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**THE UNIVERSITY OF OKLAHOMA  
GRADUATE COLLEGE**

**EFFECTS OF A LETHAL GENE ON HEMATOPOIESIS IN  
HOMOZYGOUS RESTRICTED RAT EMBRYOS**

**A DISSERTATION  
SUBMITTED TO THE GRADUATE FACULTY  
in partial fulfillment of the requirements for the  
degree of  
DOCTOR OF PHILOSOPHY**

**BY  
MARGARET LYNN PALMER  
1972**

**EFFECTS OF A LETHAL GENE ON HEMATOPOIESIS IN  
HOMOZYGOUS RESTRICTED RAT EMBRYOS**

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**TO MY PARENTS AND FRIENDS**

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# EFFECTS OF A LETHAL GENE ON HEMATOPOIESIS IN HOMOZYGOUS RESTRICTED RAT EMBRYOS

## CHAPTER I

### INTRODUCTION

A laboratory colony of rats was started in 1902 by Helen Dean King from a strain of Norway rats picked up on the streets of Philadelphia, Pennsylvania. This colony was dispersed in 1946. In 1954, some of these King animals were obtained by Dr. Allan J. Stanley, University of Oklahoma Health Sciences Center. Subsequently, they were mated with Holtzman rats and the hybrid offspring were inbred through approximately 30 generations. These so-called King-Holtzman animals now have been maintained through approximately 30 generations.

In 1962, a dominant mutant gene appeared in this colony (Stanley and Gumbreck, 1964). Males heterozygous for it have a normal XY karyotype (Allison et al., 1968), but exhibit seminiferous tubular dysgenesis by the time of puberty or shortly thereafter. Their coat color is distinctly marked in such a way that the normal coloration is restricted. For example, the color pattern in normal hooded rats extends over the shoulders. In restricted hooded

animals it is limited to a small area about the face and head. The ventral white area of the irish coat color is normally limited to the ventral body area. In the restricted irish animals, it is extended up the sides and over the back (Stanley et al., 1965). Restricted self animals have full coloration except for the white spot on the forehead.

Females carrying the mutant gene exhibit the coat color pattern marker but are not sterile. The apparent pleiotropic mutant gene for these characters in rats has been designated  $H^{re}$  (Gumbreck et al., 1971). The  $H^{re}$  females are fertile and can produce offspring when mated with normal males (+/+). Such matings result in 50% normal and 50% restricted offspring. Some of the  $H^{re}$  males remain fertile for a short time after puberty. When these males are mated with  $H^{re}$  females some of the offspring are homozygous for the mutant gene. Rather than the expected 3:1 ratio, matings of this type result in a 2:1 ratio of restricted to normal offspring (Russell, 1969). From these results, Russell postulated that  $H^{re}$  is lethal in the homozygous condition. Gumbreck et al. (1971), found that mating restricted females to normal males results in 50% normal and 50% restricted offspring, as expected. However, when  $H^{re}/+$  females are mated with  $H^{re}/+$  males, the ratio of offspring is 34% normal and 66% restricted. In this case, there are 25% less offspring than from matings between  $H^{re}/+$  females and +/+ males. This supports Russell's theory that the mutant  $H^{re}$  gene involves a lethal factor. Since the lethality has no effect on the sex ratio, Gumbreck et al. conclude that the  $H^{re}$  gene is not sex-linked. They do not rule out the possibility that two independent genes rather than one

pleiotropic one is involved. If so, the two genes would be at very closely linked loci on the chromosome involved. It has not been possible to tell whether expression of the  $H^{re}$  gene is due to a true biochemical pleiotropism, a deletion, or physical alteration of the affected genes (Gumbreck et al., 1971).

In the homozygous condition both W and SI alleles in mice may produce embryonic lethality preceded by, and apparently as a direct result of, failure in proliferation or differentiation of the hemopoietic stem cells (McCulloch et al., 1964; 1965). Also, both genetic series may produce white spotting involving failure in melanoblast migration or differentiation (Mayer, 1970). According to Wendt-Wagner (1961), in black and hooded rats the pattern series of the rat can be correlated with failure of melanoblast migration.

Gumbreck et al. (1972), have determined that body weights of the restricted and normal males are about equal at birth and after one hundred days of age. From the fifth to the tenth day the normal animals are slightly heavier and from the fifteenth to the hundredth day this difference has increased. Apparently, restricted males grow more slowly than their normal siblings, yet by 110 days post-partum, they attain the same weight as the normal.

Except for testes, there are no significant differences in organ weights between normal and  $H^{re}$  males. At birth, the testes of the  $H^{re}$  and normal rats are equal in weight. After four days of age those of the normal rats are heavier and after nine days this difference is significant at the .01 level of probability. Testes of the normal rats studied reach a maximum weight at about ninety days of age; whereas, those of the restricted animals reach a maximum weight in about

sixty days. They remain at this level until about one hundred days of age, then decline to about one-fourth of the testis weight of normal animals (Gumbreck et al., 1972).

Testes of sterile males one to ten days old do not differ histologically from those of normal male rats; however, as they become older the seminiferous tubules decrease in diameter. In tubules of older H<sup>re</sup> males, some active spermatogenesis is seen, but it is usually less extensive than normal and is absent in the old ones. Sertoli cells are present in all H<sup>re</sup> males. Fertility in the H<sup>re</sup> males that are able to produce offspring lasts for only a short time during the third month of life (Gumbreck et al., 1972).

The interstitial cells in testes of restricted male rats are similar in structure and in number to those in normal males. Prostate glands and seminal vesicles of restricted rats are similar in weight to those of the normal animals, indicating a normal production of androgens. The restricted males exhibit normal virility and copulatory behavior. It was concluded by Gumbreck et al. (1972) that tubular dysgenesis and subsequent sterility of restricted male rats is due, not to a lack of sufficient follicle-stimulating hormone from the anterior pituitary, but to a failure of the germinal epithelium to respond to this hormone to the same degree and duration as in the normal animals.

#### Development of Rat

Following fertilization, normal embryonic development of the albino rat ensues in four stages: 1) pre-embryonic; 2) embryonic; 3) early fetal, and

4) late fetal .

### Pre-Embryonic Development

In the rat, ovulation occurs spontaneously, independent of copulation (Huber, 1915). By the first through the fourth hours after sperm penetration, most of the ova have formed the second polar body and the sperm head is undergoing enlargement. The first polar body is given off within the ovarian follicle, the second in the oviduct.

During the fifth through the eighth hour after sperm penetration, the head of the sperm transforms rapidly into the pronucleus (Odor and Blandau, 1951). The first indication that the female chromosomal mass will transform into a female pronucleus is the appearance within the ovocyte nucleus of one to three minute nucleoli. After the second polar body is formed it remains closely associated with the female pronucleus.

From the ninth to the nineteenth hour post fertilization, the pronucleus grows rapidly and its nucleoli increase in number (Tafari, 1889; Sobotta and Burckhard, 1910; Kramer, 1924). From the twentieth through the twenty-fifth hours, the first segmentation spindle is formed (usually between hours 21-23) and cleavage occurs. At the twenty-fourth hour, the ova, now in the pronuclear stage, are distributed in the distal one-fourth of the oviduct. Tafari (1889) believed that the nucleoli contain chromatin which later forms into filaments. Sobotta and Burckhard (1910) thought that the nucleoli fragment and reform into a network. Huber (1915), describing relatively late pronuclear stages, found

that each nucleus contains a network with chromatin distributed upon it. There is a single large chromatoid nucleolus in each of the larger pronuclei. Also, in 1951, Odor and Blandau reported the presence of numerous nucleoli in both male and female pronuclei. These remain visible until immediately preceding the formation of the first segmentation spindle, and the first segmentation division appear to fall within a period ranging from the beginning to near the middle of the second day after insemination (probably about thirty to thirty-two hours after copulation), (Huber, 1915).

From beginning to end the two cell stage lasts about 24 hours (from about 24 hours to 48 hours post-insemination) (Sobotta and Burckhard, 1910; Melissinos, 1907; Huber, 1915). The first two blastomeres are equivalent cells of essentially the same size and structure.

As cleavage occurs migration in the uterine tubes continues to be directed toward the horns of the uterus.

The eight cell stages appear approximately three days after insemination (Huber, 1915). By the fourth day of gestation, each of the 12 to 16 cell stage has traversed the oviduct and is ready to enter the uterine horns.

When the morula stage reaches approximately thirty cells, a segmentation cavity appears. Near one pole the outermost cells have separated slightly from the more deeply placed cells, so that an eccentrically placed irregularly-shaped cavity is evident. The beginning of this segmentation cavity and early stages of the blastomeric vesicle occurs in the latter half of the fifth day. The blastocytes lie free in the lumen, and are ovoid in form. Their long axis presents no

definite relation to the long axis of the uterine horn. During the first half of the fifth day, migration of the blastocyst from the oviduct to the uterine horn appears to be completed, so that by the second half of the fifth day the vesicles are spaced in the uterine horn and partially embedded in the uterine mucosa. According to Von Brock et al. (1964), differentiation of the body axis takes place during the fourth and fifth day. The vesicles now lie with their long axis approximately at right angles to the long axis of the uterine horn.

During early stages in the formation of the blastodermic vesicle of the mouse, there is a differentiation of the inner cell mass into ectoderm and endoderm, (Jenkinson, 1900). The outer layer (trophoblast) is clearly differentiated and separated from the inner cell mass. Duval (1891) also observed that endoderm and ectoderm were present in early stages of blastomeric vesicle formation of the mouse and rat. Huber (1915), and Sobotta and Burckhard (1910), failed to recognize any ectodermal and endodermal cells at this stage. They did, however, describe the differentiation of the thicker part or the floor of the vesicle into a covering or trophoblastic layer.

During the sixth day, the blastocyst of the rat increases in size relatively rapidly owing to a distension of the cavity or blastocele. The greater portion of its wall is composed of a single layer of flattened cells. At this stage, the vesicles are not attached to the uterine wall, though the uterine mucosa shows a distinct reaction to their presence. This enlargement of the blastocyst is accompanied by a flattening and extension of the cells of the floor of the vesicle. It is reduced in thickness and forms a germinal disc having

an area equivalent to about one-fifth to one-sixth of the wall of the vesicle. This disc is about two to three cell layers thick. In the course of the development of this stage, the cells constituting the floor of the vesicle adjacent to the cavity or blastocele differentiate to form the analogue of the yolk endoderm. The remaining cells having essentially the same structure as the cells which constitute the floor of the vesicle and are irregular and compressed. Primordial germ layers cannot be recognized at this stage. They begin to make their appearance on the sixth day when the ectoderm becomes evident.

By now, the cells which extend into the cavity of the blastocyst have been designated by Huber (1915) as the ectodermal node, the analogue of the primary embryonic ectoderm of the future embryo. The ectodermal node appears as an oval mass composed of compactly arranged cells and is separated on all sides from the surrounding cells. Formation of this node is due to rearrangement and proliferation of cells of the germinal area. This ectodermal node, so far as it extends into the cavity of the blastodermic vesicle, is surrounded by yolk endoderm. Continued growth of ectodermal cells forces the yolk endoderm into the cavity of the vesicle which in turn leads to the inversion of the germ layers.

During the eighth day, the blastocyst comes into a definite relation with the maternal decidua and as an embryotroph receives maternal hemoglobin. This is done through phagocytic action of the cells of the ectoderm and partly through absorption of maternal hemoglobin. The cells increase in length and inversion of the germinal area is complete. A cavity develops in the ectodermal node. A little later a second cavity develops in the extraembryonic

ectoderm. By the end of day eight, the two cavities fuse to form a single pro-amniotic cavity, lined by primary embryonic ectoderm. The mesometrial portion is now lined by extraembryonic ectoderm. These two ectodermal entities form a continuous layer with a line of junction relatively distinguishable. As yet, there is no evidence of bilateral symmetry.

The side on which the primary embryonic ectoderm extends toward the mesometrium contains the caudal end of the future embryo. The primitive streak and groove will form from the primary embryonic ectoderm of this region. Huber (1915) states that a small group of cells at the junction of embryonic and extraembryonic ectoderm constitutes early mesodermal cells. These cells have apparently wandered from the primary embryonic ectoderm to the place they occupy. In late nine day embryos, this region constitutes the primitive streak of the future embryonic area. Duval (1891) believes that the mesoderm takes origin from a thickened part of the endoderm, probably in the region of the anterior portion of the future embryonic area. He did not recognize a primitive streak at this stage. Evidence warrants the conclusion that in the rat, cells in the caudal region of the primary embryonic ectoderm which are destined to become mesoderm, arise from a narrow zone of cells situated in the region of the future primitive streak. Cells from this region invade the potential cleft between primary embryonic ectoderm and endoderm. These eventually spread laterally in wing-like sheets.

Nine days are required for the completion of segmentation, blastodermic vesicle formation, and the formation of the primary germ layers: ectoderm,

mesoderm and endoderm. Subsequently, organ zones become visible and finally organs are formed.

Somite development is occurring by day ten and supra and infra neuropores develop in a cranial and caudal zone of the neural tube.

The cardiotube and blood vessels appear during the eleventh day and cardiac pulsations begin. Concurrently, the cranial neuropore opens and the caudal neuropore is divided into two parts by an invagination. The neural groove becomes a closed tube surrounded by somites and the cerebral hemispheres begin their development. By this time the primordia of peritoneal organs and the primitive umbilical cord can be seen. The dorsal ventral flexure of the embryo also begins during this period.

### Embryonic Development

This particular phase occurs during the twelfth to the fourteenth day. It involves considerable organ differentiation.

By now the embryo has a characteristic shape and form. The ventral curvature begins and by the end of this phase the neural tube is surrounded by thirty somites. Caudally there is a closure of the neural tube, and further development of the cerebral hemispheres takes place. The isthmus of the rhombencephalon separates the rhombencephalon from the cerebral hemispheres, and the mesencephalon continues to differentiate. The optic cup and optic canal containing the hyaloid artery come into being. The olfactory area is not folded or developed at this time. The beginning of the mandibular zone

and the analogue of the extremities appear, and the anterior extremities are formed; however, the posterior extremities are just protuberances. The cardiac tube becomes large and pulsating. The primordia of the liver exist in the embryo and the mesonephroi are being formed. At the end of the twelfth day of development the crown-rump length of the embryo is 2 to 3.5 millimeters.

On the thirteenth day, the olfactory canal arises, the mandibular arches are fully developed, the ear begins its development at the level of the hyoid fold, the cardiac tube becomes "S" shaped, and the primordia of the respiratory tract come into being. At this time the liver with its developing parenchyma is somewhat enlarged in relation to the size of the embryo. The stomach and intestines are further differentiated. By this stage the mesonephroi and reproductive organ primordia, separated from the caudal termination of the mesonephroi, have appeared. The anterior extremities have developed to an elevation of one millimeter, the posterior extremities are not differentiated.

Development into the prenatal condition begins at day fourteen. The ventral curvature remains, however, proportions are widely changed. The mandibular arches form the jaw and the olfactory groove is differentiated into the nasal septum. The telencephalon is not particularly enlarged, although it is seen to shift in a cranial direction. A lens appears lateral to each optic cup. Final transformation of the cardiac tube into a definitive heart is beginning. Lobes appear in the enlarging liver. The mesonephroi have now converged caudally. The stomach is further differentiated as is the analogue of the peritoneal organs. The vertebral column separates from the somites and

begins to assume its definitive position. Both the anterior and posterior extremities have grown a great deal. Externally a subdivision of the extremities can be noted but internally the differentiation is not as apparent.

### **Early Fetal Development**

Development is completed during the fifteenth to the sixteenth day. During the fifteenth day the prenatal form is suggested by the formation of the trunk paralleled by growth of the peritoneal organs and further development of the facial area and differentiation of the skull. Also there results further differentiation of the nasal septum and mandible. Development of the heart is continued and the lobes of the liver are enlarged. The stomach, pancreas and the metanephroi are formed. The vertebral column and bones of the extremities are in their precartilagenous state.

Maturation of the organs and precartilagenous development of the skeleton and extremities continue throughout the sixteenth day. By this time, the skin has become relatively thick.

### **Late Fetal Development**

The seventeenth day marks the period of final development. Ossification of the nasal bones, maxilla and mandible give the face its adult appearance. Final arrangement of the brain takes place with minor growth occurring. The sacral region and pelvic viscera grow and develop intensively. The seventeenth day marks the beginning of skeletal maturation with calcium deposition in bones. Ossification is initiated in the mandible, skull and the

vertebra of the trunk region. The ribs, scapula, pelvis and extremity bones are in only a precartilagenous stage. The liver and peritoneal organs further develop and are now richly supplied with blood. The epidermis has thickened and completely differentiated.

On the eighteenth day of fetal development ossification continues and is now present in the ribs, vertebral column, marginal regions of the skull bones as well as in the extremities. During the following days ossification continues, however, ossification of the sternum is relatively late. The sacral area becomes extended parallel with development of the pelvic organs; calcification of the sacral vertebra begins on the nineteenth day. The skull establishes its prenatal shape. The rhinencephalon moves to the facial area and the telencephalon surrounds the diencephalon. The metencephalon is fully differentiated and the rest of the brain has not increased much in length. The head to trunk ratio on the seventeenth day is 1:1.3; on the twenty-second day the ratio is 1:2.5.

On the twenty-first and twenty-second day only the rib attachments show ossification centers, maturation of the ribs appears to be a post-partum event. Growth during the eighteenth to the twenty-second day is accompanied by increase in weight of the fetus and considerable maturation of organs and ossification (Von Brock and von Kreybig, 1964).

The rats are born blind, hairless, with a short tail, closed ears

and underdeveloped limbs. Up to the time the young rats are twenty-one to twenty-two days of postnatal age, they are dependent upon their mothers (Donaldson, 1924).

### Development of Hematopoietic Organs

From the eighth to the fifteenth day of gestation, blood is formed in blood islands in the lateral walls of the extraembryonic coelom or yolk sac. Blood islands are aggregates of mesenchymal cells in the splanchnopleure. Hemocytoblasts appear in them before the endothelium has differentiated. They are the only source of red cells for the embryo through day eleven. This yolk sac hematopoiesis provides the generation of primitive erythrocytes which are exceptionally large nucleated cells. They may have volumes some four or five times that of erythrocytes of the adult rat, and may be compared with normoblasts of bone marrow (Rugh, 1968). During day eleven and twelve many erythroblasts may be seen in mitosis. After day fifteen these cells tend to diminish rapidly. The yolk sac erythroid cells enter the fetal circulation, and their number in the peripheral blood appears to remain constant from the twelfth to the fifteenth day of gestation and then decreases rapidly (Russell, 1963; Craig and Russell, 1964).

In the early embryo, erythrocytopoiesis appears to be largely intravascular. Later, most hematopoiesis inside the body of the embryo is extravascular in the liver, spleen and bone marrow, the cells secondarily migrating into the blood vessels where they undergo further differentiation

(Gilmour, 1941).

Experiments wherein bits of yolk sac of a young rat embryo were implanted into a neutral environment, such as the anterior chamber of the eye, proved that the first free cells of the blood islands are capable of differentiating into primitive erythrocytes, definitive red blood cells, and granular leukocytes (Arey, 1966).

From the twelfth to the sixteenth day the liver begins active hematopoiesis (Borghese, 1959; Russell and Bernstein, 1966). At nine days the liver diverticulum first forms in the region of the foregut and then begins to proliferate rapidly. The definitive liver makes its appearance at ten days of gestation when epithelial cords of the liver cells begin to differentiate. By ten and one-half days the liver acquires large blood vessels which will later join the vitelline veins. By day eleven the liver cells surround the hepatic vein and are close to the posterior vena cava. Blood cells appear in the forming blood vessels of the liver only after circulation is established on the ninth day. At this time the liver mass is full of sinusoids and is invaded by mesenchyme. By day twelve many early hematopoietic cells intermingled with the hepatic cells are seen in the liver. Some of these also lie free within the hepatic blood vessels. These cells include hemocytoblasts, myeloblasts, promyelocytes, polymorphonuclear cells, proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and normoblasts. The last three are formed in substantial numbers while the others are less than one percent (Rugh, 1968). By day thirteen

the presence of some megakaryocytes and many nucleated red cells indicate that the liver is functioning as a hematopoietic organ. By day fifteen, bile capillaries devoid of blood cells and surrounded by cords of liver cells can be distinguished from blood filled capillaries. In the sixteen day embryo, all of these elements have increased in number and volume, with the quantity of differentiating blood cells appreciably increased. The presence in the liver of numerous sinusoids and blood cells in various stages of development indicate that the function of the liver in an eighteen day embryo is more hematopoietic than endocrine. From day twelve to sixteen the liver is almost exclusively hematopoietic. Some of the hematopoietic foci remain after birth. The hepatic erythroblasts are smaller in size than blood island erythrocytes (Marks and Kovach, 1966).

During the thirteenth through the fifteenth day of gestation the spleen is contributing to hematopoiesis (Borghese, 1959). The spleen may first be identified at day thirteen located just dorsal to the stomach and near the level of the gonad. It develops in situ in the mesenchyme and is suspended by the dorsal mesogastrium into the peritoneal cavity. By seventeen days it has begun to elongate and by eighteen days it is a long slender organ. The typical pulp areas have not appeared at this time. Spleens from embryos of seventeen days gestation are found to be composed almost entirely of tissue which has a myeloid appearance. Lymphoid cells do not begin to appear until late in gestation (nineteen to twenty-one days).

From birth to thirty-five days the spleen grows rapidly and attains its adult weight during the sixth postnatal week. The white pulp sheath becomes more evident during this period. The lymphoid nodules appear during the third postnatal week.

No hematopoietic activity begins to occur in the bone marrow until the sixteenth or seventeenth day of gestation and then remains almost exclusively leukopoietic until birth (Borghese, 1959). After birth, the bone marrow becomes the primary site of erythropoiesis.

### Hematopoiesis

Two sharply contrasting views are held concerning the exact origin in mammals of the various blood elements. Ehrlich (1880), the initiator of modern hematology, considered that the erythroblasts and myeloblasts had an origin different from that of the lymphoblasts. This is the so-called "dualistic" interpretation of the origin of the blood cells. It is one of the modifications of the "polyphyletic" theory which postulates that there are two or more varieties of stem cells from which, most commonly, the erythrocytes and granular leukocytes are derived from one mother cell, while the nongranular leukocytes trace their ancestries to a separate stem cell. Most modern hematologists support the "monophyletic" theory of Maximow (1924). His view states that there is only one hematogenous stem cell, the primitive blood cell or hemocytoblast. There is still, however, a lack of agreement on the precise relationship of the different varieties of blood cells

to each other. Most investigators believe that in the early stages of development there is only one hemocytopoietic cell which gives origin, in several different situations, to myeloid (erythrocytes and granular leukocytes) and lymphoid (lymphocytes and probably monocytes) elements.

The problem of blood development is further complicated by lack of agreement on the relationships of the differentiating blood cell to the endothelium (Hamilton et al., 1962). Some workers believe that the erythrocytes arise intravascularly and the granulocytes extravascularly.

This description is based upon the "monophyletic" method of blood formation and the information given is from Hamilton et al. (1962) and Ham (1969). The mother cell from which various blood elements are thought to be differentiated is called the hemocytoblast. It is a spherical or slightly polygonal cell with a basophilic cytoplasm. Its nucleus is large and contains an acidophilic nucleolus. From such parent cells, all blood elements arise. The hemocytoblasts proliferate by mitosis so that small groups of them are formed. Subsequently, some of these acquire hemoglobin (primitive erythroblasts). Early in development hemocytoblastic cells in the yolk sac wall give origin to myeloblasts, then to the primitive erythroblasts which later indirectly transform into primitive erythrocytes. A myeloblast is a large cell. Its nucleus is ovoid, may show some indentation, and is usually acidophilic. The myeloblasts give rise to a somewhat smaller cell, the proerythroblasts. Chromatin in their nucleus is somewhat coarser than that of the myeloblast and their nucleoplasm is

basophilic. Two prominent nucleoli are usually present. The cytoplasm of the proerythroblast is also somewhat basophilic and less in amount than that of the myeloblast.

Proerythroblasts give rise to cells known as basophilic erythroblasts. The basophilic erythroblast is somewhat smaller than the proerythroblast. The chromatin of its nucleus is more dense and appears in the form of coarse granules which are often clumped. The nucleus exhibits no nucleoli and its staining reaction is still more basophilic than that of the proerythroblast. The cytoplasm is also more basophilic than that of the proerythroblast and when basic dyes are used its color ranges from a moderate to a deep blue.

Basophilic erythroblasts give rise to cells which are somewhat smaller. These are called polychromatophilic erythroblasts. The cytoplasm in these stains with polychromatic dyes. The nucleus of the polychromatophilic erythroblast is somewhat smaller than that of the basophilic erythroblast, and its chromatin is in the form of coarse granules which are commonly clumped so that the nucleus is very basophilic. No nucleoli can be seen.

Polychromatophilic erythroblasts experience two fates. Often when erythroid activity is increased because of a need for more red cells, the nucleus of the polychromatophilic erythroblast becomes pyknotic and is extruded while the cytoplasm is still polychromatophilic. This results in the formation of a polychromatophilic erythrocyte. More common, as they continue to divide, they lose their cytoplasmic basophilia. When this happens, the cell is termed a normoblast because it is going to give rise to

normocytic erythrocytes. A normoblast has a small spherical dark staining nucleus. Normally, this is lost by extrusion, at which time the cell is called an erythrocyte.

The hemocytoblasts are also capable of giving rise to granular leukocytes; megakaryocytes, monocytes and lymphocytes. In the yolk sac, in addition to hemocytoblasts and erythroblasts, some megakaryocytes, a few phagocytic cells and possibly primitive myelocytes, are present.

Three kinds of granular leukocytes develop from descendents of the myeloblasts. In forming granular leukocytes, the first cell of the line is known as a promyelocyte. This is a large cell with an appearance similar to the myeloblast. The promyelocyte differentiates into a myelocyte. The nucleus of the myelocyte exhibits a moderate degree of indentation. The chromatin of its nucleus is more condensed and the granular cytoplasm is not so basophilic as that of myeloblasts and promyelocytes.

Myelocytes form the three kinds of granular leukocytes: neutrophilic leukocytes, eosinophilic leukocytes and basophilic leukocytes.

A fairly mature neutrophil has an indented nucleus, and its cytoplasm contains a good complement of granules. These are usually confined to the bone marrow. When detected in the peripheral blood they are referred to as juvenile neutrophils.

In the formation of an eosinophil the indented nucleus generally develops a deep constriction. This deepens to divide the nucleus into two lobes that usually remain joined together by a strand of nucleoplasm.

As the constriction develops, the chromatin of the nucleus becomes somewhat condensed. The nucleus of eosinophils are paler than those of neutrophils.

In the formation of a basophilic leukocyte, the nucleus of a mature basophilic myelocyte undergoes less change than occurs in the formation of either the neutrophil or eosinophil. Irregular constrictions may appear in it to give an irregular outline, but generally it is not broken up into lobes. Since its chromatin does not become condensed, it stains only very lightly. In contrast, its granules stain deeply, and as a result the nucleus tends to be obscured.

Myelocytes differentiate along a third sub-line to form megakaryocytes; these cells give rise to the platelets of the blood. Their nuclei become so large because they go through mitosis, but the daughter nuclei do not separate and the cytoplasm does not divide. Their nuclei are deeply basophilic, whereas their cytoplasm stains lightly with basic dyes and may contain fine granules.

The lymphocytic stem cell or lymphoblast also differentiates from the hemocytoblast. Such cells are looked upon by some as large lymphocytes. It has been accepted that lymphoblasts can divide and give rise to smaller cells, which are termed large lymphocytes and that these cells divide and give rise to small lymphocytes. The chromatin of their nuclei is almost all condensed so their nuclei are very small and they have very little cytoplasm. Nucleoli are not seen in the nuclei of lymphocytes. When stained with blood stains, the cytoplasm of lymphocytes contain

reddish-purple granules.

The hemocytoblast or mother cell also gives rise to another cell type, the monocyte. The nuclei are frequently ovoid and some may be indented giving a kidney-shaped appearance. The chromatin of the nucleus is dispersed in a network of granules. The cytoplasm of the monocyte is relatively abundant and comprises a large part of the cell; its color with blood stains such as Wright's is a gray-blue. The monocytes have the ability to extend and withdraw pseudopodia which allows them motility. In tissues monocytes can develop into macrophages.

The following is a study of the development of homozygous ( $H^{re}$ ) embryos resulting from a mating between  $H^{re}$  males and  $H^{re}$  females. Peeples (1971), has indicated that approximately one-fourth of the embryos from such matings are destined to die, either before or shortly after birth. Hematocrit studies have confirmed that the 25% affected by this lethality are anemic, thus, special consideration will be given to a description of the histogenesis of the blood forming organs and cells. Emphasis will be placed on the 15 and 20 day fetuses, since prior to this time during development it is difficult to determine which embryos are homozygous.

## CHAPTER II

### MATERIALS AND METHODS

#### Experimental Animals

Adult restricted (H<sup>re</sup>) female rats were mated to adult restricted males by placing two males in each breeding cage with approximately eight females. Daily vaginal smears were performed on each female to determine the date they were inseminated. If sperm were present, it was assumed that gestation had begun. From midnight preceding the morning when sperm was discovered in the vaginal smear to midnight following, was assumed to be day-one of gestation. As soon as the females became pregnant, they were placed by themselves in a separate cage. Subsequently, the entire litter was removed from each female. Litters were removed from females at five, eight, nine, thirteen, fifteen, and twenty days of gestation.

Prior to removing embryos, the mother was anesthetized by intraperitoneal injection of 4 mg/100 g. of body weight of sodium pentobarbital. After removal, the embryos were fixed in ten percent neutral buffered formalin for at least 48 hours. A few placentae and all ovaries from the mothers were also removed and treated in the same manner. The mothers were then sacrificed.

### Histology

Embryos from five, eight, nine, thirteen, fifteen and twenty day litters were fixed, dehydrated, embedded in paraffin, and sectioned serially at ten microns (Table 1). The sections were placed on slides, stained with Harris' hematoxylin and eosin, and covered with glass coverslips.

Livers dissected from fetuses in fifteen and twenty day litters were placed in ten percent neutral buffered formalin for at least 48 hours. Some livers from normal and some from homozygous restricted fetuses in each litter were processed by the paraffin embedding technique and ultimately sectioned at five microns. These sections were placed on slides and stained with the Giemsa technique. In addition, a piece of liver from each type fetus was touch-smearred on slides, fixed in methanol and stained using the Giemsa and Wright techniques.

### Hematology

All studies under this heading were made from fifteen and twenty day old fetuses.

A comparison was made between the relative number of hematopoietic cells in livers of homozygous and normal fetuses. To do this, a slide of a section of liver from each animal studied was placed under oil immersion. The number of hematopoietic cells in each of twelve randomly chosen 43.56 square micron (the area of each square in an eyepiece grid used for counting) areas near the periphery of such a section were then counted.

TABLE 1  
ANIMALS USED

Number of litters	Number of offspring		Age of embryos in litter
	<u>Heterozygous</u>	<u>Homozygous</u>	
2	18	-*	5
2	13	-	8
2	18	-	9
3	24	-	13
5	39	9	15
3	19	6	20

\* = unable to determine homozygous condition

This was done using liver sections from two normal and two homozygous fetuses in each group. The same procedure was used for counting hematopoietic cells in the bone marrow of the extremities of twenty day animals. These counts were made in the ossification centers of long bones. In addition, the ratio of mature to developing erythrocytes present in livers from the fetuses studied also was calculated. The procedure involved counting a total of 350 hematopoietic cells in a few randomly chosen areas on each slide. Also under oil immersion, the diameter of mature erythrocytes in touch smears and in sections of livers from normal and homozygous specimens was measured with an eyepiece micrometer. Two hundred such cells were measured in smears and sections of two fetuses of each type.

In order to compare the average number of erythrocytes in the cutaneous vessels of each fetal type studied, the number of such cells was counted in a cross section of twenty different vessels of similar size in each of two fetuses of each animal type.

#### Photography

Whole fetuses were placed on an illuminator box, then photographed with a Speed Graphic camera on 3 1/4 x 4 1/4 plus X-panchromatic professional sheet film. The camera's F-stop was set at 5.6 with a six second exposure time. Negatives were developed in Kodak D-11. Prints were made on F-2 Kodabromide developed in Dektol.

Black and white and color microphotographs were made with Leitz Aristophot, 4 x 5 Bellows camera. For the black and white pictures a Leitz Macro-Dia apparatus and a Summar Macro objective were used with the camera. Four by five Kodak Ektapan film was used for these and the negatives were developed in Kodak D-76 using the time temperature method. Prints of these were made on Kodak Kodabromide F paper and developed in Dektol.

For the color photographs of livers and blood smears, a Leitz Ortholux Research microscope was used with the camera. The objective on this was 25 X Apochromatic with a 6.3 X (M) negative compensating eyepiece. A one second exposure was used on 4 x 5 Kodak Ektacolor L film. Negatives were commercially processed and printed on Kodak paper.

## CHAPTER III

### RESULTS

#### Five Day Embryos

With methods used, differentiation between embryos destined to become either heterozygous or homozygous restricted adults and those that would be normal, was impossible up to fifteen days of development.

At five days of gestation, the embryos have traversed the fallopian tubes and are in the uterine horns. Two individuals in a litter of thirteen were beginning to invade the epithelium of the uterine crypts. The remaining eleven of this litter were lying free in the uterine tubes. The decidual cells around the two implanting embryos were large and nucleated and the surrounding area was engorged with maternal blood. At this stage of development (early formation of the blastodermic vesicle), no evidence of embryonic or extraembryonic blood formation exists.

#### Eight Day Embryos

The decidual cells, containing one or two nuclei, form a massive decidual layer which completely envelops the embryos.

It is seen at this stage (see Plate II, Figs. 3 and 4, in Appendix),

that Reichert's membrane is well formed. The embryo is surrounded by a yolk sac cavity; however, there is no evidence of blood islands.

The outermost cells of the egg cylinder or inner cell mass are tall columnar, usually about one cell layer thick. The inner ectoderm exhibits several layers of cuboidal cells. Endoderm surrounds this ectoderm (see Plate II, Figs. 3, 4 and 5, in Appendix).

At the posterior margin of the egg cylinder, mesoderm can be seen forming between the ectoderm and endoderm. It appears as scattered mesenchymal cells. There is also evidence of the amniotic cavity surrounded by ectodermal cells (see Plate II, Fig. 5, in Appendix).

#### Nine Day Embryos

This stage of development in the rat is characterized by appearance of the analogue of mesoderm.

The amniotic, extraembryonic and yolk sac cavities are present (see Plate III, Fig. 6, in Appendix). The primary embryonic and extra-embryonic ectoderm lining and amniotic cavity are readily differentiated. Blood islands now present in the yolk sac contain hemocytoblasts, primitive erythroblasts and primitive erythrocytes (see Plate III, Fig. 6, in Appendix).

The primitive streak begins at one part of the margin of the egg cylinder as a thickening of embryonic ectoderm (see Plate III, Fig. 6, in Appendix). At nine days of gestation, the embryonic ectoderm at one

edge of the primitive streak thickens to begin forming the head process. The endoderm within the fold formed by this head process, is the beginning of the foregut (see Plate III, Fig. 6, in Appendix).

Mesenchyme arising from the posterior portion of the primitive streak grows into the extracoelomic cavity to form the allantois.

The circulatory system derives from blood islands, aggregations of mesenchymal cells in the mesoderm of the splanchnopleure (see Plate III, Fig. 6, in Appendix). During this stage of development, one sees the first stage of blood cell formation.

### Thirteen Day Embryos

Morphologically, mesenchymal, ectodermal and endodermal cells are differentiating and many of the internal organs can be discerned (see Plate III, Fig. 7, in Appendix). Organ differentiation occurs in seven of the nine observed embryos.

Two of the nine exhibit fibrinoid substance invading the decidua (see Plate III, Fig. 8, in Appendix).

There were no obvious differences between each of the other seven. The number of hematopoietic cells in their livers ranges from 5.7 to 26.8 cells per square mm. Differences between the number of such cells in one embryo as opposed to that in any of the others was insignificant.

In Plate III, Fig. 7 (in Appendix), the liver and its vascularity

can be seen. Already the liver seems to be establishing itself as a hematopoietic center. Also, it demonstrates definitive hepatic cells and sinusoids. The sinusoids and forming blood vessels contain primitive erythroblasts, and show evidence of developing proerythroblasts, basophilic erythroblasts and polychromatophilic erythroblasts. Megakaryocytes are not observed in the vessels or sinusoids.

### Fifteen Day Fetuses

Although there were no significant differences in crown-rump length between any members of the 48 fifteen day fetuses removed, nine were abnormally pale (see Plate I, Fig. 1, in Appendix). This color difference is not apparent in younger fetuses from heterozygous matings. At fifteen days post-fertilization, the integument and underlying body wall structures of all fetuses are so thin that deeply pigmented visceral organs, such as the liver, are visible from the outside. Thus, it is possible to see that the livers of homozygous animals are also abnormally pale. In live animals, when these are examined under a dissecting microscope, it is possible to see that the peripheral circulation in the homozygous, as opposed to normal individuals, is very underdeveloped (Table 2).

Of the two placentae in Plate I, Fig. 1, (in Appendix), the pale one is from a homozygous fetus.

### Hematopoietic Organs

Plate IV, Figs. 9 and 10 (in Appendix), represent sections of a

TABLE 2

**NUMBER OF ERYTHROCYTES IN CROSS SECTIONS OF  
CUTANEOUS VESSELS OF FIFTEEN DAY FETUSES**

	Heterozygous		Homozygous	
	<u>Diameter of vessels in <math>\mu</math></u>	<u>Number of erythrocytes</u>	<u>Diameter of vessels in <math>\mu</math></u>	<u>Number of erythrocytes</u>
<b>Animal #1</b>	18	4	27	1
	21	7	18	2
	15	5	21	0
	24	1	30	0
	27	9	15	5
	12	3	15	0
	18	4	15	0
	30	15	21	1
	18	9	24	0
	24	15	27	9
<b>Animal #2</b>	18	7	21	2
	30	7	30	3
	15	8	30	0
	18	9	18	2
	15	9	24	4
	21	6	21	1
	21	4	21	0
	15	5	15	3
	15	12	15	4
	15	8	18	4
<b>Average for both animals</b>	19 $\mu$	7	21 $\mu$	2

normal and a homozygous individual taken from approximately the same level through the liver.

Even though there is no significant difference in the average weight of a liver taken from a normal or homozygous fetus, the total relative number of hematopoietic cells contained within the sinusoids of such an organ from a homozygous embryo is significantly less than that from a normal one (see Table 3 and Plate V, Figs. 11 and 12, in Appendix). In addition, the ratio of mature to immature cells is significantly greater in livers from homozygous than from normal fetuses at this stage (see Table 3 and Plate VI, Figs. 13 and 14, in Appendix). Immature forms in peripheral blood smears from homozygous fetuses are almost all normoblastic cells. Many erythrocytes in peripheral blood from homozygous fetuses appear to be abnormally shaped. Cells so affected resemble the so-called "target" cells often described in several types of anemias in man (Wintrobe, 1956). The normal appearing ones have similar average diameters in livers from both fetal types (see Table 3 and Plate VI, Figs. 13 and 14, in Appendix).

At this stage, the bone marrow has not developed as a blood forming organ, so it will not be described.

### Liver Morphology

In normal fetuses at the fifteen day stage, epithelial cells in the liver do not form cords or plates, but exhibit a random arrangement. Most of the liver cells are round and have large round single nuclei surrounded by

TABLE 3

COMPARATIVE BLOOD STUDIES ON FIFTEEN DAY FETAL ANIMALS

	Average		Range		Standard Deviation	
	<u>homozygous</u>	<u>heterozygous</u>	<u>homozygous</u>	<u>heterozygous</u>	<u>homozygous</u>	<u>heterozygous</u>
Total number of hemato- poietic cells per mm <sup>2</sup> in liver sinusoids	13.39	59.30	3.8 - 30.6	34.43 - 80.34	+ 2.15	+ 1.14
	p = <.001					
Diameter in μ of erythrocytes in liver smears	12.29	11.37	6.8 - 17.0	5.1 - 15.3	+ .22	+ .28
	p =>.1					
			<u>homozygous</u>	<u>heterozygous</u>		
Percent of erythroid cells present (350 cells counted)	Liver smears					
	immature		86.7	71.8		
	mature		13.1	28.1		
	Peripheral smear					
	immature		66.0	36.4		
	mature		33.0	63.1		

very little cytoplasm. A few are binucleate. Some sinusoids are seen filled with developing blood cells. The portal vein and hepatic artery lie adjacent to the intestine. Other hepatic vessels are distributed throughout the liver. Megakaryocytes are present within the sinusoids, indicating the hematopoietic function of the liver at this stage (see Plate V, Fig. 12, in Appendix).

In contrast to the normal, some hepatocytes in liver sections from homozygous fetuses are arranged in cords or plates, and some of the liver cells have taken on the cuboidal shape generally associated with livers of post-partum animals. The cytoplasm of the hepatic cells in such fetuses is more distinct than that seen in the normal hepatocyte at this stage of development. However, here too, the presence of megakaryocytes indicates that hematopoiesis is still going on (see Plate V, Fig. 11 in Appendix).

At this stage of development, bile duct formation is beginning.

#### Twenty Day Fetus

Six of the twenty-five fetuses removed after 20 days of gestation were abnormally pale. There was no significant difference in the crown-rump length of any of the twenty-five. Aside from paleness, it is possible to determine the homozygous animals by three factors; these are: 1) An abnormally low number of circulating red blood cells in peripheral (cutaneous) vessels. In the heterozygous fetuses, the average

number of erythrocytes in 20 separate cutaneous vessels having an average diameter of 21 microns is 9, whereas in the homozygous the same age, the average number in 20 similar vessels, 24 microns across, is 2.2 (Table 4). 2) The observable liver seen beneath the skin occupies a smaller area in the abdominal cavity. 3) There is an apparent increase in body fluids giving the fetuses an edematous appearance. Also, the snout of these homozygous fetuses is abnormally short and blunt. This may be an artifact produced by physical changes related to the edema (see Plate I, Fig. 2, in Appendix).

Plate VII, Figs. 15 and 16 (in Appendix) represent sections of a normal and homozygous fetus taken from the same level through the liver. A comparative analysis reveals the cross sectional diameter of the homozygous fetus is smaller as is the over all size of the livers.

Noticeable in Plate VII, Figs. 15 and 16 (in Appendix) is the skin covering the abdominal cavity. Both the stratum granulosum and spongiosum of the epidermis of the homozygous individual are each one to two cell layers thick; whereas, in the normal individual they each vary from two to six layers of cells. In the homozygous fetus, even though each is composed of fewer cell layers, all strata present in the skin of normal rats are represented. In addition, the cells of the stratum germinativum of the skin from the homozygous animals appears to be less closely bound together than they do in the normal individual. The layer of subcutaneous connective tissue beneath the dermis of the homozygous

TABLE 4

**NUMBER OF ERYTHROCYTES IN CROSS SECTIONS OF  
CUTANEOUS VESSELS OF TWENTY DAY FETUSES**

	Heterozygous		Homozygous	
	<u>Diameter of vessels in <math>\mu</math></u>	<u>Number of erythrocytes</u>	<u>Diameter of vessels in <math>\mu</math></u>	<u>Number of erythrocytes</u>
Animal #1	21	10	21	2
	18	8	36	1
	30	9	18	0
	15	5	21	2
	12	5	27	16
	24	10	21	2
	15	7	15	0
	27	6	18	0
	23	8	24	0
	21	9	18	0
Animal #2	21	9	27	2
	24	10	33	4
	18	6	24	0
	30	9	30	2
	18	10	18	0
	15	10	24	2
	27	10	45	1
	27	20	21	1
	25	10	18	2
	18	10	21	7
Average for both animals	21 $\mu$	9	24 $\mu$	2.2

individual is much wider than in the normal rat.

### Hematopoietic Organs

In an attempt to detect differences between certain hematopoietic organs in the homozygous and normal 20 day fetuses, four parameters were compared. These were 1) liver weights, 2) total blood cell and differential erythrocyte counts, 3) the diameter of the erythrocytes, and 4) the structural morphology of the livers.

Liver weights. The livers of homozygous animals weigh about one-third of those from normal animals. The average weight for the homozygous animals is .135 grams. The average weight for livers of a normal individual is .340 grams.

Blood cell counts. Total population of hematopoietic cells contained within the sinusoids of the normal and homozygous livers are significantly different (see Table 5 and Plate VIII, Figs. 17 and 18, in Appendix).

With methods used, it was found that in the liver sinusoids of the rat fetus at 20 days gestation, there are significantly more blood cells in the normal animal than in the homozygous individual (see Plate VII, Figs. 17 and 18, in Appendix). It was not possible to determine, in this population of cells, whether the blood cells were circulating through the livers or being developed in situ. The ratio between such cells in normal and homozygous fetuses is approximately 4:1.

The number of mature erythrocytes in liver sections is significantly

TABLE 5

COMPARATIVE BLOOD STUDIES ON TWENTY DAY FETAL ANIMALS

	Average		Range		Standard Deviation	
	<u>homozygous</u>	<u>heterozygous</u>	<u>homozygous</u>	<u>heterozygous</u>	<u>homozygous</u>	<u>heterozygous</u>
Total number of hemopoietic cells per mm <sup>2</sup> in liver sinusoids	13.04	54.02	3.8 - 57.39	22.95 - 107.13	<u>+ 2.94</u>	<u>+ 4.29</u>
	p = <.001					
Total number of hemopoietic cells per mm <sup>2</sup> in bone marrow	4.20	25.48	0 - 26.78	7.65 - 114.78	<u>+ 2.08</u>	<u>+ 6.93</u>
	p = <.001					
Diameter in μ of erythrocytes in liver smears	11.31	8.23	6.8 - 17.0	5.10 - 13.60	<u>+ 1.78</u>	<u>+ 1.62</u>
	p =>.001					
Percent of erythroid cells present (350 cells counted)	Liver smear		<u>homozygous</u>	<u>heterozygous</u>		
		immature	38.0	71.0		
	mature	61.0	28.0			

less in homozygous than it is in normal fetuses (see Table 5 and Plate IX, Figs. 19 and 20, in Appendix).

Diameter of erythrocytes. The liver touch smears from the homozygous fetus show that the cytoplasm of the mature erythrocytes are more basophilic and have a greater average diameter than do those of the normal controls (see Table 5 and Plate IX, Figs. 19 and 20, in Appendix).

Bone marrow hematopoiesis has begun during this period in development but is limited primarily to the formation of the leukocytic series until after birth. Total relative number of hematopoietic cells contained within ossification centers in the extremities of normal and homozygous animals are significantly different. There are many more in bones of normal than there are in those of homozygous fetuses (Table 5).

Liver Morphology. At this stage of development, the sinusoids and vascular channels in livers from normal animals contain developing and mature blood cells. Thus, from its appearance, the normal fetal liver at this stage is more of a hematopoietic than a metabolic organ. Most of the hepatocytes are in randomly arranged plates of cells (some two to three layers thick). In contrast, the arrangement of these cells in livers from homozygous fetuses resembles that in livers from post-partum mature rats, in that they are in plates separated by sinusoids, and have a more distinct plasma membrane than do livers from normal fetuses. However, as indicated previously, these sinusoids contain fewer blood cells than do like spaces in livers from normal fetuses of the same age (see Plate VIII, Figs. 17 and 18, in Appendix).

## CHAPTER IV

### DISCUSSION

Hereditary anemias in the rat attract interest because knowledge gained therefrom can provide an increased understanding of the etiology of corresponding conditions in man and because of their potential value as a model for the study of gene action in man and other mammals.

The preceding study of the manifestation of anemia in rat embryos and fetuses from restricted mothers bred to restricted males is limited to certain aspects of morphology of the hematopoietic organs and of hematology.

The observed differences in effect of the gene ( $H^{re}$ ) in the homozygous condition and in the heterozygous or normal condition are probably due to differences in the genetic makeup of the two gametes involved in producing a homozygous individual. Thus, interaction between the gene complex in each gamete involved and the gene for sterility and for restriction of coat color in the gamete with which that particular one unites to form a homozygous embryo, results in variance in expression of the mutant gene between affected siblings. Only fetuses in which it was obvious that the mutant lethal gene manifested its full effect were compared with normal fetuses from heterozygous matings.

From knowledge gained with methods used, it appears that the development of the homozygous embryo and subsequent fetus remains within normal limits up to day fifteen. Hematopoietic activity begins in the yolk sac at nine days after fertilization as it does in the normal embryo and is evident in the liver of both normal and homozygous embryos by day ten. By the fifteenth day of gestation a few of all fetuses produced by heterozygous matings became pale in color. This probably represents the 25% which Gumbreck et al. (1971) have described as the lethality percentage ascribed to these litters. Hematocrit studies by these workers have confirmed the anemia (Peeples et al., 1971).

The pertinent observations concerning the effects of the homozygous condition in these fetuses are: 1) an abnormal decrease in the weight of fetal livers during development; 2) a reduction in the relative population of blood cells circulating in the liver; 3) the occurrence of an increased percentage of mature erythrocytes with a concomitant decrease in the number of developing cells; 4) an increase in size of mature erythrocytes; and 5) the histologic picture of the liver indicates that it reaches its hepatic function at an abnormally early stage of development, and concomitantly relinquishes its hematopoietic activity.

Two of the thirteen day embryos examined were being resorbed (see Plate III, Fig. 7, in Appendix). Since this frequently occurs normally in rats (Griffith, 1967), and since approximately 25% of the embryos studied were pale in color, it is assumed that this resorption was

not a reflection of activity of the gene for restriction of coat color and sterility.

Anemias in the rat have not been thoroughly investigated. Wintrobe and Schumacker (1933) and Wintrobe (1936) have recognized that macrocytic anemia in man may occur in association with certain diseases of the liver. It has been suggested that macrocytic anemia in certain cases of liver disease may be a result of defective storage and metabolism of the anti-anemic factor. This has occurred when damage to the liver leaves little functioning tissue resulting in significant depletion of the stores of hematopoietic factors.

Inherited anemias have been described in the mouse: splenomegalic anemia (Hertwig, 1942); macrocytic siderocyte anemia (Grüneberg, 1942b) and macrocytic anemia (DeAberle, 1925; 1927; Grüneberg, 1939; Russell et al., 1970). Macrocytic anemias in these animals have occurred in connection with the dominant spotting gene,  $W$ ,  $W^V W$ . The first abnormal allele of the  $W$  series was found by Little (1915) to be lethal when homozygous. Mice with a  $WW$  genotype exhibit lethal anemias (DeAberle, 1925; 1927). These animals are recognizably pale and "bloodless" as early as the sixteenth day of gestation. Gowen and Gay (1932) showed that the immediate cause of death of  $WW$  mice was their anemia. DeAberle's work demonstrated that at least in the most severely affected genotype the anemia is already present four days prior to birth.

Russell and Fondal (1951) experimented with mice of genotype  $W^a$ ,  $W^vW$ ,  $W^vW^v$ ,  $WW^v$ ,  $WW$ . They demonstrated that anemia already exists in all severely affected types by the sixteenth day of gestation. In all the genotypes examined the anemia was macrocytic. Also, the double dominant  $W$  genotypes lack pigment in the hair and are almost completely sterile. In the rat fetuses homozygous for genes controlling restriction of coat color and fertility, the gradual premature increase in hepatic function and concomitant decrease in hematopoietic activity with accompanying macrocytosis of the erythrocytes in the liver, leads to the conclusion that this phenomenon observed in rats strongly resembles the similar defect just described for mice. Russell and Fondal (1951) state that "all flowing blood cells of a sixteen day mouse embryo must come either from yolk sac or from liver hematopoiesis, since the bone marrow first becomes organized at sixteen days. Therefore, some defect in hematopoiesis is present in the liver and possibly also in the yolk sac of these anemic animals". They concluded that certain genotypes are lethal at particular stages suggesting that these deaths are due to failure of blood formation to reach the absolute level necessary for continued development and resistance to minor noxious stimuli at these critical periods of rapid growth.

In the rat fetuses studied, when the liver prematurely changes into an adult structure, its hematopoietic activity ceases to be its main function.

Possibly, with this premature maturation, the hematopoietic elements have not been given sufficient time to produce an adequate blood supply for these animals and medullary activity has not developed adequately to compensate for the decrease in the supply.

The glycoprotein erythropoietin (ESF) is a prime regulator of erythropoiesis in vertebrates (Gordon, 1959; Fisher, 1969; Gordon and Zanjani, 1970). The mechanism by which it induces differentiation of hematopoietic precursor cells has been examined in fetal mice and rat livers (Cole et al., 1968; Paul and Hunter, 1969). Adding ESF to a culture of fourteen to sixteen day fetal liver cells increases the rate of hemoglobin production in these cells up to about twenty times. Three phases of differentiation of the ESF committed stem cell have been postulated by Goldwasser (1966) and Dukes (1967). These are: 1) sensitization - this may involve the presence of a receptor protein that serves as the specific attachment site of the ESF in the target cell; 2) induction - here the ESF may serve as a depressor initiating transcription of genetic information concerned with the differentiation of the precursor cell into the earliest member of the erythroid cell series; and 3) specialization - this includes further proliferative and maturational processes associated with the development of the more distal members of the definitive red cell line.

The pale appearance of the homozygous rat fetus seems to begin around the fifteenth day of gestation. It is during this phase of

development that ESF has its greatest influence. If this substance was lacking in the homozygous animals, arrested development of hematopoiesis could be expected. This theory fails in one respect, i.e. there is no decrease in specialization of stem cells. Normally, the percentage of mature erythrocytes fall in a range of 28% with approximately 71% comprising the intermediate stages of developing blood cells. At fifteen and twenty days of gestation, the greatest percentage of erythrocytes are mature in the homozygous individual with less than 40% being immature. Possibly the decrease in the number of immature erythrocytes is the result of a cessation of hematopoiesis in the liver before the bone marrow has had the opportunity to make up for the deficit in the number of blood cells. Therefore, these animals are left in an anemic condition unable to survive.

On the other hand, abnormal hematopoiesis and accompanying abnormal liver development in fetal H<sup>re</sup> rats may result from a cause similar to that found in the flexed mutation in mice.

The flexed mutant in the mouse causes an anemia which is most severe from the thirteenth to the sixteenth days of gestation (Grüneberg, 1942a; Russell et al., 1968). These mice were found to be deficient in Delta Aminolevulinate Dehydrase (ALD) (Margolis and Russell, 1965). Since ALD catalyses the conversation of delta aminolevulinic acid to porphobilinogen (PBG), a heme presursor, it seems reasonable to assume that this relative enzyme deficiency, coupled with the inability of ALD to

respond adequately in periods of hematopoietic crisis, might be the cause of the anemia which occurs in the fetal period when blood volume is increasing at an extremely rapid rate.

Russell et al. (1968) have shown that flexed mice at all ages are smaller than normal and that the liver weight of the fetuses is only 50 to 60 percent that of normal fetuses. Thus, enzyme activity per liver is decreased. The decrease in the relative size and weight of the fetal livers from homozygous  $H^{re}$  animals at a time when hematopoiesis therein would normally be at its peak, suggests a decrease similar to that just described for flexed mice.

Russell (1949) points out a parallelism in the effect of gene of the W-series of mice on erythrocytes and on pigmentation to the extent that the three most anemic genotypes are all completely lacking in pigment in the hair. The situation in the  $H^{re}$  rat is very similar. Hematopoietic tissues come directly or indirectly from primitive mesenchyme (Bloom, 1937), and the main sites of hematopoiesis are successively the yolk sac, the liver, and the bone marrow. All hair pigment in the rat is believed to come from melanophores which migrate from the neural crest. It hardly seems possible that the pleiotropic effect of the gene responsible for these widely separate characteristics is aimed at the mesenchyme and neural crest cell, since so many other descendants of the same cells appear to be unaffected. Rather, one effect must be dependent on the other for its appearance, or both must result from independent gene action

in two types of cells, or both tissues must be in an especially susceptible state to be affected by one gene action external to both at a particular embryonic stage. The type of data presented cannot distinguish definitely among these possibilities.

The gene or genes in question have extensive pleiotropic effects in many tissues. Deeper analysis of these effects will undoubtedly increase knowledge of pathways of gene action, and may also give clues to the actual nature of the defect in hematopoietic tissue observed in the present study.

It is probable that the pleiotropic gene  $H^{re}$  in the rat finds as its target the primordial germ cells, melanoblasts or their embryonic precursors, and fetal hematopoietic cells.

## CHAPTER V

### SUMMARY

Young restricted ( $H^{re}/+$ ) male rats were crossed with ( $H^{re}/+$ ) females. Subsequently, certain aspects of the ontogeny of the offspring were examined. Particular emphasis was placed on the development of the liver and bone marrow.

No abnormalities were detected in embryos up to fourteen days of gestation. Nine of the 48 fetuses examined at fifteen days were abnormally pale. Since the  $H^{re}$  gene is an autosomal dominant, these were assumed to be homozygous embryos. The average number of blood cells in the liver sinusoids of these is  $13.39/\text{mm}^2$ , whereas that for their normal siblings is  $50.88/\text{mm}^2$ . The normal hematopoietic picture in the embryonic livers at this stage is one of a progression of developing blood cells from erythroblasts through erythrocytes. Contrary to this, the immature cells found in peripheral blood smears from fifteen day homozygous fetuses are almost all normoblasts. Some of the erythrocytes in the circulating blood of these homozygous embryos are larger than normal, however, not significantly so.

At twenty days gestation, six of the 25 fetuses examined were pale in color. Again, because this was about 25% of the total number of embryos

born, they were considered to be homozygous. Their liver weights were one-third of that of their heterozygous littermates. At this stage of development the ratio of total number of hematopoietic cells in the sinusoids in the liver of normal fetuses to that in homozygous fetuses is 4:1. Twenty-eight percent of these cells in the heterozygous and 61% in the homozygous fetuses were mature erythrocytes. Also, it is now significantly apparent that the mature erythrocytes of the homozygous fetuses are larger than those of the normal. Also, the cytoplasm of the mature erythrocytes of homozygous fetuses exhibits a different staining reaction than normal and may appear abnormal in shape. Effects of the anomalous gene are also reflected in the bone marrow of twenty day fetuses of homozygous embryos in that the number of hematopoietic cells therein is abnormally low. One seemingly significant event in the ontogeny of these homozygous fetuses is that at fifteen days their liver is starting to evolve from a hematopoietic organ to one primarily concerned with the hepatic function. By twenty days the evolution has been completed with the result that the liver has taken on the appearance of a functional hepatic unit rather than one concerned with hematopoiesis. Normally the rat liver does not relinquish its hematopoietic function until after birth.

Thus, it is apparent that when embryos are homozygous for the H<sup>re</sup> gene, they acquire a macrocytic anemia. From the histologic picture, this anemia seems to stem from early failure of the liver as a hematopoietic organ. Further studies will have to be carried out in order to confirm the observation.

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## **APPENDIX**

## LEGEND FOR FIGURES

Legends used for Figures 3 through 20 are as follows:

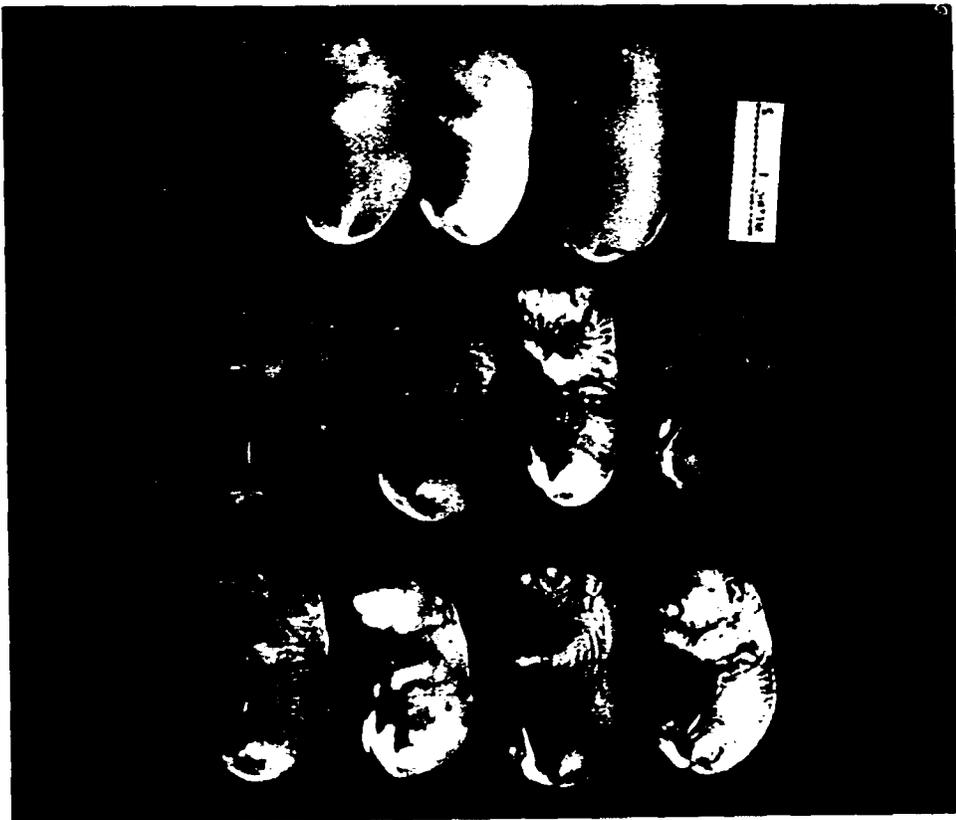
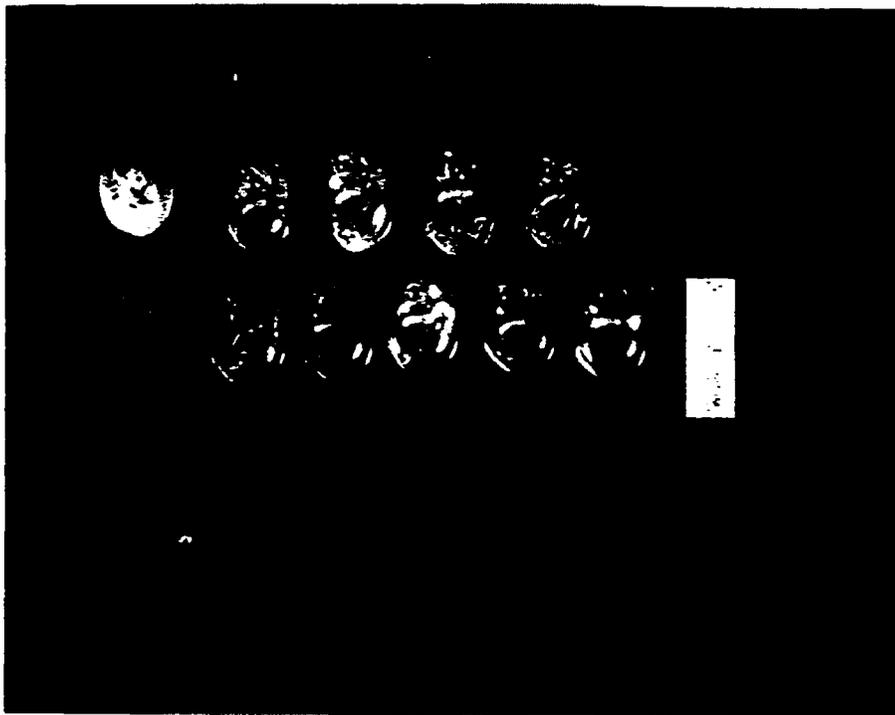
<b>AC</b>	<b>amniotic cavity</b>
<b>AL</b>	<b>allantois</b>
<b>AM</b>	<b>amnion</b>
<b>CV</b>	<b>central vein</b>
<b>EC</b>	<b>ectoplacental cone</b>
<b>ECC</b>	<b>ectoplacental cavity</b>
<b>ECT</b>	<b>extra-embryonic ectoderm</b>
<b>END</b>	<b>endoderm</b>
<b>EXC</b>	<b>extracoelomic cavity</b>
<b>HBV</b>	<b>hepatic blood vessels</b>
<b>HC</b>	<b>hepatocytes</b>
<b>HE</b>	<b>hematopoietic cells</b>
<b>HF</b>	<b>head fold</b>
<b>HPV</b>	<b>hepatic portal vein</b>
<b>M</b>	<b>megakaryocyte</b>
<b>MES</b>	<b>mesoderm</b>
<b>PC</b>	<b>proamniotic cavity</b>
<b>RM</b>	<b>Reichert's membrane</b>
<b>SCT</b>	<b>subcutaneous tissue</b>
<b>UV</b>	<b>umbilical vessels</b>
<b>VC</b>	<b>inferior vena cava</b>
<b>VM</b>	<b>ventral mesogastrium</b>
<b>YC</b>	<b>yolk cavity</b>
<b>YS</b>	<b>yolk sac</b>

**PLATE I**

**Figure 1. Fifteen day litter. Top row: homozygous restricted fetuses and placenta. Bottom row: heterozygous fetuses and placenta.**

**Figure 2. Twenty day litter. Top row: homozygous restricted fetuses, others are heterozygous individuals.**

PLATE I



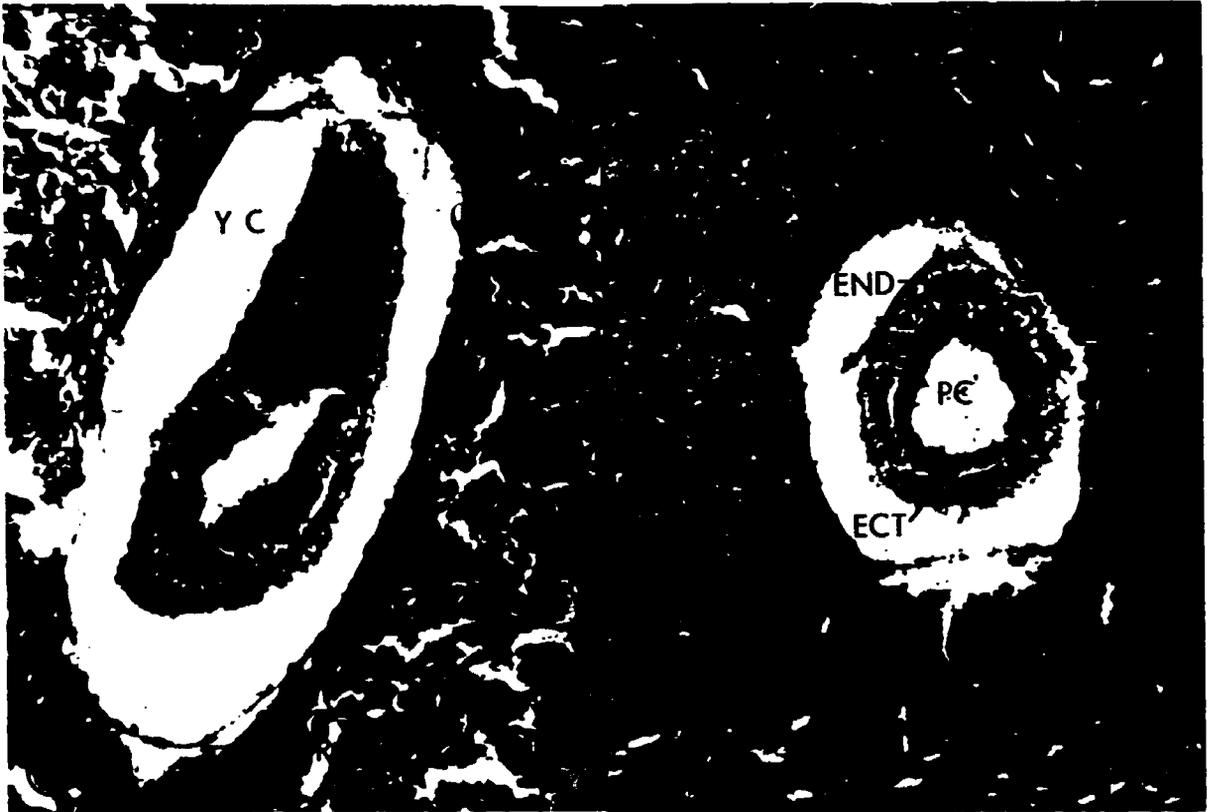
**PLATE II**

**Figure 3. Longitudinal section of an eight day embryo showing yold cavity (YC) and Reichert's membrane (RM). Stained with H & E. 75X.**

**Figure 4. Cross section of an eight day embryo showing pro-amniotic cavity (FC) and relatively thin extra-embryonic ectoderm (ECT) surrounded by endoderm (END). Stained with H & E. 75X.**

**Figure 5. Longitudinal section of an eight day embryo showing ectoplacental cone (EC), ectoplacental cavity (ECC), formation of mesoderm (MES) and amniotic cavity (AC). Stained with H & E. 83X.**

PLATE II



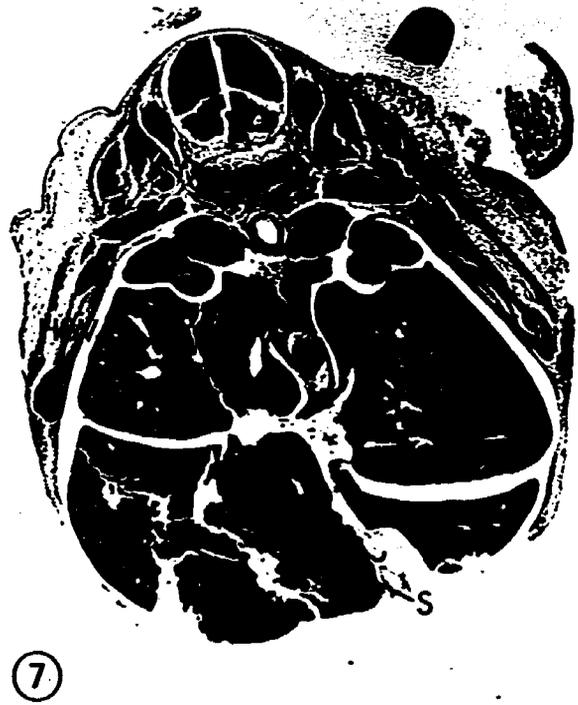
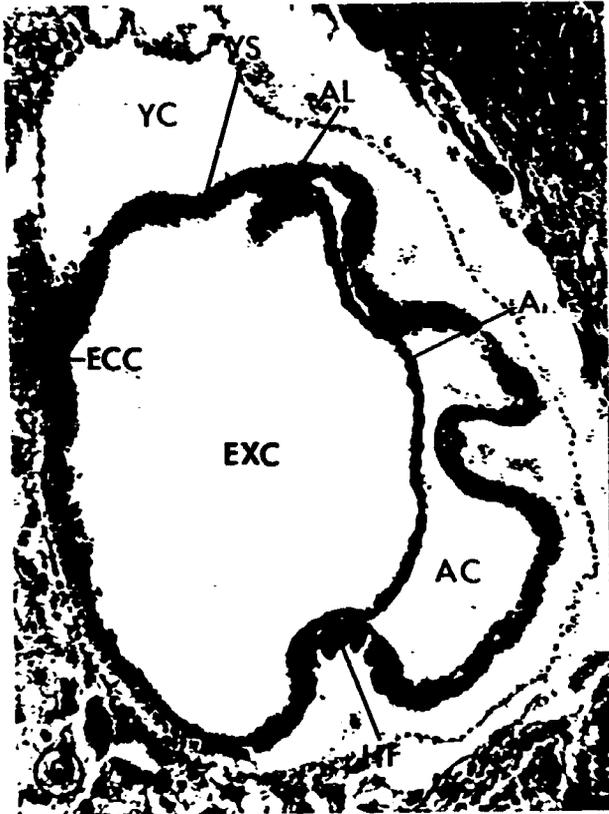
## PLATE III

**Figure 6. Sagittal section of a nine day embryo showing yolk sac (YS) and yolk cavity (YC), ectoplacental cone (ECC), extracoelomic cavity (EXC), head fold (HF), amnion (AM) and amniotic cavity (AC), allantois (AL). Close examination reveals blood forming cells in the yolk sac. Stained with H & E. 121X.**

**Figure 7. Cross section of a thirteen day embryo through the liver demonstrating hepatic blood vessels (HBV) and evidence of sinusoids (S). Stained with H & E. 18X.**

**Figure 8. A section through the uterus of an H<sup>re</sup> female that has been bred to an H<sup>re</sup> male. The fibrinoid substance in the uterine cavity is an embryo in the process of being resorbed. Stained with H & E. 18X.**

PLATE III

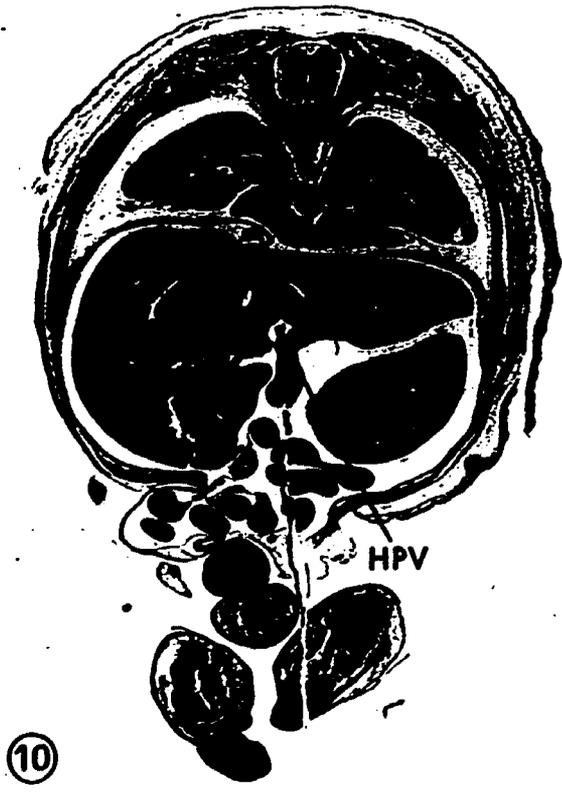


**PLATE IV**

**Figure 9. Cross section through the liver of a fifteen day heterozygous fetus showing ventral mesogastrium (VM), gut, inferior vena cava (VC) and umbilical vessels (UV). Stained with H & E. 10X.**

**Figure 10. Cross section through the liver of a fifteen day homozygous fetus showing hepatic portal vein (HPV) and gut extending into yolk stalk. Stained with H & E. 10X.**

PLATE IV



**PLATE V**

**Figure 11. Fifteen day homozygous fetal liver showing hepatocytes (HC), hematopoietic cells (HE), sinusoids (S) and megakaryocyte (M). Giemsa stain, Apo. 25X. 425X.**

**Figure 12. Fifteen day heterozygous fetal liver showing hepatocytes (HC), hematopoietic cells (HE) and sinusoids (S). Giemsa stain, Apo. 25X. 425X.**

PLATE V

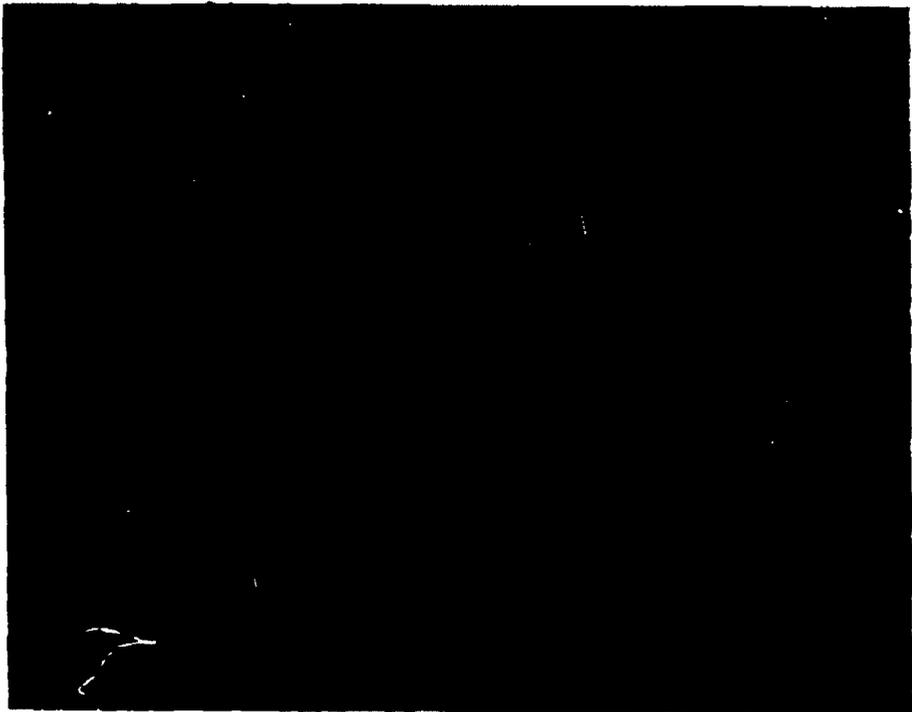


PLATE VI

**Figure 13. Fifteen day homozygous fetal liver touch smear demonstrating blood forming cells, polychromatophilic erythroblast (A) and nucleated erythrocytes (B). Wright stain, oil immersion. 556X.**

**Figure 14. Fifteen day heterozygous fetal liver touch smear demonstrating blood forming cells. Wright stain, oil immersion. 484X.**

PLATE VI



**PLATE VII**

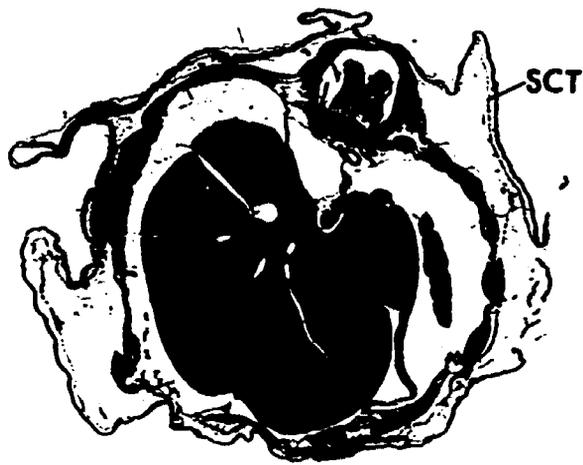
**Figure 15. Cross section through the liver of a twenty day heterozygous fetus demonstrating central veins (CV) and sinusoids (S). Stained with H & E. 8X.**

**Figure 16. Cross section through the liver of a twenty day homozygous fetus demonstrating the width of the subcutaneous tissue (SCT) and the lack of circulating blood. Stained with H & E. 8X.**

PLATE VII



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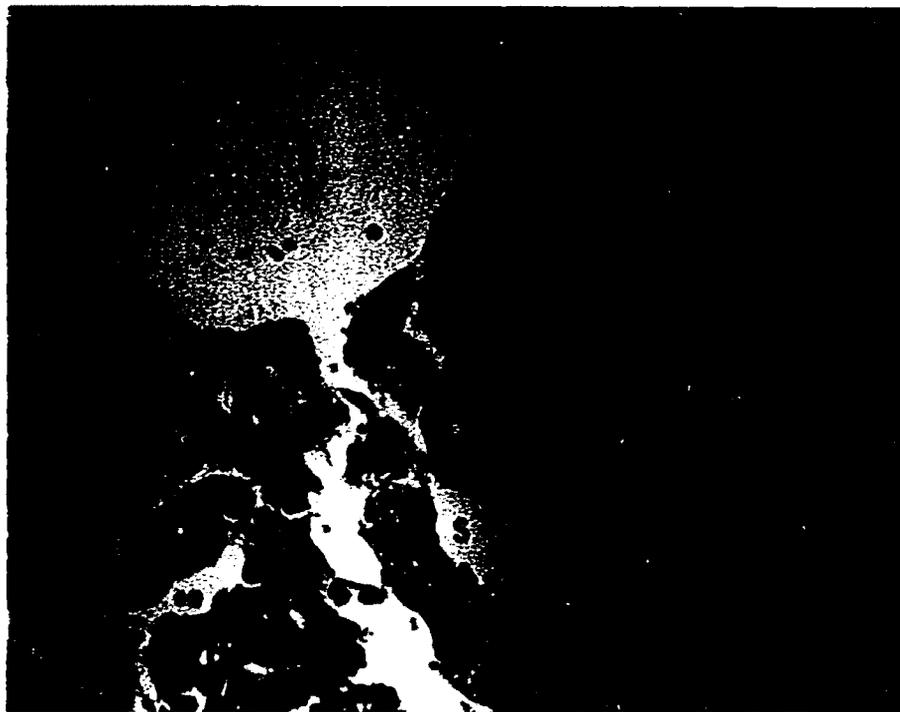
16

**PLATE VIII**

**Figure 17. Twenty day heterozygous fetal liver showing hepatocytes (HC), hematopoietic cells (HE) and sinusoids (S). Giemsa stain, Apo. 25X. 278X.**

**Figure 18. Twenty day homozygous fetal liver showing hepatocytes (HC), hematopoietic cells (HE) and sinusoids (S). Giemsa stain, Apo. 25X. 279X.**

PLATE VIII



**PLATE IX**

**Figure 19. Twenty day homozygous fetal liver touch smear demonstrating blood forming cells, showing "target" shaped cells and basophilic erythrocytes. Giemsa stain, oil immersion. 503X.**

**Figure 20. Twenty day heterozygous fetal liver touch smear demonstrating maturation of blood forming cells. Giemsa stain, oil immersion. 503X.**

PLATE IX

