AN ULTRASTRUCTURAL STUDY OF EARLY MORPHOGENETIC EVENTS DURING THE ESTABLISHMENT OF FETAL HEPATIC ERYTHROPOIESIS

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ABSTRACT

Morphogenetic events are described which characterize early stages of the interaction between mesenchyme and expanding epithelial cell cords derived from the hepatic endodermal diverticulum in the C57BL/6J mouse. This interaction culminates in the differentiation of hepatic epithelial and hematopoietic tissues. No basement membrane separates the presumptive hepatic epithelial cells from the adjacent mesenchyme, while intercellular attachments, both adherent junctions and desmosomes, are established transiently between heterologous cell types across this epithelio-mesenchymal interface. Yolk sac-derived erythroblasts found in the primitive liver are distinguished morphologically from endogenous hepatic erythroid cells; they are confined to the vascular compartment and are not, apparently, precursors for hepatic erythropoiesis. The earliest recognizable endogenous hepatic hematopoietic cells appear, extravascularly, among those mesenchymal cells in intimate contact with the endodermal epithelium between the 10¼ and 10½ gestational day. Definitive erythropoiesis commences between the 10½ and 11th fetal days. The ultrastructure of these primitive hepatic erythroid cells (proerythroblasts) and their transition to more mature forms (basophilic and polychromatophilic erythroblasts) are described.

INTRODUCTION

The erythropoietic cell line provides a useful subject for study of both morphologic and biochemical aspects of cellular differentiation and development. The site of differentiation of red blood cells changes during the course of mammalian fetal development. These sites differ with respect to the structure of the red cells produced and, at least in certain species and strains, with respect to the types of hemoglobins synthesized (1, 2). The yolk sac constitutes the primary early locus of the erythropoiesis in the fetal mouse as in other mammals. Erythroblasts developing in the blood islands of this organ synthesize characteristic embryonic hemoglobins from about the 8th day of gestation (1, 3, 4). Before the 10th day these cells enter the circulation where they mature from erythroblasts to nucleated erythrocytes over the next 4–5 days (3, 5). The fetal mouse liver assumes an active erythropoietic function after the 10th day of gestation (5, 6). The red cell precursors differentiating in this organ synthesize adult type hemoglobin; they lose their nuclei while in the liver and enter the circulation in the form of nonnucleated reticulocytes (3–5). The liver continues as an active site of erythropoiesis almost until birth, when it is supplanted by the spleen

and bone marrow (5, 6). In both the yolk sac and the liver it is believed that the erythropoietic tissue develops in an embryonal mesenchyme closely approximated to an endodermal derivative, the yolk sac endoderm on the one hand and the hepatic epithelium derived from the gut on the other (7). The over-all sequence of morphogenetic events in the fetal mammalian erythron has been explored at both the light and electron microscopic levels of resolution although ultrastructural studies, for the most part, have dealt with later, well-established stages of erythropoiesis (8–12). Important to an understanding of the development of fetal erythropoiesis and cellular differentiation in general is elucidation of the events which characterize the earliest stages of differentiation during which, in the presence of cells of endodermal origin, primitive mesenchymal cells become committed to hematopoietic pathways. In the present study evidence is presented which indicates that, in the fetal C57BL/6J mouse, hematopoiesis commences in the liver between the 10¼ and 10½ gestational days. Ultrastructural features of the hepatic epithelium and mesenchyme and of their interaction, culminating in definitive hepatocellular and erythropoietic differentiation, are described.

MATERIALS AND METHODS

Timed pregnancies in virgin female C57BL/6J mice were obtained by hormone priming and mating according to the method of Southard et al. (13). The morning following mating is designated as the zero time of the gestation (14). The livers of mice of appropriate age were dissected, fixed in cold 1% phosphate-buffered redistilled glutaraldehyde (pH 7.2), postfixed in osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812. Thick (1 μ) sections were stained with toluidine blue, and thin sections were stained with alcoholic uranyl acetate followed by lead citrate. Thin sections were examined in either a Hitachi HS-7S or HU-11-C electron microscope. Whole embryos or dissected and isolated hepatic tissues were examined by means of serial thick sections; thin sections were taken where appropriate. In this manner, the hepatic or presumptive hepatic tissues from five or more embryos were studied at each developmental stage cited.

A considerable degree of uncertainty exists in the ascription of embryologic events to specific gestational times due, in part, to variations in the time of fertilization and to differences in the state of embryos within a given litter. The assignment of gestational dates has been accomplished by the somewhat arbitrary synthesis of observations upon many mice; it is intended to establish, principally, the sequence of events and a rough chronology by which these events may be correlated with other changes during fetal life.

RESULTS

10 Day Fetal Liver

At 10 days the rudimentary liver is observed as a small thickening in the transverse septum at the anterior intestinal portal. The identity of this organ rudiment is established by its anatomic position, relationships, and cytological features (15). As seen in thick sections, the presumptive hepatic tissue is composed of a loose mesenchyme enclosing an epithelial tube of simple columnar cells, the endodermal hepatic diverticulum (Fig. 1). This diverticulum may be traced caudally, by serial sections, to its junction with another epithelial pouch which constitutes the primitive bile

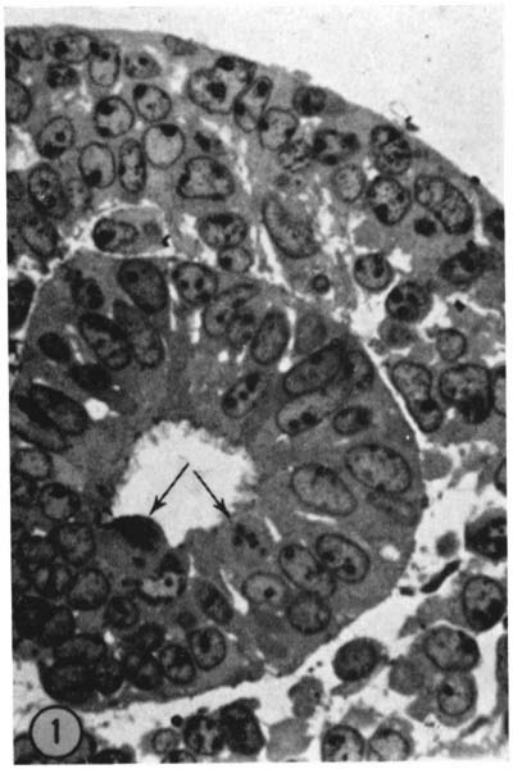


FIGURE 1 Light micrograph of a section through the endodermal diverticulum and adjacent mesenchyme of the 10 day fetus. A brush border lines the diverticular lumen. Two mitotic figures (arrows) are noted among the columnar epithelial cells. × 780.

duct and gall bladder. The columnar epithelium displays a brush border at its lumenal surface and is strictly demarcated from adjacent mesenchyme at its basal margin. Although cells in mitosis are common, at no level of sectioning is there evidence, at this developmental stage, for expansion or migration of endodermal cells into the surrounding mesenchyme. The mesenchyme itself consists of a fairly characteristic loose feltwork of extended cells which, in the immediate vicinity of the diverticulum, takes on a somewhat more compacted form. Mitotic figures are very infrequent. Although several large blood vessels (the vitelline veins) pass adjacent to the presumptive hepatic tissue, the mesenchyme immediately surrounding the diverticulum is not notably vascular. Rarely, small vascular spaces delineated by a primitive endothelium are observed (Fig. 2). These vessels contain small numbers of erythroid elements with characteristic light and electron microscopic features of the circulating 10 day yolk sac-derived polychromatophilic erythroblast (3). These features include a moderate degree of nuclear heterochromatinization and an extensive, relatively electron-opaque cytoplasm containing numerous free-lying polyribosomes. Similar cells obtained from the fetal circulation demonstrate a positive benzidine reaction for hemoglobin. Yolk sac polychromatophils constitute the only recognizable erythroid elements in the 10 day liver and are located exclusively within the vascular compartment.

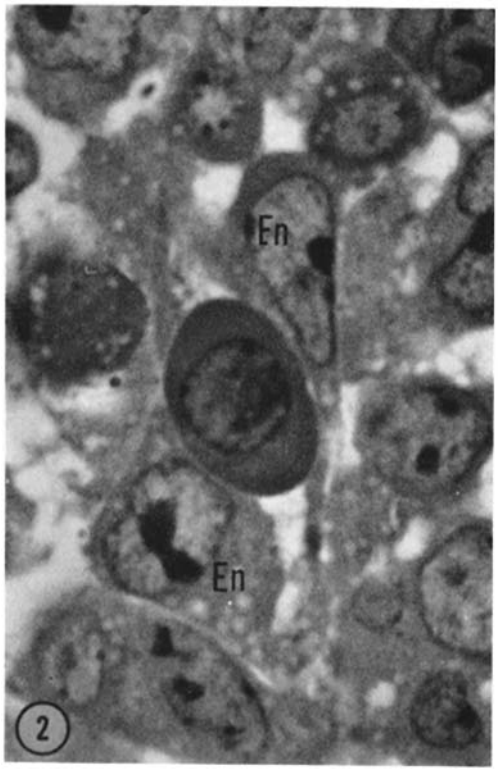


FIGURE 2 Light micrograph of a small vascular channel within the 10 day hepatic mesenchyme. Two primitive endothelial cells (*En*) delineate the vessel which contains a nucleated polychromatophilic erythroblast of yolk sac lineage. × 2000.

In the electron microscope, cells of the endodermal diverticulum display few distinctive features save for their epithelial disposition. The cytoplasm contains numerous free polyribosomes and a relative paucity of endoplasmic reticulum and vesicular elements. Junctional complexes, composed principally of adherent zones (16), are found between adjacent cells at their apical (lumenal) margins. Neither definitive desmosomes nor tight junctions are seen at this stage. The lumenal surface is amplified by numerous short cytoplasmic processes constituting the brush border. The electron micrograph illustrated in Fig. 3 includes the interface between the hepatic diverticulum (lower left) and mesenchyme. A primitive but continuous basement membrane (insert to Fig. 3) is closely applied to the basal surface of the diverticular epithelium. A fairly uniform clear zone separates this structure from the epithelial cell plasma membrane. The mesenchymal cells display both free polyribosomes and short profiles of granular endoplasmic reticulum. Adjacent cells are associated by means of short, adherent junctions. Their nuclei show rather more condensation of chromatin than is found in the epithelial cells. These fine blocks of chromatin are disposed throughout the nucleoplasm as well as along the nuclear membrane. Extracellular fibrous connective tissue elements are not observed. One clearly differentiated cellular function, cytophagocytosis, is frequently noted among the hepatic mesenchymal cells. In particular, macrophages containing cellular debris, apparently the residuum of ingested yolk sac polychromatophilic erythroblasts, are found within mesenchymal blood vessels.

10¼ Day Fetal Liver

At a slightly later time in fetal development, nominally designated the 10¼ day, the first

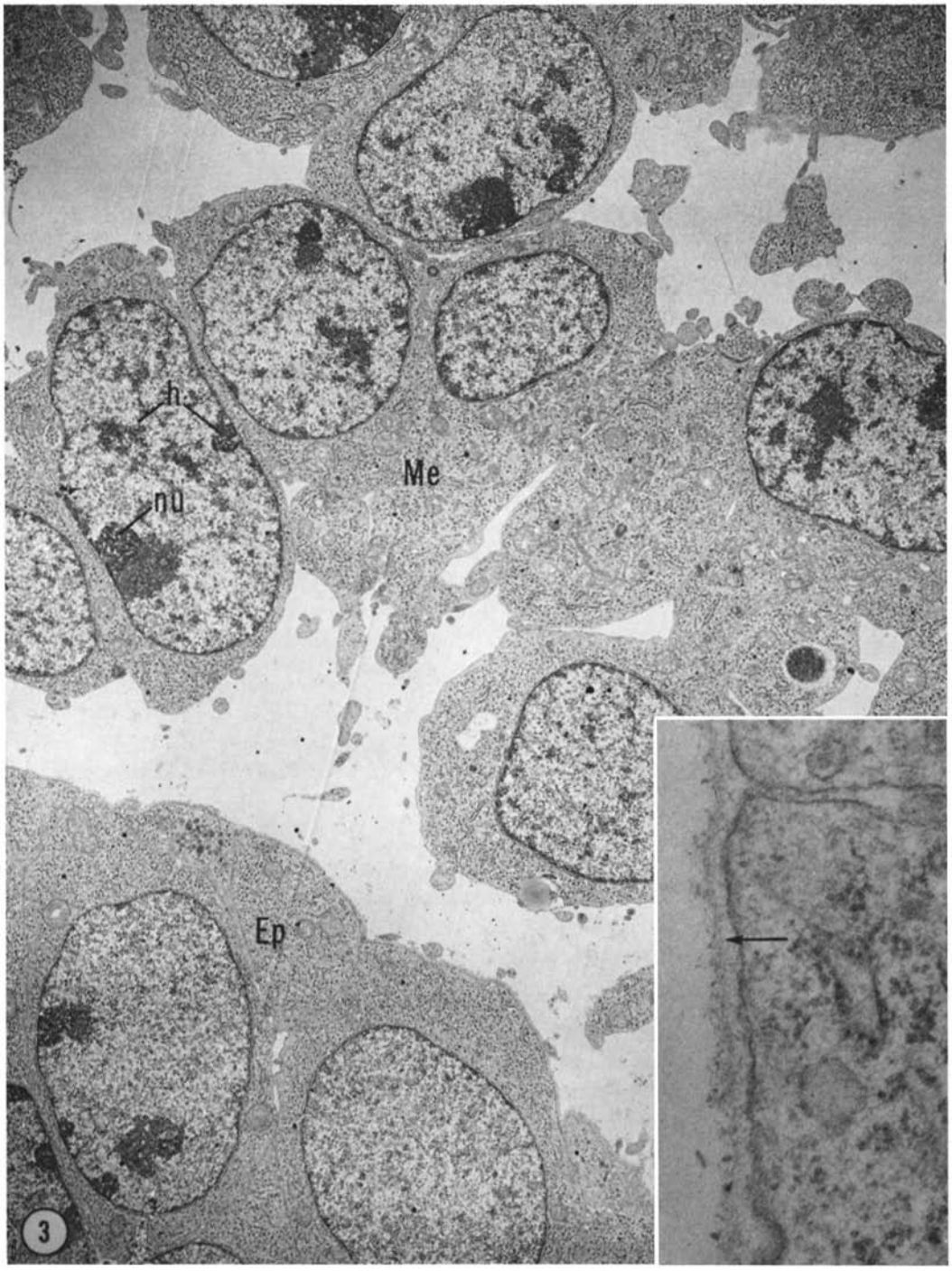


FIGURE 3 Electron micrograph showing mesenchymal (*Me*) and epithelial (*Ep*) cells and the epithelio-mesenchymal interface on the 10th fetal day. Small strands of heterochromatin (*h*) are somewhat more prominent in the mesenchymal nuclei. Multiple nucleoli (*nu*) with convoluted nucleolonemata are seen in both cell types. × 5000. Insert, the basal surface of the diverticular epithelium at higher magnification. A finely fibrillar basement membrane is indicated (arrow). × 50,000.

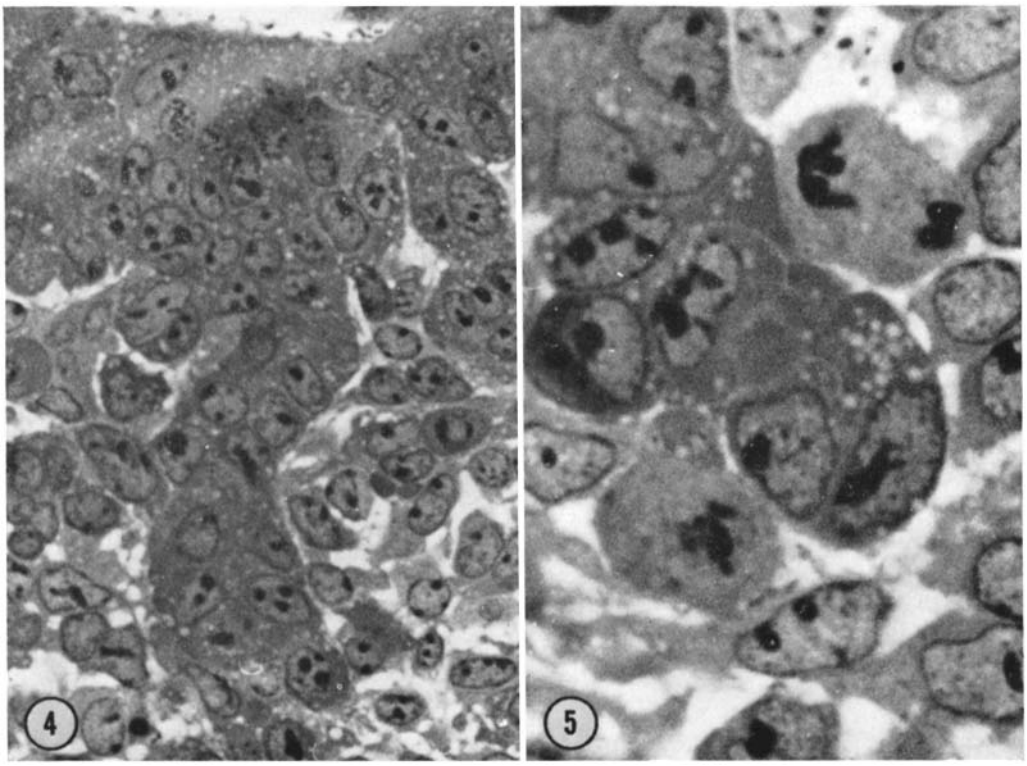


FIGURE 4 Light micrograph of a portion of the hepatic endodermal diverticulum (above) and surrounding mesenchyme (below) in a 10¼ day fetus. A cord of epithelial cells extends from the diverticulum into the mesenchyme. × 780.

FIGURE 5 Light micrograph of an outgrowing epithelial cord at the 10¼ day. Numerous small vacuoles occupy the epithelial cell cytoplasm. Two mesenchymal cells in mitosis are seen at the epithelio-mesenchymal interface. × 2000.

morphological evidence of definitive hepatic cytogenesis can be distinguished. From a relatively restricted zone in the cephalic portion of the hepatic diverticulum, cords of epithelial cells extend in a radial fashion into the surrounding mesenchyme (Figs. 4 and 5; see references 15, 17, 18). The epithelio-mesenchymal interface has been definitively identified in the electron microscope by the comparison of thin sections with adjacent thick sections examined in the light microscope. Two changes in the nature of the epithelio-mesenchymal interface are noted. In the first place, the basement membrane investing the diverticulum is penetrated by the expanding cords of epithelial cells which thus come to lie in a direct and intimate contact with the adjacent mesenchyme (Fig. 6). This situation remains constant for all later stages of hepatic growth and development; at no time is a basement membrane deposited between the parenchymal hepatic epithelial cells and their associated mesenchymal derivatives, either reticulo-endothelial or hematopoietic. Secondly, intercellular junctions, which at the 10 day stage were restricted to homologous cell types, are now established as well between unlike cells across the epithelio-mesenchymal interface. These junctions are most commonly the adherent type with an approximately 150 A gap between plasma membranes (Fig. 6). Less frequently, junctions with a 250 A gap, a plaquelike structure, and with cytoplasmic filaments characteristic of desmosomes are also encountered (Fig. 7).

It has been recognized that the expanding epithelium and the invaded mesenchyme exert a reciprocal inductive influence upon each other resulting in characteristic hepatic differentiation

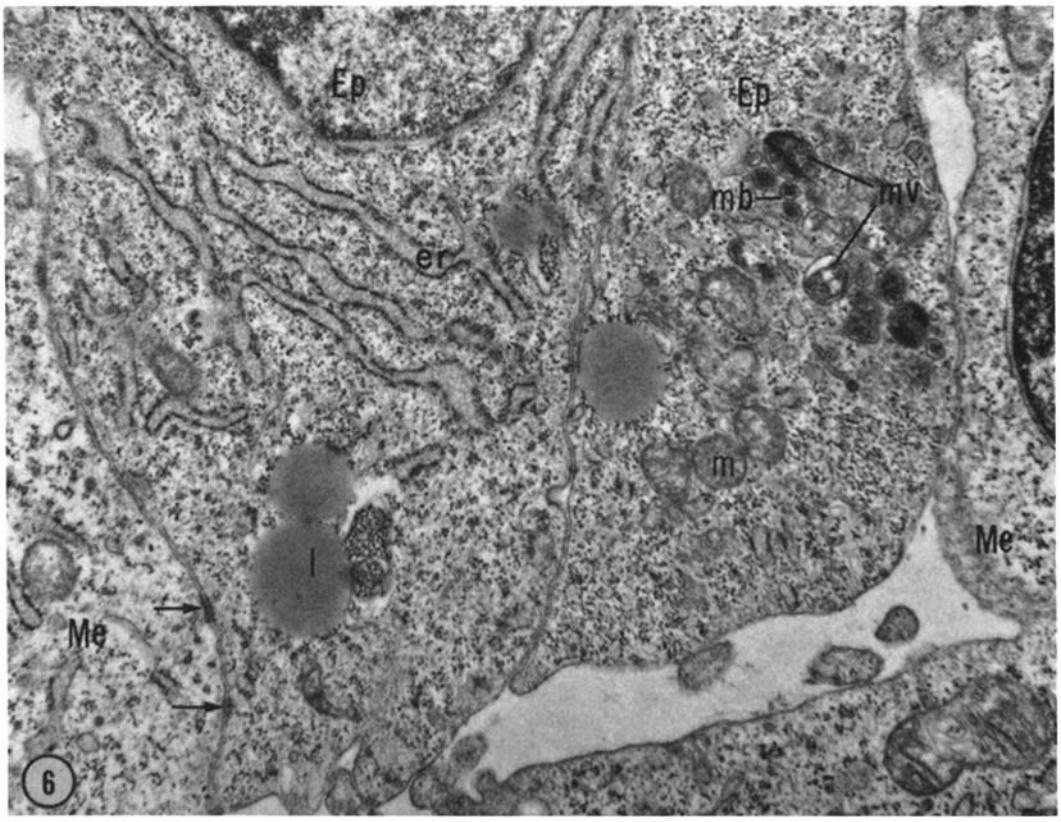


FIGURE 6 Electron micrograph displaying the epithelio-mesenchymal interface at the 10¼ day. Portions of two epithelial cells (*Ep*) contain lipid droplets (*l*) and associated dense particles, multivesicular bodies (*mv*) and microbodies (*mb*), endoplasmic reticulum (*er*), and mitochondria (*m*). Portions of two mesenchymal cells (*Me*) are in intimate contact with the epithelial cells at several places, with adherent junctions between the two cell types at several places (arrows). × 12,500.

and organogenesis (19, 20). A number of morphologic changes, noted in both of these components even at this early stage of hepatogenesis, may be considered as signs of the differentiative process. Large numbers of vacuolar or vesicular structures, not recognized in the 10-day diverticular cells, are found in the epithelium of the 10¼ day liver (Figs. 4 and 5). These are membrane-bounded structures containing a homogeneous and moderately electron-opaque material of a type commonly referred to as lipid droplets (Fig. 6). Whether, indeed, their content is lipid or other material has not been ascertained (21). Lipid droplets are not exclusively confined to developing hepatocytes; it is their large number which is characteristic of this cell and which is particularly apparent in the light micrographs (Figs. 4 and 5). Collections of densely-stained particles, slightly larger than ribosomes and disposed within irregular membrane-bounded structures, are often observed abutting upon the lipid droplets (Fig. 6). Glycogen, which the size and density of the particles might suggest, has not been reported to appear before the 16th day of gestation (21). Their relationship to the lipid droplets might also suggest that the particles represent a particulate form of lipid. Further study will be required to evaluate these structures. Additional stigmata of hepatocellular differentiation in the 10¼ day liver include increased numbers of multivesicular bodies and a heterogeneous population of microbodies (Fig. 6). The endoplasmic reticulum, insignificantly developed in the 10 day diverticulum, has undergone a modest elaboration although it is still sparse and irregular in disposition as compared with the endoplasmic reticulum in adult

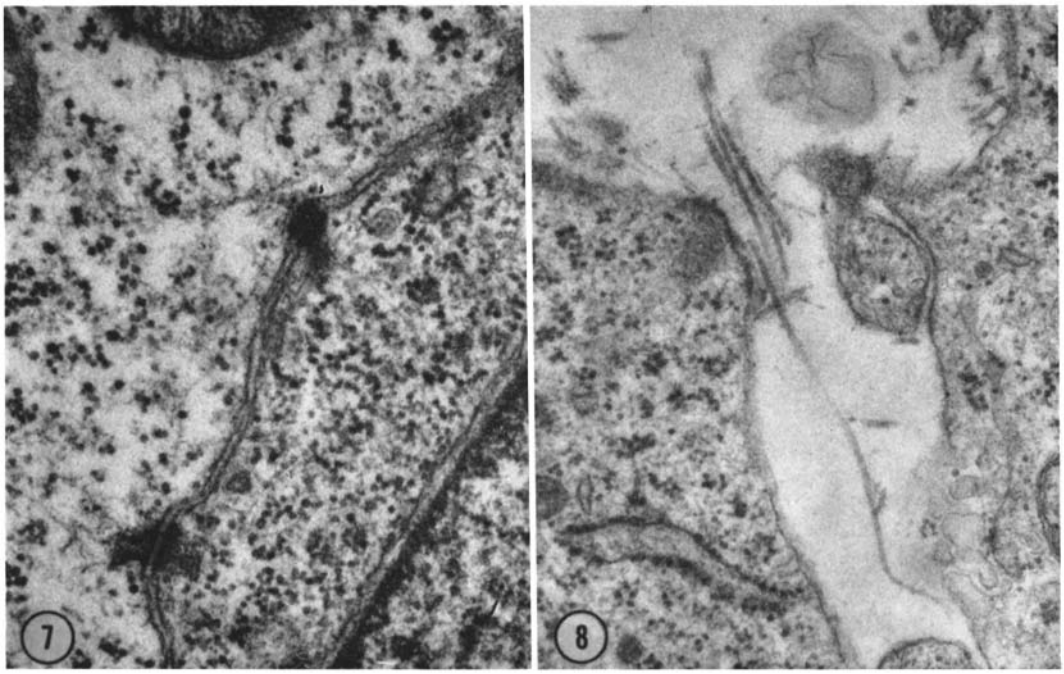


FIGURE 7 Two intercellular junctions (desmosomes) between an epithelial cell (lower right) and mesenchymal cell at 10¼ days. × 30,000.

FIGURE 8 Extracellular fibrous connective tissue elements in the intercellular space between an epithelial (left) and mesenchymal cell (right) at 10¼ days. × 37,500.

liver. The ground cytoplasm contains numerous free polyribosomes and ribosomes and an amorphous matrix, all in somewhat higher concentration than in adjacent mesenchymal cells (Figs. 6 and 7).

Changes in the mesenchyme are less definitive. Mitotic figures, present but uncommon in the 10 day mesenchyme, are distinctly more frequent at the 10¼ day stage and are often observed in direct contact with the epithelial cord (Fig. 5). At a somewhat later developmental stage (see below) the first signs of hematopoiesis will appear among mesenchymal cells in just this situation vis-à-vis the epithelial margin. The possibility that DNA synthesis and, presumably, mitotic division are required for an early stage of red blood cell differentiation has been raised by the studies of Wilt (22, 23).

It is at the 10¼ day that the first signs of an extracellular connective tissue component are recognized in the developing liver (Fig. 8). This fine fibrous material, lacking any distinct periodicity, is initially confined to the extracellular spaces at and immediately adjacent to the epithelio-mesenchymal interface. Similar fibers have been seen as well at the epithelio-mesenchymal interface in differentiating pancreas (24). The present observations cannot establish with certainty that the mesenchymal cells are involved in synthesis of this product. The fact that at a slightly later stage, indeed from the 10½ day onward, connective tissue fibrils are dispersed extensively throughout the peripheral mesenchymal portions of the liver may be adduced as indirect evidence that the synthesis of connective tissue elements constitutes one differentiated function of the hepatic mesenchyme.

Despite these very early signs of morphologic and functional differentiation in the 10¼ day liver, there is as yet no evidence for endogenous hematopoiesis at this stage. Mesenchymal cells retain their characteristic extended contours while recognizable erythroid elements are limited, as in the previous stage, to a fairly homogeneous population of yolk sac-derived polychromatophilic erythroblasts confined to the vascular channels.

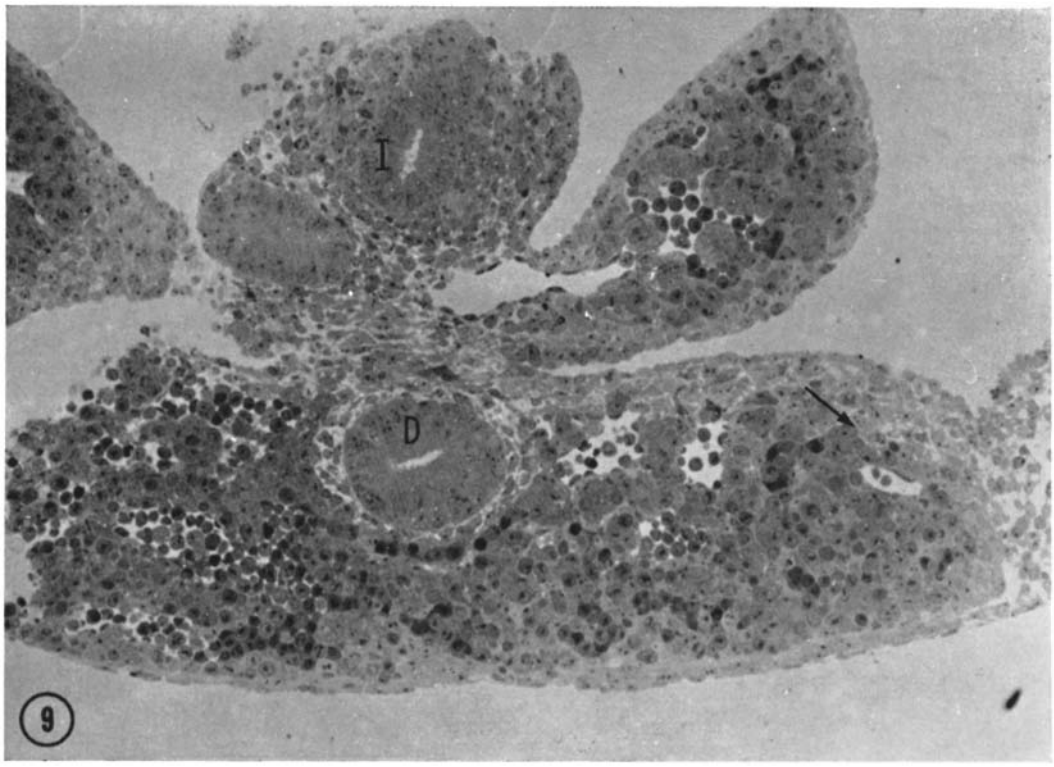


FIGURE 9 Light micrograph of a section through the dissected transverse septum of a 10½ day fetus. Intestine (*I*) and hepatic diverticulum (*D*) are indicated. The largest of three lobes lies in the transverse septum and surrounds the diverticulum. Residual mesenchyme is at far right; the epithelio-mesenchymal interface is indicated (arrow). The bulk of the liver contains epithelial, vascular, and hematopoietic elements. × 200.

10½ Day Fetal Liver

By about 10½ days early morphologic differentiation of the liver is well established, and primitive stages of hematopoietic activity are recognizable. The lobular structure of the liver, inapparent at 10 days, is now prominent (Fig. 9). One lobe, the largest, lies embedded within the mesenchyme of the transverse septum, while two others extend dorsocaudally. The endodermal diverticulum, surrounded by a thin zone of mesenchymal tissue, is still recognized within the major lobe. The disposition and configuration of the columnar epithelial cells are stabilized by addition of definitive desmosomes and tight junctions, forming typically mature junctional complexes (16). An intact basement membrane is applied to the basal surface of the diverticular epithelium. Migration of endoderm-derived epithelial cells across this boundary into the surrounding liver has ceased.

Three roughly concentric zones may be recognized in the 10½ day liver most clearly in the major lobe of the transverse septum (Fig. 9). These zones correspond to stages in the progressive morphogenesis of the definitive hepatic architecture. The zones are: (*a*) the residual mesenchyme of the transverse septum, constituting the outermost zone; (*b*) the region of the advancing margin of endodermal epithelial tissue; and (*c*) a large central zone surrounding the hepatic diverticulum, containing the most advanced stages of both hepatocellular and hematopoietic differentiation at this gestational age.

The peripheral mesenchyme is similar in most respects to its counterpart at earlier developmental stages. Although cells clearly definable as fibroblasts are not recognized, small fascicles of a fine,

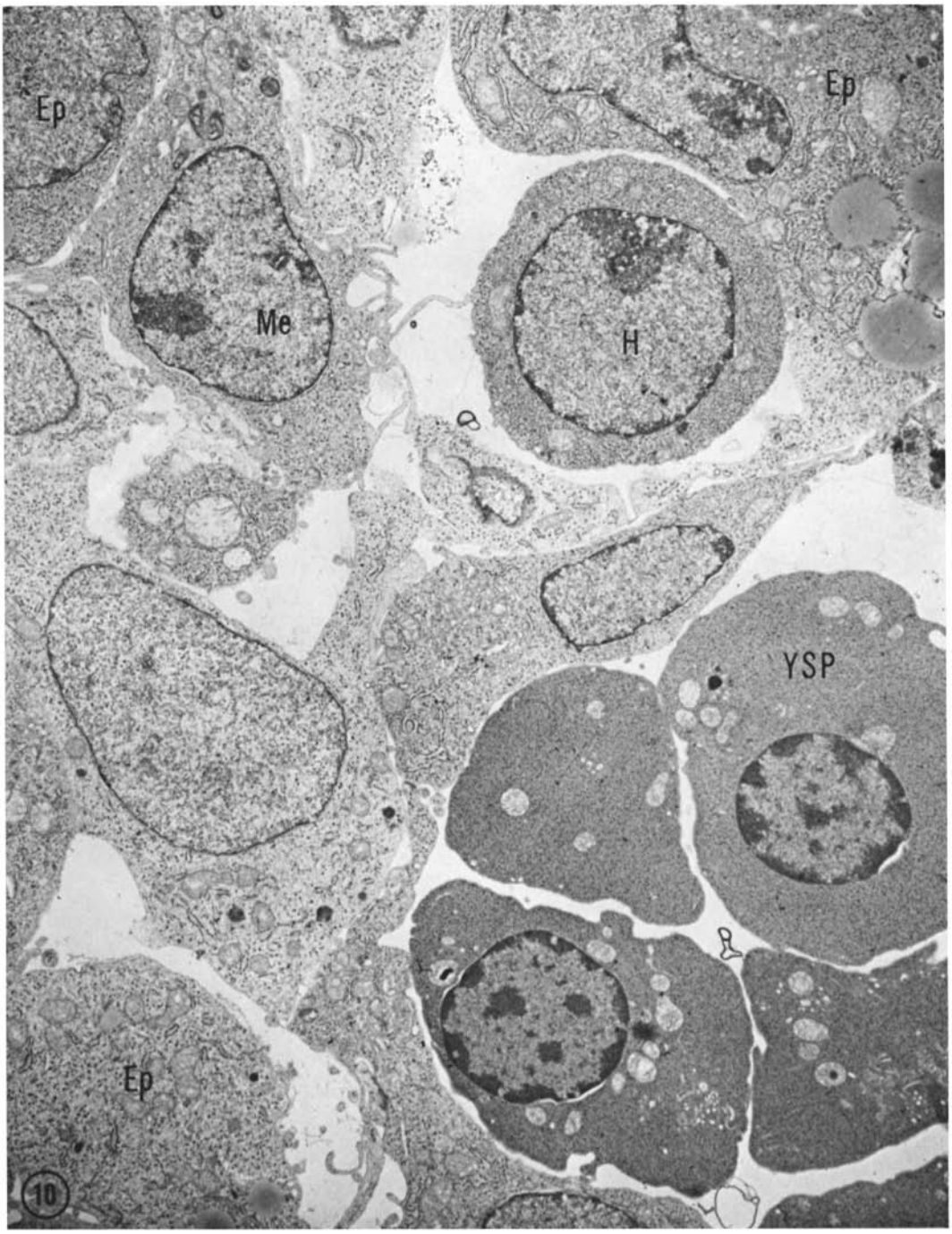


FIGURE 10 Electron micrograph of the epithelio-mesenchymal interface of the 10½ day fetus. A sinusoid containing yolk sac-derived polychromatophilic erythroblasts (*YSP*) is at lower right. Hepatic epithelial cells (*Ep*) are in contact with cells of the mesenchyme (*Me*) and presumptive hemocytoblasts (*H*). × 5000.

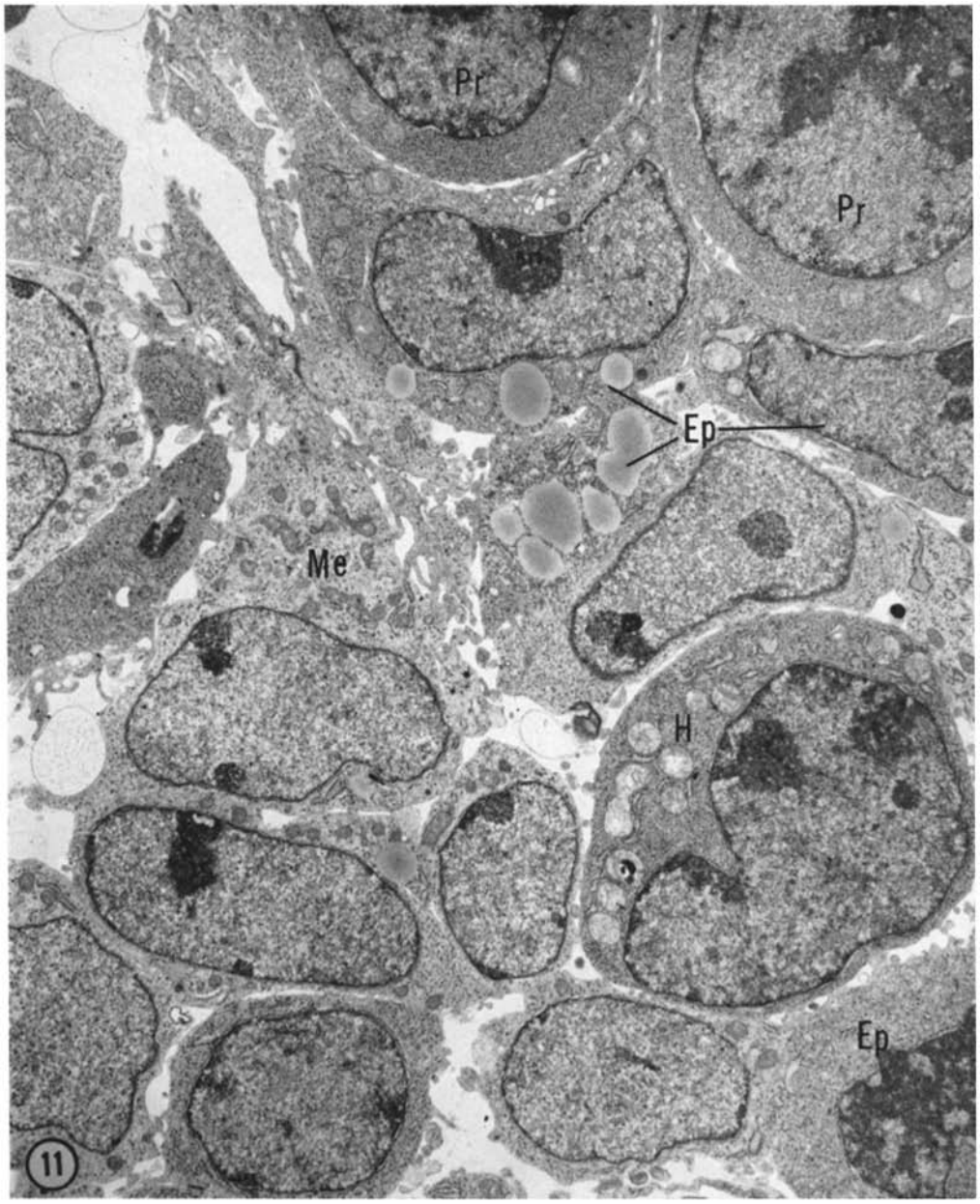


FIGURE 11 Epithelial cells (*Ep*) and mesenchyme (*Me*) at the expanding margin of a hepatic parenchymal cord (on the right). A free-lying, rounded hemocytoblast (*H*) lies at the interface. Two presumptive proerythroblasts (*Pr*) are incorporated within the epithelial tissue. × 5000.

fibrillar connective tissue are now sparsely but randomly distributed throughout the mesenchyme. Infrequent small blood vessels containing, almost exclusively, polychromatophilic yolk sac erythroblasts are seen. No recognizable hematopoietic cells are seen in the extravascular tissue.

In the region of the advancing epithelial margin three histologic elements of the primitive liver

come into proximity. These elements are the epithelium itself, the vascular structures, and the invaded mesenchyme. The disposition of these three tissues is indicated in the electron micrograph illustrated in Fig. 10. Cells of the hepatic epithelial cords, identified by their compact organization and characteristic cytoplasmic organelles, continue to display the same intimate contact with adjacent mesenchyme which was established at the previous developmental stage. No basement membrane-like material is deposited at this interface. The vascular elements, derived from penetration of the hepatic cords into the region of the vitelline veins, extend centrifugally in a fashion roughly parallel to the epithelium, in some areas just ahead and in others just behind the hepatic cords. The presence of numerous mitotic endothelial cells suggests that these vessels grow, in part at least, by extension and not only by anastomosis. Intercellular junctions between adjacent endothelial cells are preserved during mitosis, which suggests that, for the most part, the progeny of division remain endothelial in function rather than becoming free-floating intravascular elements as has been proposed (25). These advancing vascular channels are occupied by large polychromatophilic erythroblasts as in the peripheral mesenchyme. Diapedesis of these yolk sac-derived erythroid cells across the endothelium has not been seen, nor are they recognized in the extravascular compartment.

It is in this region, the epithelio-mesenchymal interface, that the first morphologic evidence suggestive of differentiation of endogenous hepatic hematopoietic tissue is discerned. At this interface cells are recognized which differ in a number of cytologic details from the hepatic epithelium, from the mesenchyme proper, and from the intravascular yolk sac erythroid cells (Figs. 10 and 11). These cells, tentatively identified as hepatic hemocytoblasts (hematopoietic stem cells), are distinguished principally by their shape and associations. The hemocytoblasts are distinctly rounded in contour, lacking both the extended cytoplasmic processes of the mesenchymal cells and the close packing of the cells in epithelial tissue. Intercellular junctions between adjacent hemocytoblasts are absent. The endoplasmic reticulum is somewhat less prominent than in mesenchyme or epithelium. The conspicuous absence of lipid droplets, microbodies, and multivesicular structures, confirmed by examination of serial thin sections, further serves to distinguish the hemocytoblast from the immature hepatic epithelial cell. Nuclear differences are less apparent. In general, hemocytoblast nucleoli are larger than nucleoli of the mesenchyme, and this increased size is accounted for principally by an increase in the thickness of the granular zone. Nucleolonemal strands are still recognizable, but coalescence of adjacent strands tends to obscure this pattern. There is a slight but consistently greater degree of condensation of chromatin at the nuclear membrane of hemocytoblasts as compared with adjacent mesenchymal cells. Both nuclear and cytoplasmic differences distinguish the hemocytoblast from yolk sac polychromatophil (Fig. 10). These differences include the smaller size and higher nuclear cytoplasmic ratio of the hemocytoblasts as well as prominent chromatin clumping, inconspicuous nucleoli, and dense cytoplasmic ground substance (hemoglobin) characteristic of the polychromatophil.

Behind the advancing epithelial margin, individual hemocytoblasts, flanked by hepatic cells and the proliferating sinusoidal vasculature, appear to be incorporated into the hepatic cords of the central zone (Figs. 11 and 12) where they continue to undergo cytologic alterations highly suggestive of differentiation along erythropoietic and megakaryocytic lines, the former vastly predominating. As recognized by Grasso et al. (10), distinction between the hemocytoblast and the earliest definitive erythroid precursor (proerythroblast) by purely morphologic criteria is not justified. Nevertheless, for the purposes of simplicity, and recognizing that a few cells (see Fig. 17, below) demonstrate features (specific granules and characteristic Golgi configuration) which identify them as being committed to the megakaryocytic lineage (11, 26), the predominant hematopoietic cell of the central hepatic zone will arbitrarily be termed a proerythroblast.

The proerythroblast is a completely free-lying cell similar in many respects to the hemocytoblast as already described. In the cytoplasm, free ribosomes in the polyribosomal configuration are abundant (Fig. 12). The few residual elements of endoplasmic reticulum are flattened and attenuated. They are most often encountered in the vicinity of the Golgi apparatus with which they maintain unmistakable connections (Fig. 13). The Golgi apparatus itself is prominent, comprising both stacked cisternal elements, generally not extensive, and a major vesicular component. The vesicles are of two sorts, plain and coated. Ex-

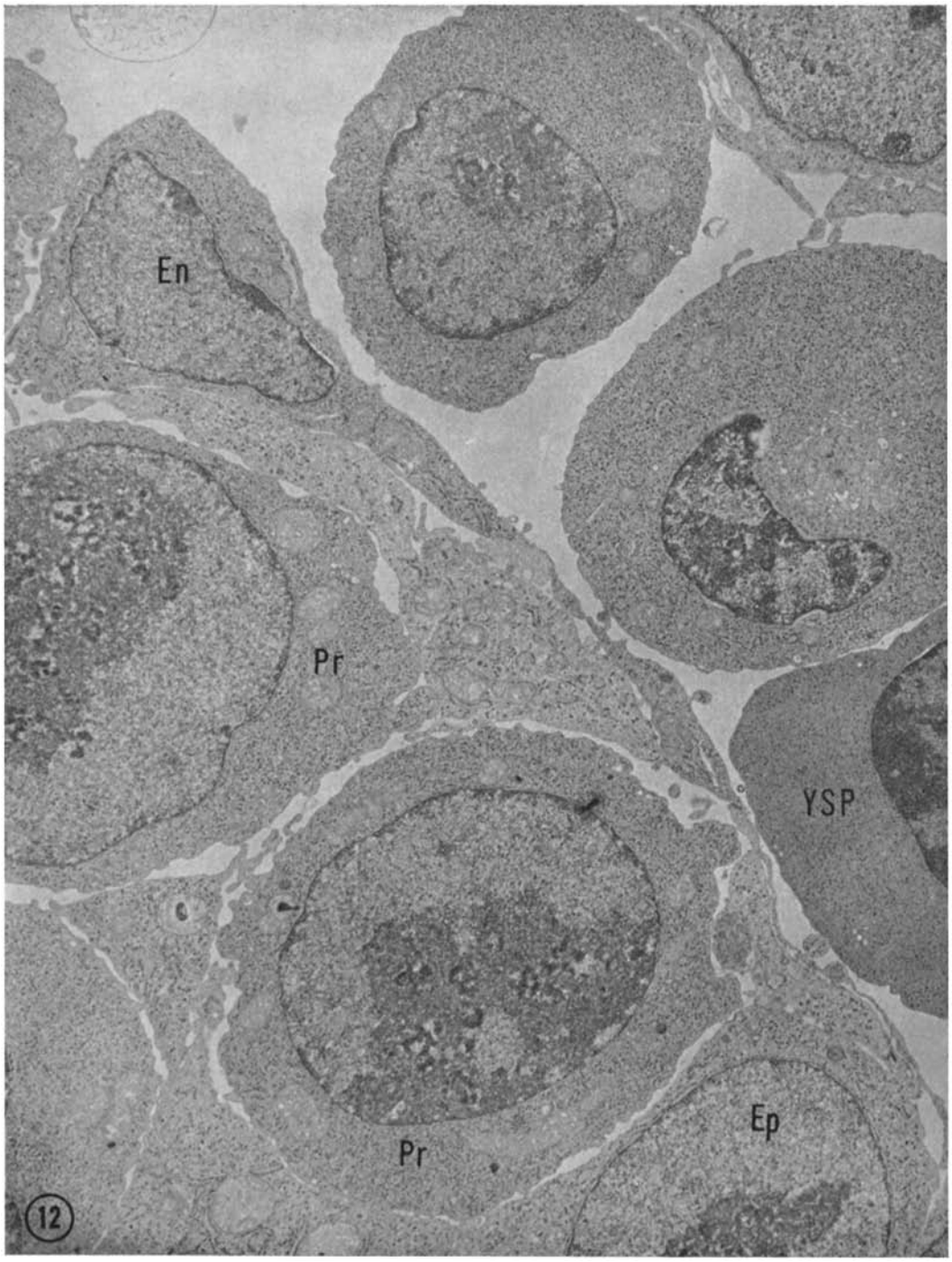


FIGURE 12 Hepatic cord and adjacent sinusoid in the central zone of the 10½ day fetus. Hepatic epithelial cells and their cytoplasmic processes (*Ep*) separate the proerythroblasts (*Pr*). Large nucleoli dominate the proerythroblast nuclei. Both yolk sac polychromatophilic erythroblasts (*YSP*) and hepatic proerythroblasts lie within the endothelium (*En*) lined sinusoid. × 6000.

amples of fusion of the two types are common (Fig. 14). The coated vesicles appear virtually identical with coated vesicles found at or near the plasma membrane in the same cells; and Fawcett (27, 28) showed them to be active in pinocytosis, specifically but not exclusively of ferritin. The demonstrated interconnections of coated vesicles, smooth vesicles, and endoplasmic reticulum clearly suggest a rôle for these membrane systems in the transport of materials to or from the cell, but they do not conclusively establish participation in iron metabolism. Indeed, in the case of the fetal mouse, ferritin granules have not been observed in association either with coated vesicles or with invaginating coated patches at the plasma membrane. Small deposits of dense granules, presumably ferritin or hemosiderin, are found, albeit infrequently, within plain vesicles in or near the Golgi apparatus of the proerythroblast (Fig. 14). Their origins have not been established. Paired centrioles, some accompanied by pericentriolar granules (29), and spindle tubules are likewise observed in this vicinity.

One large nucleolus or several confluent nucleoli are prominent features of the proerythroblast nucleus (Figs. 12 and 13). The granular zone is extremely hypertrophied, to the point that individual nucleolonemata may no longer be distinguished. Loose skeins of dense fibrillar material course throughout the nucleolus, generally within or adjacent to electron-lucent spaces or lacunae (Fig. 15). These fibrillar fascicles are accompanied by, or continuous with, small patches of a coarse electron-opaque fibrous material, tentatively identified as intranucleolar chromatin (30). Nuclear heterochromatin is restricted to a thin marginated rim along the nuclear membrane and is somewhat more prominent where the nuclear membrane approaches the nucleolus. Except at this region of attachment to the nuclear membrane, the granular zone of the nucleolus abuts directly upon the nucleoplasm without any intervening margin of heterochromatin. The bulk of the nucleoplasm consists of finely granular and filamentous material, presumably representing extended chromatin. Large granular elements, the perichromatin and large interchromatin granules (31, 32), are distinctly uncommon in this cell type as compared with later stages of erythroblast differentiation.

Differentiating proerythroblasts are mitotically active. Nevertheless, incorporation of hematopoietic precursors into the advancing epithelial cords is so rapid, being complete in approximately 12 hr, that in general, on the 10½ day, individual rather than clusters of proerythroblasts are observed, each partially separated from its neighbor by cytoplasmic processes of the epithelial cells (Fig. 12). From the 11th day onward, repeated cell divisions among the erythroblasts results in distinct clustering of these red cell precursors in a manner comparable to that seen in the erythropoietin-stimulated adult mouse spleen (33).

The sinusoidal vasculature of the central portion of the 10½ day liver is defined by a distinct, flattened endothelium as yet only incompletely separating the vascular lumen from the extravascular tissues. A mixture of yolk sac-derived polychromatophilic erythroblasts and endogenous hepatic proerythroblasts occupies the vascular spaces (Fig. 12). It would appear that these proerythroblasts develop within the hepatic cords, as previously described, and migrate through gaps in the endothelial lining into the sinusoids. Both proerythroblasts and immature megakaryocytes (Figs. 16 and 17) are observed in transit between these compartments. Although the direction of migration of these cells cannot be determined with absolute certainty from static micrographs, several features may be adduced as indirect evidence in support of a migration from cord to sinusoid. In the first place, the cells in transit almost uniformly display a cytoplasmic protrusion into the vessel; this protrusion is consistent with movement in that direction according to the normal patterns of cellular locomotion. Secondly, the endothelial and epithelial cell margins by which these migrating cells pass are uniformly evaginated into the sinusoidal lumen. Thirdly, hepatic proerythroblasts are found intravascularly only in those sinusoids adjacent to cords already containing large numbers of similar proerythroblasts. Indeed, in the 10¼ day fetus, just prior to the onset of hepatic hematopoiesis, neither hemocytoblasts nor proerythroblasts are recognized in the hepatic vasculature.

11 Day Fetal Liver and Subsequent Stages

By the 11th fetal day, the signs of established hepatic hematopoiesis are unmistakable. Serial appearance of progressively more advanced stages of erythroid cell maturation and changes in the proportion of cells at the several stages characterize the course of development from this day onwards (34). Proerythroblasts, often in small clusters (Fig. 18), constitute the principal erythropoietic element

on day 11, but in addition, smaller numbers of basophilic erythroblasts and rare polychromatophilic erythroblasts are seen. The basophil (Fig. 19) is smaller than the proerythroblast, as a consequence of a cell division during the transition (35). It owes its intense basophilia to virtually maximal close packing of cytoplasmic free-lying ribosomes; indeed, the ribosomes are so tightly packed that individual polyribosomes cannot be distinguished. Nuclear heterochromatinization is distinctly increased and no longer confined to the marginal condensations seen in proerythroblasts. The nucleolus is, over-all, more condensed; the dense fibrillar zones are less extensive, and the granular zone is reduced in thickness. The electron-lucent lacunae prominent in proerythroblast nucleoli are rarely seen and appear to be replaced by strands of condensed heterochromatin. A thin mantle of heterochromatin now envelops the nucleolus and separates the granular zone from the nucleoplasm. Large perichromatin granules are most common at this stage of erythroid cell maturation and are observed in association with both nucleolar and nonnucleolar heterochromatin.

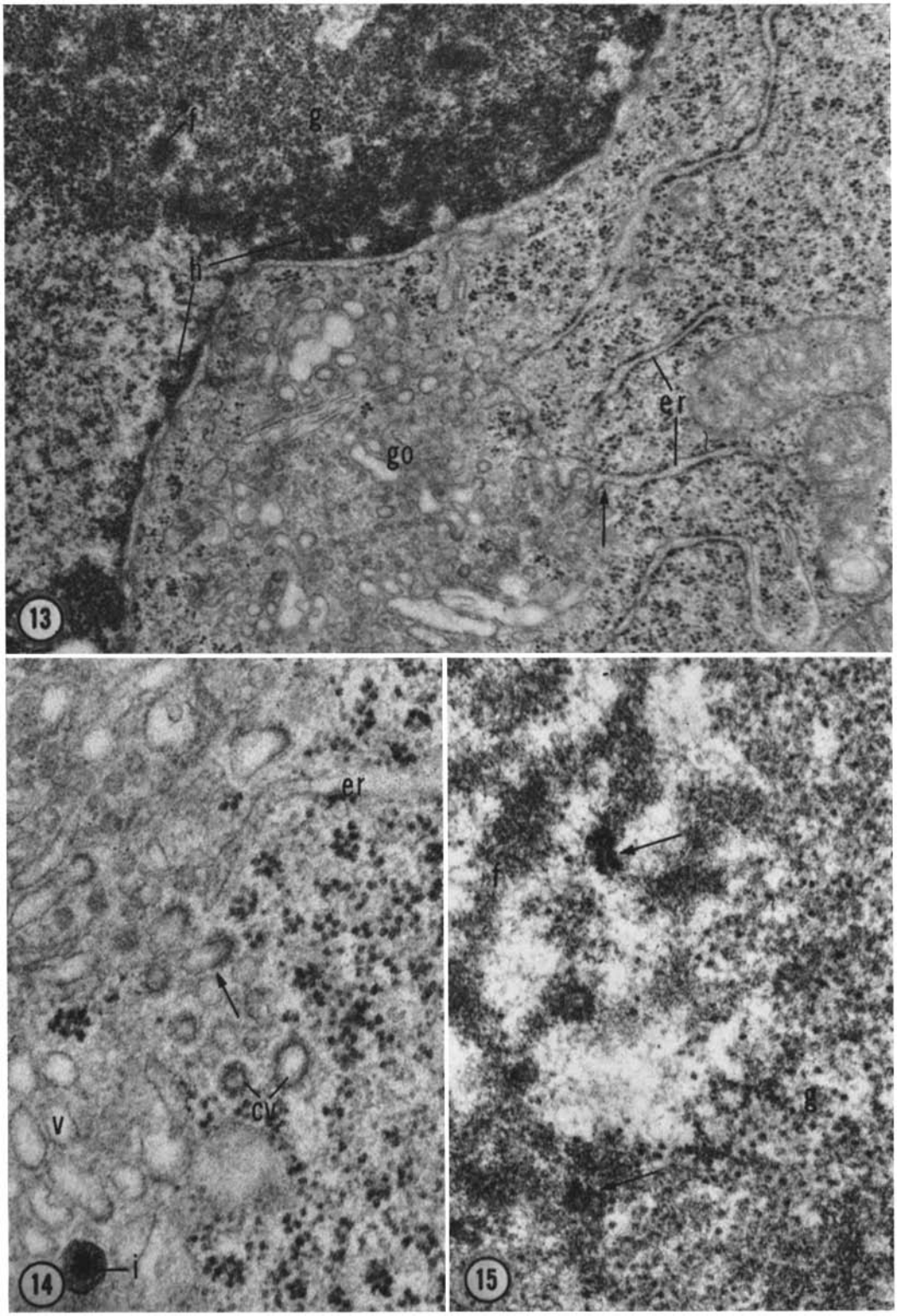
The polychromatophilic erythroblast (Fig. 20) becomes the predominant erythroid cell type by about the 14th fetal day, at which time large clusters of these cells are found within the hepatic cords, far outnumbering both the hepatic epithelial cells and the more immature erythroid precursors. The polychromatophil is mitotically active, and the cells of succeeding generations are smaller than their predecessors. The cytoplasm contains numerous free-lying polyribosomes dispersed in a progressively accumulating finely granular matrix, most likely hemoglobin. A small Golgi apparatus may persist into this stage. Vesicles containing ferritin, as well as free-lying deposits of similar material, are frequently encountered. The hepatic epithelial cells also contain recognizable deposits of ferritin at this stage. Nevertheless, morphologic evidence of transfer of these molecules between the epithelial and erythropoietic cells could not be obtained at this or any other stage.

In successive generations of polychromatophils, the nucleus displays increasing heterochromatinization while the nucleolus is reduced to a small residual nubbin of fibrillar and granular material lacking distinct nucleolonemata and embedded in a mass of condensed chromatin. Tooze and Davies (36) have quantitated the changes in nucleolar structure during amphibian erythroid cell development and point out the disproportionate decrease in the granular zone. Extrusion of this pycnotic nucleus is first seen, in small numbers, on the 12th fetal day, and at the same time the first nonnucleated reticulocytes appear in the peripheral blood among the still numerous nucleated yolk sac-derived erythroid cells. Nuclear extrusion in the fetal animal does not differ in any significant detail from the same process in the adult mammalian organism (33, 37, 38). Polarization of mitochondria alongside the separating nucleus and extensive vacuolization along the plane of cleavage between the nucleated and anucleate moieties are prominent features of this process. Discarded nuclei, enveloped in a thin rim of cytoplasm, are found both in the cords and within the sinusoids. Ingestion by macrophages appears to be the fate of these nuclei in either situation. Reticulocyte

FIGURE 13 Golgi region of a 10½ day proerythroblast. A portion of the large nucleolus is seen at upper left; extensive granular (*g*) and fibrillar (*f*) zones are noted. A thin rim of heterochromatin (*h*) marginates the nuclear membrane and becomes prominent where the nucleolus approaches the nuclear membrane. Elements of ribosome-studded endoplasmic reticulum (*er*) connect with cisternae and vesicles of the Golgi system (*go*) as indicated (arrow). × 25,000.

FIGURE 14 Golgi region of a 10½ day proerythroblast at higher magnification. Coated vesicles (*cv*), plain vesicles (*v*), and fusion of the two (arrow) are noted. A junction between endoplasmic reticulum (*er*) and an element of the Golgi system may also be seen. A plain vesicle containing ferritin or hemosiderin (*i*) is indicated at lower left. × 50,000.

FIGURE 15 Portion of the nucleolus of a proerythroblast. The granular zone (*g*) surrounds a space through which pass fascicles of the fibrillar component (*f*) accompanied by short segment of a coarse, electron-opaque, and probably fibrous material (arrows). × 50,000.



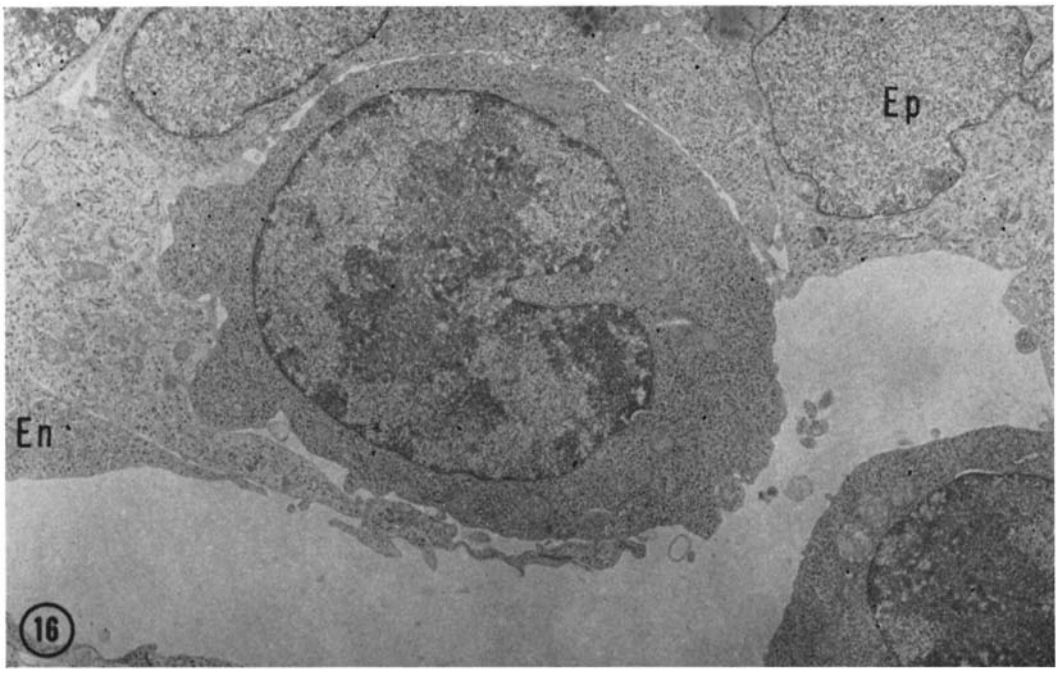


FIGURE 16 Proerythroblast apparently in passage between hepatic cord (above) and sinusoid (below) through a gap in the tenuous endothelial layer. A direction of movement, from cord to vessel, is suggested by deflection of the epithelial (*Ep*) and the endothelial (*En*) cell margins into the sinusoid lumen. × 6000.

formation accelerates rapidly from the 12th day onward, so that by the 16th fetal day nonnucleated erythroid cells of hepatic origin have virtually replaced the yolk sac erythroid cell lineage in the circulation. Maturation of these reticulocytes to mature erythrocytes entails loss of ribosomes, polyribosomes, and mitochondria in a fashion qualitatively similar to that described in other mammalian species (39–41).

DISCUSSION

The two primary sites of erythropoiesis in the fetal C57BL/6J mouse, yolk sac and liver, produce erythroid cell lines distinguished both morphologically and by the nature of the hemoglobins synthesized (1, 3, 4). A question of considerable significance is whether the precursors for hepatic erythropoiesis arise endogenously in the liver or colonize the liver from the yolk sac, the antecedent site of erythropoiesis. A variety of observations, none conclusive, bear upon this problem. Using the criterion of survival of lethally x-irradiated mice transfused with cells from donor fetuses, Wolf and Nishimura (42) were unable to demonstrate significant numbers of hematopoietic stem cells in the 10 day fetal BALB/C mouse (the gestational dating, as provided by these authors, has been reassigned to conform to the convention currently in use). Likewise Matioli et al. (43) were unable to find stem cell activity among yolk sac erythroid elements up to the 11th fetal day. The present observations establish the onset of active hematopoietic differentiation in the liver between the 10¼ and 10½ fetal days. Taken together, these observations would suggest that hepatic erythropoiesis arises *in situ* without the mediation of blood-borne stem cells from more primitive loci of hematopoiesis. Morphologic observations are consistent with this interpretation. Cells of the transverse septum, presumably of mesenchymal origin (see below), in close association with outgrowing cells of the primitive hepatic epithelium appear to provide the first recognizable blood cell precursors in this organ. Proerythroblasts appear within the 10½- and 11-day hepatic sinusoids, but only after hematopoiesis is established in the adjacent extravascular cords. A more direct approach to the cellular origins of erythropoietic tissues in am-

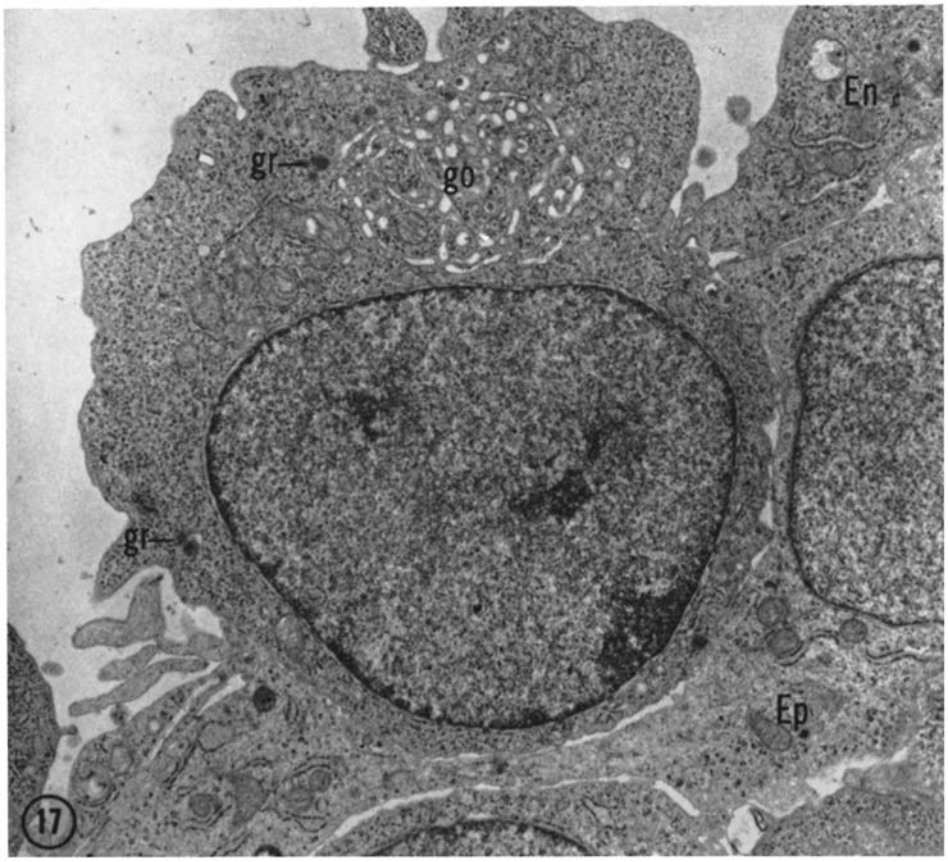


FIGURE 17 A megakaryocytic precursor, identified by its characteristic granules (*gr*) and cisternal Golgi system (*go*), apparently in transit from cord (lower right) to sinusoid. As in the previous figure, the direction of migration is suggested by the disposition of nucleus and cytoplasm, and by evagination of the epithelial (*Ep*) and endothelial (*En*) cell margins into the vascular channel. × 8000.

phibia has been attempted by Hollyfield (44) who employed a chromosomal marker and transplantation techniques. Those studies suggest, as in the present case, that blood islands do not provide stem cells for the colonization of sites of subsequent erythropoiesis.

That the mesenchyme exerts a fairly specific influence upon the endodermal tissues involved in hepatogenesis has been elegantly established in the chick by Le Douarin (19, 20). Similar dependence is recognized in other differentiating epithelial tissues (45–49). The role of the hepatic endoderm in the inductive events resulting in hematopoiesis, however, remains an area of considerable speculation. In the case of yolk sac erythropoiesis, the endoderm appears to exert at least a promoting function, since in both avian and urodele species, cultures of isolated mesenchyme undergo only abortive or incomplete erythropoiesis (50, 51). The present observations do not bear upon the mechanism of such a proposed influence but provide evidence as to the uniquely intimate nature of this epithelio-mesenchymal interaction. Unlike other endodermal derivatives (24, 52–54), the epithelio-mesenchymal interface during active hepatic morphogenesis and hematopoiesis is not delineated by a basement membrane. This situation is not unique to the liver. Yolk sac hematopoiesis likewise occurs at an epithelio-mesenchymal interface devoid of an intervening basement membrane (Chui, D., and R. A. Rifkind. Unpublished observations). Failure to synthesize a basement membrane, most likely an epithelial function (55–58), does not appear to interfere, in the liver, with the laying

down of a primitive fibrous connective tissue at the interface, a presumed mesenchymal activity (24, 59). In the absence of an intervening basement membrane separating hepatic mesenchyme and epithelium, transient adherent junctions and desmosomes are established between these two tissues during early phases of organogenesis. Interspecific cell junctions are rarely encountered in embryologic material and are virtually unknown in adult tissues. Trelstad and colleagues (60) have reported similar findings during primary inductive events in avian embryogenesis. The present observations suggest that such interactions are not unique to primary induction. Theoretical implications of cell contacts and adhesions for inductive processes have recently been discussed by Ambrose (61), but their specific significance for hepatic organogenesis and erythropoiesis remains obscure.

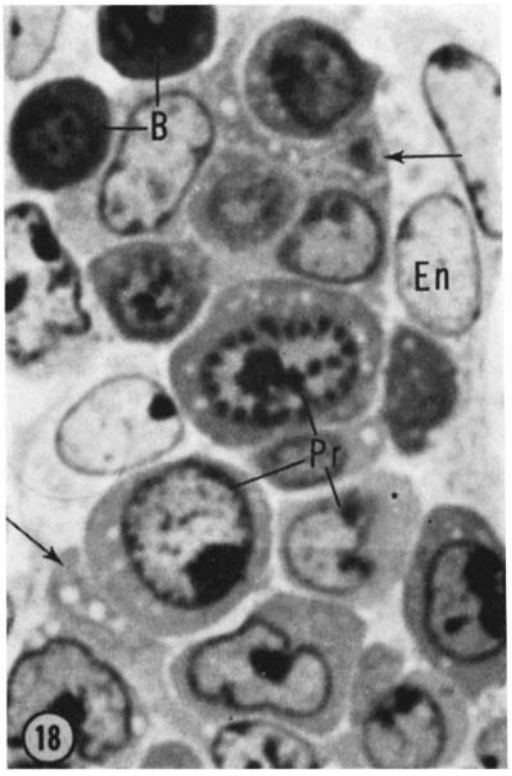


FIGURE 18 Light micrograph of a hepatic cord at the 11th fetal day. Clusters of proerythroblasts (*Pr*) as well as darkly staining basophilic erythroblasts (*B*) mingle with the cytoplasmic processes of epithelial cells (arrows) and are enclosed by endothelial cells (*En*) lining the adjacent sinusoids. One proerythroblast (center) is in prophase. × 2000.

No distinct changes in hepatic epithelial cell fine-structure can be correlated, with confidence, with the onset of hepatic erythropoiesis in the fetal mouse. Zamboni (12), in a study of the human fetus, suggests that elaboration of the endoplasmic reticulum and the accumulation of both glycogen and ferritin within the hepatic epithelial cell may have some specific bearing upon the onset of erythroid cell differentiation. In the present case, neither glycogen nor ferritin is recognizable in the epithelial cells coincident with incipient hematopoiesis, and the endoplasmic reticulum does not reach its typical adult disposition until several days later. Hepatic iron stores, as manifested by ferritin deposits, accumulate subsequent to the onset of active endogenous hepatic hemoglobin synthesis as determined by the presence of numerous polychromatophilic erythroblasts. In accord with the observations of others (12, 36, 62, 63), no evidence of transport of ferritin between erythroid and hepatic parenchymal cells could be found at any stage of fetal development in this species. Clearly, physiological and pathological factors must exist which determine the variable patterns of ferritin accumulation in erythroid tissues (10, 11, 33, 64).

The morphologic differentiation of erythroid elements within the fetal mouse liver proceeds through a series of stages, denoted in the hematologic literature as hemocytoblast, proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, orthochromatic erythroblast, reticulocyte, and mature erythrocyte (65). The over-all structural changes which characterize this sequence include progressive reduction in cell volume, heterochromatinization and expulsion of the nucleus, accumulation of hemoglobin, and gradual disappearance of other cytoplasmic organelles including Golgi apparatus, endoplasmic reticulum, ribosomes, and mitochondria (3, 10–12, 33, 34, 37–41). Certain details of this transformation deserve further comment. The hemocytoblast, classically identified as the multipotential hemopoietic stem cell (7), has, as in the present observations, generally been identified with the the most primitive cell type demonstrating structural features in common with differentiating and developing hematopoietic cells (10, 12, 33). These structural features include their rounded contours, loss of intercellular junctions, high nuclear:cytoplasmic ratio, and relatively prominent cytoplasmic baso-

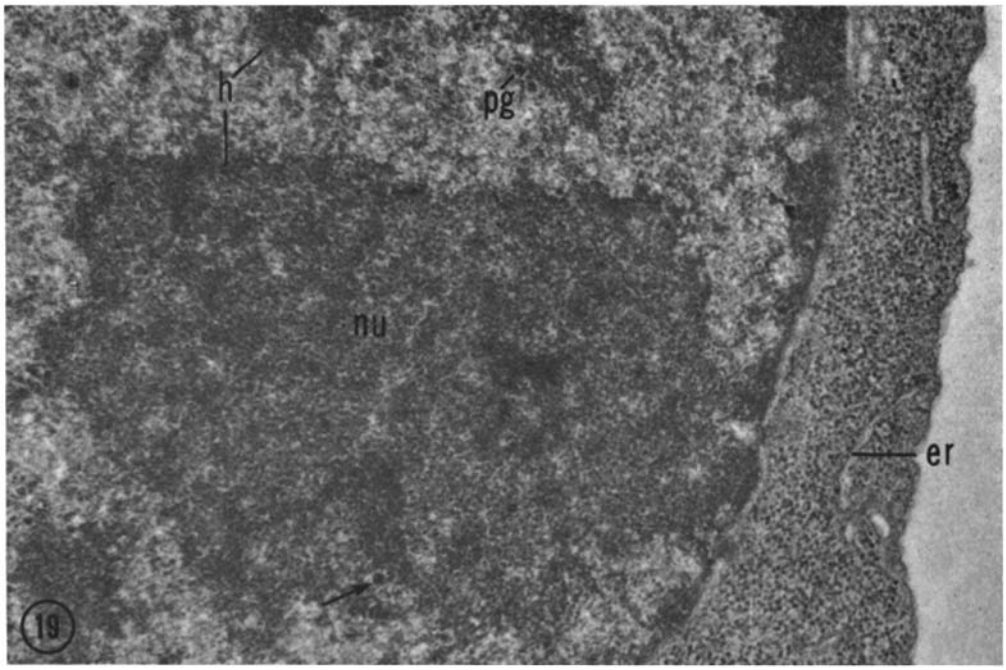


FIGURE 19 Part of the nucleus and cytoplasm of a basophilic erythroblast in the liver of an 11 day fetus. A thin rim of heterochromatin (*h*) envelops the nucleolus (*nu*). Strands of similar material lace through the nucleolus, while larger blocks of condensed chromatin are distributed in the nucleoplasm. Perichromatin granules (*pg*) lie both in the nucleoplasm and in the nucleolus (arrow). Densely packed ribosomes along with rare and attenuated elements of the endoplasmic reticulum (*er*) occupy the cytoplasm. × 28,000.

philia and ribosome concentration. The cell which we tentatively assign to this category in the fetal mouse liver is first observed between the 10¼ and 10½ fetal days at the endoderm-mesenchyme interface. By the time such cells are incorporated within the substance of the hepatic cords, their transformation into definitive hematopoietic cells is even more apparent. Arbitrarily we have termed these cells proerythroblasts, the most primitive morphologically recognizable erythroid cell precursors (65). It is clear, however, that we can not with certainty assign a functional significance to these cells by morphological criteria alone. Some cells at this stage may retain attributes of the multipotential stem cell, as could be assayed by their colony-forming and radiation-protecting capacity (42, 43). Other cells may possess sensitivity to the erythropoiesis-stimulating protein, erythropoietin, a property already recognized in hematopoietic cells of the 10¾ day mouse fetus (14). Only in the case of the infrequent primitive megakaryocyte with its characteristic granules and Golgi membranes (11, 12, 26) can definitive morphologic evidence of a differentiative commitment be provided at this early stage of hepatic hematopoiesis.

The unique cytological feature of the basophilic erythroblast is the extraordinary concentration of ribosomes in its cytoplasm. These ribosomes are, most likely, a product of the strikingly large nucleoli (66, 67) observed in the antecedent proerythroblast stage, and they account, in part at least, for the demonstrated intense RNA synthesis characteristic of the proerythroblast (68). A marked decrease in nucleolar size, principally at the expense of the granular zone, accompanies this increment in cytoplasmic ribosomes. Nevertheless, despite the massive influx of ribosomes into the cytoplasm which must occur between the proerythroblast and basophil stages, no evidence for passage of particulate ribosomes across the nuclear membranes or through the nuclear pores has been observed. It may be that ribosomes are not transferred in the form of a discrete particle of ribosomal dimensions (69, 70) or that kinetic

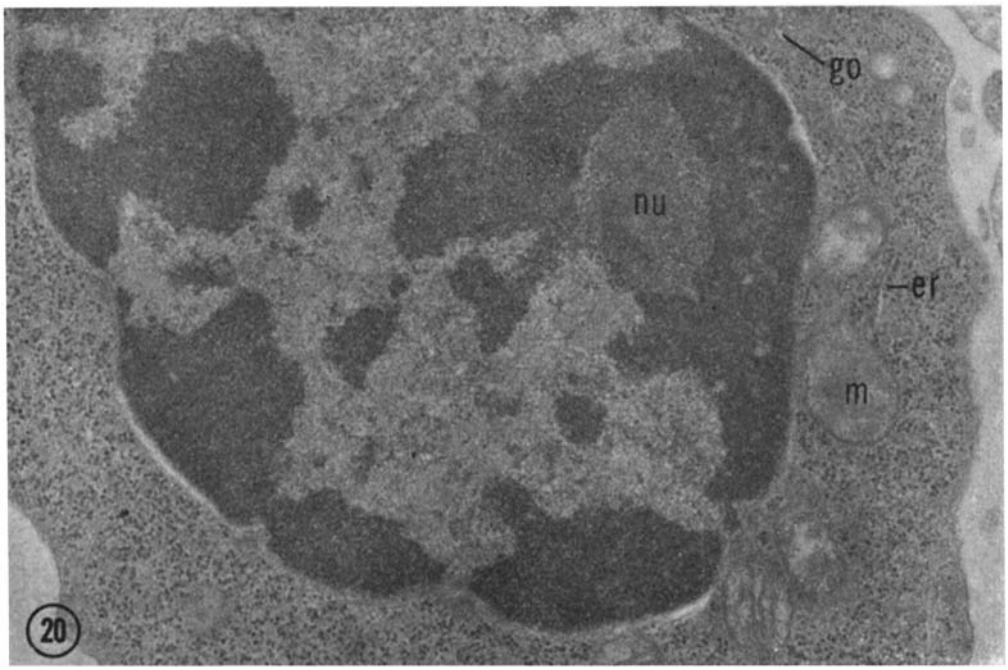


FIGURE 20 Hepatic polychromatophilic erythroblast at 12 days' gestation. Extensive heterochromatinization and shrinkage of the nucleolus (*nu*) accompany an over-all reduction in cellular size. Numerous polyribosomes are disposed in a finely granular cytoplasmic matrix. A small, residual Golgi system (*go*) is noted. Endoplasmic reticulum (*er*) is even less evident than at the basophil stage. Mitochondrion, *m*. × 28,000.

considerations make this observation unlikely. Alternatively, the bulk of cytoplasmic ribosomes or their precursors may, in the case of the proerythroblast, be released from the nucleus during a mitotic division. Such a division occurs between the proerythroblast and basophil stages (35), and other types of nuclear granule are known to be released into the cytoplasm during mitosis (71).

It is tempting to attribute the fairly rapid curtailment of RNA synthesis, which begins at the basophilic stage, to the progressive chromatin condensation which commences virtually simultaneously. Such a concept would be consistent with several observations on the relative synthetic capacities of extended and condensed chromatin in other cell lines (72, 73). As attractive as this hypothesis may be, it must be reconciled with a number of observations. The bulk of RNA synthesis is ribosomal and appears to be completed by the end of the basophilic stage (35, 68, 74), whereas progressive heterochromatinization continues until the moment of nuclear expulsion and involves extranucleolar as well as nucleolus-associated chromatin. In the same vein, although definitive data are not available, the best evidence suggests that the synthesis of messenger RNA for hemoglobin synthesis is completed prior to the basophilic stage (2, 3, 4), hence before condensation of the major portion of chromatin. Finally, it is also reasonable to correlate the observed changes in the physical state of the chromatin with those alterations in the cell's replicative capacity (75) which culminate in cessation of DNA synthesis and cell division during the polychromatophilic stage (2). The precise relationship and significance of heterochromatinization to both the transcriptional and replicative functions of the erythroblast chromosome remain to be elucidated.

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REFERENCES

1. CRAIG, M. L., and E. S. RUSSELL. 1964. A developmental change in hemoglobin correlated with an embryonic red cell population in the mouse. *Develop. Biol.* **10**:191.
2. MARKS, P. A., and J. S. KOVACH. 1966. Development of mammalian erythroid cells. *In* Current Topics in Developmental Biology. A. Monroy and A. A. Moscona, editors. Academic Press Inc., New York. 213.
3. KOVACH, J. S., P. A. MARKS, E. S. RUSSELL, and H. EPLER. 1967. Erythroid cell development in fetal mice: Ultrastructural characteristics and hemoglobin synthesis. *J. Mol. Biol.* **25**:131.
4. FANTONI, A., A. BANK, and P. A. MARKS. 1967. Globin composition and synthesis of hemoglobins in developing fetal mice erythroid cells. *Science.* **157**:1327.
5. RUSSELL, E. S., and S. E. BERNSTEIN. 1966. Blood and blood formation. *In* Biology of the Laboratory Mouse. E. L. Green, editor. McGraw-Hill Book Company, New York. 351.
6. BORGHESE, E. 1959. The present state of research on WW mice. *Acta Anat.* **36**:185.
7. BLOOM, W. 1938. Embryogenesis of mammalian blood. *In* Handbook of Hematology. H. Downey, editor. Hoeber-Harper, New York. **2**:865.
8. JONES, O. P. 1959. Formation of erythroblasts in the fetal liver and their destruction by macrophages and hepatic cells. *Anat. Rec.* **133**:294.
9. ACKERMAN, G. A., J. A. GRASSO, and R. A. KNOUFF. 1961. Erythropoiesis in the mammalian embryonic liver as revealed by electron microscopy. *Lab. Invest.* **10**:787.
10. GRASSO, J. A., H. SWIFT, and G. A. ACKERMAN. 1962. Observations on the development of erythrocytes in mammalian fetal liver. *J. Cell Biol.* **14**:235.
11. SORENSON, G. D. 1963. Hepatic hematocytopoiesis in the fetal rabbit: A light and electron microscopic study. *Ann. N.Y. Acad. Sci.* **111**: 45.
12. ZAMBONI, L. 1965. Electron microscopic study of blood embryogenesis in humans. II. The hemopoietic activity in the fetal liver. *J. Ultrastruct. Res.* **12**:525.
13. SOUTHARD, J. L., H. G. WOLFE, and E. S. RUSSELL. 1965. Artificial insemination of dystrophic mice with mixtures of spermatozoa. *Nature.* **208**:1126.
14. COLE, R. J., and J. PAUL. 1966. The effects of erythropoietin on haem synthesis in mouse yolk sac and cultured foetal liver cells. *J. Embryol. Exp. Morphol.* **15**:245.
15. RUGH, R. The Mouse. 1968. Burgess Publishing Co., Minneapolis, Minn. 262.
16. FARQUHAR, M. G., and G. E. PALADE. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* **26**:263.
17. ELIAS, H. 1955. Origin and early development of the liver in various vertebrates. *Acta Hepatol.* **3**:1.
18. WILSON, J. W., C. S. GROAT, and E. H. LEDUC. 1963. Histogenesis of the liver. *Ann. N.Y. Acad. Sci.* **111**:8.
19. DOUARIN, N. LE. 1964. Étude expérimentale de l'organogenèse du tube digestif et du foie chez l'embryon de poulet. II. Étude expérimentale de l'organogenèse hépatique. *Bull. Biol. Fr. Belg.* **98**:589.
20. DOUARIN, N. LE. 1964. Isolement expérimental du mésenchyme propre du foie et role morphogene de la composante mésodermique dans l'organogenèse hépatique. *J. Embryol. Exp. Morphol.* **12**:141.
21. PETERS, V. B., G. W. KELLY, and H. M. DEMBITZER. 1963. Cytologic changes in fetal and neonatal hepatic cells of the mouse. *Ann. N.Y. Acad. Sci.* **111**:87.
22. WILT, F. H. 1966. The concept of messenger RNA and cytodifferentiation. *Amer. Zool.* **6**: 67.
23. WILT, F. H. 1967. The control of embryonic hemoglobin synthesis. *In* Advances in Morphogenesis. M. Abercrombie and J. Brachet, editors. Academic Press Inc., New York. **6**:89.
24. KALLMAN, F., and C. GROBSTEIN. 1964. Fine structure of differentiating mouse pancreatic exocrine cells in transfilter culture. *J. Cell Biol.* **20**:399.
25. KARRER, H. E. 1961. Electron microscopic observations on chick embryo liver. Glycogen, bile canaliculi, inclusion bodies and hematopoiesis. *J. Ultrastruct. Res.* **5**:116.
26. YAMADA, E. 1957. The fine structure of the megakaryocyte in the mouse spleen. *Acta Anat.* **29**:267.
27. FAWCETT, D. W. 1964. Local specialization of the plasmalemma in micropinocytosis vesicles of erythroblasts. *Anat. Rec.* **148**:370.
28. FAWCETT, D. W. 1965. Surface specializations of absorbing cells. *J. Histochem. Cytochem.* **13**: 75.
29. ROBBINS, E., G. JENTZSCH, and A. MICALI. 1968. The centriole cycle in synchronized Hela cells. *J. Cell Biol.* **36**:329.
30. BERNHARD, W. 1966. Ultrastructural aspects of the normal and pathological nucleolus in mammalian cells. *Nat. Cancer Inst. Monogr.* **23**: 13.
31. Watson, M. L. 1962. Observations on a granule

associated with chromatin in the nuclei of cells of rat and mouse. *J. Cell Biol.* **13**:162.

32. SWIFT, H. 1962. Nucleoprotein localization in electron micrographs: Metal binding and radioautography. *In* Interpretation of Ultrastructure. R. J. C. Harris, editor. Academic Press Inc., New York and London. 213.
33. ORLIC, D., A. S. GORDON, and J. A. G. RHODIN. 1965. An ultrastructural study of erythropoietin-induced red cell formation in mouse spleen. *J. Ultrastruct. Res.* **13**:516.
34. FANTONI, A., A. DE LA CHAPELLE, R. A. RIFKIND, and P. A. MARKS. 1968. Erythroid cell development in fetal mice: Synthetic capacity for different proteins. *J. Mol. Biol.* **33**:79.
35. BORSOOK, H., J. A. LINGREL, J. L. SCARO, and R. C. MILLETTE. 1962. Synthesis of haemoglobin in relation to the maturation of erythroid cells. *Nature (London).* **196**:347.
36. TOOZE, J., and H. G. DAVIES. 1967. Light- and electron-microscope studies on the spleen of the newt *Triturus Cristatus*: The fine structure of erythropoietic cells. *J. Cell Sci.* **2**:617.
37. SKUTELSKY, E., and D. DANON. 1967. An electron microscopic study of nuclear elimination from the late erythroblast. *J. Cell Biol.* **33**:625.
38. SIMPSON, C. F., and J. M. KLING. 1967. The mechanism of denucleation in circulating erythroblasts. *J. Cell Biol.* **35**:237.
39. RIFKIND, R. A., D. DANON, and P. A. MARKS. 1964. Alterations in polyribosomes during erythroid cell maturation. *J. Cell Biol.* **22**: 599.
40. ROWLEY, P. J. 1967. Disappearance of ribosomes and polyribosomes during *in vivo* erythroid maturation. *Nature (London).* **216**:1109.
41. SIMPSON, C. F., and J. M. KLING. 1968. The mechanism of mitochondrial extrusion from phenylhydrazine-induced reticulocytes in the circulating blood. *J. Cell Biol.* **36**:103.
42. WOLF, N. S., and E. T. NISHIMURA. 1962. Protection of lethally x-irradiated mice with cells of whole embryo: A quantitative study. *Lab. Invest.* **11**:136.
43. MATIOLI, G. T., H. NIEWIESCH, and H. VOGEL. 1968. Distribution of hematologically competent stem cells in tissues of mice (fetus and newborn). *Fed. Proc.* **27**:672 (A).
44. HOLLYFIELD, J. G. 1966. The origin of erythroblasts in Rana Pipiens tadpoles. *Develop. Biol.* **14**:461.
45. GROBSTEIN, C. 1955. Inductive interaction in the development of mouse metanephros. *J. Exp. Zool.* **130**:319.
46. AUERBACH, R. 1960. Morphogenetic interactions in the development of the mouse thymus gland. *Develop. Biol.* **2**:271.
47. DAMERON, F. 1961. L'influence de divers mésenchyme sur la differentiation de l'epithelium pulmonaire de l'embryon de poulet en culture *in vitro*. *J. Embryol. Exp. Morphol.* **9**:628.
48. WESSELLS, N. K. 1962. Tissue interactions during skin histodifferentiation. *Develop. Biol.* **4**:87.
49. TADERERA, J. V. 1967. Control of lung differentiation *in vitro*. *Develop. Biol.* **16**:489.
50. FINNEGAN, C. V. 1953. Studies of erythropoiesis in salamander embryos. *J. Exp. Zool.* **123**:371.
51. WILT, F. H. 1965. Erythropoiesis in the chick embryo: The role of endoderm. *Science.* **147**: 1588.
52. KARRER, H. E. 1958. The ultrastructure of mouse lung: The alveolar macrophage. *J. Biophys. Biochem. Cytol.* **4**:693.
53. HINSCH, GERTRUDE W. 1967. Ultrastructural differentiation of the epithelium and mucous glands of the esophagus in the chick embryo. *J. Morphol.* **123**:121.
54. LOW, F. N. 1967. Developing boundary (basement) membranes in the chick embryo. *Anat. Rec.* **159**:231.
55. FARQUHAR, M. G., S. L. WISSIG, and G. E. PALADE. 1961. Glomerular permeability. I. Ferritin transfer across the normal glomerular wall. *J. Exp. Med.* **113**:47.
56. ANDRES, G. A., C. MORGAN, K. C. HSU, R. A. RIFKIND, and B. SEEGAL. 1962. Electron microscopic studies of experimental nephritis with ferritin-conjugated antibody. The basement membranes and cisternae of visceral epithelial cells in nephritic rat glomeruli. *J. Exp. Med.* **115**:929.
57. PIERCE, G. B., JR., A. R. MIDGLEY, and J. SRIRAM. 1963. The histogenesis of basement membranes. *J. Exp. Med.* **117**:339.
58. KALLMAN, F., and C. GROBSTEIN. 1966. Localization of glucosamine-incorporating materials at epithelial surfaces during salivary epithelio-mesenchymal interaction *in vitro*. *Develop. Biol.* **14**:52.
59. KALLMAN, F., and C. GROBSTEIN. 1965. Source of collagen at epitheliomesenchymal interfaces during inductive interaction. *Develop. Biol.* **11**:169.
60. TRELSTAD, R. L., E. D. HAY, and J. P. REVEL. 1967. Cell contact during early morphogenesis in the chick embryo. *Develop. Biol.* **16**:78.
61. AMBROSE, E. J. 1967. Possible mechanisms of the transfer of information between small groups of cells. *In* Cell Differentiation. A. V. S. de Reuck and J. Knight, editors. Little, Brown and Company, Boston, Mass. 101.
62. JONES, O. P. 1964. Decrease in pinocytosis accompanying maturation of erythroblasts. *Anat. Rec.* **148**:269.
63. BERMAN, I. 1967. The ultrastructure of erythro-

blastic islands and reticular cells in mouse bone marrow. *J. Ultrastruct. Res.* **17**:291.

64. BESSIS, M. 1961. The blood cells and their formation. *In* The Cell. J. Brachet and A. E. Mirsyk, editors. Academic Press Inc., New York and London. **5**:163.
65. WINTROBE, M. M. 1961. Clinical Hematology. Lea & Febiger, Philadelphia, Pa. 5th edition. 86.
66. BROWN, D. D. 1966. The nucleolus and synthesis of ribosomal RNA during oogenesis and embryogenesis of *Xenopus laevis*. *Natl. Cancer Inst. Monogr.* **23**:297.
67. EDSTROM, J., and W. BEERMAN. 1962. The base composition of nucleic acids in chromosomes, puffs, nucleoli and cytoplasm of *Chironemas* salivary gland cells. *J. Cell Biol.* **14**: 371.
68. GRASSO, J. A., J. W. WOODARD, and H. SWIFT. 1963. Cytochemical studies of nucleic acids and proteins in erythrocytic development. *Proc. Nat. Acad. Sci. U.S.A.* **50**:134.
69. VAUGHAN, M., J. WARNER, and J. E. DARNELL. 1967. Ribosomal precursor particles in the Hela cell nucleus. *J. Mol. Biol.* **25**:235.
70. STEVENS, A. R. 1967. Machinery for exchange across the nuclear envelope. *In* The Control of Nuclear Activity. L. Goldstein, editor. Prentice-Hall, Inc., Englewood Cliffs, N.J. 189.
71. SWIFT, H. 1963. Cytochemical studies on nuclear fine structure. *Exp. Cell Res. Suppl.* **9**:54.
72. HSU, T. C. 1962. Differential rate in RNA synthesis between euchromatin and heterochromatin. *Exp. Cell Res.* **27**:332.
73. LITTAU, V. C., V. G. ALLFREY, J. H. FRENSTER, and A. E. MIRSKY. 1964. Active and inactive regions of nuclear chromatin as revealed by electron microscope autoradiography. *Proc. Nat. Acad. Sci. U.S.A.* **52**:93.
74. DEBELLIS, R. H., N. GLUCK, and P. A. MARKS. 1964. Synthesis of ribonucleic acid in rabbit blood cells *in vivo*. *J. Clin. Invest.* **43**:1329.
75. LIMA-DE-FARIA, A., and H. JAWORSKA. 1968. Late DNA synthesis in heterochromatin. *Nature.* **217**:138.