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A Study of the Mesodermal Elements in The Developing, Adult and Ijured Rat Brain

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A STUDY OF THE MESODERMAL ELEMENTS IN THE DEVELOPING, ADULT AND INJURED RAT BRAIN

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CHAPTER I

INTRODUCTION

The function and origin of microglia have been a controversial subject ever since these cells were first described by Rio-Hortega (88) (89). He believed that reactive microglia were cells which showed phagocytic activity at the site of injury in the central nervous system. This response of microglia to injury has led some workers to postulate that these cells are related to the reticuloendothelial system (45)(90). Supporting this theory is their morphological similarity to certain reticuloendothelial (R-E) cells (25) and their apparent mesodermal origin (90). Yet many investigators through the years have found that the phagocytic activity of microglia is slight or absent and that other cells of the nervous system such as neuroglia (31)(33), perivascular cells (23), endothelial cells (5), leucocytes (97) (109) and even neurons (84) may have this phagocytic ability during central nervous system injury. In addition, the mesodermal origin of microglia, although accepted by many, has not been definitely confirmed. The continued introduction of new techniques and the development of criteria which identify cells and cell groups more accurately gives new hope for establishing the definitive role played by microglia in the central nervous system.

The selective demonstration of microglia in the central nervous system was discovered by Rio-Hortega (88) through the use of the silver carbonate method which he developed. With this method he found that the group of cells which was previously described as the third element by Cajal actually consisted of 2 different cells: 1) microglia and 2) oligodendroglia. The silver carbonate method selectively blackened microglia which are characterized by their elongated cell bodies from which branched, spiny processes project. The arrangement of the processes on the cell body is bipolar or multipolar with each process containing numerous branches. Most characteristic are the short spines which project perpendicularly from the surface of the processes. Oligodendroglia, on the other hand, when blackened by a modification of this silver carbonate technique demonstrate a rounded cell body from which a few long smooth processes were seen to penetrate into the surrounding tissue. The nucleus area, which is round, was only lightly blackened, and as a result, differentiation can be easily made between microglia and oligodendrolgia.

The nuclear chromatin patterns of glial cells are described by Penfield (78) who worked with Rio-Hortega. With the Nissl stain, the microglia demonstrate a characteristic elongated irregular nucleus which has large dark nuclear chromatin granules set in a dark parachromatin background. Oligodendroglia have a spherical nucleus with smaller

chromatin granules and a lighter staining parachromatin background. A third type of glial cell, the astrocyte, is the largest glial cell and has a large round to irregular nucleus with very delicate chromatin sparsely dispersed in a very light background.

The silver carbonate technique proved to be of value not only in the nervous system but also in other tissues as well. Rio-Hortega and Jimenez-Asua (91)(92) applied the silver carbonate technique to a variety of tissues and found that certain cells of these tissues also gave the silver reaction. These silver reacting cells were found to be morphologically similar to microglia and to have phagocytic properties under pathological conditions. The splenic pulp which contains numerous phagocytic cells was found to have a large number of these silver reacting cells.

At about this same time Aschoff (4) described a group of cells which he called the reticuloendothelial system. The criteria established by Aschoff for this system of cells were that they: 1) be phagocytic in the presence of large particles and 2) stain with intravital dyes. The cells which he included in this system were: 1) reticulum cells of lymphoid tissues 2) endothelial cells lining sinuses, 3) histiocytes (connective tissue wandering cells) and 4) monocytes. Glial cells were excluded from this system because they did not take up vital dyes.

Long before Aschoff (4) described this system, Metchnikoff (68) (69) studied inflamation and described the reaction of phagocytes to foreign bodies. He designated these phagocytic cells as macrophages based on their physiological activity. The term macrophage was used by Evans (29) who made an attempt to define them more clearly and to give them a specific origin. Evans (29) made note of terms given to phagocytic cells and because of their phagocytic properties counted them under the heading of macrophages. These phagocytic cells are the rhagiocrine cells described by Renaut, clasmatocytes described by Ranvier, adventitial cells of Marchand, resting wandering cells described by Maximow (polyblast) and the pyrrhol cells of Goldmann (29). There is general agreement that these cells are one and the same based on their phagocytic ability (29) (100). Much disagreement arises, however, in the origin of these cells. A group consisting of Mallory, Foot, Sabin and others holds that endothelial cells of capillaries were modified into these phagocytic forms (52)(97). Maximow, (63), on the other hand, derives his polyblast from both agranular leucocytes and fixed connective tissue cells. In recent years the endothelial origin of macrophages has lost ground to the ideas presented by Maximow (22).

The discovery of microglia by Rio-Hortega (88) immediately stimulated numerous studies on their phagocytic ability. Rio-Hortega (88) (89) was the first to observe that the cells which he had described as

microglia proliferated during injury to the central nervous system and that transitional forms could be noted between microglia and phagocytic cells. This change was the drawing up and thickening of its cytoplasmic processes with a gradual elongation of the cell body. The transitional stages appeared hypertrophied with shortened irregular processes. Many stages of transition were seen to occur between the normal and phagocytic cells which were completely rounded-up. These changes were seen to take place in the area immediately around the wound. Russell (97) reported that Metz and Spatz (70), Gallego (36) and Struwe (107) all noted the transition of microglia into phagocytic cells. Similarily, Schaffer (102) and Jimenez-Asua (45) were in agreement with Rio-Hortega's findings. Additional work by Rio-Hortega and Penfield (93) demonstrated that astrocytes were found to proliferate around the wound area but only the microglia took part in the phagocytic process.

Russell (96) was the first to relate vital staining, phagocytosis and silver reactivity to microglia. She found that microglia of the injured central nervous system were transformed into phagocytic cells which stained vitally. This ability to take up vital dyes was found to increase as the microglia became rounded into ameboid cells. Kubie (54) and Bratiano and Guerriero (12) could not find up-take of vital dyes in microglia. Lebowich (59), however, was able to extend this work and confirm the presence of vital staining in the nervous system following

the use of various stimuli. He demonstrated that vital staining was not present in normal resting microglia. Wells and Carmichael (114) and Costero (19) demonstrated vital staining in cultured cells of the brain.

Although the properties of silver reactivity and vital staining had been noted in phagocytic cells in separate studies, this relationship was first applied to cells of the spleen, kidney and the liver by Dunning and Stevenson (25). The reaction which followed the introduction of a hot trochar into these organs was studied with the silver carbonate reaction and trypan blue injection. They concluded that the cells which appeared in the wound were morphologically similar to transitional forms of microglia and that these cells reacted to trypan blue as do microglia. Dunning and Furth (24) noted that the morphological relationship between monocytes, microglia and histiocytes is strikingly similar. The phagocytes seen in lung alveoli have been studied by Marshall (61) with various silver techniques and vital dye injection. The studies show that again silver reactivity is found in cells which take up vital dyes. More recently, investigations on the central nervous system by Dougherty (22) point out the morphological relationship between brain phagocytes and agranular leucocytes. He also demonstrated that the properties of phagocytosis, vital staining and the ability to give the silver reaction can all be found in microglia following injury.

The relationship of microglia to the R-E system was first pointed out by Rezza (87) in 1924 and has been discussed by Jimenez-Asua (45) and Penfield (79). Jimenez-Asua (45) considered the similarity of silver reactivity, morphological appearance, mesodermal origin and phagocytic function to be valid criteria for relating microglia to the R-E system of cells. Penfield (79) believed that microglia resembled R-E cells and that they should be included in this system. This R-E relationship was later further established by Russell (96) who based her idea on the vital staining ability of stimulated microglia. Rio-Hortega (90) and Penfield (81) also endorsed this concept in reviews of microglial studies. Dougherty (22), in a special study utilizing all the criteria established for R-E cells, concluded that a R-E relationship exists for microglia.

Although many investigations appear to bear out the phagocytic role of microglia, this idea has not always existed, and, even presently, many findings exist which do not support such a concept. Many of the very early investigators postulated that central nervous system macrophages were of leucocytic origin. Gluge (38) in 1841 was one of the first to report that cells migrated into the brain at inflammatory sites. He called these cells "Entzuendungskugeln" (inflammation corpuscle). Strobe (106) described an early leucocytic infiltration and a later, much larger migratory cell the "kornchenkugeln" (compound granular corpuscle). Ribbert, Cohnheim and Kolbe all supported a leucocytic origin for these reactive central nervous system cells (97).

Nissl (75) lead another group which believed that adventitial cells of blood vessels formed phagocytes and that leucocytes could not leave the blood vessels to enter the nervous tissue because of a boundary membrane. He introduced the name "Gitterzellen" (gitter cells) for central nervous system macrophages because of their lace-like cytoplasm. The adventitial concept gained immediate supporters, many of whom also supported a leucocytic origin.

The idea that glial cells could possibly form phagocytes was suggested very early by Schwalbe (104), and Friedmann (35) and was the first concept which Nissl proposed (74). Robertson (94) regarded these phagocytes to be derived from glial cells which he called mesoglia because of their mesodermal origin. Numerous investigators accepted this glial origin but also preferred to include leucocytes as additional progenitors of central nervous system macrophages.

Following the discovery of microglia by Rio-Hortega, (88) many studies continued to appear which were not in accord with his concept. The transformation of oligodendroglia into compound granular corpuscles was described by Fernado and Davidoff (31). They demonstrated acute swelling and hypertrophy of oligodendroglia which were believed to be transitional forms between the normal cells and phagocytes that could ingest fat. Penfield and Cone (82) just 2 years previously, in a study of pathological changes in the brain, noted acute swelling of oligodendroglia

but did not relate them to phagocytic cells. Rio-Hortega and Penfield (93) noted a proliferation of astrocytes around the injured area but found that they did not take part in phagocytosis.

The development of compound granular cells in human nervous tissue following injury was found to be from capillary endothelial cells, advential cells of larger blood vessels and mononuclear cells of the blood by Baggenstoss, Kernohan and Drapiewski (5). They believed that microglia play only a minor role in the formation of these phagocytes and that astrocytes were not significantly involved in the scar formation until at least 6 months has elapsed. Dublin (23) studied wound healing of rabbit brains and found that adventitial cells of blood vessels are primarily involved in the formation of compound granular corpuscles. Microglia and endothelial cells were believed to play only a very small role in this activity.

The difference in opinion concerning phagocytic origins has been related to species differences by Field (33). He has shown that in the cat, microglia have little or no phagocytic activity but that rabbits and mice demonstrate a definite transformation of microglia into compound granular corpuscles. In the cat, the phagocytic cells were believed to develop from oligodendroglia which had undergone acute swelling. These cells first transformed into pseudogranular corpuscles or mulberry cells which were so-named because of their shape and absence of fat inclusions.

Forty hours following injury these mulberry cells began to take-up fatty material and were then considered to be true granular corpuscles (gitter cells). In the rabbit these changes were not seen in oligodendroglia. However, in the mouse, mulberry cell formation was occasionally present.

The advent of radioautographic techniques has brought a new method for the study of cell origins. Work with tritiated thymidine on injured brains suggests that a minimum of 50% of the macrophages appearing in the first 2 days and 70% thereafter are from leucocytes of the agranulocytic series [Konigsmark and Sidman (52)]. Similarly, Kosunen and Walsman (53) found that inflammatory cells of allergic encephalomyelitic lesions were from blood cells rather than from cells of the brain. This same finding was also demonstrated in simple injury to the spinal cord by Adrian and Walker (1). Altman (2), however, found that oligodendroglia and astrocytes may be the precursors of microglia and macrophages as shown by tritium studies.

A recent study using electron microscopy showed that perivascular cells become phagocytic in irradiated brains (65). Blunzinger and Hager (9) demonstrated, however, the transformation during injury of microglia to phagocytic cells whose ultrastructure is similar to that of tissue phagocytes of histocytic origin. Russell (97) observed mononuclear cells in stab wounds of the brain as early as 12 hours after the

wound and the phagocytosis of fatty material by 18-24 hours. These cells contained a typical monocyte type nucleus. Russell (97) believed that microglia may be nothing more than blood monocytes which can migrate freely in and out of the brain through the vascular system. The shapes of microgiia which occur are believed to be due to the nature of the intercellular space which is found throughout the brain.

The origin of microglia from mesodermal elements was described by Rio-Hortega (88) at about the time he discovered these cells by selectively reacting them with silver carbonate. Although Rio-Hortega (90) admitted that the exact origin of these cells could not be definitely determined, numerous workers immediately accepted this mesodermal concept which gained further recognition in the ensuing years. Rio-Hortega (90) found that certain surface areas of the nervous system are the sites from which these cells entered the nervous system. These areas are the pial folds which make their way toward the tela choroidea of the ventricles, the pia which overlies the white matter and the pial adventitia of large and medium sized blood vessels. The time of microglial appearance corresponds to the development of the vascular system within the nervous system and continues until after birth. Numerous microglial cells were found at specific migratory sites and, therefore, these sites were called microglial fountains or microglial nests.

Even before microglia were identified, suggestions had been made regarding a mesodermal origin for glial cells or certain unidentified glial elements.

According to Glees (37), microglia were first called "Stabchenzellen" (rod cells) by Nissl as early as 1899. He described these cells which had a rod shaped nucleus with a faint thread of cytoplasm projecting from the two poles of the nucleus. The pia was found to contribute to the formation of these cells, and, as a result Nissl considered them to be of mesodermal origin. Boll (10), as early as 1874, noted ameboid cells entering the nervous system from the surrounding connective tissue in 17 day chick embryos. Schwalbe, in 1881, suggested both a mesodermal and ectodermal origin of glia (104). Eichorst (26) and His (43) both noted mesodermal corpuscles which entered the nervous system of human fetuses and their transformation into glial cells.

The work of Robertson (94) has been highly regarded in the identification of and determination of the origin of microglia. The previous findings had been based entirely on Nissl stains and glial staining which was found in the Golgi technique. Robertson (94) introduced the platinum technique with which he was able to demonstrate processes on glial cells which until that time had been considered adendritic. He could distinguish between macroglia which were attached to blood vessels and his newly discovered group of cells which he called mesoglia (94). These cells which were believed to be of mesodermal origin were later discovered to be microglia and oligodendroglia. The recognition of this error by Robertson (94) is based on the structure of the cells which are shown in the illustrations used in his publications (31)(37). Other investigators such as Capabioneo and Fragnito,

Alzheiner, and Hatai endorsed this mesodermal origin and believed that these cells have a vascular origin (37).

Metz and Spatz (70) disagreed with the findings of Rio-Hortega in regard to the origin of microglia as they did not observe a migration of these cells into the nervous system. Pruijs (86) and Rydberg (98) related the development of microglia and oligodendroglia to cells which arise from the ependymal zone. Both investigators supported the neuroectodermal origin of these cells. Belezky (37), on the other hand, held that mesodermal cells develop into both microglia and oligodendroglia.

Because of the time relationship of microglial development and the development of the vascular system, Juba and Santha, reviewed by Kershman (46), have expressed the opinion that microglia were from vascular adventitial cells which in turn were derived from monocytes. They found that the cells which migrated from the blood vessels were of a primitive type and gradually differentiated into mature microglia.

Studies on the appearance of microglia in human embryos have been made by Kershman (46) whose work agrees with that of Rio-Hortega (90). Kershman makes special note of the ameboid forms which gradually go through transitional stages to mature microglia. This wandering type of cell was found in the mesenchymal tissue of the choroid plexuses, around large blood vessels and in the meninges which overlie tracts. A gradual maturing of these cells was noted as they passed farther out into the nervous tissue.

Dougherty (22) has made similar findings on the rat with the use of both the silver reaction and morphological stains. He related the ameboid cells (microglioblast) which migrated into the nervous system of the newborn to gitter cells and histiocytes because of their morphological similarity. In addition he could find no difference between macrophages of lymphocytic origin and microglioblasts except for the large size of the latter cells. The microglioblast was considered to be the cell from which microglia develop.

The time that microglia first occurred in the central nervous system of different species, was studied by Field (32). The earliest microglial cells were found to appear at the time of vascularization. However, the presence of ameboid type cells did not occur until later in development (near birth). These cells were found in fountain arrangements which were especially prominent in new born rats. He doubted the role of ameboid cells in the formation of microglia and considered them to have a relationship to myelinization which was beginning to occur at this time. The origin of microglia was thought to be from blood cells which migrated from the blood vessels as mature-like microglia rather than cells from the fountains (32).

Having reviewed the literature on microglia, one can readily see that the origin and role of microglia are still in question. Because of this, the basis for relating microglia to the reticuloendothelial system has been placed on unstable ground. Although radioautography and electron microscopy have given new insight into the problems of microglia, additional techniques must

be used and new concepts must be formulated in order to solve the problems which have plagued the interpretation of microglia since their discovery.

Recent investigations on the enzyme chemistry of cells has possibly uncovered new criteria for the identification of R-E cells. The enzymes nonspecific esterase and acid phosphatase have been shown to be present in phagocytic and possibly progenitor cells of the R-E system. Chessick (17) found that, in a number of species, the largest histochemical activity for nonspecific esterase was in the cells of the liver, pancreas and kidney. The spleen was found to contain scattered cells which contain the enzymes and corresponded to the macrophages of this organ. The Kupffer cells of the liver were found to have a slight enzyme reaction. Wells (113) showed that macrophages in the skin disease, leishmaniasis, gave a nonspecific esterase reaction while Braunstein et al. (14) demonstrated this enzyme in the sinus lining cells and macrophages of lymph nodes.

Acid phosphatase was demonstrated in splenic macrophages as early as 1941 by Gomori (40). Weis and Fawcett (112) linked the presence of acid phosphatase with phagocytic activity. They found that the monocytes were negative for acid phosphatase and that as these cells developed acid phosphatase activity they also became phagocytic. Little more work was done until Braunstein et al. (14) demonstrated acid phosphatase activity in the sinus lining cells and macrophages of lymph nodes. Howard (44) and Novikoff (76) were probably the first to relate acid phosphatase to the R-E system. The

work of Barka (6)(7) has shown similar results. Barka (7) found that R-E cells of the liver, spleen, thymus and lymph nodes of the rat have a high acid phosphatase activity. He has shown that acid vital dyes are stored in the cells which show acid phosphatase activity. Stimulation with typhoid vaccine produced an increase in the acid phosphatase of lymph nodes and the thymus while in the spleen it is decreased in the initial stages of stimulation (7).

A correlation between phagocytosis and enzyme activity (both acid phosphatase and nonspecific esterase) has been postulated by Snook (105). He felt that the use of these 2 enzymes may be of importance in identifying more precisely the cells of the R-E system, or at least, certain elements of this system. This concept has been pursued in studies on the thymus and the spleen (27)(83) with a good correlation existing between phagocytic activity and enzyme content. In the spleen, it was believed that as macrophages developed from primative cells they also developed nonspecific esterase activity (83).

Many questions remain unanswered in regard to the relation of enzyme activity to R-E cells, yet, the indications are that acid phosphatase and nonspecific esterase have a definite importance in this regard. The use of enzyme studies for the identification of phagocytic cells, is an important tool for investigation.

The studies which have been done on acid phosphatase and nonspecific esterase in the central nervous system have been largely on a topographical

basis with only a few studies indicating the type of cell in which they occur. The work which has been done on nonspecific esterase has been carried out for the most part in conjunction with specific esterase. These studies were done by Koelle (47)(48)(49)(50) with the use of the metal precipitation technique. The results showed that nonspecific cholinesterase was found in glial nuclei of the cat and rat brain. Gomori and Chessick (40) and Chessick (17), using the diazo dye method with Alpha naphthol and Naphthol AS acetates as substrates, found that rat central nervous tissue had minimal or no glial activity for nonspecific esterase while vascular pericytes were strongly reactive. However, in the dog and cat they found that glial cells were positive for the nonspecific esterase reaction (40).

The studies of acid phosphatase in the central nervous system are considerably more numerous than those on nonspecific esterase. Here again 2 techniques have been used and both have given different results. The majority of the studies have been done using the metal precipitation technique with glycerophosphate as the substrate. Except for 2 studies, acid phosphatase was shown to be found in the nuclei of glial cells. One of these studies demonstrated cytoplasmic staining in glial cells [Naidoo and Pratt (72)] while the other study [Meath and Pope (66)] found that glial cells were negative except for subpial astrocytes. Only one metal precipitation study has listed all 3 glial cells as giving an enzyme reaction (116).

Burstone, in 1958 (15) developed the diazo dye technique which utilized Naphthol AS phosphate derivatives as the substrates. By use of this technique, the nuclear staining has been eliminated and as a result nuclear staining has been determined to be an artifact. The azo-dye technique has been applied to human brain tissue and shows that acid phosphatase and non-specific esterase are found in neurons and glial cells (Schiffer et al. (103)). Anderson and Song (3), using the diazo dye technique on rat, rabbit and guinea pig brain tissue, found light acid phosphatase activity in glial cells while vascular pericytes were very active. These workers also made a brief note of an increase in acid phosphatase activity in microglia, macrophages and perivascular cells after injury to the nervous system.

The enzyme studies on the nervous system, with the exception of the last-mentioned study and a few tumor studies, have all been done on normal tissue. The majority of the studies listed these enzymes as being found in glial cells in general and make no attempt to determine which glial cells show the enzyme reaction. Anderson and Song (3) have utilized a nuclear counterstain for identifying cells of the nervous system that have been treated for acid phosphatase, however, this technique has only been slightly utilized. In addition, the studies which use metal precipitation techniques have been considered to be invalid. Existing experimental studies which test these enzymes have been few and developmental studies are lacking.

The present study has been done to extend the knowledge on the relationship of microglia to the reticuloendothelial system. This work has been done by making use of the enzyme relationships which have been recently developed for the reticuloendothelial system. The enzymes, nonspecific esterase and acid phosphatase, have been studied in the normal adult rat brain during development of the nervous system from fetal life to young adults and during the healing process following injury in the adult rat central nervous system.

The purpose for considering these 3 groups of animals is to demonstrate under what conditions these enzymes are found in the mesodermal elements of the nervous system. A summary of these purposes is given in outline form below.

A. The normal adult animals are used in an attempt to confirm the presence or absence of these enzymes in normal resting microglia.

B. The developing animals are used to demonstrate whether these enzymes are present:
   1. In the cells which give rise to microglia (meningeal cells).
   2. In the ameboid cells which have been described to enter the nervous system to form microglia.
   3. In the microglia which appear as normal resting cells of the developing nervous system.

   In addition this group serves to demonstrate the possible origin of microglia and their course of entry.

C. The injured tissue is used:
   1. To demonstrate the development or increase of enzyme activity in stimulated microglia around the wound.
   2. To demonstrate enzyme activity in gitter cells (phagocytes).
   3. To attempt to demonstrate from what cells gitter cells take origin.

CHAPTER II

MATERIAL AND METHODS

The animals used in this study were albino rate of the Holtzmann strain. Some animals were obtained directly from the Holtzmann farm while others were bred in the Anatomy Department animal colony from Holtzmann stock animals. Fetal stages, newborn animals, young animals (newborn to 42 days), adult animals and experimentally injured animals comprised the groups studied. The young and adult animals were sustained on Purina Laboratory Chow and water ad libitum. The newborn animals were sustained by the mother until 21 days when they were weaned.

Rat fetuses of 15 days gestation to birth were obtained from stock animals. The fertilization day was determined by the presence of sperm in vaginal smears which were taken each morning. Gestation time was counted as the number of days beginning with day zero when sperms were observed. In most cases, 2 fetuses from 2 animals were studied for each day of gestation (see Table I). Postnatally, at least 2 animals were studied for the periods of newborn, 1,2,4,6,8,10,12,14,20,25,30,35 and 46 days (Table II). Ten adult animals were used. In the injured series, adult animals were studied at various times after injury as indicated in Table III.

TABLE I

FETAL ANIMALS

Days of Gestation	Number of Animals
15	2
16	4
17	7
18	6
19	7
20	6

TABLE II

POSTNATAL ANIMALS

Days of Age	Number of Animals
Newborn	4
1	4
2	2
4	2
6	3
8	3
10	3
12	3
14	2
20	2
25	2
30	2
35	2
46	2

TABLE III

INJURED ANIMALS

Time after Injury	Number of Animals
12 hrs.	2
24 hrs.	3
36 hrs.	4
2 days	4
3 days	7
4 days	3
5 days	4
6 days	3
8 days	3
15 days	2
20 days	3
30 days	2
40 days	2

Injury was produced by inserting a number 22 hypodermic needle to a depth of 5 mm through the skin, skull and into the brains of animals anesthetized by sodium pentabarbital (Nembutal) supplemented by ether. This puncture was made just lateral to the mid-sagittal line in such a manner that it penetrated both the cortex and the corpus callosum.

The animals were anesthetized and then sacrificed by perfusion with saline through the abdominal aorta or left ventricle. As soon as the blood was removed (saline return was clear) the animals were perfused with cooled 15% unbuffered formalin for 1 hour. For fetal animals the perfusion was limited to 15 to 20 ml of formalin. Following perfusion, the brains were removed, sectioned into 3 or 4 mm sagittal or coronal slices and fixed further in formalin

for 12 to 24 hours. However, for the fetal material the entire head was fixed.

All the specimens were prepared for the frozen section technique by first quenching the tissue in isopentane which was previously cooled to freezing (approximately ±160°C) with liquid nitrogen. This method was used to prevent ice crystal artifact which is seen in the nuclei of these lightly fixed specimens when they are frozen by conventional methods. Following the quenching process the tissues were stored for not more than 2 days at -30°C or immediately frozen to cryostat tissue holders (chucks) with minimal or no thawing of the frozen tissue.

The developing and adult normal tissues were cut sagittally and the experimental brains were cut coronally in the cryostat at temperatures of -20°C to -25°C. The normal adult and injured series were cut at 12 micra while the entire fetal heads were cut at 48 micra. The removed brains of the newborns up to 14 days of age were cut at 24 micra. Sections were free floated in 1% formalin and others were placed on lightly albuminized cover glasses and allowed to air dry.

The techniques carried out on these tissues were a silver reaction, the Nissl stain, an asure-eosin method and enzyme techniques for acid phosphatase and nonspecific esterase.

The silver reaction was done according to Marshall's metalophil method (62). The sections were first washed by floating in 2 changes of distilled

water, after which they were impregnated in Marshall's silver solution for 5 to 15 seconds. The Marshall silver solution was made fresh by titrating 2 cc's of ammonium hydroxide with 10% silver nitrate until a slight cloudiness remains. Following the silver treatment, the sections were reduced in 1% formalin with agitation. The sections were dehydrated in 2 changes of 95% alcohol, cleared in carbolxylene and mounted on slides.

The Nissl stain was carried out in Columbia staining dishes and was modified for coverslips from a cresyl violet acetate stain for frozen sections given by Powers and Clark (85). The air dried sections were washed with 70% alcohol for five minutes and rinsed in distilled water. They were stained in 0.1% cresyl violet acetate in 0.1M acetate buffer at a pH of 3.5. The excess dye was removed with distilled water and the sections were passed through 70% and 95% alcohol. The final differentiation was carried out with an equal mixture of absolute alcohol and chloroform after which they were cleared in butyl acetate and xylene.

Lillie's azure-eosin method (59), which uses azure A and eosin-B in a citric acid buffer at a pH of 3.5, was used for routine staining. The sections on cover glasses were used for this technique. This method was used most effectively in identifying cells of the injured series.

The acid phosphatase technique was done according to Burstone (15). Following cutting, sections were placed in the incubation medium with a clean glass rod. The incubation medium was prepared fresh from stock

solutions prepared with double distilled water. This medium consisted of 4 mg Naphthol AS-BI phosphate (substrate) and 20 mg Garnet GBC (azo dye) in 25 cc of a 0.2M sodium acetate buffer at a pH of 5.4. The substrate, was first dissolved with 4 drops of N,N-dimethylformamide because it is not water soluble, and the buffer was added followed by the dye. The medium was then filtered and the sections were incubated at 37°C for 2 hours. After incubation the sections were floated onto slides from distilled water and air dried. After drying was sufficient for the sections to adhere to the slide, they were remoistened with distilled water and mounted with glycerol gel. Control sections were prepared simultaneously in an incubation medium which differed only in that it lacked the substrate (Naphthol AS-BI phosphate).

The nonspecific esterase reaction was done using Pearse's (77) modification of the Gomori Azo-dye method. The incubation medium for nonspecific esterase was prepared fresh. Four mg Naphthol AS acetate was dissolved in 1 cc of acetone. Two-tenths cc of the acetone-substrate solution was added to 10 cc of 0.2M sodium phosphate buffer at a pH of 6.8 which contained 0.01% propylene glycol. Ten mg of Garnet GBC dye were added to this solution and the medium was filtered. The sections were transferred to the incubation medium with a clean glass rod and were incubated at room temperature of 30 minutes. At this time the sections were placed in distilled water and mounted on slides. These sections were mounted and treated as those in the acid phosphatase technique. Control

sections were incubated at this same time in a similar medium which differed only in that it did not contain the substrate.

Another method of study was the use of enzyme techniques and the silver reaction on the same sections. This was used primarily in studying the needle puncture series. In this method, recent enzyme reacted sections were mounted with water and photographs were made of the needle tract. These sections were floated off into water and treated by the silver reaction. Following mounting these sections were again photographed and the photographs were compared.

CHAPTER III

RESULTS

The findings presented in this investigation are based primarily on enzyme techniques augmented by morphological stains and the silver reaction in order to more clearly identify enzyme positive cells and structures. These morphological stains and the silver reaction of Marshall are also used to demonstrate cells and structures important to the present study, but which are not shown by the enzyme techniques. First, a normal description of the structures as they are seen with each of the various techniques is given to show the applicability of these techniques and also to establish control pictures. This description includes the results of the enzyme reactions as applied to the microglia. Secondly, the developing central nervous system is described in animals from 15 days gestation to young adults. In this group, the development of the meningeal layers from which microglia are considered to originate will be described. This will be followed by a description of the development of enzyme activity in the brain in general. Lastly, in this group the apparent migration of meningeal cells into the nervous tissue both directly and indirectly as they follow ingrowth of blood vessels will be considered. The third topic for consideration is the relationship of enzyme activity to reactive cells of the central nervous tissue following experimental injury.

Application of the Techniques

The metalophil reaction results in some black deposits on all cells of the nervous system, but a total cell reaction is usually seen only in microglial cells (fig. 1) and in perivascular cells (fig. 2). The cell body of a microglial cell is elongated with processes, usually 1 or more, projecting from each end. These processes are branched and have rough irregular surface. The perivascular cells appear as large blackened areas in the adventitia of blood vessels. They are large cells which are elongated in the axis of the vessel. Short, blunt processes are seen to project from the ends of these cells.

The Nissl stain is used to identify the nuclei of microglia, oligodendroglia and astroglia. In addition to neuroglial nuclei, perivascular cell nuclei, endothelial nuclei, smooth muscle nuclei of the blood vessels and neuronal cell nuclei can be identified. The dark staining nature of the perivascular cell nuclei makes them stand out among the very light staining endothelial nuclei and elongated smooth muscle nuclei (fig. 3). The dark staining of the nuclei is characteristic of perivascular cells, microglia and cells of the pia and arachnoid trabeculae. The nuclei of the astrocytes, oligodendroglia and neurons stain lighter. The neurons are most easily identified by the Nissl substance in their cytoplasm and their very light staining nuclei.

The nuclear chromatin pattern of the neurons, astrocytes and oligodendroglia is not demonstrated in the Nissl preparations. The nuclei of these cells is stained very lightly. Ordinarily the Nissl technique demonstrates a nuclear chromatin pattern in these cells. It is believed the lack of this chromatin staining is due to the very short fixation time used in order to preserve enzyme activity. The nuclear chromatin of microglia, perivascular cells and some cells of the pia and arachnoid trabeculae is stained very darkly. These dark granules are set in a dark parachromatin background. This staining difference aids in the identification of these mesodermal elements.

The azure-eosin method stains all nuclei in addition to background staining. This stain clearly delimits the white matter from the gray matter. This stain was most useful in the experimental injury study because the cytoplasm of the macrophages could easily be seen and the leucocytes could be identified.

The enzyme techniques were useful for identification of those cells and structures which contain enzymes. However, they were not useful in identifying the various neuroglial cells in the adult. The reaction seen with the nonspecific esterase method is the most useful of the 2 techniques employed. The coupled dye in the cytoplasm leaves a clear nuclear outline. The cells which are best demonstrated are certain neurons (fig. 4) and perivascular cells (fig. 5). Other neurons of the nervous system have less activity and are only slightly marked (fig. 5). Perivascular cells are the

most intensely reactive cells found in the nervous system. They demonstrate a distinctive dye color (deep violet) which is not found in any other structure of the adult brain. These perivascular cells are distributed uniformly along all blood vessels larger than capillaries. The interval between perivascular cells is usually as seen in figure 5. The intimal lining of blood vessels larger than capillaries gives a slight nonspecific esterase reaction. The capillaries do not appear to react and are not observed with this technique. Perivascular cells (Rouget cells) of capillaries are not enzymatically reactive with this technique.

Cells are seen in the pia which show an activity similar to that seen in the perivascular cells. These cells are present only occasionally within the pial layer. In pial invaginations a number of these positive cells are seen because 2 layers of the pia are present in addition to the intervening arachnoid trabeculae and blood vessels which pass through the area. The blood vessels within the subarachnoid area also have nonspecific esterase positive cells in or on their wall.

Neuroglial cells are not well-demonstrated by the nonspecific esterase technique. This is demonstrated by the very slight activity which is shown in the white matter where a number of oligodendroglia, a few astrocytes and microglia are to be found (fig. 5). Repeated attempts to demonstrate microglial cells by this technique have failed. The various slight reaction seen in neuroglial cells makes it impossible to identify which neuroglial cells have the activity.

With the acid phosphatase technique, a reaction occurs in possibly all neuronal and neuroglial elements. Reaction products could be seen in a paranuclear position in neurons with an occasional reaction in the processes near the cell body. The intensity of the reaction is, in general, more uniform than that found in the nonspecific esterase reaction. Like the nonspecific esterase reaction, occasional, very intensely reacting neuronal groups are observed. The neuroglial cells give a slight reaction. The identification of specific neuroglial cells is again not possible by means of this enzyme technique. The nature of the final reaction product (dye-substrate complex) is very granular, and, as a result, small slightly positive structures are very hard to identify. The perivascular cells, which react intensely with the nonspecific esterase technique show only a moderately intense reaction with the acid phosphatase technique. The endothelial lining of the blood vessels does not give an acid phosphatase reaction.

The enzyme reaction pattern of these 2 techniques in the normal cerebral cortex and corpus callosum, will also be described at this time because this area has been used for study in experimental injury. This site was chosen for injury because of the uniform enzyme activity in the cells throughout the cortex. The reaction for nonspecific esterase is slight in all the neurons noted. Within the neuroglial elements the reaction is absent or very slight at the most. The perivascular cells of the area are the only intensely enzymatically active cells present.

In the acid phosphatase technique, a similar uniform reaction is found among the neuronal and neuroglial elements. The neuroglial elements have a slight reaction as seen in both the corpus callosum and among the neurons of the cortex. The perivascular cells have a moderately, intense reaction and are readily identified by their position along blood vessels.

Developing Central Nervous System

The meninges have been most often regarded as the tissue which gives rise to microglial cells (46). The enzyme techniques demonstrate a relationship between certain meningeal cells and cells which have migrated into the nervous tissue. Cells which appear to have migrated into the nervous system react positively to the silver technique of Marshall. These cells are thought to arise in the meninges, but similar cells in the meninges show no reaction to silver. The enzyme techniques demonstrate the cells in the course of migration as follows: 1) certain meningeal cells can be identified, 2) these same meningeal cells can be seen to penetrate the nervous tissue and 3) the resulting distribution of cells can be observed.

The development of enzyme activity in meningeal cells has been studied, beginning with the fifteenth day of gestation. At this time, the brain is surrounded by a layer of condensed mesenchyme except in the vicinity of the cisterna magna, where a breakdown of this tissue to form the subarachnoid space can be seen. In the Nissl stain, the condensed mesenchyme shows metachromatic staining of the intercellular material. The

The condensed meningeal layer contains fusiform cells which have a very slight activity with both the nonspecific esterase (fig. 6) and acid phosphatase techniques. The enzyme activity is seen in the cytoplasm of these fusiform cells which are evenly distributed throughout this layer.

The nonspecific esterase reaction produces coupled dye masses surrounding a large nucleus which has a constant characteristic indentation. The size and number of these dye granules are variable and therefore they simulate vacuoles of ingested material. The enzyme positive granules which are of a smaller diameter, are commonly found in the bipolar processes of these fusiform cells. In addition, the entire cell cytoplasm is faintly outlined by a very slight homogeneous nonspecific esterase reaction. The over all nonspecific esterase reaction in these cells is by and large not very intense. The shape of these cells is almost exclusively fusiform, although occasional stellate forms can be found. In some cases, the processes are very short so that the cell has an almost rounded appearance.

The homogeneous reaction product seen with the nonspecific esterase technique cannot be seen in the acid phosphatase technique. This is because of the granular nature of the final reaction product (dye-substrate complex) of the particular acid phosphatase technique. In addition a diffuse reaction is found throughout the tissue of the head. This reaction appears to be the result of a general enzyme activity possibly occurring in the tissue fluid.

In the area of the cisterna magna the metachromatic staining substance seen in the Nissl stain is lost. The tissue network has become very loose with many large open areas. In this area, the 2 enzyme techniques demonstrate fusiform cells within the meningeal tissues. In addition, rounded-up enzyme positive cells are caught or trapped in the meshes of the tissue network. These cells also have an indented nucleus which is best observed with the nonspecific esterase reaction. The size of the nuclei of these rounded-up cells is consistently smaller than those of the fusiform cells which are variable in size. In the Nissl stain, the rounded-up cells can easily be identified by the rounded cell outline. The nuclear staining of these rounded cells is as dark as any of the cells in the area except for erythroblasts. Within the tissue network itself many large light-staining indented nuclei are found.

At 17 days gestation, the condensed mesenchymal layer of the meninges is still present in localized areas around the brain. Three prominent areas in which this condensed layer still exists are the regions of the olfactory bulbs, all pial invaginations and in the meningeal area on the ventral surface of the brain at the cervical flexure. In these areas metachromatic staining of the intercellular substance is commonly seen. The remainder of the brain is for the most part surrounded by a loose tissue network. Immediately adjacent to the neuroectoderm a condensed layer of mesodermal tissue (pia) remains. Also lining the developing cranial

vault is a similar but thicker layer (dura and arachnoid membrane). The metachromatic staining with the Nissl technique is completely absent from the meningeal area which has lost the compact mesenchymal tissue arrangement. At the tight/loose mesenchymal junction a "breakdown front" occurs (fig. 7). At this site, the metachromatic intercellular substance is absent. Many rounded-up cells are found in the recently-developed loose area.

In the enzyme techniques, the fusiform cells and rounded-up cells are easily recognized by their distinct positive reaction. These areas of the meninges in which a breakdown of the condensed mesenchyme has not yet occurred contain fusiform cells which are distributed throughout the area. In the areas where a breakdown has occurred, fusiform cells are prominent in the layer of mesoderm (pia) which adjoins the neuroectoderm (fig. 8). These same fusiform cells are also found in the loose tissue network of the subarachnoid spaces and fewer numbers are found in the layer of tissue which lines the cranial vault. The rounded-up cells are seen in the subarachnoid space area and are caught in the trabeculae. Because these enzyme techniques involve free floating of the sections the rounded-up cells which remain are those cells which are caught in or attached to the arachnoid trabeculae, and, therefore, a true picture of the activity of free rounded cells can not be obtained.

In the enzyme techniques, the fusiform and rounded-up cells have a definitely increased activity over that seen at 15 days gestation. The

most marked increase in activity is seen in the fusiform cells with the nonspecific esterase technique. These fusiform cells have an over-all intense reaction. This intensity is due to a general homogeneous cytoplasmic reaction accompanied by an intense granular deposit in the cytoplasm. The rounded-up cells as seen with the nonspecific esterase reaction are also as intensely reactive as the fusiform cells (fig. 9). In the acid phosphatase reaction the fusiform cells are variably reactive from moderate to intense while the rounded-up forms are intense.

The nuclear outlines seen in the nonspecific esterase reaction show that these fusiform and ameboid cells have characteristically indented nuclei. These nuclear outlines can be seen even at the low magnification in figures 8 and 9 while the very detailed shape of these nuclei of fusiform cells can be seen at a high magnification in figure 10. The nuclei of the rounded-up cells are found to be eccentrically placed as shown by figure 8.

In the Nissl stain, these characteristically indented nuclei are found throughout the meningeal area (fig. 11). In addition, very large pale-staining nuclei are found among those nuclei which have indentations. The nuclei which have an indentation are found to have varying degrees of nuclear chromatin dispersal. Also the size of these indented nuclei was found to vary considerably. In comparing the size of the nuclei with the amount of chromatin granules, it was found that the very large indented nuclei lacked visible chromatin granules. The medium-sized indented nuclei had a finer nuclear chromatin pattern while the smallest nuclei had the heavier nuclear chromatin

pattern. The large pale nuclei are considered to be undifferentiated mesenchymal cells. In figure 11, the progressive development of these undifferentiated mesenchymal cells into the indented nuclear forms which in turn develop a visible nuclear chromatin pattern is illustrated. As these indented cells decrease in size, the nuclear chromatin pattern becomes denser.

In the loose areas of the meninges, the cells with the dark-staining nuclei are found to have a rounded-up cytoplasm (fig. 12). Occasionally these rounded-up cells are found to have hemosiderin-like material in their cytoplasms.

The correlation between the cells which give the 2 enzyme reactions and the nuclei seen in the Nissl sections is difficult to determine. The rounded-up cells seen in the enzymes techniques can be considered to correspond to those cells seen in the Nissl preparations which also show indented nuclei and a rounded cytoplasm. The indented nucleus of the fusiform cell is the only characteristic which can be used to make a correlation between these cells and the nuclei seen in the Nissl slides. Therefore it cannot be determined at what point along the maturation process described in the Nissl stain that the enzyme activity first develops in these cells.

At 20 days gestation, the meningeal layer has for the most part remained like that seen at 17 days. The areas which have not broken-down can now be seen to begin their breakdown. This is noted especially in the areas of the cervical flexure where only a small bridge of this condensed layer

remains. The 3 meningeal layers were not studied beyond 20 days because the brains had to be removed from the cranial vault to facilitate sectioning and this process disrupted the membranes. The pia and attached arachnoid trabeculae could easily be observed, especially in the pial invaginations. The appearance of the pia remained constant until about 30 days when it began to become very well-defined and the enzyme-reacting cells were found to decrease. By 46 days a normal adult appearance has developed so that only a few enzyme positive cells occur along the very thin pial layer.

The development of enzyme activity within the brain itself follows a definite pattern. Here the enzyme activity differs greatly between the 2 techniques. At 15 days the most distinct feature of the nonspecific esterase reaction is a lamina of spongioblastic-like processes which pass from the ependyma to the pia in the basal plate region of the brain stem (fig. 13). The lamina extends rostrad as far as the cephalic end of the fourth ventricle. The extent of this lamina has not been determined at 15 days, but in 16 day animals, where sections have been cut slightly obliquely in the mid-sagittal plane, it can be distinctly noted to be a thin lamina in the midline.

The ependymal lining of the ventricles demonstrates a varied nonspecific esterase response from area to area. This reaction at most is only moderate. In the ependyma which is in contact with the spongioblastic lamina the nonspecific esterase activity is the most distinct of any region of the ependyma.

In the acid phosphatase reaction the ependymal lining is also positive and variable. In the nervous tissue, a diffuse tissue reaction is found. In addition, groups and scattered cells which are only slightly positive are found throughout the nervous tissue. These cells are difficult to distinguish from the general tissue reaction as they have the same intensity. The spongioblastic processes similarly have the same intensity as the other positive reaction in the nervous tissue except that a greater intensity can be noted near the ependymal layer. It is thought that this activity may be found in all spongioblastic processes rather than being limited to the spongioblastic lamina seen in the nonspecific esterase reaction. The spongioblastic elements may be represented by some of the diffuse reaction seen throughout the nervous tissue.

At 17 days the nervous tissue has the same nonspecific esterase-reactive structures as it did at 15 days. In addition, the blood vessels of the brain are outlined by a slight but distinct nonspecific esterase reaction. At this time, mesodermal cells are entering the nervous system. This migration will be described later.

In the acid phosphatase reaction the same slight activity is seen throughout the nervous tissue. The blood vessels do not give a reaction with this technique. The mesodermal cells which correspond to those in the nonspecific esterase reaction have a greater activity than other reactive elements of nervous tissue.

At 20 days the enzyme pattern remains the same as at 17 days with the exception of an increase in mesodermal elements to be described. In the new born series of 1-2 days of age the nonspecific esterase activity begins to develop in the neurons of the lower brain stem. At this same time, the acid phosphatase activity is found to increase in these same elements. By 4 days this activity has spread throughout the brain stem but is not seen in the cerebral cortex. The cerebral cortex begins to develop the nonspecific esterase activity at 12 days and increases in intensity until a mature enzyme pattern is seen at 30 to 35 days. Concurrently with this development of nonspecific esterase activity, the acid phosphatase reaction also shows an increase in activity.

Migration of Mesodermal Cells into the Nervous System

The migration of mesodermal cells into the developing nervous system from the meninges and the blood vessels of the nervous tissue is strikingly demonstrated by the nonspecific esterase reaction. This migratory process can be followed with the acid phosphatase technique but less easily because of the slight general reactivity found throughout the tissue. With these 2 enzyme techniques, fusiform cells which have been described in the meninges are found to enter the nervous tissue both directly and indirectly by following blood vessels. These fusiform cells are found to enter the nervous system, directly, at points along the pial invagination and more especially when this

mesenchymal layer comes into a close relationship with the ependymal lining of the neuroectodermal tube. After they have entered the nervous system these fusiform cells lose much of their activity and take on a more rounded appearance. Although the shape of these changed fusiform cells appears rounded, a number of small processes are seen to project for shorter distances into the surrounding nervous tissue than do the processes of resting microglia.

In animals of 15 days gestation, the enzyme techniques do not demonstrate any migrating cells which are entering the nervous system. The morphological stains demonstrate that very small blood vessels have penetrated to all depths of the brain. These vessels are outlined by the metalophil reaction. At 16 days gestation the same findings are observed.

The brains at 17 days gestation demonstrate a migration of fusiform cells into the nervous tissue. This is most easily observed with the nonspecific esterase reaction. Because this migration is so readily observable with the nonspecific esterase reaction and the full course of the apparent migration can be observed, the description in this section is given primarily according to that observed with this technique. The presence of similar cells has been observed with the acid phosphatase reaction and metalophil reaction, and the nuclei have been identified with the Nissl technique. These techniques will be introduced into this description in areas where they can aid in the description and give further support to the identity of these migratory elements.

The migration pattern of these mesodermal cells is that they usually arise from a rather localized area of the meninges and spread out in the nervous tissue to their characteristic locations. As a result, many times due to the one plane of section and the limited number of sections, groups of cells occur in the nervous tissue which can not be related to any surface area but still have all the characteristics of mesodermal cells. In some of these sites the resulting cells of the migration are observed before the origin is found.

Beginning at 17 and 18 days gestation, 4 areas of the meninges have been found in which an apparent migration of nonspecific esterase-positive fusiform cells into the nervous tissue is found. Three of these areas are where the pia comes into close contact with the ependyma while the other is where the pia comes in contact with the corpus callosum in the longitudinal fissure. The areas of close contact between the pia and ependyma are: 1) the pia on the posterior margin of the fourth ventricle (taeniae of the fourth ventricle); 2) the pial invagination which separates the cerebellum from the quadrigeminal plate (anterior medullary velum); 3) the pia in the areas of the third and lateral ventricles (fig. 14).

The criteria which this observer has used to establish these migration sites is based on the appearance and enzyme activity of these migrating cells with the nonspecific esterase reaction. These criteria are necessary because the shape of these cells changes and the enzyme activity decreases

at the time of or after these cells have entered the nervous system. These rules are: 1) Nonspecific esterase positive cells are present in the pia at the migration site; 2) Fusiform cells or a slightly changed fusiform cell (has developed additional process) with an enzyme intensity like that of the meningeal cells is found penetrating or just beneath the surface of the nervous tissue; 3) Changed fusiform cells with enzyme activity are present in the nervous tissue beneath the migration site. The criterion for the changed fusiform cells is that in the nonspecific esterase reaction they demonstrate an indented nucleus like that seen in fusiform cells. These changed fusiform cells are further identified by comparing the areas where they occur with these same areas in the metalophil reaction, Nissl stain and acid phosphatase reaction.

The migration of cells into the taenia of the fourth ventricle is first seen at 17 days gestation. Here the nonspecific esterase positive fusiform cells are seen after they penetrate the nervous tissue of the taenia. The penetrating fusiform cells retain their fusiform shape and distinct nonspecific esterase activity. Under the floor of the fourth ventricle, the changed forms of these mesodermal cells are scattered in the nervous tissue to the deepest part of a central recess in the rhomboid fossa (fig. 14). These cells are probably the ones which accumulate in the nervous tissue around this central recess (fig. 14) and also laterally along its lateral extentions.

The changed fusiform cells which occur under the floor have a decreased activity which is slight or moderate. These cells show a homogeneous cytoplasmic activity but in addition nonspecific esterase positive granules of various sizes are found in the cytoplasm. An indented nucleus is outlined in the cytoplasm by the majority of these cells. In the accumulation sites, just beneath the ependyma, some of these cells have a rounded-up appearance, however, in the nervous tissue all of these cells retain processes.

In the metalophil reaction, a distribution pattern of metalophil positive cells (fig. 15) is seen which is similar to the distribution of the nonspecific esterase positive cells. In the Nissl stain, cells of this area are found which have nuclear staining like that of the meningeal cells.

This migration site demonstrates cells which fulfill all the established criteria for migration until 4 days after birth. The number of animals in which this apparent migration is found (based on these criteria) is listed for each day in Table IV. This table is given to show that this apparent migration is not a spot finding and that it occurs over a certain time period in the developing brain.

During this period of migration the number of changed fusiform cells is found to increase until 20 days gestation at which time the approximate number remains constant. These cells are found in the area until 10 days of age or 6 days after the migration is no longer apparent. At 20 days, many

TABLE IV

The number of animals for each day of age and migration site which demonstrate migration according to the established criteria.

Days	Migration sites			
	taenia of fourth ventricle	anterior medullary vellum	transverse fissure and third ventricle	sub-callosal area
17	2	-	2	-
18	2	3	4	1
19	1	2	2	2
20	3	2	1	1
newborn	1	1	2	-
1	2	1	3	1
2	-	-	1	1
4	1	1	1	-
6	-	-	2	-
8	-	-	2	-
10	-	-	1	-
12	-	-	1	-

of the changed fusiform cells are found to have an intense activity like the fusiform cells of the pia. The cells which show this distinct activity are found just beneath the ependyma of the fourth ventricular floor. At 20 days

the acid phosphatase activity also becomes intense in the cells which appear to be fusiform cells and lie just beneath the ependyma. The acid phosphatase activity of the fusiform cells and changed fusiform cells before 19 days is slight or moderate so they are barely recognizable.

The second migration site is the pial invagination which meets the developing anterior medullary velum (fig. 14). The migration of the fusiform pial cells is first seen here at 18 days. The fusiform cells penetrate the nervous tissue. These cells also retain the distinct nonspecific esterase activity which is seen in the fusiform cells of the pia. The changed fusiform cells are found around the cerebral aqueduct and out beneath the floor of the fourth ventricle. These cells remain close to the ependymal lining. The nonspecific esterase activity is found to be intense in some of the changed fusiform cells while others have only a slight or moderate activity. These changed fusiform cells demonstrate a characteristic indented nucleus like that seen in the fusiform cells of the pia.

This apparent migration process is found in animals up until 6 days of age (Table IV). During this period a change of the entire area is found to occur due to the development of the cerebellum. The anterior medullary velum is found to increase in its anterio-posterior length. At 19 days gestation, no distinct change is noted from that seen at 18 days. The fusiform cells penetrate the nervous tissue as seen in figure 16. They are found in the ependyma and the nervous tissues of the area. At 20 days the penetration of the fusiform

cells is like that seen in the previous stages. The number of changed fusiform cells has increased in the area around the cerebral aqueduct and anterior recess of the 4th ventricle. The nonspecific esterase activity is intense in a number of these cells. In the acid phosphatase technique, some cells are found which have an intense reaction and correspond to the distribution area where changed fusiform cells are seen with the nonspecific esterase technique.

In the newborn and one day animals the anterior medullary velum begins to lengthen, and the cerebellum increases in size. The fusiform cells are seen to penetrate the anterior medullary velum. The changed fusiform cells are found near the ependyma both above and below the neural canal. A number of these changed fusiform cells have an intense activity like that seen in the fusiform cells of the pia. Again the acid phosphatase activity is intense in cells which appear to correspond to the changed fusiform cells of the area.

At 4 days the anterior medullary velum has further increased in length and has thinned so that the pia lies almost directly on the ependyma. Fusiform cells can be seen to penetrate the anterior portion of the medullary velum into the quadrigeminal area. Just beneath the floor of the anterior recess of the fourth ventricle intensely nonspecific esterase reactive cells are seen to line this layer and also agregate into small groups. Again, these same cells (changed fusiform) show a very intense reaction with the acid phosphatase technique. The presence of the changed fusiform cells has been observed as late as 12 days of age.

The third site of apparent migration is seen in the region where the pia of the transverse fissure meets the ependyma of the third ventricles. Here the area is very extensive and can be seen in almost every section. The area which has been considered is that area where the pia meets the ependyma in the anterosuperior aspect of the third ventricle as shown in figure 14. This sight does not show fusiform cells which have penetrated the nervous tissue but only changed fusiform cells which have an intensity like that seen in the fusiform cells of the pia. These cells are seen at the very outer extent of the nervous tissue immediately adjacent to the pia. Further away from the pia the changed fusiform cells show a progressive decrease in activity. These cells are found distributed in the nervous tissue in the anterior aspect of the third ventricle. Near the ependyma of this anterior wall of the third ventricle changed fusiform cells are distributed to the inferior recess of the third ventricle. At 19 days and after, these changed fusiform cells along the ependyma have an intense nonspecific esterase reaction. Similarly in the acid phosphatase reaction this intensity is seen in cells of the same region.

The most concentrated mass migration of cells into one area is seen immediately beneath the developing corpus callosum in fetal animals as young as 18 days. This migration site occurs in and near the midline (longitudinal fissure) where the pia comes into contact with the genu of the recently developed corpus callosum. In the nonspecific esterase technique, mesodermal cells showing a positive reaction are seen to migrate from the

meninges into the nervous tissue so that they pass under the genu and accumulate as a group beneath the corpus callosum (figs. 14 and 17). In these mid-sagittal sections the corpus callosum appears to be cupped over this group of cells.

The corpus callosum develops first in the midline as a small mass of white matter. At 18, 19 and 20 days gestation the corpus callosum has not extended laterally for any distance and is also very short in the anteroposterior aspect (fig. 17). As the brain develops further, the corpus callosum grows laterally and lengthens anteroposteriorly so that a layer of white matter is present beneath the cerebral cortex. This layer as it extends from the midline laterally passes superior and then lateral to the lateral ventricle thereby separating the lateral ventricle from the cortex.

In the midline at 18 days the migration of cells into the area under the corpus callosum occurs as fusiform cells from the pia (fig. 18). These fusiform cells have a nonspecific esterase reaction like that seen in the fusiform cells of the pia. These intense fusiform cells are found in the nervous tissue between the pia and the mass of changed fusiform cells which have accumulated beneath the corpus callosum. These changed fusiform cells have an activity which is slight. Both the fusiform and the changed fusiform cells have characteristically indented nuclei.

From 18 to 20 days the corpus callosum doubles its length in its anteroposterior aspects. In the subcallosal area an increase in the number of changed fusiform cells accompanies this change. The nonspecific esterase positive fusiform cells are found between the pia and this subcallosal area in all 3 of these ages (fig. 18). The intensity of the changed fusiform cells also remains the same at these 3 ages.

The corpus callosum begins to develop rapidly at one day, and the adult shape is acquired by 6 days (fig. 19). This development is an anteroposterior lengthening and the formation of a distinct genu at the anterior end and a splenium at the posterior end. This body of white matter develops laterally by projecting superior to the lateral ventricle and then passing lateral to it thus following the internal surface of the cerebral cortex.

Concurrent with this development of the corpus callosum, the mesodermal cells which had accumulated beneath the corpus callosum in the midline, form a layer of cells on the lower surface of this large body of white matter. At 8-10 days after birth the changed fusiform cells can be seen in coronal sections to extend as a layer from the midline laterally to the lateral ventricle. This is demonstrated by the nonspecific esterase reaction in figure 20. In the area of the lateral ventricle these mesodermal cells are found to follow the corpus callosum as a layer between it and the ventricular ependyma. In addition, some of these cells are found to migrate medially and ventrally around the lateral ventricle. In a para-sagittal section of the brain

in the region of the lateral ventricle, the anteroposterior extent of this subcallosal layer of changed fusiform cells can be noted at this later age (fig. 19). These cells extend as a layer from the genu to the splenium.

While following the development of this subcallosal area it was noted that the mesodermal layer of cells was found to occur in the area of the obliterated lateral ventricle which had collapsed due to the increase in nervous tissue. Around the lateral ventricle these mesodermal cells are seen in the nervous tissue near the ependyma. Also, mesodermal cells are found within the corpus callosum itself.

These changed fusiform cells have a round cell shape in the Nissl stain and the nucleus stains darkly (fig. 21). Occasionally these changed fusiform cells also are seen to have phagocytized red blood cell material in their cytoplasm (fig. 22). With the acid phosphatase reaction the subcallosal layer also demonstrates a layer of cells like that seen in the nonspecific esterase reaction. The metalophil reaction demonstrates this subcallosal layer of cells very distinctly (fig. 23). With this reaction, the irregular cell body can be noted from which slim processes project. Near this layer on both sides small metalophilic elements occur which appear to be microglia (fig. 23). Because of the relationship of the large metalophilic cells of the subcallosal layer and the numerous microglial elements which are in the area, it appears that the microglia are developing from the large metalophil cells. With the enzyme techniques, none of these 4 areas

demonstrate a complete transition of fusiform cells to mature microglia. However, with the metalophil reaction and on the basis of nuclear characteristics in the Nissl stain this transition of enzyme active cells into microglia becomes complete.

Other possible means of entrance of cells into the nervous tissue are the migration of mesodermal cells along penetrating blood vessels as perivascular cells, from blood vessels (as leucocytes) and as cells of the nervous tissue blood vessels themselves. In this study evidence for the possible migration of fusiform cells on blood vessels has been found. In addition, cells have been observed on blood vessels whose origin cannot be determined by any technique used. These latter cells are being considered because of their possible bearing on cells which appear to arise from cells of the pia.

These cells of unknown origin can be recognized on blood vessels of fetal animals as early as 19 days with the Nissl stain. They appear as perivascular cells on all blood vessels as is shown by their dark nuclear staining like that seen in mesodermal cells. The recognition of these cells in the Nissl stain prompted the finding of these cells in the metalophil reaction. In the metalophil preparations they appear as a definite cell reaction on the blood vessel wall which also shows a reaction. The nonspecific esterase reaction does not readily demonstrate these cells. The nonspecific esterase reaction which has been found in these cells is the

same intensity as that in the blood vessels. These cells only become recognizable as a cell when they are projecting from the side of the vessels. However, they can also be recognized with the nonspecific esterase reaction by the indented nuclear outline which is barely visible in the reaction of the blood vessel. These cells can be found on the blood vessels after birth until the age when the blood vessels have acquired nonspecific esterase positive perivascular cells which have been described in the adult.

Beginning at the time of birth or soon thereafter, intensely enzyme active perivascular cells can be found on the larger vessels just at the point where they enter the brain (fig. 24). In the 2, 4 and 6 day old animals these highly active perivascular cells are found at increasingly greater depths in the nervous tissue. In figure 25 the presence of these cells is not seen when the diameter of the vessel decreases. By 8-10 days they can be observed in all depths of the brain stem. The cerebral cortex is the last area to develop these active perivascular cells. They begin to occur in 12 to 14 day old animals and are not prominent until 25-30 days. These cells appear just before or at the time nonspecific esterase enzyme activity is developing in the neurons of the areas. It is also related to a marked increase in the diameter of the blood vessel which also occurs during this period.

The structure of these perivascular cells is well demonstrated by the nonspecific esterase reaction. One can observe the fusiform and stellate shape of these cells which also contain nuclei that appear round to oval and

demonstrate an indentation similar to that seen in the meningeal cells (fig. 26). In the Nissl stain they have a definite mesodermal-appearing nucleus. These cells also give a silver reaction. There is a direct size relationship of vessel diameter to the shapes of these cells. In the larger vessels the slightly elongated stellate shape is prominent while in smaller vessels they become very much elongated and stretch for great distances along the vessel wall (figs. 25 and 26). A crowding of these cells on the blood vessels occurs at the age of 8-12 days, however, by 20 days they begin to decrease. In 35 day old animals a normal adult distribution is found.

Evidence that perivascular cells have a meningeal origin is shown by their progressive occurrence along the blood vessels with increased age. Another finding which is even more impressive is demonstrated in figure 24. Here one can note the direct relationship between the meningeal cells and those which appear to be migrating onto the blood vessel from the meninges. Some of the meningeal cells are rounded-up and could easily be making their way to the vessels. The enzyme intensity of these meningeal cells is the same as the perivascular cells. The cell shape and nuclear outlines are also similar.

In the interpeduncular fossa very slightly enzyme positive cells can be seen in the nervous tissue between the blood vessels which have recently acquired enzyme positive perivascular cells (fig. 26). In addition some of

these cells appear to be on the small blood vessels near the surface of the nervous tissue (fig. 26). With the silver reaction, cells are seen in this area which are mature and immature microglia.

In one area of the brain stem a direct relationship between these perivascular cells and microglia can be observed. This area is in the spongioblastic lamina which passes between the ependyma and the ventral surface of the brain stem. In figure 27 the thickness of this lamina can be seen because it is obliquely cut, while its connection to the ependyma can be seen because it is obliquely cut, while its connection to the ependyma can be seen in figure 28 where it is cut parallel. Large blood vessels which carry numerous perivascular cells enter the ventral surface of the brain stem and pass along this spongioblastic lamina (figs. 27 and 28). The perivascular cells on these vessels are very rounded.

In the area between the large blood vessels and among the spongioblastic processes, cells are moderately enzyme positive and have mesodermal characteristics (fig. 28). These are identified in the silver reaction as transitional (immature) microglial cells (fig. 29). In a preparation of nonspecific esterase followed by the metalophil reaction, mature-appearing microglia are seen to give the silver reaction (fig. 30). Other cells are seen which are nonspecific esterase positive but do not give a definite silver stain of their processes in this preparation (fig. 30). In the silver reaction some of these cells appear to be immature microglia. These latter cells may be immature microglia which have developed from the perivascular cells or from cells which have migrated directly from the meninges.

In the Nissl stain a number of the nuclei in this lamina have the characteristic indented nucleus and nuclear stain which is consistently seen in the mesodermal cells in this study. The numerous microglia seen here are not found outside of this lamina in this age group.

The direct migration of mesodermal cells from vessels is seen from small and medium sized vessels and has been found occasionally in relation to large vessels. In figure 31, 2 vessels of the same size appear in the same area. A normal perivascular arrangement is seen on one while the other appears to be giving off numerous ameboid cells of lesser activity. A few of these cells which are still closely related to the blood vessel demonstrate an enzyme intensity like that of perivascular cells. In the area of the spongioblastic lamina, the migration of these cells is massive. A migration which fulfills all the criteria for direct migration, can be found in animals from 6 to 10 days of age.

Injured Animals

The injury produced in this series of animals did not appear to cause any visible behavioral signs which could be considered as being abnormal. Immediately following recovery from the anesthesia the animals were as active and as curious as before the needle puncture was performed. The process of healing proceeded in a normal and orderly fashion. Infectious processes were not seen in the skin or in relation to the meningeal layers. The wound was easily noted on the surface of the exposed brain in the early stages

by the presence of a hemorrhagic area and later by a depression on the surface of the brain. When seen in sections, it could be verified that the site of injury was small in diameter and extended through the cerebral cortex, corpus callosum and for a short distance into the underlying brain stem. The needle tract was filled with blood in the earlier stages and was marked by a small hemosiderin containing tract in the later stages.

In the first period of injury studied, which was 12 hours past operative some changes had already begun to occur. Scattered along the needle tract within the marginal tissue are occasional polymorphonuclear cells. The most striking cells around the wound are medium sized pyramidal neurons which have hyperchromatic nuclei and a very brilliant eosinophilic cytoplasm, as shown by the azure-eosin stain. In the white matter, pecular, round and oval shaped bodies are well demonstrated by the nonspecific esterase reaction (fig. 34). These bodies are found in the normal appearing areas of the corpus callosum which are close to the wound. Smaller bead-like bodies of nonspecific esterase reactive material are scattered in rows in which these larger round or oval bodies are also found. In figure 34 a large number of these bodies can be noted between the vessel in the area and the wound.

By 24 hours the neurons which had hyperchromatic nuclei at 12 hours have completely lost all nuclear staining, and are seen as eosinphilic cell bodies. At least 1 or 2 polymorphonuclear cells are found to come into direct contact with these neurons. With the acid phosphatase and nonspecific

esterase reaction many of the neurons in the area have a distinct increase in activity of these 2 enzymes. At this stage an increase in the number of nuclei is noted which is most marked near the surface of the brain. The nuclear staining seen with both the Nissl and azure-eosin methods demonstrates a preponderance of very darkly staining nuclei of various sizes. Many can be identified as polymorphonuclear cells, while others are large and have a characteristically indented nucleus. The phagocytosis of debris by some of these large nuclear cells is indicated because of their foamy cytoplasm. In the 2 enzyme techniques, a slight activity is seen in cells of the injured area. In the metalo phil reaction, the microglia appeared normal in shape and number.

The 36 hour period shows an increase in the number of phagocytes. The largest influx of these cells is from the surface of the brain (fig. 35). The majority of these phagocytic cells have large indented nuclei with a foamy cytoplasm, however, numerous polymorphonuclear cells are also found among this group of cells. Further down in the wound the number of phagocytes is much less numerous. They are commonly seen in groups around vessels and scattered throughout the injured tissue. The majority of cells near the surface of the brain consistently show large nuclei while deeper in the wound the phagocytic cell nuclei are small, medium and large sized. Blood mononuclear cells are beginning to appear at this time as seen in the blood vessel in figure 36.

In regard to the 2 enzyme reactions, the activity is slightly stronger in the phagocytic cells and small infiltrating cells (PMN's) at this period than in cells in the surrounding normal cortex, with the exception of perivascular cells (fig. 37). The nonspecific esterase reaction is not as intense in the cells of the inflammatory site as the acid phosphatase reaction. The phagocytes in the nonspecific esterase reaction appear as large cells which demonstrate a granular cytoplasmic reaction. A large nucleus is outlined by this cytoplasmic final reaction product. Also in the area, smaller enzyme positive cells can be seen with these 2 enzyme reactions. These cells do not demonstrate a granular reaction with the nonspecific esterase reaction but have a homogeneous reaction of their cytoplasm. With the acid phosphatase reaction this distinction is not evident. The majority of these small cells have been identified as polymorphonuclear cells based on the nuclear outline which can be seen by the cytoplasmic reaction.

The blood vessels in and very near the injured area demonstrate an increase in the number of perivascular cells. This is seen in figure 38 with the nonspecific esterase reaction.

The nonspecific esterase and acid phosphatase reactions also demonstrate this cell influx from the surface of the brain (fig. 37) while deeper in the wound a lesser number of these phagocytic cells are found. The large nuclear outline is evident in many of the cells which are migrating internally from the surface. The pia remaining over and near the wound does not demonstrate

intense, nonspecific esterase reactive cells similar to those seen in the normal pia. Away from the wound for a variable distance, however, an increase of these intensely enzyme positive cells can be seen in the pia. Many are related to the areas where blood vessels penetrate the cerebral cortex. A number of rounded pial cells in this area surrounding the wound are seen among the remaining arachnoid trabeculae.

In the metalophil reaction, an immediate impression is created that the surrounding nervous tissue is not reacting by flooding the area with microglial cells (fig. 39). The only microglia to be observed which demonstrate a reactive change are a few cells which are seen at the line of demarcation between the apparently normal and injured tissue. In the wound area (needle tract and surrounding injured tissue) many silver positive cells are seen which correspond to the enzyme reactive cells. The shapes of these cells are variable in the silver reaction. They occur as rounded cells which have short slim processes extending from the cell body. Among these rounded-up cells are other cells which have an irregular shape with many processes. This later type of cell is most commonly seen inareas where the injured tissue has experienced an early breakdown. These areas are commonly seen in the corpus callosum very close to the wound.

At 3 days postoperative the phagocytosis of the injured tissue is very evident. Numerous large mononuclear macrophages are filled with debris and additional new phagocytes are entering from the surface and

appear around blood vessels in the area. These findings are best demonstrated in the enzyme reactions which both show increased activity over that seen at 36 hours. The enzyme reactions of these phagocytic cells are much more intense than those seen in the surrounding neuronal cells. However, in the nonspecific esterase reaction, the reactivity is not as intense as that seen in the perivascular cells with this same technique.

The vessels of the injured area demonstrate numerous nonspecific esterase and acid phosphatase-active cells in or near the wall of the vessels. The wall of the vessels in the wound are heavily infiltrated with these active cells (fig. 40). Adjacent to the wound the number of these cells on the vessels is less so that one can see individual cells. The vessels which course to the injured area have numerous perivascular cells distributed between the surface of the brain and the wound. In the pia around the wound, enzymatically active cells occur in greater numbers than seen in normal animals. These cells appear to have a direct relationship to the increase in perivascular cells on the vessels.

In the metalophil technique at this same period, the silver reaction shows round cells with short processes similar to those seen at 36 hours. These cells are distributed in the injury and are also found in groups around blood vessels. Among these cells and out toward the normal nervous tissue reactive microglial cells are beginning to become prominent (fig. 41). These reactive microglial cells are especially prominent in areas which show little

remaining debris but are filled with these reactive cells (fig. 41). Among these cells a few round macrophages can be seen. By comparing figure 46 with a similar area demonstrating acid phosphatase in figure 42, the cells which are the apparent reactive microglia are only recognizable by the few granules of enzyme activity seen in the area between the active rounded macrophages.

At 6 days the most striking finding is the enzyme active cells which infiltrated the blood vessel wall. This is demonstrated in figures 43 and 44. The phagocytosis of debris is still very prominent as shown by the numerous phagocytes in the enzyme techniques and morphological stains. Among these round, phagocytic cells numerous enzyme positive granules are seen in the background which do not appear to be in cells. When these non-specific esterase and acid phosphatase reacted sections are compared to the metalophil reaction, the cells which have been called reactive microglia are found to contain these enzyme active granules in their cytoplasm. The amount of this enzyme active material in the cytoplasm varies from cell to cell. It is very evident that the cells which have the longest processes and the smallest amount of cytoplasm around the nucleus either have very little or no enzyme active granules in their cytoplasm in the area of the nucleus. Those with a larger cytoplasmic area around the nucleus have enzyme reactive areas in the cytoplasm of the nuclear area. This relationship of the enzyme reactive particles, to reactive microglia is demonstrated by comparing

figure 45 to 46 and 47 and 48. These are paired photomicrographs, one of which is the acid phosphatase reaction the other which is this same section followed with the silver reaction of Marshall.

In the nonspecific esterase reaction 2 degrees of enzyme activity have been noted among the enzyme active cells which have entered the injured area. The difference is primarily in the color and intensity of final reaction product of these 2 types of cells. In figure 44, 2 types of cells, which are demonstrated by dark and light cells in this photomicrograph, appear both in the mass of macrophages of the area and around the blood vessel in the area. The light cells have an enzyme reaction which is pinkish-red while the dark cells have a deep violet stain similar to that seen in the perivascular cells. The light cells have a reaction similar to that seen in the polymorphonuclear cells described earlier. Very commonly, around blood vessels near the injured area, cells are seen which are about the size of blood monocytes or lymphocytes (fig. 43). These cells also have this light pinkish-red reaction. This difference in the enzyme reaction of the larger phagocytes is seen as early as 2 days after injury and is found in the later stages of healing as well. This distinction is not seen in the acid phosphatase reaction.

At about 10 days the phagocytosis of tissue debris is nearly completed. The majority of the macrophages which are present are concerned with blood cells which have been closed off into vacuoles. The phagocytosis of red

blood cells begins very early but is not a prominent feature until this later stage. The removal process of the red blood cells and completion of wound healing continues slowly as the addition of new macrophages to the area decreases sharply after the destroyed tissue has been removed. By 15 days the wound begins to demonstrate fibrous tissue among the cells which fill the destroyed area. At 30 days the connective tissue fibers appear to give the wound area a contracted appearance with a connective tissue core. Macrophages laden with hemosiderin are seen in this contracted connective tissue core. The enzyme activity of some of these hemosiderin-containing macrophages has been lost. At 42 days the majority of these macrophages which contain hemosiderin have lost their enzyme activity.

During these later stages, the enzyme activity which has been shown to be in the reactive microglia continually decreased so that at 42 days only occasional enzyme reacting cells are found in the scar. Reactive microglia are only occasionally identifiable in the metalophil reaction at this late stage.

CHAPTER IV

DISCUSSION

The enzyme techniques used in conjunction with the silver reaction and nuclear stains, give additional criteria for the identification of those mesodermal cells that are related to the nervous system. The enzyme activity of certain meningeal cells during development, selectively demonstrates these cells so that one can follow their activity within the developing meninges and also, their progressive migration into the developing nervous system. These mesodermal cells migrate directly into the nervous tissue and also move along the blood vessels as perivascular cells (adventitial cells). In both instances, these cells are related to the development of microglia. The mature microglial cells do not contain sufficient enzyme activity to permit identification of these cells on an enzyme basis. As a result, the transition of these mesodermal cells to mature microglia can only be suggested by a decrease in the enzyme activity of the mesodermal forms and the development of microglial-type cytoplasmic processes by these cells. In the adult, mesodermal elements are found to enter injured nervous tissue in large numbers. These cells appear to come from the

meninges and also from the blood vessels. The cells migrating from the blood vessels appear to be from the blood proper as well as the perivascular (adventitial) cells.

The development of the meningeal layers (pia, arachnoid and dura) has been described by His (42) and Kolliker (51). They described the development of two thickened layers, an inner and an outer layer. The outer layer was attached to the bone and was considered to be the periosteum. This layer became divided into 2 layers in some regions and became separated by fat. The resulting space was called the epidural space. The inner layer (leptomeninges) also was divided into 2 layers; 1) the pia which lies on the brain and 2) the arachnoid which is connected to the pia by a loose network of trabeculae. The loose area, occupied by the trabeculae, made up the subarachnoid space while the space between the dura and the arachnoid layer (membrane) is the subdural space. They attributed the origin of this layer to mesenchymal tissue (42)(51).

The next pertinent work was that done by Weed (110). He also described the development of an inner and outer layer. Between these 2 layers a loose tissue network was found in the space between the brain surface and the cranial vault. This description differed from the original works of His (42) and Kolliker (51) in that the arachnoid membrane developed by a separation of the outer layer into the arachnoid and dura. The result was a subarachnoid space between the pia of the brain and the arachnoid

membrane which lies next to the dura. The formation of the subarachnoid space was found to start in the cisterna magna and develop from this point and to eventually surround the central nervous system. The development of the subarachnoid space was related to the flow of cerebral spinal fluid from the brain ventricles. These findings, except for the cerebrospinal fluid, are similar to those observed in this study.

Cytological studies of the meninges during development are almost nonexistent. Weed (110) mentioned that the origin of the flattened cells which line the fibrous structures of the meninges was from large mesenchymal cells. Other than this, the cellular differentiation has not been described.

In the adult, however, investigations of dye uptake by cells of the meninges have been studied by a number of workers. The most outstanding investigation is that of Essick (28), who demonstrated the phagocytic ability of the pavement or flattened lining cells. He showed that the flattened cells which covered the fibrous structures or the meninges could phagocytize erythrocytes and could round up into ameboid-like cells. Wolland (117) found that two types of phagocytic cells occurred in response to dye injection. One type originated from the lining cells and could phagocytize dye as a fixed cell or this cell could become an ameboid cell. The other type was from within the arachnoid trabeculae and the membranes themselves. These latter cells were found to take up much more dye and

to have the characteristic appearance of macrophages. In addition, perivascular cells were found to be in the latter category. This dual origin later encountered difficulties in that it could not be determined if the phagocytic lining cells, which lined the cavity, were possibly cells from within the meningeal tissue.

The injection of both irritating toxins and dyes has also shown the migration of polymorphonuclear cells and blood monocytes into the meningeal spaces. The work of Kubie and Shultz (56) demonstrated 2 types of phagocytic cells in the meninges: 1) 1 from blood cells and 2) 1 from the meningeal cells.

Enzyme positive perivascular cells are shown in this study to develop from enzyme positive meningeal cells, both in the developing nervous system and during injury. These enzyme positive perivascular cells, probably better called adventitial cells, are found on all vessels larger than capillaries. Perivascular cells which are on capillaries were first described by Rouget (95) and hence are known by the eponym, Rouget cells. He believed that these cells had smooth muscle properties which could cause the vessels to contract. At present the origin and function of these cells are still in doubt. However a few studies have given some further indications as to their nature. Clark and Clark (18) have shown in amphibian larvae that these pericapillary cells developed from connective

tissue cells. They also demonstrated that capillaries contracted independently of these pericytes or Rouget cells.

More recently, Rouget cells have been studied on the capillaries of the brain in relation to the blood brain barrier. These cells, as demonstrated by electron microscopy are embedded in the basement membrane of the capillaries (21). Donahue and Pappas (21) studied the development of capillaries by the use of electron microscopy in the rat cerebral cortex. In animals from 20 days of fetal age to 14 days post partum, a basement membrane was found around all capillaries. At 14 days thickening of this basement membrane could be seen. Pericytes embedded within the basement membrane, were found to lie along the capillaries of all animals studied (21). Other studies by Torack (108) and Luse (60) showed that in brain tumors the pericapillary cells responded to the tumor. Farquhar and Hartmann (30) suggested that the perivascular cells are histiocytes.

A histochemical study of cerebral capillaries by Landers et al. (56) showed that pericapillary cells have different enzymes than do advential cells. Also these cells lack phagocytic ability. The capillary pericytes were found to lack acid phosphatase activity while perivascular cells (advential cells) of larger vessels were found to have this activity. Also the advential cells were phagocytic while pericapillary cells were not. They (56) concluded on the basis of these 2 findings, on other enzyme results and on their morphological similarity to smooth muscle that the pericapillary cells are modified smooth muscle cells.

From this review there is a suggestion of 2 types of perivascular cells, 1) pericapillary cells and 2) adventitial cells. The present investigation shows that the adventitial cells are positive for both acid phosphatase and nonspecific esterase. They also originate from meningeal cells and possibly become phagocytic during injury. Pericapillary cells do not possess sufficient activity or else lack nonspecific esterase so that they do not react to this technique. The perivascular cells noted in this study during early development, lacked a distinct enzyme activity and may be similar to the pericapillary cells mentioned above. However, they are on vessels larger than capillaries. An explanation of the difference between the 2 types of perivascular cells may be that the enzyme activity difference is due to a different origin of these cells. The distinct difference in the nonspecific esterase activity of capillaries and larger vessels themselves also may indicate a reason for the difference in these cells. Capillaries do not react to nonspecific esterase or acid phosphatase (56). Large vessels, on the other hand, have nonspecific esterase in their endothelial cells (56).

The migration of mesodermal cells into the nervous system has been the object of many studies. The silver technique has been the prime means of identifying these cells. With this technique Rio-Hortega (88) first demonstrated silver reactive cells which entered the nervous system. Rio-Hortega (90) stated that he could not determine for sure if these cells

had a mesodermal origin or not. One reason for this is that with the silver reaction it becomes impossible to identify cells in the meninges from which these cells might have originated (90). Numerous investigators since then have conducted similar studies using a silver reaction (97).

The enzyme techniques used in this study, make it possible to demonstrate the mesodermal cells in the nervous system and also the cells from which they take origin. As a result the exact cellular relationship between the meningeal cells and mesodermal cells of the brain has been determined in this study during development. In addition, implications of phagocytic activity can be made on the basis of their enzyme activity.

The mesodermal cells which are found in the developing nervous system occur as ameboid cells. They were first noted by Virchow (109) during development and were identified by Rio-Hortega (90) as mesodermal cells. In comparing the appearance and occurence of these ameboid cells in this study with that of various workers (22)(32)(45)(46) it is found, that they are the same cells which have been described. The development of these ameboid cells into microglia is not clearly shown with the enzyme techniques used. Mature microglia lack demonstrable enzyme activity and, therefore, it becomes nearly impossible to follow the transition from ameboid cells to mature microglia by these enzyme techniques. However, transitional forms of microglia and rounded-up cells have been noted in the midline area of the brain stem, during the period of 8-12 days of age.

Here the migration of cells into the nervous system is in large numbers so that, as a result, the chance for observing transitional cells is greater. In this area the loss of activity during the maturation process is well demonstrated and tends to support the contention of transition of all ameboid cells into mature microglia. This maturation of microglia has been described in the literature (45)(88) and also observed in this study with the use of the silver technique. The origin of the microglioblastic cells in this midline lamina appears to be from the highly enzyme positive perivascular cells. This is demonstrated by their similar nuclear size, shape and enzyme activity which is seen in both of the enzyme techniques. A migration of perivascular cells into the nervous system has been described by Rio-Hortega (90). He has shown that larger vessels can be found which show a mass migration of these cells from the vessel surface. This finding has also been confirmed in the present study.

The origin of the ameboid cells of the nervous tissue from perivascular cells is entirely possible. This is based on enzyme activity and origin from the meningeal cells of the perivascular cells. Their nuclear morphology and enzyme activity are especially similar to cells in the meninges.

The meningeal migration of cells into the nervous system has been considered by Field (32) to be related to certain areas of stress occurring during development. Such areas are the angles made by the developing

cerebellum and by the cerebral cortex. The results, in this study indicate that the ependyma appears to have an attracting property for these cells or vice versa. In many cases the ameboid type cells are found to be in and around the ependymal layer. This tactic nature is demonstrated by the fact that these cells are found to pass into the neuroectoderm in areas where the pia comes into close relationship with the ependyma. These areas are especially prominent where the wall of the neural tube thins and the pia and ependyma meet to form the choroid plexus. This migration, however, also is prominent in areas along the neural tube which do not possess a tela choroidea, for example the cerebral aqueduct.

In other areas mesodermal cells migrate directly into the white matter. This was first described by Rio-Hortega (90) and by numerous workers since then. The areas which have been described are those in which the pia overlies the white matter. In the present study the migration of cells beneath the corpus callosum occurred in the mid-line where the pia comes into contact with this mass of white matter. The presence of this subcallosal layer of mesodermal cells can be seen in the illustrations by Kershman (46). However, he made no mention as to their origin. Here again, the present author feels that the subcallosal ameboid cells result from a taxis-relationship between the ependymal and mesodermal cells. This is believed because the area in which this layer is found represents the site of the obliterated lateral ventricle as it protruded toward

the mid-line during development. As the brain tissue increases in amount the lateral ventricle becomes collapsed in this area leaving a layer of ependymal cells. One could argue that these cells are ependymal cells because ependymal cells also have enzyme activity for these 2 enzymes. It is definitely felt that some cells in the area during the earlier stages of ventricular collapse are ependymal cells. The ameboid features and the nuclear appearance of other cells indicate that mesodermal cells are present. This collapsing feature of the lateral ventricle is also noted in the anterior and posterior projections of the ventricle. Here again these ameboid cells are found among other cells of the area.

This relationship of mesodermal cells to the ependyma is an interesting phenomenon. Very recently, Levi-Montalcini (58) found that macrophages present in the nervous system during the development of neuronal elements serve to ingest certain of these degenerating cells. The reason for the degeneration of these elements appears to be due to the fact that many neuronal cells develop but only a few become functional. On a similar note, Kershman (46) has shown what appears to be the phagocytosis of spongioblastic elements by these ameboid mesodermal cells which are believed to form microglia. Earlier studies have shown ingested fatty material within these ameboid cells (22), however, this finding has also been more recently denied (32) at least in the period before myelination occurs.

It appears to the present author, that these ameboid cells which are seen during development have ingested some sort of material. This is based on the large amount of cytoplasm and the actual presence of red blood cells in some of these cells. The possibility exists, that the relationship of these ameboid cells to the corpus callosum and lateral ventricle is to remove ependymal and even spongioblastic elements or debris which resulted from the obliteration of the ventricle. Around the ventricular ependyma they may be concerned with spongioblastic elements. Many of these phagocytic cells have been noted within the corpus callosum itself. This finding could represent a migration of these cells through the corpus callosum or even possibly the phagocytosis of the developmental nervous elements which were present when the neuronal processes grew into the area (such as spongioblastic processes etc.). Even more striking is the finding of numerous mesodermal ameboid elements within the spongioblastic lamina described in the brain stem of 8-12 day old animals. At this period the spongioblastic processes are very evident but after 20 days not a remnant of these structures could be found. The occurence of the mesodermal cells at the 8-12 day period may have completely removed the structures of the lamina.

The function of microglia during injury has been the object of numerous investigations. With the use of the silver carbonate reaction, Rio-Hortega (88) was the first to describe the proliferation of microglia and

and their subsequent changes into rounded ameboid cells. This change was described as a reduction in size of the cell processes with an increase in the cytoplasm in the area around the nucleus. The end result was the formation of a round ameboid cell which has been called a number of names; gitter cell, compound granular corpuscle etc. Since the original study (88), numerous investigators have shown similar results (22)(45)(74)(96). Occasionally, studies have indicated that blood vessel advential cells (23) or oligodendroglia (31)(33) took part in phagocytic activity during injury. More recently, autoradiography (52) and electron microscopy (65) have shown that cells other than microglia respond to injury. Two recent studies (1)(52) using autoradiography have indicated that certain blood cells give rise to cells with phagocytic activity during injury. One electron microscopic study (9) found that phagocytic cells arose from cells which were thought to be microglia. Another study (108), however, demonstrated that perivascular cells were the cells of origin.

The study which probably is the most reliable is an autoradiographic study of injured mouse brains [Konigsmark and Sidman (52)]. This study was designed to demonstrate the contribution of cells from the blood which took part in the reaction to injury. The blood cells were labeled before the puncture was made in the brain. Since the labeling in the brain is minimal, this technique permits a selective labeling of the blood cells. By comparing the percentage of labeling of the blood to counts of the injured area an

estimate of the cells which have entered the brain from the blood during the injury can be determined. The findings show that during the first 2 days after injury as many as 50% of the phagocytic cells of the area were from the blood. After 2 days the blood cells were found to contribute at least 70% of the macrophage population. These authors believed that lymphocytes did not become phagocytic, and in addition, they noted that many macrophages underwent mitoses in the wound.

The origin of phagocytic cells, other than from the blood has not been determined by tritiated thymidine studies. Other possible origins of phagocytes which can be considered are from oligodendroglia, microglia, perivascular cells (pericapillary and/or adventitial cells), endothelial cells and meningeal cells. Astrocytes, have been for the most part ruled out by the above tritium study (52).

The tritiated thymidine study (52) shows that very little labeling occurs in endothelial and adventitial cells. However, around these blood vessels of the injured area labeled cells were observed, which were called transitional macrophage cells. This latter finding was seen in a series of animals which were first injured and then given tritiated thymidine 1 hour before death. From this tritium study, the authors (52) concluded that adventitial cells did not significantly contribute to the phagocyte population.

In the present study, the specific origin of phagocytic cells cannot be directly related to any specific cell or cells. The study does show that 2 possible areas contribute phagocytic cells. These areas are the blood vessels of the injured site and the meninges in the region of the puncture. The origin of phagocytes from blood vessels is indicated in the acid phosphatase and nonspecific esterase reactions by the enzyme positive cells in and around blood vessels. This finding is very prominent at 3 days after the initial injury. In addition, the perivascular cells are found to increase in number on those blood vessels which supply the injured area. Around these blood vessels of the injured area, large enzymatically active cells are found, as well as smaller cells about the size and shape of lymphocytes and monocytes. The last mentioned cells are also positive for these enzymes.

This interpretation of phagocytes arising from cells which appear in and around blood vessels is based on a postulation (to be discussed later) that enzyme activity is present in phagocytes or potential phagocytes when they enter the injured area. Also, one can conclude that after phagocytosis has taken place, these cells do not migrate back to the blood vessels but remain in the area, digest their debris and subsequently lose their enzyme activity.

From these blood vessels 2 possible groups of mesodermal cells may arise which could develop into phagocytes. These groups could be from

the blood or from cells of the blood vessels. The blood cell origin appears evident from the tritium studies (1)(52)(53). The extent to which blood cells are involved is in excess of 50% (52). This concept of blood cells forming phagocytes has existed for many years (52)(97). The origin of ameboid cells in the brain was described by Virchow (109). He thought these ameboid cells were leucocytes, a finding which was mentioned earlier by Gluge (38). This concept was endorsed by many early workers before Rio-Hortega's discovery of microglia (52)(97). Since then other workers have expressed this leucocytic relation for phagocytes (52)(97).

The cells which appear to be the most logical precursors of phagocytes in nervous tissue are the perivascular cells which give a very intense reaction with both enzyme techniques. The evidence which supports this thesis is that: 1) they have the 2 enzymes nonspecific esterase and acid phosphatase; 2) they are found to respond to injury by increasing in numbers on the blood vessels of the area; 3) these cells are seen to enter the nervous tissue as ameboid cells during development. These ameboid cells in turn have been shown to be phagocytic; 4) during development these cells originate from cells of the meninges which also demonstrate phagocytosis and 5) during injury the fusiform cells of the meninges are found to increase in the meninges around the wound and demonstrate a relationship to the blood vessels of the wounded area, apparently becoming perivascular cells.

In the literature we find that the phagocytic ability of perivascular cells was first described by Nissl (75). This idea was held by many workers until Rio-Hortega (88) introduced the microglial concept of phagocytosis. Since then studies utilizing both electron microscopy (108) and light microscopy (23) have shown that perivascular cells are phagocytic. In the tritium study (52) discussed previously, the investigators found no evidence of an involvement of perivascular cells. However, this study was done by labeling the cells of the brain by introducing the label 1 hour before death into previously injured animals. Sections were cut transversely to the needle tract and the tissue which was observed was probably from deep in the wound. One hour labeled cells of the meninges, where the proliferation is taking place, was probably not studied due to the plane of section. Any labeling of perivascular cells will not be prominent until these perivascular cells have entered the nervous tissue (52).

The other possible phagocytic forming cells of the blood vessels themselves, are the endothelial cells and the pericapillary cells. The endothelial cells have nonspecific esterase activity but do not have acid phosphatase activity. In the early literature, endothelial cells were thought to have the ability to develop into phagocytes (5). The pericapillary cells have been studied much more extensively in regard to their phagocytic potential. The enzyme activities, if present, have not been identified in the adult animal in this study. These cells have been noted in electron

microscopy to become phagocytic in the area of brain tumors (60)(108) and to demonstrate lysosomes which are seen in phagocytic cells (30).

In the present study another portal of entry of cells into the injured area is evidenced by the migration of cells from the meningeal area. From this meningeal area migratory cells could possibly arise from the blood and from the meningeal cells. Since the arachnoid and pia have vessels passing through them and the vascular dura lies very close to this surface area, this area should have a blood leucocytic response similar to that of inflammation in connective tissue. Also the tissue cells should respond by a proliferation of macrophages and fibroblasts.

In this study the cell migration from the surface demonstrated polymorphonuclear cells in addition to numerous large phagocytic cells. The nuclei of these large cells appeared much larger than those which were expected from monocytes. The enzyme activity was found to develop slowly in the phagocytes migrating from the surface and also in those originating deeper in the wound. These cells appeared at 24 hours, but a distinct enzyme reaction was not obtained by either technique until 2 days.

The transformation of these meningeal cells into phagocytes has been well documented in the literature by Essick (28), Weed (111) and Wolland (117) and has already been discussed in this paper. In the present study, the large phagocytic response which appeared early at the brain surface is believed to be from the mesenchymal cells of the pia and arachnoid.

Also, during development mesodermal cells from the meninges are seen to round-up and show phagocytosis. It is also felt that, among these phagocytes from the meninges, blood mononuclear cells contribute to a number of these phagocytes. This is seen especially after 36 hours when mononuclear cells become prominent in and around the blood vessels of the injured site.

A finding, which is interesting, is the presence of enzyme active meningeal cells in the pia away from the wound of 24 hours and 36 hours post-operative animals while in the pia around the immediate injured area no such distinctive enzyme activity was found for the enzymes in question. This activity, however, developed in this pial area when the enzyme activity increased in the phagocytic cells of the injured area. The slow development of a distinct enzyme activity within the cells of the injured area is also an interesting finding. Although the cells show phagocytosis at 24 hours they have only a slight activity as compared to that seen at 3 days.

The macrophages which are not derived from blood are believed by Konigsmark and Sidman (52) in their tritium study to be from the cells of the injured area. The cells which may be involved are the oligodendroglia and microglia. This relationship to oligodendroglia has been supported by some workers (31)(33). More evidence, however, points to microglia as a possible contributor (81)(90). In the present study, the microglia

away from the injured area were not seen to become activated in any manner and migrate to the area. Within the injured area and especially at the margin of this area and the supposedly normal tissue, reactive microglia were noted. The origin of these microglia cannot be definitely attributed to microglia of the area. They could represent mesodermal cells which have migrated into the brain, and their shape is the result of the tissue space which they inhabit. Also these cells could be phagocytic cells which have come to rest as they digest their debris.

The problem concerning the origin of reactive microglia has been created by the introduction of the blood macrophage concept in brain injury. The possibility that reactive microglia originate from phagocytic cells has been briefly suggested by Konigsmark and Sidman (52). In the present study, the enzyme methods demonstrate that a progressive loss of enzyme activity occurs as the reactive microglial cells take on a more resting-like appearance. There is no certainty, however, that this process is not reversed as Rio-Hortega (90) described it. Considering that enzyme activity develops in the cells when they form from their cell of origin (primative reticular cell), the direction of this process should be from the blood cells and/or meningeal cells toward the reactive and resting microglia. The idea that enzyme activity develops during maturation of the cells is supported in this study by the presence of the enzyme activity in the cells as they enter the wound area.

This study supports the development of reactive microglia from phagocytic cells. The time of appearance of the phagocytic cells and the reactive microglia is demonstrated both in the silver reaction and the 2 enzyme techniques. The macrophages are found to definitely appear in the area before any significant number of reactive microglia are seen. Later in the repair process, the phagocytic cells have decreased to only a few while the reactive microglia are numerous. Early investigators described the progressive development of microglia into ameboid cells which demonstrated an increase in phagocytic debris as they rounded-up (81)(90)(96). The enzyme reactions in this study show that the rounded-up phagocytes appear first and after these cells have ingested debris they digest this material. As the ingested debris is broken down, these cells develop increasingly longer processes, the cytoplasmic area around the nucleus decreases and the enzyme activity decreases. The result is reactive microglial cells of various shapes and sizes.

Support of the concept that phagocytes have enzyme activity when they enter the nervous system and lose it as they digest their debris, can also be found in the present study. At the 24 hours stages, enzyme positive cells are seen to enter the wound area. The enzyme activity is very slight but appears to be always present. The cells which contain this enzyme activity are the polymorphonuclear cells and also the early phagocytes. This enzyme activity is distinctly increased at 3 days and is very

prominent in those cells which appear around the blood vessels. Furthermore in the later stages of healing this enzyme activity is found to become lost from the blood-containing macrophages remaining in the wound.

In the developing brain series, the enzyme activity of the 2 enzymes is present in those cells which migrate into the nervous system. This enzyme activity is found to be acquired as these cells differentiate from the undifferentiated cells of the meninges. In the spleen the development of nonspecific esterase was noted in macrophages as they developed from the primative cells (83).

It appears to this investigator that the shape of reactive microglia is the result of the tissue space in which these cells reside. The concept that resting microglia have a shape which is the result of the limited intercellular space of the central nervous system has recently been proposed by Russell (97). In the present study the shape of reactive microglia are related to the state of the area in which they reside. In areas where the injured tissue has been recently removed by phagocytosis the reactive cells have short thick processes with a considerable amount of cytoplasmic area in the region of the nucleus. In these same sections reactive microglia out near the normal tissue have processes which are long and thin. In later stages when the injured area is resolved the reactive microglia have very long processes. Here again out toward the undisturbed tissue, the microglia have a more resting-like appearance.

Russell (97) had postulated that monocytes may migrate freely in and out of the nervous system. He feels that while in the nervous system they are microglia and have a shape that depends on the intercellular space. The present author has made no observation which would agree or disagree with this concept. The opinion of this investigator is that perivascular cells from the meninges probably maintain the microglial population.

The relationship of microglia to the reticuloendothelial system has been stated by a number of investigators as being based on the apparent reactive state of microglia during injury. At this time these cells become phagocytic and take up vital dyes (22)(57)(96). In addition, the morphological similarity of reactive microglia to cells of the reticuloendothelial system both with the silver reaction and nuclear stains is evident (22)(24). According to the recent findings with autoradiography, electron microscopy and the present study, the phagocytic criterion is no longer valid. The cells which have been considered to represent reactive microglia in the injured site are very likely phagocytes from the blood, perivascular cells, meninges and possibly microglia which were in the wound area. There is no proof that resting microglia have the ability to become phagocytic although the possibility definitely exists. In tissue culture Bornstein (11) has recently shown the uptake of lithium carmine in microglia. The cells which are involved here can hardly be resting microglia but either

"reactivated microglia" or reactive microglia from perivascular cells or from the blood. The reticuloendothelial characteristics of microglia which are undisputed are their morphological similarity (both shape of the cell and nuclear characteristics), silver reactivity, and mesodermal origin.

In regard to the enzyme relationships to reticuloendothelial cells, normal microglia are considered to contain acid phosphatase (3). This has been established in tissue culture with a hexazonium dye. However, the photomicrographs used for evidence show reactive type microglia (3). The origin of these tissue culture cells could easily be from the blood or perivascular cells which were introduced into the culture with the tissue. In this same study (3) with fresh tissue, it was stated that microglia and the other neuroglial cells have a slight acid phosphatase activity around the nucleus. In the present study, it is felt that the proper identification of resting microglia cannot be made on the basis of this slight activity and therefore it is felt that a more sensitive technique is necessary to determine for certain the extent of acid phosphatase and nonspecific esterase in resting microglia. The enzyme activity of developing microglia, however, does indicate that a possible R-E relationship exists. The origin of microglia from enzyme positive cells of the meninges and the subsequent loss of activity during maturation supports the fact that sometime during their life microglia are related to reticuloendothelial cells. In this study, the R-E relationship is also supported by the phagocytic

ability of the migratory cells before they become microglia. The relationship of resting microglia to the R-E system can hardly be established with the present knowledge of these cells.

The criteria of the R-E system have not been well established and as a result it becomes difficult to delimit a specific group of cells. The original concept of the macrophage system by Metchnikoff (67) probably defined a much more exact group of cells, however, this system was not designed to include cells which might become macrophages. The original criteria of the R-E system (4), phagocytosis and vital staining, were extended to those cells which reacted with the silver carbonate technique (87). The result was a group of cells which could not easily be defined on the bases of 3 criteria. The demonstration of acid phosphatase activity in relation to phagocytic cells was more recently introduced (7) with hopes of giving the R-E concept a new prospective. More recently nonspecific esterase has been added to these criteria (105).

The problem which has resulted from the new enzyme concepts is the understanding of the extent that these enzymes can be found in the R-E system. From studying the numerous observations with these enzymes, it becomes evident that all cells which give the silver reaction do not necessarily demonstrate enzyme activity. This can be seen in this study where microglia give a definite silver reaction, but they do not demonstrate

distinct enzyme activity. Other cells of the nervous system have the enzyme activity but they do not give the silver reaction.

The need exists for the further establishment of the enzyme role in the R-E system. Some work has been done in this regard on acid phosphatase. This enzyme has been localized in the lysosomes of the macrophage cytoplasm (76). These lysosomes represent ingested material which is surrounded by a unit membrane (73). The problems which exist are: What does this enzyme activity represent? Is it a property of the unit membrane which may be from the plasma membrane of the cell? Does the phagocytic property exist in cells which show no acid phosphatase or as soon as this enzyme property is developed does the cell immediately phagocytize material? In other words, do the properties of being able to ingest material and of having the enzyme property exist together or possibly are they one and the same? If cells occur, which have the acid phosphatase potential or the possibility of developing the potential then these cells must be true R-E cells but do not show phagocytosis during this particular period of their life. Also, are cells which have lost the enzyme activity, still R-E cells, as is seen in microglia in this study? Along this same line of thinking, are macrophages which have been claimed to remain in the wound and become fibroblasts (34), also R-E cells? Normally fibroblasts are not considered in this system. Carrel and Ebeling (16) have shown that fibroblasts and macrophages are both morphologically and

physiologically different (34). Gropp and Hupe (41) found that acid phosphatase and nonspecific esterase were slight or absent in fibroblasts.

The other problem which arises is: What does the acid phosphatase represent which is found in cells which are not normally considered phagocytic, e.g., neurons? Could this be a different type of phosphatase enzyme or even possibly represent the presence of the enzyme without the property of being able to phagocytize foreign material?

The time of the development of enzyme activity or the potential for enzyme activity in relation to the maturity of the cell presents another problem. In this study, enzyme activity was seen to appear in the developing meninges as the cells matured from undifferential mesenchymal cells. The injury study shows that blood elements are positive after they enter the nervous system. In lymphoid tissue, however, neither acid phosphatase nor nonspecific esterase were found, in lymphocytes or monocytes (14). More recently, nonspecific esterase activity has been found in monocytes (13). The point at which these cells develop this enzyme activity remains to be demonstrated.

The relationship of nonspecific esterase to the R-E system is even less well understood. Here, there is the problem of a number of enzymes being involved. In the brain nine such enzymes have been demonstrated with starch gel electrophoresis (8). Many of the same questions which pertain to acid phosphatase also pertain to nonspecific esterase.

CHAPTER V

SUMMARY

The relationship of microglia to the reticuloendothelial system has been studied in the rat by the use of nonspecific esterase and acid phosphatase in conjunction with the silver reaction and nuclear staining techniques. This relationship was studied in developing rats from 15 days gestation to adult animals. In addition, it was studied following injury to the nervous system. The results show that during development mesodermal cells migrate into the nervous system as ameboid elements and as perivascular cells which follow the blood vessels.

In animals of 15 days gestation, certain mesenchymal cells of the premeningeal area show a slight nonspecific esterase activity in their cytoplasm. This activity increases so that at 17 days gestation, numerous enzymatically active, fusiform cells are seen throughout the developing meningeal area. In addition to fusiform cells, enzyme positive ameboid cells are also seen in the loose meshwork areas of the meninges which will become the meningeal spaces. As the meningeal spaces are formed within the condensed meningeal area, ameboid cells are seen to develop from these positive enzyme fusiform cells. In the nonspecific

esterase reaction, the fusiform and rounded-up cells demonstrate characteristic indented nuclei.

By using the Nissl stain, characteristic indented nuclei with varying densities of nuclear chromatin are found in the meninges. Nuclear changes are found which correspond to a maturation of these cells from a primitive cell. The first change is the indentation of the large pale-staining mesenchymal nuclei. This is followed by the development of a characteristic nuclear chromatin pattern. As the chromatin pattern becomes denser, the nuclear size decreases, the indentation remaining. The enzyme positive fusiform cells appear to correspond to cells with medium-sized nuclei which have developed moderately dense nuclear chromatin granules. The ameboid cells usually had a smaller indented dark-staining nucleus.

With the nonspecific esterase reaction, the fusiform meningeal cells are found to penetrate the nervous tissue where the pia and ependyma approached each other in animals of 17 days gestation. The areas which demonstrate a consistent migration are the taenia of the fourth ventricle, the anterior medullary velum, the transverse fissure as it adjoins the rostrad wall of the third ventricle and the pia which contacts the genu of the corpus callosum. After the fusiform cells enter the nervous tissue they apparently change to cells with many processes and a rounded cell body. This is usually accompanied by a decrease in the nonspecific esterase activity. Two or 3 days after the onset of migration was noted, many cells

highly active for both nonspecific esterase and acid phosphatase are found in the nervous tissue, adjacent to the ependymal layer.

Fusiform cells also enter the nervous system from the pia which contacts the developing corpus callosum in the longitudinal fissure. These cells migrate into the lower aspect of the corpus callosum (subcallosal area) and, as the corpus callosum develops laterally and anterio-posteriorly, a layer of these mesodermal cells is formed on the lower surface of the corpus callosum. The enzyme activity of these migrating cells within the subcallosal area is less than that seen in the meningeal cells from which they originated. However, in the nervous tissue between the pia and the subcallosal area fusiform cells like those of the meninges are found. The actual development of these migrating cells into mature microglial cells do not contain sufficient enzyme activity to allow for their identification.

Perivascular cells are found to develop from positive meningeal cells. They begin to occur on the blood vessels in the area of the brain stem at the time of birth. These cells are first seen near the surface of the brain. The migration of enzyme positive fusiform meningeal cells is seen to proceed along the larger vessels to increasingly greater depths of the brain stem. At about 12 days of age, perivascular cells are found on all large vessels throughout the brain stem. In the cerebral cortex, this process is seen to occur at a later period of development which begins at

about 12 days of age. In the brain stem and the cerebral cortex the appearance of enzyme positive perivascular cells appears to be closely related to the development of nonspecific esterase activity in the neuronal elements. As maturity is reached, all vessels have attained enzyme positive perivascular cells. In the Nissl stain these perivascular cells have the same mesodermal characteristics as the enzyme positive cells found in the meninges.

The migration of perivascular cells into the nervous tissue is seen to occur occasionally along the vessels. These cells which enter the nervous tissue have the same rounded appearance as those cells which have migrated directly from the pia. In addition, mass migrations of these cells have been noted from larger vessels. This has been observed in the medulla of 8-10-12 day old animals where a midline lamina of enzyme positive processes extend from the ependyma to the pia. Accompanying this lamina are many large blood vessels which possess numerous round enzyme positive perivascular cells. Between these vessels, rounded cells and immature microglial cells are demonstrated with the enzyme techniques. In the silver reaction, the rounded cells give the characteristic appearance of microglioblasts described by earlier workers. The perivascular cells appear to be entering the nervous tissue as rounded cells which in turn develop into mature microglia.

Long before the appearance of enzyme positive perivascular cells on the vessels, perivascular cell nuclei can be seen in the Nissl stained section from animals as early as 17 days gestation. These cells give the silver reaction and appear to be forming microglia seen in the nervous system at this early age. In studying the nonspecific esterase reacted sections, nuclei which are indented are seen in the enzyme reaction of the vessels and of the cells, themselves, when they are positioned on the slide of the vessel. The origin of these cells, unfortunately can not be determined.

Mesodermal cells appear in injured nervous tissue following a needle puncture into the cortex and corpus callosum. Twelve hours following injury polymorphonuclear cells are seen around neurons of the ischemic area. After 36 hours many phagocytic cells with large nuclei appear to be migrating into the wound from the meningeal surface of the brain. A lesser number of these cells are found in the depths of the wound. In the enzyme stains these phagocytic cells have a slightly greater enzymatic activity than the surrounding neurons of the cerebral cortex. At this time, an increase in the number of perivascular cells is noted around the wound. A further increase in the number of polymorphonuclear cells is demonstrated while mononuclear cells are beginning to appear in the interrupted vessels. In the silver reaction very few so-called reactive microglia can be seen.

At 3 days post operatively the enzyme activity of the ameboid phagocytic cells has increased to such an extent that they are considerably more delineated than the surrounding neuronal cells. These phagocytic cells are found to be distributed throughout the wounded area. The vessels which are in the wound area have, associated with them, numerous intensely enzyme-positive cells in their walls. Phagocytic cells appear to be entering the wound from these vessels and from the surface of the brain. The differential contribution of cells from the blood and from perivascular cells cannot be determined by the enzyme techniques. Some differences in cell shape and staining in the nonspecific esterase reaction may indicate a difference in the origin of these cells.

In the wound area many degrees of activity are seen among the cells in the areas which have been actively attacked by phagocytic cells. Rounded very active cells like those which are found around vessels are seen in the wound. Among these cells, others of various shapes, sizes and of lesser enzyme activity can be seen in the background. In the silver reaction, the round phagocytes are positive to silver staining but these cells are not definitely outlined. Those cells with lesser enzyme activities have corresponding shapes and similar positions in the wound to reactive microglia seen in the silver reaction.

It is thought that these cells represent a process whereby phagocytic cells engulf debris and retire in the wound at least for a time while

they digest their debris. As the debris is digested the enzyme activity decreases and is lost. Finally a scar results in which there is no demonstrable enzyme activity. This theory is supported by evidence from the early stages where many phagocytes were observed and very few reactive microglia were found. At later stages a more distinctive origin of phagocytes from blood vessels occurred and an increase in reactive microglia was distinct. During the final phagocytic stages enzyme positive rounded macrophages are few and reactive microglia are found with decreased enzyme activity. In addition, a loss of enzyme activity is demonstrated in hemosiderin-containing macrophages.

Microglia are thought to be directly related to the reticuloendothelial system because of their origin from mesenchymal cells, their migratory and enzyme activity during development and their reaction to silver. The role of microglia during injury could not be determined. It is entirely possible that microglia in the immediate injured area take part in phagocytosis.

CHAPTER VI

CONCLUSIONS

From the results of this investigation it can be concluded that: 1) during development microglia appears to come from meningeal cells. These meningeal cells are positive for the enzymes nonspecific esterase and acid phosphatase. These mesenchymal cells migrate into the nervous system directly from the meninges and along penetrating blood vessels as perivascular cells. As these 2 types of cells enter the nervous system they lose some of this enzyme activity and, as the resulting migratory cells develop into mature microglia, the enzyme activity is decreased so that the cells can no longer be identified by these techniques. 2) The meningeal cells which show activity for nonspecific esterase and acid phosphatase show phagocytic activity and ameboid properties during the development of the meningeal spaces. 3) The development of these meningeal cells which migrate into the central nervous system can be traced through a group of cells which in turn originate from undifferentiated mesenchymal cells. 4) During injury, nonspecific esterase and acid phosphatase positive macrophages are found to enter the injured area from the surface of the brain and from blood vessels. The enzyme positive cells

which are seen on and around the blood vessels are believed to be of 2 types: 1) perivascular cells and 2) blood leucocytes. Those from the surface of the brain are believed to be from meningeal cells and blood leucocytes. 5) The macrophages which enter the wound are found to engulf debris and then reside in the area for a time as cells which appear similar to reactive microglia while they digest this material. Concurrent with the digestion of the debris, the nonspecific esterase and acid phosphatase activity diminished and becomes undetectable. Macrophages containing RBC material are rounded-up. Those which remain in the wound eventually lose their enzyme activities. 6) The presence of nonspecific esterase and acid phosphatase in mesodermal cells of the nervous system during development supports the relationship of microglia to the R-E system.

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PLATE I

Explanation of Figures

Figure 1. Normal animal; metalophil reaction. (m) Microglia in the corpus callosum. 900X

Figure 2. Normal animal; metalophil reaction. Perivascular cells (P) on the blood vessels (V) show a positive reaction in the entire cell. Neuroglia and neurons (N) in the background show deposits in their perinuclear areas. 900X

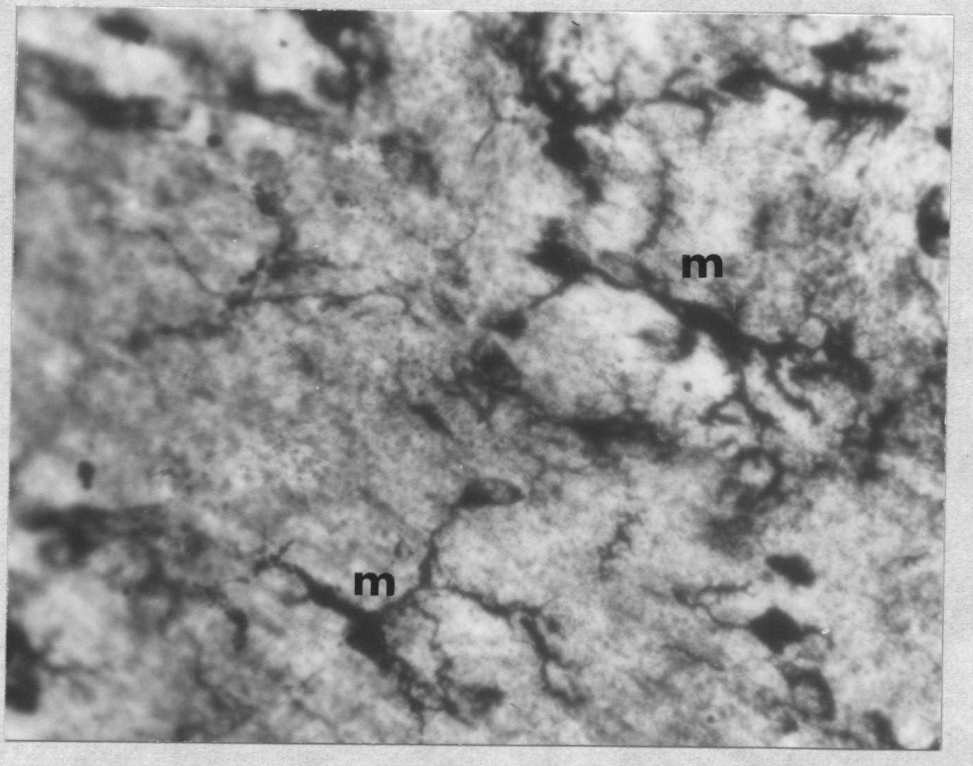

Figure 1

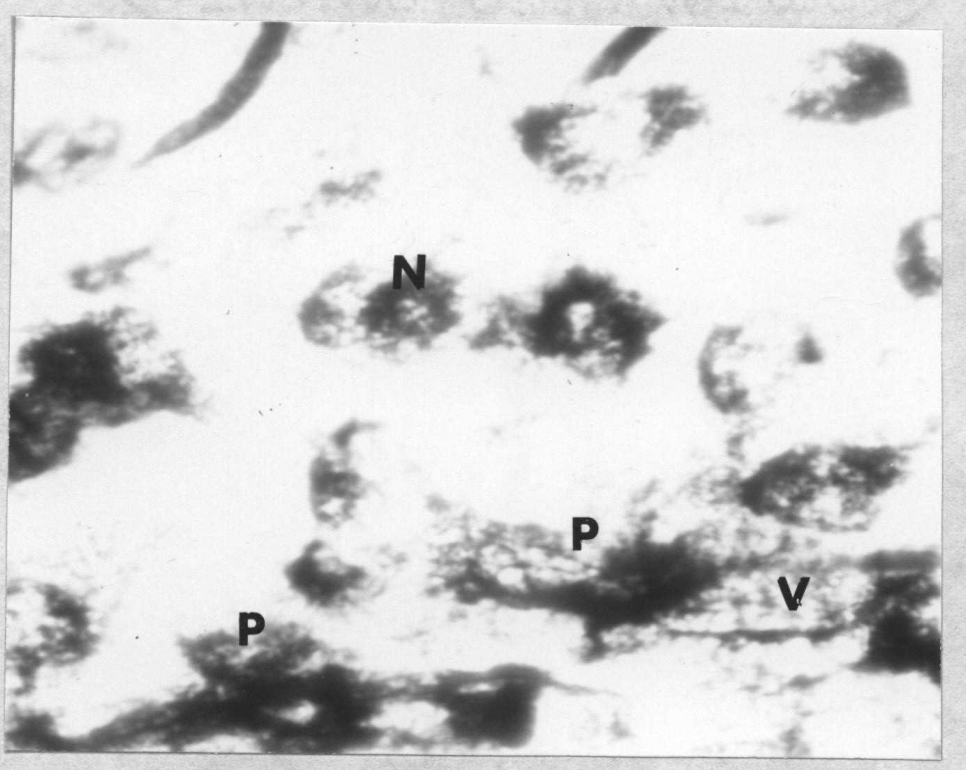

Figure 2

PLATE I

PLATE II

Explanation of Figures

Figure 3. Normal animal; cresyl violet stain. Oblique section of vessel in the hippocampal cortex. (s) smooth muscle nuclei, (E) endothelial nuclei, (P) perivascular nuclei. 630X

Figure 4. Normal animal; nonspecific esterase reaction. The brain stem of a normal animal showing the various degrees of enzyme activity in neurons. Note the variable neuronal activity (*) and lack of identifiable glial cell reaction. 160X

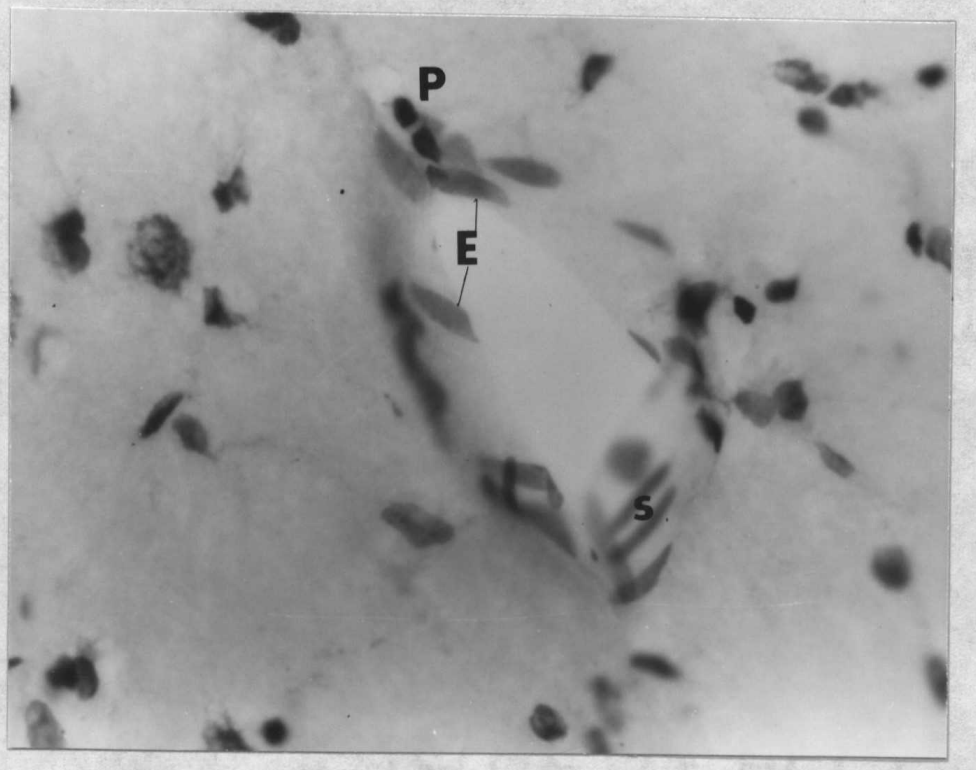

Figure 3

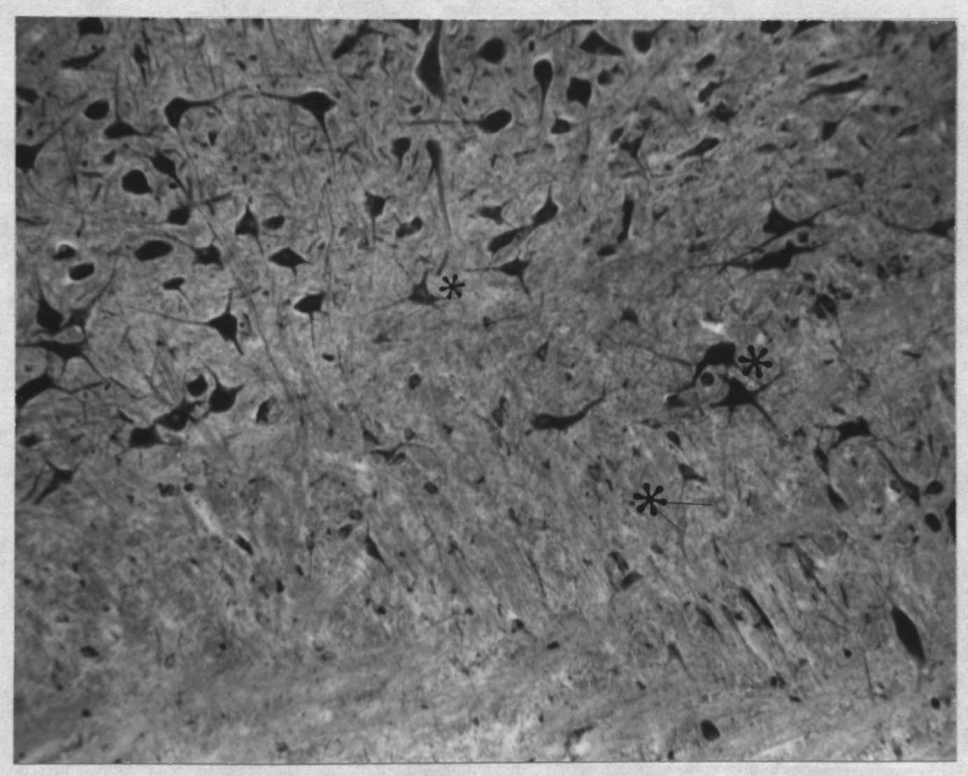

Figure 4

PLATE II

PLATE III

Explanation of Figures

Figure 5. Normal animal; nonspecific esterase. The brain stem of a normal animal showing perivascular cells. (p) Very positive perivascular cells on a vessel, (n) note light neuronal staining, (W) the white matter shows no identifiable cells except for perivascular cells of small blood vessels. 190X

Figure 6. Fifteen days gestation; nonspecific esterase. Meningeal space on the ventral surface of the brain stem. (m) Meningeal area, (N) nervous tissue. Note faint activity of a fusiform cell with an (i) indented nucleus. 190X

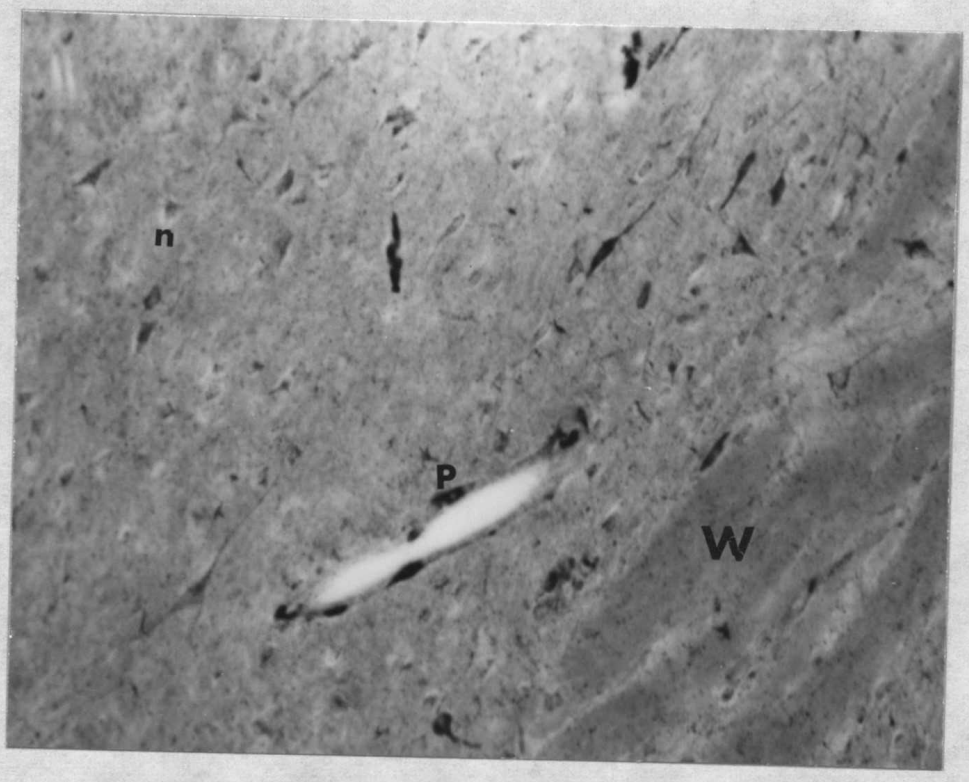

Figure 5

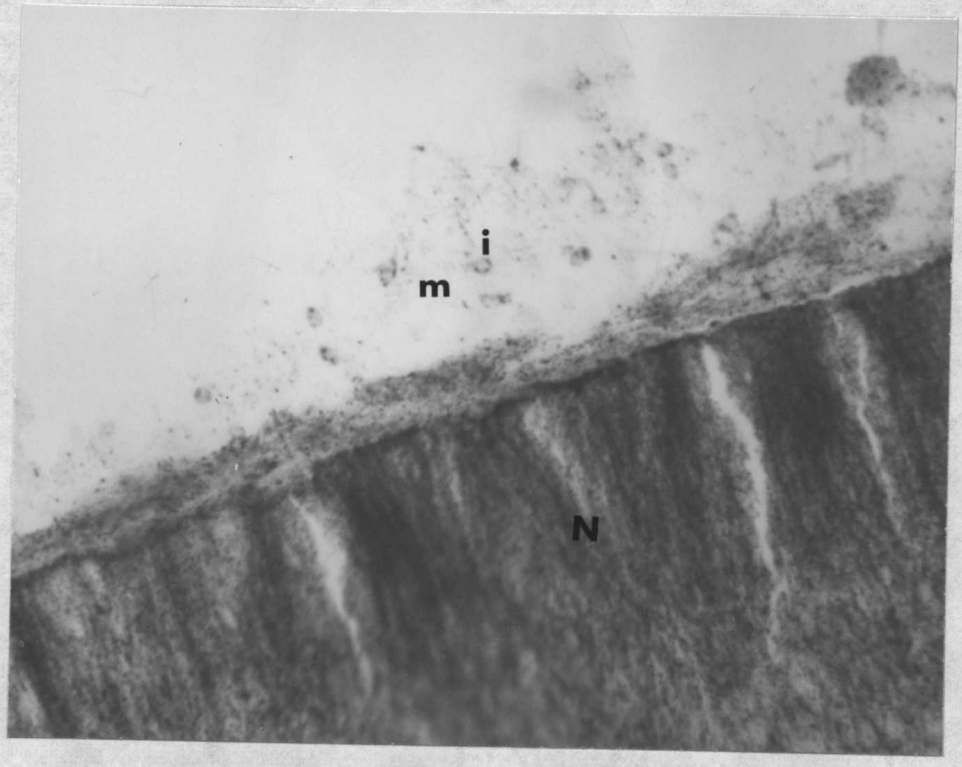

Figure 6

PLATE III

PLATE IV

Explanation of Figures

Figure 7. Seventeen days gestation; cresyl violet. The breakdown of the meningeal area to form the subarachnoid space. (N) Nervous tissue, (C) cartilage, (m) metachromatic area, (S) subarachnoid space. 160X

Figure 8. Six day postnatal animal; nonspecific esterase. The demonstration of fusiform cells in a layer on the neuroectoderm (pia) where the cut is oblique to the surface. (S) Surface area obliquely cut, (f) note the fusiform and (a) ameboid cells in the loose portion of the meninges (subarachnoid space). 160X

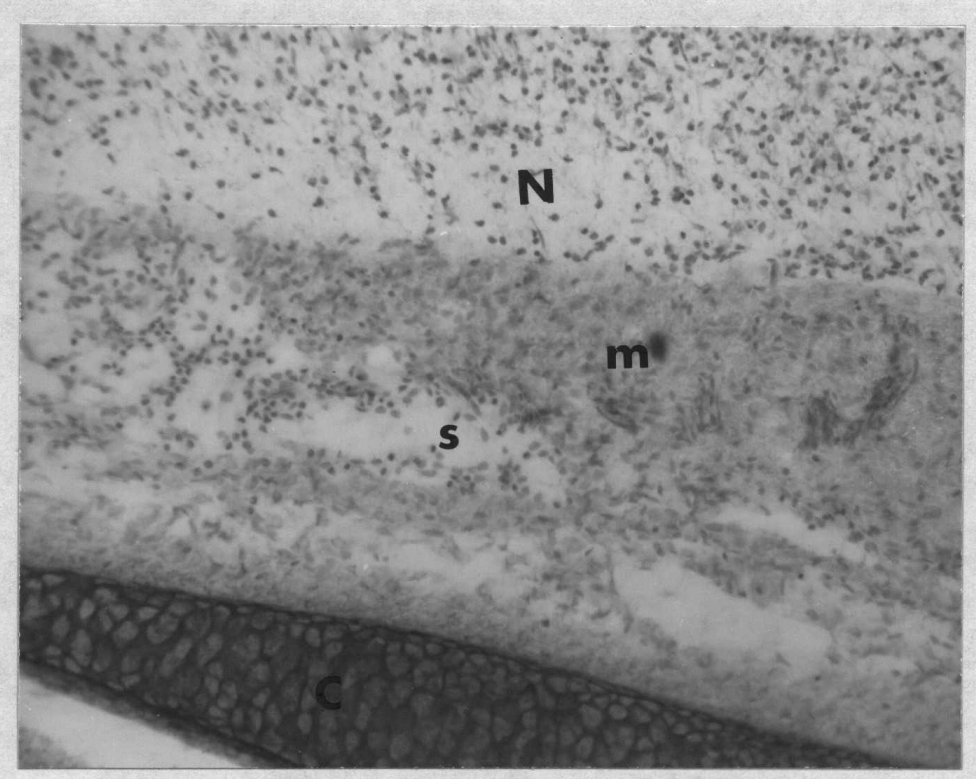

Figure 7

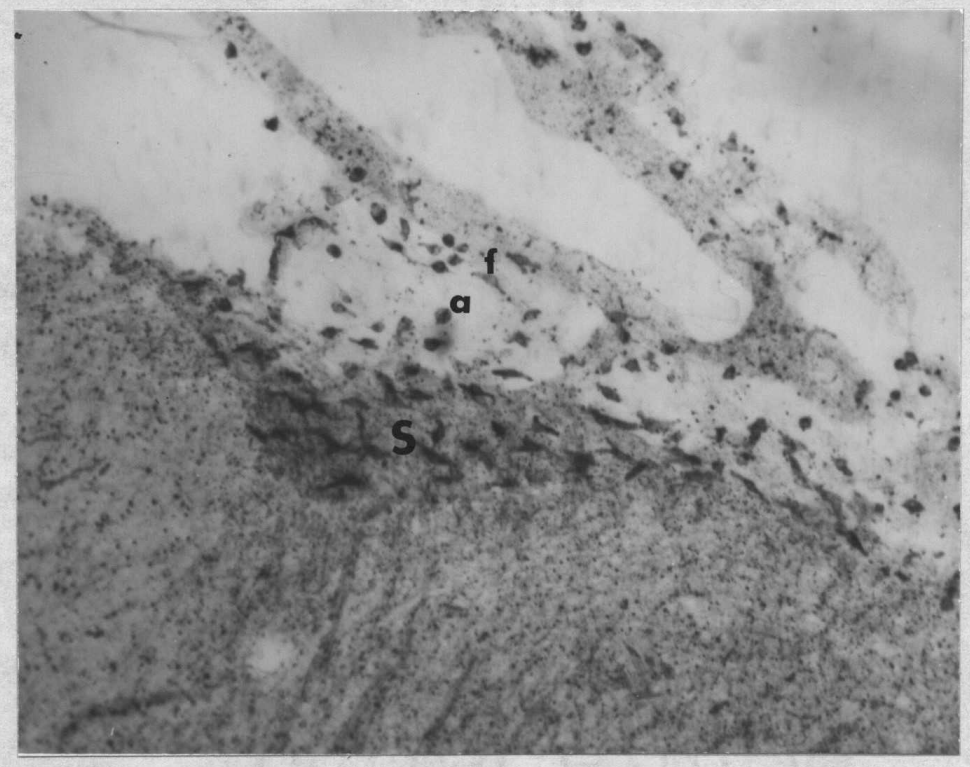

Figure 8

PLATE IV

PLATE V

Explanation of Figures

Figure 9. Nineteen days gestation; nonspecific esterase reaction. Meningeal space showing nervous tissue (N) and connective tissue (T). Meningeal space (m), fusiform cells (f), Note the numerous rounded-up cells in the space. 160X

Figure 10. Six day old animal; nonspecific esterase. High power view of figure 8. Note the indented nuclei of the fusiform cells. 730X

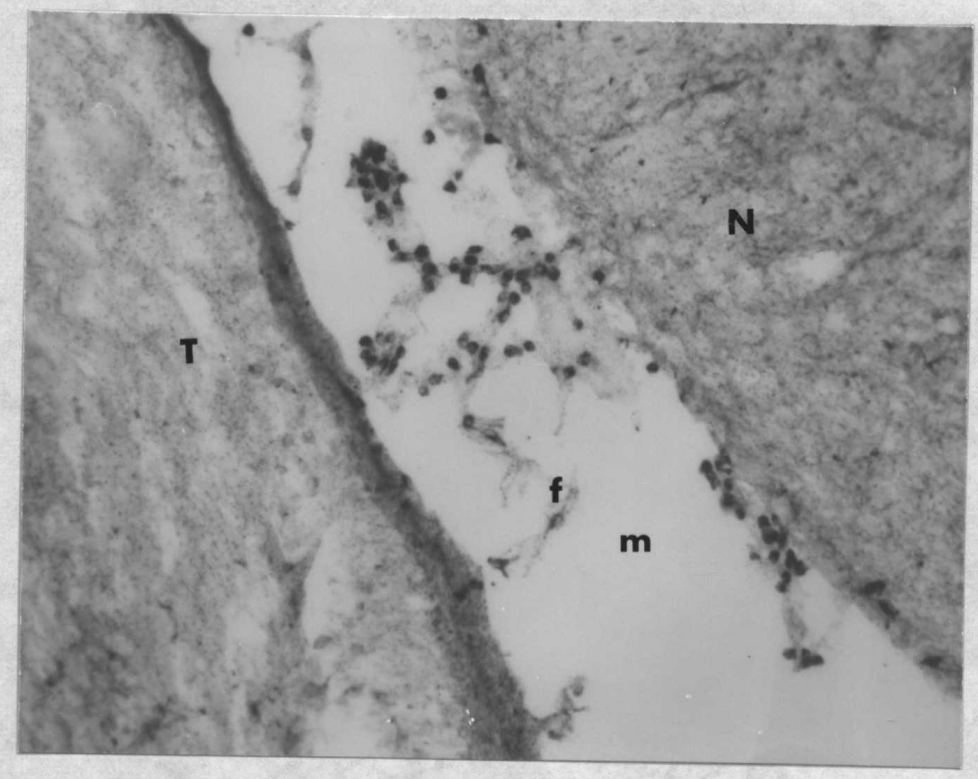

Figure 9

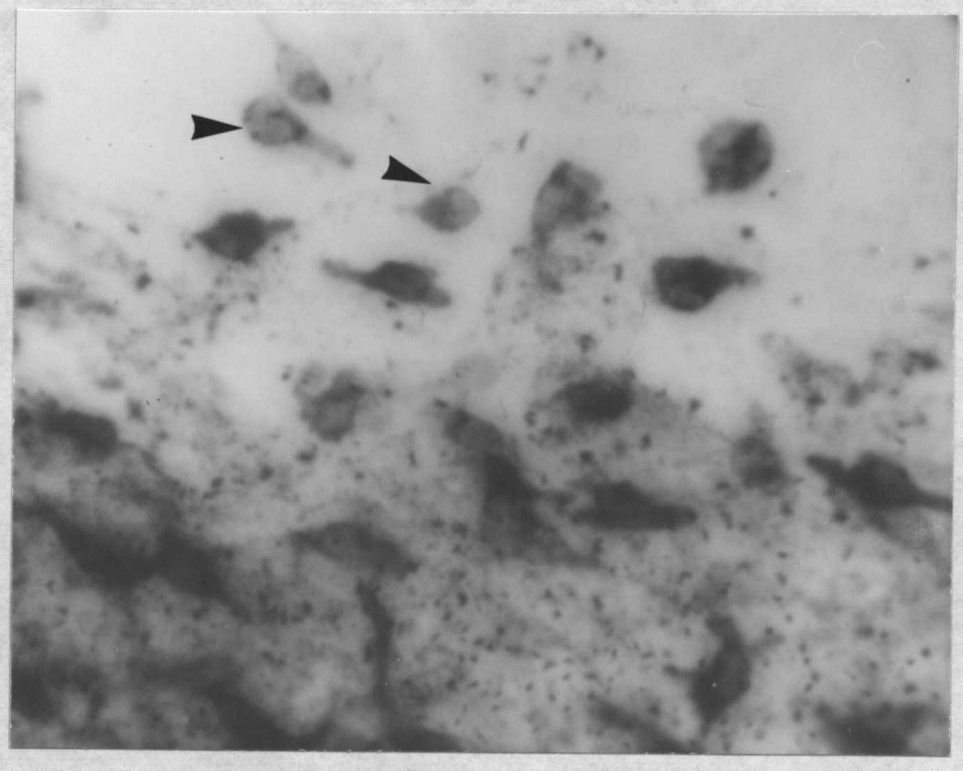

Figure 10

PLATE V

PLATE VI

Explanation of Figures

Figure 11. Seventeen day old fetus; cresyl violet. Meningeal area showing the various nuclei found in this layer. Many dark nuclei are seen of which many show indentations. Some are rounded-up (7) others are in tissue network. The possible maturation of these cells from primative mesenchymal cells (1) is seen by following the numbers. Note the first change to an indented nucleus and then the development of a nuclear chromatin pattern while at the same time the nucleus becomes smaller. 190X

Figure 12. Seventeen day gestation; cresyl violet. Meningeal area showing rounded-up cells in the meningeal space. Many of the cells with dark nuclei also demonstrate a rounded cytoplasm (arrow); also note the indented nuclei of these cells. 630X

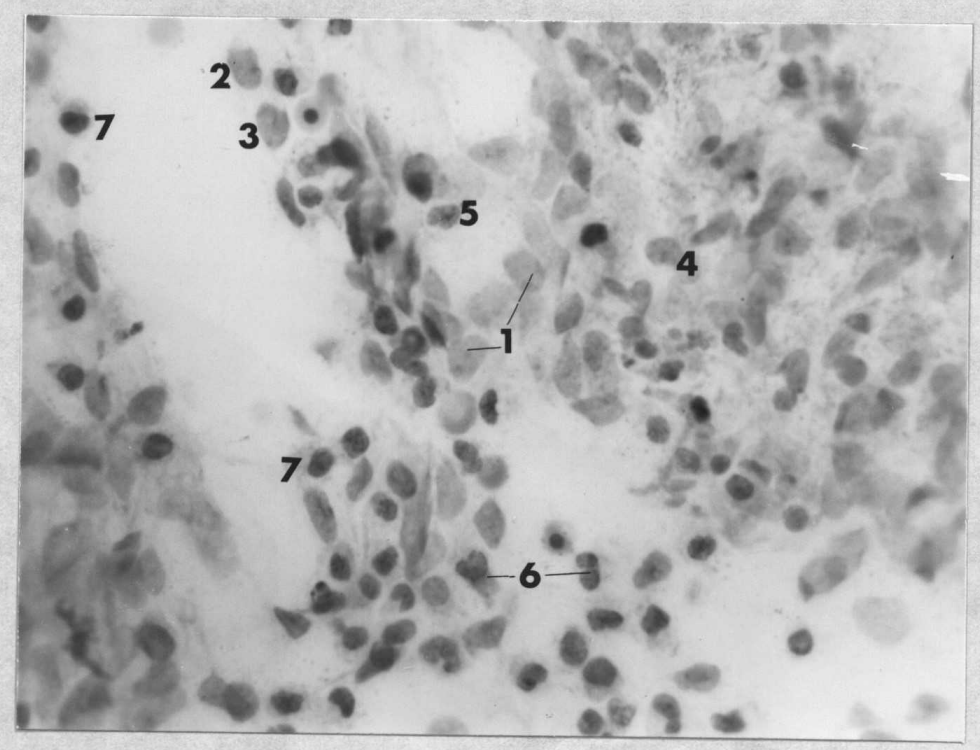

Figure 11

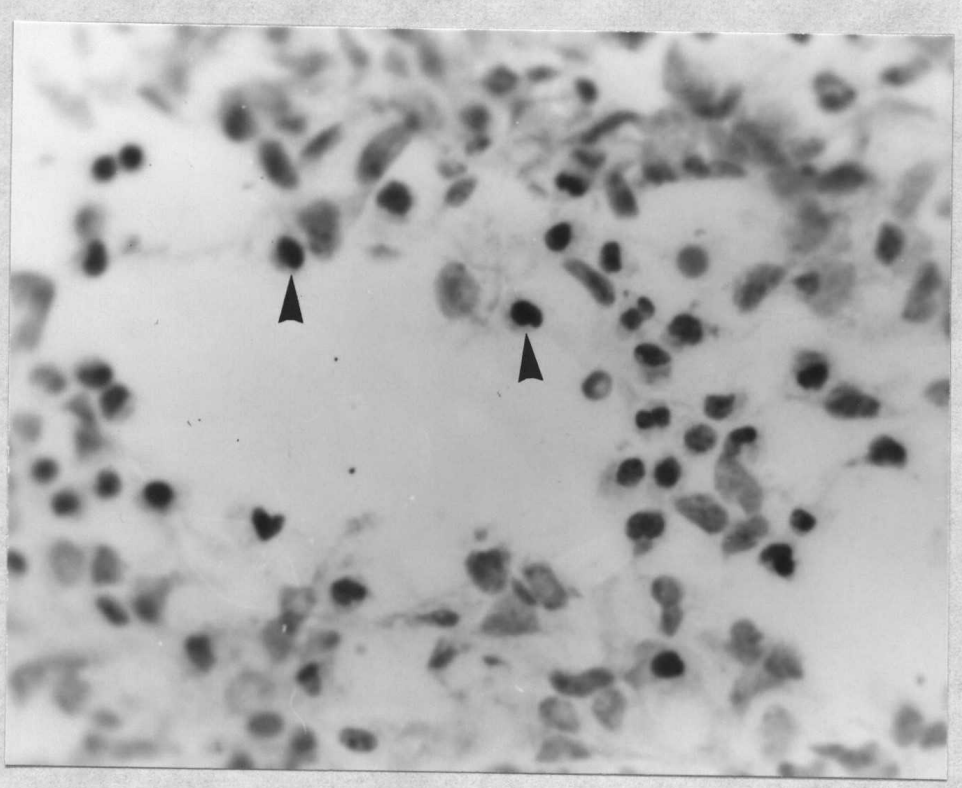

Figure 12

PLATE VI

PLATE VII

Explanation of Figures

Figure 13. Fifteen days gestation; nonspecific esterase. Basal plate of the nervous system at the cervical flexure. Note the intensity of the structures which pass from the ependyma (e) to the (M) meningeal surface (X) neural canal. 630X

Figure 14. Diagram of a near mid-sagittal section at 20 days gestation showing sites of migration. The cerebral cortex (Y) adjacent to the longitudinal fissure has been slightly sectioned as compared to the full size (Z). Sites of migration: (T) taenia of the fourth ventricle, (A) anterior medullary velum, (Tv) transverse fissure and the third ventricle and (CP) subcallosal area and longitudinal fissure. Stippling indicates their origin and their probable accumulation sites. (TF) Transverse fissure, (cc) corpus callosum, (P) pia-arachnoid, (v) third ventricle, (Q) quadrigeminal plate, (C) cerebellum, (FV) fourth ventricle, (AQ) cerebral aqueduct, (Pg) pineal gland and (IP) interpeduncular fossa.

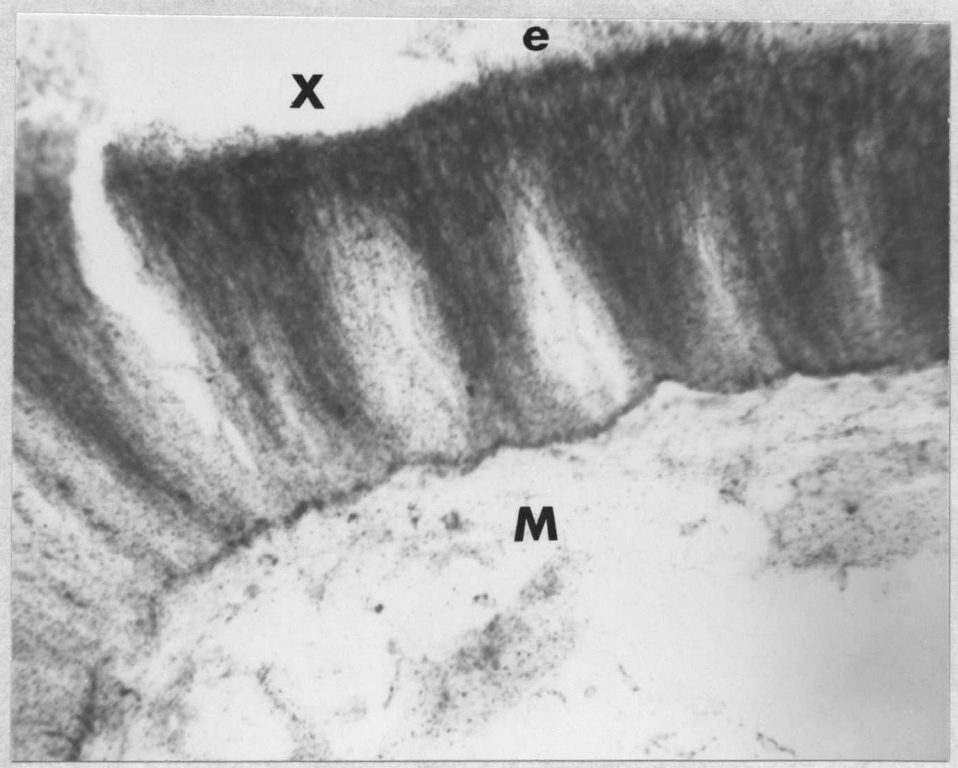

Figure 13

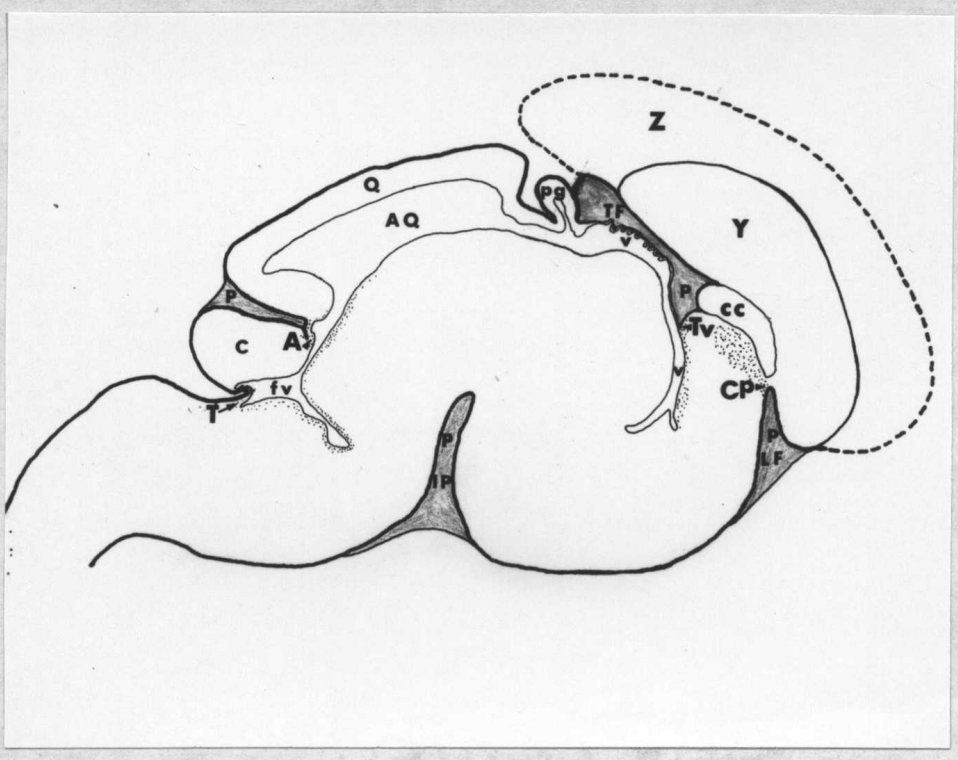

Figure 14

PLATE VII

PLATE VIII

Explanation of Figures

Figure 15. Twenty days gestation; metalophil reaction. Silver reacting cells which appear-like and correspond in distribution to migrating cells observed with the nonspecific esterase reaction. 630X

Figure 16. Nineteen days gestation; nonspecific esterase. The migration of cells into the nervous tissue of the anterior medullary velum. (P) Pia-arachnoid, (C) cerebellum, (Q) quadrigeminal plate, (*) migrating fusiform cells.

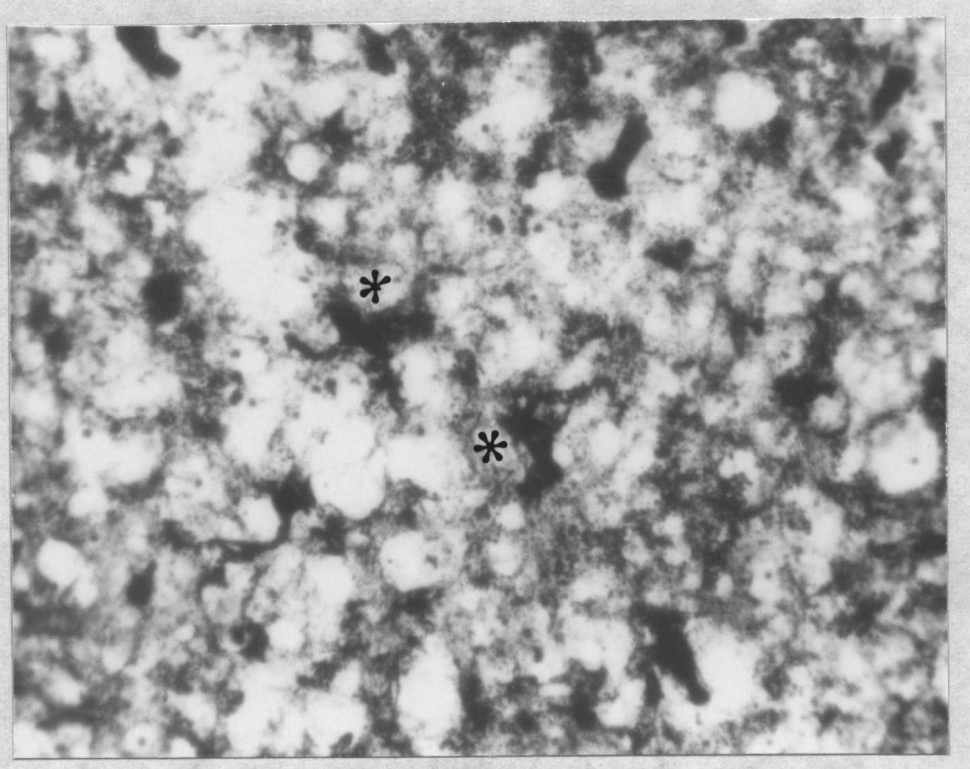

Figure 15

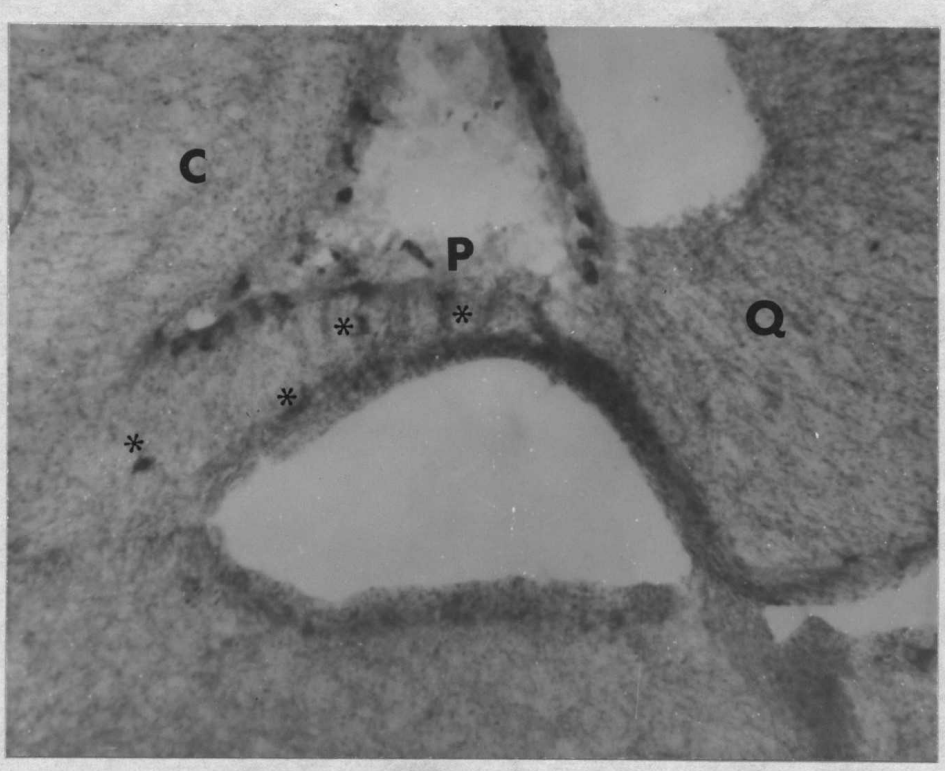

Figure 16

PLATE VIII

PLATE IX

Explanation of Figures

Figure 17. Twenty days gestation; nonspecific esterase. Migration of cells from longitudinal fissure to the subcallosal area. (c) Corpus callosum, (P) longitudinal fissure. 80X

Figure 18. Nineteen days gestation; nonspecific esterase. Migration of cells from the pia to the subcallosal area. (C) Corpus callosum, (arrows) fusiform cells which are migrating to the subcallosal area. (O) A cell which has migrated into the area. 630X

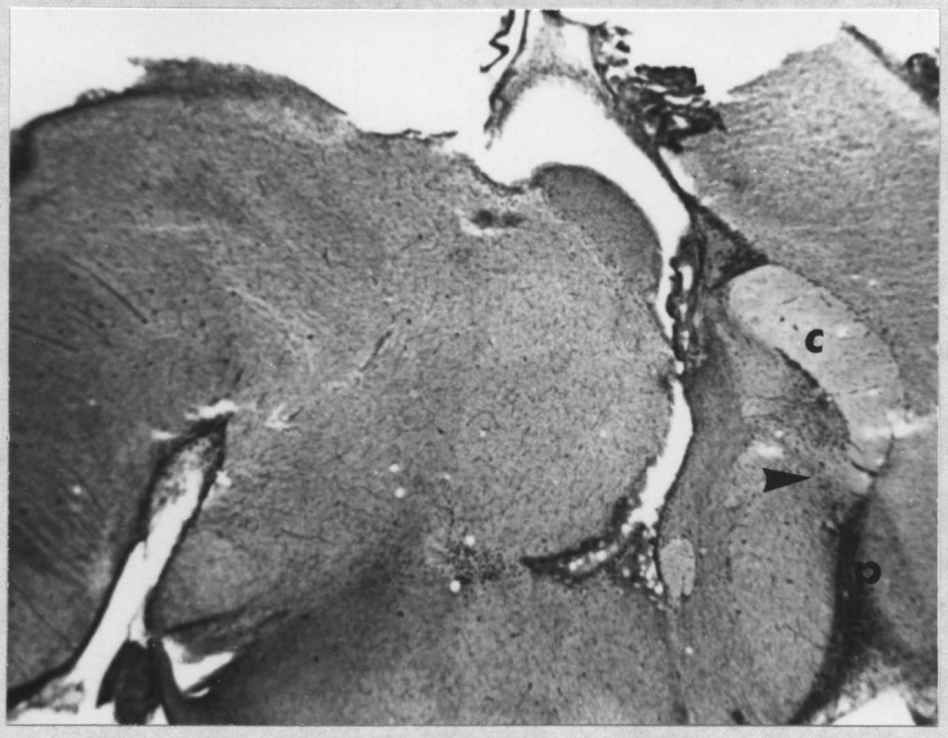

Figure 17

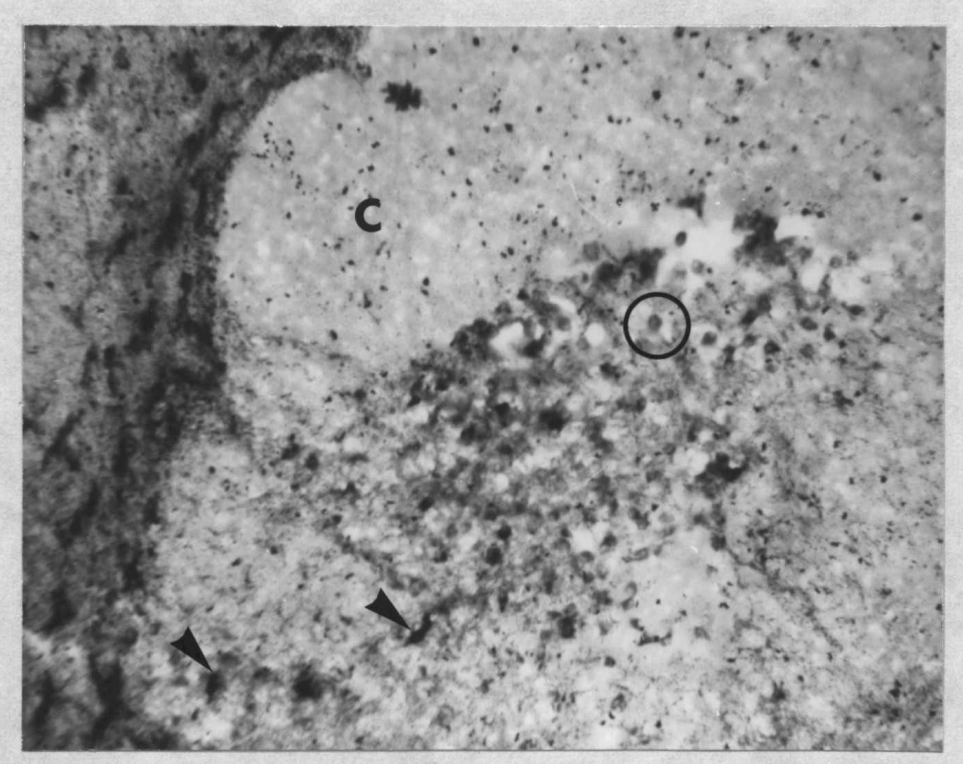

Figure 18

PLATE IX

PLATE X

Explanation of Figures

Figure 19. Six day old animal; nonspecific esterase. A para-sagittal section through the lateral ventricle (v) showing the corpus callosum (c) and the subcallosal layer of cells. The subcallosal layer of cells passes between the corpus callosum (c) and the lateral ventricle. The transverse fissure (t) passes between the cortex and the brain stem. 80X

Figure 20. Six day old animal; nonspecific esterase. A coronal section through the region of the genu (c) showing the lateral ventricle (L) with the subcallosal layer of mesodermal cells. Note that the layer of cells is continuous from lateral ventricle to pia (lower right hand corner) of the longitudinal fissure. 160X

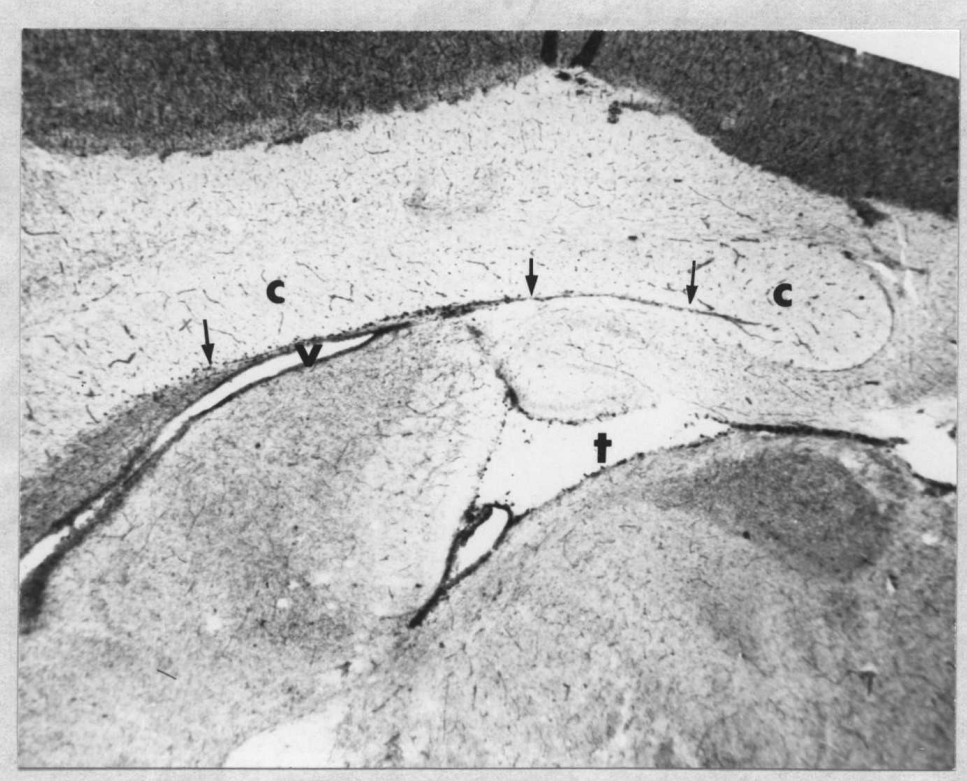

Figure 19

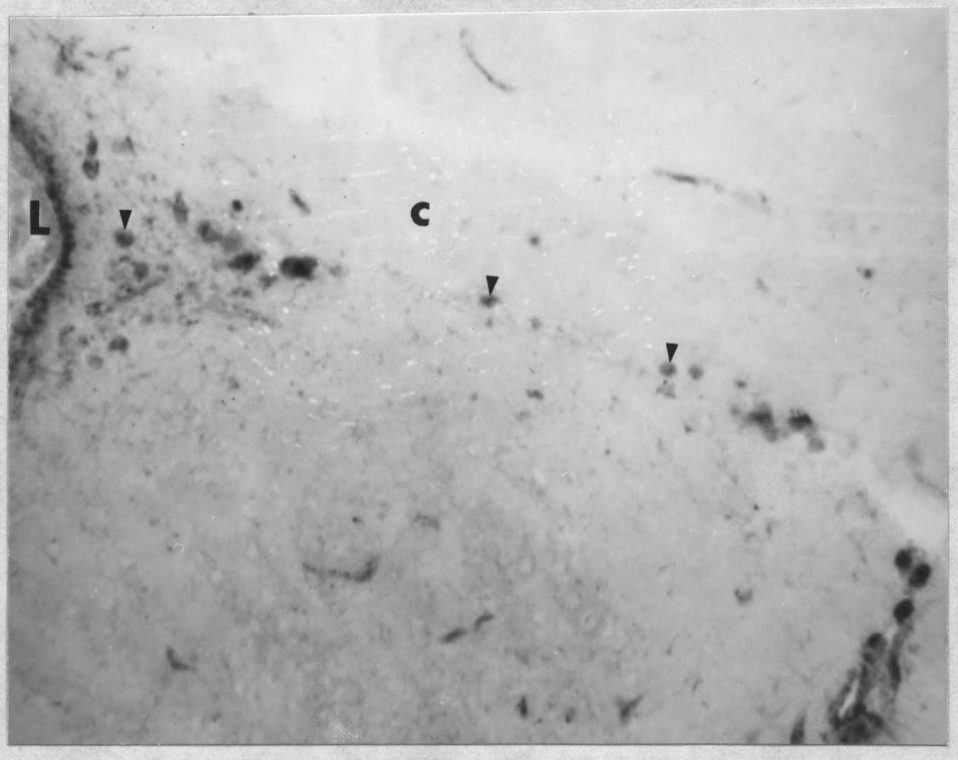

Figure 20

PLATE X

PLATE XI

Explanation of Figures

Figure 21. Eight day old animal; cresyl violet. Rounded-up cells which are seen in the subcallosal layer. Note the dark staining of their nuclei. 630X

Figure 22. Twelve day old animal; cresyl violet. Cell with dark nuclei which is found in the subcallosal layer. Two of these cells containing debris of erythroblasts. Some nuclear debris is seen in the cytoplasm.

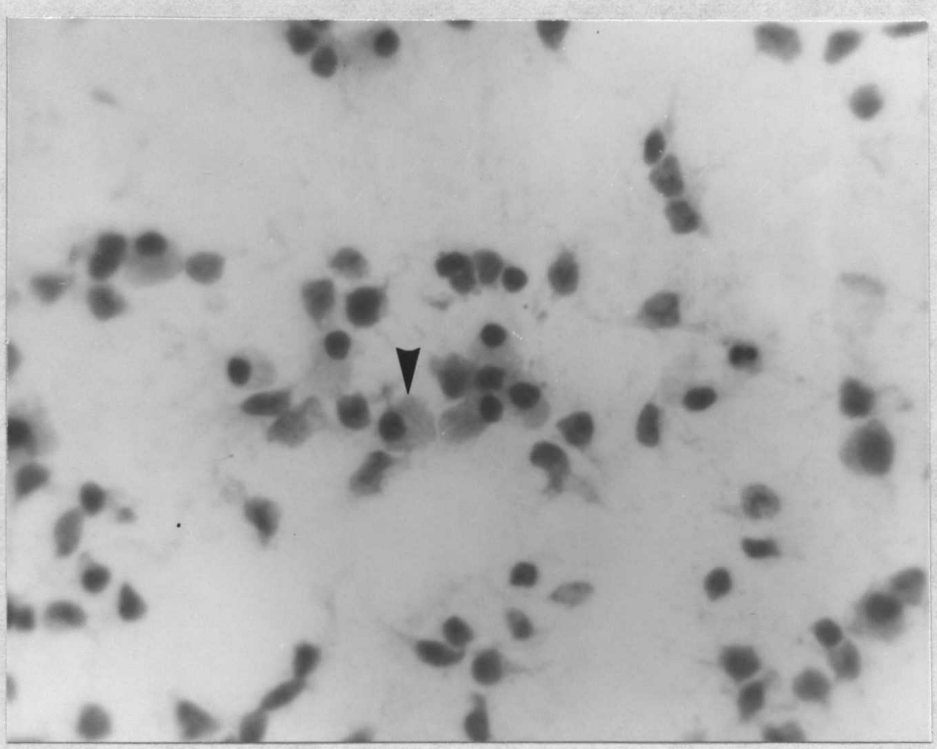

Figure 21

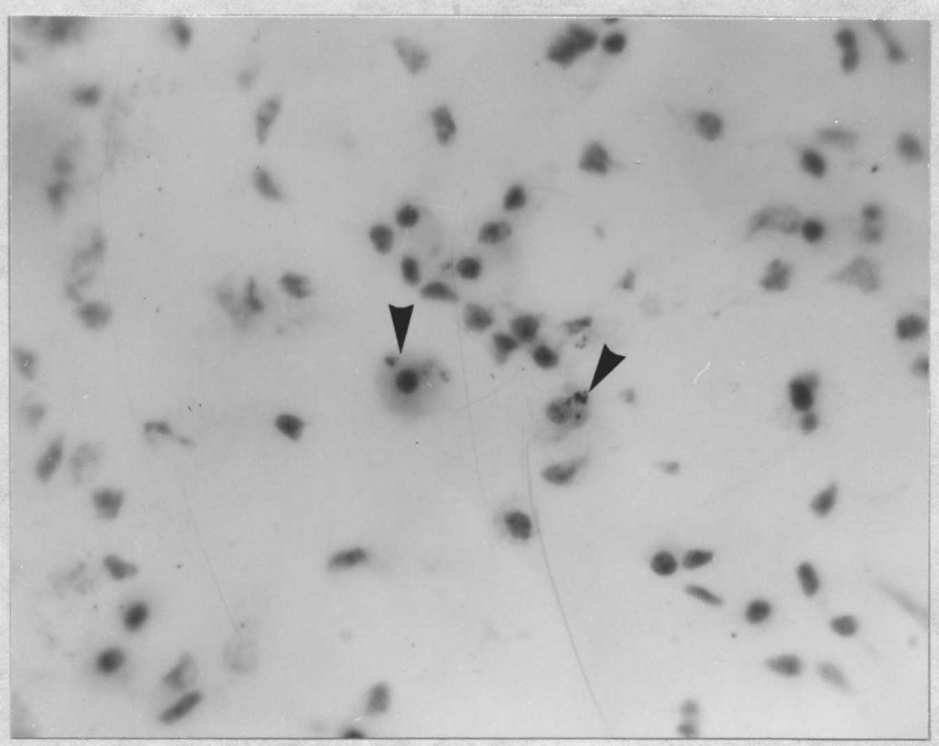

Figure 22

PLATE XI

PLATE XII

Explanation of Figures

Figure 23. Six day old animal; metalophil reaction. A coronal section through the corpus callosum (W) demonstrating a positive silver reaction in the layer of mesodermal cells which migrated into the area. (H) Mesodermal cell layer. Note the numerous maturing microglia which appear to be migrating out from the area (arrows). 160X

Figure 24. Six day old animal; nonspecific esterase reaction. Migration of the perivascular cells from the interpedunuclear fossa. Note how the meningeal cells appear to be migrating down on to the vessels as perivascular cells.

Figure 23

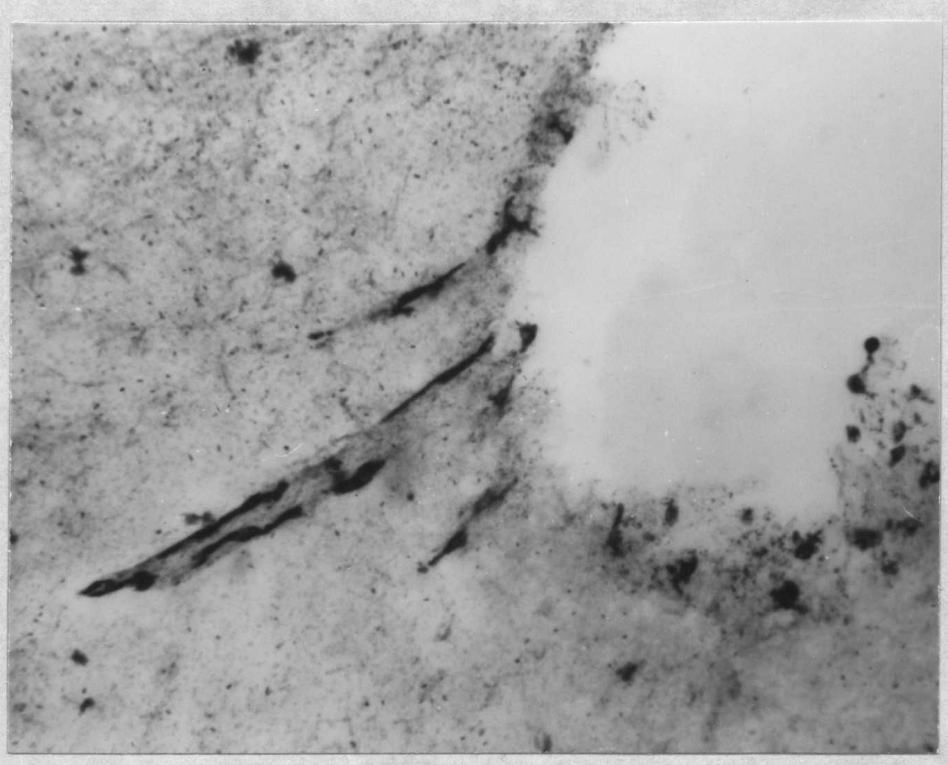

Figure 24

PLATE XII

PLATE XIII

Explanation of Figures

Figure 25. Eight day old animal; nonspecific esterase. Perivascular cells during migration along the vessels. Note that the migration has stopped when the diameter of the vessels has decreased. 190X

Figure 26. Eight day old animal; nonspecific esterase. Vessel penetrating from the interpeduncular fossa. Note the shapes of the perivascular cells. Lightly positive cells are seen on smaller vessels and between the vessel (arrows). 190X

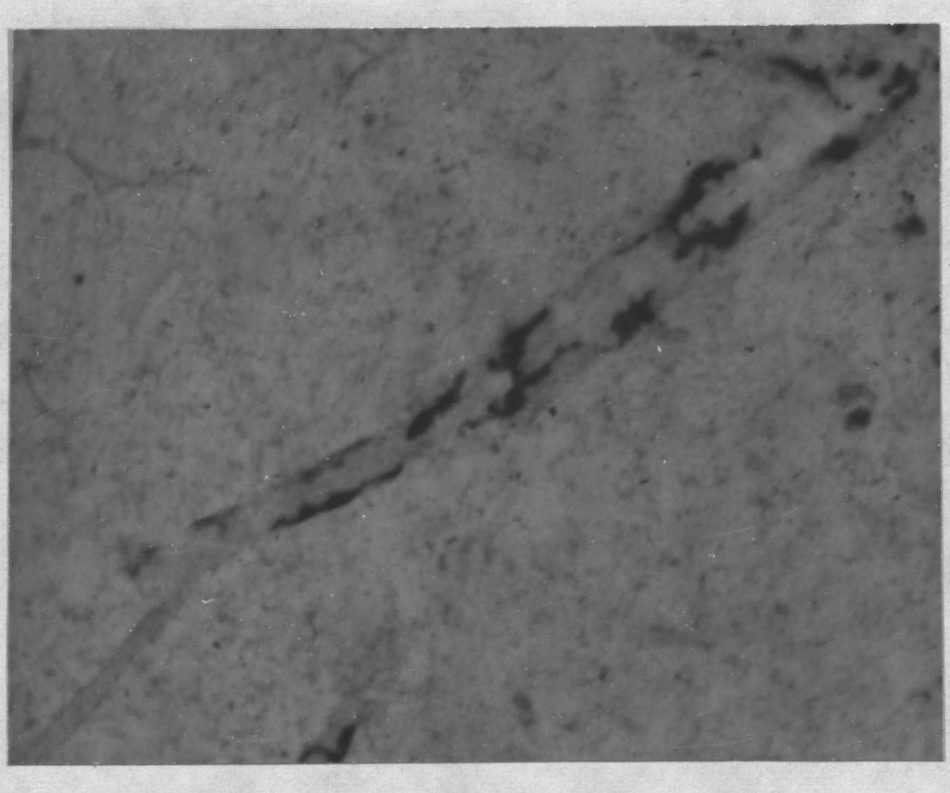

Figure 25

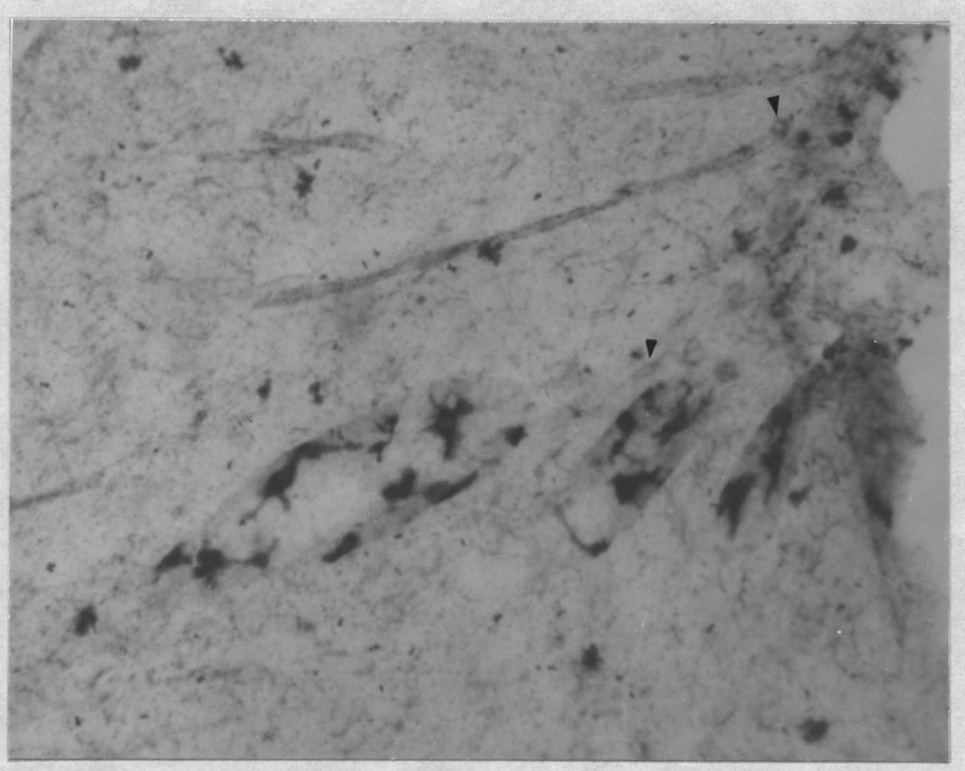

Figure 26

PLATE XIII

PLATE XIV

Explanation of Figures

Figure 27. Ten day old animal; nonspecific esterase. Oblique cut of the spongioblastic lamina. Note vessels with perivascular cells which are accompanying these processes. Note the rounded appearing cells between the spongioblastic elements. 190X

Figure 28. Ten day old animal; nonspecific esterase reaction. Spongioblast network cut parallel to the processes. Note that some cells in this area have processes like microglia. 160X

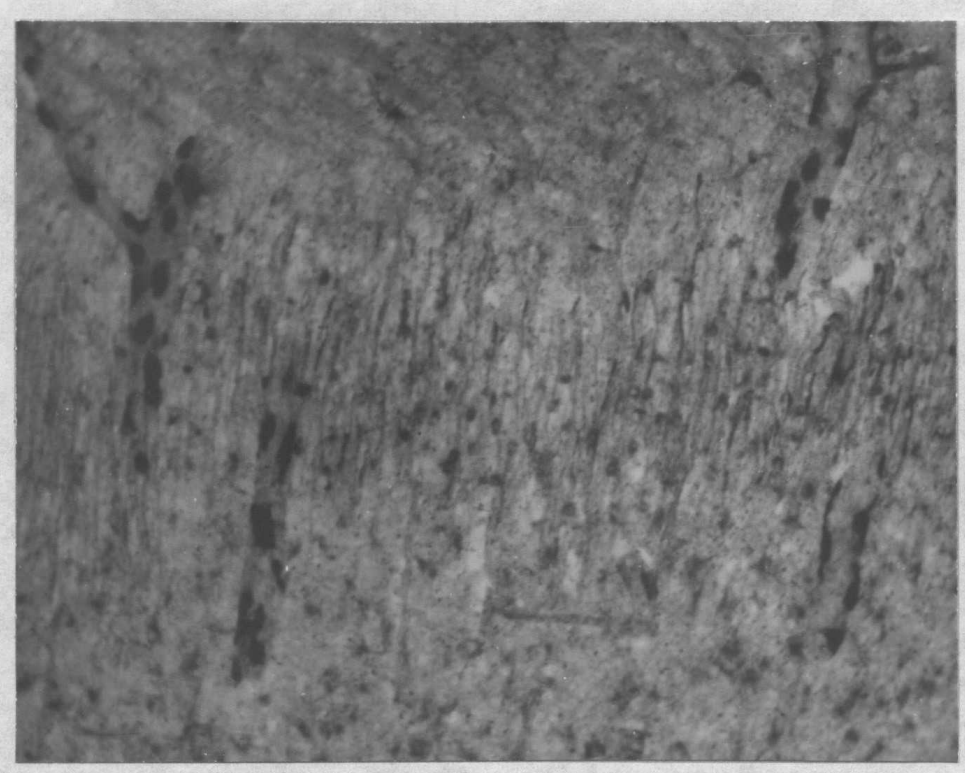

Figure 27

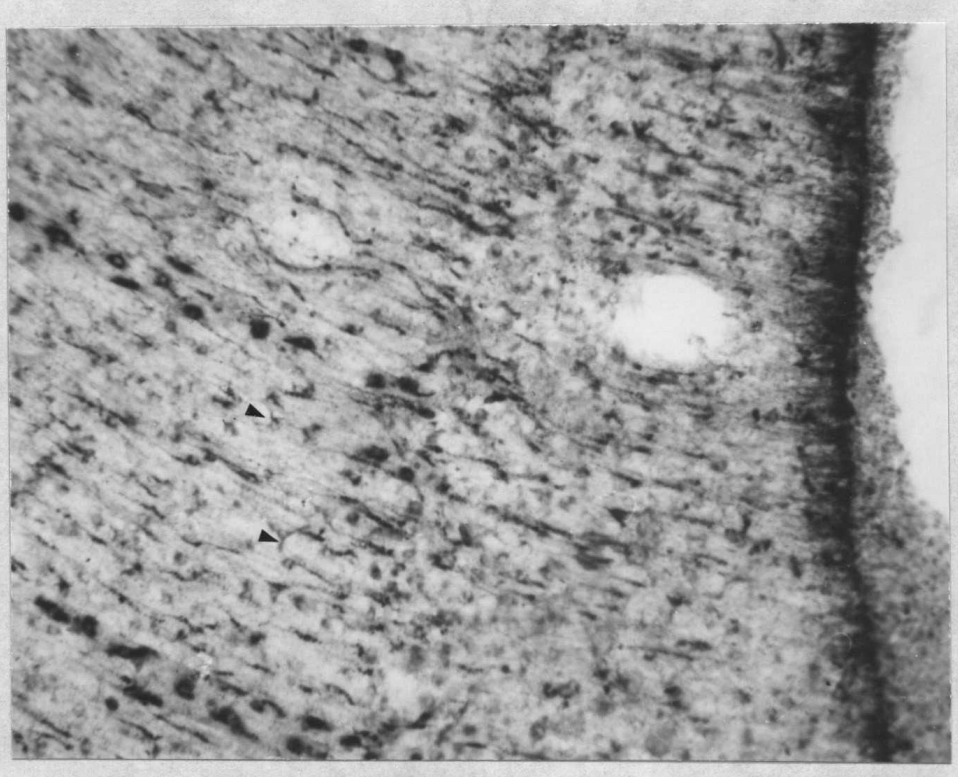

Figure 28

PLATE XIV

PLATE XV

Explanation of Figures

Figure 29. Eight day old animal; metalophil reaction. Section through the spongioblastic lamina showing very immature microglia. At this early stage few mature-appearing microglia are found. (M) Immature microglial cell, (V) vessel. 630X

Figure 30. Twelve day old animals; nonspecific esterase - metalophil reaction. Mature-appearing microglia are shown by the demonstration of their processes. These same cells have nonspecific esterase enzyme activity. The intense enzyme positive perivascular cells and those cells which appear to be immature microglia are not silver positive in this preparation. 160X

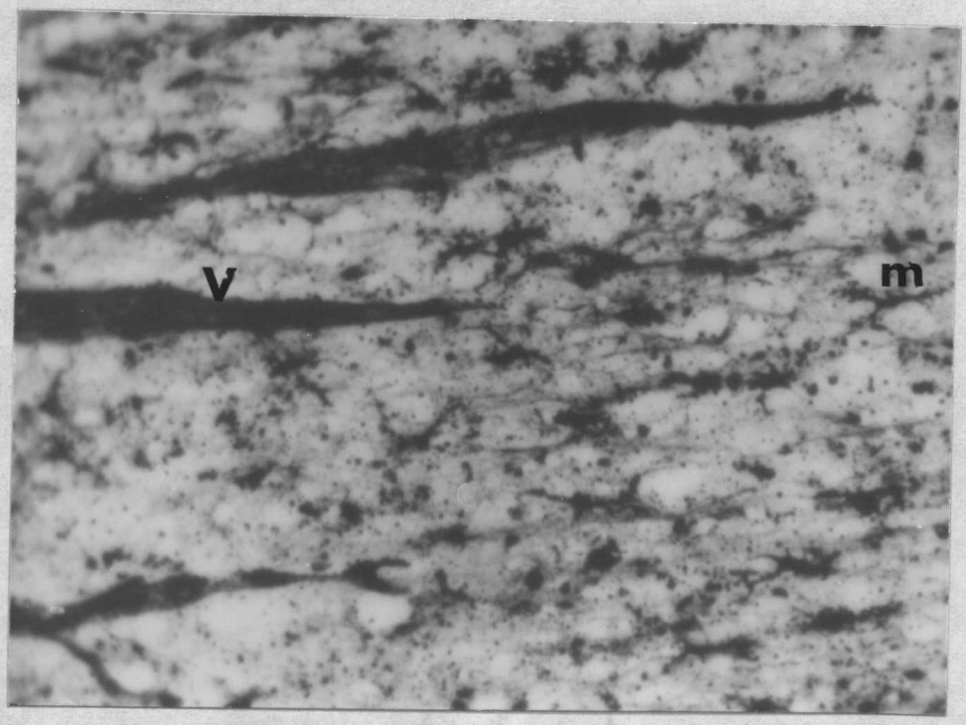

Figure 29

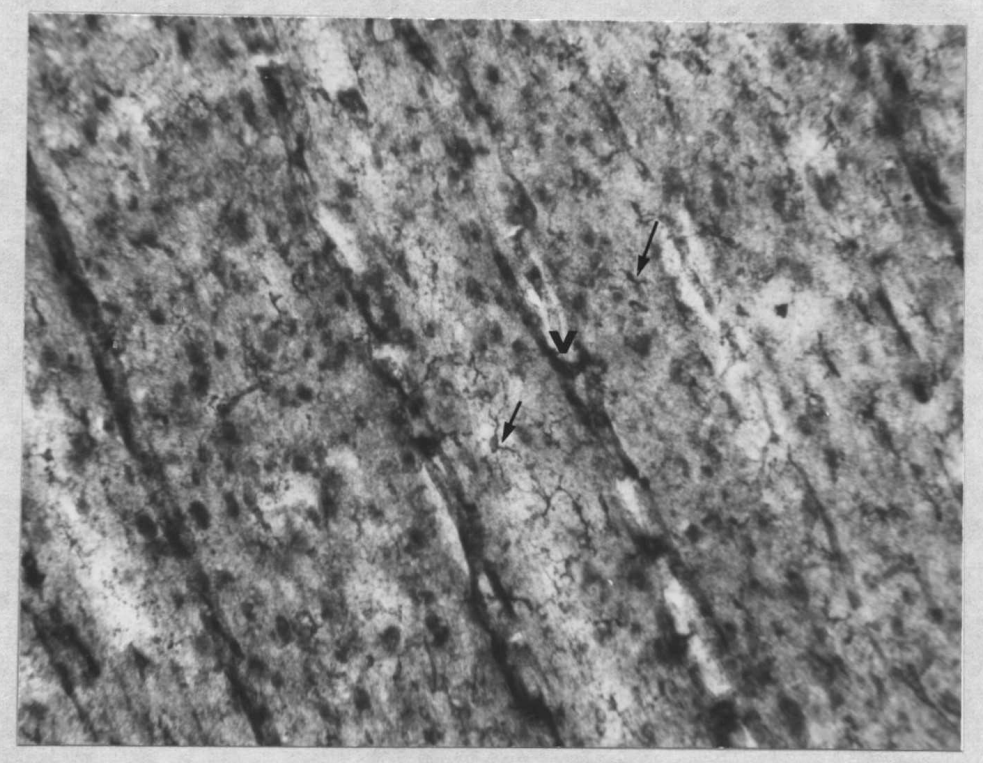

Figure 30

PLATE XV

PLATE XVI

Explanation of Figures

Figure 31. Six day old animal; nonspecific esterase. The direct migration of cells is noted from one vessel while the other is laden with perivascular cells. Note (*) perivascular cell still very positive.

Figure 32. White matter near an injury of 12 hours duration. Nonspecific esterase reaction. Note the numerous positive bodies streaming toward the wound area from the vessel area.

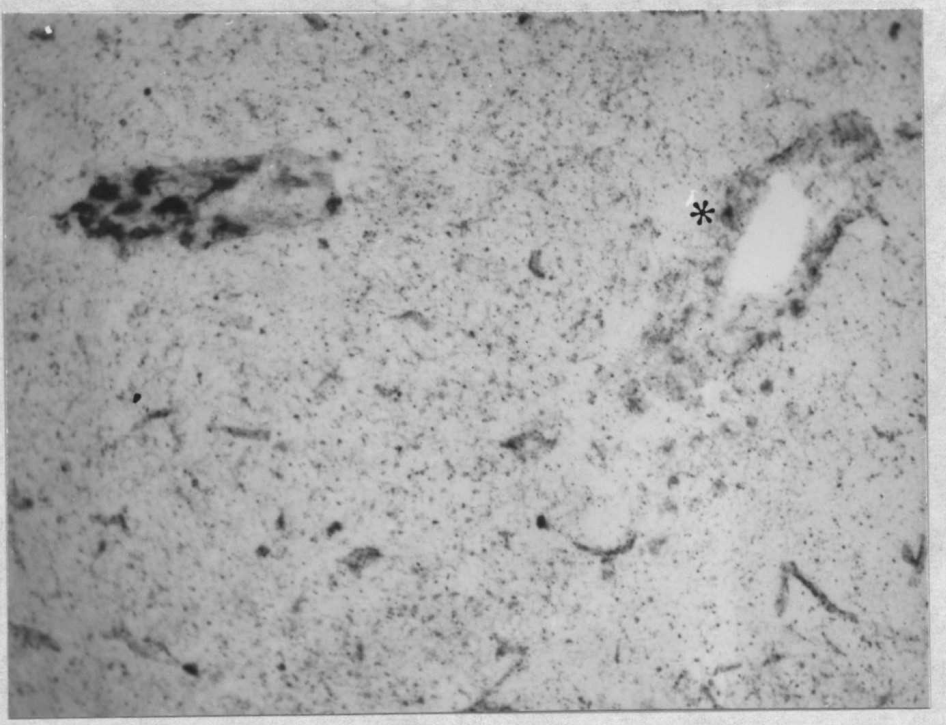

Figure 31

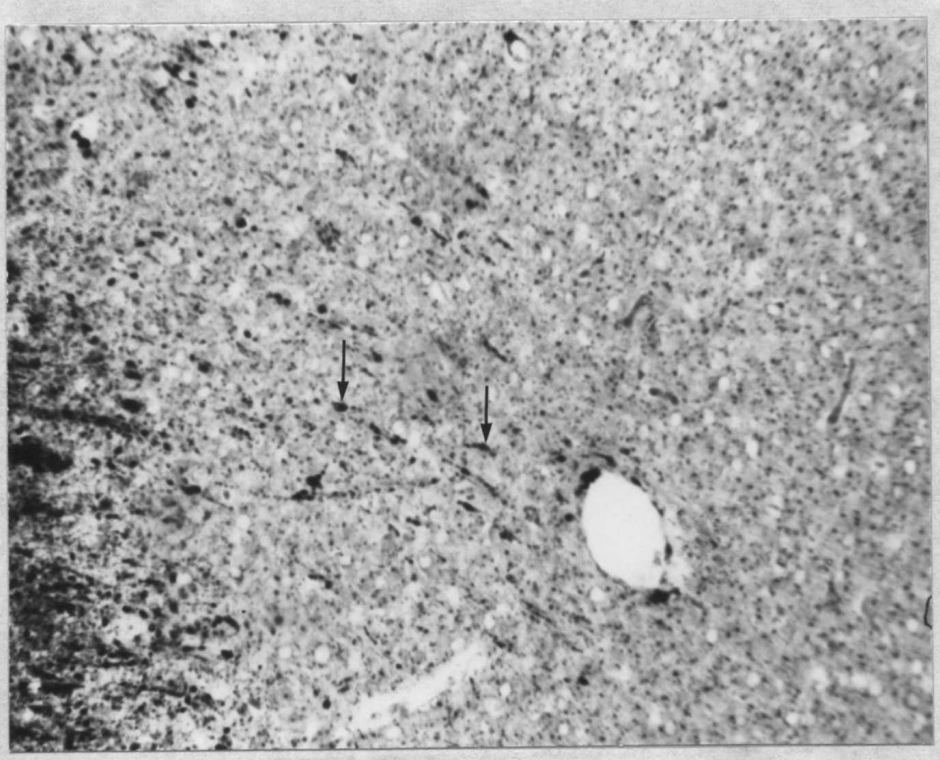

Figure 32

PLATE XVI

PLATE XVII

Explanation of Figures

Figure 33. Thirty-six hours following injury; cresyl violet. The wound near the surface of the brain showing numerous phagocytes which appear to arise from the pia. Note the numerous cells in and near the pia. (arrows) Phagocytes. 160X

Figure 34. Thirty-six hours following injury; azure-eosin. Blood vessel near the wound which contains many lymphocytes and monocytes. Near the vessel are a number of phagocytes. One has a round nucleus while the others have various shapes. 630X

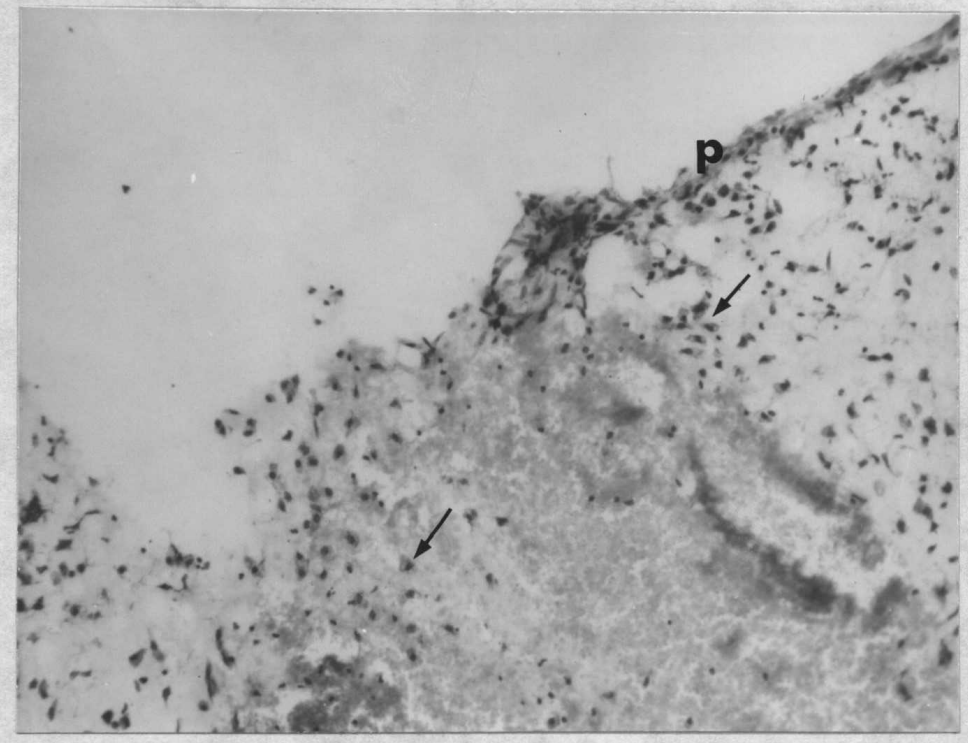

Figure 33

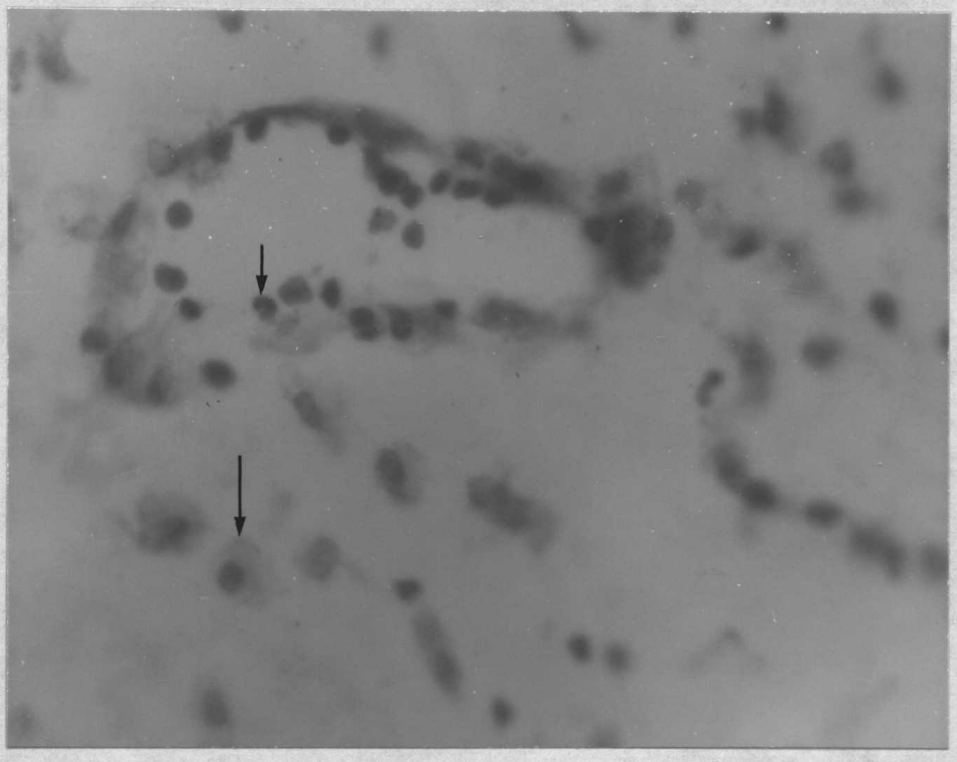

Figure 34

PLATE XVII

PLATE XVIII

Explanation of Figures

Figure 35. Thirty-six hours following injury; nonspecific esterase. The activity of phagocytes is very light at this early period. The cells with the large nuclear (arrows) areas are phagocytes like those seen in figure 33. In addition activity is seen in small cells which do not have a nuclear outline. These have been identified as PMN's. 160X

Figure 36. Thirty-six hours after injury; nonspecific esterase. Two vessels of the cerebral cortex which lie near the wound. The one has a normal distribution while the one close to the wound has an increased number of perivascular cells. 230X

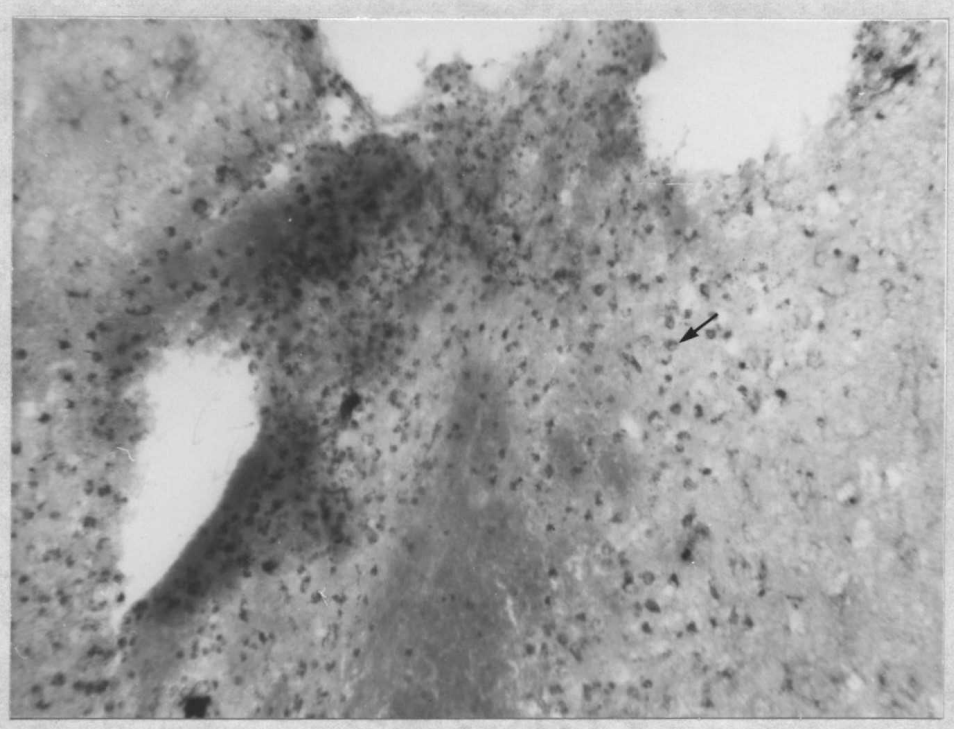

Figure 35

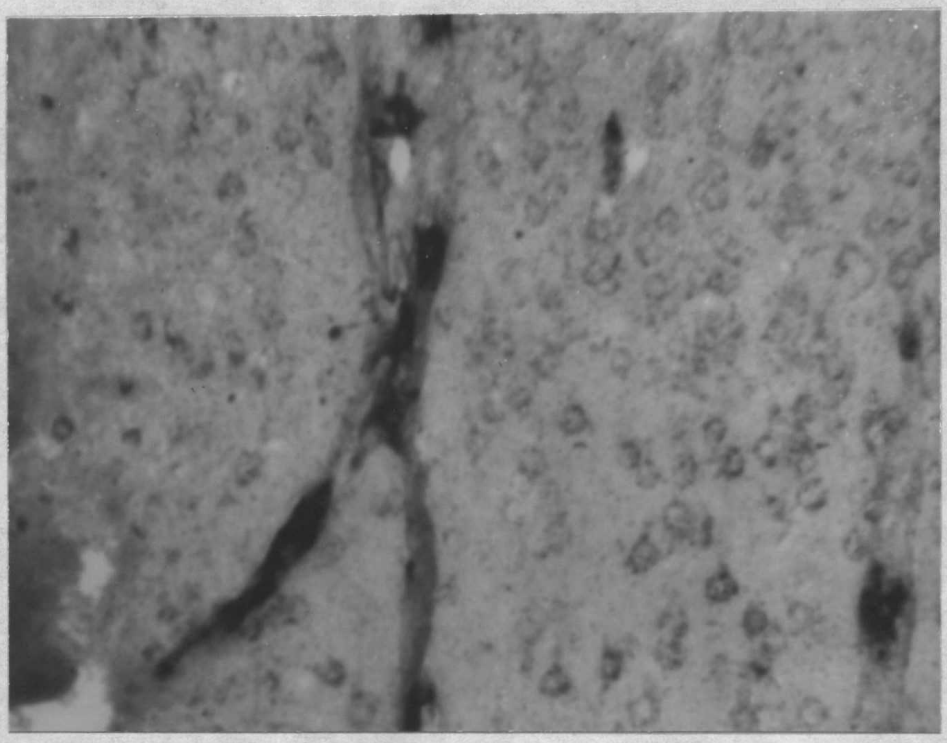

Figure 36

PLATE XVIII

PLATE XIX

Explanation of Figures

Figure 37. Thirty-six hours following injury; metalophil reaction. Only a few microglia are seen around the wound which show a reactive change (arrow). Other positive cells of the wound area correspond to phagocytes demonstrated in figures 33 and 35. 160X

Figure 38. Three days following injury; nonspecific esterase. The blood vessel in the injured area is heavily surrounded by enzyme reactive cells. 230X

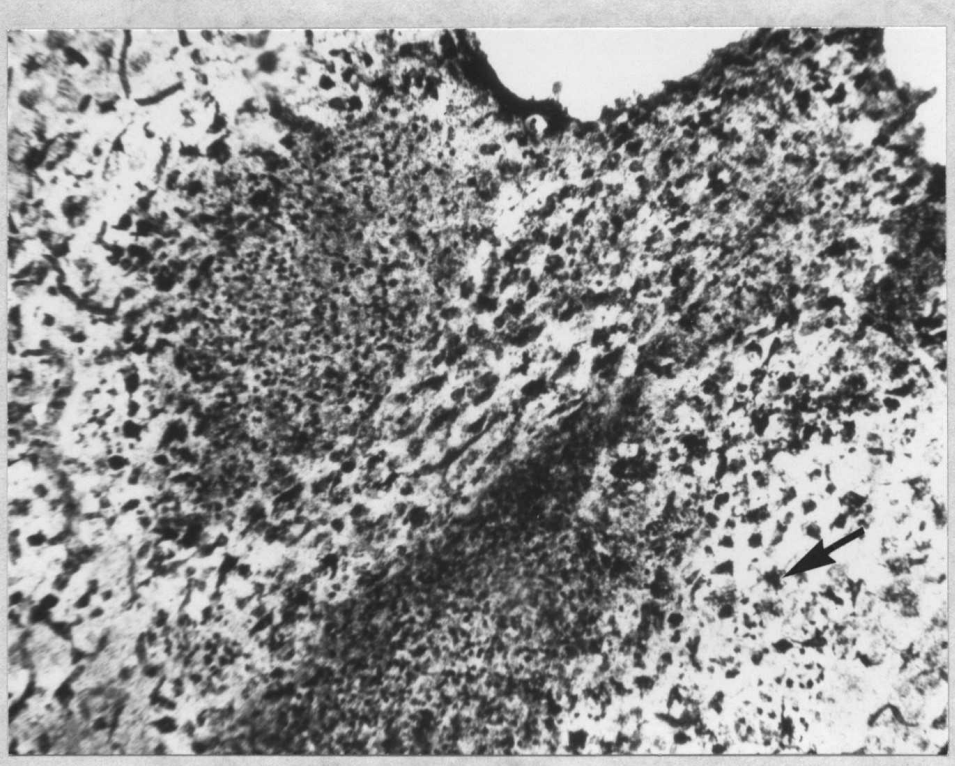

Figure 37

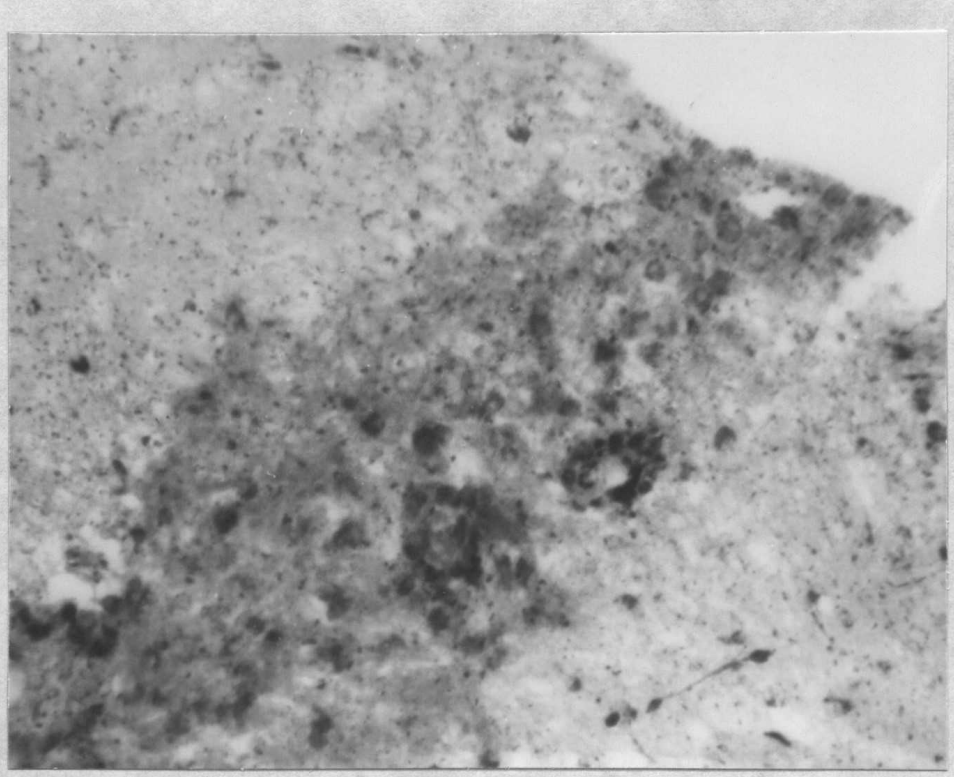

Figure 38

PLATE XIX

PLATE XX

Explanation of Figures

Figure 39. Three days following injury, metalophil reaction. Margin of the needle tract just above the corpus callosum (X). Positive rounded cells (Y) near the wound (p). Among these are reactive microglia (t). An area which has been heavily infiltrated by macrophages and is nearly cleaned up (R). Note the entire area is filled with reactive microglia and only a few round macrophages. 160X

Figure 40. Three days following injury; acid phosphatase. Area similar to that of figure 39. Round macrophages are very positive (Y). In the background numerous lightly reacting cells are seen which have various shapes from rounded cells with short processes to those with long processes (t). 160X

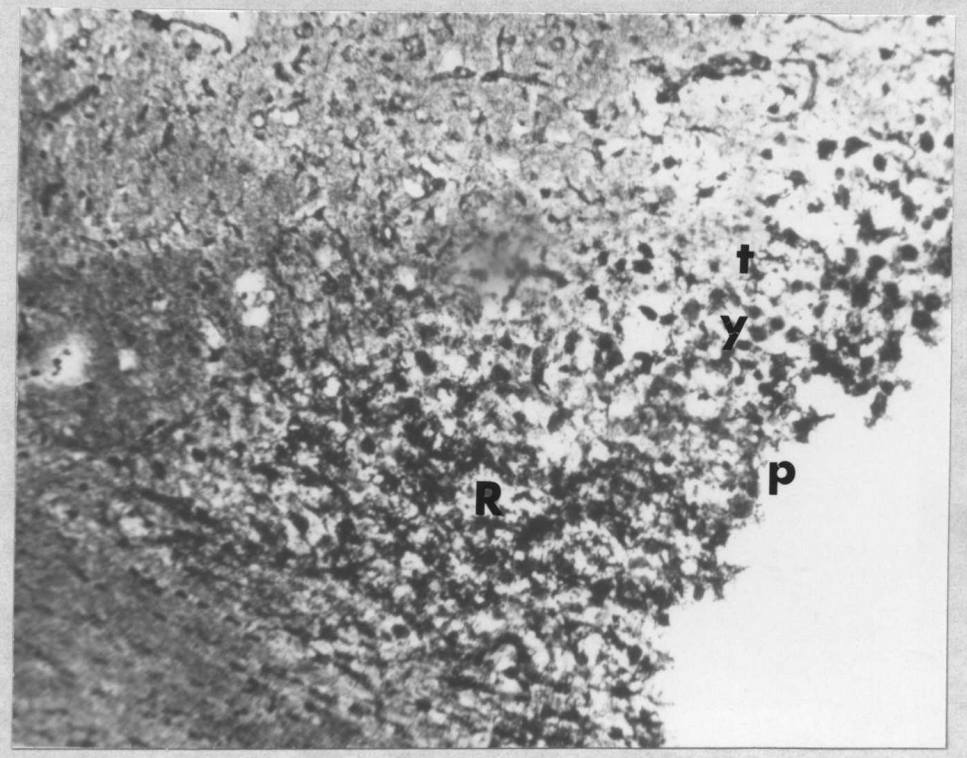

Figure 39

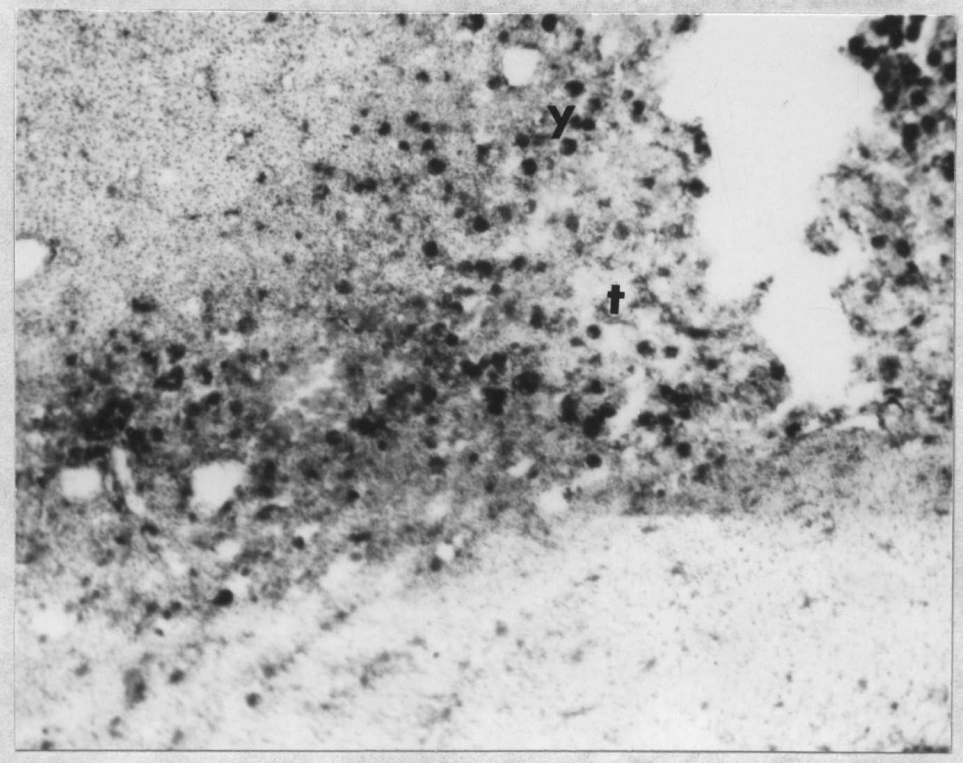

Figure 40

PLATE XX

PLATE XXI

Explanation of Figures

Figure 41. Six days following injury, nonspecific esterase. Dark reacting cells in the vessel wall which are probably both pericytes and blood cells. Small round cells which have migrated out into the tissue appear to be lymphocytes or very small monocytes. Note no nuclear outline can be seen even though these cells have little cytoplasm. This may be due to the fact that the section thickness does not allow cutting of the cytoplasm. (A) Open area, which was filled with blood cells. 230X

Figure 42. Six days following injury; nonspecific esterase. Many macrophages are in the wound and many are accumulated around blood vessels in the wound area which appear to be the source of these cells (B). Note the dark perivascular cells (P). Two types of cells appear to be coming from these vessels, dark reacting cells and light reacting cells. Although there may be no difference in these cells, it is possible that the dark ones are from perivascular cells as they have the same staining as the perivascular cells while the light ones are from blood cells. This differentiation in staining is especially prominent after 2 days, although, this light pink staining is seen in the cells described in fig. 31 at 12 hours (PMN's). This differentiation is not seen in the acid phosphatase reaction. 160X

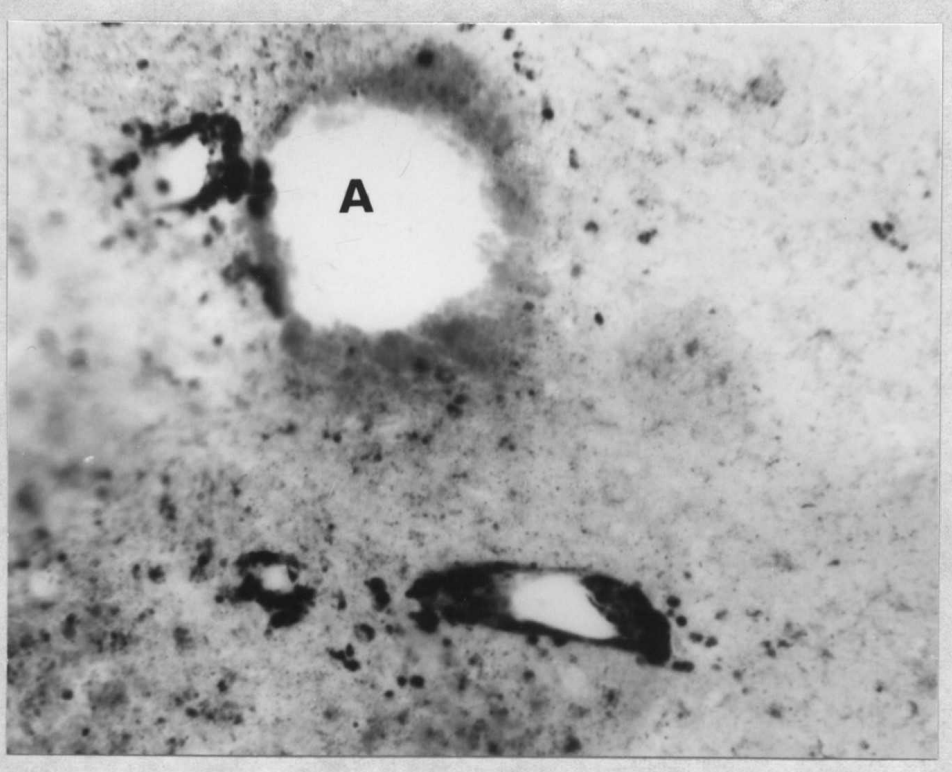

Figure 41

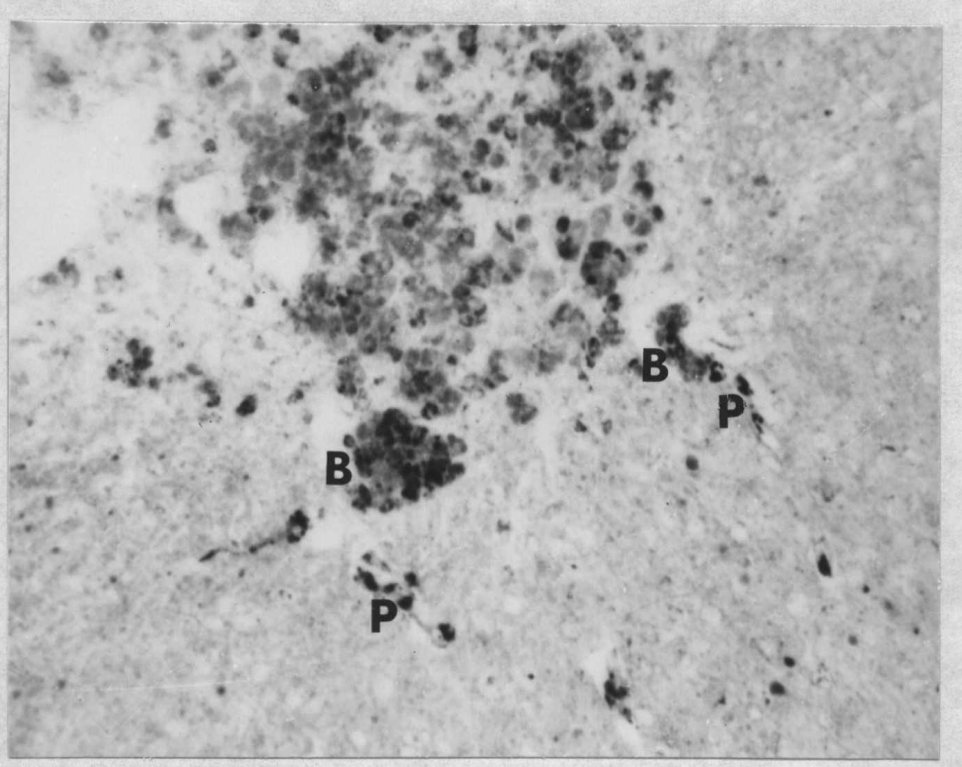

Figure 42

PLATE XXI

PLATE XXII

Explanation of Figures

Figure 43. and Figure 44. Six days following injury; nonspecific esterase (fig. 43) followed by the silver reaction in figure 44. Note the enzyme activity and silver staining of the marked area. Enzyme activity is variable in cells which appear as reactive microglia. 190X

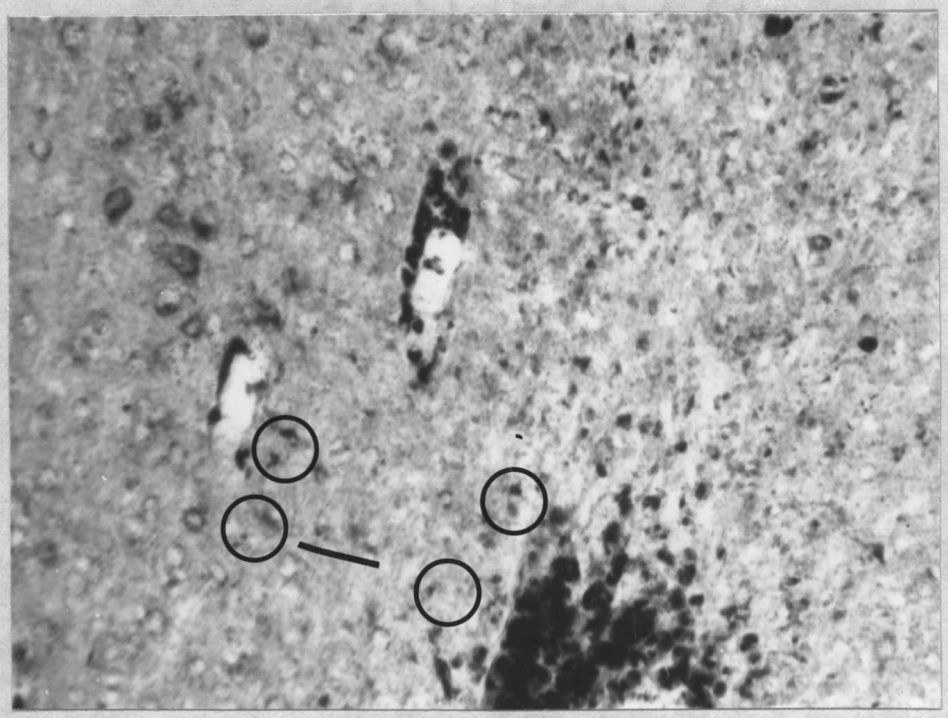

Figure 43

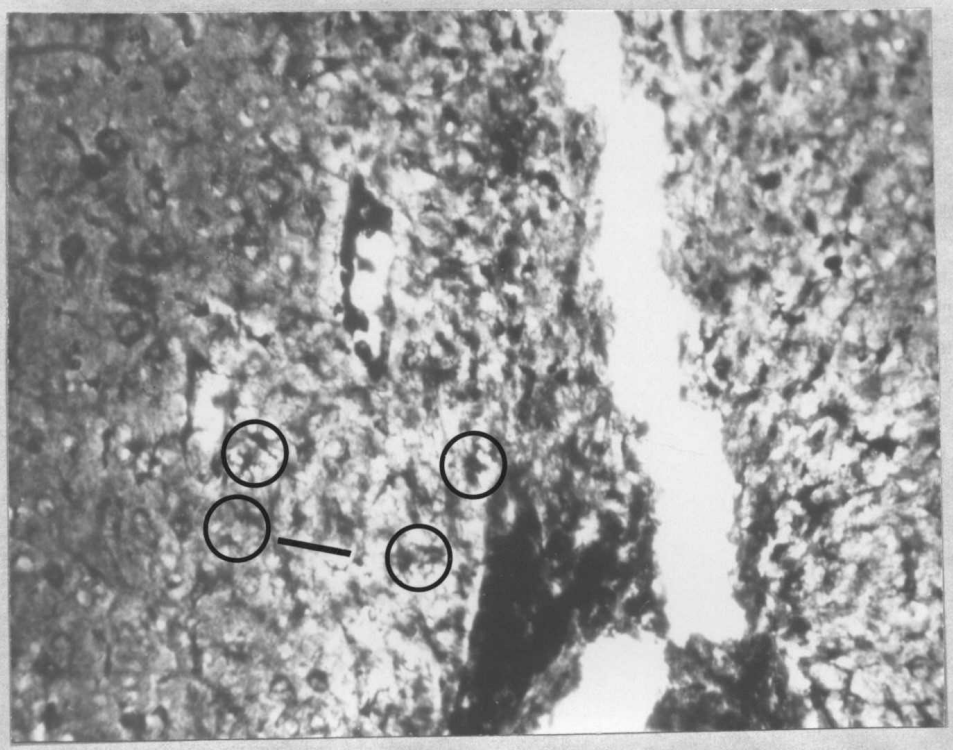

Figure 44

PLATE XXII

PLATE XXIII

Figure 45. Twenty day old animal; acid phosphatase (fig. 45) and followed by the metalophil reaction (fig. 46).
Figure 46. Compare the marked areas of the two photomicrographs. Note reactive microglia have small areas of activity within their cytoplasm. 230X

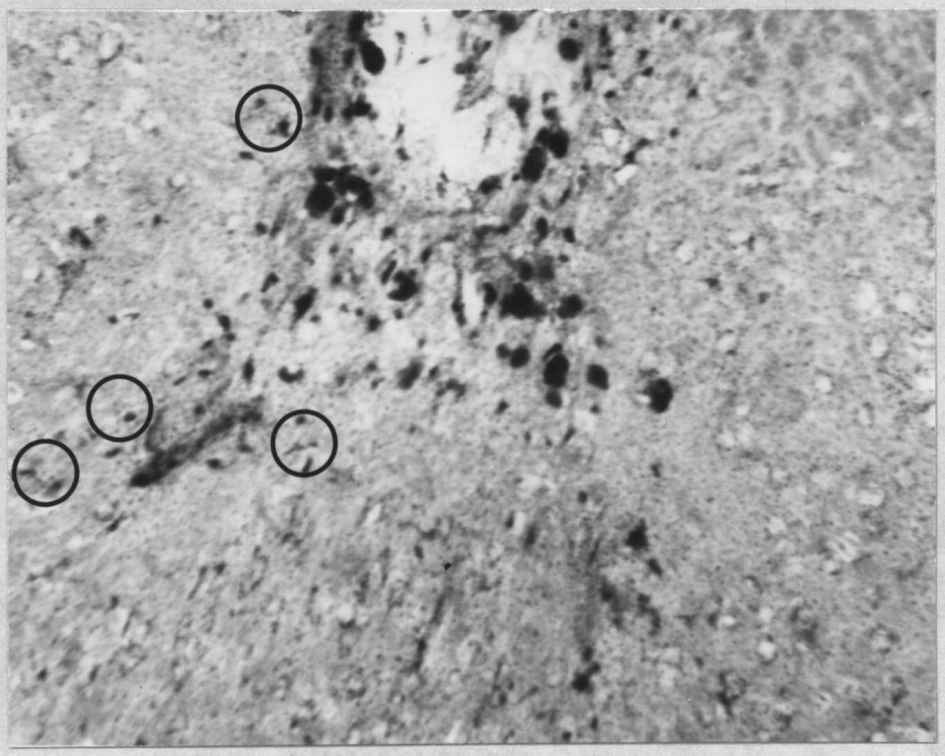

Figure 45

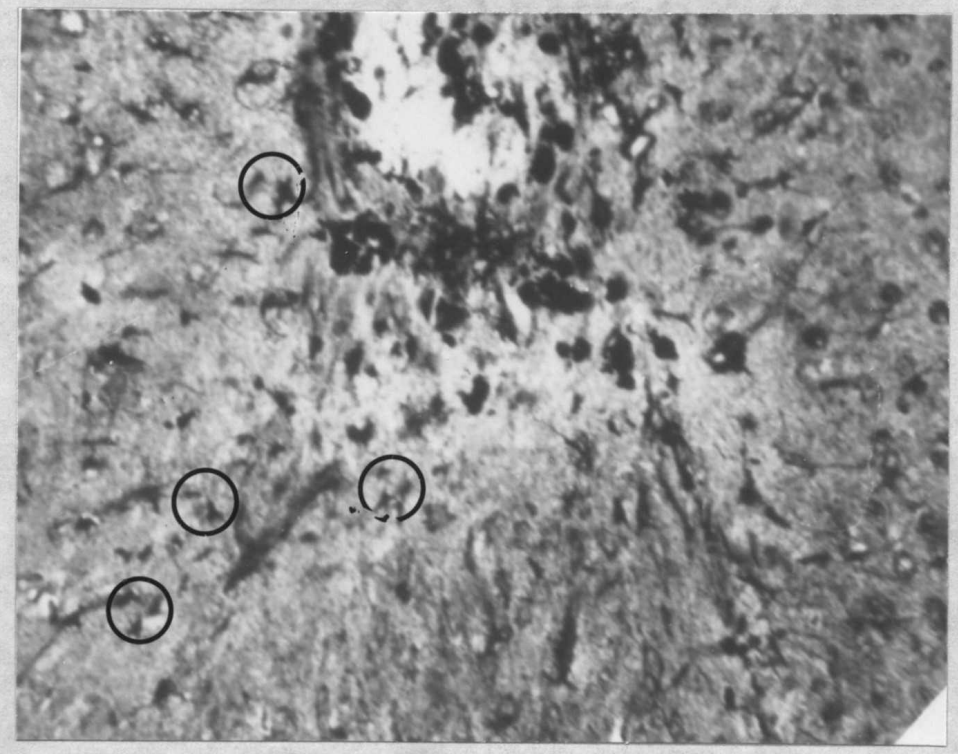

Figure 46

PLATE XXIII

A STUDY OF THE MESODERMAL ELEMENTS IN THE DEVELOPING, ADULT AND INJURED RAT BRAIN

Dean E. Hillman, B. S., M. S., Ph. D.

The dissertation here abstracted was written under the direction of John J. Taylor and approved by Vernon L. Yeager, Theodore Snook, Wilber F. Potter and Charles Hatfield as members of the examining committee, of which Dr. Taylor was Chairman.

Microglia have been related to the reticuloendothelial (R-E) system by previous workers on the basis of their morphological characteristics, ability to react with silver, apparent mesodermal origin and reaction to injury by becoming phagocytic. Microglia were believed to originate from cells in the meninges, however, this relationship could not be demonstrated with the silver reaction and was based on migration sites which were present near the surface of the brain. More recently, autoradiographic studies have demonstrated that cells from the blood form at least 65% of the phagocytes encountered following brain injury. This finding and the occurrence of a characteristic indented nucleus in gitter cells as demonstrated by electron microscopy has led at least one investigator to speculate that microglia may arise from monocytes which migrate into the brain. In addition, the finding that blood cells are largely involved in the

phagocytic process during central nervous system injury raises some questions in regard to the role of microglia during injury.

The enzymes acid phosphatase and nonspecific esterase have recently been shown to be contained in R-E cells. These enzymes are reported to be particularly active in cells which contain phagocytized material. Although many studies have been made to demonstrate these two enzymes in cells of the central nervous system, the results of these studies are conflicting. Histochemical tests for these two enzymes during injury to the brain are incomplete, and are lacking for the developing central nervous system. The present study was designed to demonstrate the possible relations between cells of the meninges and migration fountains, cells involved in the reaction to injury, and the possible demonstration of enzyme activity in mature microglia.

In this study, the following animals were used -- rat fetuses from 15 to 20 days gestation, young rats from birth to 46 days, and normal and injured adults. Experimental injury was produced by a needle puncture through the cortex and into the corpus callosum. The animals were sacrificed at intervals from 12 hours to 40 days. All groups of animals were fixed by perfusion with formalin and entire fetal heads and brain slices were fixed further in formalin for 12-24 hours. Quenched tissues were cut in a cryostat and sections were floated on formalin or mounted on cover slips. The techniques for nonspecific esterase using Naphthol AS acetate, and acid phosphatase using Naphthol AS-BI phosphate, in

addition to Marshall's metalophil reaction were carried out on free-floating sections. The cover-slip mounted sections were stained with a Nissl technique and Lillie's asure-eosin stain.

The results show that two types of cells in the developing meninges were positive for the two enzymes techniques. Fusiform cells were found in the tissues of the meninges, and rounded-up forms were among the arachnoid trabeculae. In the nonspecific esterase reaction, a characteristic indented nucleus was outlined by the cytoplasmic reaction of these cells. The rounded-up cells appeared to develop from the fusiform cells which were especially prominent when the condensed meningeal area was breaking down to form the meningeal spaces between 15 and 17 days gestation.

With the Nissl stain, indented nuclei with varying densities of chromatin were readily identified in the meninges. Some of the cells with the most dense nuclei exhibited rounded-up shapes and had the smallest nuclei. A maturation of meningeal cells appeared to occur from undifferentiated mesenchymal cells. Pale-staining nuclei changed to large pale indented nuclei which in turn developed dense chromatin granules and also decreased in size. The nuclei with the dense chromatin and characteristic indentation correspond to fusiform and rounded-up cells seen in the nonspecific esterase material.

During development from 17 days gestation to 6-12 days after birth, cells from the meninges migrate into the brain tissue. This

migration took place at sites where the pia-arachnoid comes into contact with the ependyma. Some areas have been considered because of their consistent demonstration of this migration over a specific time period. These areas are the taenia of the fourth ventricle, the anterior medullary velum, the transverse fissure, the lateral ventricle, and the longitudinal fissure and a subcallosal area. After entering the nervous tissue, these cells change from a fusiform shape to one with a rounded cell body from which processes project for short distances into the nervous tissue. With this change a distinct decrease in nonspecific esterase was noted over that seen in the fusiform cells of the meninges. These changed cells were found in the nervous tissue adjacent to the ependyma. In the case of the subcallosal migration, these cells spread laterally and antero-posteriorly beneath the corpus callosum in the area of the obliterated lateral ventricle and also lateral to the lateral ventricle. These cells accumulate in sites which are most prominent along the ependyma. The enzyme activity of the majority of these para-ependymal cells is distinctly increased 2 days after the onset of the migration. The majority of cells with the increased activity have lost their processes and have rounded-up. In the nonspecific esterase reaction the same characteristic indented nuclei can be found at all stages of migration. In the metalophil reaction the shapes of these cells correspond to those seen in the nonspecific esterase reaction. The Nissl stain demonstrates dark staining indented nuclei which have been described in the meninges. The complete

transition to mature microglia could only be studied by the use of several staining techniques, since mature cells lack the enzyme reaction.

Meningeal cells have also been related to the formation of intensely enzyme positive perivascular cells which are seen in the adult. Beginning in the brain stem at birth the nonspecific esterase reaction demonstrates perivascular cells which have the same intense reactions as the meningeal cells described. These cells are first seen on the large penetrating blood vessels on the ventral surface of the brain. Later (about 4-6 days), they are found on the large vessels for a considerable distance into the nervous tissue. By 8-12 days the walls of the large vessels contain these cells and at 35 days they are seen on all vessels larger than capillaries. These enzymatically active perivascular cells do not appear in the cerebral cortex until 10-12 days post partum. The appearance of these perivascular cells corresponds to an increase in vessel diameter and also to an increase in the thickness of the basement membrane of cerebral capillaries which is described in the literature.

A migration of enzyme positive perivascular cells appears to take place from the blood vessels into the nervous tissue. This apparent migration can be found at sites along most blood vessels. The majority of these migrating cells have an indented nucleus and also immature and mature appearing microglial cells can be identified with the silver technique. Cells containing nonspecific esterase are seen which have little perinuclear cytoplasm but which have long processes. In preparations

which have been reacted for nonspecific esterase followed by the silver reaction, these cells demonstrate typical microglial processes. These cells appear as the most mature microglial cells seen during this period of development.

In the injured animals, the techniques used demonstrated a migration of phagocytic cells from the meninges into the wound by 36 hours. Also, phagocytes were scattered throughout the deeper part of the wound. At this time, reactive microglia were only occasionally present in the silver preparation. The nonspecific esterase technique demonstrated an increase in the number of perivascular cells very near the wound. At 3 days the vessels of the area demonstrated a distinct cuffing with intense enzyme positive cells which also appear to be streaming into the injured site. Many rounded phagocytes, also intensely positive, are found in the wound. Among the rounded phagocytes of the wound other cells which have shapes similar to reactive microglia are found but which have slightly less enzyme activity. In the metalophil reaction cells are found which appear as reactive microglia and correspond to the same cells seen in the enzyme preparations. This relationship has been demonstrated by doing the metalophil reaction on cells which had previously been treated with one of the enzyme techniques. Later in the repair process, the rounded phagocytes disappeared except for those cells which had phagocytized erythrocytes. At this stage, the reactive microglia are seen within and around the wound. In the enzyme-metalophil preparations, these cells

demonstrate small areas of activity which appeared as a reaction in the perinuclear region. At still later stages the enzyme activity within these reactive cells is decreased to a granule near the nucleus. Phagocytes which contained hemosiderin were found to lose enzyme activity late in the repair process.

It is concluded: 1) that at least some reactive microglia are formed from phagocytes which have originated from leucocytes and/or perivascular cells and 2) that at least some reactive microglia represent phagocytes which have developed processes and are digesting debris.