A STUDY OF THE CHRONOLOGY OF ERYTHROPOIESIS IN THE EMBRYO OF THE PIG (SUS SCROFA)

By

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INTRODUCTION

Study of the development of blood in the embryo has centered around three principal areas of investigation: (1) the controversy concerning the role of the angioblast, (2) tissues and stem cells from which blood cells are derived, (3) the question of what organs and/or regions of the body play dominant roles in blood cell formation.

The embryo of the domestic pig, Sus scrofa, has assumed importance as a laboratory specimen for study in pre-medical and medical embryology courses. No chronology for the formation of red blood cells in this mammal has been presented so far as can be found in the literature. The purpose of this investigation, therefore is to record the sequence of appearance, ascendency, and decline of erythropoiesis in various regions and organs in the domestic pig.

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PART II

REVIEW OF LITERATURE

Almost any discussion of the literature of erythropoiesis involves a review of the controversy over the angioblast theory of His (1900); according to this theory, endothelial cells of the embryo originate as layers between the yolk mesoderm and the entoderm, but are derived from entoderm. It is suggested that the angioblast persists throughout life and is the sole and direct precursor of all vessels and blood cells formed intra-embryologically (Minot 1912). Evans (1912) describes the aorta descendens as being formed from the vitelline capillary plexus. Although he indicates no final source of the endothelial cells from which the anterior end of the aorta is formed, he states that all vessels within the embryo are formed from sprouts that, growing out from the aorta and from the vitelline veins, first form plexuses of capillaries, and then vessels, as channels of circulation become established. Evans states that "It can only be said that the histological appearances are inconclusive. On the other hand the series of sections does not permit one to exclude the strong possibility that these (intra-embryonic vascular cells) constitute a connected unit which have invaded the embryo from the splanchnopleure of the yolksac." (page 590) Bremer (1912) believes that in the network of angioblastic cords there are hollow spaces

unconnected with each other at first and hence not reached by injection; that it was the observance of these spaces, which later fuse, that led to the observations of Ruckert and Mollier (1906) that the dorsal aortae arise in situ. In this summary Bremer states that in the rabbitt the first aortic arch, the conus arteriosus, and the lateral heart are all parts of an original network of angioblastic cords derived from the extra-embryonic plexus of blood vessels. These views and statements are derived from the theory that vasculogenic cells (vascular endothelium) do not arise from non-differentiated cells within the body of the embryo but invade from extra-embryonic regions. Hence the question is: Can vascular cells and blood-forming elements arese within the embryo from embryonic tissues? or are these elements derived solely from the extra-embryonic area vasculosa or the angioblast?

Stockard (1915) compares the central mass of Fundulus with the yolksac of other vertebrates, stating that in many teleosts the first red cells appear here when there is no yolksac island area. In normal teleosts there are islands formed on the posterior and ventral surfaces of the yolksac by groups of red cells which are swept away into the circulation. Stockard believes that the endothelium of a blood vessel never shows any cell in the process of forming an erythrocyte and that there is no evidence that blood corpuscles ever arise from vascular endothelium. In support of his contention that red blood cells arise extra-embryonic-

ally, he states that, when circulation in the teleost is prevented, the vessels of the heart are empty. Emmel (1915) describes formation of red cells by the endothelium in the region of aortic arches in the dorsal aorta. He suggests that a toxic condition due to the degenerating vessels of the arches may provide the stimulus to the formation of the hemoblast clusters in the lumen of the aorta, ventral to such vessels. Jordan (1916) cites two sources of origin of hemoblasts, mesenchyma and endothelium of the earliest blood vessels. Sabin (1917) states that endothelial cells give rise to red blood cells by developing hemoglobin. Doan, Cunningham, and Sabin (1925) describe the endothelium as undergoing two types of division: the first in an axis parallel to the wall of the sinusoid which increases the endothelium; the second, in an axis at right angle to the endothelial wall, which gives rise to a megaloblast and to an endothelial cell.

In contrast to the theory of His, Reagan (1917) presents the following summary of the local origin theory of Ruckert and Mollier: Vessels are formed by proliferation of cells already formed, by the addition of solid aggregates, by the addition of locally differentiated endothelial cavities, and by the active migration of mesenchyme cells to form vascular cavities. According to this theory, mesenchyme in any part of the body may become transformed into vascular tissues and the cells which form an intra-embryonic vessel are not in direct lineage with those of the angioblast; they have not come into being as ingrowths from the yolksac nor from any

pre-existing endothelial cells. Reagan summarizes his experimental work on Fundulus by stating that mesenchyme in many parts of the body becomes converted into endothelium; prevascular tissues come from more than one germ layer; mesenchyme cells that form a given type of blood cell are not confined to any one region of the body; and endothelium becomes transformed into blood cells. He demonstrates that erythrocytes develop from endothelium in the region of the anterior end of the nerve cord, the ducts of Cuvier, the heart, and liver of the teleost (Reagan and Thorington, 1914). Reagan thus differs from Stockard who maintains that nothing has ever been seen in the study of wandering mesenchyme cells in the living embryo to indicate that an endothelial cell has the power to produce a blood cell or to change into a blood cell of any type, but much has since been seen which indicates the contrary.

According to Haff (1915) peritoneum covering the embryonic liver may give rise to cells within the liver that may differentiate into blood cells. Sabin (1917) states that "angioblasts" and later endothelial cells give rise to red blood cells. Sabin defines "angioblasts" as blood formhg cells derived from mesenchyme. These cells would, therefore, be hemoblasts. Dantchakoff (1916) concludes that new erythropeoitic organs are formed by local differentiation of hemoblasts both in bone and liver. She states that an endothelial member split from the mesenchyme, surrounds some of the cell masses and marks them for erythrocytes (page 405). In sheep, megaloblast proliferation takes place in vessels and

mucoid tissues throughout the body, the proliferation in liver and kidney in no way differing from that in vessels (Goodall, 1908). Maximov (1909,b) states that it is known that embryonic vessels are formed from vessel cells in situ, which have that special function. One can see the formation of empty vessels within the body mesenchyme. According to Maximov blood vessels are formed from vaso-formative cells. Such vessels may form prior to derivation of blood cells, or may form simultaneously with red cells (page 512). He adds that blood forming cells can originate from free wandering cells, mesenchyme, and from endothelial cells. This contradicts Stockard but supports Reagan's theory of local origin. Dantchakoff (1909) states that in earliest states of erythropoiesis within the chick, there are sites of red cell formation in the head and in the vicinity of the aorta where such cells arise by an intensive proliferation of the endothelium. She states that vascular endothelium arises from mesenchyme which in the early embryo is an indifferent tissue, and from its non-specific beginning and later differentiation arise all the blood elements. Dantchakoff (1918) finds that the endothelium is a true anlage of the blood cells and states that this polyvalence is characteristic of both endothelium and mesenchyme. McWhorter and Whipple (1912) cut the chick embryo from one side of the blastoderm, and found that intra-embryonic vessels developed within that side of the embryo severed from the area vasculose. Miller and McWhorter (1914) state that identical processes are at work within the embryo

and within the membranes, that blood vessels within the embryo are not derived from an ingrowth of a formed endothelium but arise locally from an indifferent mesenchyme.

The preponderance of the more recent literature bearing upon the subject of the origin of angioblastic tissues within the embryo supports the idea of local origin of erythropoietic elements. However, so far as can be determined from the evidence presented in the literature there is no apparent reason why many of the hemoblasts within the embryo could not have originated extra-embryonically. With the break-up of the islands and the establishment of the vitelline capillary system, hemoblasts and megaloblasts from the islands could have been swept into the general circulation and have later established themselves within organs where venous systems are undergoing proliferation. To such an extent would extra-embryonic angioblast serve intra-embryonic erythropoiesis.

The literature dealing with erythropoiesis uses a confusing variety of terms for each type of cell identified with progressive stages of red cell formation. Because of this, it is necessary to indicate the specific term we shall use and to describe it so that its synonyms in literature may readily be associated with it.

Within the pig embryo erythropoiesis consists of two generations or series of cell maturation. The first series to appear, after passing through specific stages of maturation culminates either in an ovoid cell the nucleus of

which is extremely pyknotic and in the cytoplasm of which the full complement of hemoglobin is in the formation of granules, a cell 12-16 microns in diameter, or, toward the termination of first generation, ends in a non-nucleated plastid of similar size and shape. The second series of red cells appears first in the liver of the pig, and then in the spleen, and finally in the bone marrow. At the first appearance within the vascular system, the second generation cells appear along with those of the first generation and first generation plastids are present in the blood of pigs from a length of 15mm. to 43mm.

A similar situation is described for the human embryo by Knoll (1932) who clearly describes human embryonic erythropoiesis as consisting of the two generations. Knoll's chart, (ibid)(page 560), indicates that the second generation cells have appeared in the blood of the embryo of 6.5 length. Knoll, on page 561, states that the first appearance of second series hemoblasts within the liver occurs in embryos of 5mm. to 7mm. length. The subsequent development of groups or masses of developing cells within the liver substance Knoll terms "erythroblastennestern".

Not only are the two series definitely separated, and not only are the cells of each series different from the cells of corresponding stages of the other (Kirschbaum, 1937), but the manner of enucleation within each series is different. Within cells of the first generation erythroblast stage nuclei become increasingly pykotic with the

age of the cell and with each generation (mitotic) so that progressive stages of reduction in the diameter of the nucleus may be demonstrated, from maximum diameter to a diameter of almost pin-point size (at magnifications of 900-1000). The enucleated first generation plastid cell first appears in blood films bearing this maximum degree of pyknosis of the nucleus.

Although one does find more or less pyknotic nuclei undergoing mitosis, evidence of fragmentation and extrusion are not observed.

In pig fetuses that are nearing the end of the gestation period, fragmentation of the nucleus of the second generation erythroblast is readily observed within the liver. The phenomenon observed is not mitosis since more than two particles are frequently observed within the single cell, and the particles are not of a similar diameter nor are they regular in outline.

Kölliker (1854) believes that the nucleus of the red blood cell is absorbed by the cytoplasm; Jordan (1919) states that it is dissolved (undergoes karyolysis); Howell (1890) states that it is extruded in its entirety, to be engulfed by lymphocytes or dissolved by the plasma. Maximov (1909, b) believes that the nucleus first becomes fragmented and that the great fragments are then extruded. Emmel (1914) though he states that there is no concrete evidence, believes that the process is one of unequal amitotic division, the result of which is a nucleated and a non-nucleated daughter

cell. Jordan (1919) and Sabin (1922) have described intra-cellular erythropoiesis by endothelial cells in which such cells have given rise to cytoplasmic separation into one or more plastids of the second generation type within their cytoplasm. Jordan (1919) states that the intra-cellular type of erythropoiesis is active within the umbilical cord of the pig throughout fetal life.

According to Bremer (1944) the terms "first" and "second" generation cells have no real foundation since several investigators maintain that continued mitosis of the megaloblast of the first series is thought to be the mode of formation of the second or definitive series. Kirschbaum (1936) definitely states that the yolksac-vascular line of erythropoiesis is separate and apart from the "normoblastic" line of the spleen-liver-marrow activity. He believes that the yolksac vascular series is "megaloblastic" and that the spleen-liver-marrow series is "normoblastic." Kirschbaum based his studies upon pig and rabbit embryos. His separation of erythropoiesis into the two series is supported by Knoll (1932) for man, and by the findings and observations within this paper.

CLASSIFICATION OF BLOOD CELLS OF THE ERYTHROCYTE SERIES:

First Generation Red Blood Cells:

Hemoblast: This stem cell from which all blood cells are derived, according to the monophyletic school, has a diameter of about 15 microns and possesses a nucleus

containing a minimum of chromatin. The nucleus is resistant to staining and, with hemotoxylin-eosin, is pale blue in color. The cytoplasm also stains with basic stains and the outer margin is irregular with indications of pseudopodia-like protrusions. This cell is the lympthoidocyte of Pappenheim, the main hematoblast of Dantchakoff, and the hematocytoblast of Maximov. Knoll (1932) terms this stem cell the basophilic primary blood cell. We shall use the term hemoblast in referring to this hemopoietic stem cell.

Megaloblast: The term "megaloblast" is a poor one to use in an account of normal erythropoiesis in the adult because of its application in accounts of pathological blood. If used for erythropoiesis in the embryo, it must be defined. Because of the creation of a regenerative situation following the pathological condition of hypoplasia in the marrow of the pigeon, Doan, Cunningham and Sabin (1925) are correct in their use of the term when speaking of the type of cell which they describe as a megaloblast. In their conclusions, however, they suggest that the megaloblast is the antecedent of the successive stages of marrow erythropoiesis in the normal pigeon--that it plays this role under non-pathological conditions. This application of the term to normal conditions seems hardly justifiable. The term megaloblast is used herein to indicate a first generation blood cell in erythropoiesis derived by the immediate differentiation of a hemoblast of the yolk-sac-vascular system since such use seems to be the prevalent

one in recent literature. The megaloblast is a cell approximating that of the hemoblast in size. The nucleus contains chromatin material arranged in clumps so that the effect is that of a "checkerboard". These clumps have great affinity for basic dyes and stain deeply with hematoxylin-eosin, being nearly black in color. The cytoplasm of the megaloblast also is basophilic due to the slight amount of hemoglobin present. The margin of the megaloblast is regular and the entire cell is ovoid in outline. Maximov designates this cell as a basophilic erythroblast; Knoll calls it a "young nucleus megaloblast." It should be emphasized that this is strictly a first generation cell when the term megaloblast is used.

Erythroblast of the First Generation: The term erythroblast is applied by Maximov and Bloom (1942) to a red blood cell having a pyknotic nucleus and cytoplasm that possesses a full complement of hemoglobin. Maximov and Bloom make no distinction between a first and second series cell of these characteristics, since they are not discussing embryonic erythropoiesis. The word "first" will be used with erythroblast to indicate the cell of the first series, derived by further differentiation of the megaloblast. The first generation erythroblast is approximately of the same diameter as the megaloblast; it has a nucleus that becomes increasingly pyknotic with increase in the age of the cell and throughout succeeding mitoses. The amount of hemoglobin

within the cytoplasm reaches a maximum; the nucleus is small and stains blue-black with hematoxylin-eosin and pale blue with Wright's stain, and the cytoplasm stains pink-red with either stain. The hemoglobin is present in the form of granules. The cell is ovoid in shape. The first generation erythroblast corresponds to the ichthoid cell of Minot (1912).

First Generation Plastid: Toward the culmination of first generation erythropoiesis enucleation of the first generation erythroblast results in the appearance of a non-nucleated red corpuscle similar to the definitive cell of adult mammalian blood. However, the first generation plastid is as large as 16 microns in diameter and is ovoid in shape. Diameter is not as uniform as it is in the second generation plastid. The first generation plastid persists for only a short period in the general vascular system of the embryo, and that is at the end of the first generation series of red cell formation. Hemoglobin is granular.

Second Generation of Red Blood Cells:

Hemoblast: This is similar to that of the first generation. It may arise by the differentiation of a mesenchyme cell (spleen) or from an endothelial cell (liver, bone marrow). The "endothelial" cell is identical with the primitive wandering cell of the mesenchyme. According to Maximov and Bloom (1942, page 103) these primitive wandering cells establish a thin layer of mesenchyme between the epithelial cells of the liver and the vascular endothelium and give rise

to the hemoblast of the second series which, in this organ, undergoes a prolific erythropoeisis.

Normoblast: The normoblast cell is a second generation cell derived from the hemoblast and corresponds to the megaloblast of the first generation. However, the normoblast cell is similar in size to the final or adult form of plastid--6 to 8 microns. The nuclear chromatin is "checkerboard" in arrangement and the nucleus almost fills the cell leaving only a rim of hemoglobin surrounding it. This cell is called a basophilic normoblast by Knoll (1932) and a basophilic erythroblast by Maximov and Bloom (1942). The cytoplasm stains blue with May-Giemsa and with Wright's stain and blue-black with hematoxylin-eosin, so that the nucleus and the cytoplasm stain with the same dye fraction and are distinguished by differences in intensity of staining reaction rather than the color of the dye with which they react. The term normoblast, with adjectives to describe staining reactions due to increasing amounts of hemoglobin in the cytoplasm, has been used in the literature to indicate all second series cells following the hemoblast to the stage wherein the cell becomes enucleated. Along with this the term "erythroblast" and "erythrocyte" have been used interchangeably so that the greatest of confusion results. To avoid this confusion of terms we shall confine the use of the term normoblast to the cell that has an amount of hemoglobin insufficient to produce an acidophilic staining and is immediately derived from the second gener-

ation hemoblast.

Erythroblast of the Second Generation: The further differentiation of the normoblast produces a cell the nucleus of which is so dense that separate chromatin clumps can no longer be distinguished. The cytoplasm possesses such an amount of hemoglobin that it stains pink to red with the usual stains. The size of the nucleus compared with the size of the entire cell does not become reduced as it does within the first generation series.

Plastid of the Second Generation: Enucleation of the second generation erythroblast results in the first definitive plastid or "adult type" of red blood corpuscle. This cell possesses the full amount of hemoglobin which is present in a non-granular form, and stains pink with eosin. The diameter of the plastid of the second generation series of cells is quite constant.

It is to be noted that in our definition and use of the terms designating second series blood cells we reversed the terms normoblast and erythroblast as customarily used in the literature. Customary use of the term normoblast designates a second generation red blood cell that contains not only a dense nucleus but also a full complement of hemoglobin, and that, consequently differs from the second generation plastid only in the presence of its nucleus. The term erythroblast has been used in literature to represent the non-hemoglobin-bearing precursor of the normoblast, a cell that stains with a basic cytoplasmic

reaction and the nucleus of which contains chromatin material arranged in checkerboard fashion.

Our use of the term normoblast to refer to the cell with scant hemoglobin and checkerboard nucleus, and the special application of the term secondary or second series erythroblast to the cell with dense nucleus and rich amount of hemoglobin is deliberate so that megaloblast of the first series and normoblast of the second correspond in cytological characteristics and so that the same is true of the use, within this paper, of the terms erythroblast of both the first and of the second generation of forming red blood cells. It seems illogical that the erythroblast of the first generation should be a cell terminating a series while the erythroblast of the second series is definitely an intermediate and immature stage that contains such scant amounts of hemoglobin that the cytoplasm does not stain with eosin.

For the purposes of this paper we shall therefore use the terms as defined under description of terms.

Erythropoietic Organs: Formation of erythrocytes begins extra-embryonically within the fetal membranes. Jordan (1916) and Sabin (1917) describe the yolksac as the first location of such formation. Patten (1931) attributes an erythropoietic activity similar to that of the yolksac to the allantois of the pig prior to specialization of the latter as a nutritive organ following its expansion into the blastocyst between the amnion and the chorion where it forms the inner lining of the chorion. In the platyrrhine monkey

first evidence of red cell formation is in the chorionic villi (Wislocki, 1943). Bremer (1914) presents evidence that in the human embryo first formation of blood islands occurs in the chorion and the umbilical cord. Vasiformative cells differentiate from mesenchyme and form either endothelium or hemoblasts according to Schulte (1914). If the mesenchyme forms endothelial cells, these in turn may give rise by mitosis to hemoblasts, additional endothelium, or may revert to a mesoderm cell (Schulte, 1914). Schulte (1914, page 36) quotes Bonnet (1899) as saying, "endothelium is the result of mechanical factors, blood from biochemical factors, both consequent upon position and neither of them rigidly pre-determined to a special cytomorphogenesis!" Islands of hemoblasts surrounded by endothelial cells form within the mesenchyme of the area vasculosa of the fetal membranes (Dantchakoff, 1918), and junctions of endothelium by anastomosis form vessel-systems within which, and outside of which, lie hemoblasts. Those hemoblasts which lie outside the vascular system form leukocytes if they have not progressed too far toward their differentiation into megaloblasts, in which case they are doomed to destruction, according to Dantchakoff (1918) and to Schulte (1914). Red cells found in the mesenchyme outside the vessels are cells which have escaped and are degenerating (Emmel, 1915). This statement by Emmel is difficult to resolve against the evidence that mesenchyme not enclosed by endothelium or by vessel-walls

within the yolksac and in the spleen differentiates hemoblasts which, through maturation processes, develop into red blood cells.

Vascular erythropoiesis continues actively until the time of the formation of the liver (Howell, 1890). According to Patten (1931) the liver diverticulum in the pig appears at about the 3.5 mm stage and erythropoiesis in that organ begins at about 8 mm. At this time, although nucleated cells are almost exclusively predominant in the circulatory system, the normoblast of the definitive series is soon the only type of cell in evidence with the mammal (Knoll, 1932).

Jordan (1916) describes hemopoietic activity by the endothelium of the kidney. Emmel (1916) and Haff (1914) describe abortive attempts of the peritoneum over the liver to form red blood cells.

The spleen becomes hemopoietic in its earliest stages of formation, at first being erythropoietic but later serving as a place for the differentiation of white corpuscles, according to Howell (1890). Our evidence (page 69) does not support application of this statement to the pig spleen. Howell (1890) and Sabin (1916) give Kölliker credit for first describing (in 1854) the erythropoietic activity in the spleen. Sabin considers that splenic erythropoiesis is intra-vascular, the splenic pulp between the artery and vein serving as a vascular bed of sinusoids. Thiel and Downey (1922, page 306) state that "in the pig spleen, erythropoiesis is largely extra-vascular".

With the formation of marrow within the long bones, erythropoiesis becomes established as a primary function of these structures. The importance of marrow erythropoiesis in the pig increases until at the time of birth all red cell formation normally takes place within this tissue (Maximov, 1909, B). With culmination of hepatic erythropoiesis and establishment of marrow red cell formation, only non-nucleated red cells --(plastids)--are found in the circulating blood, according to Knoll (1932).

Doan, Cunningham, and Sabin (1925) studied erythro-cyto-genesis in bone marrow of pigeons by first inducing a hypo-plasia by means of starvation, and then observed changes occurring within the marrow during regeneration of that marrow. These investigators conclude that the circulatory system of the marrow is a closed system of sinuses and intersinusoidal capillaries, the latter being simply collapesed sinuses. Doan, Cunningham, and Sabin (1925) state that within the sinusoidal spaces erythrocytes are derived from megaloblasts, but it is possible that they do not differentiate between endothelial wall and the initma reticular stroma as the source of the hemoblast from which these megaloblasts are formed. It is also quite possible that they, as pointed out by Jordan and Johnson (1935) and Kindred (1942), failed to note the discrepancy between blood cells needed by the normal growing embryo and the hemoblastic supply within the sinusoid.

It should also be pointed out that these findings and conclusions of Doan, Cunningham, and Sabin are based upon a pathological condition and need not necessarily be applic-

able to the normal process of bone marrow erythropoiesis. Thus their use of the term "megaloblast" for the basophilic erythrocyte of the second generation (a term ordinarily of pathological significance) is perhaps correct but it is likely that the term should not be used under normal fetal processes in application to a second generation blood cell.

According to Jordan and Johnson (1935) hemoblasts for maintaining marrow erythropoiesis are supplied by differentiation of the relatively unspecialized small lymphocytes. They describe the latter cell as being similar to the small lymphocyte of splenic origin and state that they originate from histiocytes or peri-sinusoidal reticulum cells. The reticular stroma is intimately associated with the endothelial wall of the venous sinusoids. Under tension, fenestrae form a direct connection between sinusoids and stromal tissue spaces, according to Jordan and Johnson. Stromal spaces contain other stromal cells--histiocytes--or reticulo-endothelial cells. Jordan and Johnson state that these are the cells that differentiate into hemoblasts and that those in spaces connected by fenestrae with the sinusoids differentiate hemoblasts and then normoblasts and plastids, and that they found no differentiation of hemoblasts within the marrow spaces and conclude that such essential cells in bone marrow erythropoietic regions arise extra-vascularly. This may seem to disagree with the statement of Dantchkoff, Schulte, and others that extra-vascular hemoblasts form granulocytes and only intra-vascularly

develop into erythrocytes, but it is only after the hemoblasts have entered into the sinusoidal or circulatory environment that the route toward erythrocyte formation is begun. Those hemoblasts that remain within the stromal spaces form granulocytes.

Jordan and Johnson (1935) also point out that there is a great discrepancy between the need for red blood cells by the blood and the degree of proliferation of hemoblasts within the marrow. They suggest that, in view of the morphologic identity of the lymphocyte and the hemoblast, and the evidence that more lymphocytes enter the blood from lymph channels than are present in the blood at any time, these lymphocytes are filtered out in the intersinusoidal capillaries of the bone marrow where they differentiate into hemoblasts within the stromal spaces, augmenting those hemoblasts formed from stromal cells.

Kindred (1942), after calculating mitotic time and percent of cells formed and of cells needed by the blood of the embryo, concludes that the number of hemoblasts in mitosis within the sinusoids of the bone marrow is insufficient to meet the red cell needs of the organism and that the supply of hemoblasts must therefore be augmented from an extra-sinusoidal source.

Maximov and Bloom (1942) describe a source of hepatic hemoblast from mesenchymal cells located as stromal tissue between hepatic sinus endothelium and the entodermal parenchymal cords of the liver. Thus a similar source of

hemoblasts has been indicated in the hepatic and marrow erythropoiesis--stromal cells, or fixed (primitive) wandering cells, or histiocytes, with perhaps a utilization by adult marrow of small lymphocytes which, freed from the spleen, and removed from the circulation by the marrow, undergo conversion into hemoblasts from which erythrocytes are formed.

Ringoen (1935) states that the crucial point in the solution of the intersinusoidal concept is the relationship of the intersinusoidal capillaries to the venous sinuses. He contends that the evidence supports the claim that the inter-sinusoidal capillaries of avian bone marrow are not genuine capillaries but are stromal spaces, not lined with endothelium but with histiocyte elements, which open into venous sinusoids. According to Ringoen these histiocytes exhibit phagocytic properties on depleted or regenerating bone marrow. Thus Ringoen supports the contention of both Kindred and Jordan and Johnson that the precursors of the red blood cells of bone marrow arise outside the vascular system.

One interested in following the literature on hemo-poiesis will find an excellent bibliograph of 728 titles in Knoll (1932).

PART III. MATERIALS AND METHODS

Collection and Preparation of Materials: Embryos removed from uteri of sows at the eviscerating platform in abattoires of packing plants were taken to the plant laboratory. Whole embryos of 25 mm. crown-rump length or less were placed in Bouin's fixative fluid. From embryos of a length greater than 25 mm. pieces of liver, spleen, hind limb, as well as pieces of yolksac and umbilical cord from specimens of all sizes were removed and likewise placed in Bouin's fixative. The embryos secured ranged in size from 3.5 mm. to 256 mm. Liver, spleen and bone marrow were removed from an adult hog to serve as an index to full development.

Embryos up to and including 25 mm. length were embedded by the xylol-paraffin method, serially section, mounted and stained by Harris' modified hematoxylin-eosin method, Wright's technique and May-Giemsa. Tissues from the larger embryos were given the same processing. With Wright's stain, the stock stain remained on the tissue 2.5 minutes before dilution 1-1 with fully buffered dilutant, following which staining proceeded for 4 minutes. Differentiation was with 50/50 solution of acetone and absolute alcohol, followed by xylol and mounting in balsam. In the May-Giemsa staining, stock stain was diluted 1/1 and the stain remained

on the tissue for 15 minutes, then the slide was washed, dried, and the covered glass mounted in balsam. See Table I.

Mode of Approach: Examination and study of tissues from each size group of pig embryos was made to determine; (1) onset of erythropoiesis; (2) degree of development of the erythropoietic function; (3) types of red blood cells present, and (4) in what size pig erythropoiesis either ceased to be a function of a region, an organ, or a tissue or culminated in an adult mode of red blood cell formation. Records were made by descriptive notes, by drawings under camera lucida, and by photomicrographs.

In the study of vascular erythropoiesis of embryos of less than 25 mm. CR length observations were made of the contents of vessels within cross-sections of whole pigs. Beyond an embryo length of 25 mm. the umbilical cord was first carefully dried to remove superficial materials and moisture. A drop of blood from the vessels of the cord was placed at one end of a standard microscope slide and drawn into a thin film with the edge of another slide. The dried blood film was stained with Wright's stain.

Marrow from the femur was smeared upon microscope slides and stained with either Wright's stain or with May-Giemsa stain. Using the oil immersion lens and a 10x ocular, counts of types of erythropoietic cells within the blood were made by the technique used in taking a "white cell differential count." When development of vascular activity had reached

TABLE I

Size Pig mm. CR	Number of specimen	Serial section	Liver	Spleen	Bone (limb)	Yolk sac	Umbilical cord
3.5-5 mm.	10	x				x	
8-12	16	x				x	
13-15	12	x				x	
17-22	12	x				x	
23-25	4	x				x	x
30-40	13		x	x	x	x	x
45-65	12		x	x	x		x
75-100	4		x	x	x		x
115-150	8		x	x	x		x
160-200	3		x	x	x		x
201-225	6		x	x	x		x
226-256	6		x	x	x		x
New Born	3		x	x			
Adult	1		x	x			

Blood smears from pigs larger than 30 mm. were made from umbilical blood.

Marrow smears from femur "pulp" of pigs larger than 100 mm.

Total specimen -- 110 pig embryos.

a stage where only normoblasts and second generation erythroblasts were present, and in the midst of such numbers of second generation plastide that they could not be counted, only nucleated red blood cells were counted in ten fields of each smear. Results of these counts were tabulated. (Table II.) While such technique does not give truly quantitative figures, yet trends are indicated for the development and regression of vascular erythropoiesis.

PART IV. OBSERVATIONS AND DISCUSSIONS

The Yolksac

Formation of the Yolksac: The yolksac is that part of the primitive gut which is not included within the body when the embryo is folded off from the blastoderm. In the young pig the peripheral extent of the yolksac is closely correlated with the unusually elongated blastoderm, thus extending anteriorly beyond the head, posteriorly beyond the tail, and laterally beyond the dimensions of the embryo, actually extending nearly to the ends of the blastoderm. During this period of separation of embryo from blastoderm, the yolksac serves the primary purpose of securing nutriment for the embryo since it is separated from the uterine wall only by the thin outlay of the blastocyst, and its blood vessels (the omphalomesenteric) are in a position to facilitate absorbtion from the uterine bed.

When the allantois has undergone development and differentiation, the nutritive functions of the yolksac are assumed by that organ, which, in the pig, persists throughout fetal life. It extends as a thin sheet of tissue lying between the amnion and the chorion (figure 55,B) and is readily separable from the other fetal membranes. The yolksac rapidly shrinks to become a small shrivelled body lying between the allantois and the amnion. This condition is fully attained in the 30 mm. pig.

The foldings that bring about the establishment of the gut with its lining of entoderm and its mesenchymal external layer establish the yolksac of like construction (figure 7). The entoderm in the yolksac of the 3.5 mm. pig is composed of a single layer of low cuboidal cells each of which contains a large spherical, vesicular nucleus which has a poor affinity for basic dyes. The cytoplasm in heavily granular. The external layer of mesoderm is derived from mesenchyme and is composed of squamous cells that from a lateral view are fusiform in outline and are distended at the locus of the nucleus. The nucleus is similar in appearance to that of the entodermal cell. Between these marginal tissues of entoderm and mesoderm (figure 4) is a more or less extensive mass of mesenchyme, in some places quite broad and in others only a strand. Cytoplasmic cell boundaries are not apparent within this mass. The nuclei of the mesenchyme are similar in size, shape and internal composition to those of the mesothelium and entoderm, in that they are large, spheroidal, resistant to stains, and contain chromatim granules aggregated into small clumps. A fine reticulum may be observed within the nucleus.

Observation and Discussion: At those locations of greater area of mesenchyme scattered cells may be seen in the 3.5 pig undergoing certain changes in appearance, the chromatin clumps in these cells increase in size and in the intensity of their staining reaction to basic dye, and one or two nucleoli appear. The outstanding develop-

ment is found in the cytoplasm where differentiation withdraws the cytoplasm of these cells from the syncytial mass. Thus the cytoplasmic outlines of the hemoblast cell are now discernable (figure 1). The outline of the cells is not, however, defined by any observable membrane and irregular protuberances similar to pseudopodia indicate the possibility of ameboid activity. The amount of cytoplasm present compared to the size of the nucleus is relatively small. At first these specialized cells are of a size approximating that of the cuboidal entodermal cell. Through active mitosis, the single cells form around them a cluster of similar cells (figure 2) so that in sections of the 5 mm. pig's yolksac clusters of these hemoblastic cells form islands (figure 3). The mesenchyme thus is the fundamental source of the hemoblast of the yolksac islanda as they originally form. Peripheral hemoblasts of the islands have a greater diameter than those toward the center of the island. Those hemoblasts forming the outer margin of the island become flattened into endothelium which continues to give rise by mitosis to additional hemoblasts, (figure 5a). With the formation of endotheloium the islands elongate and merge so that within the 10 and 12 mm. pig (figure 5) channels are becoming well outlined. With extension of endothelium to form connected boundaries, the islands then are contained within vessels which become the capillary termini of <u>omphalomesenteric</u> or <u>vitelline</u> veins. With the establishment of circulation the islands break up,

hemoblasts and megaloblasts enter a circulating medium, and further production of erythroid parent cells-(hemoblasts) is dependent upon the elaboration from andothelim rather than from upon mitosis of preciding generations of hemoblasts. By the time this stage has been attained, almost all cells of the mesenchyma have been converted either into hemoblasts or into mesoderm (figure 6). Erythropoiesis is now fundamentally a vascular rather than an "island" phenomenon.

Withinthe islands of the 5 mm. and 8 mm. pig yolksac, hemoblasts rapidly develop into megaloblasts by the accumulation of chromatin into such large masses that the nucleus has the checkerboard effect, and by the development of a cytoplasmic membrane and a non-granular cytoblasm. At first these cells contain such a slight amount of hemoglobin within the cytoplasm that this portion of the cell stains blue with hematoxylin-eosin. Later generations of megaloblasts develop sufficient amounts of hemoglobin to stain pink with eosin and are erythroblasts of the first generation.

Beyond the 15-16 mm. stage of growth the yolksac rapidly loses its erythropoietic function and becomes a shrunken, shrivelled diverticulum composed of columnar entoderm and a well developed mesoderm. The cells of the blood within the vessels are megaloblasts and first generation erythroblasts. The nucleus of the latter stains dark blue and is pyknotic while the cytoplasm stains yellowish-

red with eosin because of the hemoglobin present. With Wright's stain granules of hemoglobin stain red and the nucleus a bright blue, (figure 7a). One entire cluster of cells observed within the mesoderm of the yolksac of the 25 mm. pig consisted of 19 red cells (figure 8). No red cells other than first generation erythroblasts with pyknotic nuclei were present in this cluster. The cytoplasm, though it did not stain deep blue, on the other hand did not give indication of well developed hemoglobin by staining heavily with eosin. The hemoglobin is in the form of granules. While these cells might be capable of mitosis no nuclei were observed that were in a state of division.

Since hemoblasts are not observed in sections of the yolksac of pigs of a length greater than 16 mm. and since none but first generation erythroblasts typical of the general circulating blood of this stage are found within the 25 mm. pig yolksac, it is concluded that blood island erythropoiesis has reached an end before the 25 mm. stage. With the progression of island constituents to the first generation erythroblast stage, the establishment of channels of circulating blood, and the disappearance of islands as such, erythropoiesis within the yolksac differs in no way from that of intra-vascular red cell formation where the cells are freely circulating within the blood fluids.

Conclusions: Yolksac erythropoiesis is initiated within the 3.5 mm. to 5.0 mm. stage of the pig embryo by the differentiation of the mesenchyme cells into hemoblasts; it rapidly increases in intensity by mitosis of hemoblasts

and endothelium cells to a maximum in the 10-15 mm. pig, when vascular channels have become established and the islands break up with the beginning of circulation in the vitelline capillaries of the omphalomesenteric vessels. Cells within the islands progressively differentiate from hemoblasts through checkerboard-nucleated young megaloblasts to pyknotic-nucleated cells rich in granular hemoglobin--the mature megaloblast or first generation erythroblast of the general circulation. No non-nucleated red cells of the first generation are found in the yolksac prior to cessation of island erythropoiesis.

Yolksac blood island erythropoiesis is initiated in the pig of 3.5-5 mm., reaches a maximum of activity in the pig of 10 mm. and ceases when the last of the remaining islands break up prior to the 15-16 mm. pig. Beyond 16 mm. erythropoiesis within the yolksac is intra-vascular and similar to that being maintained within the intra-embryonic blood vessels of pigs of this size.

The Umbilical Cord

Embryology: Following formation of the central cell mass and its development into the embryonic disc, cephalic and caudal elongations produce invaginated folds that lift the embryo off the blastocyst. Subsequent continuation of this folding process separates the primitive gut from the yolksac except for the yolk-stalk by which connection between gut and yolksac is maintained; and likewise there is left the body-stalk which connects the hind

end of the embryo with the chorion. Growth of the amnion encloses the embryo within its membrane and pushes the yolk-stalk and sac ahead of it, so that the stalk with its arteries and veins, the vitellines, as well as the body-stalk, become enclosed by the amnion. The allantois evaginates as a diverticulum from the hind-gut into the body-stalk and elongates beyond the distal end of the stalk to emerge extra-embryonically whence it extends between the amnion and the chorion. The arteries and vein of the allantois persist as the umbilicals and, by their relationship to the placenta, provide transportation for nutritiment and wastes of the embryo. The complete umbilical cord thus is made up of Wharton's jelly, a type of embryonal connective tissue, which includes within its mass the belly-stalk, the yolk-stalk, and the stalk of the allantois; it is covered by the amnionic membrane (figure 10). The amniotic sheath of the umbilical cord is continuous distally with the amnion. The yolk-stalk extends through the cord to the chorionic cavity where it expands into the remnant of the yolksac, an organ that rapidly becomes shrivelled and vestigial subsequent to the 15 mm. stage. Beyond the 125 mm. stage the yolk-stalk may have disappeared entirely from the umbilical cord. The allantois of the pig continues to transport nutrients for this animal throughout fetal life. It extends in both directions beyond the limits of the embryo to within a short distance of the ends of the blastocyst; it is a thin

membrane lying against the inner surface of the chorion; and as it contains branches of the umbilical vessels it serves as the principal organ through which nutritive elements are absorbed and the wastes of metabolism are eliminated (figure 55). In the pig, therefore, the allantois serves as a fetal membrane in addition to the amnion and the chorion which are the usual ones in mammals.

The umbilical cord of the pig is only slightly twisted. In the full term pig it is oval in cross section and has a diameter of about one centimeter. The length of the cord of the full-term pig varies greatly (table II) in comparison to the CR length of the Pig from which cord measurements are taken. This variation may be due to the difficulty of properly measuring such a jelly-like structure, but seems better explained as a natural condition due to such variables as food, health, breed of animal, climate, etc.--all those variables that result in deviations of size and weight of the new-born of any species.

The study of umbilical cords was based upon sections taken through the proximal, distal and central regions of each cord from a total of 56 pigs. These pig embryos were distributed as to size as follows: 23-25 mm, 4 pigs; 30-40 mm, 13 pigs; 45-65 mm, 12 pigs; 75-100 mm, 4 pigs; 105-150 mm, 8 pigs; 160-200 mm, 3 pigs; 201-225 mm, 6 pigs; and 226-256 mm, 6 pigs. In none of the sections of these cords was there any evidence of the occurrence of erythropoiesis; there were to be seen only the same intra-vascular

TABLE II

Length of Pig - CR	Length of cord	Length of Pig - CR	Length of cord
100 mm.	30 mm.	235 mm.*	170 mm.
190 mm.	120 mm.	240 mm.	240 mm.
215 mm.*	180 mm.	240 mm.**	320 mm.
220 mm.*	160 mm.	255 mm.***	380 mm.
220 mm.*	150 mm.	260 mm.***	375 mm.
220 mm.	220 mm.	275 mm.**	340 mm.
220 mm.*	240 mm.	275 mm.***	335 mm.
230 mm.	210 mm.	280 mm.**	360 mm.
230 mm.*	215 mm.	295 mm.***	440 mm.

* pigs from the same uterus.

Crown-Rump length of new born pigs: 266 mm.; 267 mm.; 295 mm.

FETAL PIG LENGTH COMPARED TO UMBILICAL CORD

stages characteristic of the general embryonic circulation of the pig. Red blood cells were observed extra-vascularly in a single area of one section of the distal region of the cord of the 25 mm. pig and at the extreme border of the cord peripheral to the umbilical arteries. These cells consist of first generation erythroblasts and possess extremely pyknotic nuclei. Because of the stage of development of the cells, the appearance that these cells have of being superimposed upon the tissues of the cord, and their similarity to the stage of development of cells within adjacent vessels, it seems logical to consider this cell mass the result of hemorrhage rather than of extra-vascular erythropoiesis. Cross sections of vessels within the cord contain megaloblasts, erythroblasts of the first generation, and plastids of the first generation. No hemoblasts were observed within any of the vessels of the cord of the 25 mm. pig embryo.

In sections from all three regions of the cord of the 45 mm. pig erythroblasts of the first generation occur singly and in groups of three or four throughout areas that, in cords of older pigs, contain many small capillaries. None of these cells are in a mitotic state, nor is there any evidence that they are formed within the cytoplasm of endothelial cells to which might be ascribed an erythropoietic function as Jordan (1919) asserts. Since the umbilical vein of the cord of this size pig contains no megaloblasts but does contain only first generation erythroblasts and also normoblasts of the second series of red cells, it is possible

that these extra-vascular erythroblasts have been freed through regression of an earlier capillary and thus they must remain within the tissue of the cord until destroyed. They apparently play no role in erythropoiesis. One section of an umbilical vein contained the following cells: 5 first generation erythroblasts, 6 first generation plastids, 22 normoblasts, 7 second generation erythroblasts, and several hundred adult type of plastids of the second generation. This is the blood picture typical within the general circulation of the embryo of this size and tends to indicate that no contribution to the number of any particular type of red cell is being made by the umbilical cord of the pig of 45 mm. length.

In cords of all pigs from 105 mm. to 256 mm. there is an area of loose connective tissue within which are many small vessels and capillaries located peripherally to the umbilical arteries and lying somewhat between them and the umbilical vein containing both second generation arythroblasts and second generation plastids (figure 10). There also occur many connective tissue cells whose tytoplasmic strands enclose from one to several second generation plastids. In one such cell (figure 10,a) the enclosed blood cell is in the first generation erythroblast stage, the nucleus is greatly shrunken, stains pale blue and shows no mitotic figure. The cytoplasm stains a faint pink with Wright's stain. Within cell "b" (figure 10) of the same area of the same section, the connective

tissue cell has undergone recent division of its nucleus. Adjacent to the two cell nuclei there is a second generation plastid within the cytoplasm of the connective tissue cell. The cytoplasm of this plastid stains with eosin to an intensity indicative of a full complement of hemoglobin.

Such a plastid may have been derived, as Jordan (1919) believes, from a division within the tissue cell. However, to our mind, the fact that this cell has all the characteristics of a mature plastid, plus the fact that cells "a" and "b" are near capillaries "c" and "d", of which "d" contains an erythrocyte of the same degree of maturation as cell "a", would lead us to believe that these cells might just as well indicate that the capillaries are undergoing regression and that escaping plastids and erythrocytes (erythroblasts) are being phagocytized.

If cells "a" and "b" are producing erythroplastids by intra-cellular erythropoiesis then it seems that the cells should be of the nucleated normoblast type and that some of this kind of cell could be seen in this area. No normoblast cells were observed. If cells "a" and "b" are serving an erythropoietic function of importance as Jordan maintains, then what function is afforded the organism when such activity confines the plastid to an intra-cellular rather than a vascular existence?

A total of only three second generation erythroblasts were observed within the capillaries of sections from the three regions of the cord of the 130 mm. pig. All other

cells were second generation plastids. Sections of the vein of the cord contained a total of three second generation erythroblasts and two normoblasts. The normoblasts are mitotic. One of the normoblasts contained two nuclei and an elongated cytoplasm with a partial equatorial constriction. Many second generation plastids occur extra-vascularly in an area between two capillaries in one section of the cord. There is no evidence, however, of erythropoiesis occurring within any areas of cords of the 105-150 mm. group, nor within the extra-vascular tissues of any cords of any of the pigs.

Within the area described above as lying near the umbilical arteries and somewhat between them and the umbilical vein and consisting of rather loose connective tissue, there is a progressively greater number of capillaries in cords of the older pig embryos. Sections taken from cords of pigs of 200 mm. length (figure 11) and of 250 mm. (figure 12) contain capillaries in greater number than do the cords of smaller pigs. Since the pig of 250 mm. length approximates the completion of the gestation period, we conclude that extensive vascularization of the cord continues throughout the fetal life of the pig.

Discussion: Morphological studies would hardly determine whether intra-cellular erythropoiesis does occur within the tissues of the umbilical cord or an alternative interpretation - - phagocytosis. Even though intra-cellular erythropoiesis should occur, we would hesitate to include the cord as an organ playing a significant role throughout

fetal life as an originator of red cells. It is difficult to believe that Jordan (1919) was observing a full term specimen in his pig of 160 mm. length with a cord of 40 mm. Table II compares CR length of pigs and of umbilical cords as well as showing variations of fetal dimensions within a single uterus. Cords of these pigs were measured from the hilus of the belly to the point of separation of the vessels in the fetal membranes.

According to Jordan (1919) the umbilical cord of the pig remains erythropoietic until full term is reached, the mesenchyme cells of the cord become vaso-formative by hollowing out to form an endothelial cell which differentiates erythro-plastids intra-cellularly, and capillaries arise in situ and become connected secondarily with existing vascular units (page 8). Jordan states (page 11) that the pig cord at the time of birth is extensively vascular and that its intensive hemopoietic activity supplies valuable data with respect to the initial steps of vasculogenesis.

Jordan based his conclusions upon the study of the morphology of an umbilical cord 4 cm. long taken from a pig whose CR length was 16 cm., and calculated that the pig lacked from one to two weeks of being full term. (Throughout his paper and his conclusions as well, Jordan speaks of this as "the full term cord".) In addition to this one cord, Jordan studied another acquired from Huntington, which he describes as being nearly full term

but gives no measurements; he had, as well, cords from several 9 mm. to 21 mm. pigs. In his discussion under the topic "connective tissue", Jordan states that the connective tissue surrounding the allantoic duct is less differentiated--that it resembles young mesenchyme--that in this region there are numerous vasofactive cells, and refers to his figure 15, which illustrates nine types of cells found in varying degrees of conversion into endothelium. According to Jordan the connective tissue cells along the periphery are stellate or fusiform and are widely separated, and it is in this region that the capillaries are most abundant. Here also are found, according to Jordan, abundant initial stages in the formation of blood channels. He states that in the area between the lower umbilical artery on the left and the vein on the right the connective tissue is in part of the type characteristic of the human cord and that the area around the yolk-stalk contains vascular connective tissue of an intermediate type. In the narrow space between the two umbilical arteries there occur several blood islands (Jordan's figures 6-7). On page 12, Jordan states, "Though this study can throw no light on the cause of the vascularized condition of the umbilical cord of the pig, the intense hemopoietic activity of its connective tissue supplies valuable data with respect to the initial steps in vasculogenesis. This is the dief point of value of this specimen." On page 13, Jordan further states, "The method of erythrocytogenesis here described for the speci-

men of the umbilical cord of the pig, however, differs radically from that described by Ranvier and Schaefer in that erythro-plastids in this case differentiate from a typical erythroblast in the usual mode. The nucleus of the erythrocyte disappears by keryolysis". Jordan states that the plastid within the "vasoformative cell" does not indicate degeneration or phagocytosis since these cells are radically different from those described by Ranvier and Schaefer and so, he believes, are not subject to the criticism of Spuler (1892), but Jordan does not describe how these intra-cellular plastids differ. He states that no free erythrocytes are available for phagocytosis within the cord, and that there is no indication of a disintegration of the blood vessels; that on the contrary, the full term cord is relatively more extensively vascularized than the cords of the 21 mm. fetus. Jordan states that this intra-cellular mode of erythropoiesis is strictly comparable to that described for other hemopoietic organs, e.g., the yolksac of the 10 mm pig (Jordan, 1916), the yolksac of the mongoose (Jordan, 1917), and the red bone marrow (Jordan, 1918).

Conclusions: The fact is true that the entire length of the umbilical cord of the pig is highly vascularized to the end of the gestation period (figure 9-10-11) and that such a supply of small vessels seems to be augmented rather than diminished toward the close of the period. The continuance of vasculogenesis to the end of term is supported by my observations but this does not

support the hypothesis of intra-cellular erythropoiesis.

The Vascular System:

Vascular erythropoiesis: Endothelium produced from the mesenchyme may not only proliferate additional endothelium but also may give rise to parent cells of the erythroid series (figure 5a, 14). Therefore, in regions of vessel formation endothelium may be accompanied by or preceded by the formation of red blood corpuscles (Maximov, 1909, b). Kirschbaum (1936) states that this mode of origin of primitive red blood cells parallels yolksac erythropoiesis, and belongs to a type distinct from that of the spleen, liver, and bone marrow. According to my observations the basis for such distinction lies in the size of the megaloblast and of the "final stage" cell of each series, as well as in the physical appearance of the hemoglobin within the cytoplasm. In the yolksac-vascular series the megaloblast is in size comparable to the first generation erythroblast, i.e., from 12 to 16 microns in diameter. In each series the megaloblast and its counterpart of the second series, the normoblast, has a checkerboard nucleus; that is--the nucleus is vesicular with chromatin clumps taking an intense stain. The megaloblast possesses a more abundant cytoplasm than does the normoblast.

When the separate chromatin granules can no longer be seen within the nucleus and the cytoplasm has developed an amount of hemoglobin sufficient to stain with eosin, the

red blood cell is termed not a megaloblast but an erythroblast of the first generation. The diameter of the nucleus of the erythroblast decreases with increase in age. Similar reduction in the size of the nucleus of the normoblast does not occur.

Accompanying the progressive reduction in the diameter of the nucleus of the erythroblast, the hemoglobin content of the cytoplasm increases in the form of granules until the culmination is a cell from which the nucleus has completely disappeared and which possesses a full complement of hemoglobin. Thus, by karyolysis, the plastid of the first series is formed (figure 54,e). This plastid differs from the definitive plastid of the liver-spleen-marrow series in that while the size is not as uniform as is that of the comparable cell of the second series, it is much greater in diameter. The plastid is ovoid in shape, and with Wright's stain the hemoglobin has the form of eosin-stained granules. The plastid of the second series is formed by loss of the erythroblast nucleus by fragmentation and extrusion (karyorrhexis), is fairly uniform in size and 6 to 8 microns in diameter and is circular in outline. A non-granular hemoglobin is evenly distributed throughout the cytoplasm (figure 54,f).

Nucleated red blood cells of the vascular stream may have come from two types of sources, maturation of megaloblasts derived from hemoblasts within the circulating medium and from cells escaping into the general circulation from organs that are in an erythropoietic state. Hemoblasts

may enter the circulating blood from the yolksac blood islands; they may be produced by the endothelium of the nearly early vessels, and they may be produced by division of a parent hemoblast cell. Hemoblasts disappear from the circulating blood at an early age (page 46). Beyond this age blood may be thought of as a passive medium wherein differentiation and maturation of the blood cells originating within other erythropoietic foci takes place. In one sense, blood and any developing capillary bed constitute an eroythropoietic region so long as hemoblasts are present in them, and are being derived from vascular endothelium--that is, to the 15 mm. stage in the pig--and when only the formation of cells of the first series is concerned. In a passive role blood serves as a medium within which nucleated red cells of the second series (erythroblasts) occur in small numbers to the end of the gestation period.

Questions to be answered concerning vascular erythropoiesis are:

1) in what size pig do hemoblasts cease to appear in the circulating blood?

2) in what size pig do first generation cells disappear from the blood?

3) in what size pig do second generation red blood cells appear within the blood?

4) in what size pig does the blood reach a state of composition that will be fairly constant to the culmination of gestation?

Observations: Blood cells within the dorsal aorta of the 3.5 mm. pig embryo consist of hemoblasts and

and megaloblasts. Both hemoblast and megaloblast have a vesicular nucleus. The nucleus of the hemoblast stains only faintly and is of greater diameter than that of the megaloblast; the cytoplasm of the hemoblast is basophilic, contains no hemoglobin, and possesses no distinct cytoplasmic membrane. Only a few of the megaloblasts possess a nucleus of such dense staining reaction that chromatin aggregates cannot be seen. Along the ventral wall of the aorta the mesenchymal-endothelial area is giving rise to new hemoblasts (figure 14). Those megaloblasts that possess a densely staining nucleus fail to show any indication of hemoglobin formation, granular or otherwise.

The picture within the dorsal aorta of the 5 mm. pig is essentially the same as that of the 3.5 mm. embryo except that the number of megaloblasts has increased. These cells not only possess a more compact nucleus but also have granules of hemoglobin within the cytoplasm. Only a few scattered hemoblasts can be seen.

No hemoblasts are seen within the aorta of the 10 mm. pig and the megaloblasts contain a greater amount of hemoglobin granules. First generation erythroblasts are present. These latter possess nuclei so compact that separate internal chromatin clumps cannot be observed, and cytoplasm that contains a full amount of hemoglobin for this series. The nucleus is no longer spherical but is mis-shapen and more ovoid. Many of these cells are in the process of division (figure 19). Within the aorta of the 10 mm. pig hemoblast formation has ceased but

multiplication and maturation of megaloblast first generation erythroblast continue.

Within the aorta of the 15 mm. pig embryo the megaloblast of the first series is still the predominant cell. Several first generation erythroblasts per field have the extremely pyknotic nucleus of the "old" erythroblast (figure 17). The first generation erythroblast is a large, oval cell, 15 by 20 microns in diameter. An occasional normoblast can be observed in the blood for the first time. Such a cell corresponds to the megaloblast of the first series; that is, it has a checkerboard nucleus containing clumps of densely staining chromatin and a cytoplasm that contains only a slight amount of hemoglobin. The cytoplasm is scantier in comparison with the nuclear size then is that of the megaloblast and first generation erythroblast, and the hemoglobin is non-granular. The diameter of the normoblast approximates that of the definitive second series plastid (6-8 microns).

In an area midway between the cartilagenous centrum of the vertebrae and the aorta, in a section posterior to the heart and through the liver and the mesonephros in the 21 mm. pig there is a mass of red blood cells. These consist primarily of large, oval cells with pyknotic nuclei and a cytoplasm containing hemoglobin, typical erythroblasts of the first generation. In addition to a few normoblasts of the second series, there is present a number of plastids of the size of the first generation erythroblast. These plastids possess a hemoglobin that

is distinctly granular when stained by Wright's method. The plastids vary in width from 8 to 12 microns, are ovoid in outline, and are derived from first generation erythroblasts. Erythroblasts with nuclei of such degree reduction in diameter that they are not much more than a blue staining speck within an eosin stained cytoplasm (figure 18) are seen in blood smears from pigs 20 mm. in length. At this time first generation plastids first appear in the blood. No fragmentation and extrusion of nuclear material nor any unequal division is observed wherein the nucleus remains with the parent call and a non-nucleated daughter cell (a plastid) is formed. Therefore the first generation plastid is formed by the gradual dissolution or reduction in size of the highly pyknotic nucleus until a non-nucleated cell remains. Karyolysis resulting in the formation of plastids is first observed in the pig 21-25 mm. long.

Sections of the liver were examined to ascertain whether the normoblasts present within the blood stream of the young pig embryo (15-21 mm.) are of hepatic origin or are derived from vascular megaloblasts by differentiation beyond the erythroblast stage of the first series. The distribution of normoblasts and second generation erythroblasts within the liver of the 20 mm. pig is so profuse and their presence within the capillaries and sinusoidal spaces is so abundant that there is no doubt that many of these cells are escaping into the general circulation prior to enucleation, and are not of vascular origin

(figure 37). There is no observable production of first generation cells within the liver.

Results of Cell Counts: Beyond a length of 35 mm., smears of blood from the umbilical vessels contain second generation plastids too numerous to count. Therefore ten fields from each slide were studied under high power magnification and enumerations were made of all erythroid cells other than plastids within each field. Averages were calculated per field per type of cell (Table III). In the blood smears of the 25 mm. pig, 100 cells of each of three smears were counted. In smears of the 35 mm. pig, 250 cells were counted. After the 35 mm. stage, the relative numbers of nucleated cells (normoblasts and erythroblasts) became so reduced that percentage expressions were no longer statistically valid. Therefore, the following figures are numbers per field at 970 x magnification. Normoblasts are present as follows: 1.7 per field at 43 mm., 9.5 perfield at 80 mm. and none are seen in blood smears from pigs of a length greater than 80 mm. Since my attention was directed to the formation of cells within the blood stream and since absence of all cell types of the red cell series except the plastid and the non-mitotic erythroblast indicates cessation of vascular erythropoiesis, observations were continued only through the 160 mm. pig where examination of the blood smear indicates the presence of only these two types of red cells. The erythroblast, with pyknotic nucleus and full complement of non-granular hemoglobin, persists in the blood of the

TABLE III

Length of embryo	Megablasts (1st series)	Erythro-blasts	Plastids (1st series)	Normoblasts (2nd series)	Erythro-blasts	Plastids (2nd series)
20 mm.	4.0%	80 %	11.7%	2.9%	1.5%	---
25 mm.	23.0	50	13.0	10.0	4.0	---
25 mm.	15.0	48.0	37.0	7.0	---	---
25 mm.	13.0	52.0	18.0	17.0	---	---
35 mm.	---	24.0	42.0	10.0	20.0	4.0%
* * * *						
43 mm.	---	---	0.1/field	0.5/field	24/field	TNTC*
75 mm.	---	---	---	0.2	12	"
80 mm.	---	---	---	1.4	6.6	"
115 mm.	---	---	---	---	3.0	"
135 mm.	---	---	---	---	2.1-2.5	"
160 mm.-250 mm.	---	---	---	---	0.6/field	"

* Too numerous to count (TNTC)

* * Average number of cells per field

CELL COUNTS WITHIN BLOOD SMEARS

embryo in small numbers to the time of birth.

Hemoblasts are present in the blood of 15 mm. pigs but are not evident in the smears from 20 mm. pigs. Within the blood of 20 mm. pigs, megaloblasts of the first series comprise only 4%, in the 25 mm. pig they comprise 12% of the cells, but megaloblasts have disappeared from the blood of the 35 mm. pig. First generation erythroblasts constitute 80% of the cells in the 20 mm. pig blood, 50% in the 25 mm. pig, 24% in the 35 mm. pig, and have disappeared from the blood of the 43 mm. pig embryo. Erythroplastids of the first series increase from 12% in the 20 mm. pig to 23% in the 25 mm. pig, and to 42% in the 35 mm. pig, but drop to 0.1% in the pig of 43 mm. length. Beyond this length no first series plastids are observed within blood smears. Within the second series, normoblasts are present in the proportion of 3% at 20 mm., 10% at 25 mm., and 10% at 35 mm. per field. Secondary erythroblasts, cells of the second generation, comprise 1.5% of the total cells per field in the 20 mm. pig, 4% in the 25 mm. pig, with total numbers of nucleated red blood cells so reduced that only cells per field were counted, 24 second generation erythroblasts per field are present in the 43 mm. pig, 12 per field in the 75 mm. pig, 6.6 per field in the 80 mm. pig, 3.0 per field in the 160 mm. pig, and less than one per field within the blood smears of pigs from 160 mm. length to the close of term at about 256 mm. length.

Conclusions: Aortic blood within sections of pig embryos contains hemoblastic cells through 10 mm., but not

15 mm. length pig. Hemoblasts are observed in process of formation from aortic endothelium-mesenchyme in the 3.5 mm. and 5.0 mm. pig. The definitive normoblast first appears in the circulating blood (within cross-sections of vessels) of the 15 mm. pig. In the 20 mm. pig, megaloblasts of the first generation lose their nuclei by karyolysis and form plastids of first-generation-erythroblast size that contain hemoglobin in the form of granules. These large plastids of the first generation persist in the blood of all embryos studied by section, from 20 to 25 mm. in length.

The study of blood smears stained by Wright's method shows that hemoblasts remain in the general circulation until the embryo has reached a length of 15 mm. Megaloblasts are absent in the blood of the 35 mm. pig. First generation erythroblasts are present in greatest number in the 20 mm. pig, but have disappeared from the circulation of the 43 mm. pig. Erythroplastids derived from first generation erythroblasts first appear in pigs 21 mm. long, amount to 42% of the blood cells at 35 mm., and are absent in the blood of the pig of 45 mm. length. Normoblasts appear first in the blood of the 15 mm. pig, comprise 20% of the cells in the 35 mm. pig, and are present in diminishing from 6 per field to an average of 2.5 per field through the 135 mm. pig. Erythroblasts are present up to the time of birth of the pig and are found 1 in each of three out of five fields examined. Definitive plastids appear at 35 mm. and, within the blood of the 43 mm. pig are too

numerous to count. In the blood from pigs 200-250 mm. in length it is very easy to confuse erythroblasts with small lymphocytes which comprise more than half the nucleated cells having a diameter of 6-8 microns. However, these two cell types may be differentiated by the apparent lack of cytoplasm in the small lymphocytes and the presence of non-granular hemoglobin in the extremely small rim of cytoplasm surrounding the nucleus of the erythroblast.

The Liver:

Embryology of the Liver: The hepatic diverticulum of the primitive gut is evident at the level of the duodenal region in the pig of 3.5 mm. length. A disorganized maze of parenchyma growing ventrad and cephaled extends from this diverticulum into the transverse septum. The proximal portion of these parenchymal cords forms the wall of the hepatic ducts and the distal portion gives rise to the secretory tubules. The space between the cords of cells form the hepatic sinuses which become lined with endothelium and succeed the breaking up of the omphalomesenteric (vitelline) veins within the substance. This occurs in the 5 mm. pig. From the endothelial lining of the sinusoidal spaces hemoblasts and normoblasts are derived that form the second generation of red blood cells--the definitive normoblast. Johnson (1919) states that septa separating hepatic lobules begin to appear in the pig of 254 mm. length (at the time of birth).

Hepatic lobules develop, according to Johnson (Op, cit.) by segregation of parenchyma centering around a bifurcation of the hepatic vein. As new bifurcations of branches of this vein appear, neighboring strands of parenchymal cells align themselves with it and form a new lobule, separated from those around it by extension of the connective tissue septum. This lobule formation is determined by the development of the circulatory system and not by ingrowth of septal tissue from the capsule. The collagen tissue forming the septa arises from the portal canals, according to Johnson (1919), who finds such fibers differentiating within the pig liver as early as 80 mm. but that they are confined to the portal canals, especially in the region of the porta hepatis. The size of the lobules remains fairly constant from 80 mm. pig to that of the 230 mm. Beyond 230 mm. the size of the lobules increases rapidly, with rapid increase in size of the liver.

Observations and Discussions: The hepatic mass of the pig of 5 mm. contains only a few clearly discernable cords separated by intervening sinuses (figure 33). Cells possessing the typical checkerboard nucleus and size of the megaloblast are found toward the center of the liver, within vascular spaces and within the veins and capillaries (figure 34). An occasional cell with hemoblastic characteristics is seen. Because of the location of these cells, the structure of the nucleoplasm and cytoplasm, their diameter, and the fact that they are typical of the general vascular

system in pigs of this size, it is concluded that erythropoiesis within the liver of the 5 mm. pig is vascular rather than extra-vascular. Within the liver of the 5 mm. pig numerous endothelial cells of vessels are developing and proliferating hemoblasts.

The condition found within the liver of the 5 mm. pig is duplicated within that of 8 mm. except that the area of parenchyma containing sinuses has increased. Red blood cells are within capillaries and veins and are typical first generation megaloblasts and erythroblasts of vascular origin.

Within the liver of the 10 mm. pig extra-vascular islands of normoblasts have begun to appear. Such islands are few and scattered, only one or two being present per field at 100 diameters magnification (figure 35). In the 11 and 12 mm. pig extra-vascular islands have increased in number and in area of each island.

In the 15 mm. pig only a few scattered first generation megaloblasts and erythroblasts are present. Scattered among the parenchymal cords are clusters of normoblasts which have a scant, neutral cytoplasm but a nucleus of dense composition and strong basic reaction to stains. Many of these normoblasts, which are of a size approximating that of definitive plastids, seem to be imposed upon the parenchymal cords and to lie between the cords of parenchyma and the sinus. Maximov and Bloom (1942) state that hemoblasts of the liver, from which the normoblast nests originate, are derived from stromal or

littoral cells that lie between the endothelium of the sinus and the parenchyma. According to them this stroma is persistent mesenchyme that remains in an embryonal state throughout life as reticulo-endothelial tissue and may serve as a source of blood cells whenever a physiological condition demands, as in the case of extreme loss of blood. There arises the question as to whether the walls of the sinuses of the fetal pig remain constructed of an endothelium that has not progressed beyond mesenchymal activity through the gestation of the pig, or whether the endothelium "matures" and the erythropoietic function of the liver is continued by the littoral cells or reticular tissue. Whatever the ultimate source of the parent cell of normoblasts, they form clusters within the substance of the liver and not within the sinusoids of this organ.

Inspection at a low magnification indicates somewhat the degree of hepatic erythropoiesis achieved by the 15 mm. pig (figure 37). All blood cells within the vessels of the liver are first generation erythroblasts and megaloblasts. A few normoblasts are escaping into the smaller capillaries and into the sinuses.

In the 34 mm. pig the cells within the hepatic islands consist primarily of normoblasts but in the sinusoids the cells consist of secondary erythroblasts and second generation plastids, both fairly uniform in size (6-8 microns in diameter). The erythroblast contains a densely pyknotic nucleus and a maximum of non-granular

hemoglobin. When the liver of the 34 mm. pigs are viewed at a magnification of 100 diameters an early indication of the arrangement of the parenchyma around a central vessel is seen (figure 41). A few scattered first generation erythroblasts and plastids are present in the blood of the large vessels within the liver. Counts made within the cross-sections of capillaries and small veins indicate that the ratio of second generation erythroblasts to second generation plastids is about One:Two, although the range is anything but uniform (Table IV). Total counts were made within six different small veins within single sections of liver from each of two 34 mm. pig embryos.

The number of normoblast-erythroblast clusters among the cords increases until, within the substance of the liver of the 135 mm. pig, the parenchyma is crowded into thin strands (figure 42). Within the vessels there are only a few scattered erythroblasts among the plastids.

The number of islands within the liver of the 210 mm. pig has become reduced. Parenchyma has increasingly become arranged into lobules. Extra-vascular erythropoiesis within the liver reaches a maximum in the 135 mm. pig and retains that rate until the 190 mm. stage. Within the liver of the 250 mm. pig clusters of normoblasts and erythroblasts are still present, and erythroblasts are present in small numbers within the hapatic vessels. Under a magnification of 100 diameters each field of liver tissue observed contains from 20 to 60 islands of nucleated red

TABLE IV

Erythroblasts (2nd series)		Plastids (2nd series)		Erythroblasts (2nd series)		Plastids (2nd series)	
Number	%	Number	%	Number	%	Number	%
20	47	38	53	8	29	28	71
2	40	5	60	6	40	15	60
3	28	11	72	14	40	32	60
2	25	8	75	2	7	30	93
3	43	7	57	7	46	15	54
5	28	18	72	3	17	17	83

Average percent erythroblasts of second generation: 32.5%

Average percent plastids of the second generation: 67.5%

Erythrocytes present in the capillaries of the liver in the 34 mm. pig.

cells per field. Many of the nuclei are undergoing fragmentation (karyorrhexis)(Figure 58).

Liver tissue of three new born pigs was examined. According to Bremer (1914) erythropoiesis continues in the pig liver for some time following birth. Results of our investigation show that this condition of post-natal hepatic erythropoiesis is extremely variable and probably does not exist in a truly full-term fetus. In one pig of not less than 18 hours post-natal age, parenchymal cords are arranged into lobules but are not set off by connective tissue septa. According to Johnson (1919), if this pig were truly post-natal and not a pre-maturely dropped pig, trabeculae should be present. Yet all red blood cells observed in sections of this liver, either within sinuses or in central veins, are plastids. Careful examination by sweeping fields of entire sections (12 sections) failed to reveal the presence of any nucleated red blood cells. (Figure 59).

In a second new born pig of not less than 12 hours post-natal age, a similar sweep of 16 sections disclosed only one erythroblast. Lobule formation around a central vein is definite, cords of parenchyma are arranged vertically to the central vein, which appears more as a collecting reservoir than a vessel because of an apparent absence of a well defined vessel wall. Between two neighboring lobules the parenchyma still is of a haphazard arrangement. Between such cords the sinuses contain masses of second series plastids.

The liver of a third pig, at least 12 hours post-natal, is erythropoietic but on a very much reduced scale. An examination of 12 sections of the liver under a magnification of 970 diameters reveals two islands within one section but in two different fields. One island contains six erythroblasts and the other contains eight. The nuclei are extremely pyknotic and the cytoplasm stains heavily with eosin. The nuclei are irregular in outline, and indicate the early stages of karyorrhexis (figure 59). Why do the three post-natal pigs show a difference in the degree of maturation of the liver? Why has the pig been reported to have continued hepatic erythropoiesis beyond the gestation period (Johnson, 1919)? Our post-natal pigs were collected in the pens of the stock yard the morning following the deposition of the sow the preceding evening. These sows had been hauled by truck from the farm to the stock pen, and had been crowded and pushed around in the pen all night. With the completion of the term at hand, it is entirely possible that new born pigs dropped by these sows are really prematurely dropped fetuses. The actual time from completed gestation would vary and result in new born pigs whose livers likewise vary in degree of maturation.

Conclusions: Until the embryo is 10-12 mm, in length the liver of the pig contains only red blood cells derived from vascular erythropoiesis. Beyond a length of 12 mm. extra-sinusoidal erythropoiesis increases by elaboration of hemoblasts and second genera-

ion normoblasts from sinusoidal endothelium (the "littoral cells" of (Maximov and Bloom, 1942) with the consequent establishment of extra-sinusoidal islands or clusters of second generation cells, until a maximum rate of erythropoiesis is attained in the 135 mm. pig. This maximum rate is maintained to the 190 mm. stage after which erythropoiesis is rapidly reduced. At the time of birth erythropoiesis in the liver has ceased in two out of three post-natal pigs studied. The erythropoietic function of the liver in the pig differs radically from that in the liver of man. Knoll (1932) states that from 70 mm. to the end of the 6th month the liver and bone marrow compete in the production of red cells but that, beyond the 6th month, the liver of the human fetus ceases its erythropoietic function.

The Spleen:

Embryology of the Spleen: The spleen develops in the dorsal mesogastrium at the level of the stomach. When first observed it appears as a dense mass of tissue in the left dorso-lateral portion of the mesogastrium occupying about one-half the extent of that structure. There are a few scattered first generation erythroblast cells within the mesenchyme of this rudiment and a few capillaries that contain first generation erythroblasts are present.

In the 15 mm. pig the epithelium of the spleen is distinctly set off from the mesenchyme and cells have

so far differentiated that they are clearly distinguishable from the typical mesenchyme cell. The nuclei are almost uniformly rounded structures, the nuclear membrane is distinct, and the chromatin is distributed as fine granules throughout the nucleus. Nucleoli, when present, are not so adequately defined as in the nucleus of the mesenchyme cell. In the 15 mm. pig the splenic rudiment, 0.2 to 0.3 mm. in diameter, shows little differentiation within its tissues.

Circulation within the Spleen: Thiel and Downy (1921) state that most workers consider the circulatory system of the spleen to be an open one and that in the pig embryo of less than 60 mm. the only vascular elements in evidence within this organ are narrow, irregular, branching capillaries through which erythrocytes pass, and that these capillaries possess a well defined endothelium lining. They state that there are no large unlined sinuses within the spleen during the first five weeks of fetal life, and that as early as 12 mm. it is possible to trace the mesenteric artery along the midventral margin of the dorsal mesogastrium. According to Thiel and Downy (1921) capillaries branch off from this vessel to penetrate the mesenchyme of the spleen and form a system of blood supply for the splenic mesenchyme in the embryo to a length of 40 to 60 mm. Sabin (1916) states that there are both arterial and venous capillaries within the spleen of the 30 mm. pig. Thiel and Downy (Op,cit.) believe that the sinuses of the spleen

constitute no part of the vascular system since, according to them, these sinuses lack a true endothelium. Sabin (1916) states that to a length of 75 mm. the spleen of the human fetus is no more than undifferentiated mesenchyme and that red blood cell development lies within the capillary bed. She refers to Lifschutz (1906) as stating that in the human fetus hemopoietic activity reaches its height at 180 mm.; Sabin considers the sinuses as a capillary bed intervening between arterial and venous capillaries.

Whether or not the sinuses of the spleen constitute a part of the vascular system (that is--whether they are bordered by a true endothelium) our purpose is to determine the erythropoietic activity of that organ within the pig.

Toward the end of gestation it is difficult to differentiate between normoblasts and small lymphocytes in the spleen. Since the normoblast is formed from the hemoblast and the small lymphocyte occurs with the large lymphocyte one might suppose that the presence of hemo blasts would indicate a continuance of erythropoiesis. However, there is no obvious cytological difference between the large lymphocyte and the hemoblast. One must, therefore, look for differences between the small lymphcyte and the normoblast. Lambert (1938, page 114) describes the lymphocyte as being from 6-8 microns in diameter, their size being that of an erythrocyte (plastid).

The lymphocyte contains a nucleus filled with a mass of granular chromatin which stains intensely with the basic dyes. The nucleus is surrounded by a cytoplasm so small in amount that it is impossible to see it with ordinary magnifications ... in ordinary preparations the nucleus is the only part of a small lymphocyte which can be identified. The fundamental observable difference then between the small lymphocyte and the normoblast is the visible but tiny rim of cytoplasm surrounding the nucleus of the red blood cell. The occurrence of erythropoiesis in the spleen is indicated not only by the fact that normoblasts are observed in its titsues, but also because they are present within the spleen beyond the size of embryo when this type of red cell can no longer be found in the general circulation.

Observations and Discussion: The formation of red blood cells begins in the spleen of the 33 mm. pig (figure 22) with the differentiation of mesenchyme cells into typical hemoblasts. These, in turn, differentiate into normoblasts of the second generation of red blood cells, having a checkerboard nucleus similar to that of the first generation megaloblast but a diameter similar to that of the second generation plastid (6-8 microns). The normoblast progressively develops hemoglobin within a cytoplasm which itself is scant. With the development of a pyknotic nucleus and a full complement of hemoglobin the normoblast differentiates into an erythroblast (figure

21). No plastids are seen within the spleen until the pig reaches a length of 80 mm. In the spleen of the 43 mm. pig a total of 8 centers of hemoblast formation have developed.

In the 80 mm. pig the spleen not only contains second generation erythroblast cells with a fringe of cytoplasm that possesses a maximum amount of hemoglobin and a pyknotic nucleus, but there are also many non-nucleated plastids with a diameter of 6-8 microns. The mass of the spleen is still mesenchyme enclosed by a developing connective tissue capsule. This latter structure consists of several layers of cells the cytoplasm of which is drawn out over the surface of the mesenchyme. Although the nuclei of these capsular cells are vesicular to a degree comparable to those of the mesenchyme cells, they stain a bright blue while those of the mesenchyme are very resistant to stain.

The spleen of the 115 mm. pig contains a myriad of islands of small cells 6-8 microns in diameter that have a densely stained nucleus (figure 25). The nucleus is so pyknotic that separate chromatin clumps cannot be observed. The cytoplasm contains a maximum amount of hemoglobin. These cells are second generation erythroblasts. Along with these islands and filling intra-cellular spaces within the pulp there is a vast number of second generation plastids. Whether all of these have stemmed from normoblasts within the spleen or whether a portion have entered with the blood through the

sinuses into the pulp, it is impossible to say. However, the size of the nucleus of the normoblast seems to remain about the same; it does not seem to be decreasing in diameter with the age of the cell. If these definitive plastids, or any portion of them, are arising from the normoblasts and erythroblasts, then enucleation is most rapid if it occurs by karyolysis. No fragmentation of the nucleus of the erythroblast is observed within the islands nor within the pulp of the spleen at this stage. It is condluded, therefore, that at this stage of development, the plastids are entering the splenic pulp from the general circulation. Whatever the source of the plastids, they now greatly outnumber the erythroblasts within the splenic pulp.

At the surface of the spleen more mesenchyme cells have arranged themselves lengthwise and are being transformed into connective tissue fibroblasts of the capsule. The nuclei of these cells, while still vesicular and staining rather faintly, are oval to elongate in shape. Growths inward from the capsule indicate potential trabeculae. Cells near the capsule consist of second generation erythroblasts, normoblasts, hemoblasts, mesenchyme cells, and a few scattered lymphocytes.

Deep in the substance of the spleen the clumps of cells consist of hemoblasts, normoblasts, second generation erythroblasts and many second generation plastids. In spite of the numbers of plastids present in the pulp

definite islands of normoblasts and erythroblasts are observable under a magnification of 100 diameters.

In the 135 mm. pig the groups of normoblasts and erythroblasts have so increased in size and number that separate islands have lost their identity. The connective tissue and pulp now has the appearance of areas of pulp and plastids surrounded by masses of normoblasts (figure 28). This condition of very large masses of normoblasts and erythroblasts continues through the 190 mm. stage of the pig (figure 30). The numbers of such cells in relation to the plastids shifts until about half the cells of the pulp appear to be plastids and half erythroblasts. Cells of the size of erythroblasts but with no clearly observable hemoglobin--(small lymphocytes) abound in areas near the periphery of the gland.

Toward the border of the spleen in the 190 mm. pig trabeculae from the connective tissue of the capsule are growing into the splenic substance and nodules are forming. Although arteries are seen within the trabeculae or septa, none are present as central arteries within the nodules. Occasional groups of three or four "splenic" cells are seen. Although these are of a size comparable to that of megakaryocytes they differ from the latter in that hemotoxylin-eosin stains the cytoplasm faintly pink and the nucleus, while vesicular with chromatin arranged upon a fine reticulum, is not lobate. The cytoplasm of the "splenic cell" is non-granular and the outline of the cell is irregular.

Erythropoiesis continues actively in splenic tissue of the 250 mm. pig. Within the pulp at least half the nucleated cells are present as normoblasts (figure 56). Groups of cells similar in size to the normoblast, erythroblast and plastid are present near splenic nodules. The cytoplasm is not observable. These cells are small lymphocytes. Numerous cells were observed that are approximately twice the diameter of the small lymphocytes and contain a nucleus that stains a deep blue with the chromatic granules taking a yet deeper stain. The staining reaction and chromatin arrangement suggests a hemoblast. The cytoplasm consists of a narrow rim and takes a stain no more intense than does the nucleoplasm. These cells are large lymphocytes.

Connective tissue trabeculae extend well into the pulp of the spleen in the new born pig. Toward the periphery of the gland and somewhat scattered into the red pulp are numerous nodules of white pulp consisting of large and small lymphocytes. The nodules are distributed along the trabeculae and surround a central artery. A "germinal center" or an area less dense than the rest of the nodule immediately surrounds the central artery. Groups of small lymphocytes are located near the nodules in areas near the border of the gland (Figure 57).

The internal appearance and composition of the spleen of the new born pig is similar to that of the adult pig. Erythropoiesis has ceased. Red blood cells are present only in the form of second generation plastids.

The capsule of the spleen is composed of connective tissue with scattered smooth muscle fibers--the tunica albuginea--covered by a layer of squamous cells--the peritoneum or tunica serosa. There is greater penetration of trabeculae and there are more nodules of white pulp in the adult spleen, but beyond that, the spleen of the new born pig is similar to that of the adult.

Erythropoiesis within the spleen of the pig differs from that of the human embryo. Arey (1942) states that in the latter erythropoietic function of the spleen is limited to the middle third of fetal life. Sabin (1916) quotes Lifschutz (1906) as stating that erythropoiesis extends from 150 mm. to 300 mm. in the human spleen, reaching a maximum at 180 mm. Knoll (1932) states that there is a recession of erythropoiesis in the spleen of the human after the fifth month but does not indicate a termination of this activity.

Conclusions: The spleen becomes erythropoietic with the formation of hemoblasts from mesenchyme in the 34 mm. pig. Production of normoblasts and secondary erythroblasts increases until at the height of activity in the 135 mm. pig the mass of the spleen seems to be composed of nucleated red cells separated by strands of splenic tissue. This height of activity continues until the pig is 190 mm. long, when, following a rapid diminution, normoblasts are present in large numbers only in the deep pulp. Erythropoiesis continues within the spleen of the pig until the close of the gestation period.

In this the pig differs from the human fetus where the spleen is an erythropoietic organ only through the middle third of fetal life.

The Bone Marrow:

Embryology: The impending onset of ossification can be recognized by the re-arrangement that takes place among the cartilage cells. By the erosion of the cartilagonous matrix these cells form into transverse rows. By extension of the erosion the cartilage becomes honeycombed. Cells derived from the perichondrium by rapid mitosis begin to invade the honeycombed cartilage carrying blood vessels with them. This invasion of blood vessels marks the disintegration of the cartilage, and onset of ossification, etc.

The bone marrow is formed from the mesenchyme remaining within the spaces of the diploe of the bone during the time of ossification and following it.

Observations and Discussions: Mesenchymal cells in the fore-limb of the 15 mm. pig are being transformed into chondroblasts and are beginning to deposite early cartilage in the intracellular matrix (figure 47). The first indication of this deposition is an apparent migration of cells away from each other. The thin cartilage strands stain red with hematoxylin-eosin. Cartilage cells remain within lacunar spaces and possess nuclei that stain a bright blue. The cytoplasm of the chondroblast is difficult to observe. Mesenchymal cells at the periphery of the bone-forming anlage have begun to flatten

to form the perichondrium (Figure 47-c).

Costal cartilage is completely laid down in the 33 mm. pig (Figure 48). The perichondrium is well formed as a connective tissue capsule surrounding the cartilage.

The earliest erosin of the cartilage of the femur occurs in the pig of 80 mm. (Figure 49); when areas of destruction of cartilage appear in separate portions of the cartilagenous limb structure. Cells of the capsule, the periosteum, begin to erode the boundary of the cartilage and, proliferating at a greatly accelerated rate, penetrate into the eroded areas (figure 52). These invading cells align themselves along the strands of cartilage and begin to deposite calcium salts as laminations around the trabeculae that survive erosion (Figure 51).

The first blood cells appear in marrow smears from the limb of a pig 115 mm. in length (Figure 50). Most of the cells observed are in the erythroblast stage but a few normoblasts are seen.

Erosion of cartilage in the femur of the 210 mm. pig results in the formation of a large area of honeycomb appearance. Mesenchyme that first deposits a layer of bone over the trabeculae remains as a layer of cells that lies against the spicules and forms a lining for the marrow spaces. Endothelium of reticular cells as seen in cross section in the marrow spaces form sinusoids that appear as a circular band of cells, each cell consisting of elongated cytoplasm and a fusiform nucleus. Most of

the sinusoids contain from a few to many secondary generation plastids. Normoblasts are present both within and outside these circles of endothelium (Figure 51). Numerous second-series erythroblasts in the sinusoids and in the extra-sinusoidal spaces are undergoing karyorrhexis as the nucleus consists of several particles that are deeply stained with basic dye. Hemoglobin is fully developed within the erythroblasts but is of a scant amount within the cytoplasm of the normoblasts. In erythroblasts where karyorrhexis is not underway, the pyknotic nucleus is eccentrically located and is irregular in outline. Marrow erythropoiesis is well established within the femur of the 210 mm. pig (Figure 51).

Knoll (1932) states that in the human fetus the onset of medullary erythropoiesis occurs at about the 5th month or the middle of the gestation period, and increases as it replaces hepatic and splenic erythropoiesis until it is the sole source of red blood cells. In the pig the marrow shares erythropoietic responsibility with both spleen and liver after the pig reaches a length of 115 mm. and until the close of gestation.

Conclusions: Cartilage begins to form within the limb anlage of a length of 15 mm. with the apparent separation of mesenchyme cells (chondroblasts) form from each other due to the deposition of cartilabe in the inter-cellular matrix. Erosion of cartilage begins within the femur of the 80 mm. pig and evidence of erythropoiesis is first obtained in marrow smears from the

femur of the 115 mm. pig. Erythropoiesis is well established within the marrow of the femur of the 210 mm. pig.

PART V SUMMARY

1. The yolksac is an active erythropoietic organ in pigs of 3.5 to 15 mm. length, reaching a maximum of activity in the 10 mm. pig. Blood channels begin to form anastomoses in the 10-12 mm. pig yolksac. "Island" production of hemoblasts has ceased in the yolksac of the 16 mm. pig. Any red cell production within this organ beyond this stage is typically vascular.

2. The umbilical cord of the pig increases in vascularity throughout the fetal life of the pig. There is no evidence to indicate that this organ plays a role in erythro poiesis.

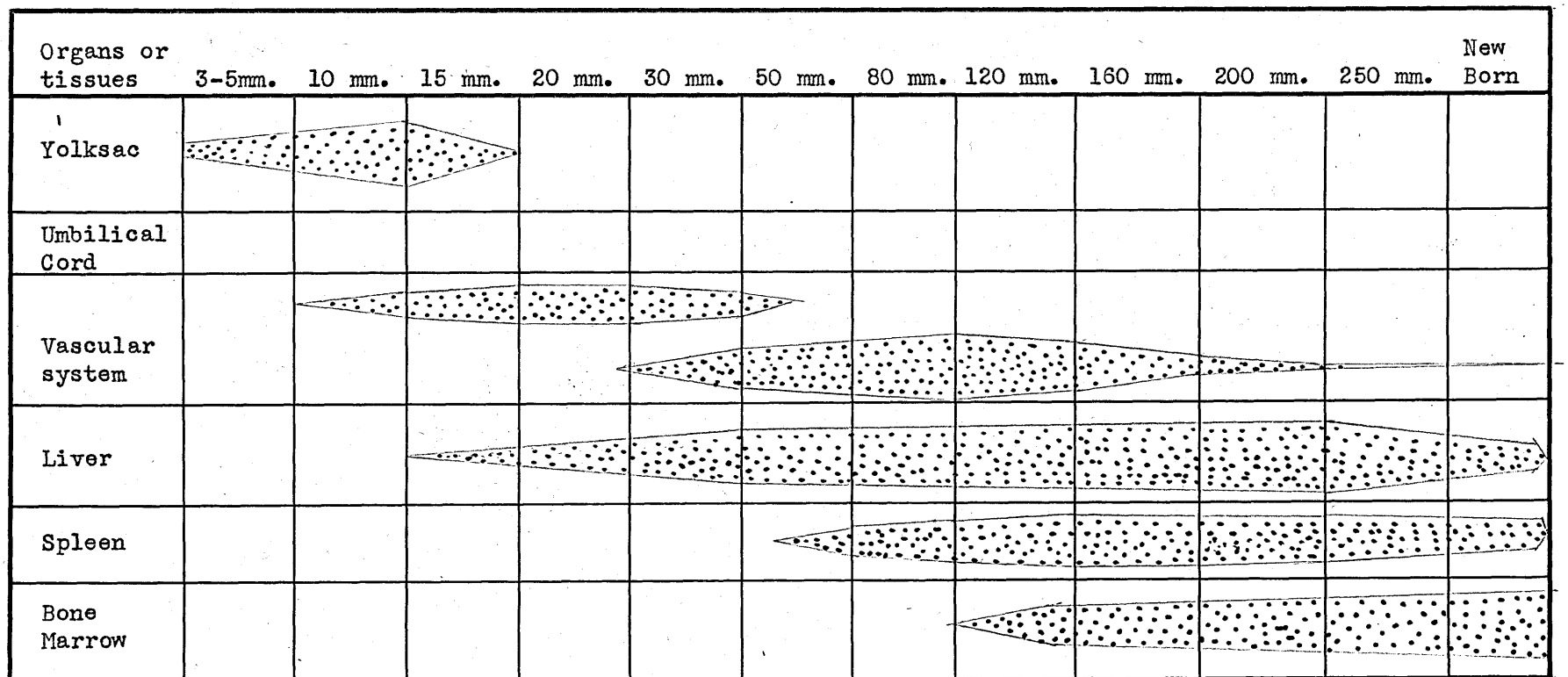
3. Vascular erythropoiesis has begun in the 3.5 mm. pig. The formation of hemoblasts from endothelium of the blood vessels ceases in the 15 mm. pig. Normoblasts first appear in the circulating blood of the 15 mm. pig. First generation red blood cells are gone from the circulation at 43 mm. Second series cells of the normoblast type continue to be present through the 80 mm. stage but are gone from the vessels beyond this stage. Erythroblasts of the second generation appear in the general circulation at 20 mm., become most numerous at 43 mm., and are present in small numbers from 160 mm. to the close of term at 256 mm.

4. Blood islands begin to form in the liver of the 10-12 mm. pig. Height of erythropoesis in the liver is attained in the 135 mm. pig, and is maintained until 190 mm. after which there is a gradual reduction until the end of gestation. All erythropoietic activity has ceased in the liver in two out of three post-natal pigs.

5. Splenic erythropoiesis begins with differentiation of mesenchyme cells into hemoblasts in the 33 mm. pig. The maximum of normoblast production is attained in the pig of 190 mm. after which the rate of production is reduced. Splenic erythropoiesis continues through the 250 mm. pig, but is not present in the new born.

6. Cartilage is first observed in the 15 mm. pig. Erosion begins in the femur in the 80 mm. pig. The first evidence of marrow erythropoiesis is in the marrow smear from the femur of the pig of 115 mm. In the 210 mm. pig the femoral marrow is in an active state of erythropoiesis.

DEVELOPMENTAL STAGES IN CROWN-RUMP LENGTH



HISTOGRAM SHOWING ORIGIN, RISE, and DECLINE OF ERYTHROPOIESIS IN VARIOUS ORGANS

PART VI

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PART VII

EXPLANATION OF PLATES

Plates I to VIII inclusive consist of figures that were drawn by aid of camera lucida.

Plate IX is made up of four figures consisting of microphotographs.

PLATE I

EXPLANATION OF FIGURES

Figure 1: Section of yolksac from a pig of 3.5 mm. length. a. mesoderm. b. differentiated hemoblast. c. hemoblast in process of differentiation. d. mesenchyme. x 970

Figure 2: Island formed from mesenchyme of the yolksac of 3.5 mm. pig. a. mesenchyme. b. hemoblast. c. megaloblast. x 970

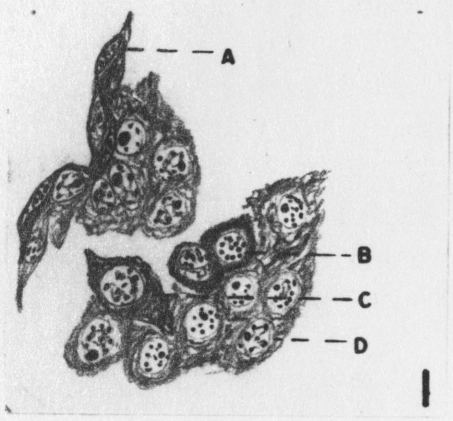
Figure 3: Blood island within the yolksac of a 5 mm. pig. The cluster of cells is composed of megaloblasts. x 970

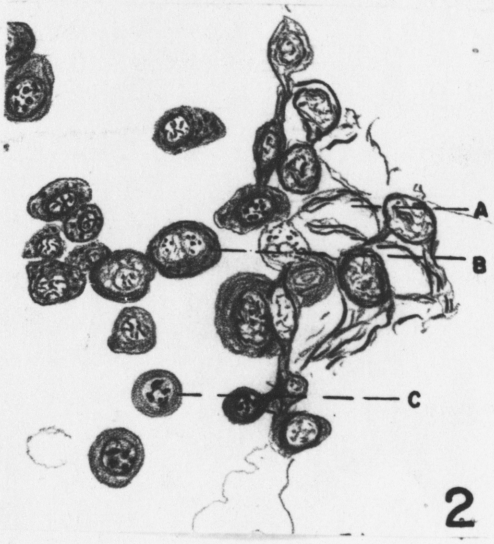
Figure 4: Yolksac of the 5 mm. pig embryo. a. entoderm. b. mesoderm. c. megaloblast. d. erythroblast. e. hemoblast. f. mesenchyme. The nucleus of the erythroblast is dense and stains almost black. The cytoplasm of this early stage of erythroblast stains basophilically due to the absence of hemoglobin. Entodermal cells are cuboidal and mesodermal cells are fusiform. x 970

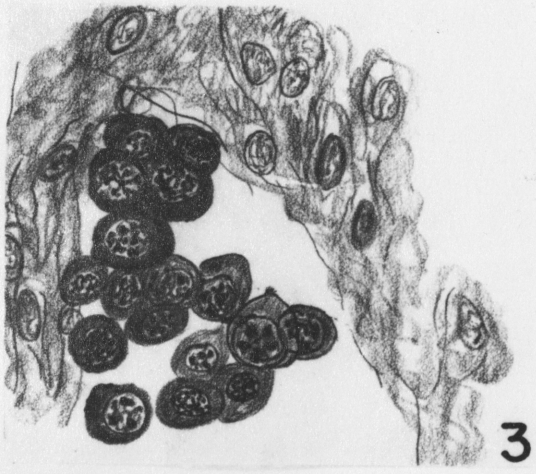
Figure 5: Yolksac of a 10 mm. pig indicating the extent of island formation at this stage of development. x 100

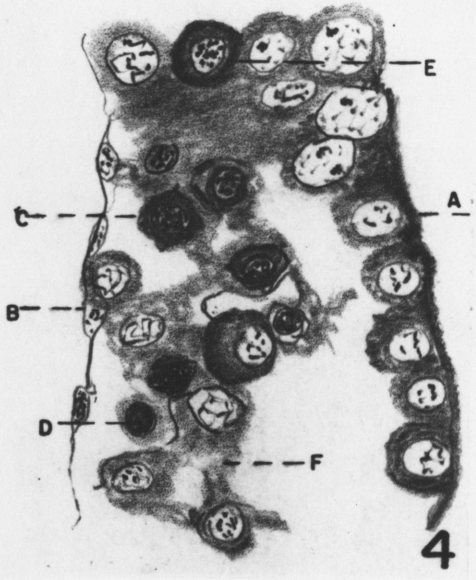
Figure 5a: Yolksac of a 10 mm. pig. a. mesoderm. b. megaloblast. c. erythroblasts. d. entoderm. x 450

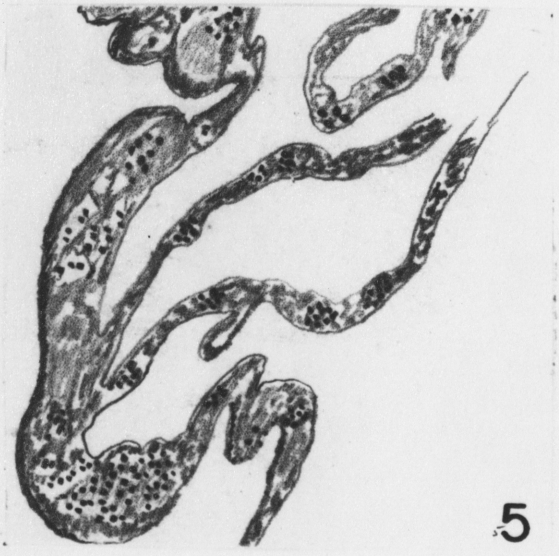
PLATE I











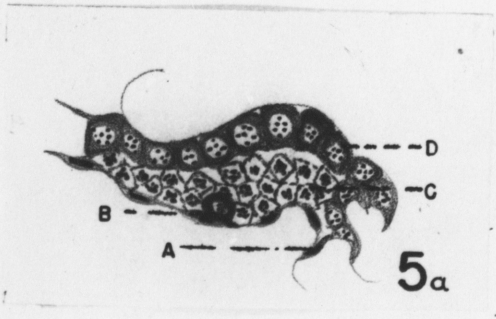


PLATE II

EXPLANATION OF FIGURES

Figure 6: Yolksac of 16 mm. pig. The surface of the sac was wrinkled so that sections having been cut across these invaginations appear to contain glandular structures or villi in cross section. a. folds of entoderm. b. islands of erythroblasts. c. mesenchyme. d. entoderm. x 450

Figure 7: Yolksac of 25 mm. pig embryo. a. mesoderm. b. columnar entoderm. x 430

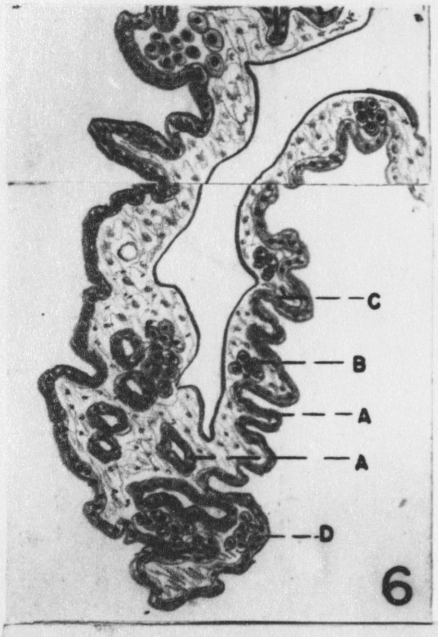
Figure 7a: Group of "old" erythroblasts within the yolk-sac of 25 mm. pig. Nuclei are pyknotic and stain blue, cytoplasm stains red and is granular when tissue is stained with Wright's stain. x 970

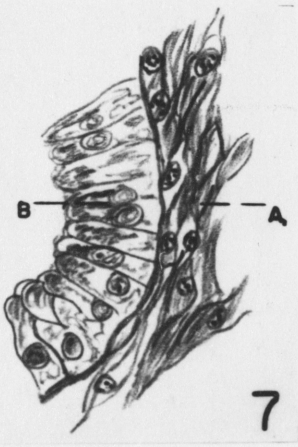
Figure 8: Group of erythroblasts within the mesodermal tissue of yolksac of 25 mm. pig. Nuclei are larger and the hemoglobin of the cytoplasm stains less dense than in those cells of Figure 7a. x 970

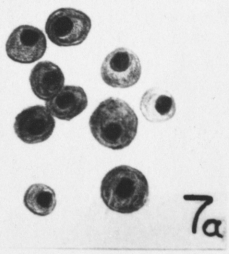
Figure 9: Sections of capillaries and tissue cells from the umbilical cord of a 105 mm. pig. a. endothelial cell containing an erythro-blast of the first generation in addition to its own nucleus. b. endothelial cell pre-ceding division, containing two cell nuclei and one erythroplastid of the second genera-tion. c. capillary containing one normo-blast and ten plastids. d. blood capillary containing one first generation erythroblast, six second generation erythroblasts, and fourteen second series plastids. x 970

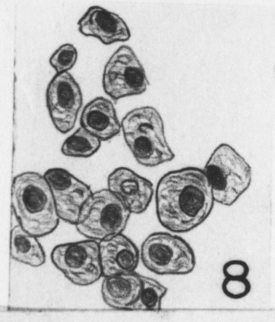
Figure 10: Cross section of umbilical cord of 105 mm. pig. a. Peripheral capillaries b. um-bilical artery c. vein d. allantoic stalk x 16

PLATE II









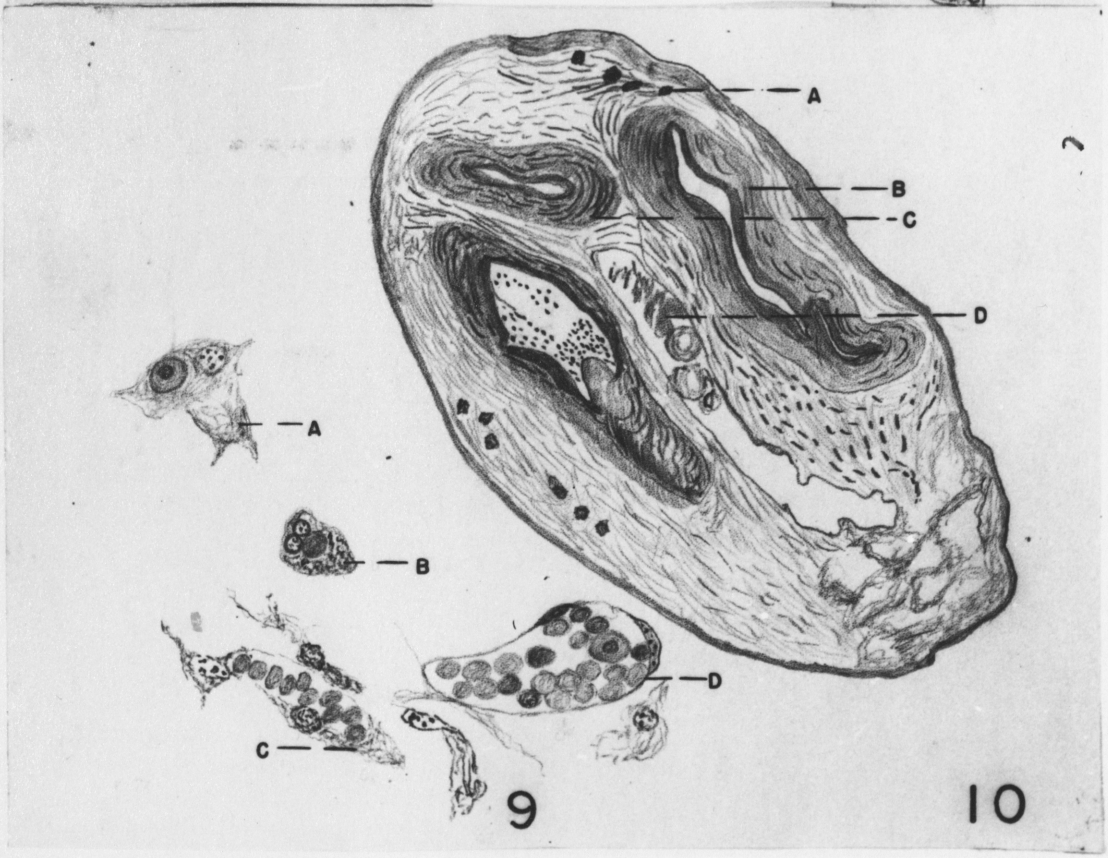


PLATE III

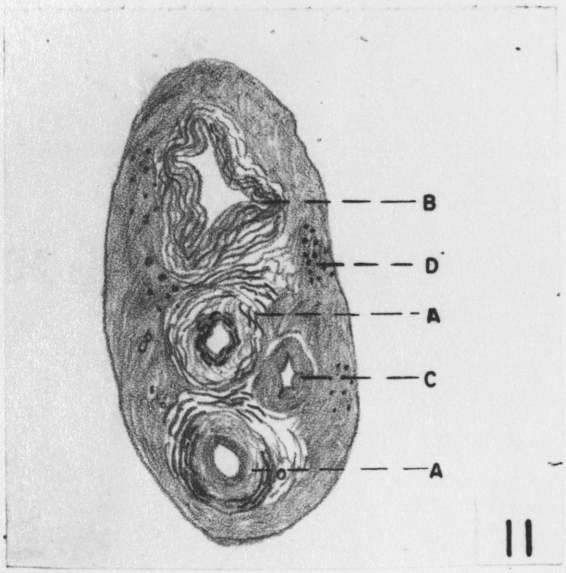
EXPLANATION OF FIGURES

Figure 11: Umbilical cord of 200 mm. pig. The area of cord that contains capillaries has increased over that of the 105 mm. embryo. a. arteries b. vein c. allantois d. peripheral capillaries x 8

Figure 12: Umbilical cord of 250 mm. pig embryo. This pig had practically reached the end of gestation. The abundance of capillaries in this cord indicates that the extensive vascularization of the cord is continued throughout the gestation period. a. arteries b. vein c. allantois d. capillaries x 8

Figure 13: Cross section through the head of 3.5 mm. pig embryo. Blood cells of erythroblast stage are already present within the capillaries. a. blood capillaries containing erythroblasts. x 100

PLATE III



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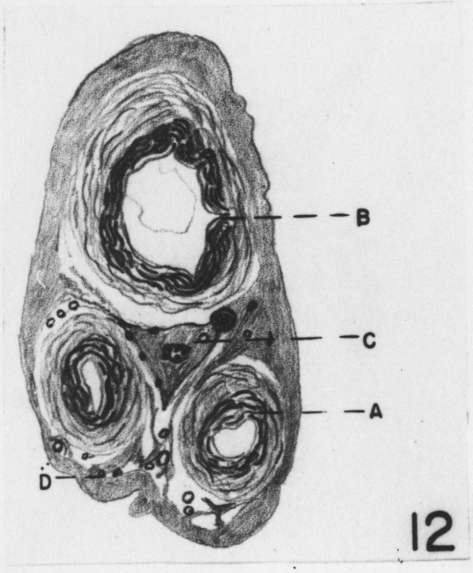

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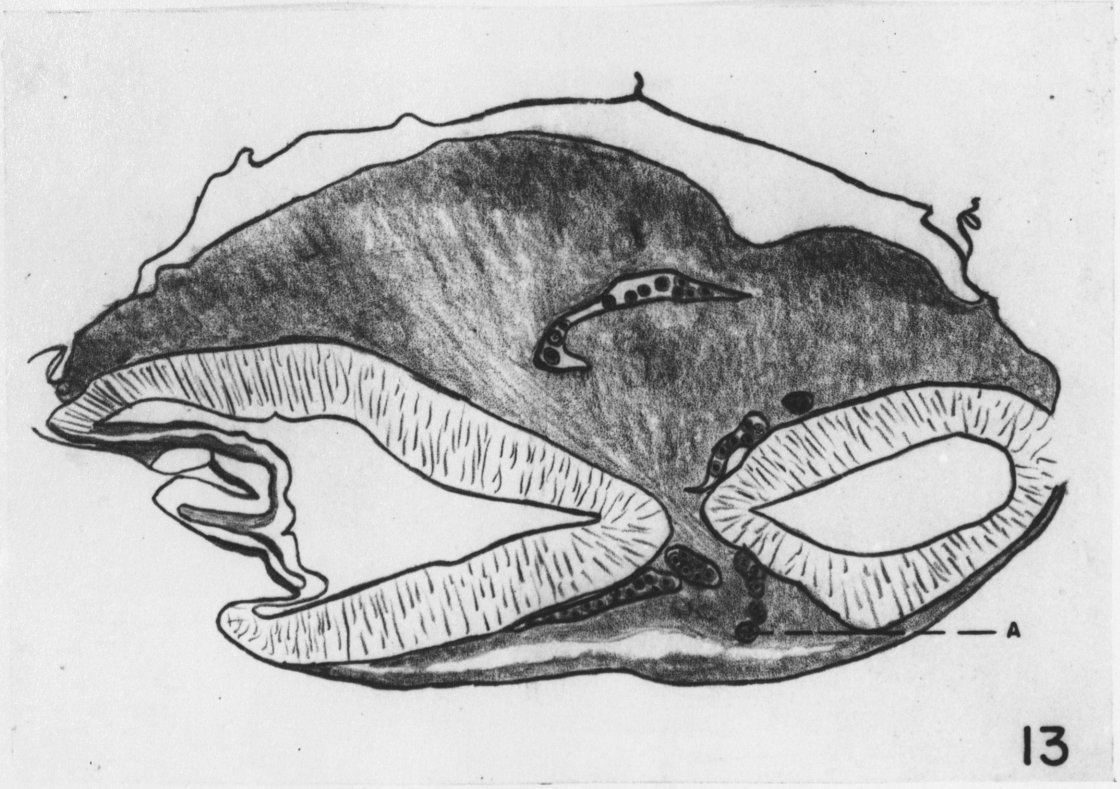

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PLATE IV

EXPLANATION OF FIGURES

Figure 14: Dorsal aorta of 3.5 mm. pig. a. mesenchymal cells forming aortic wall. b. hemoblasts. c. lumen of aorta. d. megaloblast. e. erythroblasts. x 970

Figure 15: Blood cells within dorsal aorta of 5 mm. pig. a. hemoblast. b. megaloblast. c. erythroblast. x 970

Figure 16: Mitotic second generation erythroblasts in vitelline vein within the liver of 10 mm. pig. x 970

Figure 17: Types of blood cells found in dorsal aorta of 15 mm. pig. a. megaloblast. b. first generation erythroblast. c. normoblasts of the second series. x 970

Figure 18: Cells from blood smear from umbilicus of 25 mm. pig. a. megaloblast. b. first generation erythroblast with large pyknotic nucleus. C. erythroblast with small (minute) nucleus. d. large plastid of first series of cells. x 970

Figure 19: mitotic first generation erythroblasts in blood of a 10 mm. pig. x 970

Figure 20: 21 mm. pig. Blood cells in aorta. a. mitotic first generation erythroblasts. b. plastids of the first series. c. normoblasts. x 970

Figure 21: Blood cells within a vessel between the spleen rudiment and the mesogastrum of a 34 mm. pig. a. normoblast. b. erythroblasts of second series. x 970

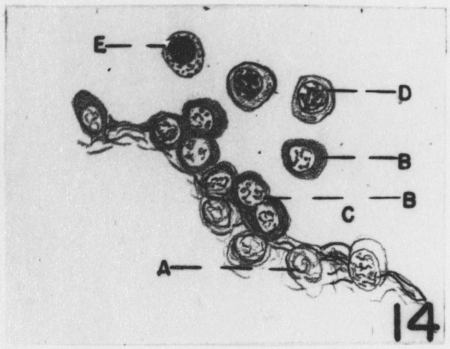
Figure 22: Hemoblasts being differentiated from splenic mesenchyme in the 33 mm. pig. x 970

Figure 23: Erythrocytes within one of eight islands in the spleen of 43 mm. pig. a. hemoblast. b. normoblast. c. erythroblast. x 970

Figure 24: Erythroblasts from splenic island of 80 mm. pig. x 970

Figure 25: Appearance of sinusoids, showing extent of islands of erythroid cells within spleen of 115 mm. pig. x 100

PLATE IV



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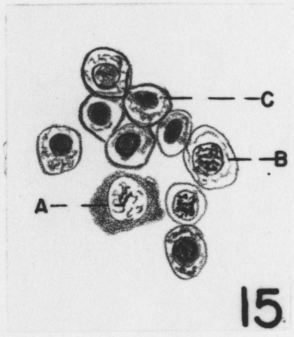

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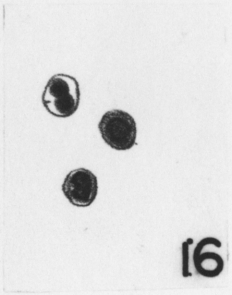
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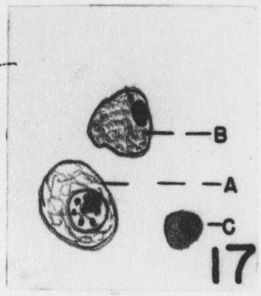

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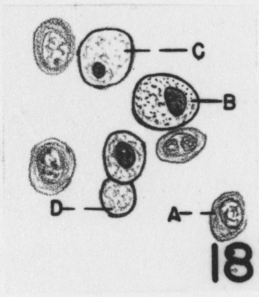

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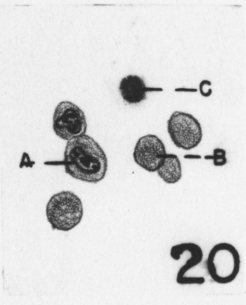

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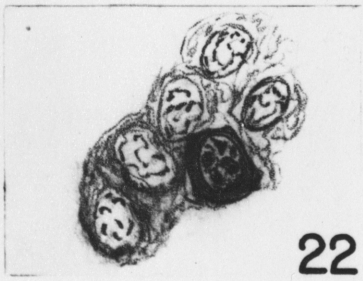
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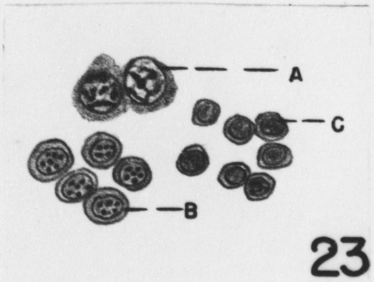

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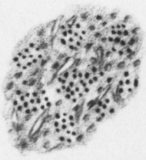
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PLATE V

EXPLANATION OF FIGURES

Figure 26: Splenic capsule and beginning of formation of trabeculae in 115 mm. pig. a. capsule. b. trabecula. c. plastids. d. erythroblast. e. normoblasts. X 970

Figure 27: Cells from splenic pulp of 125 mm pig. a. erythroblasts. b. normoblasts.x 970

Figure 28: Spleen from pig of 135 mm. Erythroid cells islands are so extensive that mesenchyme appears as strands. x 100

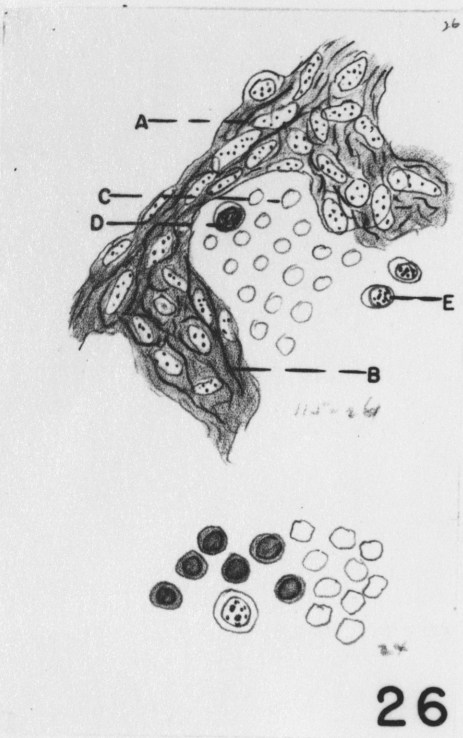
Figure 29: 135 mm. pig. Spleen as seen under 970 times magnification. a. hemoblasts. b. mesenchyme. c. normoblasts. d. erythroblasts. x 970

Figure 30: Splenic nodule of 190 mm. pig. a. splenic cells. b. normoblast. c. small normoblast. d. erythroblast (x 970) e. trabecula. f. plastids. g. lymphocytes. x 100

Figure 31: Spleen of 240 mm. pig. a. connective tissue trabeculae. b. erythroblasts. c. plastids. x 430

Figure 32: Spleen of new born pig. a. capsule. b. nodule with central vein. c. trabecula. d. red pulp. x 100

PLATE V



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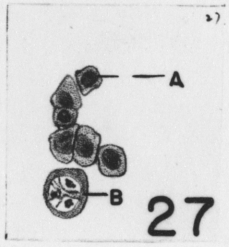

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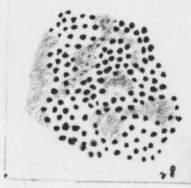

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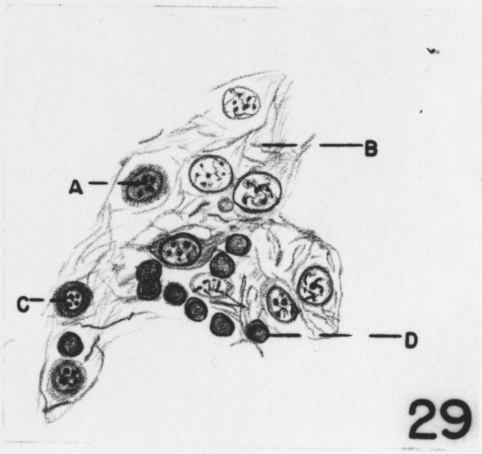

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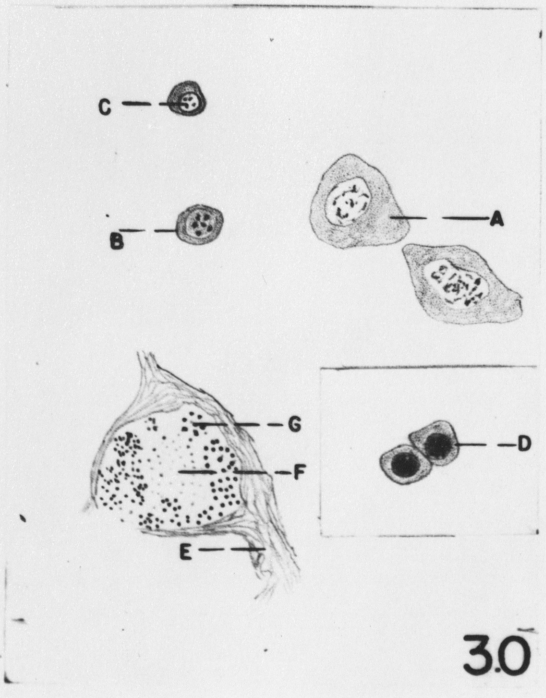

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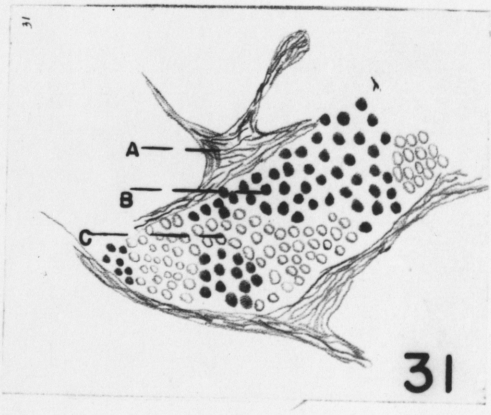

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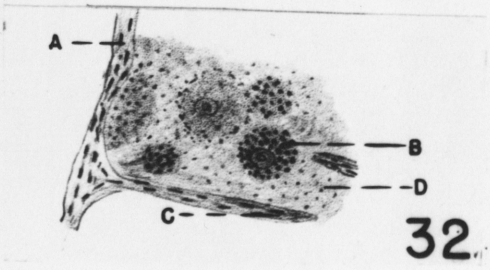

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PLATE VI

EXPLANATION OF FIGURES

Figure 33. Liver of a 5 mm. pig. a. capillaries containing first generation erythroblasts. b. parenchymal cords. x 100

Figure 34. Liver of a 5 mm. pig. a. parenchyma not clearly organized as cords and sinuses. b. erythroblast blood cells. x 450

Figure 35. Liver of 10 mm. pig. a. parenchymal cords b. erythroblasts of first generation. c. islands of normoblasts. x 100

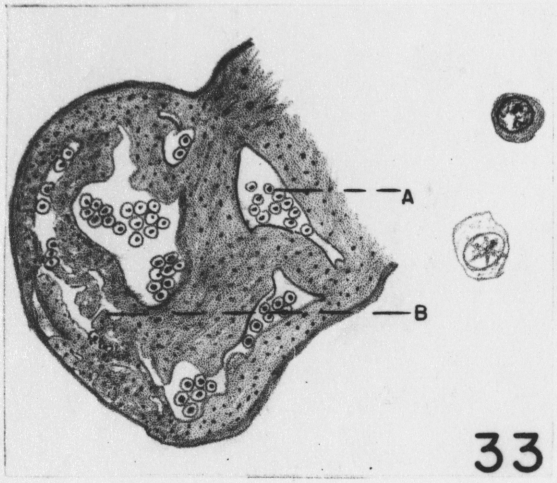
Figure 36. Hepatic sinus in 12 mm. pig. a. parenchyma b. sinus c. normoblasts. x 950

Figure 37. Liver of 15 mm. pig. a.parenchyma b. islands of normoblasts. c. vessel containing first generation erythroblasts. x 100

Figure 38. Hepatic sinus of 20 mm. pig. a. parenchyma b. endothelium c. normoblast. d. erythroblast of second generation x 430

Figure 39. Hepatic sinus of 34 mm. pig filled with second generation erythroblasts. x 970

PLATE VI



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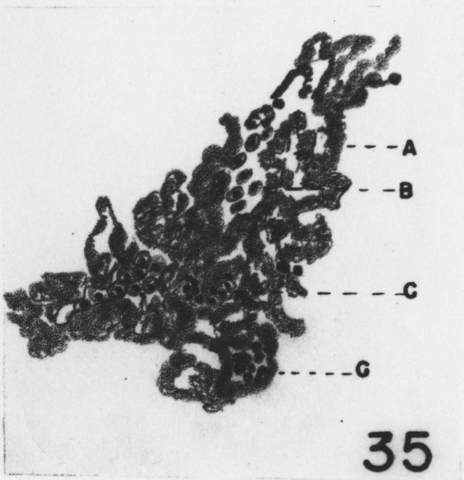

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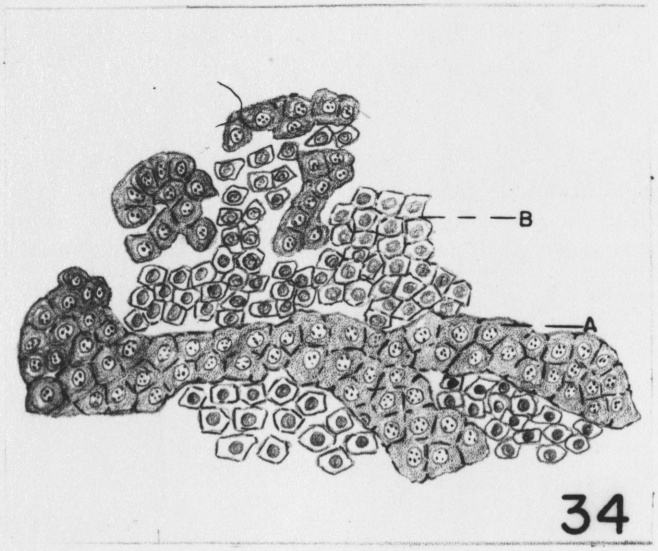

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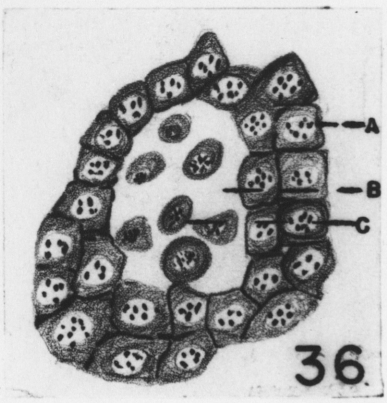

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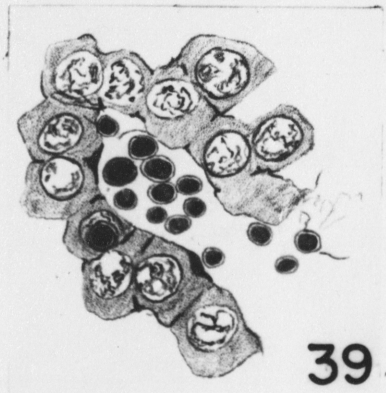

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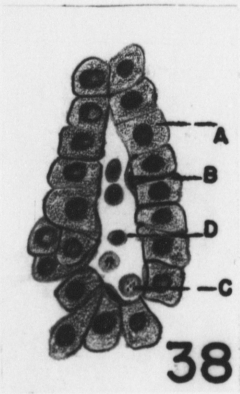

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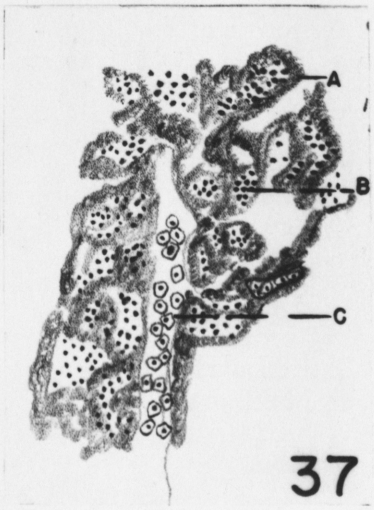

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PLATE VII

EXPLANATION OF FIGURES

Figure 40. Hepatic sinus from 43 mm. pig. a. parenchyma. b. normoblast. c. erythroblasts x 970

Figure 41. First indication of lobulation of liver. 34 mm. pig. a. parenchyma b. central or intra-lobular vein x 20

Figure 42. Liver of 135 mm. pig showing degree to which erythropoiesis has occupied the hepatic areas. x 100

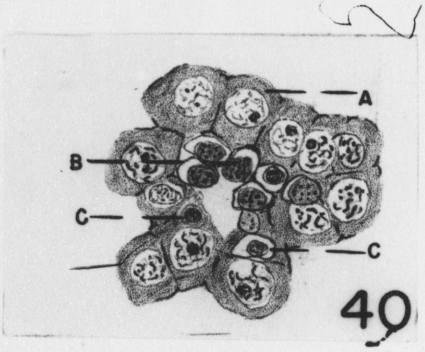
Figure 43. Cells within vein in the liver of a 135 mm. pig. a. plastids b. normoblasts. x 200

Figure 44. Hepatic capsule (Capsule of Glisson) in new born pig. x 970

Figure 45. Liver lobule in new born pig. a. central vein. b. sinus. c. cord. x 100

Figure 46. Septal bifurcation in liver of new born pig. a. portal vein. b. hepatic artery c. bile duct. x 970

PLATE VII



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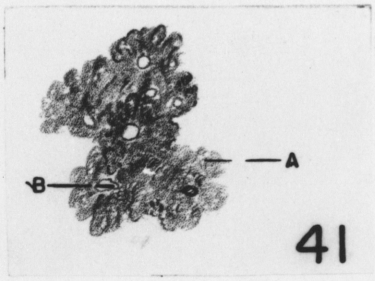

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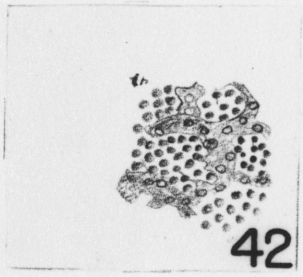
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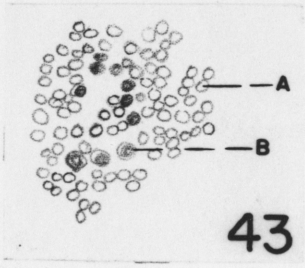

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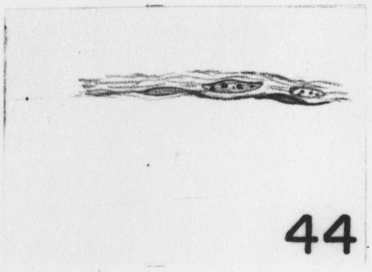
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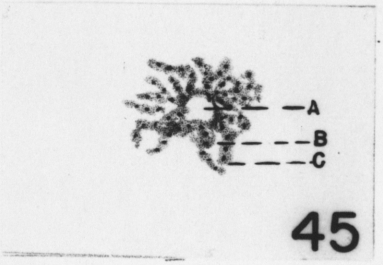

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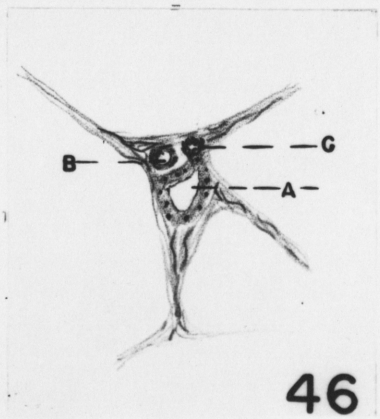

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PLATE VIII

EXPLANATION OF FIGURES

Figure 47: Cartilage being formed in the limb of 15 mm. pig. a. chondroblasts. b. new cartilage. c. perichondrium. x 970

Figure 48: Costal cartilage in the 33 mm. pig x 100

Figure 49: First appearance of erosion of cartilage. Femur of an 80 mm. pig. a. area of erosion. b. cartilage. c. perichondrium. x 430

Figure 50: Blood cells in marrow smear from femur of 115 mm. pig. a. second generation erythroblasts. b. normoblast. x 970

Figure 51: Bone deposited along cartilage trabeculae in 210 mm. pig femur. a. cartilage. b. trabeculae. c. bone. d. marrow. x 100

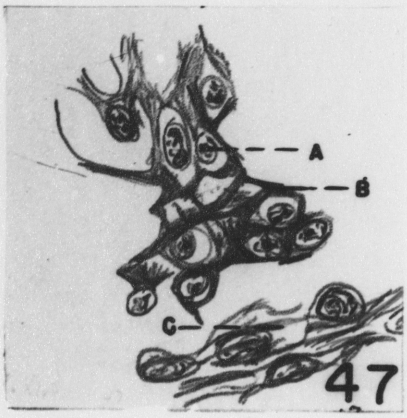
Figure 52: Cartilage undergoing destruction and invasion by the periosteum in the femur of 210 mm. pig. a. periosteum. b. cartilage. c. chondroclasts. x 100

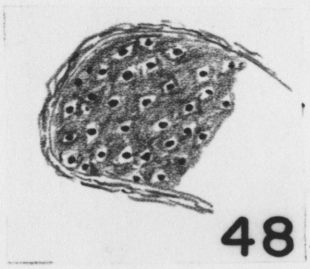
Figure 53: Hemoblasts in marrow smear from femur of a 210 mm. pig. x 970

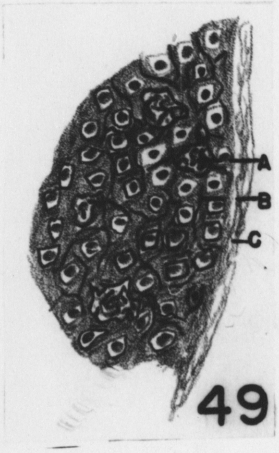
Figure 54: Types of erythrocytes: a. hemoblast. b. megaloblast. c. first series erythroblast. d. first series plastid. e. normoblast. f. second series erythroblast. g. second series plastid. x 970

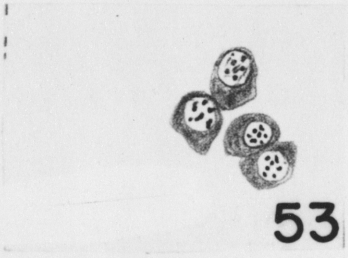
Figure 55: Fetal membranes of 65 mm. pig. a. chorion. b. allantois. c. nodules of diffused placentation. d. umbilical cord. e. amnion. f. yolksac rudiment. x 1/4

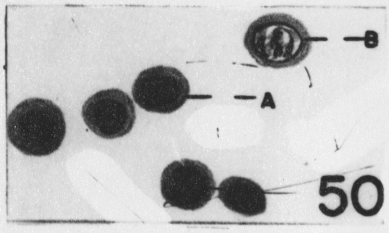
PLATE VIII

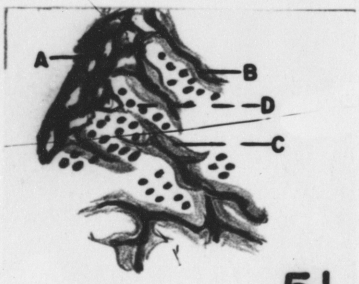


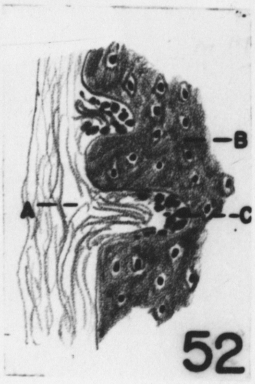


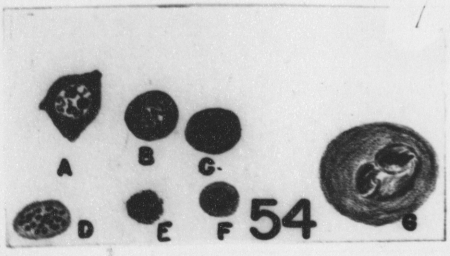












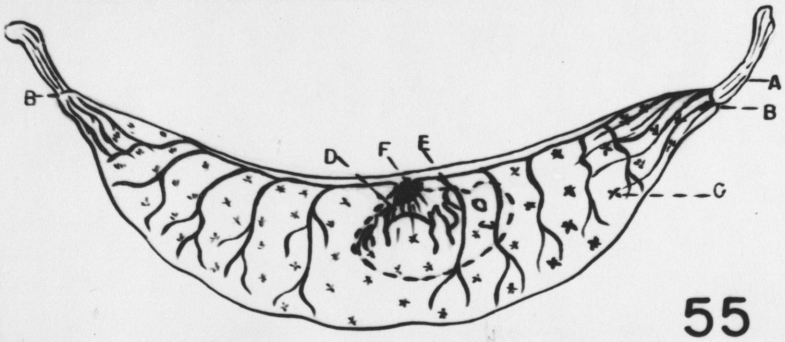


PLATE IX

EXPLANATION OF FIGURES

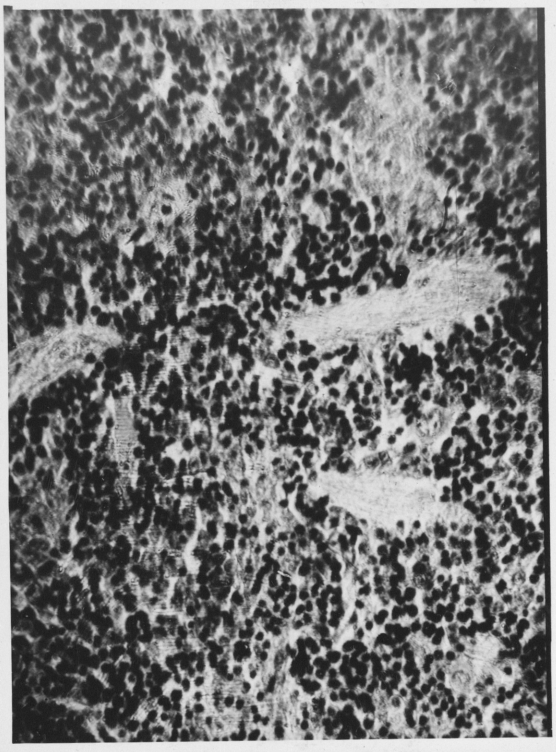
Figure 56: Microphotograph of a portion of a section of spleen from pig of 250 mm. length, indicating the continuance of erythropoiesis. Nucleated red blood cells are present in large numbers within the sinuses of the red pulp. x 450

Figure 57: Microphotograph of a portion of a section of spleen of a new born pig of 266 mm. length. Nucleated red blood cells are absent from the sinuses of the spleen; lymphocytes are present in the modular areas. x 450

Figure 58: Microphotograph of a portion of a section of liver from a pig of 250 mm. in length. Eight islands of nucleated red blood cells occur within the one field shown. x 450

Figure 59: Microphotograph of a portion of a section of liver from a new born pig 266 mm. in length. An apparent "island" visible within this portion is in reality a zone of mitotic activity among the hepatic parenchyma. No nucleated red blood cells are observed within the liver of this pig. x 450

PLATE IX



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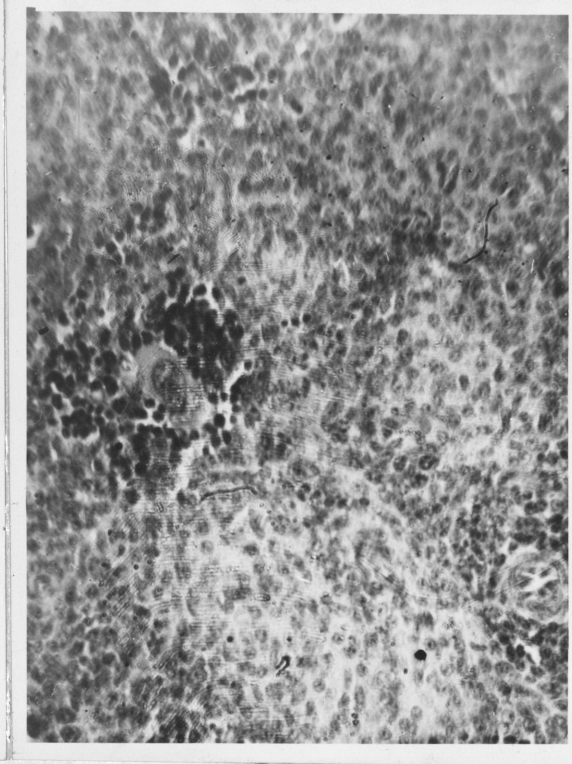

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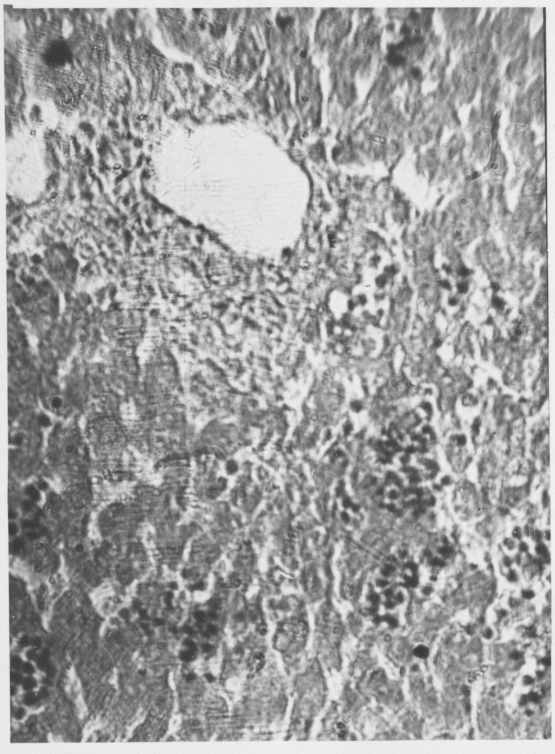

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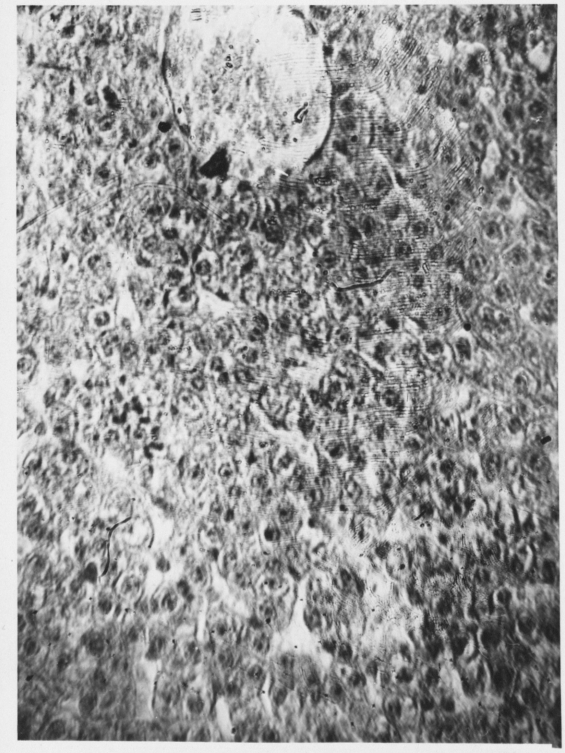

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