stage 40. The number of leukocytes subsequently increased, and extravascular clusters were observed at Stages 43 and 44. Granulopoietic cells are easily identified in histological sections (Fig. 2). The promyelocyte, myelocyte, metamyelocyte, band cell, and leukocyte are spherical cells.

A promyelocyte has an eccentric nucleus containing dispersed chromatin and one or two nucleoli (Fig. 2A). Eosinophilic (specific) granules begin to accumulate in the periphery of the cell, at the side opposite to the nucleus. The cytoplasm is moderately basophilic. Compared to a promyelocyte, a myelocyte has more specific granules, and its cytoplasm has stronger basophilia (Fig. 2B). Its ovoid nucleus is often indented, and there are clumps of condensed chromatin beneath the nuclear envelope. The nucleoli, although still present, are no longer prominent in photomicrographs. Metamyelocytes may differentiate alone or in masses which include other granulopoietic cells (Fig. 2C). The nucleus of a metamyelocyte is typically reniform and anucleolate, and condensed chromatin is apparent throughout the nucleoplasm. The essential difference between a metamyelocyte and a band cell relates to nuclear morphology. The nucleus of a band cell is horseshoe shaped, with thick bridges ("bands") joining the lobes (Fig. 2D). As the bridges narrow, the 3-4 nuclear lobes of a mature eosinophilic leukocyte can be clearly delimited (Fig. 2E).

Basophilic and heterophilic leukocytes have not been observed in the liver. Circulating eosinophilic leukocytes can be found in Stage 34, but evidence of granulopoiesis is not detected until Stage 35, indicating that the earliest cells in the bloodstream originate outside the liver. Upon commencement of granulopoiesis in the liver, granulopoietic cells are found individually and in groups within the connective tissue sheaths of blood vessels. From Stage 35 onward, granulopoietic blood islands become well established extravascularly (Fig. 2F). These observations indicate a much earlier appearance of granulocytes and granulopoietic blood islands than recorded previously (Kingsbury et al., 1956).

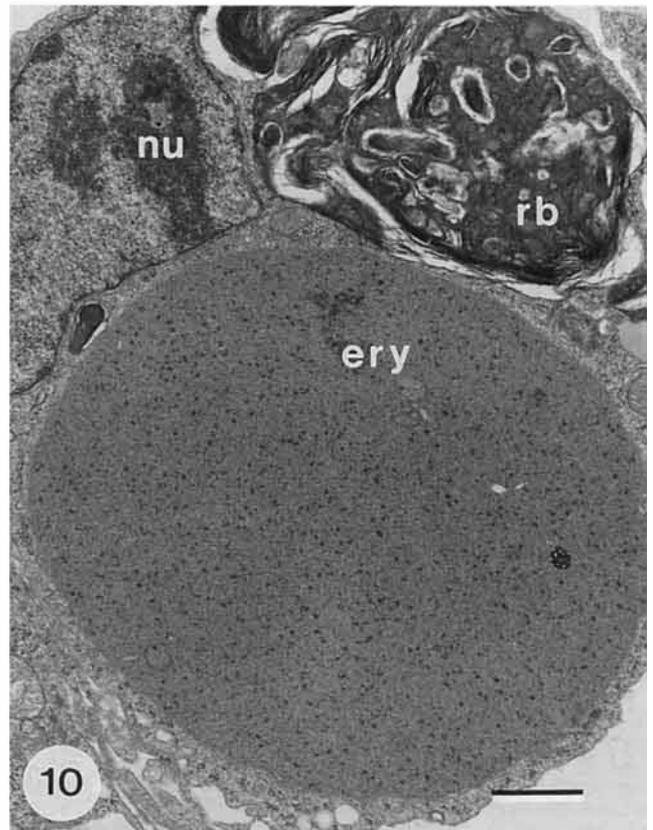
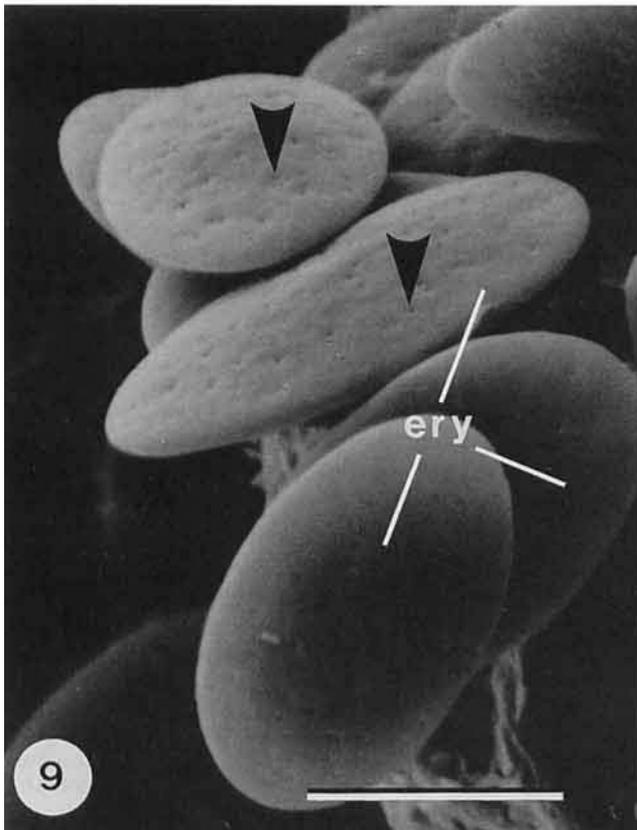
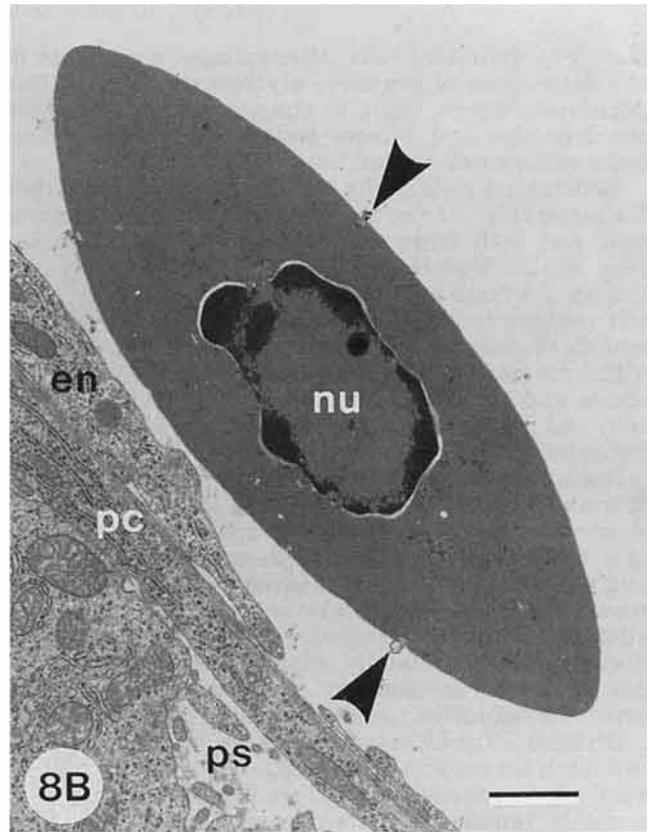
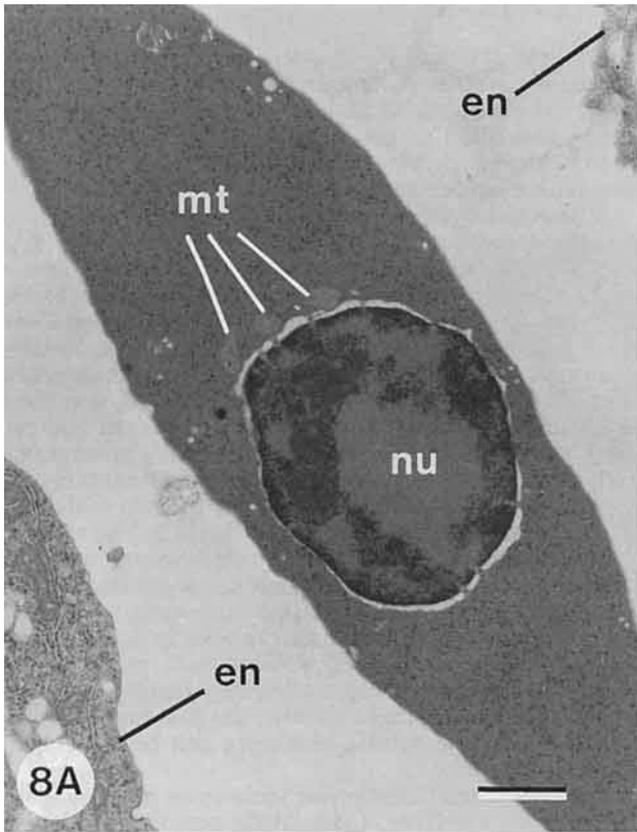
The specific granules of mature eosinophilic leukocytes are rod shaped (Fig. 12). Endoplasmic reticulum, mitochondria, and ribosomes are rather scarce in these cells. The nucleus contains a high proportion of condensed chromatin. Extravascular granulocytes may come into close contact with one another, but there is no evidence suggesting that the cells were ever physically linked.

#### *Other Cells in the Hepatic Circulation*

Lymphocytes, monocytes, and thrombocytes are found in the hepatic circulation. The circulating lymphocyte has a spherical nucleus and a thin rim of basophilic cytoplasm. Chromatin condensation is apparent in the central nucleoplasm and subjacent to the inner membrane of the nuclear envelope. The perinuclear cytoplasm is rich in free ribosomes and polysomes (Fig. 13). Membranous organelles, such as mitochondria and endoplasmic reticulum, are scarce. Lymphocytes can be distinguished from proerythroblasts and basophilic erythroblasts by lower concentrations of ribosomes and mitochondria and by a greater fraction of cell volume occupied by the nucleus. Lymphocytes may

Fig. 6. Polychromatophilic erythroblasts. **A:** Ferritin granules (fe) occur throughout this polychromatophilic erythroblast (er, endoplasmic reticulum; nu, nucleus). Numerous cytoplasmic vesicles may be the remnants of membranous organelles such as mitochondria (mt). ery, erythrocyte.  $\times 16,300$ . Bar,  $1\ \mu\text{m}$ . **B:** A lowly differentiated polychromatophilic erythroblast (er, endoplasmic reticulum; mt, mitochondrion; nu, nucleus) reveals a subplasmalemmal band of microtubules (tu) and small accumulations of ferritin granules (fe).  $\times 17,200$ . Bar,  $1\ \mu\text{m}$ .

Fig. 7. Acidophilic erythroblasts. **A:** An early acidophilic erythroblast (er, endoplasmic reticulum; nu, nucleus) is assuming the biconvex shape of an erythrocyte. The cytoplasm contains large aggregations of ferritin granules (fe), and the mitochondria (mt) are undergoing degenerative changes.  $\times 17,700$ . Bar,  $1\ \mu\text{m}$ . **B:** Hemoglobin in a late acidophilic erythroblast (er, endoplasmic reticulum; mt, mitochondrion; nu, nucleus) accounts for the cytoplasmic density. A few degenerating organelles can still be identified. Cell shape and nuclear morphology are approaching their final forms.  $\times 20,400$ . Bar,  $1\ \mu\text{m}$ .



Figs. 8-10.

also reside in extravascular locations, but these cells lack the rounded contours of circulating cells. Kingsbury et al. (1956) encountered a large number of lymphocytes in the hepatic blood vessels at Stage 43.

Monocytes, like lymphocytes, have a basophilic cytoplasm. The nucleus of a monocyte, however, is indented. Clumps of condensed chromatin appear in the central nucleoplasm and beneath the nuclear envelope (Fig. 14). The cytoplasm contains a few mitochondria, polysomes, and a small number of spherical non-specific granules.

Thrombocytes display dense nuclei, similar to those of erythrocytes. The cytoplasm has a slight acidophilia. Thrombocytes from the circulation are commonly found in clumps, but individual cells do sometimes occur.

## DISCUSSION

Developing extravascularly and in close association with the hepatocytes, erythropoietic cells must gain entry to the sinusoids secondarily (Ackerman et al., 1961; Bankston and Pino, 1980; Asano et al., 1987). Movement into the lumina involves a transmural migration of erythropoietic cells across the sinusoidal linings in mammalian embryos (Bankston and Pino, 1980). Endothelial cells form temporary "migration pores," either de novo or by the widening of fenestrae, which close after erythropoietic cells have moved into the lumina. A comparable route of cell migration in the bird is doubtful. Endothelial cells in the avian liver lack fenestrae, and blood cells have ready access to and egress from the general circulation through gaps in the sinusoidal walls.

Endothelial cells are able to phagocytose small particles (Bankston, 1974; Le Douarin, 1975; Fukuda, 1976; Bankston and Pino, 1980). Le Douarin (1975) discovered that hepatic mesenchymal cells can take up ink particles injected into the circulation of Stage-40 embryos, and Fukuda (1976) noted phagocytic activity among the endothelial cells in the vitelline veins and ductus venosus. The ink-ingesting mesenchymal cells developed characteristics like those of Kupffer cells, leading Le Douarin (1975) to consider that endothelial cells and Kupffer cells might be functional variants of

a single cell. Sandström and Westman (1971) reported the phagocytosis of erythrocytes by endothelial cells, but it is quite possible that they were observing immature Kupffer cells, rather than endothelial cells, in the sinusoidal lining.

Karrer (1961), in an early electron-microscopic study of erythropoiesis in the chicken embryo, observed the rounding of endothelial cells and their projection into the sinusoidal lumina. Organelles of the rounded cells were displaced basally, leaving the apical cytoplasm with a homogeneous ground substance devoid of organelles. When these "bulging cells" divided, the basal cell supposedly remained in contact with the parenchyma as an endothelial cell and the apical cell entered the circulation. Finding no extravascular erythropoietic cells, Karrer (1961) speculated that cells released to the circulation were proerythroblasts and, thus, the sole source of erythrocytes. While cell release from the hepatic sinusoids has been confirmed by several studies, including our own, the identity of the liberated cells has never been determined conclusively.

Mammalian erythropoietic cells have been identified extravascularly as individual cells and as members of blood islands (Ackerman et al., 1961). In the bird, only individual proerythroblasts and basophilic erythroblasts have ever been observed extravascularly. Proerythroblasts in mammalian embryos are capable of mitosis (Jones, 1970), as are those in the avian embryo (Karrer, 1961; present study). Since extravascular proerythroblasts do not occur in groups, they could be quiescent interphase cells or, if mitotically competent, one daughter cell of each division might immediately enter the circulation. Polychromatophilic erythroblasts were never observed extravascularly in our study, intimating that proerythroblasts and basophilic erythroblasts are the cells which actually enter the circulation.

Kingsbury et al. (1956), also finding intravascular and extravascular erythroblasts at early stages of hepatic development, discovered a subsequent increase in the number of intravascular erythroblasts and a decline in the number of extravascular cells. As hepatic hemopoiesis draws to a close, the proerythroblasts must either enter the circulation or differentiate into basophilic erythroblasts which then cross the sinusoidal walls.

As the earliest site of hemopoiesis in amniote embryos, the yolk sac is the source of the first circulating blood cells. It may also be the source of stem cells which colonize the intraembryonic organs of hemopoiesis (Bruns and Ingram, 1973; Dardick and Setterfield, 1978; Houssaint, 1981). Other studies, however, indicate that stem cells which colonize intraembryonic organs have an intraembryonic, rather than extraembryonic, origin (Rifkind et al., 1969; Dieterlen-Lièvre et al., 1976; Martin et al., 1980). Dieterlen-Lièvre et al. (1976) concluded that the primitive erythrocyte line was the only series derived from the yolk sac and that stem cells for the definitive line originated intraembryonically. The site of origin of the extravascular stem cells for the erythrocyte line remains controversial. Like endothelial cells, the extravascular stem cells may originate from mesodermal cells of the septum transversum (Rifkind et al., 1969).

The close association of hepatocytes and hemopoietic cells in mammals has led some investigators to con-

Fig. 8. Primitive and definitive erythrocytes. A: A primitive erythrocyte displays a dense nucleus (nu). Ribosomes and a few degenerating mitochondria (mt) are identifiable in the cytoplasm. en, endothelial cell.  $\times 11,400$ . Bar, 1  $\mu\text{m}$ . B: A definitive erythrocyte also exhibits a dense nucleus (nu), but the cell is distinctly smaller than a primitive erythrocyte. The dense cytoplasm is devoid of most organelles and inclusions, and plasmalemmal blebbing is apparent (arrowheads). en, endothelial cell; pc, perisinusoidal cell; ps, perisinusoidal space.  $\times 11,400$ . Bar, 1  $\mu\text{m}$ .

Fig. 9. Circulating blood cells. This scanning electron micrograph illustrates the surface deformations (arrowheads) of developing erythrocytes (ery) from the general circulation. Flattened cells, which are more mature than rounded cells, show more plasmalemmal activity.  $\times 6,750$ . Bar, 5  $\mu\text{m}$ .

Fig. 10. Macrophage with a recently phagocytosed blood cell. The nucleus (nu) of the macrophage is displaced by an engulfed erythrocyte (ery). A forming residual body (rb) also assumes an eccentric position. Note the contact between the macrophage and endothelial cell (lower left corner of the field).  $\times 11,600$ . Bar, 1  $\mu\text{m}$ .

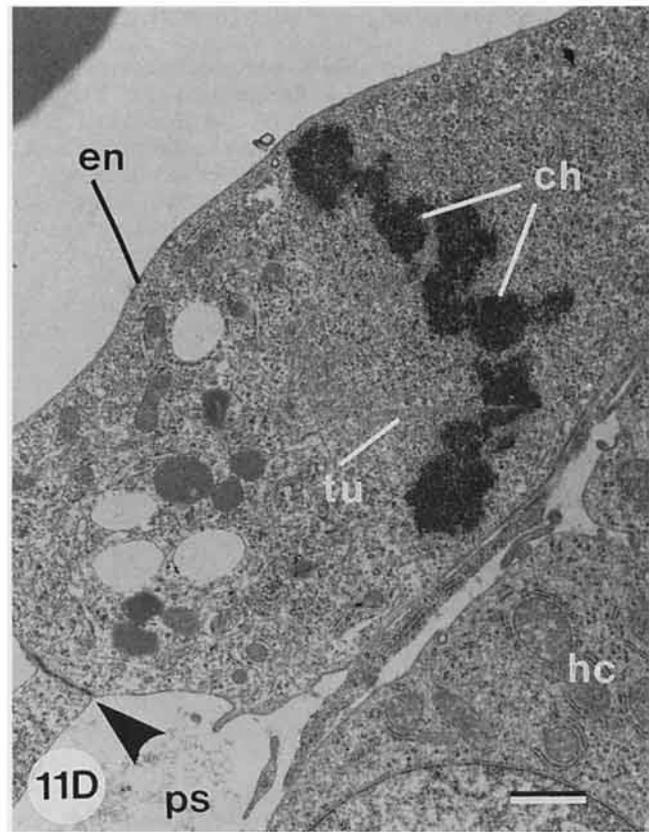
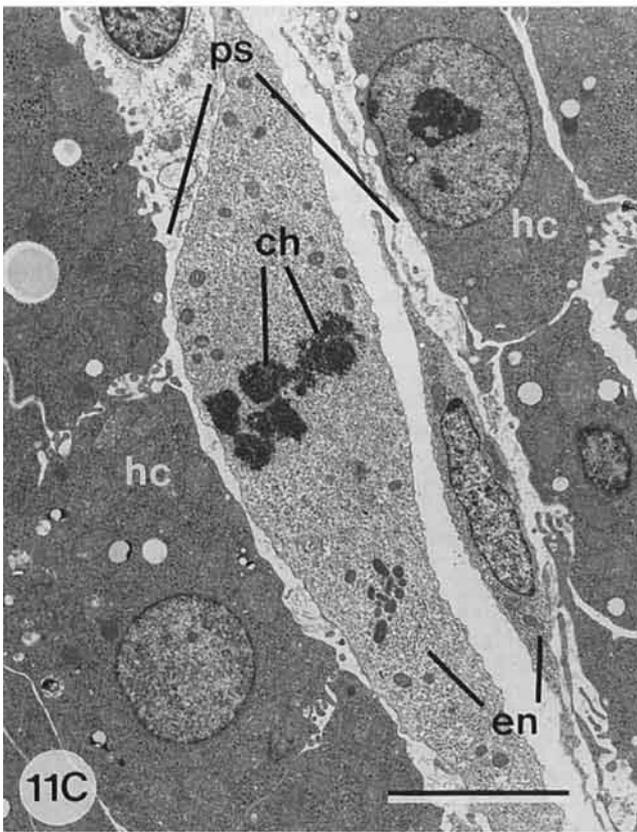
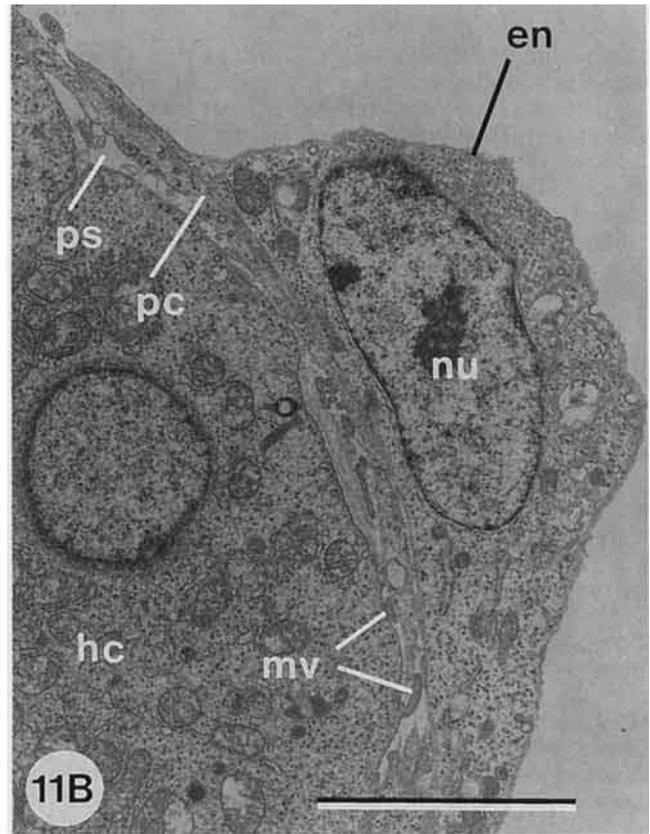
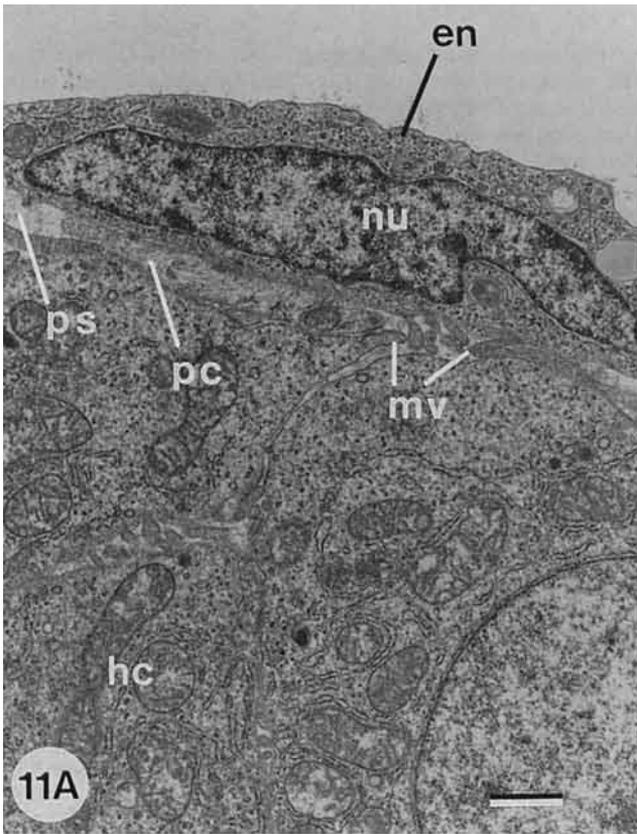


Fig. 11. Endothelial cells. **A:** Flattened endothelial cell (en) in close association with hepatocytes (hc). Part of a perisinusoidal cell (pc) and microvilli (mv) of the hepatocytes occupy the perisinusoidal space (ps). nu, nucleus.  $\times 9,100$ . Bar, 1  $\mu\text{m}$ . **B:** Rounded endothelial cell (en) projecting into the sinusoidal lumen. Microvilli (mv) of hepatocytes (hc) and processes of perisinusoidal cells (pc) appear in the perisinusoidal space (ps). nu, nucleus.  $\times 6,850$ . Bar, 5  $\mu\text{m}$ . **C:** If the mitotic apparatus is centrally located, both daughter cells of an endothelial

cell (en) division will likely remain in the sinusoidal lining. ch, chromosome; hc, hepatocyte; ps, perisinusoidal space.  $\times 4,700$ . Bar, 5  $\mu\text{m}$ . **D:** Should the mitotic apparatus situate eccentrically, endothelial cell (en) division can release one daughter cell to the general circulation and retain the other in the sinusoidal lining. arrowhead, junctional zone; ch, chromosome; hc, hepatocyte; ps, perisinusoidal space; tu, spindle microtubule.  $\times 9,050$ . Bar, 1  $\mu\text{m}$ .

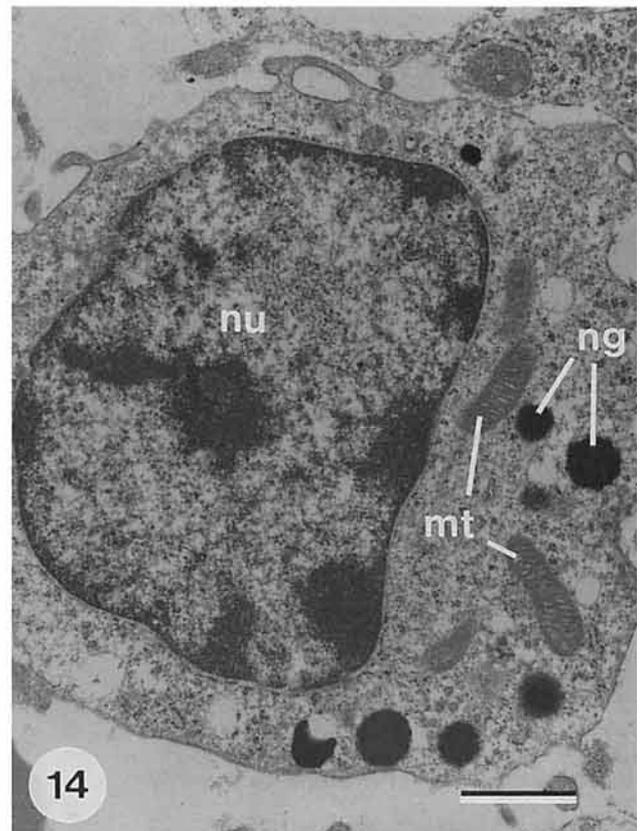
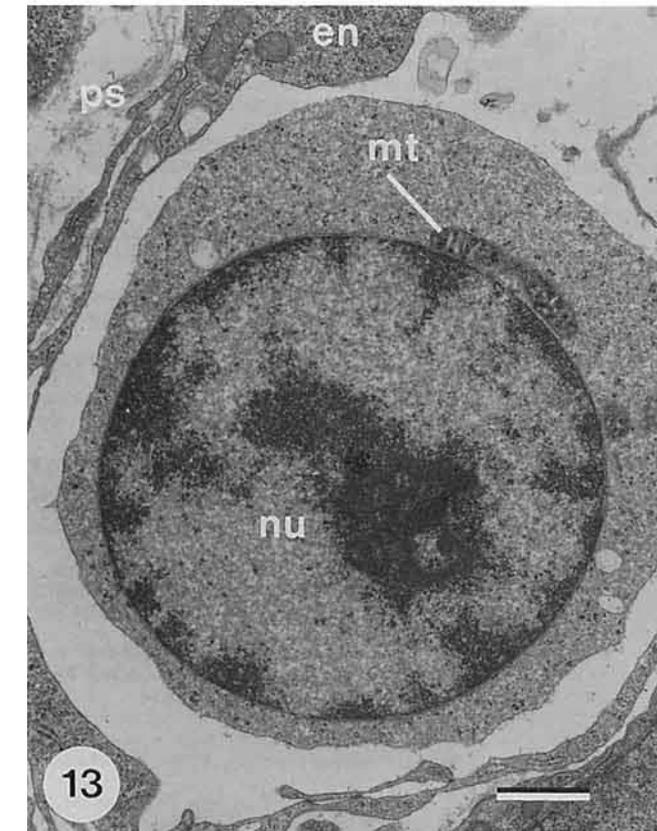
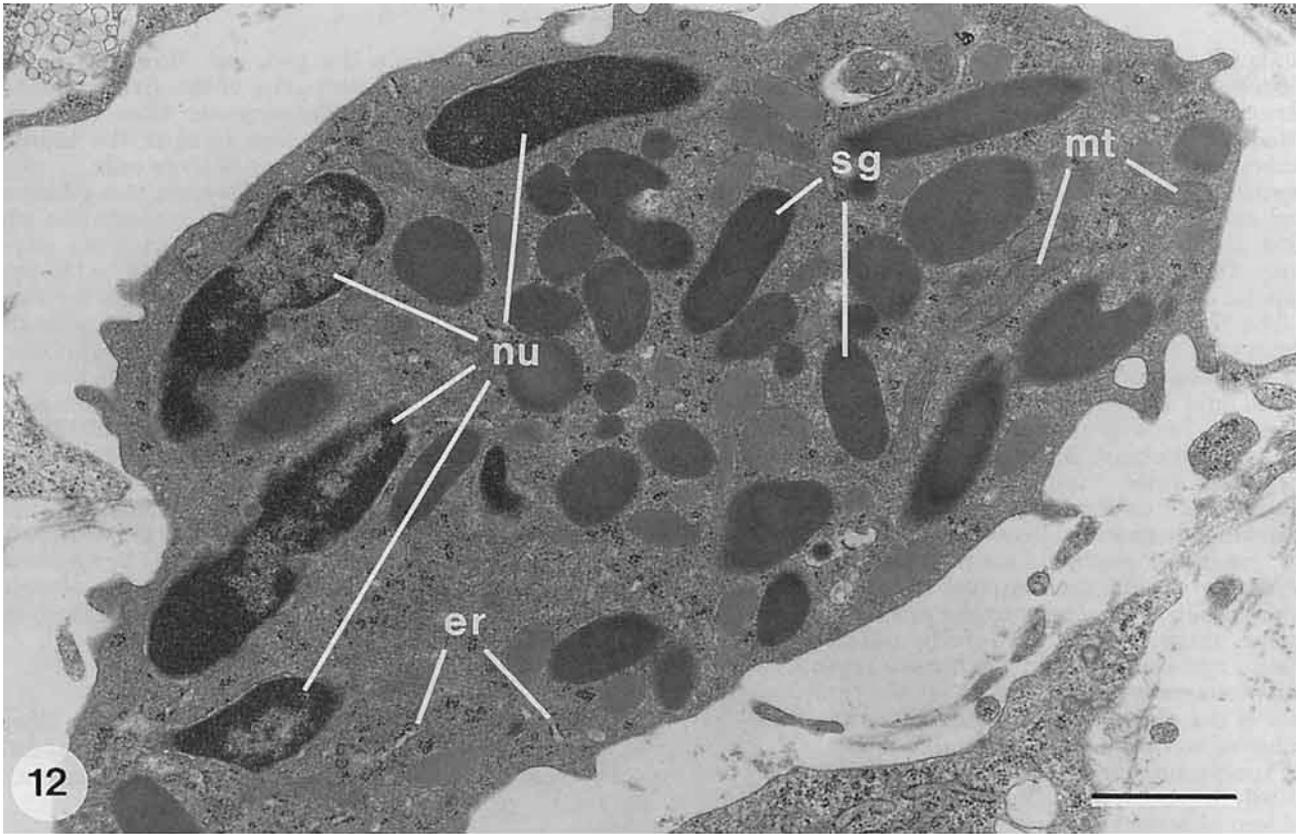


Fig. 12. An eosinophilic leukocyte in the general circulation shows several rod shaped specific granules (sg). Four lobes of the nucleus (nu) can be discerned. Granular endoplasmic reticulum (er), ribosomes, and mitochondria (mt) are scarce.  $\times 18,600$ . Bar, 1  $\mu\text{m}$ .

Fig. 13. A lymphocyte from the general circulation has a spherical nucleus (nu) with an expansive nucleolus. The nucleus is surrounded by a thin rim of cytoplasm containing ribosomes and occasional mi-

tochondria (mt). en, endothelial cell; ps, perisinusoidal space.  $\times 11,900$ . Bar, 1  $\mu\text{m}$ .

Fig. 14. A monocyte (mt, mitochondrion; ng, non-specific granule) in the sinusoidal lumen has an irregular shape. The indented nucleus (nu) is displaced toward one side of the cell. Polysomes are evident in the cytoplasm.  $\times 15,400$ . Bar, 1  $\mu\text{m}$ .

clude that hepatocytes regulate the differentiation of hemopoietic cells (Asano et al., 1987). Such regulation occurs without the benefit of intercellular junctions (Medlock and Haar, 1983). Hepatocytes in the avian embryo can situate quite close to extravascular proerythroblasts, but they likely exert little, if any, control over the hemopoietic cells. Erythropoiesis in the bird, it must be remembered, also occurs intravascularly, far removed from any influence of cells in the hepatic cords.

The involvement of microtubules in the shape changes of erythropoietic cells was suspected by previous investigators (Ceresa-Castellani and Leone, 1969; Small and Davies, 1972). These workers detected a marginal band of microtubules in the polychromatophilic erythroblast. Microtubules may also perform cytoskeletal roles, maintaining the shape of erythroblasts and conferring upon them elasticity and resistance to external pressure (Grasso, 1966). The reduction in the number of microtubules in mature avian erythrocytes and their virtual absence from mature mammalian erythrocytes argue against any role in the maintenance of shape in fully differentiated cells (Small and Davies, 1972). In human erythrocytes, a filamentous network of proteins on the cytoplasmic surface of the plasmalemma is implicated in the maintenance of cell shape (Lange et al., 1982).

Pinocytotic uptake of ferritin granules, a process called rhopheocytosis (Policard and Bessis, 1958), is seldom observed in birds (see also Ceresa-Castellani and Leone, 1969; Small and Davies, 1972). Coated pits and vesicles, commonly observed in proerythroblasts and basophilic erythroblasts, suggest that receptor-mediated endocytosis is a potential mechanism for uptake of macromolecules (Goldstein et al., 1979). A serum-chelating protein may facilitate the entry of iron into erythroblasts (Small and Davies, 1972). Transferrin, a serum glycoprotein in mammals, transfers iron from absorption or storage sites to other locations for uptake. Conalbumin in the chicken is a transferrin analogue, so the process of receptor-mediated endocytosis is also available to this group of organisms. The transferrin-receptor complex releases iron into the lumen of an endocytotic vesicle, and the iron is then shunted to the cytoplasm for processing into ferritin granules (Dautry-Varsat et al., 1983). As hemoglobin production tapers off during erythropoiesis, and the erythropoietic cells thus require fewer ferritin granules, the prevalence of coated pits and vesicles declines.

Granulopoiesis can be detected in the embryonic liver of the Stage-31 chicken embryo, although this process is not pronounced (Romanoff, 1960). Intense granulopoietic activity, which does not occur until Stage 37, peaks at Stage 40 and terminates shortly after hatching (Romanoff, 1960; Bruns and Ingram, 1973). Eosinophilic leukocytes, which purportedly arise from perivascular and peritoneal cells, are the first cells produced by granulopoiesis. Basophilic granulocytes, lymphocytes, and monocytes have been described in the liver of a Stage-44 embryo (Romanoff, 1960).

The origin of granulocytes from the vascular connective tissue is unlikely. It was originally believed that granulopoietic stem cells, like erythropoietic stem

cells, originated from the yolk sac. However, recent studies indicate that stem cells of the liver probably migrate from other intraembryonic sites of origin (Martin et al., 1980). The mesoderm of the septum transversum is a possible source of stem cells.

Hepatic hemopoiesis in the avian embryo is different in several respects from that in the mammalian embryo. Since blood cells of the bird can originate intravascularly as well as extravascularly, neither the prehepatocyte population nor the compartments for stem cell differentiation hold the same relevance as in the mammal. Furthermore, there seems to be no subdivision of the hepatic environment in the bird into microenvironments for the erythrocyte and granulocyte lines. Hemopoiesis in the avian liver is essentially intravascular (Metcalf and Moore, 1971), and since all major blood vessels of the embryo may form blood cells, hemopoiesis could indeed be more a reflection of vascular development generally than a feature of hepatic organogenesis specifically. Indeed, hepatic hemopoiesis may be just a transient stage between the hemopoietic decline in the yolk sac and the maturation of the bone marrow (Le Douarin, 1975).

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