8-1-1964

A Histo;pgical and Histochemical Study of Developing Rat Spleen: With Special Reference to the Macrophage System

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A HISTOLOGICAL AND HISTOCHEMICAL STUDY OF THE DEVELOPING RAT SPLEEN: WITH SPECIAL REFERENCE TO THE MACROPHAGE SYSTEM.

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A Dissertation

Submitted to the Faculty

of the

Graduate School

of the

University of North Dakota

in partial fulfillment of the requirements

for the Degree of

Doctor of Philosophy

Grand Forks, North Dakota

August

1964

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Acknowledgements

The author wishes to express his appreciation to Dr. Theodore Snook, Department of Anatomy, for his advice, constructive criticism and for the preparation of the photographs used in this paper.

Grateful acknowledgement is also give Dr. John Taylor, Department of Anatomy, for suggesting the original problem and for his advice in regard to iron metabolism.

Acknowledgement is given to Dr. Vernon Yeager, Department of Anatomy, for his suggestions on enzyme histochemistry and constructive criticism of this paper.

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CHAPTER I

INTRODUCTION

The reticuloendothelial or macrophage system which is so widely distributed throughout the body has received considerable attention since Metchnikoff (1,2) first gave his description of a general defense system of cells which he divided into microphages (polymorphonuclear neutrophils) and the highly phagocytic cells of the connective tissue which he called macrophages. He was first to mention the fact that these cells, widely scattered throughout the body, possess in common the power of phagocytosis. Metchnikoff was among the first to employ vital dyes which the macrophages could phagocytose and segregate without injury to their physiologic activities.

Various other investigators had called attention to cells of the body which engulfed foreign matter but had not defined them as a specific system. Among these was Ranvier (3,4) who studied the "milk-spots" of the omentum of animals and found in them large phagocytic cells which he called clasmatocytes because they seemed capable of breaking off bits of their protoplasm. Jaffe (5,6) believed that this process was an artifact. Mallory (7) emphasized the occurrence of phagocytosis of red cells and lymphocytes in the lymphoid tissue of the intestine, lymph nodes and especially in the spleens in his work on typhoid fever in 1898. The phagocytes in the spleen were found mainly in and lining the blood vessels including the venous sinuses. He believed that the phagocytes arose by

proliferation and desquamation from the endothelial cells lining the vessels.

A more precise delineation of this system was given by Aschoff (8). He called this system of phagocytic cells the reticulo-endothelial system. He defined it as a group of cells related to both reticular tissue and endothelium whose most striking characteristic was their ability to phagocytose particulate matter. Aschoff included in this system the reticular cells of the spleen and lymph nodes, cells lining lymph sinuses, sinusoids of the liver and bone marrow, also similar cells in the suprarenal and pituitary glands, ameboid wandering cells of the connective tissues, cells within the sinuses of the spleen as well as macrophage-like cells in the blood. He did not include lymphocytes.

At the turn of the century acid vital dyes and colloidal suspensions were being used extensively for the study of macrophages. According to Patek and Bernick (9) and Wislocki (10) the use of colloidal suspensions was first introduced by Ponfick (1869) and Hoffman and Langerhan (1869) who injected cinnabar granules into the circulation of living animals and observed the intracellular deposition of granules of cinnabar in the bone marrow. They thought that the cells concerned with the phagocytosis of the particulate matter were lymphocytes. Patek and Bernick (9) gave credit to Ribbert (1904) for being the first to successfully inject

animals with lithium carmine and to von Kupffer who in 1899 was probably the first to use India ink as a colloidal suspension of carbon to demonstrate the stellate macrophages of the liver. Cousin (11) injected suspensions of carmine or litmus blue into animals and reported that the pigment was phagocytosed by the endothelial cells lining the capillaries of the bone marrow. Bouffard (12) injected isamine blue into mice to determine its toxicity. He demonstrated that the Kupffer cells of the liver took up the dye in abundant quantities while the macrophages in the spleen had fewer granules. Bouffard seemed to think that the splenic macrophages had ingested dead polymuclear cells which contained lesser amounts of the isamine blue. This he believed was the reason that splenic macrophages gave a weaker response than the Kupffer cells of the liver.

Goldman (13), using the vital dyes developed by Ehrlich (14), was able to show that the various forms of cells which so avidly ingested particulate matter were the same as those previously described by Metchnikoff (1,2) and others as being phagocytic for tissue debris.

Sabin, Doan and Cunningham (15,16), who had the opportunity to work in Metchnikoff's laboratory, introduced the vital dyes such as trypan blue and pyrrhol blue and found that these dyes were deposited in the cytoplasm of sinus-lining cells of liver, bone marrow,

lymph nodes and the spleen. Other phagocytic cells also concentrated these dyes; these are the reticular cells of lymph nodes, splenic pulp and bone marrow and great masses of cells in the connective tissue designated variously as clasmatocytes or resting wandering cells. It was also noted by Evans (17) that the polymorphonuclear cells of blood were able to phagocytose particles of ordinary microscopic dimensions but not the finer ultramicroscopic particles which the macrophages were able to "drink in" and store.

Cappell (18) has given an excellent review on the subject of vital staining. He divided the vital stains into two categories, those absorbed rapidly such as trypan blue and vital new red and those absorbed slowly such as pyrol blue and diaminefast scarlet. The more diffusible dyes, i.e. trypan blue and vital new red, show relatively little tendency to accumulate in the spleen. The degree of participation of the spleen in the vital staining process was shown to depend on the agent used and the mode of administration. Administration of vital dyes by the intravenous route produced a more extensive participation of the splenic tissue than do subcutaneous or intraperitoneal injection of similar doses. Suspensoids such as India ink and saccharated iron oxide were removed from the circulation by the spleen with extreme rapidity and accumulated in the spleen progressively after intravenous injection. Cappell (18) also found that the cells lining the sinuses of the spleen took

took up very little of the vital dye, whereas the stellate and free cells of the red pulp readily picked up the dye particles. He also observed a few cells in the white pulp that contained vital dye aggregates. Cappell felt that the dyes best suited for intravital staining were those of the acid group, such as trypan blue.

Although vital dyes have been used extensively for determining cells of the R. E. system, this method has been shown by Bouffard (12), Evans and Scott (19) and Evans (17) to have the drawback in that they are not always specific for macrophages. Bouffard (12) and Evans (17) demonstrated that polymorphonuclear leukocytes ingest dye but to a lesser extent than the R. E. cells. The morphological difference here was enough to prevent any confusion. Evans and Scott (19) presented evidence that fibroblasts also stored dye although to a lesser degree than the macrophages. The fact that the fibroblasts and the macrophage at times are somewhat similar morphologically could thus cause some confusion in the study of R. E. cells by the use of vital dyes.

Marshall (20) thought that vital staining has left many questions unanswered and created much controversy in regard to the R. E. system. He has suggested that the method of ammoniacal silver or silver carbonate impregnation offers an opportunity for a better understanding of the R. E. system.

This silver impregnation method was first employed by Del Rio-Hortega and de Asua (21) in 1921 for the demonstration of microglia. Rio-Hortega and de Asua (22,23) described similarly reacting cells in the spleen using the silver carbonate method. Del Rio-Hortega (24) in 1927 also described a ferric chloride method for the impregnation of the cells of the splenic pulp. This same year, according to Marshall (25), de Asua also demonstrated argyophilic cells in the spleen. Cone (26) described microglia-like cells in degenerating areas of tumors and in the adventitia of vessels using the silver carbonate method. He considered the impregnation of the microglia and transitional forms as being selective and specific for these cells. In the areas of degeneration the microglia became actively phagocytic and he thought that the phagocytes, besides arising from the microglia, also arose from the walls of vessels or from cells in the perivascular spaces. In both cases he was able to specifically demonstrate these macrophages using the silver carbonate method. Belezky (27) was able to demonstrate reticuloendothelial cells in the red pulp of the spleen of mice, man, rabbit and dog and in the white pulp of mice using the pyridine-soda silver impregnation procedure. Dunning and Stevenson (28) showed that these microglia-like cells in the spleen and other tissues respond to injury by forming rounded macrophages which segregate vital dyes. Dunning and Furth (29), in further

studies using the Del Rio-Hortega silver carbonate method concluded that microglia and histiocytes located in other parts of the body constitute a related cell type. This latter conclusion is in agreement with that of Wells and Carmichael (30).

The first complete and accurate study of the R. E. system of the rat spleen, using the Del Rio-Hortega silver method was done by Pellegrino and Iraldi (31). This work apparently has been overlooked by many investigators because it was written in their native Spanish and published in an Argentinian journal. They described the nodule as having a germinal center which contained only a few argyrophil cells. The nodule itself had a halo of reticular cells, Snook's marginal metalophils (32,33,34), at its periphery. Outside of this is a pale ring with very few argyrophil or reticular cells (the marginal zone). The elements of the red pulp were condensed around the periphery of the marginal zone.

Marshall (20) employed a modification of Del Rio-Hortega's silver carbonate method in a study of the R.E. cells in the human lung and spleen. He found that the cells blackened by this technique (metalophils) correspond to the R.E. cells in the strict sense as defined by Aschoff (8), that is, those cells which possess both phagocytic ability and the property of storing acid vital dyes. Marshall (20,25) and Marshall and White (36) also pointed out that cells which store vital dyes are always demonstrated by the silver

impregnation methods but the converse is not true. For this reason the use of the silver impregnation techniques on the spleen demonstrates a much larger number of R.E. cells than may be seen after the most intense vital staining. Snook (32) stated that this illustrates that some cells which are metalophilic do not show phagocytic activity. This may be due to either of two reasons: "First, they may not be fully differentiated, and second, the vital dye or particulate matter may be prevented from reaching their cell membranes." Snook, Linford and Bache (35) thus considered these "extra" cells stained by silver impregnation but not showing phagocytic activity as potential macrophages (relatively undifferentiated reticular cells) and in order for them to become full-fledged macrophages they must develop further naturally or be appropriately stimulated to do so. They may possess the capability to become active phagocytic reticular cells when the need arises.

In 1950 Marshall and White (36), employing Marshall's (20) silver technique, were able to demonstrate four types of argyrophilic cells in the rabbit spleen. The first type comprises the spheroidal macrophages lying free in the lumina of the sinuses; these cells can be heavily stained with vital dyes.

The second type is composed of cells of an elongated spindle form which constitute the lining cells of the sinuses, one surface

of the cell is thus directly exposed to the blood stream. Marshall (25) stated that the impregnation of these cells is often difficult.

The third type of cell comprises most of the interstitial R.E. tissue of the spleen. They lie in the stroma of the red pulp and possess numerous irregular and coarse branching processes. In vitally stained animals only a few of the cells of this type contain the vital dye.

The fourth cell type is confined to the white pulp and is composed of cells with much finer and more extensive branching processes than the previous types. These cells closely resemble the microglia of the brain. Guzman (37), using Del Rio-Hortega's double silver impregnation method, has also described four groups of cells which correspond in location and in cytological description to those described by Marshall and White (36).

Pellegrino and Iraldi (31) studied the rat spleen using silver impregnation methods and arrived at similar results. They concluded that the silver method is an excellent procedure for the delineation of the R.E. system in the spleen.

White (38), while reporting on the immunologically competent cell, used the Weil-Davenport silver technique to demonstrate that the marginal metalophils of the rat spleen are much more prominent than the corresponding cells of the rabbit spleen. Snook (32), using

Marshall's (20) metalophil reaction, found that the distribution of metalophil cells in the rat was different from Marshall's (25), for the rabbit and man. In the rat there are many metalophils aggregated to form a dense ring around the margin of the white pulp at the level of the marginal sinus. Snook (33,34,39) had previously demonstrated these marginal metalophils by using Marshall's (20) method and had suggested that these cells were probably potential macrophages. He thought, because of their position near the marginal sinus, that they would be the first R.E. cells of the spleen to encounter the splenic blood and that these cells could become actively phagocytic when the need arises. In his most recent paper, Snook (32) was unable to stimulate the marginal metalophils to take up India ink. The aggregation of metalophils at this area, because they are metalophilic cells, still suggests that it is a zone of potential phagocytic activity.

The marginal metalophils as described by Marshall and White (36) and Guzman (37) for the rabbit have fine and extensive branching processes, while those described by Snook (32) for the rat are rounded and have blunt pseudopodia. This seems to indicate a case of species difference and should be kept in mind when studying these cells in different animals. Snook (40,41) has dramatically demonstrated in his study of the vascular arrangements in mammalian spleens the need for caution in studying similar structures in different animals because species differences are many.

Snook (41) stated, "-----conclusions based on the study of one animal cannot always be applied to another animal."

Between the lymphoid follicles and the red pulp proper of the spleen is an intermediate, pale staining zone. This area immediately surrounding the follicle has been called the "marginal zone" by MacNeal (42) and Altschul and Hummason (43). This area has been described previously by Pellegrino and Iraldi (31), Andrew (44,45) and Baillif (46). Later it was described in more detail by Snook (40) in the spleens of the mouse, rat, horse, cow and man. Snook (32) stated that the marginal zone was nearly devoid of metalophil cells, a few specimens showed scattered metalophils in the outer part of the zone. These findings on the scarcity of metalophils in the marginal zone are in agreement with those of Pellegrino and Iraldi (31), White (38) and Guzman (37).

Immediately surrounding the lymphoid follicles in the rat and mouse spleens is a sinus called the "marginal sinusoid" by Baillif (46). Other names have been applied to this sinus; Andrew (44,45) called this space an intermediate sinus. Altschul and Hummason (43) used perifollicular space, a term also used by Snook (40). In his most recent publication Snook (32) preferred to use the term "marginal sinus." Certain cells (marginal metalophils) related to this "marginal sinus", as has been previously

stated in the introduction, are considered to be potential macrophages and as such will be considered in this research.

Enzyme histochemical techniques have been employed by many investigators for the identification of R.E. cells. Of the various enzymes studied in relation to macrophages, there are two that manifest strong activity in the reticuloendothelial cells. These are acid phosphatase, an enzyme which hydrolyzes phosphate monoesters and liberates orthophosphate in an acid medium, and non-specific esterase, an enzyme which hydrolyzes simple (short chain) common esters.

The first histochemical method for the demonstration of a hydrolytic enzyme in tissue sections was independently described by Gomori (47) and according to Barka and Anderson (48) by Takamatsu in 1939. Both of these investigators reported finding alkaline phosphatase in sections of various tissues of dogs and rabbits. Since this time there has been a growing interest in the demonstration of enzymes in tissue sections. Our discussions will be limited to those studies related to the macrophage system.

Braustein, Freiman and Gall (49) were able to demonstrate very strong esterase and acid phosphatase and moderately strong phosphoamidase activity, mainly in the cytoplasm of the macrophages of lymph nodes. They believed the study of enzyme systems possess

greater potentiality for the identification of macrophages than does the affinity of histiocytes for metals (especially Marshall's silver reduction method). Barka, Schaffner and Popper (50) also considered that enzyme patterns were more reliable for the study of R.E. cells than metalophilia. They thought metal impregnation methods were a source of artifact (false positives).

Acid phosphatase activity of macrophages of the spleen was described by Gomori (51). In all species studied (human, dog, gopher, guinea pig, cat, mouse and groundhog) he found that macrophages and monocyte-like cells of the spleen were the most reactive. Occasionally lymphocytes showed some activity. The Malpighian bodies were entirely unstained except at their periphery. Barka, Schaffner and Popper (50), using a modified azo-dye method, were able to demonstrate that the R. E. cells of the liver, thymus, lymph node and spleen have a high acid phosphatase activity. He concluded that this method gave more reliable localization and consistent and reproducible results than that obtained by Gomori's (51) method or by Marshall's silver impregnation techniques. His acid phosphatase response (his fig. 2) in the rat spleen was very similar to that obtained by Pettersen (52) for acid phosphatase, non-specific esterase and metalophil reactions for the rat spleen and by Snook (32), using the metalophil reaction for the rat spleen. Burstone (53), using an

azo-dye procedure, was able to demonstrate acid phosphatase activity in the splenic macrophages, especially in the red pulp of mice and rats. Rutenberg and Seligman (54) demonstrated high acid phosphatase activity in the prostate, kidney, liver, spleen and ovary of the rat. Weiss and Fawcett (55) cultured chicken leukocytes and stained these cells hitochemically in order to study the cytochemical changes associated with the transformation of monocytes to macrophages, epitheloid cells and multi-nucleated giant cells. Monocytes were found to be devoid of enzymes esterase and acid phosphatase. Macrophages, giant cells, and epithelioid cells which developed from monocytes were negative for esterase but strongly positive for acid phosphatase. The acquisition of acid phosphatase by monocytes during their transformation to macrophages appeared to be related to their enhanced phagocytic activity. The negative results obtained for the esterase was probably due to the substrate used, beta-napthyl acetate (48,56). Wolf, Kabat and Newman (57) using Gomori's original technique were able to demonstrate acid phosphatase in splenic red pulp cells and in a few cells of the Malpighian corpuscles.

The first and most extensive histochemical studies for esterases were made by Nachlas and Seligman (58), using beta-naphthyl acetate as a substrate. They were unable to demonstrate esterase activity in the spleen but as pointed out by Chessick (59), the

substrate they used is subject to diffusion artifacts and results obtained are not reproducible. Chessick (59), using alph-naphthyl acetate and naphthol AS acetate, reported intense activity in the macrophages of the splenic pulp with variation according to the species of animal used. The staining of the macrophages was more intense with naphthol AS acetate than with alpha-naphthyl acetate. With the alpha-naphthol technique, only the rat and mouse showed a moderate activity in the macrophages. When the naphthol AS technique was used, intense activity in these cells appeared in the rat and mouse and moderate activity was seen in the human, cat, and rabbit. Smith, Wharton and Gerhardt (60) studied normal and irradiated thymus, lymph nodes and spleen. They reported esterase positive cells in the periphery of the white pulp while the macrophages in the center were less active. Wells (61) described esterase activity in the macrophages of earthworms and noted a positive correlation between phagocytosis of carbon particles and esterase activity. In this same research he also worked with skin biopsy of a boy infected with Leishmania tropica. It was noted that the dermal histiocytes engorged with Leishmania bodies are also rich in non-specific esterase. These results suggest that non-specific esterase is generally present in actively phagocytic macrophages. These and other reports in the literature such as Doyle and Liebelt (62), Barrnett (63), Barrnett and

Seligman (64), Pearson and Grose (65) and Wachstein and Wolf (66) have led to the conclusion that cells of the R.E. system contain large quantities of the enzymes non-specific esterase and acid phosphatase.

Petterson (67,68) in his study of the distribution of non-specific esterase and acid phosphatase in rat spleens has shown that there was strong activity for both enzymes in cells of the red pulp cords and in scattered, isolated macrophages within the white pulp nodules. Acid phosphatase was found in cells at the level of the marginal sinus, while these cells had only a slight activity for esterase. Lining cells of the red pulp sinuses showed a constant but weak esterase activity but no activity for acid phosphatase. Pettersen (52) demonstrated a weak non-specific esterase activity in sinus lining cells of rat spleens before and after treatment with typhoid vaccine and adjuvant but was never able to demonstrate the presence of acid phosphatase. He did not consider this esterase activity as being significant.

The ellipsoid cells of the dog and cat spleens have been shown by Jacobsen (69) to give a positive reaction to both the metalophil reaction and acid phosphatase, but he found that the dog and cat spleens responded differently to the non-specific esterase technique. The non-specific esterase activity was lacking in the dog ellipsoid cells while it was present in cat ellipsoid cells.

Jacobsen (70) also observed that the sinus lining cells of the dog spleen gave occasional positive acid phosphatase, non-specific esterase and metalophil reactions.

Dorfman (71,72), employing the histochemical techniques described by Pearse (56,73) for acid and alkaline phosphatase and non-specific esterase obtained results contrary to those of Braunstein, Freiman and Gall (49) concerning the splenic sinus lining cells. Dorfman's work was done on human, guinea pig and monkey spleens. He demonstrated non-specific esterase and acid phosphatase in the stellate cells in the cords of Billroth and in the Malpighian corpuscles, but found the sinus lining cells were consistently negative for these enzymes. He also noted that Marshall's (25) results with silver impregnation showed that the sinus lining cells of the spleen are not true histiocytes (macrophages). Hosoda and Takase (74) noted that subcutaneous histiocytes gave a non-specific esterase response. After injection of trypan blue it was observed that the esterase activity was located in the same areas where the ingested trypan blue granules were located.

Weiss and Fawcett (55) and Nachles and Seligman (58) used beta-naphthyl acetate as a substrate for the non-specific esterase procedure whereas Chessick (59) used alpha-naphthyl acetate and naphthol

AS acetate in studying esterase activity in various organs. The findings of these investigators varied widely depending on what substrate they used.

It appears from the literature that the substrate used for a particular enzyme has considerable bearing on whether there is a positive response or not. This is particularly true in the case of non-specific esterase (49,58,59,74). There seems to be less disagreement as far as acid phosphatase is concerned. This may be one reason that acid phosphatase is a more common finding in macrophages than non-specific esterase.

As mentioned previously the splenic sinus lining cells are considered by some (71,72) not to be true histiocytes because they fail to give a positive metalophil reaction and do not always exhibit acid phosphatase or esterase activity. On the other hand, many researchers have been able to demonstrate their phagocytic activity by the injection of colloids.

Snook (75), in unpublished research has demonstrated that the splenic sinus lining cells will phagocytose saccharated oxide of iron and chlorazol black E two or three days after injection but will not phagocytose India ink. Moore et al (76,77) also demonstrated by Perls test for iron, that these cells were able to take up saccharated oxide of iron. They also found that the intensity of the Perls reaction and the number of cells containing iron increased

as a function of the number of doses of iron oxide. Injection of adjuvant had no effect. In contrast to Snook's (75) belief that sinus lining cells do not take up India ink, Patek and Bernick (9) report phagocytosis by the macrophages of the spleen 5 minutes after injection of the carbon. The cells taking up the carbon appeared to be firmly fixed to or within the sinus wall.

Weiss (78,79), after the injection of thorotrast into rabbits found a slight amount of this substance in the sinus lining cells. In a previous study Weiss (80), demonstrated that the endothelium of the patent splenic sinuses in normal untreated rabbits seldom showed evidence of phagocytosis or cytopoiesis, although there were many "filled" macrophages in the sinal lumina. On the other hand he found that the sinus lining cells in normal untreated rat spleens were actively phagocytic after treatment with silver nitrate by subcutaneous injection. The sinus lining cells and the macrophages of the red pulp cords in both the rabbit and rat were engorged with what he presumed to be silver. While working on human and rat spleens Weiss (81) found markedly dense bodies in the sinus lining cells, which he thought were hemosiderin or ferritin.

The first studies on the developing spleen were confined primarily to the early stages of development and were concerned essentially with the germ layer origin of this organ. The old controversy concerning the germ layers involved in the developing

spleen has apparently been settled, the generally accepted view today being that it is entirely of mesodermal origin (Radford, 82; Sabin, 83; Thiel and Downey, 84; Holyoke, 85; and Yasui, 86).

Other workers concerned themselves with the developing circulatory system of the spleen. Barta (87), in perfusion studies of human and calf spleens, demonstrated that the spleen in early stages of development has a closed circulatory system which is gradually converted into the open system of the adult. The change from the closed to the open system takes place in human fetuses of 20-25 cm. in length. Thiel and Downey (84), in a study of the development of the spleen in the pig embryo, found that initially there is a closed capillary network of endothelial-lined vessels but that an open circulation is established before the middle of embryonic life. Lewis (88), while studying the development of the circulatory system of the fetal rabbit by histiological sections and by the injection of monostral fast blue was able to show that from earliest stages the circulation in this animal is open.

Although there have been numerous investigations on the macrophagic or reticuloendothelial system of adult mammals, relatively few studies have been made on this system in fetuses and young mammals. Wislocki (10), in studies of newborn rabbits, has demonstrated by injecting India ink that there is phagocytic activity of the R.E. cells in the liver, spleen, lung and bone marrow.

Culbertson (89), after injection of trypan blue into rats, came to the conclusion that the phagocytic activity of young rats ("nursling animals") is much less developed than that of adult animals. His work was concerned primarily with the action of the Kupffer cells of the liver.

Italian investigators have contributed extensively to our knowledge of phagocytosis in fetuses. Battoglia (90) injected carmine into 5 dog fetuses through the umbilical arteries. Carmine collected in the pulmonary arteries but phagocytosis by alveolar phagocytes was not evident and the Kupffer cells segregated carmine granules only rarely. Dellepiane (91) injected colloidal mercuric sulphide into human fetuses of 3, 5 and 6 months gestational age and found phagocytosis in the liver and spleen beginning in the third month. Nizza (92), using Dellepiane's method studied human fetuses older than 6 months gestational age. He confirmed Dellepiane's findings and concluded that phagocytic activity was more marked in the liver than in the spleen. Bracco (93) compared the macrophagic potentialities of fetal placental blood and term babies and concluded that the premature child has less phagocytic potentialities.

The only other work that deals specifically with the development of phagocytic activity in the rat, besides Culbertson's (89) was that of Suzuki (94). After injection of Thorotrast he was able to identify the segregated thorium salt in two 21 day fetuses and

in one newborn animal. He also observed hemosiderin in two 19 day fetuses and one 21 day fetus (no specific stain for hemosiderin was used). The phagocytosis was restricted to the red pulp and was rarely found in fixed macrophages. Andrew (44,45) was unable to demonstrate pigment-containing macrophages in 21 day old animals (immature animals). He employed a specific stain for hemosiderin but could not show the presence of this material in the splenic macrophages of this age group. He also showed that the Malpighian follicles of these young animals are in the processes of formation and that no reaction centers (germinal centers) are present.

The purpose of this research was to make a histochemical and histological study of the developing rat spleen with special emphasis on the macrophage system. The study of the splenic macrophage system was made, as far as possible, using the three criteria as set down by Snook, Linford and Bache (35).

By employing the enzyme, metalophil and acid-ferrocyanide procedures it may be possible to trace the origin of the macrophage cells and to determine at what time in development certain members of this system appear, such as, the marginal metalophils and other intrafollicular cells. Also of interest will be the activity of these in relation to age changes. Will they be more or less active with advancing age? Our histochemical techniques should enable us to determine specific patterns of activity in the spleen and relate this to the

general morphology as determined by the histological techniques.

Morphological descriptions of the spleen will be given with emphasis on the appearance of Malpighian follicles and their secondary nodules, general nature of the red and white pulp, and some qualitative and quantitative information on the megakaryocytes which are so prominent in some spleens.

CHAPTER II

MATERIALS AND METHODS

A total of 318 albino rats of the Holtzman strain was used in this study. Some animals were obtained directly from the Holtzman Company while others were bred and raised in the Anatomy Department colony from Holtzman stock animals. The animals were divided somewhat arbitrarily, for convenience in description, into four groups according to age.

Group I comprises the fetal animals. There were 109 animals in this group. Two litters were used for each day studied. The age of the animals was dated from the time spermatozoa were found in vaginal smears. A male and female rat were caged together overnight, vaginal smears were obtained the next morning using mammalian saline. Wet mounts were prepared and observed with the light microscope. Those animals found to have spermatozoa in the smears were presumed pregnant and this day was considered as "day zero" in determining the age of the fetuses.

It was found in pilot studies and from the work of Suzuki (94) that spleens of animals younger than 17 days gestational age were extremely small and delicate and thus were not suited to this research. The fetal group therefore contained animals of 17 to 21 days gestation age.

Group II are the definitely immature animals. They range in age from the newborn to 21 days. Twenty-one days of age was taken

as the cut-off day for this group because, in our laboratory, this is the age at which the animals are weaned. A total of 165 animals was studied in this group.

Group III is composed of the very young and young adult animals. There were 35 animals in this group ranging in age from 22 to 270 days. This group was used primarily to trace out the development of specific structures until a definite adult form was obtained.

Group IV included 11 senile animals. All animals of this group were over two years of age. The addition of this group made it possible to study the process of senescence in the spleen thus enabling us to make a study of the age changes that are taking place at different stages of the rat's life cycle and relating these structural changes to possible changes in function. All adult and weanling rats were sustained on Purina Laboratory Chow and water ad libitum. The immature animals were sustained by the mother until 21 days when they were weaned.

Pregnant rats used to provide the fetuses were anesthetized with ether fumes at the determined times. Fetuses were removed from the uterine horns and the fetal spleens were removed with the aid of a dissecting microscope. These spleens were fixed whole in cold (0-4°C) 15% neutral unbuffered formalin (12-16 hrs.), Carnoy's fixative (3-6 hrs.), or 10% neutral buffered formalin (12-16 hrs.). The rats of the other three groups were also sacrificed with ether

fumes at the appropriate times. The spleens were removed rapidly and small pieces (2-3 mm.) of the tissue were fixed in the previously mentioned fixatives for similar lengths of time.

Sections from the cold formalin-fixed tissues were cut at 15 micra on the freezing microtome and free-floated in 1% formalin prior to staining. These sections were used for the following procedures: (1) the metalophil reaction, (2) tests for non-specific esterase content, (3) tests for acid phosphatase content, and (4) the Prussian blue reaction for iron.

Marshall's (25) silver impregnation method was used as outlined by him. The sections were removed from the 1% formalin, washed in three changes of distilled water, after which they were impregnated in the silver solution for 5 to 10 seconds. This silver solution was made fresh by titrating 2 ml. of fresh concentrated ammonium hydroxide against 10% silver nitrate until a slight cloudiness remains. This titration usually required about 20-23 ml. of the 10% silver nitrate solution. Following the treatment with silver, the sections were reduced in 3% formalin solution with agitation (usually less than 2 minutes). The sections were then washed in 2 changes of 95% alcohol, cleared in carboxylene and mounted on slides with permount. Some of the 10% formalin fixed, paraffin imbedded tissues were also impregnated with silver. This tissue was also cut at 15 micra on the rotary microtome, deparaffinized,

brought to water, stained and mounted as described above for the frozen sections.

Pearse's (73) modification of Gormori's technique was used for non-specific esterase. Naphthol AS acetate was used as the substrate and Garnet GBC was the diazonium salt used. The sections were removed from the 1% formalin solution, washed in distilled water and then incubated at 37°C in the substrate medium for 30 minutes. The incubation medium for non-specific esterase was prepared as follows. Eight mg. of naphthol AS acetate was dissolved in 1 ml. of acetone. One-tenth ml. of the acetone substrate solution was added to 10 ml. of sodium phosphate buffer at a pH of 6.8. This buffer contained 0.1 ml. of propylene glycol. Ten to 20 mg. of Garnet GBC dye was added to this solution. The medium was thoroughly mixed and then filtered. Following the incubation period the sections were transferred to distilled water, free-floated onto clean slides and mounted with glycerine jelly.

Burstone's (53) method was used to test for acid phosphatase activity. Naphthol AS-BI phosphate was used as the substrate and Garnet GBC as the diazonium salt. The sections for this procedure were transferred from the 1% formalin into distilled water, then incubated in the substrate medium at room temperature for 15 to 30 minutes. The incubation medium for the acid phosphatase reaction

consisted of 4 mg. Naphthol AS-BI phosphate, as the substrate, dissolved in 0.25 ml. of dimethylformamide. To this was added 25 ml. of 0.2 M sodium acetate buffer at a pH of 5.3. Thirty-five mg. of the diazonium salt, Garnet GBC, was added along with 2 drops of 10% $MnCl_2$ (activator). The medium was then stirred and filtered. The sections were placed in distilled water after incubation and mounted in the same manner as that described for non-specific esterase.

Control sections were used for both of these enzyme procedures to distinguish false-positive staining from genuine enzyme reactions. Substances in the splenic tissue, such as hemosiderin and ferritin, may be confused with positive staining reactions of the enzymes. Hemosiderin usually appears as a yellow to yellow-brown pigment and should be easily distinguished from the dark brown to red-purple reaction products of the enzymes, but if the enzyme activity is low, then some confusion may result. For this reason some sections from each animal were incubated in a substrate-deficient medium and these controls were used to better evaluate the results obtained from the enzyme techniques.

Gomori's (95) Prussian blue reaction was performed on both frozen and paraffin embedded tissues cut at 15 micra on the freezing microtome or the rotary microtome. The frozen sections were washed

in three changes of distilled water and then passed into the staining solution. The paraffin sections were first deparaffinized and brought to distilled water and stained. The staining solution for this procedure is made of equal parts of 20% HCl and 10% potassium ferrocyanide.

It is known that iron occurs in two forms in animal tissue. The first group are complexes in which iron is loosely bound to proteins and easily released by mild acid treatment to react as ferric iron. The second group are complexes in which iron is more strongly bound (masked iron) and cannot be released by mild acid hydrolysis. Hemosiderin is the best known representative of the first group and hemoglobin of the second. Part of the masked iron can be made reactive by more drastic treatment with "unmasking agents".

One unmasking agent used in this study was hydrogen peroxide as suggested by Gomori (96) and Pearse (56). The tissues were treated for 30 minutes with a 30% solution of hydrogen peroxide, alkalized with some sodium carbonate. After this treatment the tissue sections were washed in distilled water, then subjected to the Prussian blue staining solution. The other unmasking agent employed was 3% HNO_3 in 95% alcohol (Taylor, 97). After washing in distilled water the sections were placed in the acid-alcohol solution for 24-36 hours

at 35°C. The tissues were then washed in distilled water and treated with the acid-ferrocyanide solution.

All tissue sections treated with this reagent were rinsed in 2 changes of distilled water, dehydrated in 2 changes of 95% alcohol, cleared in carboxylene and mounted with permount.

Sections, 8 micra thick, from all of the specimens studied were stained with routine Harris' hematoxylin and eosin. In addition, some slides from 20 animals, representing the four groups studied, were stained with the Giemsa stain which was prepared as outlined by Wolbock (98). The routine hematoxylin and eosin stain was performed on Carnoy-fixed tissue and the Giemsa stain was performed on tissue fixed in 10% buffered neutral formalin. Slides prepared by these two procedures were used to study the cellular morphology of the spleen.

Sections from about 25 animals in groups I and II, which had been fixed in 10% formalin or Carnoy fixative, imbedded in paraffin and cut at 8 micra, were stained for reticular fibers and counter-stained with van Gieson by the method given by Snook (99). Tissue sections from these same animals were also stained by the Periodic Acid-Schiff procedure according to McManus (100). These latter two stains were used primarily to corroborate the findings of the other stains used in regard to the white pulp, marginal zone and red pulp of the spleens of fetal and immature animals.

CHAPTER III

RESULTS

The general microscopic appearance of the spleen, particularly the cellular make-up of the white and red pulp, presents certain definite qualitative differences in the various stages of this developmental series. The histology of the developing spleen will be described from tissues stained with routine hematoxylin and eosin, Giemsa and PAS. Special histochemical techniques were used to demonstrate the presence of the enzymes non-specific esterase and acid phosphatase; acid-ferrocyanide reaction was used to demonstrate iron and the silver impregnation of Marshall for the demonstration of the metalophil reaction. The histochemical findings will be given separately.

White Pulp and Neighboring Structures

In the earliest fetal material studied, 17 and 18 day gestational animals, lymphoid tissue has not yet appeared in the adventitia of the arteries. The arteries at this developmental level are very small and few in number. Beginning lymphoid sheaths are foreshadowed in some sections as a condensation of the primitive reticular cells around the arteries (fig. 2). These are large cells having oval to round nuclei with a stippled chromatin pattern, the cytoplasm is variable in outline and is usually colorless.

By days 20 to 21 of gestation lymphoid cells are arranged

around the arteries in definite concentric rings, usually 2 to 5 layers thick (fig. 3). These cells appear to be medium lymphoid cells and are definitely the beginnings of the early forms of the splenic white pulp. It should be pointed out that these developing sheaths are not abundant, sometimes only 1 or 2 in a section can be found.

At birth the white pulp is composed of 4 to 6 layers of medium lymphocytes (fig. 4). The sheaths were no larger in the three day spleen than in the newborn but by 5 to 7 days the lymphoid masses had grown to as much as 10 cell layers deep around the arteries. The lymph sheaths had grown to 20 cell layers in some sheaths by the 10th day with the artery still centrally located. During the period of 15 to 21 days the central artery has its characteristic eccentric position in the many layered follicle (fig. 5).

A marginal zone is slightly apparent around some sheaths in the immature animals. An occasional "slit" around some of these sheaths in the very young postnatal rats is observed which may represent the beginning of the marginal sinus. The marginal sinus is, however, not a definite finding until the 19th postnatal day and is consistently found only after the 30th day (fig. 6).

The secondary nodule was first seen in a 26 day old animal but this structure was more definitive at 46 days (fig. 6) and could be found with regularity in animals from this age to 270 days.

These structures were a variable finding in the senile animals, thus indicating a definite decline in the number of these centers with advancing age.

Phagocytic activity was the main function of the secondary nodules and for this reason the author considers them to be of the reaction center type. None of the animals examined showed the germinal type of secondary nodule although some appeared to contain questionable mitotic figures. In the youngest immature animals mitotic figures were observed periodically in the white pulp but these figures were wide spread throughout the nodule and could not be considered as constituting a true secondary nodule. The material phagocytosed by the cells of the reaction centers appears to be degenerated cells, probably lymphocytes (fig. 7). In the older animals, hemosiderin is also found in the cells of the reaction centers.

Animals varying in age from 46 to 270 days had follicular nodules of the "adult" or mature histological structure (fig. 6). They have an eccentrically placed "central" artery. The marginal zone, which was indicated very early in postnatal life and a definite feature by the twenty-first day of life, now appears to be more prominent. This prominence of the marginal zone is probably due to the fact that it is bracketed by the marginal metalophils and marginal sinus on its inner surface and a condensation of red pulp cells which circle the marginal zone laterally. The marginal zone

has attained its maximum width in group 3 and is composed of small cells which have light staining nuclei. They appear to be medium lymphocytes. Mitotic figures are occasionally seen in the marginal zone of some animals, and phagocytic activity in this area was never observed. The cell population appears to be less dense than that of the red or white pulps.

In a few of the animals studied, vessels which appeared to by lymphatics were found in the white pulp proper. These lymphatics were not closely approximated to the central artery but were somewhat removed and branches from them seemed to be directed toward the marginal sinus (figs. 8 and 9). Similar vessels have been previously described in the guinea pig, mole, mouse, horse and monkey by Snook (101). More recently Kellner (102) has given a rather complete description of lymphatics in the human spleen.

The marginal sinus hinted at in the immature rats is a distinct structure in animals of group 3. Again in the senile animals the marginal sinus, as is the case with other structures, is a variable finding. Erythrocytes were usually found in the marginal sinus and scattered throughout the marginal zone.

The spleens of the senile animals showed considerable variation in the size and make-up of the white pulp nodules. The amount of white pulp in these was greatly reduced and the nodules (follicles)

were indistinct. Earlier quantitative studies have established this fact for man (103, 104) and qualitative studies on rats, cats and rabbits (45, 105) are also in agreement with our findings.

The most prominent cells in the white pulp of the oldest animals studied are large phagocytic cells that are located around the central artery. These perivascular cells give the white pulp a "clear" appearance in the area of the central artery. These cells have relatively clear staining cytoplasm except for the inclusion bodies that are present. Some of these perivascular cells were also observed in the 270 days old animals of group 3. In senile animals these large cells are more numerous and are found in the majority of follicles (fig. 10). This general area appears to be one of degeneration and phagocytosis.

Another definite feature of the senile animal's white pulp was the greatly thickened walls of the central artery, thus markedly narrowing the lumen of that vessel and in some cases occluding it, (fig. 11). This could possibly be one factor in the appearance of the large perivascular cells in the senile animals. In some longitudinal sections of the arteries they appear very tortuous in their course (fig. 12,13), which is a change from the relatively straight condition found in the lymphoid follicles of younger animals. The narrowing and occluding of the central artery apparently

caused some arterial branches to hypertrophy and take on the appearance of being "supernumery" central arteries (fig. 12).

Red Pulp

The spleen of the 17 day fetuses was composed almost entirely of myeloid appearing tissue, that is, it is primarily a primitive type of tissue. The most prominent cells in these spleens were the developing fetal erythrocytes, mostly normoblasts, located in foci throughout the sections (fig. 14). These cells were considerably larger than similar cells which had been observed in adult bone marrow. Smaller foci of developing granulocytes, usually in groups of 2 to 6 cells, are also seen on occasion but the most abundant cell type was the primitive reticular cell forming the greater mass of the splenic tissue.

The reticular and PAS stains showed that the reticular net of the fetal spleen was of uniform dimensions. There is no sign of differentiation of the reticulum into fine, medium or coarse fibers as is observed in the adult spleen (fig. 14). The uniform thickness of the reticular fibers and their even distribution throughout the spleen gives the general reticulum pattern that is seen in adult bone marrow.

The fetal splenic sinuses are irregular channels which permeate throughout the general reticulum background. The pattern of the

lattice-like sinus reticulum as Snook (40) has described for the sinuses of the adult rat is non-existent in the fetus. These primitive sinuses are filled with fetal erythrocytes and normoblasts. The sinuses themselves are lined with flattened endothelial-like cells. There did not seem to be any set pattern to the arrangement of the fetal sinuses, they appeared simply to occupy the spaces in the meshes of the reticulum.

The reticular network has started to differentiate toward the adult pattern of fibers by late gestation (fig. 15). At 20 to 21 days gestation the first concentric fibers around the central arteries appear. These fibers are heavier than those that could be observed in the red pulp. In postnatal animals 7 to 21 days old, occasional fine reticular fibers can be seen outside the concentric rings of reticular fibers of the white pulp (fig. 16). These were considered to be the fine fibers of the marginal zone. The reticular and PAS stains failed to bring out the reticular fibers of the splenic sinuses and for this reason it was impossible to determine at what time in development these structures took on their characteristic "barrel-stave" configuration as described by Snook (40). The development of the splenic sinuses had to be followed using the hematoxylin and eosin and Giemsa-stained tissues.

By the twenty-first day the sinus shape was of the "cucumber" variety as described by Knisely (106). Although "adult" in form,

the simuses were not plentiful enough to consider these spleens as simusoidal in type (fig. 17). After the twenty-first postnatal day the simuses gradually become more numerous until the simusoidal type of spleen is reached. Some animals had developed this type of red pulp patternby the 45th to the 125th day, but it is only in the 270th day and senile animals that the red pulp becomes primarily of the simusoidal type.

Only an occasional megakaryocyte was seen in the earliest fetal material studied but in the older fetuses, 19 to 21 days, they became more prominent and their number continues to increase up to the 26th postnatal day. Their number remains fairly constant until the 36th day when they start to decrease in number. This decrease continues until the 270th day when very few megakaryocytes were found, as few as 6 per 30 high power microscopic fields. A slight increase in the megakaryocyte count was noted in the senile group. The numerical results of the megakaryocyte counts are plotted on a graph (fig. 1). These cells are often in groups of 5 to 8 as well as being scattered singly throughout the red pulp (figs. 18 and 19).

There is a great diversity in the nuclear pattern of the megakaryocytes, varying from a single spheroidal, light-staining nucleus with one or two nucleoli to a multilobed irregular, dark-staining nucleus without any visible nucleoli. Between these two

extremes are multinucleated cells whose nuclei appear separated from each other and stain like those megakaryocytes which have a single nucleus. Most of the apparent multinucleated cells could be explained on the basis of tangential sections. By tracing the cell in serial sections the "separate" nuclei can be seen to be connected to each other as illustrated by Hamre (107), his figure 5, and by Copenhaver, (108), his figure 7-10. There are some multinucleated cells that are observed which cannot be explained by tangential cuts, these will be considered more fully in the discussion section.

Megakaryocytes were also seen to undergo mitosis, especially in the early postnatal animals (figs. 20 and 21). No sequence of mitotic events ranging from prophase to telophase was observed in the megakaryocytes, however, chromosome arrangements comparable to a "multi-metaphase" are seen. It is obvious from figure 20 that the number of chromosomes is greatly increased as compared with mitoses in other cell groups in the spleen. In figure 20 illustrating megakaryocyte mitosis another cell type can be seen with a "typical" mitotic figure. By comparing the two mitotic cells it is easy to see that the megakaryocyte mitosis is of the polyploid variety.

From the earliest specimens studied until about the 45th to 51st postnatal day, the most striking characteristic of the red pulp, besides that of the megakaryocytes, was the many foci of developing erythrocytes (fig. 22). These erythropoietic centers

of the spleen contain red blood cells in various stages of development of which the normoblast was the most obvious. Occasionally foci of developing erythrocytes are found in older animals but these are infrequent. Some foci of developing granulocytes are seen in animals throughout the developmental series but these are never abundant.

Capsule and Trabeculae

The capsule of the fetal spleen is composed of a single layer of low cuboidal epithelial cells (fig. 23). No signs of trabeculae are present. This is the general condition in all fetal material examined. The newborn animal exhibited a capsule that was two cell layers thick. The outer layer is squamous epithelium (mesothelium), and the inner layer is made up of elongated connective tissue cells (fig. 24). Delicate filaments of the connective tissue were seen projecting from the capsule into the red pulp; these of course are the beginnings of the future trabeculae. The capsule has thickened noticeably and the trabeculae are readily observable in the red pulp of animals 10 days old. From the 15th to the 21st day the capsule and trabeculae become more prominent. The reticular stain shows that reticular fibers now make up the greater part of the capsule and that the trabeculae extend considerable distances into the red pulp. In studying the Giemsa and hematoxylin and eosin stained tissues, it is evident that smooth muscle cells are now a constituent of the capsule and trabeculae (fig. 25). In animals older

than group 2, the thickness of the capsule and trabeculae are variable from one animal to another. Some have very thick capsules while others have capsules not much different than the 21 day old animals. Trabeculae are numerous and prominent in a few specimens but in others they appear thin and sparse.

The most obvious change in these structures in old age is the wrinkling of the splenic capsule. This change is especially well illustrated in the reticular stained tissues (figs. 26 and 27). The fibers in the capsule and trabeculae are densely packed together and their path is much more tortuous than is found in younger animals.

Trabeculae of the senile animals are more numerous than in the other 3 groups observed, and their structure was similar to that of the capsules seen in this group.

Observations From Histochemical Techniques

It has been demonstrated by Pettersen (52), for the spleen, and by Hillman (109) for the brain, that the same cell types are demonstrated by the metalophil, non-specific esterase and acid phosphatase reactions. This is well illustrated in figures 5 through 10 of Pettersen's paper and figures 43 through 46 of Hillman's dissertation. These same procedures were used in the present study, and although they were never used on the same section as the two investigators mentioned above have done, it was evident that throughout the entire growth series that the cellular pattern

demonstrated by these 3 procedures closely parallel each other. In addition, the acid-ferrocyanide method for the demonstration of iron also produced results in the adult animals similar to those of the enzymes and silver impregnation reactions.

The results of these four histochemical techniques, that is, metalophil reaction, non-specific esterase, acid phosphatase and acid-ferrocyanide reaction, are given separately.

Prussian Blue Reaction

Splenic tissue from the animals of groups 1 and 2 was found not to store hemosiderin. This finding was based on the negative results obtained when the acid-ferrocyanide reaction was performed on these tissues. Even after treating the tissue from the fetal and immature animals with the unmasking agents described in the section on materials and methods, a positive Prussian blue reaction was unattainable in these two groups of animals.

The first animal to show the presence of hemosiderin by the method employed was a 51 day old female (fig. 28). In this case the final reaction products were in the cytoplasm of red pulp cells. The majority of these hemosiderin containing cells were massed near the splenic capsule in an area that had numerous sinuses. As the age of the various animals increased there was a corresponding increase in the number of cells containing hemosiderin (fig. 29).

This was a qualitative observation. From the 51st day on throughout the rest of group 3 the cells positive for hemosiderin were almost exclusively confined to the red pulp cords. There were a few iron containing cells found occasionally in the white pulp. These were mostly confined to the reaction center area with an occasional positive cell in other parts of the nodule (fig. 30). In the oldest animals of group 3 (270 days), other very large cells containing iron were sometimes found surrounding the central artery. In the "old age" or senile animals of group 4 these large perivascular cells were a frequent and characteristic finding (fig. 10). It is believed that these large cells are the same ones that have previously been described in these results from the hematoxylin and eosin stained sections.

At the edge of the white pulp nodule there was a ring of cells that usually gave a very intense reaction for hemosiderin in all animals of group 4. This ring of hemosiderin containing cells was first observed in some of the older animals of group 3 (fig. 31). In most of these, the rings were only partial, whereas in the senile animals it was not uncommon to find complete rings of Prussian blue positive cells. The reaction in these cells of group 3 did not have the intensity of those observed in the animals of group 4 (fig. 32). The position of these iron containing cells corresponds very

closely to the marginal metalophils as described by Snook (34) for the rat.

At no time in the development of the rat spleen was iron ever observed in the sinus-lining cells. Another group of cells that was consistently free from hemosiderin were the cells of the marginal zone.

The macrophages of the red pulp cords store the majority of the iron brought to the spleen. As the age of the animal increases some cells in the reaction centers and around the central artery also segregate hemosiderin and lastly, in the oldest animals, the marginal metalophils take in iron and store it.

Non-Specific Esterase

The development of enzyme activity in the spleen was studied starting with the 17th day of gestation. At this time there was a diffuse reaction throughout the splenic tissue for both the non-specific esterase and the acid phosphatase enzymes. Hillman (106) observed a similar reaction in brain tissue of fetal rats for these two enzymes and attributed it to a general enzyme activity occuring in the tissue fluid.

In the early fetal specimens the number of cells giving a non-specific esterase response were few, sometimes less than 10 in a section (fig. 33). Those cells containing the final reaction product were round to fusiform in shape and had oval to round nuclei.

The diffuse enzyme response made it difficult to determine if the primitive sinus-lining cells were positive for esterase or not. It was finally determined that they contained little or no final reaction product and could be considered negative or only slightly reactive for this enzyme.

The round or fusiform cells that did give a positive response for this enzyme were thought to be primitive or potential macrophages. These cells were usually located in the central areas of the sections, very few were found near the periphery.

In the late gestational animals the diffuse background granules of final reaction product had greatly diminished (fig. 34). The developing white pulp was outlined by the now more numerous positive responding cells of the red pulp (fig. 35). An occasional, rounded, enzyme containing cell was found in the developing white pulp.

The newborn animals had the same general esterase pattern as that of the late fetal specimens. About day 5 the white pulp was more obvious and the number of follicles had increased but there were still relatively few positive reacting cells in the nodules. The endothelium of the central artery was also observed to give a slight positive response to the non-specific esterase reaction. The positive cells of the red pulp were numerous. Their shape was generally of the rounded or stellate type. The megakaryocytes were

also strongly positive for non-specific esterase. The sinus lining cells were now slightly positive for esterase.

There was at this time an evident condensation of esterase-containing cells around the periphery of the developing sheaths, that is, at the marginal zone - red pulp junction. These perimarginal cells gave a somewhat more intense response than the rest of the cells of the red pulp.

There was not much change in the enzyme activity of the spleen until the 20th postnatal day. At this time there was seen an occasional hint of a ring of cells at the outer margin of the white pulp (fig. 36). This ring of cells appeared somewhat diffuse and it suggested that these cells were condensing from the interior of the white pulp to the periphery of the nodule (fig. 37). Along with this "hint" of a marginal esterase-containing ring of cells there also appears an irregular marginal zone which is generally free of positive esterase reacting cells.

The marginal ring of esterase containing cells was more evident by the 35th to the 40th postnatal day (fig. 38). These cells do not give an intense response but the presence of the final reaction product is constant at this stage of development.

For the animals from the 40th to the 81st days the non-specific esterase containing cells varied in form from oval to stellate,

with the majority of them located in the red pulp cords. Esterase reactive cells were found scattered throughout the lymphoid nodules, with the reaction centers having the greatest density of these cells (fig. 39). The marginal ring of esterase-containing cells located at the level of the marginal sinus and on the nodular side of it was more definite in their response than in the younger animals. It was still weak in activity when compared to the cells in the red and white pulp. This ring of cells was described by Pettersen (52) as "a faint thready rim of final reaction product," which is an apt description of these nodular esterase positive cells when viewed under low power of the microscope. These perinodular cells constantly outlined the marginal sinus. Beyond this sinus is the marginal zone which is generally free of esterase-containing cells. Immediately beyond the marginal zone is observed the densely packed intensely reacting perimarginal cells. At this stage of development these cells are probably more intensely reactive than any other cells in the spleen (fig. 39).

Large esterase reactive cells were observed in the lymphoid nodules of animals 122 to 270 days old. These cells were seen either near the central artery or at the level of the reaction center (fig. 40). The appearance of these cells is not a consistent finding in this age group and it is not uncommon for them to be entirely absent from the tissue sections.

The spleens of the senile animals present a striking enzyme picture. The cells that contain esterase are in the same general pattern as seen in younger animals but the final reaction product is much denser in the cells, especially the perinodular cells, than observed in the younger animals. A consistent finding in the old animals was the large esterase positive cells located around the central artery and a few large cells in the reaction center, when it is present (figs. 10 and 41). As mentioned above there was a noticeable increase in the enzyme activity in cells at the level of the marginal sinus, that is, in the perinodular cells. This indicates a maturation of these cells from potential macrophages to "mature" macrophages. The marginal zone in this group of animals was also generally free of enzyme containing cells. As in the younger animals the cells at the edge of the marginal zone (perimarginal cells) form a ring of intensely reacting cells around the follicles. The enzyme activity in these cells again appeared more pronounced than in other cells of the red pulp.

Throughout the entire developmental series the sinus-lining cells gave a slight or no esterase reaction.

In summary, the esterase activity in the spleen and the number of cells containing this enzyme increase with the advancing age of the animals.

Acid Phosphatase

Acid phosphatase containing cells were distributed in essentially the same pattern as the cells containing esterase (figs. 42, 43, and 44). There were, however, some differences. The number of acid phosphatase containing cells were more numerous, especially in the nodules. In the red pulp of the fetus there were obviously more cells containing acid phosphatase than esterase (figs. 35 and 43).

With the appearance of the perinodular ring of cells (marginal metalophils), these cells were very reactive for acid phosphatase (fig. 45). The final reaction product of these cells approached the intensity of activity of cells seen in the red pulp cords. The perimarginal ring of cells, those at the marginal zone - red pulp junction, which were very active for esterase were not as prominent with the acid phosphatase technique.

In the fetal animals the sinus-lining cells were only slightly positive for the acid phosphatase. This slight activity is not observed in any postnatal animals.

The marginal zone was free generally of acid phosphatase activity as it was for the esterase. The red pulp appeared to contain more cells with acid phosphatase than those with esterase (figs. 46, 47, 48 and 49). Megakaryocytes were also seen to give a uniform positive response for this enzyme.

Metalophil Reaction

Marshall's metalophil procedure demonstrated cells in patterns similar to the two enzymes described previously and also similar to the cellular response demonstrated with the acid-ferrocyanide technique in mature animals.

In all stages of development the metalophil reaction demonstrated a greater number of cells than the other three methods employed. This was the case in both the red and white pulp. The marginal metalophils, as described by Snook (32), were especially prominent in the mature animals.

The metalophil impregnation of the fetal tissues at first appeared to be an unrecognizable mass of impregnated cells. However, upon further observation it was seen that the sinus-lining cells were deeply impregnated, in many cases completely obscuring the erythrocytes within the sinuses (fig. 50). The rest of the fetal sections were packed with mostly rounded cells or cells with blunt pseudopodia. The impregnation of these cells was so complete that it was impossible to see nuclear configurations. The entire section had a fine black granular deposit over it. This granulation becomes much less toward the end of gestation. It was also noted in the group 1 animals that the epithelial cells forming the splenic capsule were outlined by what seemed to be an intercellular impregnation.

The general pattern of the silver impregnated cells was similar

to that of the two enzymes (figs. 51 and 52). The most important difference was that the metalophil reaction always demonstrates more cells than are shown by either enzyme or the acid-ferrocyanide procedure. This difference becomes much less in the senile animals where the esterase, acid phosphatase activity and the storage of hemosiderin has increased.

The early white pulp nodules are generally free of metalophil cells but with the development of these structures there is a gradual increase in the number of metalophil cells contained in them, (figs. 51 and 53). The marginal metalophils become evident by the 25th postnatal day and appear to be a condensation of cells on the nodular side of the marginal sinus (fig. 52). These marginal metalophils are somewhat rounded with blunt psuedopodia whereas the cells in the main nodule proper are stellate (fig. 54). In the senile animals large cells in the nodules were also impregnated. These large cells compared favorably in size and position with those which gave positive responses to the two enzymes and the Prussian blue reaction (fig. 55). These cells are, of course, the large cells of the reaction centers and the large perivascular cells of the central artery.

As reported above the sinus-lining cells of the fetal animals were blackened by the metalophil reaction but from early postnatal life these cells were rarely if ever impregnated.

CHAPTER IV

DISCUSSION

The only previous publications dealing specifically with the age changes in the spleen of the rat are those of Suzuki (94) and of Andrew (44,45). Suzuki's (94) work dealt with the developing of phagocytic activity in the reticuloendothelial system from fetal stages to 70 days postnatal. Along with the main theme of phagocytosis he gave some histological descriptions of the fetal and newborn rat spleens. For the most part, his description of the splenic histology for these stages corresponds generally to that observed in the present study. Andrew (44, 45) studied the histological changes in the spleen as produced by the aging process. He had a group of 21 day old animals and the rest ranged in age from 50 days to senile animals (over 762 days).

Neither of these studies covered a developmental series from fetal through senile ages as has been done in this research, nor did they do any enzyme histochemistry. Andrew did do a Prussian blue reaction for iron. Along with the histological changes, the histochemical findings will be discussed for the various structures of the different age groups.

Sinus and Sinus-lining Cells

Apparently Suzuki (94) had difficulty in determining if the fetal splenic sinuses were lined with stellate or flattened endothelial-type cells. This difficulty seemed to be due to the

masses of red blood cells in the sinuses, quoting from his paper--- "these blood cells made the sinusoidal lining cells difficult to observe." At another point he mentions finding, "occasional stellate macrophages lining sinusoids." In our study of the Carnoy-fixed tissue stained with routine hematoxylin and eosin, the sinus-lining cells were obvious because the fixative had caused hemolysis of the erythrocytes so that the structure of the fetal sinus was not obscured. The sinus-lining cells in all specimens were found to be of the flat endothelial type, from fetal through senile animals.

The electron microscope has recently revealed that the littoral cells in the splenic red pulp of the rat are of 2 distinct types and a third type that is an inter-grade between the first two. The work by Galindo and Freeman (110) delineates one cell form that has no phagocytic activity and the other with phagocytic capabilities. These two types of littoral cells were found to be distributed at random. Roberts and Latta (111) were also able to classify the sinus-lining cells of the rabbit splenic red pulp into 3 types. Weiss(78), on the other hand, with the electron microscope could only see one type of littoral cell in the rabbit splenic red pulp. He described these as elongated reticular cells which are attached to the basement membrane, but free of attachments to adjacent cells. It is obvious from the above discussion that there is some difference of opinion as to what type or types of cells line the splenic sinus,

but most investigators agree that there are flat elongated cells forming at least part of the sinus wall. Whether other cell types are present will have to be left to the skills of the electron microscopists.

In a number of publications, Weiss (80,81,112) stated that Billroth's cords were nothing more than collapsed splenic sinuses. This same view is held by Galindo and Freeman (110) and to some extent by Roberts and Latta (111). Weiss (78,113) changed his hypothesis to state that the channels and walls still existed but the "cord channels" could be identified from "sinal channels" by a different type of lining cell. He described the "cordal" lining cell as having long cytoplasmic projections and the "sinal" lining cells as being elongated without these cytoplasmic projections.

Histochemistry offers a solution to this problem, at least as far as the rat is concerned. It will be recalled that Snook, Linford and Bache (35) have postulated that in order for a cell to belong to the macrophage or R. E. system it had to give a positive response for the enzymes non-specific esterase and acid phosphatase as well as being phagocytic and blackened by Marshall's metalophil reaction.

These sinus-lining cells have consistently failed to give a response to either of the above named enzymes. Dorfman (71) (72),

using alpha-naphthyl acetate was unable to demonstrate any esterase activity in the littoral cells. Pettersen (52) (68) used both alpha-naphthyl acetate and naphthol AS acetate for the esterase and naphthol AS-BI phosphate for the acid phosphatase. The author also employed these two procedures for this research. The sinus lining cells were found to give only a slight response for the esterase and were completely negative for the acid phosphatase.

The present investigation and that of Pettersen (52) and Marshall (25) have shown that these littoral cells are not always impregnated with silver and when they are it is only to a slight degree. The only exception to this is in the fetal material where the littoral cells are strongly impregnated by Marshall's metalophil reaction.

The phagocytic activity of these cells is extremely variable. Suzuki (94) was unable to find any thorium in the littoral cells after injecting thorotrast into fetal and newborn animals. Baillif (46) found this to be the case even in the adult rats. Pettersen (52) observed no phagocytic activity on the part of these cells even after splenic stimulation with typhoid vaccine and adjuvant. He did, however, see increased macrophage activity in the white pulp. As given in the results the sinus lining cells were consistently negative for hemosiderin. Snook (75) has demonstrated that the littoral cells

can sometimes be "primed" to take up a substance. Thus, after a number of injections over a period of 2 to 3 days, he observed that these cells would segregate iron and chlorazol black E but not India ink. Moore et al (77) were able to get the sinus-lining cells to phagocytose iron after repeated injections. Dorfman (72), on the other hand, failed to observe any phagocytic activity by these cells in normal rats or in humans suffering from lymphomas, granulomas or leukemia.

The cells of the pulp cords in Dorfman's (71, 72), Pettersen's (52,68), and according to our results always gave positive non-specific esterase and acid phosphatase responses. These cells were also impregnated with silver and segregated hemosiderin. See chapter on results and Marshall's monograph (25).

Roberts and Latta (111) believed that the same cell types line the splenic sinuses as fill the red pulp cords. Weiss (113) also believed this at one time but has since changed his opinion. If this were true one would expect that these cells would respond in the same way to the various histochemical treatments. As is readily seen from the above discussion, this is not the case. If the Billroth cords were collapsed splenic sinuses as Roberts and Latta (111) and Galindo and Freeman (110) still believe, then, again one would expect the cells of the splenic cords to respond to the histochemical technique in a similar fashion as do the cells lining the

the splenic sinuses, but this is not the case.

Through the use of histochemical techniques it is easy to see that the cells of the splenic cords and those lining the splenic sinuses are different and therefore it is reasonable to conclude that Billroth's cords are not collapsed splenic sinuses as has been suggested by some.

In addition to this it is also apparent that the sinus-lining cells do not fulfill the criteria as set forth by Snook, Linford and Bache (35) and therefore cannot be considered as true macrophages as far as the rat is concerned. It has been shown by Pettersen (52) and by Dorfman (72) that these cells fail to become active macrophages even after stimulation and for this reason it is questionable if they can be considered even as potential macrophages.

It is true that the littoral cells were strongly metalophilic in the fetus but this metalophilia became greatly diminished or absent in postnatal life. This investigator has no explanation for such a finding, other than the fetal material simply reacted differently to the silver impregnation due to it's primitive nature. Whether these cells are potential macrophages in the fetus is a possibility, but it should be recalled that Suzuki (94) was unable to get these cells to pick up injected thorotrast and therefore it appears that even in the fetus these cells are not true histiocytes.

It is difficult to reconcile the negative or very slight enzyme, metalophila and acid-ferrocyanide responses in sinus-lining cells of the rat spleen with the fact that these cells are supposed to be members of the reticuloendothelial system. In other animals, such as the rabbit, these cells are clearly members of the R. E. system (77,79,114). In the rat it would appear that the littoral cells have differentiated into more specialized lining cells, possibly approaching true endothelial cells. Maximow (115) believed that macrophages could differentiate into fibroblasts or endothelial lining cells but the reverse is not possible. It is easy to speculate that this is what has happened in the case of sinus lining cells of the rat spleen. Perhaps these cells are highly specialized macrophages which become active only when acted upon by very specific stimuli. Further work is certainly necessary to resolve the question of the true nature of these cells.

Marginal Zone and Sinus

The marginal zone surrounds the Malpighian follicles. It is composed of cells which usually stain lighter than those of the white or red pulp. This area is devoid of sinuses and contains only an occasional macrophage. Some mitotic figures have been observed in this zone.

Krumbhaar (116) seems to have been the only one that has done a study exclusively on the structure and function of the

marginal zone. Most of his material was routine paraffin sections stained with hematoxylin and eosin. These were augmented by fresh splenic imprints stained with Giemsa. He concluded, after consulting with several colleagues, that the cell type found here were lymphocytes. Andrew (44,45) had arrived at a similar conclusion 2 years previous to this. Baillif (46) also believed these cells to be lymphocytes.

Snook (32) has shown that the white pulp capillaries empty into the marginal sinus. From the sinus the blood passes into the marginal zone and then into the red pulp. MacNeal (42) thought that "----this marginal zone about the follicle is the most important part of the lobule, as far as action on formed elements of the blood is concerned. Here, phagocytosis of worn out blood cells and foreign matter, such as microbes, occurs most abundantly." Lillie, cited by Krumbhaar (116), had this same idea for he stated, "It (marginal zone) is often the principle site of hemosiderin accumulation." Pettersen (52) and Snook (32) have found only an occasional metalophile cell in this area and the author never observed any hemosiderin containing cells in this zone. It is of interest that non-specific esterase and acid phosphatase positive cells are for the most part absent from the marginal zone. On the basis of these findings the author agrees with Snook (32) that some other function than phagocytosis may occur in the normal functioning

of the marginal zone of the rat.

Separating the marginal zone from the white pulp proper is the marginal sinus. This space has previously been described by many investigators and given a variety of names. Andrew (45) called it an intermediate sinus and mentioned that it frequently surrounded the entire follicle. He found that it was lined with endothelial cells. Altschul and Hummason (43) used perifollicular space to describe this sinus and Baillif (46) called it the marginal sinusoid. Snook (32), in his most recent publication, called it the marginal sinus and gives a rather complete picture of its structure. He described it as consisting of a series of anastomosing spaces lying between the white pulp proper and the marginal zone. Follicular capillaries usually empty directly into the marginal sinus. It would be expected that erythrocytes should normally be present in this space and this was the case in our research. In practically all spleens showing a marginal sinus erythrocytes were found to be present in this sinus. This latter finding is contrary to that of Andrew (45), ---"Usually, no red cells are seen within this sinus." It is difficult to reconcile Andrew's finding in the light of Snook's (32, 40) description of the marginal sinus with follicular capillaries emptying into it and the fact that red blood cells were so obvious in this sinus as our results have shown.

White Pulp Lymphatics

A most interesting but infrequent finding was the appearance of lymphatic vessels in the white pulp nodules. Of all the tissue sections viewed these lymphatics were seen in about 6 specimens. Snook (101) has described these structures in detail for the guinea pig, mole and mouse. He described them as following the white pulp artery and opening into the lymphatic vessels of the hilus. For the horse he was able to trace these vessels into the trabecular and capsular lymphatic plexuses.

In the few animals that we saw these lymphatics it was impossible to trace them to their terminations. A personal communication from Snook revealed that he had also observed these occasional lymph vessels in the rat spleen but could not trace their course satisfactorily. Kellner (102) has recently reported finding lymphatic channels in white pulp of the human spleen. He described four ways in which the lymph can flow away from the spleen; periarterial, perifollicular, intra-follicular and pretrabecular. The most frequent course was found to be the latter because it is the shortest. Concerning the intrafolicular lymph vessels, he found that these parallel the central artery and he believed that they carry lymphocytes away from the reaction center as well as drain lymphatic fluid from the follicle.

The lymphatic vessels observed in the splenic white pulp

of the rat were not as close to the central artery as those described by Snook (101) for the guinea pig, his figure 1 or by Kellner (102) for the human, his figure 16. These two researchers were able to trace these lymphatics to the hilus of the spleen. For the rat it was impossible to describe any pattern for these vessels. They apparently are not aswell developed in this animal as in others. The study of deep lymphatics of the spleen deserves and warrants further study and is a challenge for future research.

Reaction Centers

The secondary nodules first appeared in the splenic nodules at about 26 days. This is about 2 weeks earlier than Andrew (44,45) first observed them. He, of course, did not study any animals between the ages of 21 to 50 days. This time period is a transitional period in the rat life cycle. As pointed out in the results it is during this time that the maturation of many splenic structures takes place.

These secondary nodules become a consistent and conspicious structure in animals from 50 to 270 days. They are large and well developed in this age group while in the senile animals they may be absent. The main cells in these centers are large phagocytic cells which generally contain tissue debris, possibly pyknotic lymphocyte nuclei and in many cases hemosiderin. They were

impregnated by Marshall's metalophil reaction and gave strong enzyme response for non-specific esterase and acid phosphatase. For these reasons the centers are considered as being reaction centers as opposed to germinal centers. None of the animals examined showed the germinal center type of secondary nodule, some appeared to contain mitotic figures but their presence could not be positively identified. Even in those centers that may have continued mitotic figures, the large phagocytes were still the most striking feature of the secondary nodule. Our results of studies on normal rats agree with those of Andrew (44,45), that the process of phagocytosis was more prominent than the lymphocytopoietic activity in these nodules. For our material, at least, it would seem that the term reaction center would be more appropriate than germinal center.

Because of the controversy that has been carried on over whether the secondary nodules are "reaction centers" or "germinal centers", it might be well to review some of the literature pertaining to this subject and relate it to our findings.

Latta (117,118) considered the lighter staining areas of the lymphoid follicles as areas of cellular degeneration rather than of cellular proliferation. He based his conclusions on the fact that the central artery is never found in the secondary nodule and that the capillary supply to this area is also much less than to other areas of the follicles. This poor vascularity causes a

deficiency in the nutritive supply which lowers the metabolic rate to the point that degenerative changes take place in the lymphocytes of this area. He felt that these centers were points of lowered resistance and could possibly become foci for infection. In this respect he compared the secondary nodules to the process of caseation necrosis as seen in tubercular nodules.

Cannon (119) suggested that the secondary nodules are areas reacting to pathological stimuli rather than centers for the active production of lymphocytes and preferred the term reaction center to germinal center. He used all human material, much of it coming from people who had had acute or chronic infections, leukemia or hypertension. His results, therefore, cannot be considered the normal finding.

West (105) thought that the centers were regions of active lymphocyte production but that at the end of a cycle of production and outward migration of cells a diffuse condition of the center occurs because of faulty nutrition due to its remoteness from blood vessels. He concluded that "the germinal center---is both a region of proliferation and cellular disintegration, the fate of any particular cell or group of cells being determined by the proximity of the vascular supply."

Conway (120, 121, 122) also ascribed to the idea of a cyclic activity normally occuring in these centers. She stated,

"In any series of normal animals a cycle of growth, maturation and regression of lymphatic nodules --- can be found." The primary function of the center, as far as she was concerned, was that of lymphocyte production and only secondarily is it a reaction center.

The idea of a cyclic activity of the secondary nodules is the view most commonly given in the histology textbooks; see Bloom and Fawcett (123) and Copenhaver (108). The germinal center phase is considered by these authors to be the most important process while the action of phagocytosis is considered secondary.

Conway (121, 122) has emphasized that the only time the reaction center becomes the primary phase of the cycle is during disease conditions and that this is not the case in normal tissue.

The present research and that of Andrew (44, 45), all done on normal rats, contradicts Conway's theory. In both of these studies the most prominent feature of the secondary nodule was that of phagocytosis with cell proliferation being secondary. Pettersen (52) did not see any striking phagocytic activity in the nodules of his normal control animals nor was mitotic activity predominant. He concluded that it would be reasonable to accept both observations as being part of the function of the secondary nodules, because both these activities are occuring side by side. For the enzymes and the metalophil reaction, he found that rounded, enlarged cells were

rarely seen in the nodules of his untreated rats. As we have stated previously in this discussion, the large cells of the reaction centers gave a very definite response to non-specific esterase, acid phosphatase and the metalophil reaction.

It is difficult to reconcile Pettersen's findings with the authors and that of Andrew (44, 45) but it is the author's conviction, based on the present research and that of Andrew (44, 45), that in normal rat spleens the primary function of the secondary nodule is that of degeneration with a secondary function function of lymphocyte production.

Phagocytic Activity

Phagocytic activity of the spleen, as based on the presence of hemosiderin, does not become manifest until about the 50th postnatal day. At this time in development the amount of pigment present is more scanty than abundant and it is confined to the macrophages of the red pulp. Andrew (44, 45) also found this to be the case in his study of age changes in the spleen.

The phagocytosis of other cellular debris is evident much earlier than this, although it is not a common finding. It will be remembered that the reaction centers were first observed at 26 days and so this is the earliest period at which active macrophages are observed in the white pulp. As far as phagocytic activity in the red pulp is concerned it was not seen in fetal

or immature animals. Andrew (44, 45) did not observe any active macrophages in his 21 day old animals and only a few in his 50 day animals. Suzuki (94) was able to demonstrate active macrophages in the fetal and immature animals only after the injection of thorotrast. He reported that phagocytic activity in fetal spleens was minimal. Of 136 fetuses injected he found only 5 where macrophages had segregated the thorium salt. Up to 7 days postnatal only occasional macrophages in the red pulp contained thorotrast and, although the sinus lining cells were clearly visible at this time, he did not find thorotrast in any of them. A continuous increase in the number of active macrophages was observed with increasing age until an "adult" activity was reached, which he considered to be between 40 to 70 days of age. Culbertson (89), after injecting trypan blue into rats of different ages, concluded that there is a difference in the capacity for phagocytic function between young and old rats. Nursling rats, 6 to 21 days old, were less able to phagocytose particles of trypan blue than macrophages of older animals. He believed as Suzuki did that the phagocytic activity levels off at about the age of 40 to 70 days. The work of Andrew (44, 45) indicates that phagocytic activity of the splenic macrophages continues to increase up to and including old age. His table 4 shows a very definite increase in the number of active macrophages visible in spleens of animals ranging in age from 50 days to 1170

days of age. Our qualitative findings based on the up-take of hemosiderin by splenic macrophages also indicate that phagocytic activity increases with advancing age.

The histochemical techniques employed in this study also support the contention that phagocytosis on the part of the spleen, does not reach its peak until old age. It will be recalled from the results that the number of enzyme containing cells and the intensity of the enzyme response in many of these cells increased with advancing age. Markert and Hunter (124) made zymograms of esterases using starch gel electrophoresis on homogenates of various organs from mice of different ages. They demonstrated that embryos gradually acquired their enzyme systems as they developed. They were able to identify more than 10 different esterases in splenic tissue. In a previous study Hunter (125) did a study of the distribution of esterase in mouse embryos using histochemical techniques. He was able to show that embryos have no enzyme activity but by the fetal stages (17 days gestation) esterase activity becomes apparent.

Based on the acid phosphatase, non-specific esterase, acid-ferrocyanide and metalophil reactions, the changes in the phagocytic activity brought about by increasing age may be summarized as follows: (1) the number of reactive cells in the red pulp was increased and the intensity of the esterase response, especially in the perimarginal cells (cells at the marginal zone - red pulp junction),

was also increased; (2) large phagocytic cells were present in the reaction centers; (3) in the older animals of group 3 and in the senile animals large perivascular cells developed around the central artery; (4) the marginal metalophils increase in esterase activity and the uptake of hemosiderin in old age. If Snook, Linford and Bache (35) are correct in stating that a full-fledged macrophage should be metalophilic, phagocytic and contain the enzymes acid phosphatase and non-specific esterase, then many of the cells that are metalophilic and contain acid phosphatase do not fulfill the other two criteria until the age of senility, at which time they do become mature macrophages.

It is obvious from the discussion and the results that phagocytic activity is almost non-existent in the fetal animals, but the number of active macrophages becomes a more common finding with increasing age. Macrophage activity is observed earlier in the life-cycle of the rat only after an injection of some particulate matter has been made. In normal rats the first sign of phagocytic activity was seen in the reaction center of a 26 day old animal. These large macrophages appeared to have engulfed remnants of disintegrating cells. In the red pulp the first definite sign of active macrophages was seen in a 51 day old rat which had segregated hemosiderin. Some free cells which may have been active macrophages

were seen in sinuses of some younger animals but their identity could not be positively ascertained.

Marginal Metalophils

The marginal metalophil cells of the rat were first described by Pellegrino and Iraldi (31) in 1946 and later in more detail by Snook (33, 34). These cells are demonstrated by Marshall's ammoniacal silver nitrate method of impregnation. They form a dense aggregation of rounded and slightly-branched metalophilic cells found bordering the white pulp. Snook (32), in further studies of these cells, was unable to determine the exact relation of these cells to the marginal sinus. He stated, "They appear to be draped around the sinus with the majority lying along the follicular surface." These cells have been previously described by Marshall (25), who called them "reticulum cells" and by Krumbhaar (116), who referred to them as "a young type of fibroblast." These cells were first observed about the 20th postnatal day. At this time they had a slight esterase activity, a more pronounced acid phosphatase response and were blackened by the metalophil reaction.

Pettersen (52) has previously shown that these marginal cells give a weak esterase response and that this enzyme activity can be increased by stimulating the R.E. cells of the spleen with typhoid vaccine and adjuvant. The rats used in his studies were young adult males approximately 60 days old.

It has been stated by Marshall (25) that, "While all cells that possess such dye storing properties as would qualify them for membership in the reticuloendothelial system are metalophils the converse is not true." Therefore, some cells that are metalophilic are not active phagocytes. Snook (32) feels that this inability to phagocytose particulate matter is possibly due to either of two reasons. "First, they may not be fully differentiated, and, second, the vital dye or particulate matter may be prevented from reaching their cell membranes. Snook (32) has also demonstrated that in sexually mature male rats these marginal cells do not readily take up injected materials such as India ink, chlorazol black E and saccharated oxide of iron.

The literature cited above indicates that the marginal metalophils may be considered immature macrophages in the group 3 animals. They are blackened by Marshall's silver reaction, are not phagocytic, give a weak esterase response but have a relatively strong acid phosphatase activity. Pettersen (52) has shown that they can become active phagocytes when stimulated or at least he has shown that their enzyme content is greatly increased. The present research indicates that the normal growth pattern of the rat causes these marginal cells to become "mature" R.E. cells. In the senile group they were found to have increased esterase and acid phosphatase activity and had also segregated hemosiderin, thus demonstrating their phagocytic

ability. It would appear from this that these marginal metalophils have undergone a change and may now be considered as Full-fledged members of the R.E. system.

Iron

As has been stated in the results the spleen of fetal animals gave a negative response to the acid-ferrocyanide reaction even after being treated with unmasking agents, thus indicating a lack of iron storage at this stage of development in this organ as determined by the procedures employed.

Suzuki (94), in his study of phagocytic development in the rat, reports finding hemosiderin in free macrophages in the spleen of fetal animals of 19 and 21 days gestation. This phagocytosis was restricted to macrophages of the primitive red pulp. In his work Suzuki employed 136 fetal animals of which only 3 had macrophages containing hemosiderin. In the present study 120 fetuses were used and none of these was found to contain stored iron in their spleens. It is well to point out that Suzuki (94) had injected his fetal material with Thorotrast, either into the amniotic cavity or directly into the body of the fetus, intraperitoneally or intrapleurally. Such a procedure would have a very traumatic effect on such delicate organisms and there is the possibility that such harsh treatment will produce hemorrhage and other abnormal processess and thus upset

the normal activity of the body. This could explain the segregation of iron by a few macrophages. The fact that Suzuki (94) failed to employ a specific stain for the presence of iron made it impossible for him to verify his findings. It is interesting to note that in the other animals employed in Suzuki's research, those ranging in age from the newborn to 30 days were free of any splenic stored iron. This latter finding is in accord with those of the author and of Andrew (44, 45) for this age group. It would be expected that if iron is stored in the fetal spleen it would also be stored in the spleens of the immature animals but this was not the case. Judging from the research discussed above it is believed by the author that under normal circumstances neither the fetal nor the young rats store hemosiderin in the splenic cells. The finding of iron in so few animals, if indeed it was iron as reported by Suzuki, must be regarded as unusual and not the normal finding in the spleens of fetal and immature rats.

The results have shown that iron storage in the splenic macrophages usually starts to take place between the 45 to 50 days. Andrew (44, 45) was also able to demonstrate hemosiderin in the spleens of this age group. By this time in the life of the rat erythropoiesis has greatly diminished or stopped in the spleen, as observed in this study and that of Suzuki (94). Issacson (126) has stated, "The storage of iron in the macrophages of the spleen will

not take place until the production of erythrocytes is markedly decreased or stopped." This seems logical and it appears to be what takes place in the rat spleen. As long as erythrocytes are being produced by this organ the iron brought to it will be utilized immediately by the developing red blood cells and consequently none will be left to store. When the production of red blood cells ceases, the iron brought to the spleen will be stored in the macrophages.

As has already been pointed out in a previous part of this discussion, the phagocytic activity in fetal and young animals is poorly developed and so the possibility exists that at these ages the spleen lacks the capacity to store much iron. On the basis of literature to be cited it would appear that decreased phagocytic activity on the part of animals in groups 1 and 2 is only a minor factor in their inability to store hemosiderin. Erythropoiesis is the major factor in preventing iron storage in the spleens of young rats.

Recently Schjeide et al (127, 128) have shown that the red blood cells of fetal and newborn chickens and rabbits take up from 2 to 20 times more iron than do adult cells. In nucleated cells this iron is changed into hematin by the nuclei and then passed to the cytoplasm as hemoglobin. It was not determined by them what the erythrocytes of the adult does with the absorbed iron because these cells are devoid of cytoplasmic structures and so no conclusions

were made on the fate of the absorbed iron.

On the basis of this finding iron released in the spleen from destroyed erythrocytes or brought there in the form of plasma iron will be more quickly utilized in the fetal and immature animals than in the adults and will not be taken up as storage iron by the splenic macrophages.

With the stoppage of erythropoiesis in the spleen, the iron from red blood cell break down will be stored in this organ. Under normal circumstances this hemosiderin will continue to be stored in the splenic macrophages and the amount stored will increase with age, as shown by our results. As more room is needed for the iron storage more potential macrophages, such as the marginal metallophils, will be stimulated to transform into full-fledged macrophages and help with the storage of this metal.

Moore and Dubach (129), in their extensive review of iron metabolism, point out that stored iron contributes only a minor portion of the iron used in hemoglobin synthesis where as the majority comes from plasma iron. It was also reported by them that plasma iron is added to primarily by the absorption of iron from the gastrointestinal tract and to a much lesser extent from the iron stores. They further stated that storage iron will be utilized only in cases of emergency, such as hemorrhage, inability on the part of the animal

to absorb iron from the intestine or if the animal is on an iron deficient diet. This indicates and indirectly confirms our results that the storage of iron in the splenic macrophages increases with the age of the animal.

No reaction for iron was ever found in the marginal zone. Snook (32) reports finding iron in the marginal zone only after the injection of saccharated oxide of iron but this was an intercellular deposition which was only transitory in this area of the spleen. The lack of cellular-contained iron in the marginal zone is contrary to the view of Lillie, cited by Krumbhaar (116), "It is often the principle site of hemosiderin accumulation". MacNeal (42) also considered the marginal zone or perifollicular envelope as a site of phagocytosis of worn out red blood cells. In view of our findings and those of Snook (32) it is unlikely that the marginal zone is an area for iron storage.

Another group of cells which are free of iron pigment are the sinus-lining cells. These cells can be "primed" into taking up iron by means of colloidal iron injections as carried out by Moore et al (77) and by Snook (75).

Snook (32) has found that hemosiderin, as demonstrated by the Prussian blue reaction, is found in variable amounts in the normal rat spleen, where it is usually confined to the red pulp. He also found a few specimens that gave a fairly strong Prussian

blue reaction in the marginal metalophils. This is in agreement with the findings of Altschul and Hummason (43), who were able to demonstrate iron in the marginal cells in 1 animal of the 12 stained for hemosiderin. Both of these studies were done on sexually mature animals which would correspond to animals in our group 3. It has been shown by the results that the storage of hemosiderin in the marginal cells is only an occasional finding in this age group.

In senile animals a strong Prussian blue reaction in the marginal metalophils is a common finding. In fact, all 11 animals studied in this age group had hemosiderin in these cells to a greater or lesser extent. Apparently the aging process plays a part in the activation of these cells, causing them to store the hemosiderin.

The red pulp and marginal metalophils are not the only locations that have stored iron. Cells located at the level of the reaction center and some large perivascular cells of the central artery also store hemosiderin. Age seems to be a factor here, too. The perivascular cells are occasionally positive for iron in some animals of group 3 but these cells are again more common in the follicles of the senile animals.

It would appear from what has been said, that the storage of iron in the splenic macrophages increases with changes brought

about by advancing age. The major factors contributing to this increased storage of iron in animals of groups 3 and 4 are the stoppage of erythropoiesis and the greatly developed phagocytic activity of the splenic macrophages.

Megakaryocytes

Although the megakaryocytes were not a main concern of this research their prominence made it impossible to overlook them entirely. These large cells were present in all the rat spleens studied except the very early fetuses. Their frequency in the various age groups has been plotted on the graph in the chapter on results.

Megakaryocytes occur in spleens of many normal adult mammals. DeKervily (130) has reported finding them in the spleens of adult hedgehogs, rats, mice, guinea pigs, rabbits, cats, dogs, dolphin, bat, and rarely, in the bear. He found that the fetal hedgehog and new born cats had the greatest number per square millimeter of all animals examined. Chatterjee and Cruickshank (131) have reported finding megakaryocytes in the red pulp of mouse spleens but did not see any in the guinea pig, rabbit or dog. These investigators made no mention of the origin or development of megakaryocytes in the spleen. Andrew (44, 45), in his study of age changes in the spleen, felt that the origin of splenic megakaryocytes was by fusion of smaller cells. He based this belief on the point that there are large

numbers of groups of cells with relatively clear nuclei and considerable cytoplasm in the spleen. He states, "In such a group of cells the cytoplasmic bodies frequently seem to fit together rather closely and the possibility of fusion seems credible." He also pointed out that these masses of cells are often in close apposition to megakaryocytes and the nuclear characteristics, as well as the cytoplasm of the smaller cells and that of the megakaryocytes appear very similar. The great diversity in the degree of separation of the nuclear parts also lead him to the fusion theory of development. In our study most megakaryocytes that exhibited these "distinct" and "separate" nuclei could be explained on the basis of tangential cuts. Those that could not be explained on this basis are believed to be the results of mitosis before fusion of the nuclei has taken place.

Andrew (44, 45) apparently discarded the possibility of mitosis as a means of producing megakaryocytes because he stated, "It may be of significance that the megakaryocytes are rather seldom found two together, but, like the groups of cells which seem to form them, are scattered among the masses of deeper staining, smaller nuclei." He did not mention finding any megakaryocytes in mitosis in any of the 100 animals studied in his research.

At least 2 dozen mitotic figures were observed in our splenic tissue sections. No concerted effort was made to locate

them; those found were seen during routine observation of the entire tissue section. Most of these mitotic figures were observed in the immature and young adult animals. The megakaryocyte has a polyploid type of mitosis, where the nucleus undergoes division without cytoplasmic splitting. Nuclei thus formed fuse and eventually through further differentiation form the complex nucleus of the mature megakaryocytes. No sequence of mitotic figures, ranging from prophase to telophase was observed in cells of the megakaryocyte series. The large number of chromosomes present made it impossible to determine exactly at what stage of development these cells were in. However, chromosome arrangement comparable to a metaphase or early anaphase were seen. It was obvious that the number of chromosomes in these cells was greatly increased compared with mitotic figures in other cell groups of the spleen (figure 20).

An excellent description of megakaryocyte mitosis is given by Feinendegen et al (132) in their account of megakaryocyte proliferation in the bone marrow of rats. Their figure 4 compares favorably with those mitotic figures seen in this study. It is reasonable to conclude that the development of megakaryocytes in the bone marrow and in the spleen of the rat is through the process of mitosis and maturation and not by fusion as suggested by Andrew (44, 45).

The results have shown that the number of megakaryocytes

increases per unit area until about the 26th to 27th day of life, at which time their number decreases until the senile stage when there is again a slight increase in their number. The decrease in the number of megakaryocytes after 26 days corresponds to the fact that this is the approximate time that the white pulp is increasing in amount and the follicles are becoming more numerous. The amount of white pulp reaches its maximum amount at 75 to 270 days and this is the point at which there were the fewest megakaryocytes observed. The slight rise in the number of megakaryocytes in old age again appears to be correlated to the decrease in the white pulp in this age group.

In regards to the amount of lymphoid tissue at different ages, measurements were not made in this study because the changes in the amount of white pulp from one group to another were obvious enough. Actual measurements of the amount of lymphoid tissue have been made for man by Hwang, Lippincott and Krumbhaar (103) and Krumbhaar and Lippincott (104). In the rat, qualitative descriptions have been given for the amounts of splenic lymphoid tissue present at different ages by Suzuki (94), Andrew (44, 45) and West (105). Their findings compare favorably with those of the present study, that is, the amount of lymphoid tissue in the spleen increases with age until old age when there is a decrease in the lymphatic tissue.

Another factor which may have a bearing on the variation in the number of megakaryocytes is the compactness of the red pulp, whether it is sinusoidal or non-sinusoidal. Here again the decline of the megakaryocytes is at a time when the red pulp is becoming sinusoidal. In other words, with an increase in the amount of white pulp and the development of a large number of sinuses there is a corresponding decrease in the red pulp content and consequently a decrease in the number of megakaryocytes present. Downey, Palmer and Powell (133), in a case of atypical myelosis, have shown that the density of the red pulp definitely is related to the production of megakaryocytes.

The location of the rat splenic megakaryocytes needs some clarification. Andrew (44, 45) has described them as abundant and more conspicuous in the immature animals because they are commonly surrounded by white pulp, while he finds that in older animals they are almost invariably in the red pulp. Our study supports the fact that these giant cells are more abundant in the immature but these were never found in the white pulp. From fetal to senile animals, the megakaryocytes were confined solely to the red pulp. At no time in development were any of these cells observed in the white pulp or even in the marginal zone. Similar results as ours were obtained for the rat and other animals, fetal to adult, by DeKervily (130) and for the young mouse by Chatterjee and

Cruickshank (131) and Krumbhaar and Lippincott (104).

Galindo and Imaeda (134), in their electron microscope study of the white pulp of the mouse spleen, go into great detail in their description of cell types found in the follicles but made no mention of finding megakaryocytes.

The literature cited and the authors' findings indicate that megakaryocytes are a normal finding of the red pulp and not a constitutent of the white pulp.

Histochemically the megakaryocytes give an intense response to non-specific esterase, acid phosphatase and the PAS procedures. This latter technique has been used by Ackerman and Knouff (135) to trace the origin and development of megakaryocytes in the embryonic liver. Pettersen (52) reported that megakaryocytes are uniformly pyroninophilic but did not elaborate on the significance of this. In the present study it was noted that they were not impregnated with Marshall's metalophil technique but sometimes were impregnated during the impregnation of reticular fibers. These cells were also found to be negative for the acid-ferrocyanide test for hemosiderin. The histochemical results given above indicate that the chemistry of these cells, if studied more completely, could possibly resolve some of the controversial problems concerning the origin, development, potentialities and interrelationships of these giant cells. The fact that the megakaryocytes do not "take" the metalophil reaction eliminates them as members of the R.E. system,

even though there have been reports that these cells can phagocytose particulate matter.

Phagocytosis by megakaryocytes has been reported by a number of authors. Filho (136), who studied various animal species, found that megakaryocytes were especially prone to phagocytosing erythrocytes and this function was increased after intra-vital staining. Andrew (44, 45) thought that the inclusion bodies he observed in the megakaryocytes were truly phagocytosed material. On the other hand some authors have opposed this idea of the phagocytic ability of these cells. Wuyts (137) believes that what is actually being seen is not phagocytic activity but cells or debris within a niche of the megakaryocyte's cytoplasm which have been cut tangentially giving the illusion that these structures are within vacuoles in the cytoplasm. Fieschi (138) feels that such activity on the part of a megakaryocyte is not the expression of a normal function of this cell. Of the thousands of megakaryocytes counted and observed in this study, less than a dozen appeared to contain foreign material. From so few a number of possible cases of phagocytosis it was impossible to determine if this was true phagocytic activity or an illusion, as Wuyts believed. This observation led the author to agree with Fieschi, that splenic megakaryocytes do not normally phagocytose blood cells or other foreign material.

CHAPTER V

SUMMARY AND CONCLUSIONS

The spleens of 318 albino rats of different ages were studied using both histological and histochemical techniques. For convenience of study and description these animals were divided, somewhat arbitrarily, into four groups according to age. Group 1 was comprised of 109 fetal animals ranging in age from 17 to 21 days gestation. Group 2 were the definitely immature animals. They ranged in age from the newborn to 21 days. Twenty-one days of age was taken as the cut-off day for this group, because in our laboratory this is the age at which the animals are weaned. There were 165 animals in this group. Group 3 was composed of 35 very young and young adult animals. These animals varied in age from 22 to 270 days. This group was used primarily to trace out the development of specific structures until a definite adult form was obtained. Group 4 included 11 senile animals, all of which were over 2 years old. With this group it was possible to study the process of senescence in the spleen.

The animals were sacrificed on the appropriate days. Tissues were removed and prepared for both histological and histochemical study. The fetal spleens were fixed and sectioned in their entirety, while for the other groups only portions of the spleens were used. Histological staining procedures employed were, routine hematoxylin and eosin, Giemsa (97), Snook's reticular stain (98) and the PAS technique (99). Histochemical techniques employed were Marshall's

metalophil reaction (25), Pearse's modification of Gomori's non-specific esterase reaction (73), Burstone's technique for acid phosphatase (53), and Gomori's Prussian blue reaction for hemosiderin (94). Along with this latter procedure, unmasking agents were used in an effort to uncover iron not normally demonstrated when just the acid-ferrocyanide reaction is used.

In the fetal animals very few cells contain the enzymes non-specific esterase or acid phosphatase although many are impregnated with silver. The enzyme activity and the number of cells containing the two enzymes increases with the advancing age of the rats. The most important difference between the enzyme techniques and the metalophil reaction was that the silver impregnation always demonstrated more cells. This difference becomes much less in the senile animals where the esterase, acid phosphatase activity and the storage of hemosiderin has increased.

White pulp sheaths are just beginning to form in the fetal animals and the first nodules are observed about the 21st postnatal day. This white pulp is generally free of enzyme activity and has only a few metalophil cells. The marginal metalophil cells have started to make their appearance in these immature animals. The white pulp nodules consistently have reaction centers after the 50th postnatal day and can be considered as "adult" in structure. The white pulp areas undergo some degenerative changes in the senile

animals. The enzyme activity and iron storage is increased markedly in the nodules of these old animals.

Fetal animals have red pulp which is of a myeloid type. The main function of this tissue is the production of red blood cells. By the newborn and immature stage of development certain changes have taken place in the appearance and arrangement of the red pulp. There is the development of the "mature" type of sinus, the marginal zone and the extension of the trabeculae into the red pulp. Even with these changes the dominant activity of the spleen is erythropoiesis. Another function of the spleen at this time appears to be the active proliferation of megakaryocytes.

With the decline or stoppage of red cell production the function of the spleen is changed from the primary purpose of proliferation to phagocytosis and storage of iron. This becomes evident about the 51st postnatal day. This activity increases up to and including old age. Most of this hemosiderin is stored in the red pulp with some located at specific areas of the white pulp.

As demonstrated by the enzyme, acid-ferrocyanide, and metalophil reactions, the spleen is in a state of constant change of structure and function from the fetal stages to the age of senility.

From the results of this research it may be concluded that: 1) The lymphoid sheaths are in the process of developing during the latter part of gestation but lymphoid nodules are not apparent until

the 21st postnatal day. These "young" nodules do not possess reaction centers. 2) Reaction centers are first seen at 26 days but do not become numerous until after the 50th postnatal day. From 50 to 270 days these centers are a characteristic finding. In senile animals they are less common. 3) The amount of lymphoid tissue increases from the fetal animals to those of 270 days. The lymph nodules of the senile animals are variable, usually there is a decrease in the amount of this tissue. 4) The marginal zone and sinus first are seen in the immature animals of group 2, but these structures are better defined and consistently found only after the 30th day. 5) Mitotic figures are observed periodically in the white pulp especially while the lymphoid nodules are developing. Mitosis was never seen in isolated foci of the nodule. 6) Phagocytosis is considered the main function of the secondary nodule and for this reason it is considered that "reaction center" is more appropriate for this part of the nodule. 7) Animals between the ages of 46 to 270 days are considered as having follicular nodules of the "adult" or "mature" histological structure. 8) Lymphatic vessels were found in the splenic nodules and appeared to be related to the marginal sinus. They were not close to the central artery as has been described for other animals. 9) The marginal sinus was found to contain erythrocytes in all cases observed. Red blood cells were also seen scattered throughout the marginal zone.

10) The most prominent cells in the white pulp of the senile animals are the large perivascular phagocytic cells. These contain esterase, acid phosphatase, hemosiderin and are impregnated with the metalophil reaction. They are, therefore, considered part of the splenic macrophage system. 11) Malpighian follicles of senile animals usually show a number of sections of vessels with thick walls and occasionally the central artery appears occluded. In young animals there is just one, thinned-wall central artery. 12) Spleens of the 17 day fetuses were composed almost entirely of myeloid appearing tissue. The reticulum at this stage was of uniform dimensions. There is no differentiation into fine, medium or coarse fibers as there are in the adult spleen. 13) Fetal splenic sinuses are irregular channels which permeate throughout the general reticulum background. By late gestation the reticular network has started to differentiate toward the adult pattern and forms concentric rings around the arteries. In group 2, the immature animals, the sinuses are of the adult "cucumber" shape but the spleen does not generally become sinusoidal until late in group 3 and group 4. 14) Megakaryocytes are almost absent in the early fetal spleen but increase in number until early in group 3, at which time their numbers decrease until the senile age when there is again a slight increase. This growth pattern of the megakaryocytes seems to be related to the development of the white pulp and the sinusoidal type of spleen.

Megakaryocytes are never found in the white pulp. 15) The fetal capsule is a single layer of epithelial cells. This thickens progressively by the addition of reticular fibers and muscle cells until an "adult" type of capsule is reached at about the 21st postnatal day. In group 3 the capsule and trabeculae are variable in structure. Wrinkling of the splenic capsule is the most obvious change that takes place in the senile animals. The first sign of developing trabeculae are seen in the newborn animals. As these develop it is obvious that they are continuous with the splenic capsule. The trabeculae become more numerous in group 4 rats. 16) Hemosiderin is not stored in the spleen until erythropoiesis has stopped. The first sign of iron in macrophages was at 51 days. Throughout the rest of group 3 iron was stored mainly in the red pulp. The older animals of this group periodically had iron stored in cells, the reaction center and at the level of the marginal metalophils. A characteristic of senile animals was the large hemosiderin ladened cells around the central artery and the strong Prussian blue reaction on the part of the marginal metalophils. Sinus-lining cells and cells of the marginal zone were free of any stored hemosiderin. 17) Increased esterase activity is observed in the marginal metalophils as the age of the animals increases. There are also more esterase positive cells in the nodules. This increased esterase activity coupled with the increased storage of hemosiderin

is taken to indicate that immature macrophages have differentiated into mature forms. 18) The increasing presence of non-specific esterase, acid phosphatase and hemosiderin in the cells of the spleen indicates that there is a difference in capacity for phagocytic function between young and old rats.

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PLATE XIV

Explanation of Figures

Figure 28. and Figure 29. Iron is not stored in the spleen until erythropoiesis stops. The first sign of hemosiderin storage was in a 51 day old rat (fig. 28). This iron was confined solely to the red pulp. Iron storage in the spleen is progress, and figure 29 shows a 54 day old rat with hemosiderin storing cells outlining the white pulp. WP, white pulp; RP, red pulp; H, hemosiderin containing cells. Prussian blue reaction. X285.

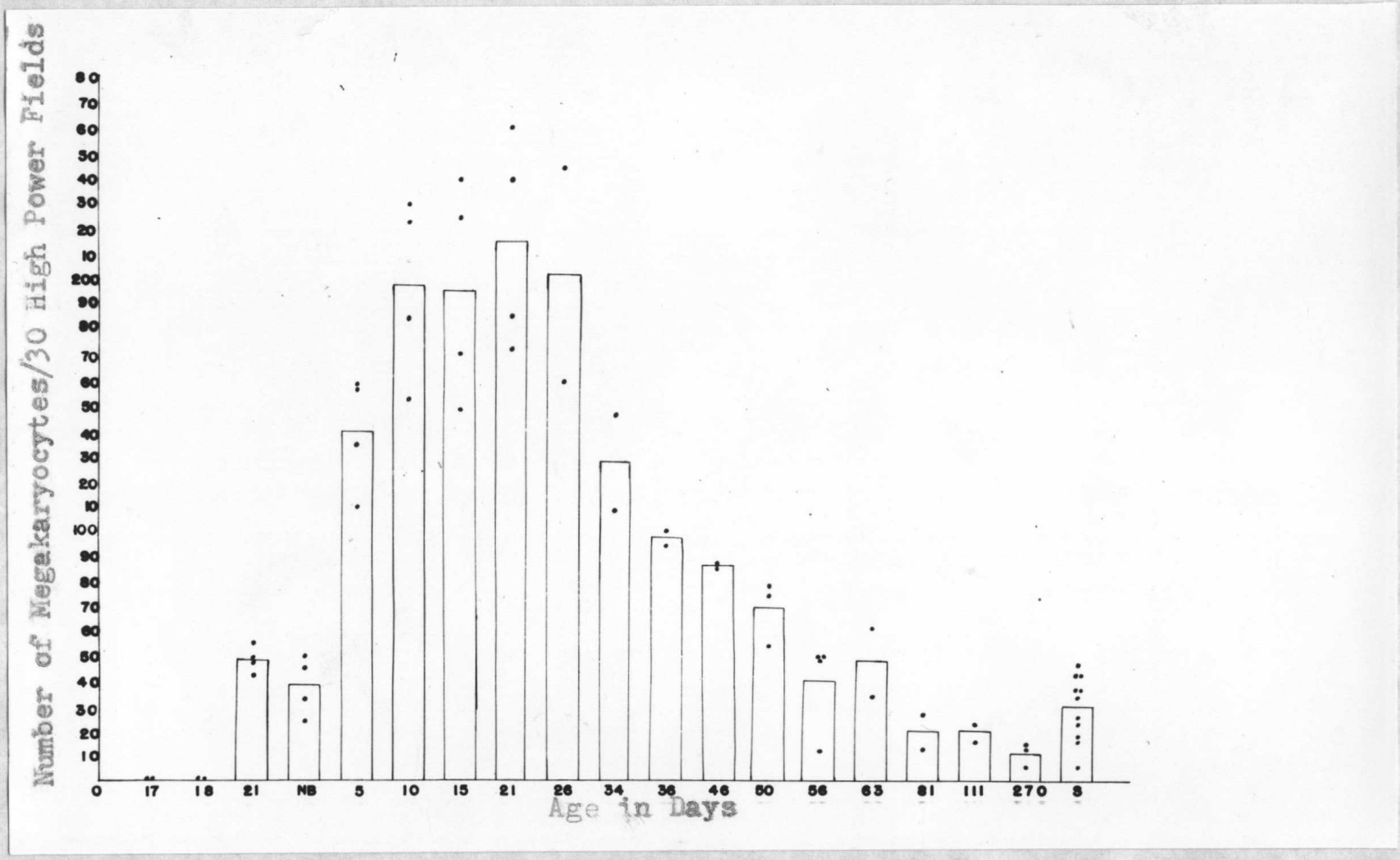

Figure 1

Number of megakaryocytes in the spleens of albino rats varying in ages from 17 days gestation to senile animals. The number of cells for each animal counted in 30 high power fields (10X ocular, 43X objective) is represented by a dot. The bar shows the average count for each group of animals. NB, newborn animal and S, senile animal.

PLATE I

Explanation of Figures

Figure 2. Lymphoid sheath is foreshowed as a condensation of the primitive reticular cells around the arteries in early fetal animals. A, artery; R, reticular cell; M, megakaryocyte. Hematoxylin and Eosin. X1260.

Figure 3. By late gestation the lymphoid cells are arranged around the artery in definite concentric rings. Hematoxylin and Eosin. X1260.

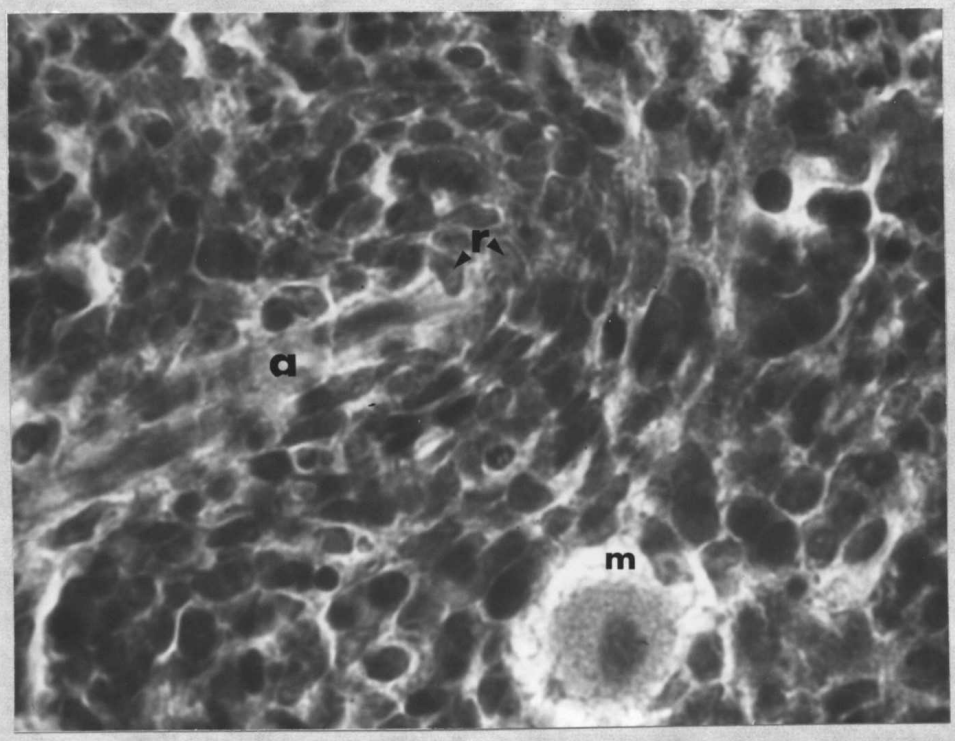

Figure 2

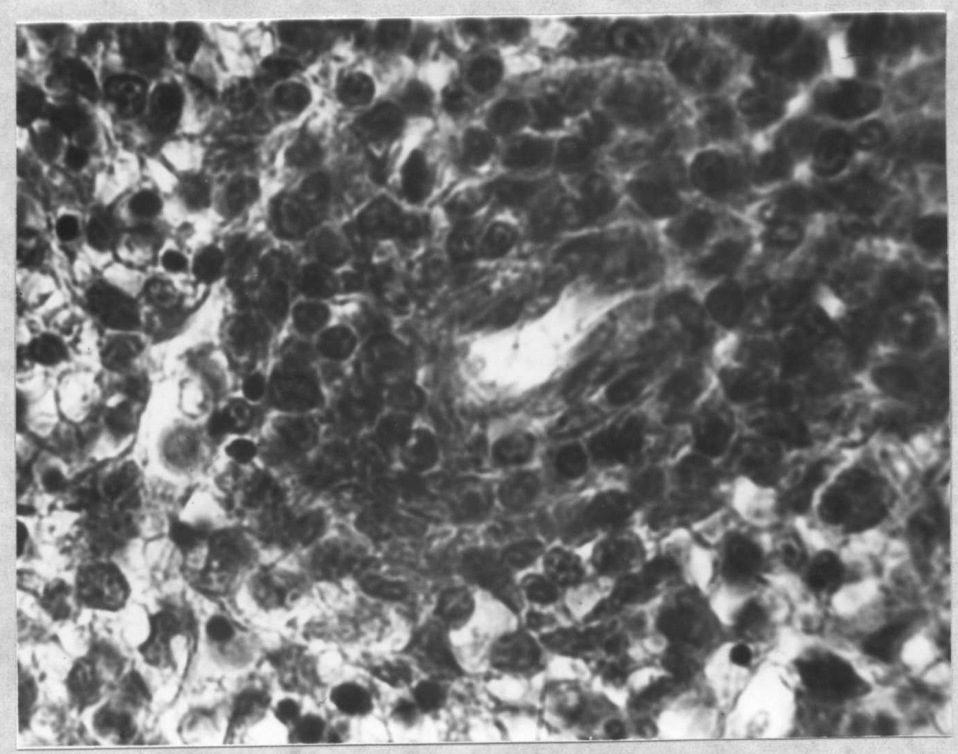

Figure 3

PLATE I

PLATE II

Explanation of Figures

Figure 4. White pulp sheaths in newborn. These are somewhat more common at this stage than in fetal animals. Hematoxylin and Eosin. X285.

Figure 5. A 20 day old animal exhibiting an eccentric "central" artery. This indicates that actual nodules have formed. A, artery; L, limit of follicle. Hematoxylin and Eosin. X1260.

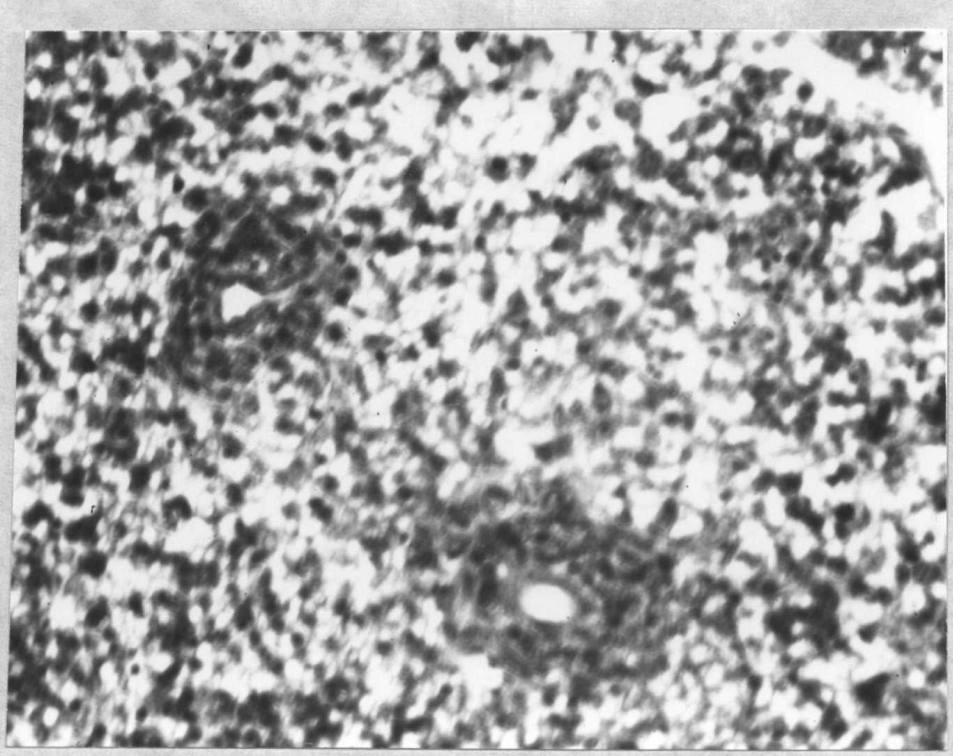

Figure 4

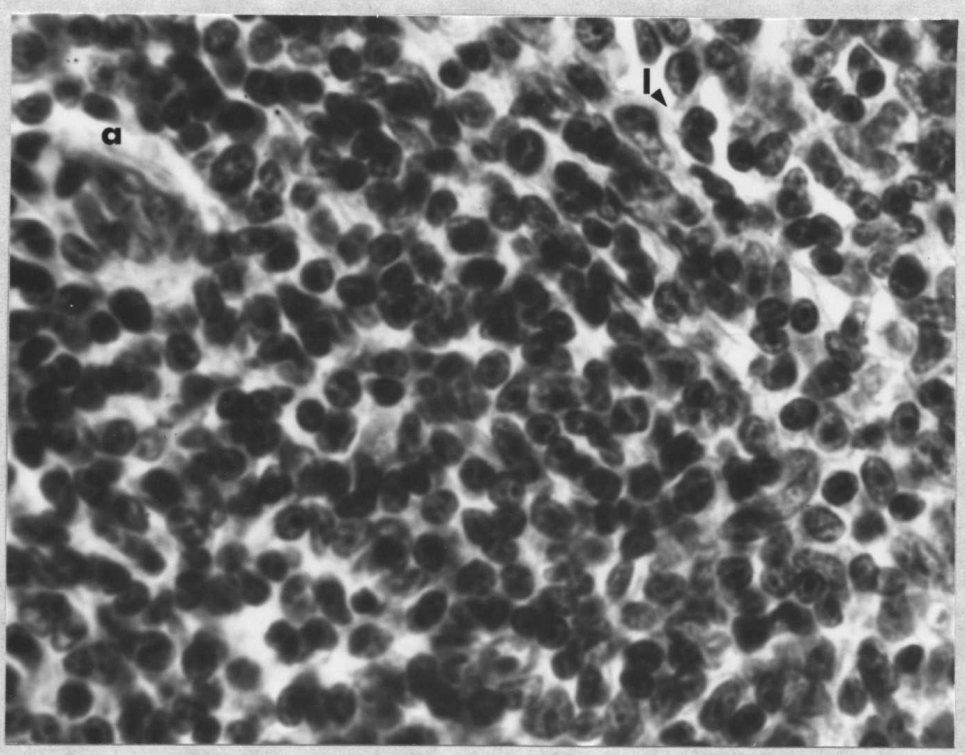

Figure 5

PLATE II

PLATE III

Explanation of Figures

Figure 6. This is a 46 day old animal illustrating a "mature" follicle. A, central artery; R, reaction center; MS, marginal sinus; MZ, marginal zone; RP, red pulp. Hematoxylin and Eosin. X285.

Figure 7. The clear areas are the large phagocytic cells found in the reaction center. Hematoxylin and Eosin. X1260.

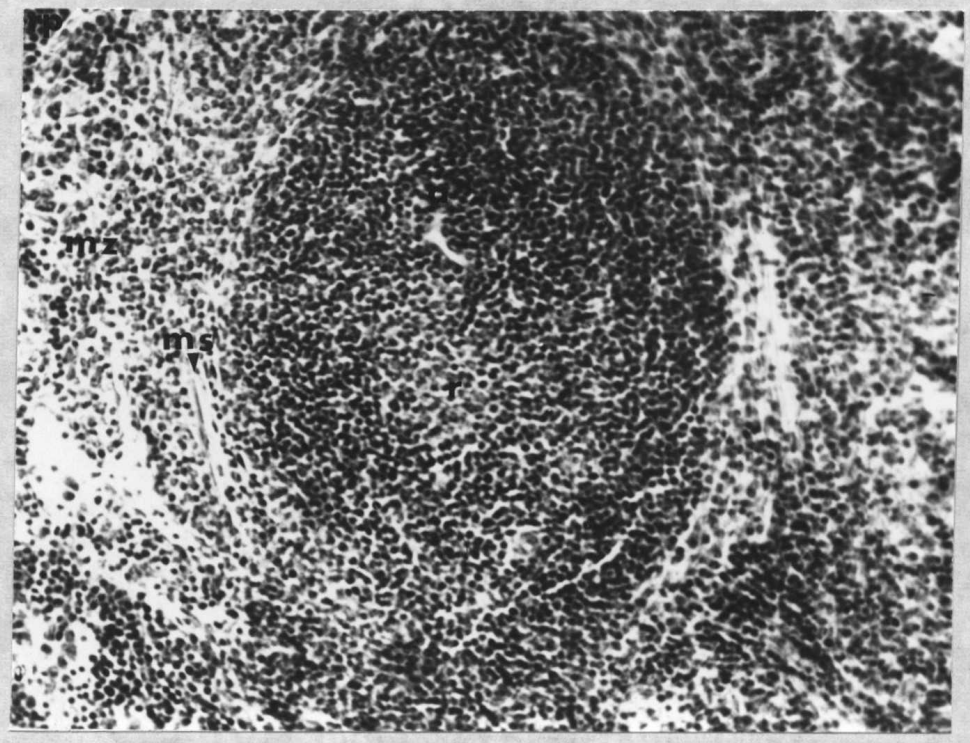

Figure 6

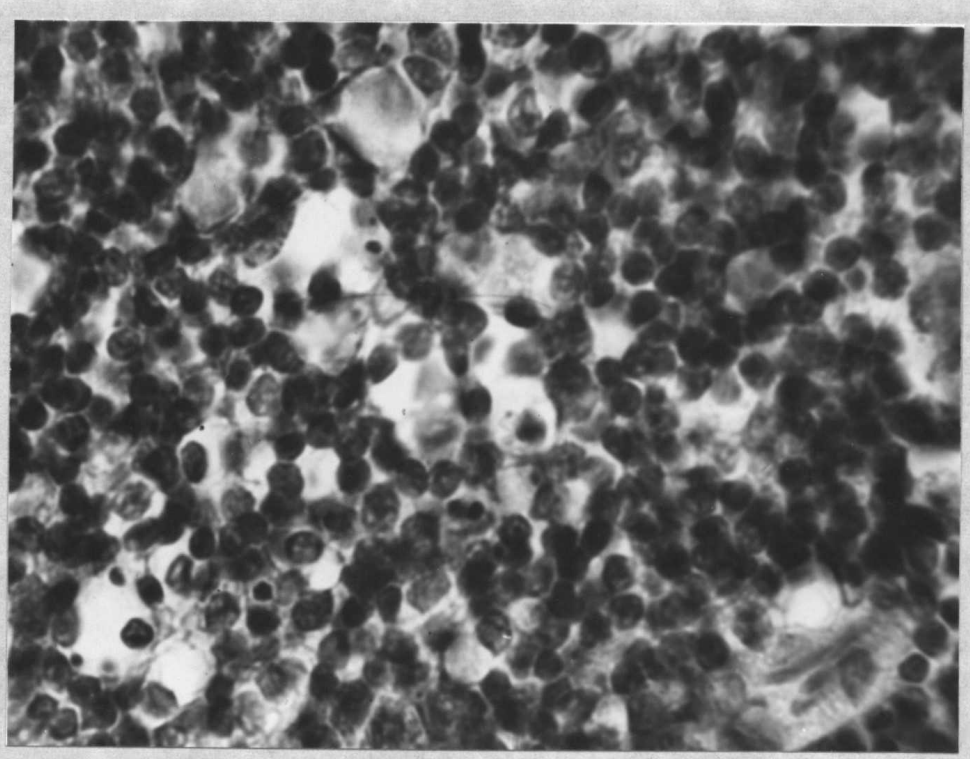

Figure 7

PLATE III

PLATE IV

Explanation of Figures

Figure 8. and Figure 9. These are lymphatic vessels located in the splenic white pulp. The arrow shows how close they could be traced to the marginal sinus. WP, white pulp; L, lymphatic; MS, marginal sinus. Hematoxylin and Eosin. X1260.

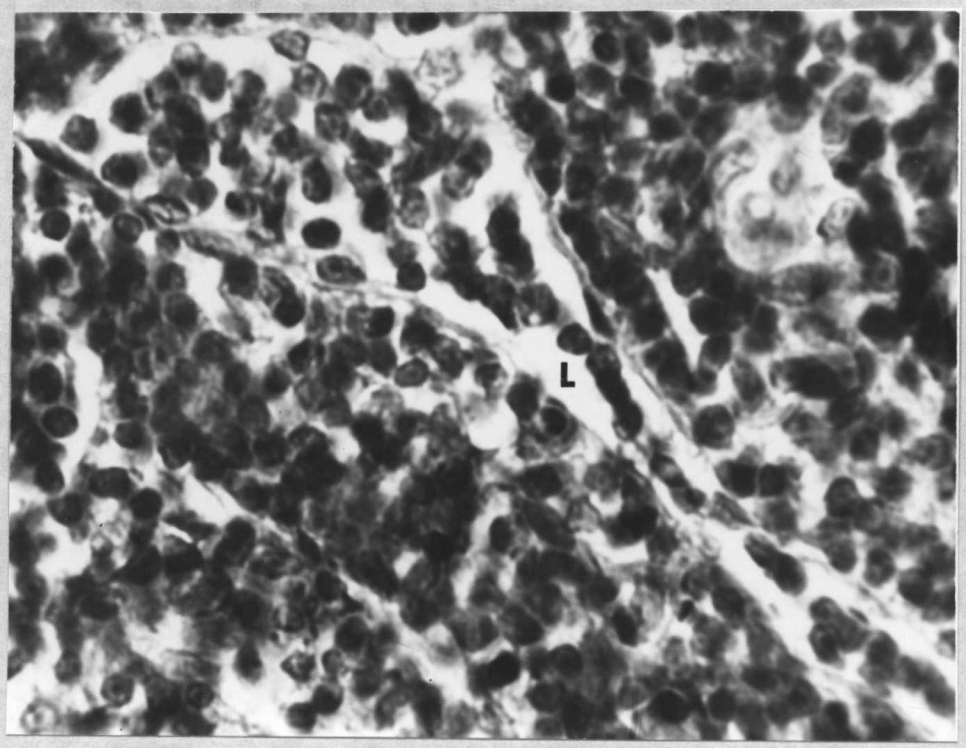

Figure 8

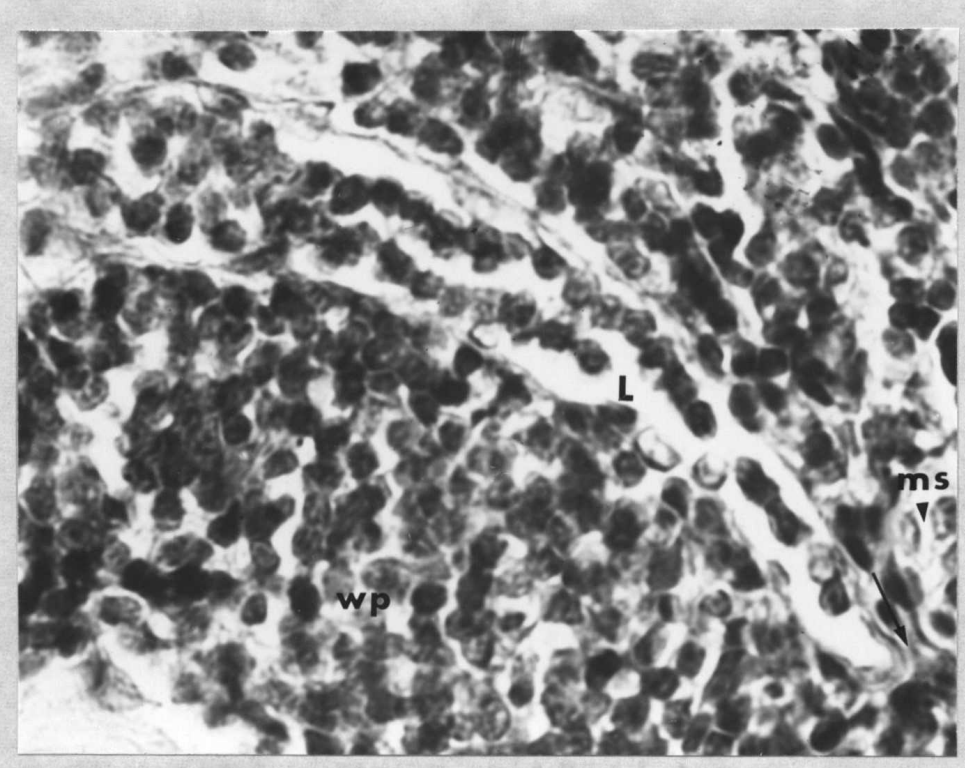

Figure 9

PLATE IV

PLATE V

Explanation of Figures

Figure 10. Senile animal with a thick walled artery and many perivascular phagocytic cells containing hemosiderin. The arrows indicate the large macrophages. Hematoxylin and Eosin stain. X285.

Figure 11. This is also a senile animal. The central artery is noticeably thickened as compared to younger animals, see figure 5. Hematoxylin and eosin. X285.

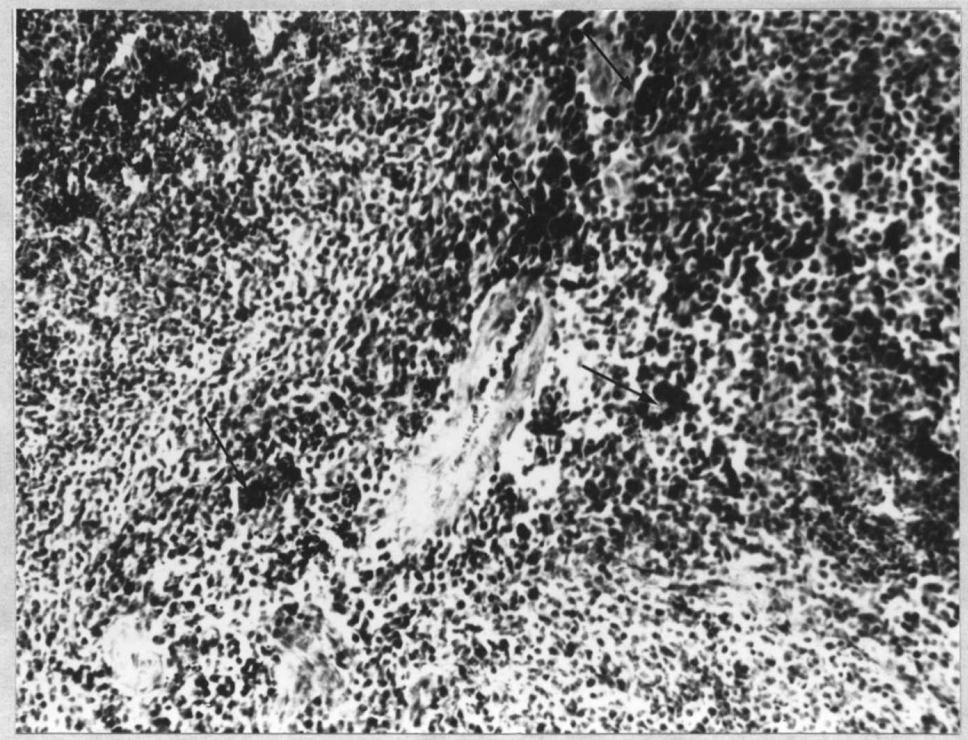

Figure 10

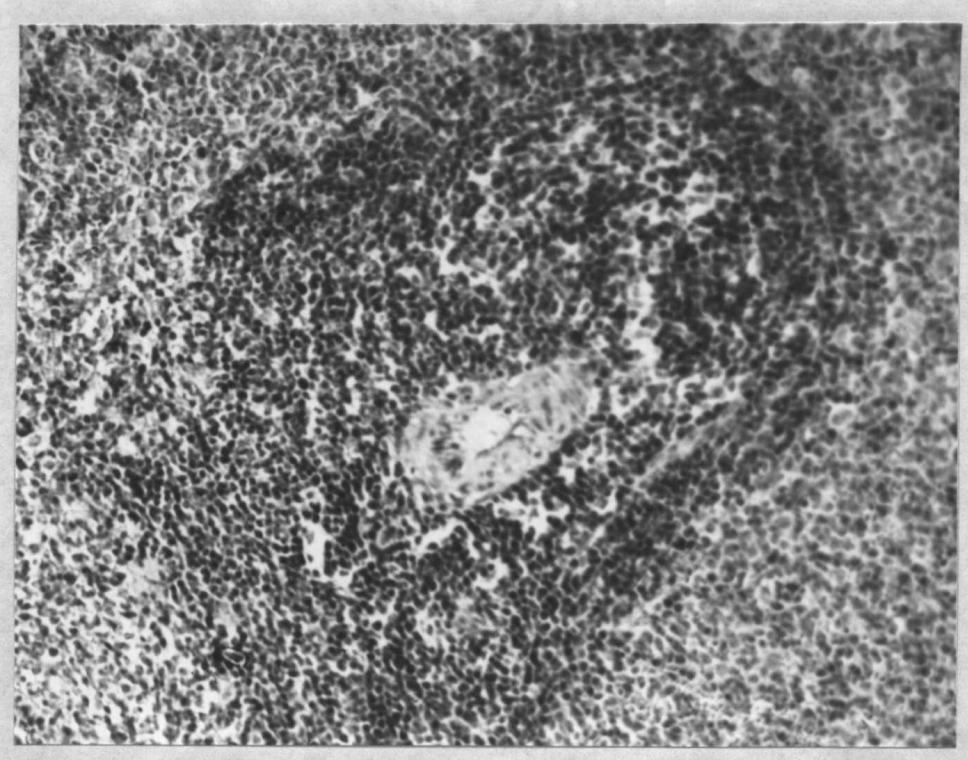

Figure 11

PLATE V

PLATE VI

Explanation of Figures

Figure 12. and Figure 13. Longitudinal sections of the white pulp arteries of senile animals showing a tortuous course and possible "supernumary" central arteries. S, supernumary artery; T, tortuous artery. Hematoxylin and Eosin. X285.

Figure 12

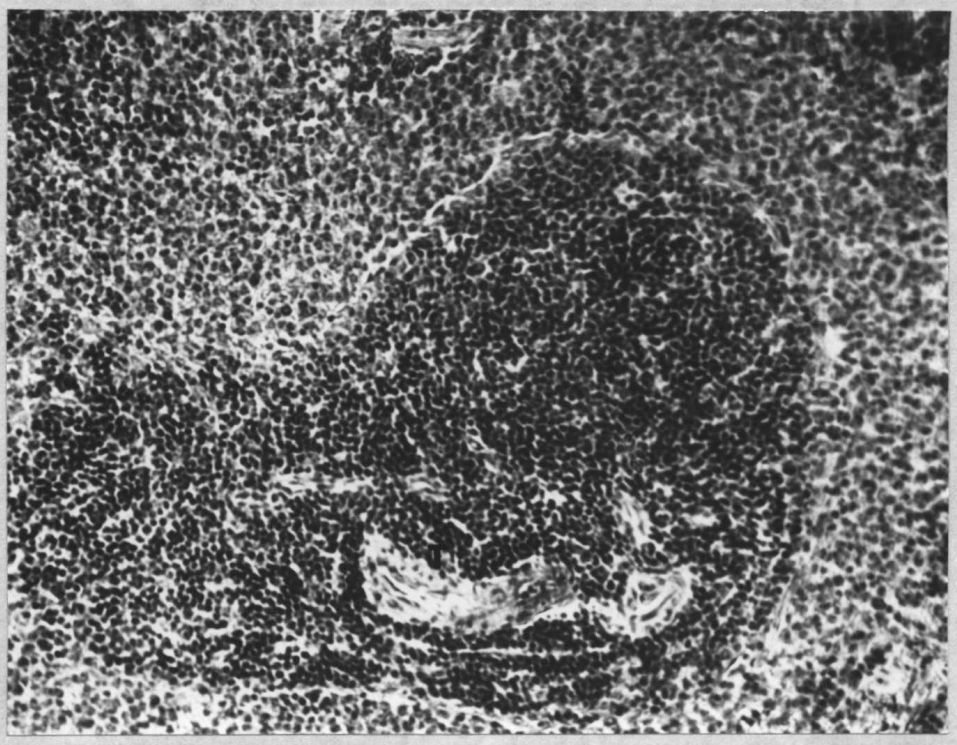

Figure 13

PLATE VI

PLATE VII

Explanation of Figures

Figure 14. Shows the reticular net of the early fetal spleen to be of uniform dimensions. The simuses seem to permeate the meshes of this net. Some developing erythrocytes can be seen. S, simus; E, erythrocyte. Reticular stain. X1260.

Figure 15. A 21 day gestation animal illustrating that the reticular network has started to differentiate and form rings of fibers around the artery. A, artery; R, reticular fibers around arter. Reticular stain. X1260.

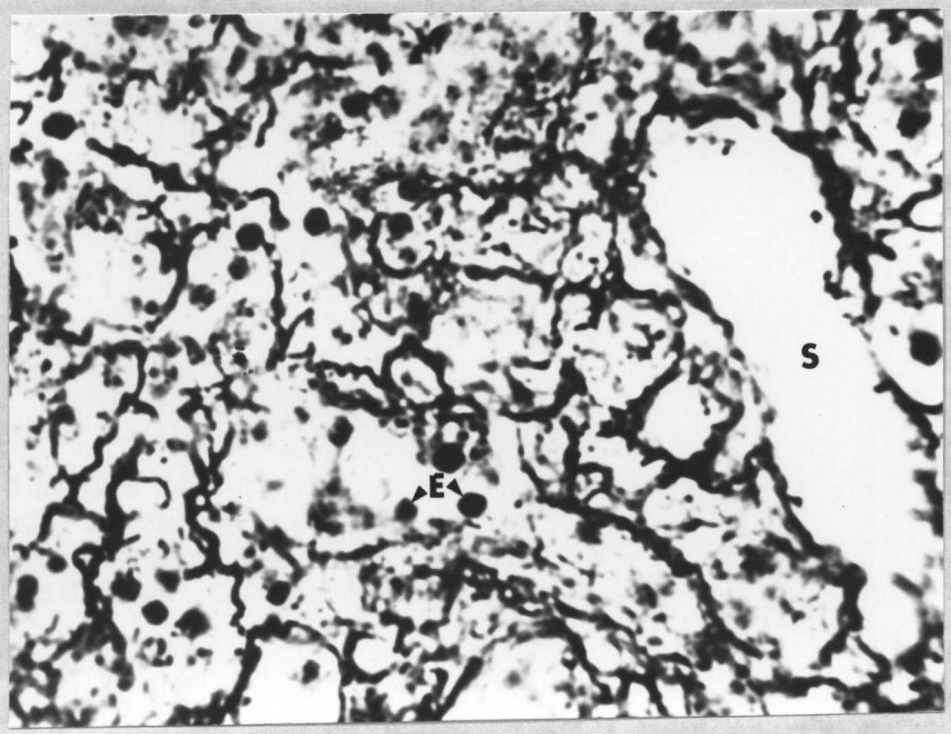

Figure 14

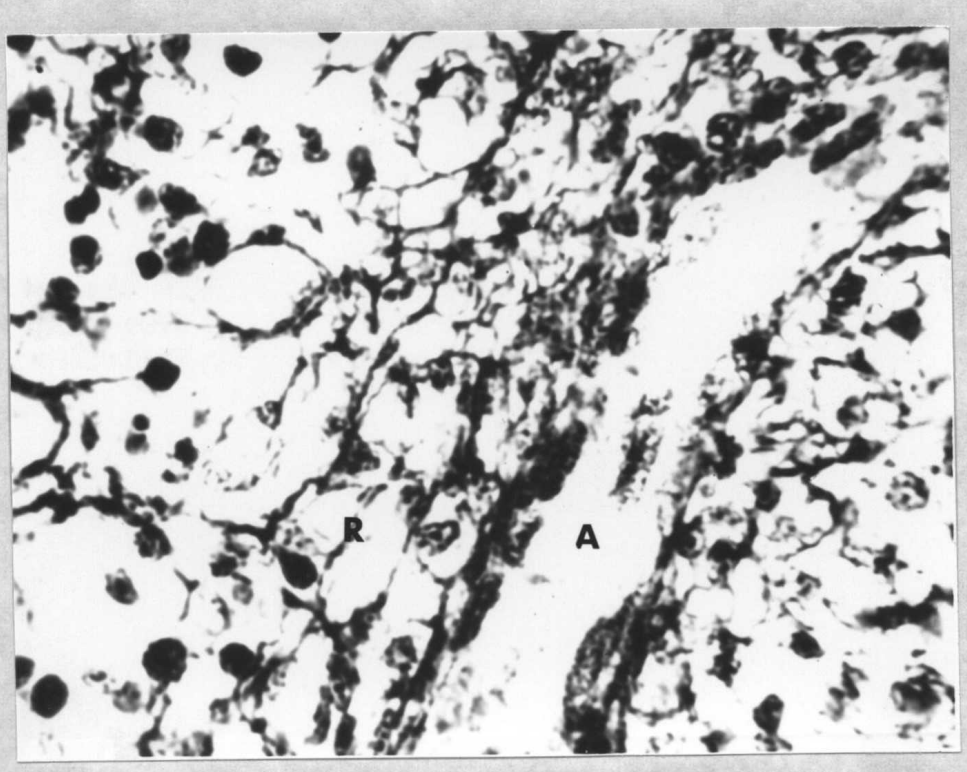

Figure 15

PLATE VII

PLATE VIII

Explanation of Figures

Figure 16. Postnatal animal of 10 days. Here the fine reticular fibers of the marginal zone are visible along with the concentric thick fibers of the lymphoid sheath. F, fine fibers of the marginal zone; T, thick fibers of nodule. Reticular stain. X1260.

Figure 17. This figure of a 21 day old rat shows a "typical" "cucumber" shaped sinus, although the fibers of the sinus have not been deeply impregnated. Note the heavy concentric fibers of the nodule and the fine fibers of the marginal zone. S, sinus. Reticular Stain. X285.

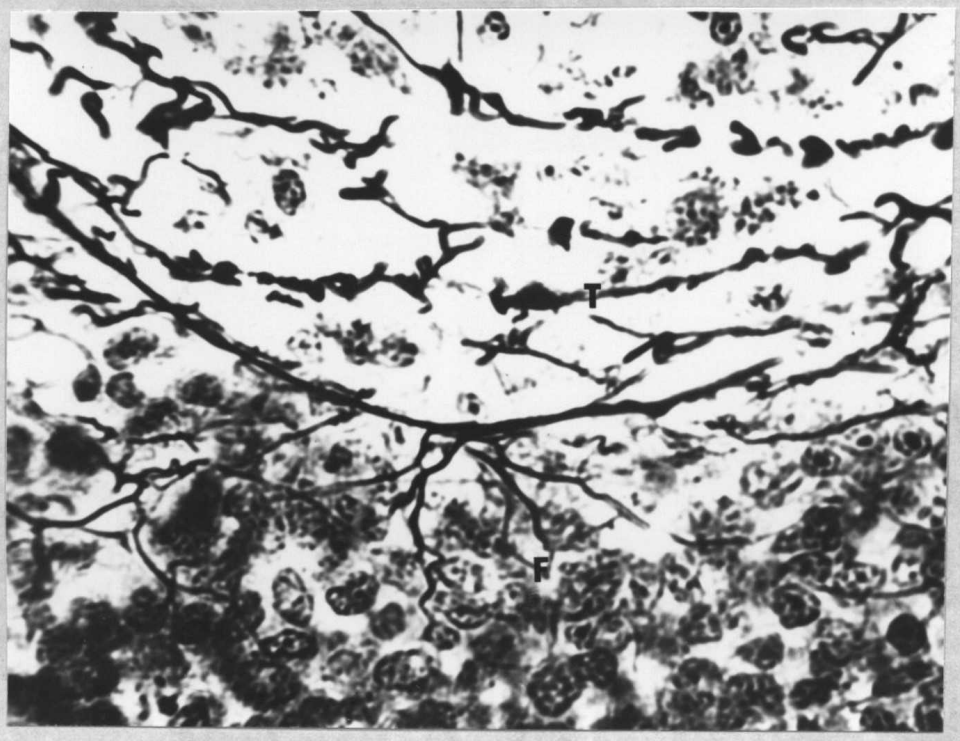

Figure 16

Figure 17

PLATE VIII

PLATE IX

Explanation of Figures

Figure 18. and Figure 19. These figures indicate how prominent and numerous megakaryocytes are in rat spleens, especially immature animals. Figure 18 shows that these cells are confined to the red pulp. WP, white pulp; M, megakaryocyte. Hematoxylin and Eosin. X285.

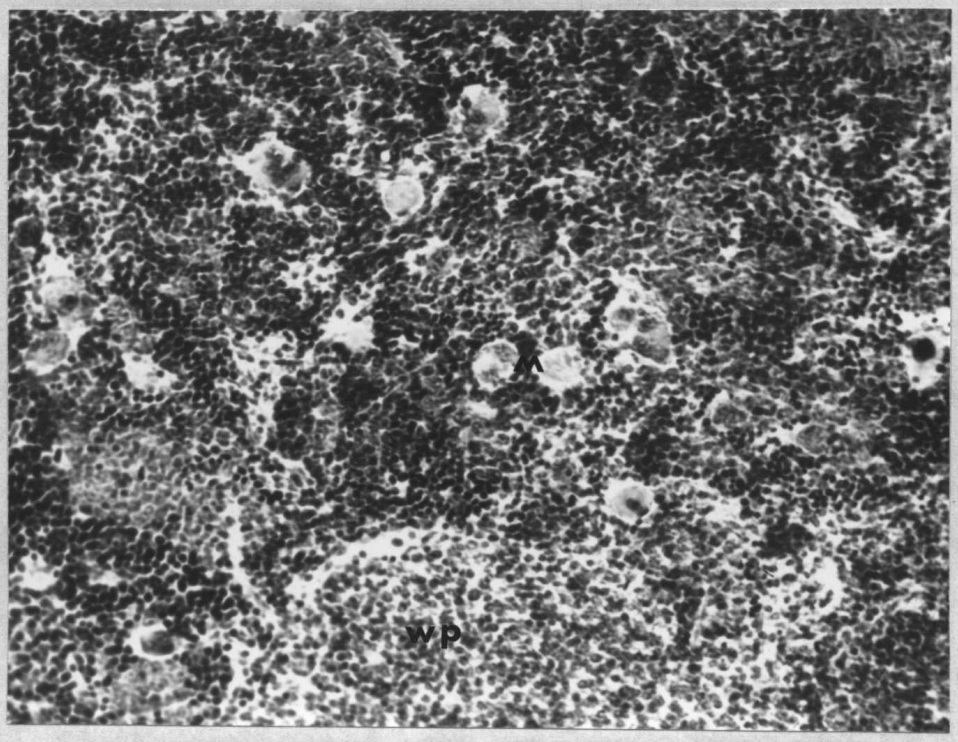

Figure 18

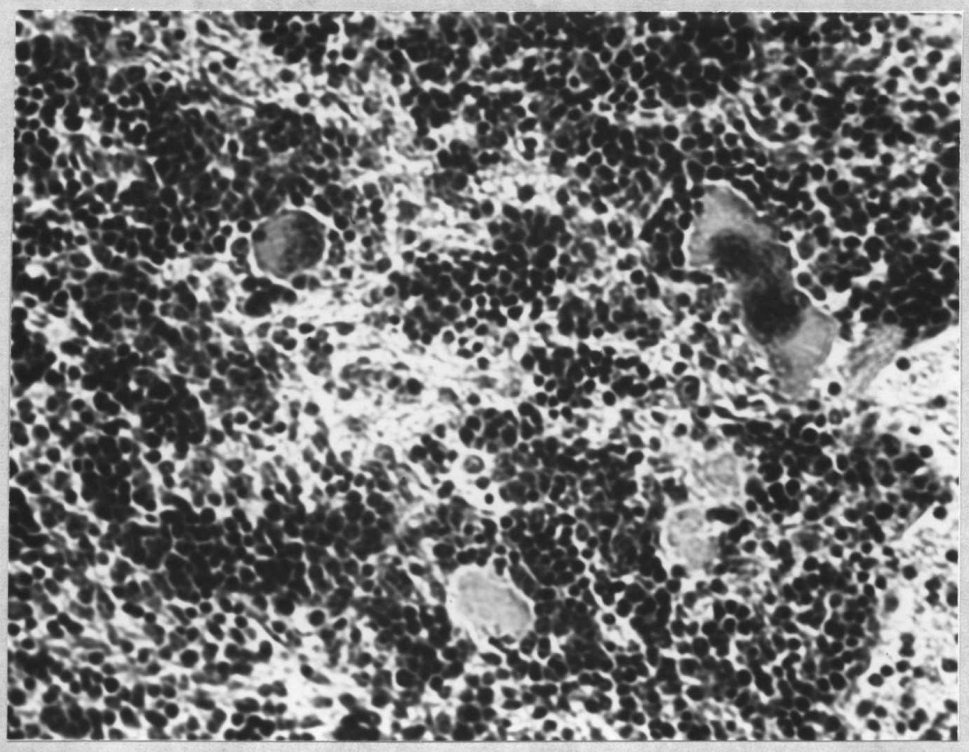

Figure 19

PLATE IX

PLATE X

Explanation of Figures

Figure 20. and Figure 21. Megakaryocytes in mitoses are an obvious feature of these two figures. In figure 20 compare the polyploid type of mitosis of the megakaryocyte with the "normal" mitotic figure in telophase. N, normal mitosis; M, megakaryocyte in mitosis. Hematoxylin and Eosin. X1260.

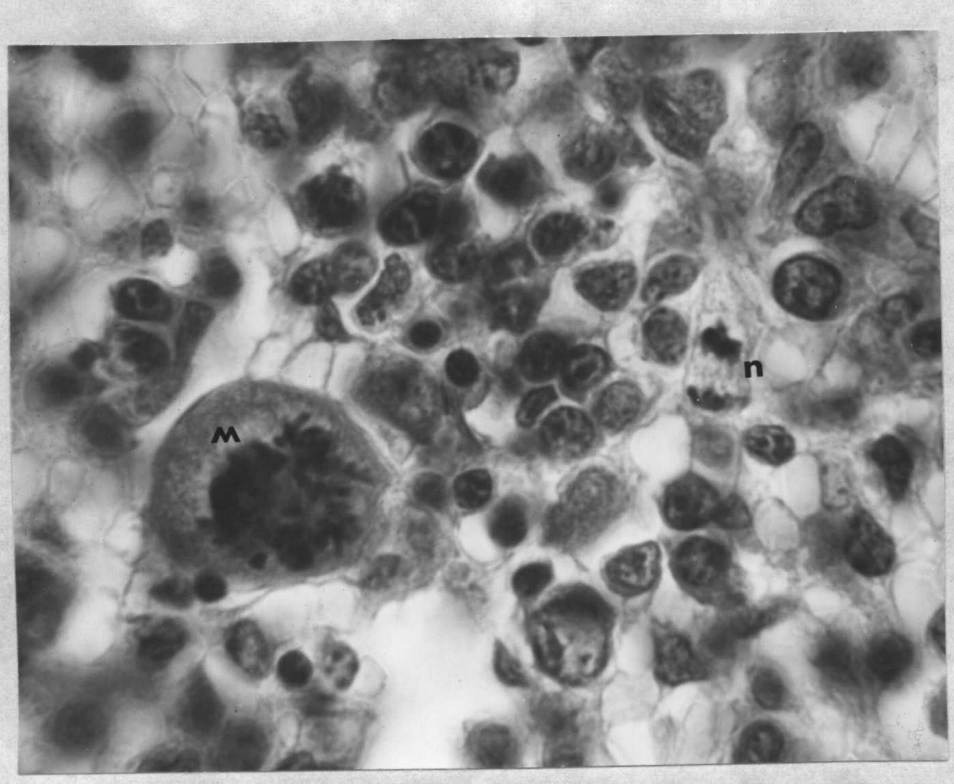

Figure 20

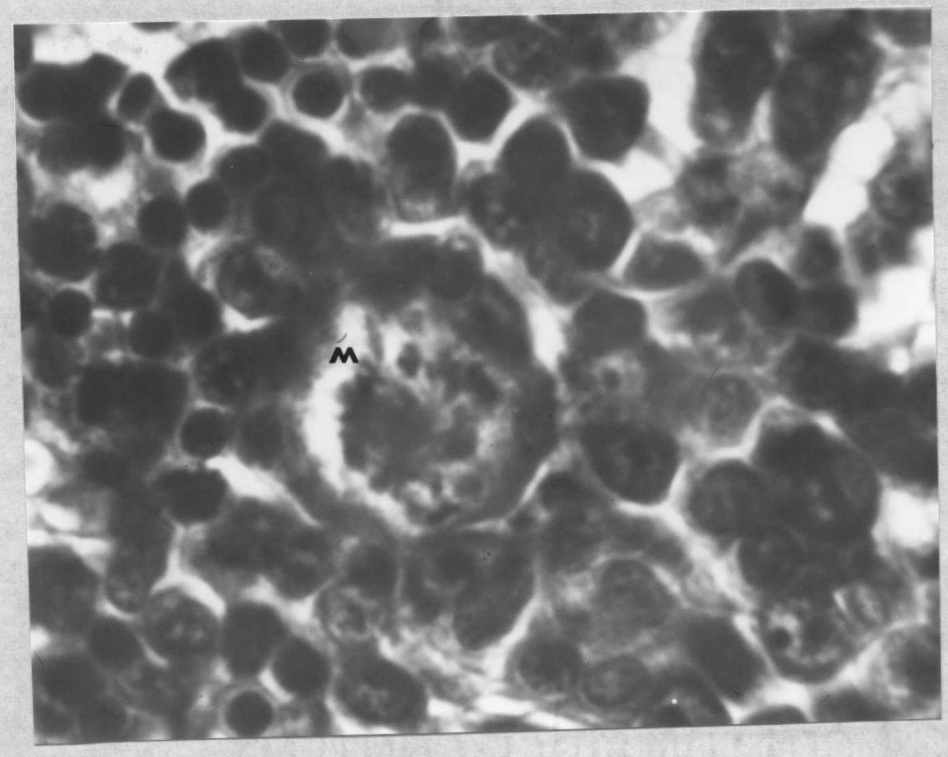

Figure 21

PLATE X

PLATE XI

Explanation of Figures

Figure 22. Erythropoietic centers are numerous in this figure and are the dark staining cells. The main function of the fetal and immature spleens is that of erythropoiesis. Giemsa. X285.

Figure 23. Capsule of the early fetus is composed of a single layer of mesothelial cells. This section also illustrates one of the very few megakaryocytes found in a 17 day gestation animal. C, capsule; M, megakaryocyte. Hematoxylin and Eosin. X285.

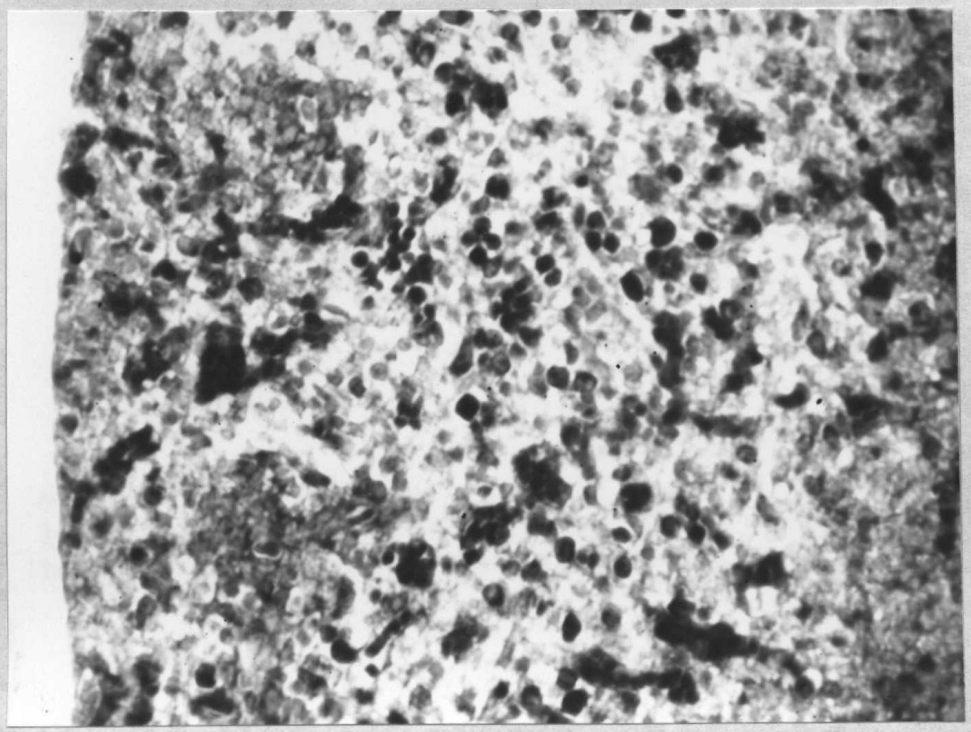

Figure 22

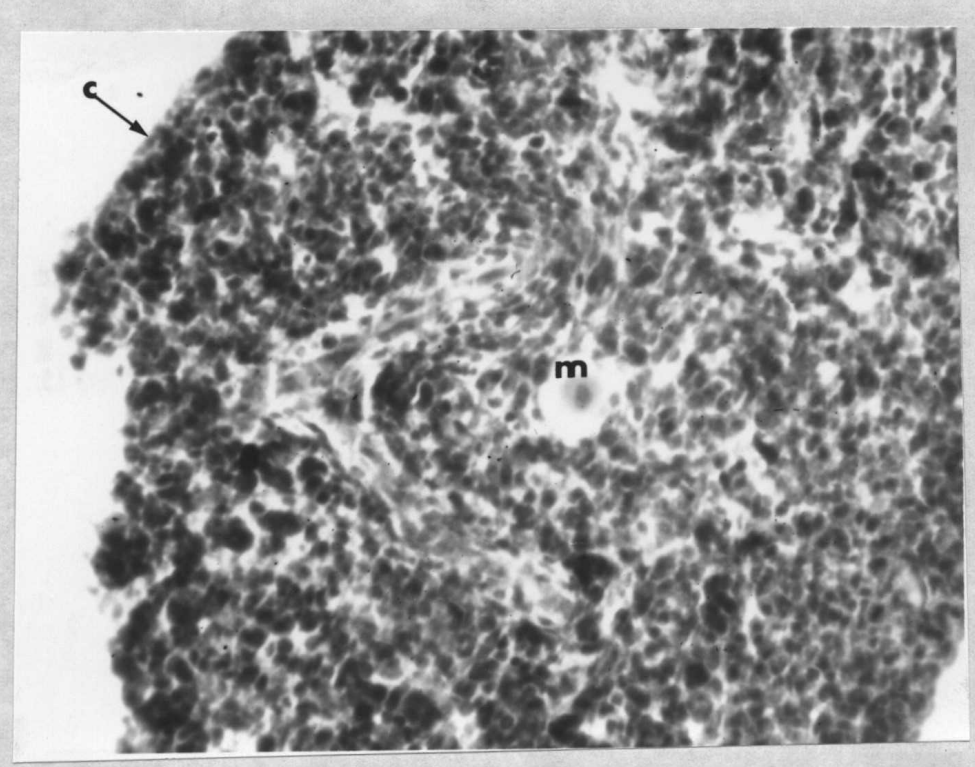

Figure 23

PLATE XI

PLATE XII

Explanation of Figures

Figure 24. The capsule at birth is two cell layers thick and there are indications of developing trabeculae. T, trabeculae; C, capsule layers. Hematoxylin and Eosin. X1260.

Figure 25. The capsule at 21 days is very close to the structure of the capsule of adult animals. The trabeculae extend long distances into the red pulp and contain blood vessels. T, trabeculae; B, blood vessel; C, capsule. Hematoxylin and Eosin. X285.

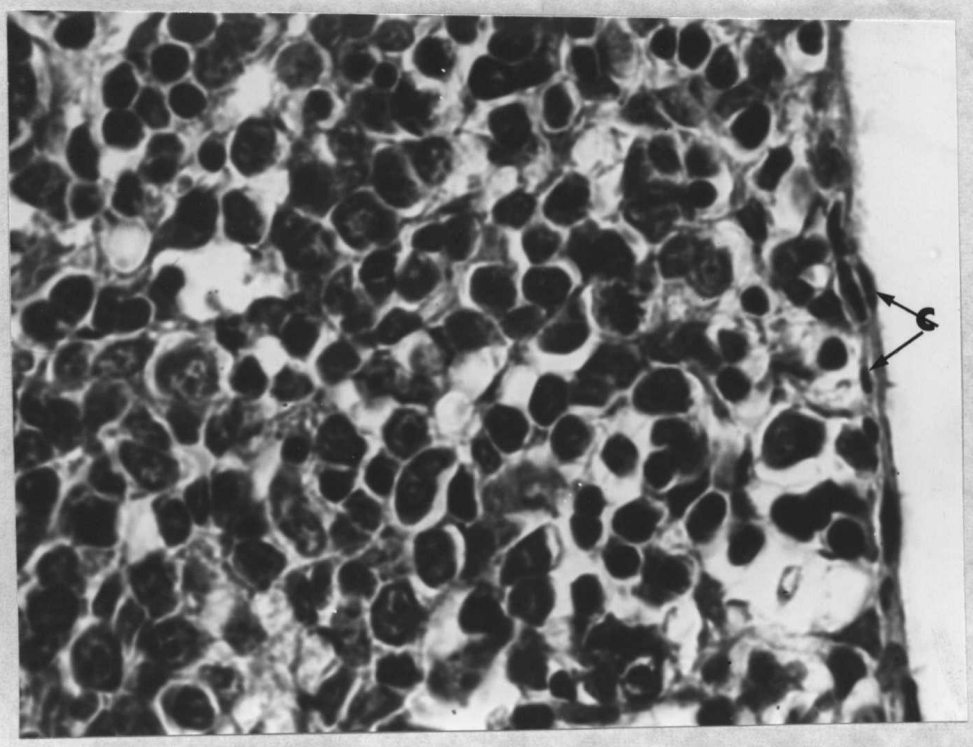

Figure 24

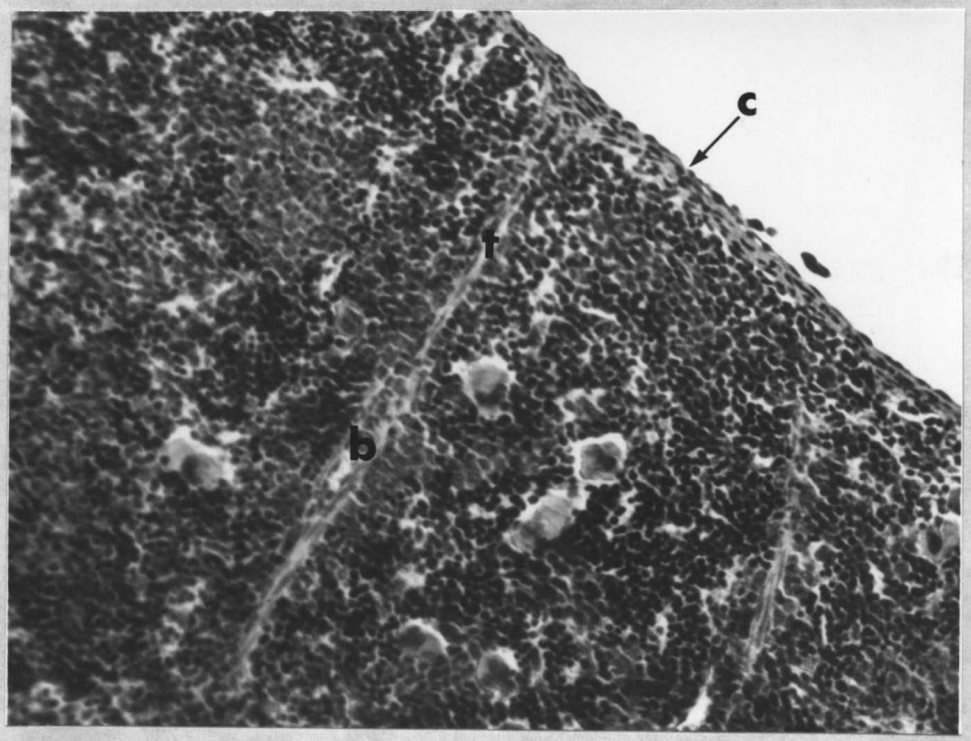

Figure 25

PLATE XII

PLATE XIII

Explanation of Figures

Figure 26. and Figure 27. These figures illustrate very well the age changes that take place in the splenic capsule. Figure 26 is from a 21 day old rat, notice how relatively smooth it is compared to the wrinkled and thickened capsule from a spleen of a senile animal. Reticular stain. X285.

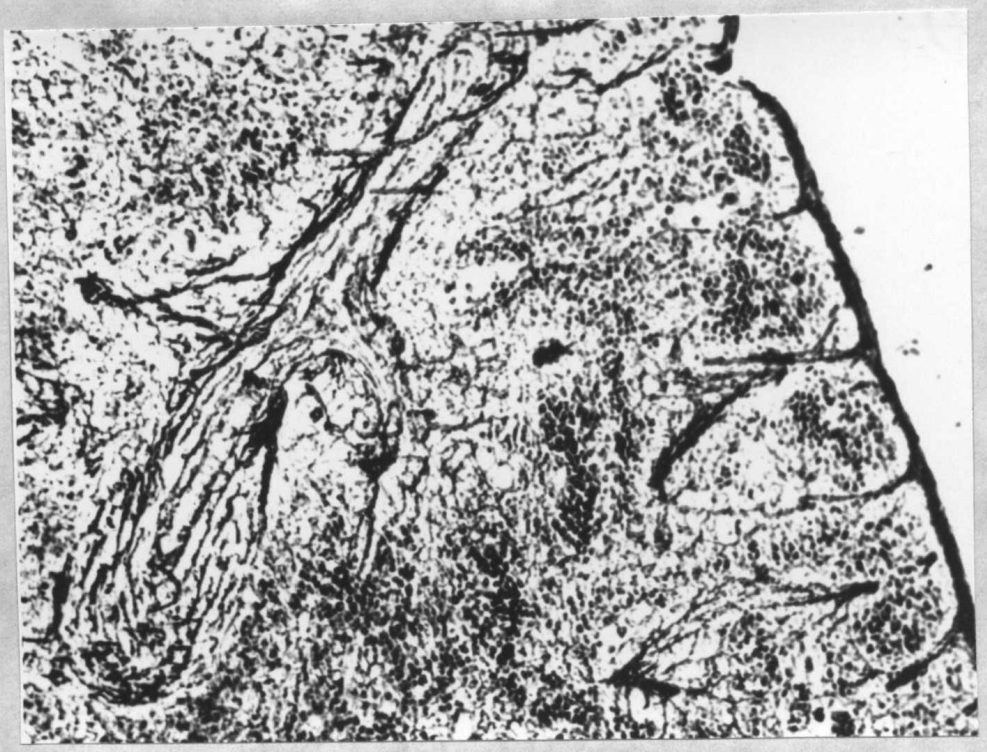

Figure 26

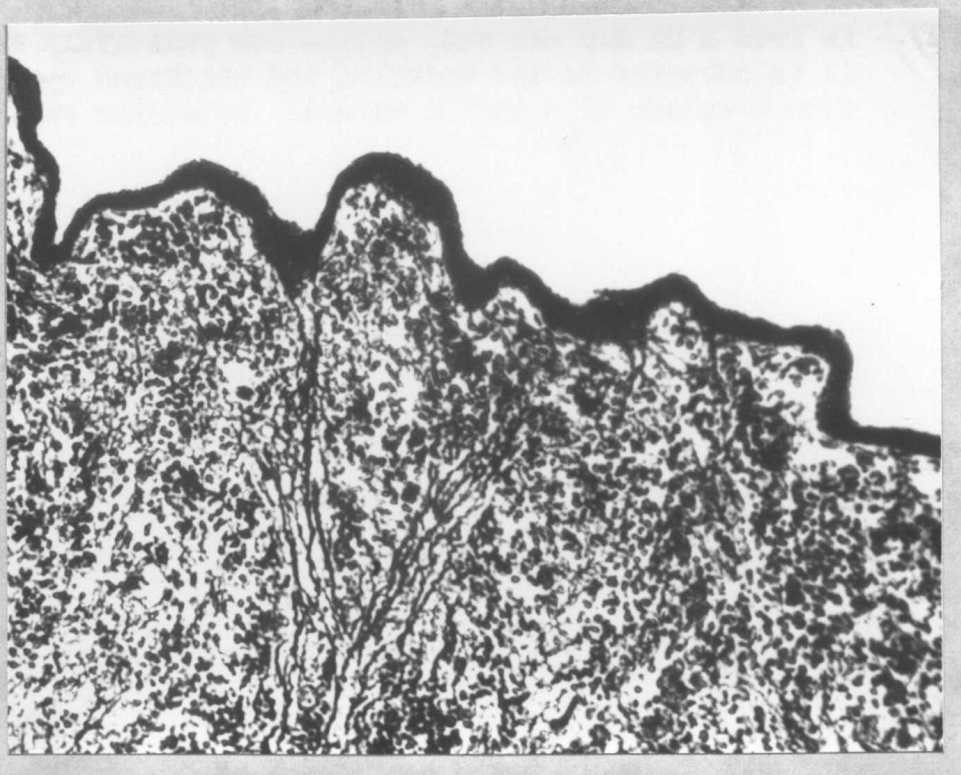

Figure 27

PLATE XIII

PLATE XV

Explanation of Figures

Figure 30. Some animals in group 3 were found to store hemosiderin in the white pulp. This 81 day old animal was the only one under a 100 days old that had such large amounts of iron in the reaction center cells. Red pulp was the main storage area for iron. Arrows indicate large phagocytic cells of reaction center. WP, white pulp; MZ, marginal zone; RP, red pulp. Prussian blue reaction. X285.

Figure 31. The perinodular cells (marginal metalophils) first showed signs of storing iron in this 270 old animal. The reaction is weak when compared to the cells in the red pulp. WP, white pulp; RP, red pulp; P, perinodular cells; MZ, marginal zone. Prussian blue reaction. X285.

Figure 28

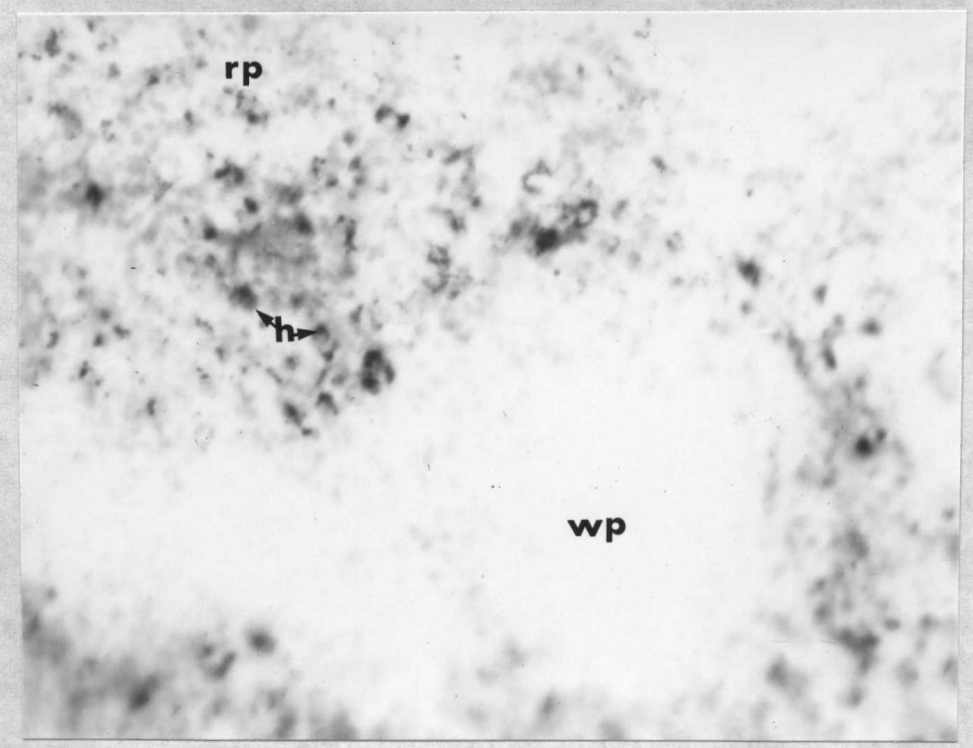

Figure 29

PLATE XIV

PLATE XVI

Explanation of Figures

Figure 32. Characteristic of the senile animals is the great amount of iron they store in the perinodular cells (marginal metalophils) and the large perivascular cells. Note how clear the marginal zone is, clearly indicating that its function is not phagocytic activity. P, perinodular cells; MZ, marginal zone; PV, perivascular cells; WP, white pulp; RP, red pulp. Prussian blue reaction. X285.

Figure 33. Fetal animals exhibited a diffuse tissue fluid reaction to the esterase procedure. As seen in this case, it is very difficult to see cells that actually contain the enzyme. The arrows indicate some cells that the author thought were truly positive for the enzyme. Non-Specific Esterase. X1260.

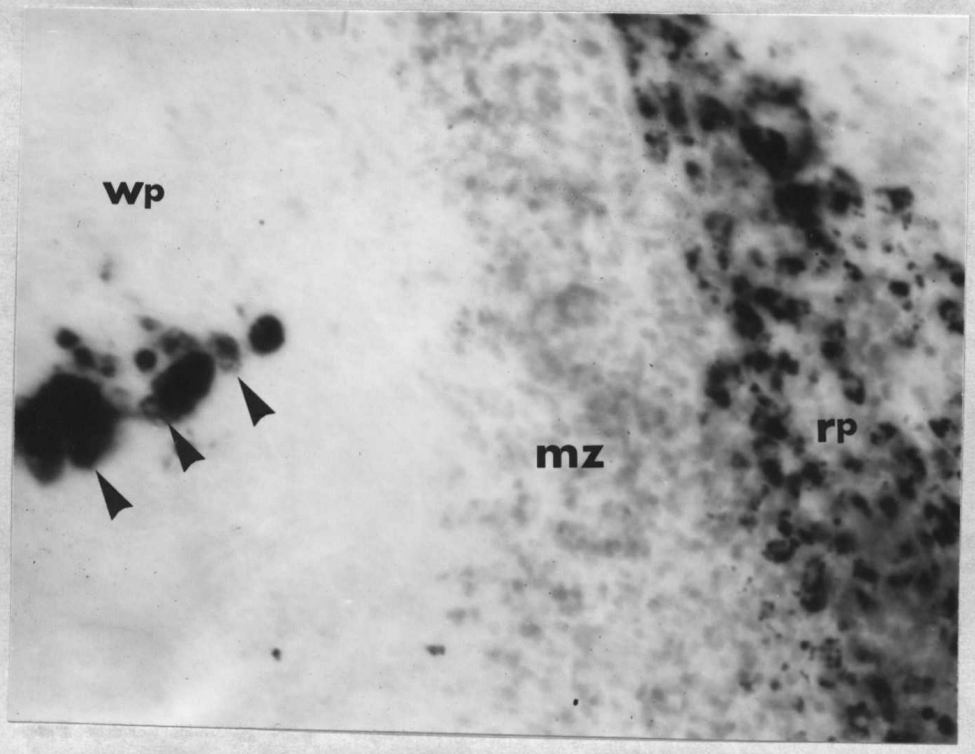

Figure 30

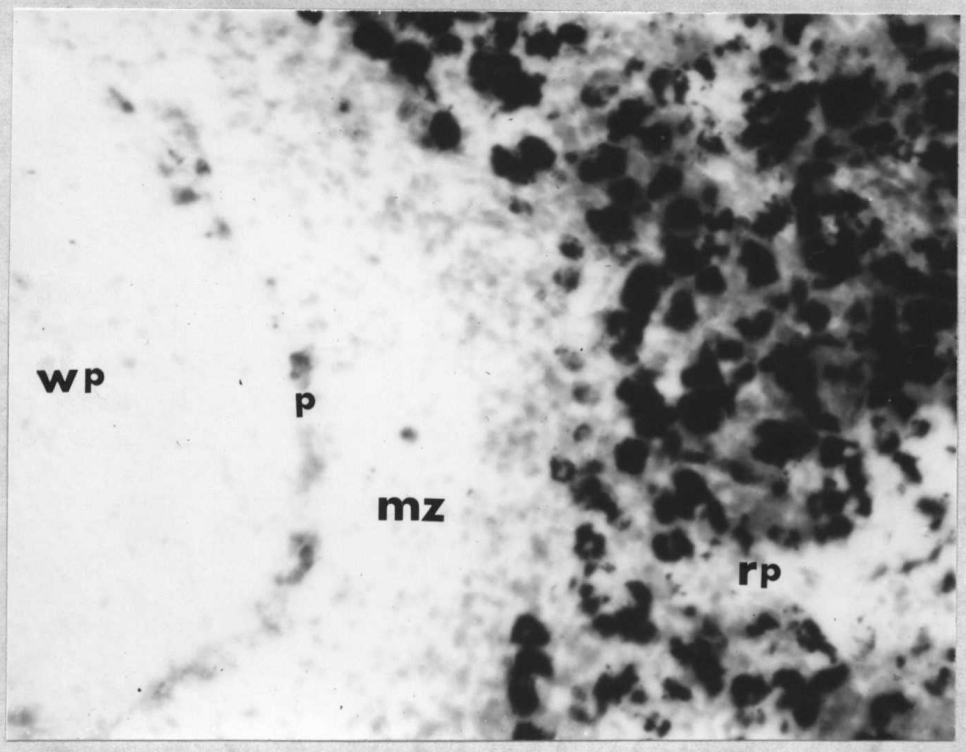

Figure 31

PLATE XV

PLATE XVII

Explanation of Figures

Figure 34. Cells reacting for esterase are clearly visible in this 21 day gestation animal. Note most of the diffuse reaction product is gone. Non-Specific Esterase. X1260.

Figure 35. This esterase section shows that the developing lymphoid sheath is relatively free from esterase positive cells, although in this specimen there are many coarse granules of final reaction product in the lymphoid area. LS, lymphoid sheath; A, artery. Non-Specific Esterase. X1260.

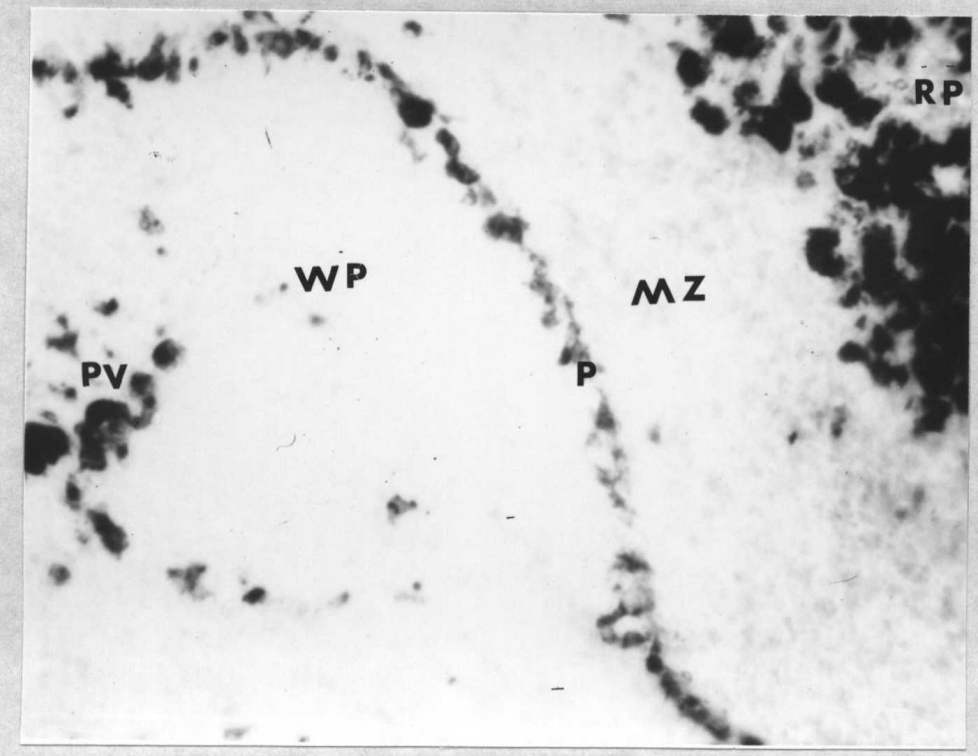

Figure 32

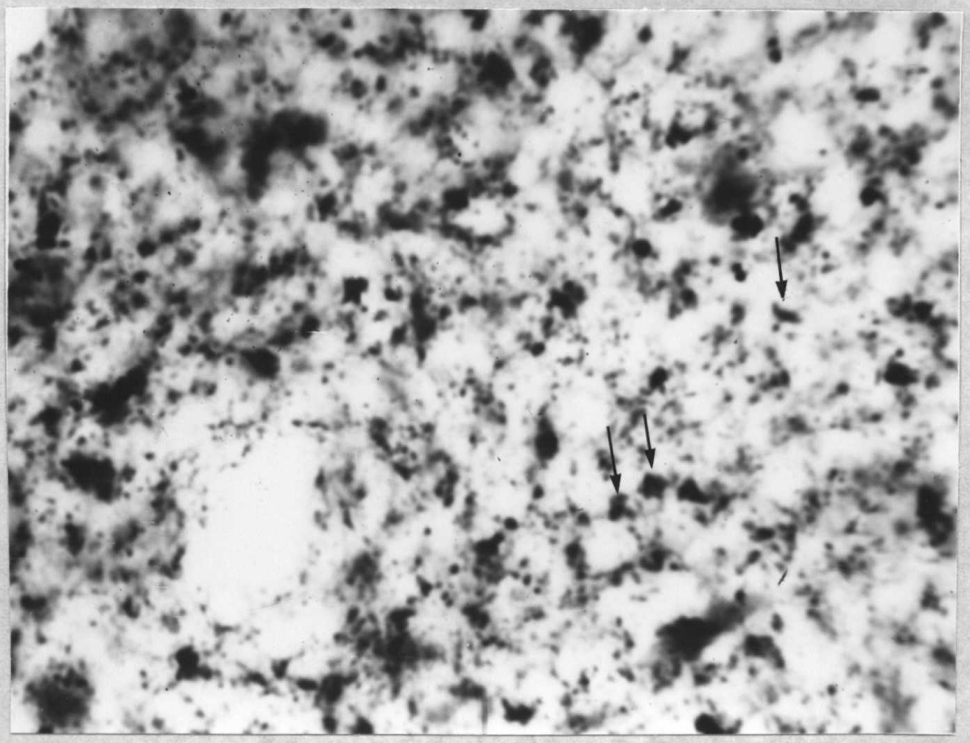

Figure 33

PLATE XVI

PLATE XVIII

Explanation of Figures

Figure 36. Perinodular esterase positive cells can be seen rather faintly. They appear to arise from the white pulp nodule. Note the strong activity of the red pulp macrophages and the relatively non-reaction marginal zone. WP, white pulp; RP, red pulp; P, perinodular cells; MZ, marginal zone. Non-Specific Esterase. X285.

Figure 37. This section was taken from a litter mate of figure 36. These animals were 20 days old and although the perinodular cells are somewhat spread apart it is possible to see that a ring of cells is developing. This high power view shows more clearly the positive cells of the red pulp, perinodular region and some in the white pulp. The marginal zone is clear. WP, white pulp; RP, red pulp; P, perinodular cells; MZ, marginal zone. Non-Specific Esterase. X1260.

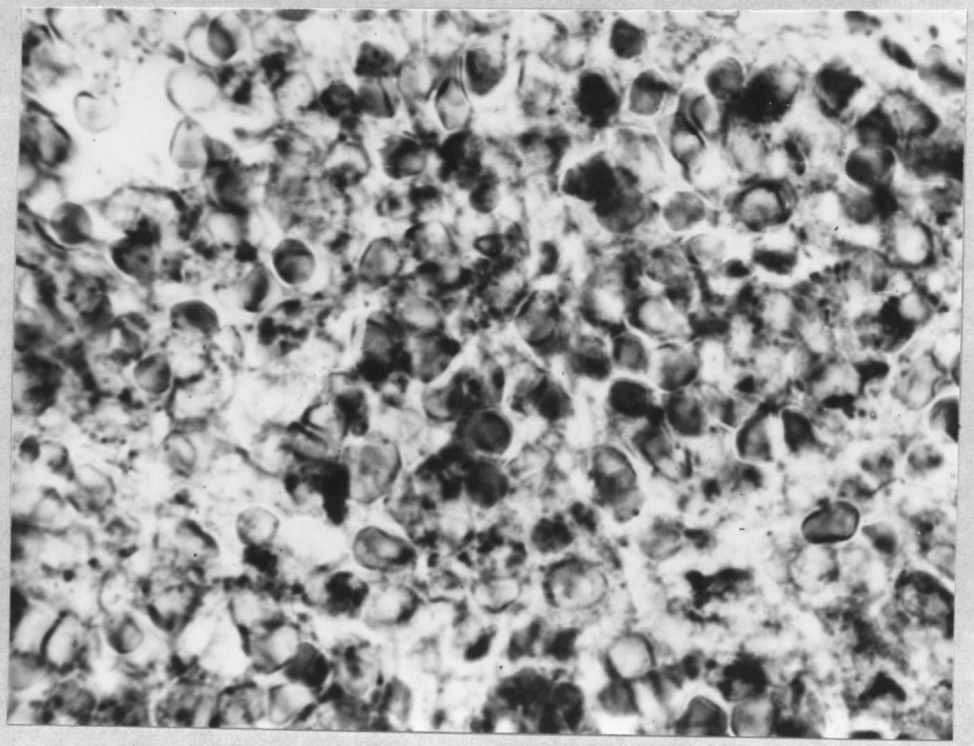

Figure 34

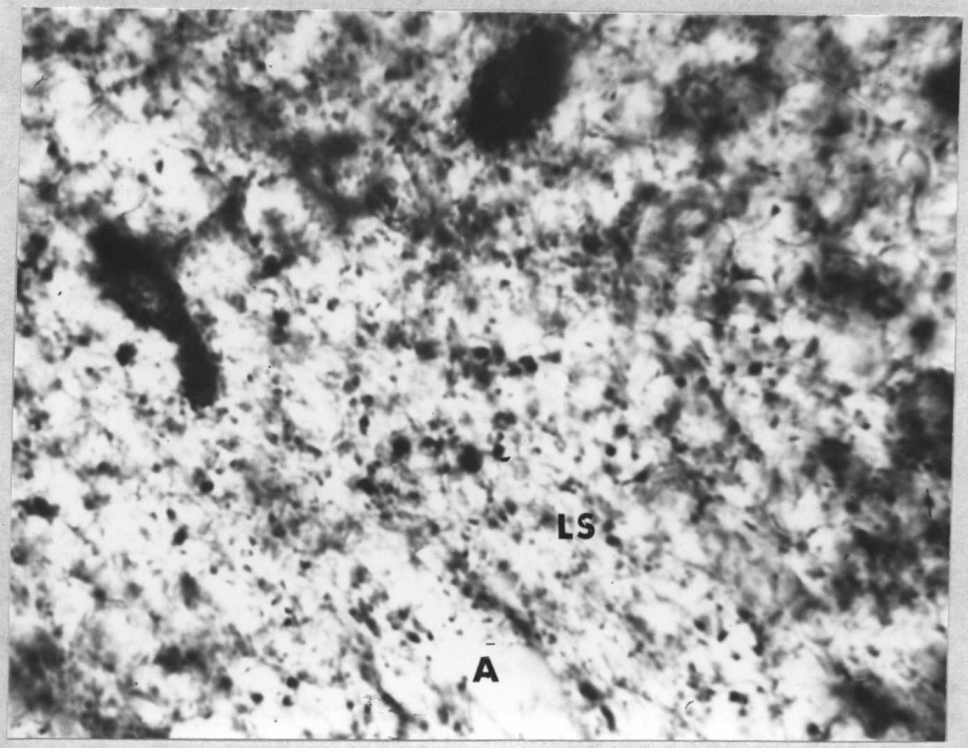

Figure 35

PLATE XVII

PLATE XIX

Explanation of Figures

Figure 38. and Figure 39. The esterase pattern in these two animals is the type normally seen in adult animals. The perinodular cells are obvious but weak reacting when compared to the cells of the red pulp. Figure 38 shows some positive cells scattered throughout the white pulp. This 40 day old animal has no reaction center while the 81 day old animal of figure 39 has many esterase reactive cells in the reaction center. R_s reaction center; P, perinodular cells. Non-Specific Esterase. X285.

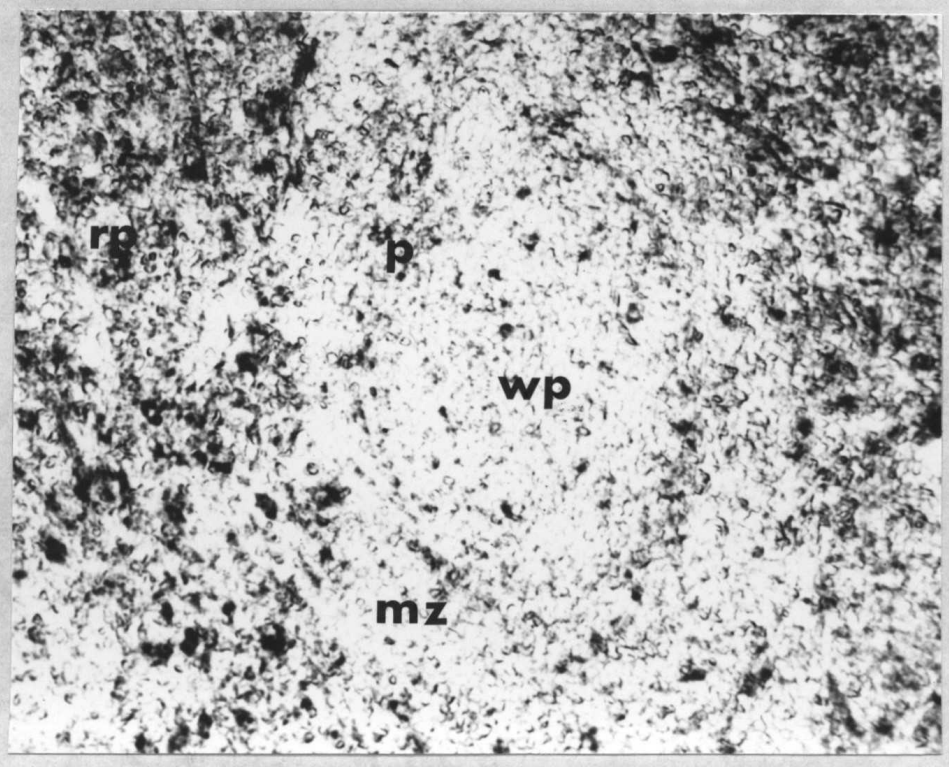

Figure 36

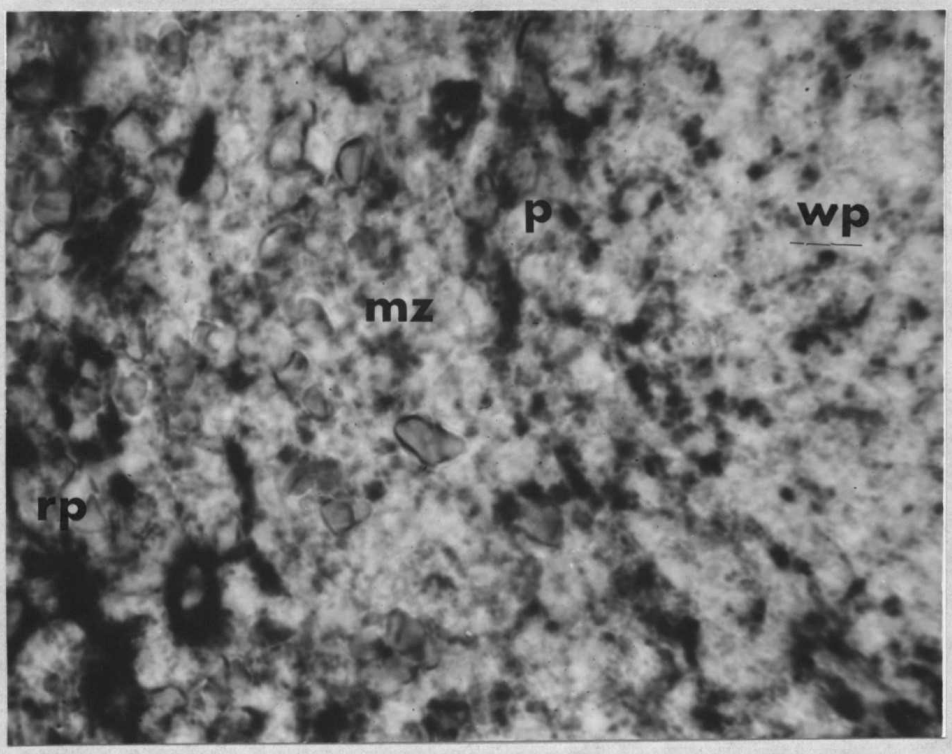

Figure 37

PLATE XVIII

PLATE XX

Explanation of Figures

Figure 40. A 270 day old animal with large esterase positive cells in the reaction center and some large esterase containing perivascular cells. The enzyme activity in the perinodular cells is increased over that seen in figures 38 and 39. R, reaction center; PV, perivascular cells; P, perinodular cells. Non-Specific Esterase. X285.

Figure 41. The main characteristic of the senile animals were the intense esterase response of the large phagocytic cells of the white pulp especially the perivascular cells. The perinodular cells and the red pulp cells have also increased their esterase activity. R, reaction center; P, perinodular cells; RP, red pulp, MZ, marginal zone; WP, white pulp. Non-specific Esterase. X285.

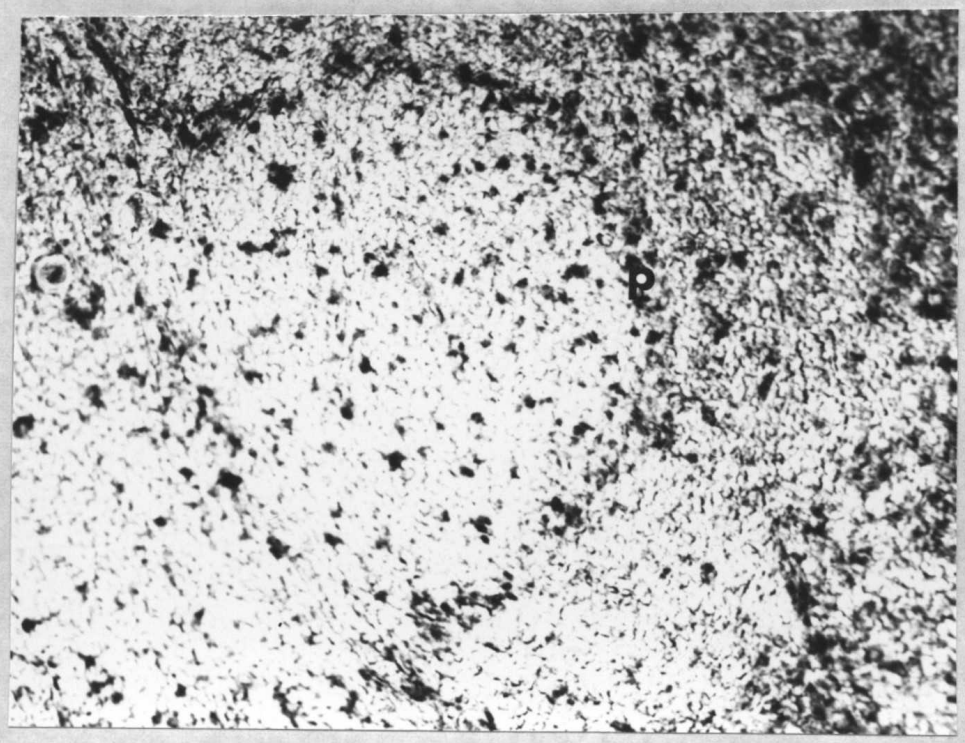

Figure 38

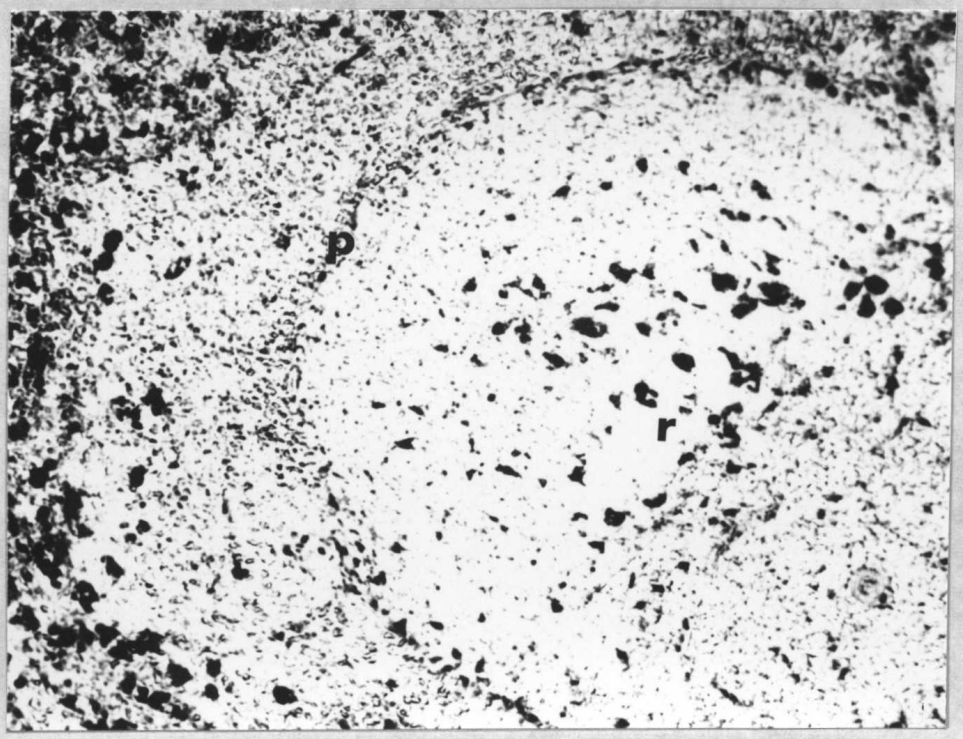

Figure 39

PLATE XIX

PLATE XXI

Explanation of Figures

Figure 42. Compare this acid phosphatase reaction with figure 33, the esterase response on the same animal. Here again is illustrated the diffuse tissue fluid response. Acid phosphatase. X1260.

Figure 43. The developing lymphoid sheaths are relatively free of phosphatase containing cells. The diffuse background reaction is much reduced in this 21 day gestation animal. Positive cells are clearly visible in the red pulp. LS, lymphoid sheath. Acid Phosphatase. X285.

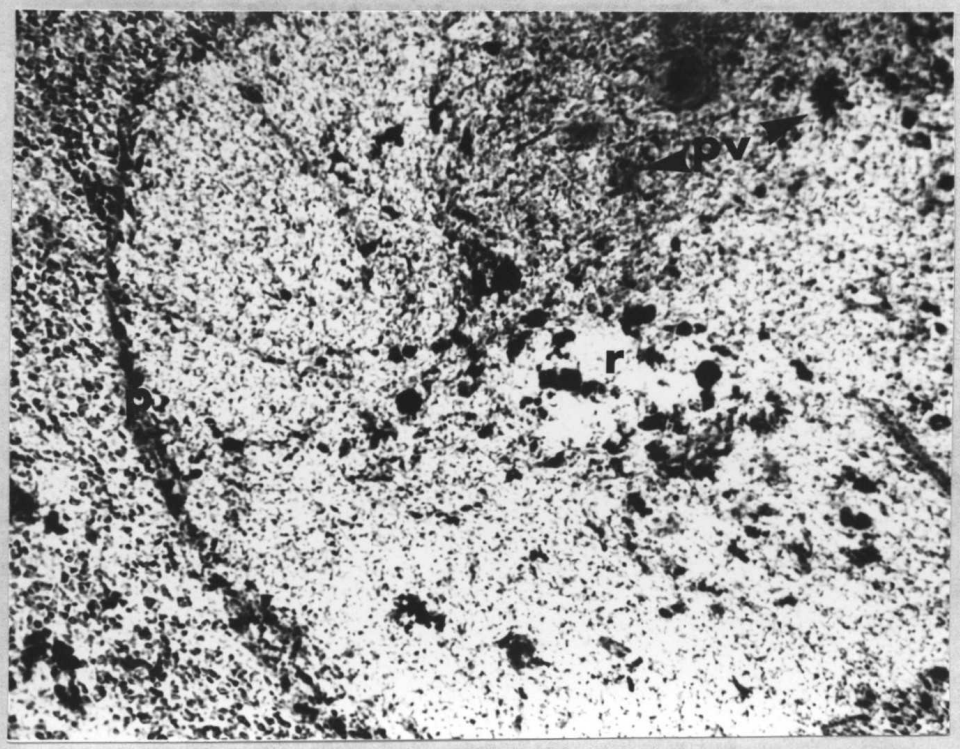

Figure 40

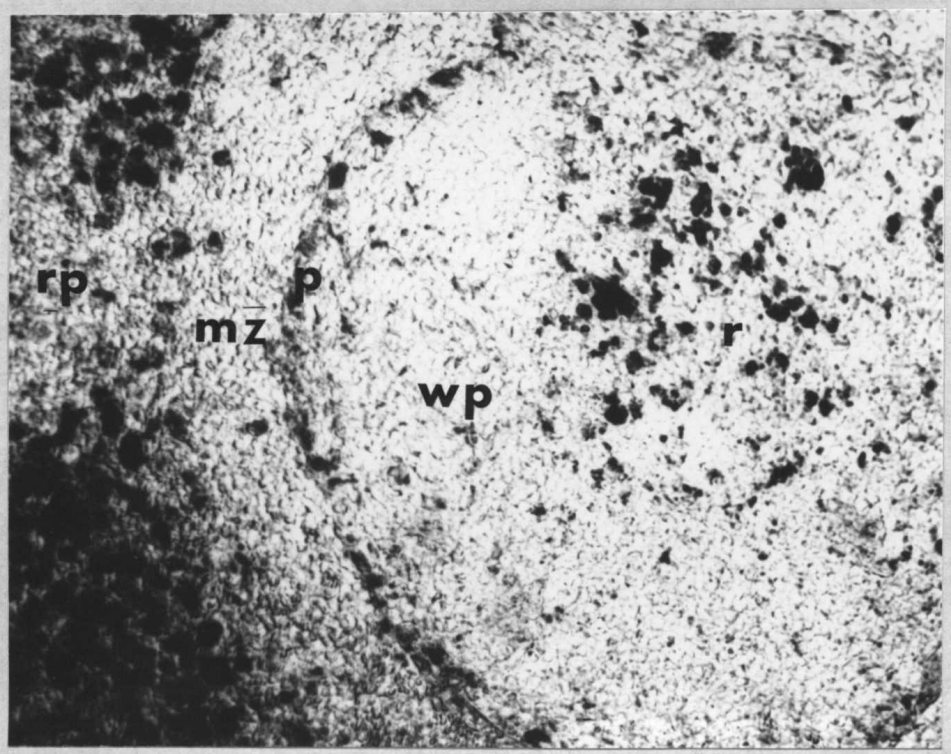

Figure 41

PLATE XX

PLATE XXII

Explanation of Figures

Figure 44. Longitudinal section through an artery with its developing white pulp. Note the intense activity of the cells in the red pulp while the white pulp is relatively few active cells. This was a one day old animal. A, artery; LS, lymphoid sheath; RP, red pulp. Acid Phosphatase. X1260.

Figure 45. In contrast to the weak esterase activity in the perinodular cells, the acid phosphatase response in these cells is very intense. Compare this figure with figure 39. Acid Phosphatase. X285.

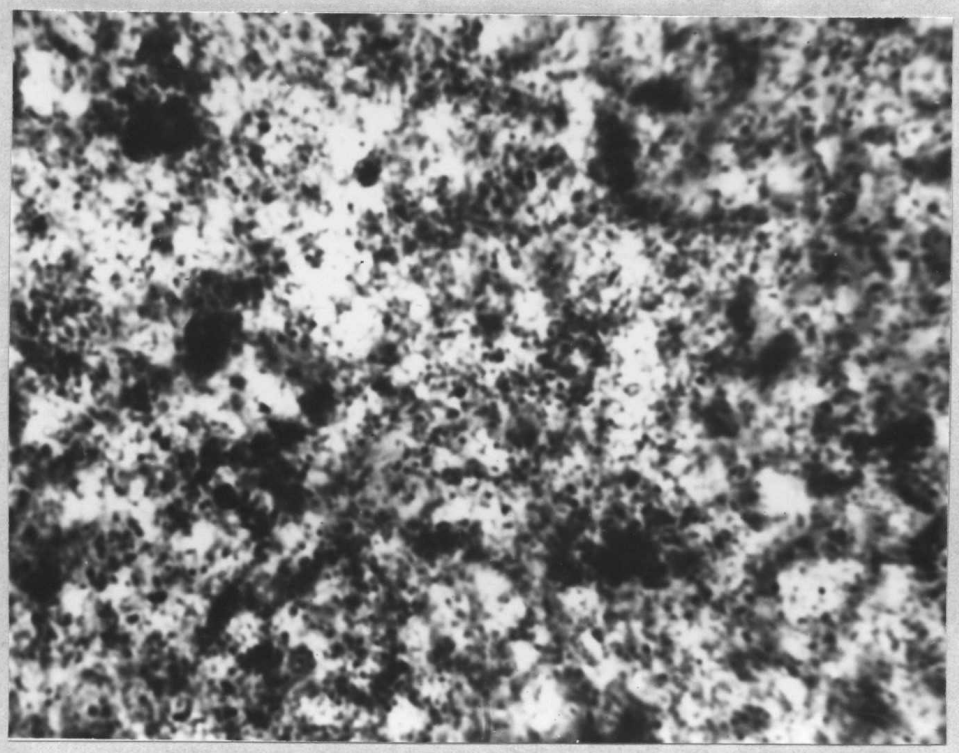

Figure 42

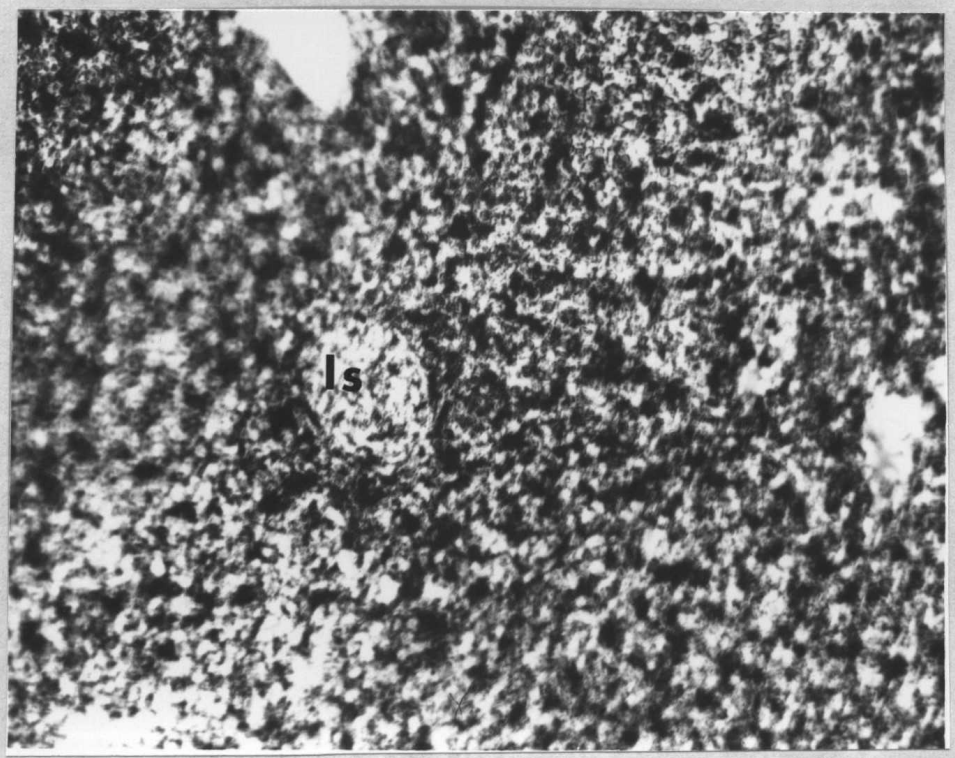

Figure 43

PLATE XXI

PLATE XXIII

Explanation of Figures

Figure 46. and Figure 47. Compare these figures with figures 28 and 39, here again is illustrated the similarity in the action of these two enzymes. The perinodular cells are very reactive for acid phosphatase in both these animals. Figure 47 is from an 81 day old animal and thus has a reaction center which contains acid phosphatase containing cells. R, reaction center; MZ, marginal zone; P, perinodular cells. Acid Phosphatase. X285.

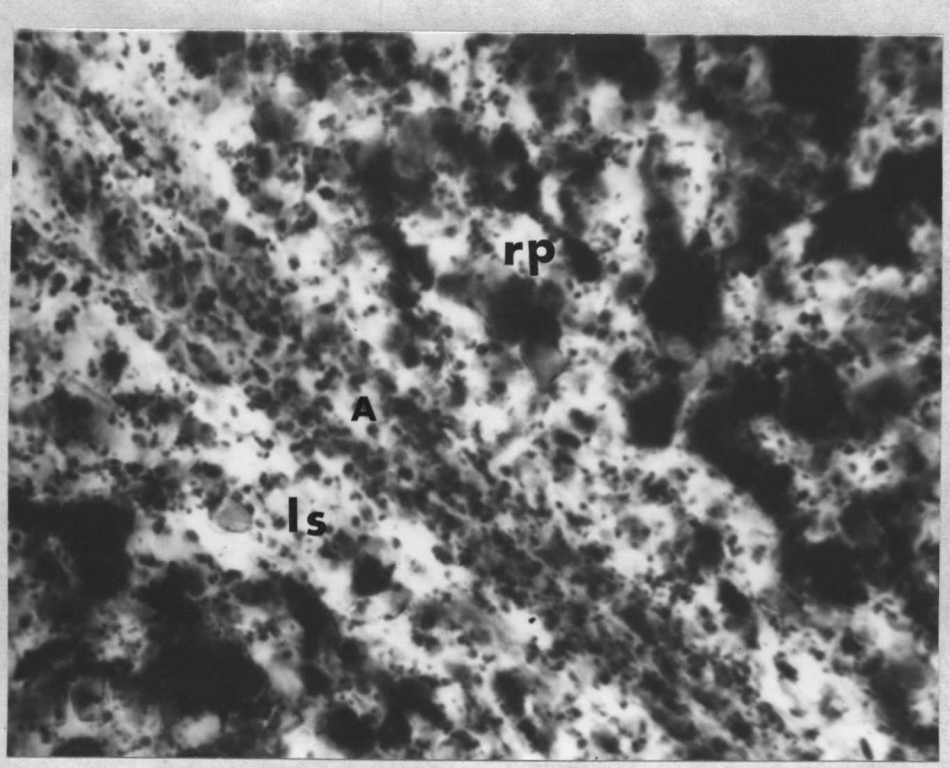

Figure 44

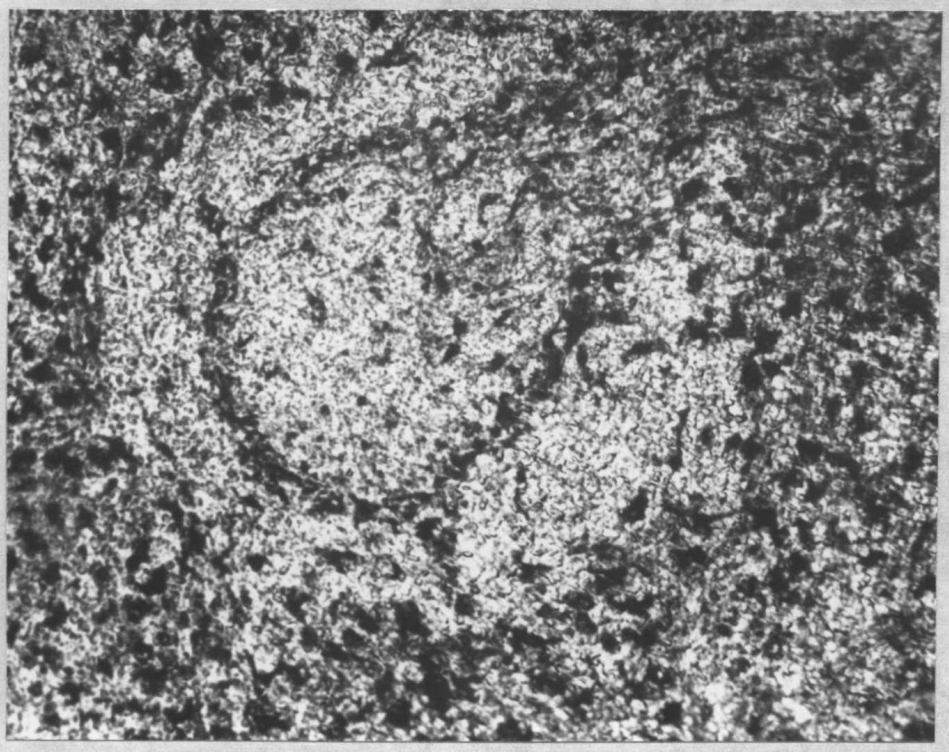

Figure 45

PLATE XXII

PLATE XXIV

Explanation of Figures

Figure 48. A 270 day old rat illustrating acid phosphatase activity in both the reaction center cells and the largr perivascular central artery. This latter finding is not common for this age group. R, reaction center; PV, perivascular cells; P, perinodular cells; WP, white pulp. Acid Phosphatase. X285

Figure 49. Senile animal demonstrating intense acid phosphatase activity in the perinodular cells, reaction center cells and the perivascular cells. The presence of the perivascular cells is characteristic of the senile animals. R, reaction center; PV perivascular cells; P, perinodular cells; WP, white pulp; MZ, marginal zone. Acid Phosphatase. X285.

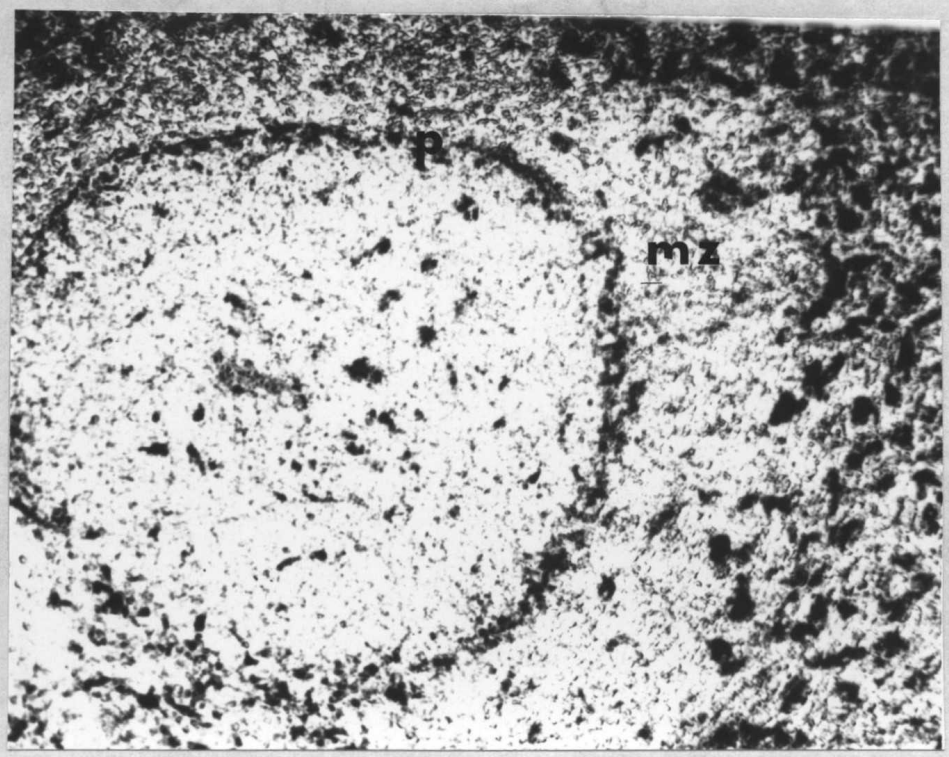

Figure 46

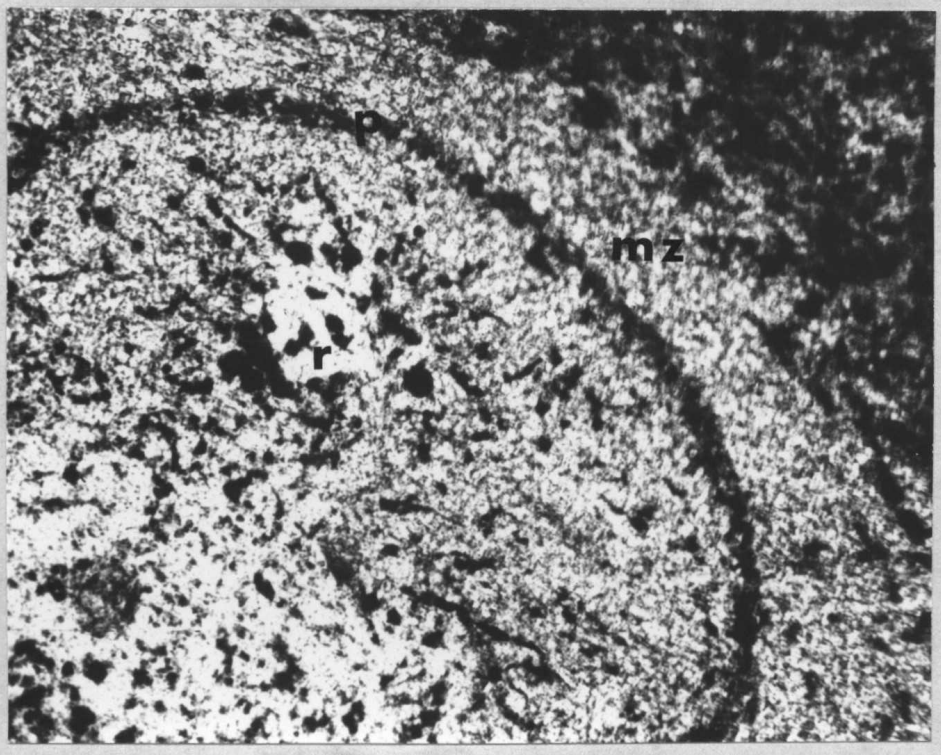

Figure 47

PLATE XXIII

PLATE XXV

Explanation of Figures

Figure 50. The dense black areas are the sinus-lining cells in the 17 day fetal rat. They are so deeply impregnated that it is impossible to see the erythrocytes in the sinus. This is not a normal reaction for these cells and the only time it is seen is in the early fetuses. Marshall's Metalophil Reaction. X1260.

Figure 51. As with the esterase (fig. 35) and acid phosphatase (fig. 43) the metalophil reaction does not impregnate many cells of the developing lymphoid sheath. There are numerous blackened cells in the red pulp. LS, lymphoid sheath. Marshall's Metalophil Reaction. X285.

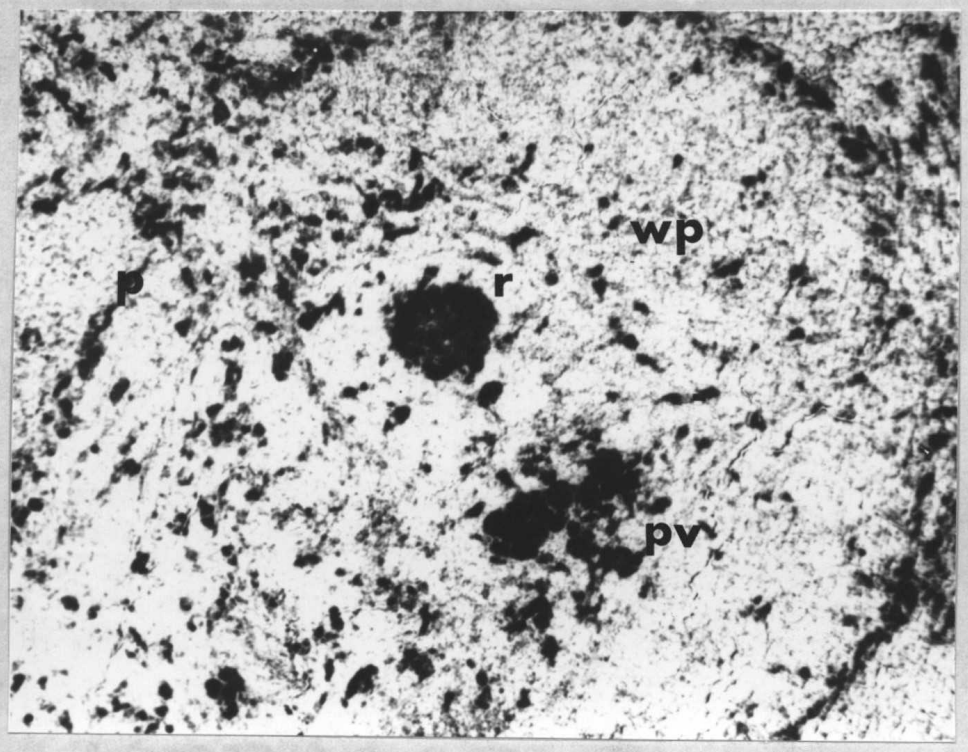

Figure 48

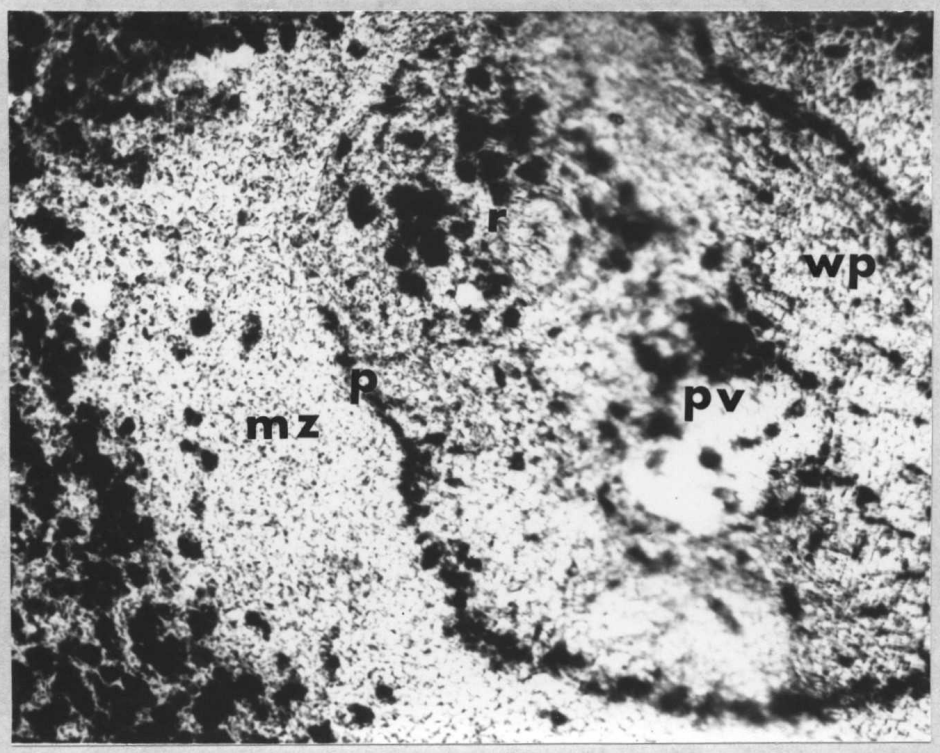

Figure 49

PLATE XXIV

PLATE XXVI

Explanation of Figures

Figure 52. Demonstrates the formation of the marginal metalophil cells in this 20 day old animal. The cells appear to be migrating from the center of the nodule to its periphery. With this change in cellular arrangement the marginal zone becomes evident. Marshall's Metalophil Reaction. X285.

Figure 53. A six day old rat with a few metalophils in the developing lymphoid sheath. This section is similar to those obtained with esterase and acid phosphatase, figures 35 and 43. Marshall's Metalophil Reaction. X285.

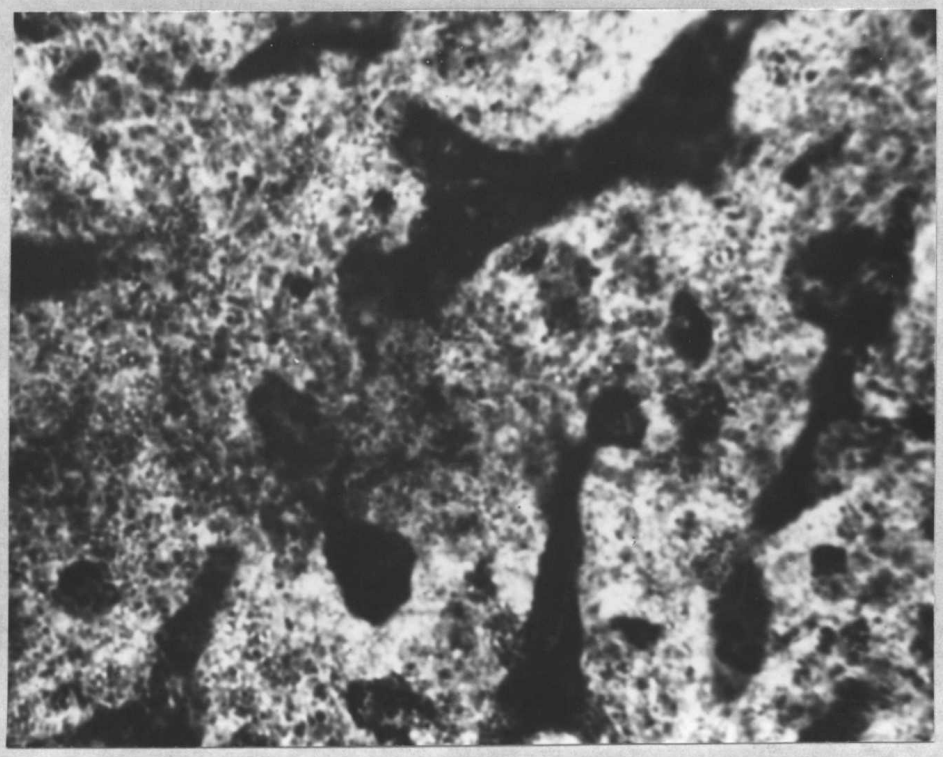

Figure 50

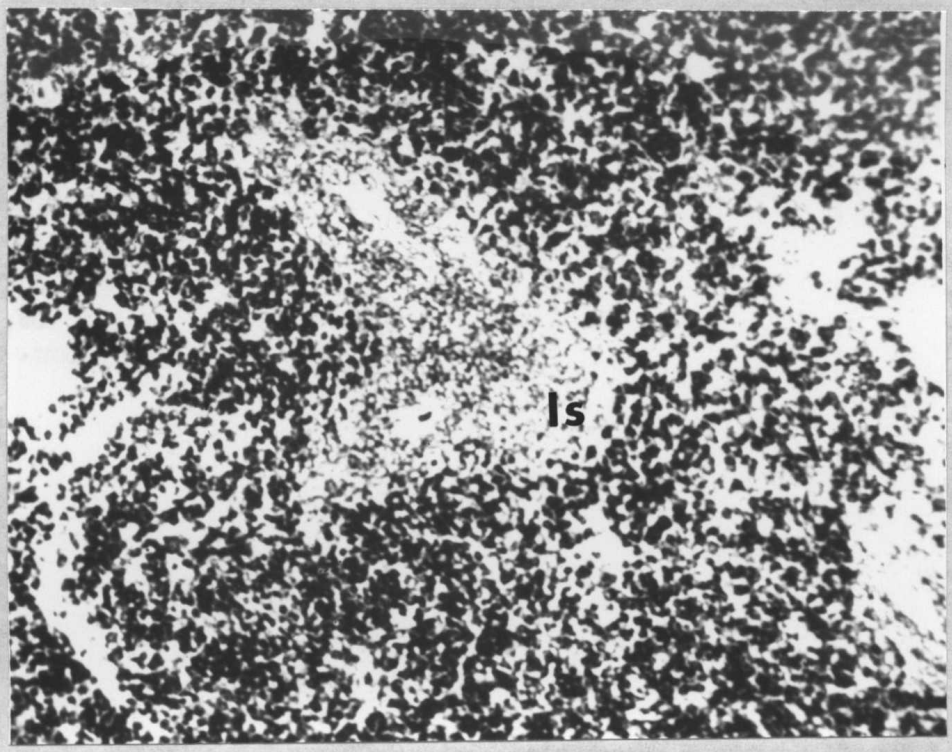

Figure 51

PLATE XXV

PLATE XXVII

Explanation of Figures

Figure 54. This is a 63 day old rat showing a typical "adult" response to the metalophil reaction. The marginal metalophils are clearly visible. There are more reactive cells in the white pulp than is found with either enzyme. The reaction center is visible with its blackened cells and part of the marginal zone can be seen. R, reaction center; M, marginal metalophils; MZ, marginal zone; RP, red pulp. Marshall's Metalophil Reaction. X285.

Figure 55. Senile animal with the large perivascular cells around the central artery and many other impregnated cells throughout the white pulp. The marginal metalophils are very prominent but the marginal zone is not well delineated. PV, perivascular cells. Marshall's Metalophil Reaction. X285.

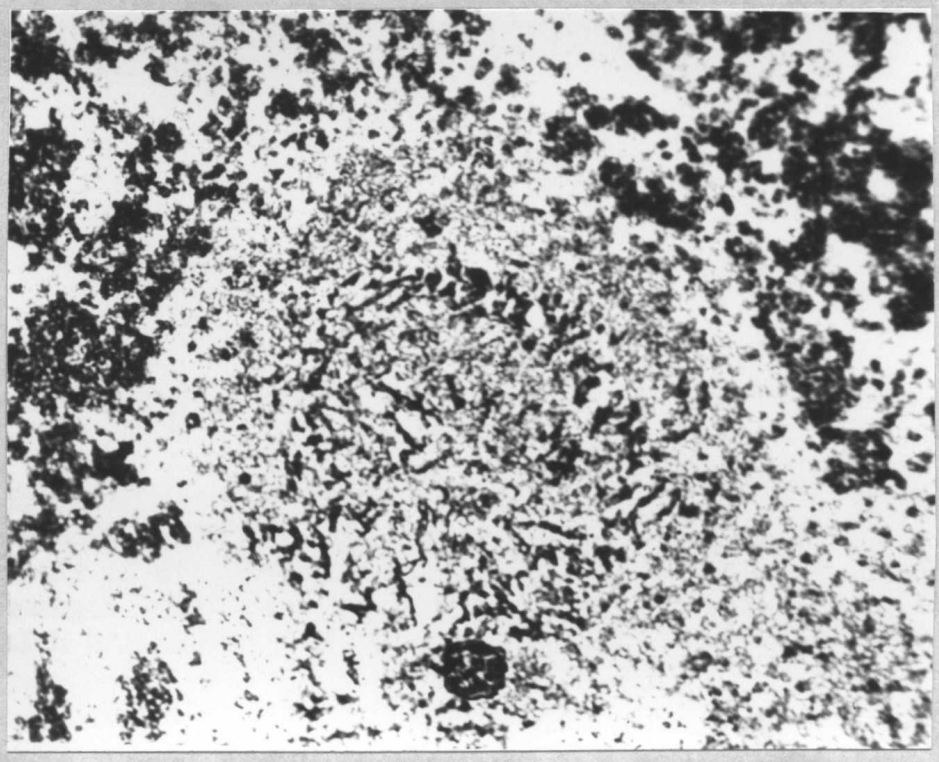

Figure 52

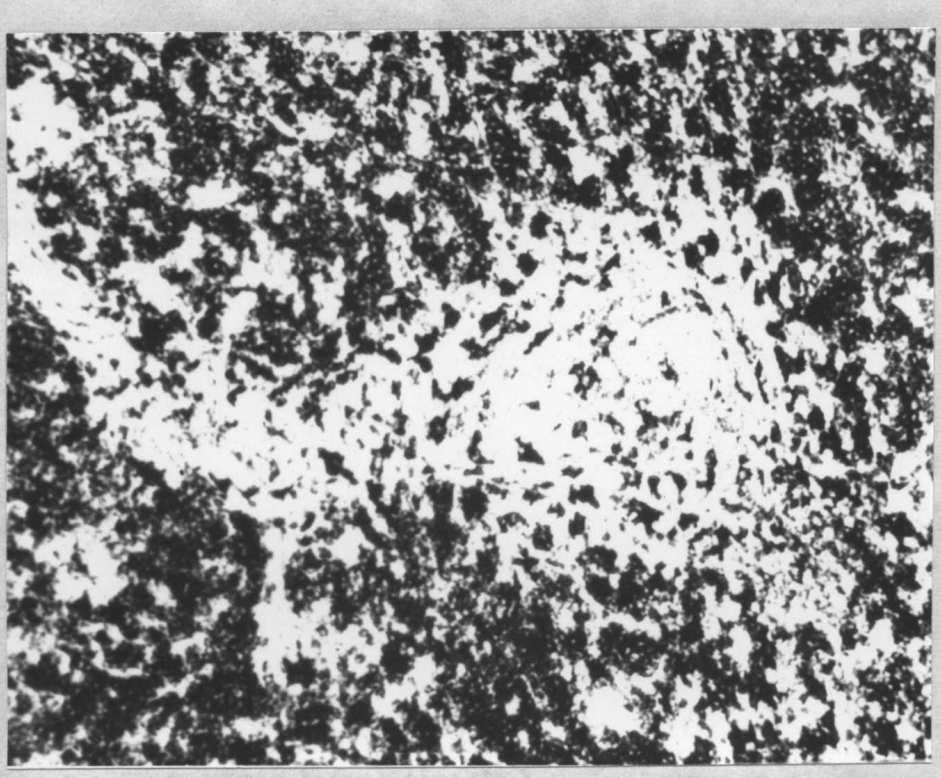

Figure 53

PLATE XXVI

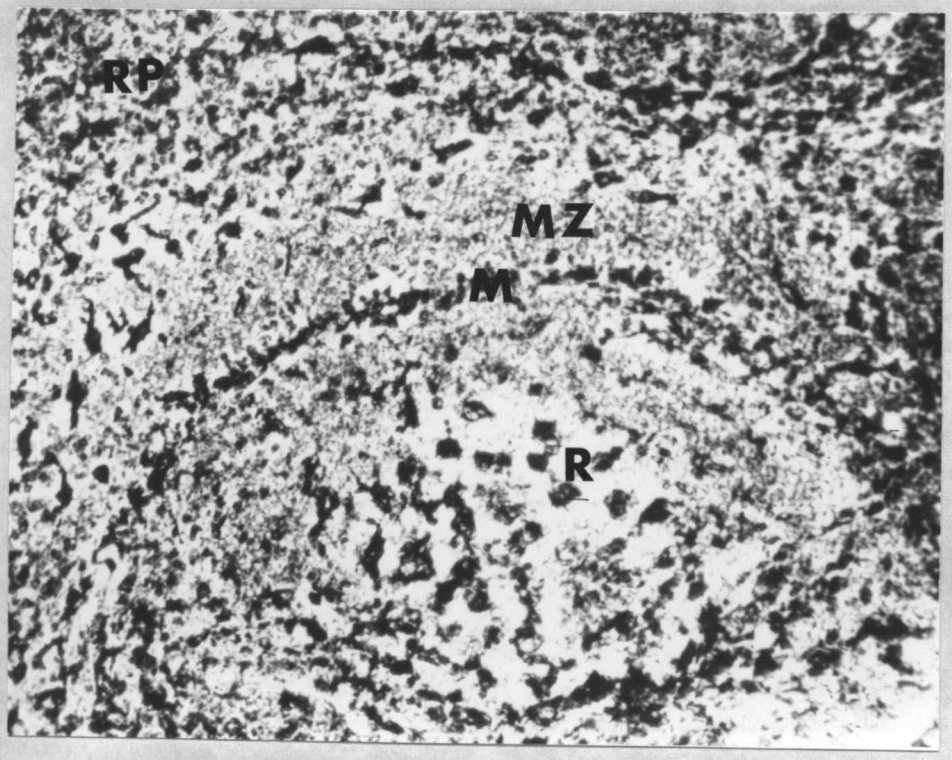

Figure 54

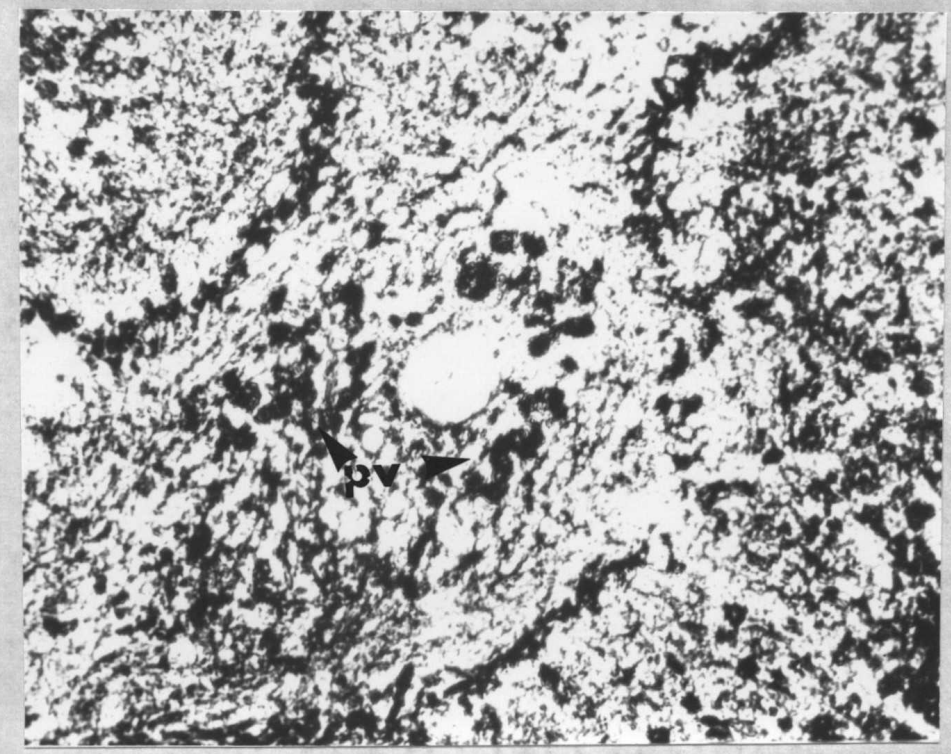

Figure 55

PLATE XXVII