STUDIES ON THE BLOOD BRAIN BARRIER

I. Effects Produced by a Single Injection of Gram-negative Endotoxin on the Permeability of the Cerebral Vessels *

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The existence of a so-called "blood brain barrier" has been postulated since 1913, when Goldmann[1] demonstrated the peculiar impermeability of the central nervous system vasculature to vital dyes. He noted that trypan blue, injected intravenously, failed to stain the brain while other organs were deeply stained. It seemed apparent that the central nervous system possessed some mechanism for selective exchange of substances from the circulating blood to the nervous tissue. The anatomic location of this barrier has been difficult to establish. Walter,[2] in 1930, pointed out that the unique impermeability of the central nervous system vasculature must reside either in the vessel walls, their pial covering, or in the glial perivascular membrane. Recent studies[3,4] by electron microscopy indicate that the cytoplasmic foot processes of astrocytes are closely applied to the endothelium of capillaries of the central nervous system. This unusual relationship is currently considered to be an important factor in the blood brain barrier.

The functional integrity of the blood brain barrier is said to be maintained under conditions of anoxia, vasodilatation, and alterations in osmotic pressure and pH of the blood.[5] Certain toxic agents such as bile salts[6] or radio-opaque contrast media[7] have been demonstrated to produce a temporary alteration of the barrier so that passage of dye substances into the brain may occur.

In previous studies[8] it was shown that a single intravenous injection of gram-negative bacterial endotoxin in rabbits produced alterations in the intramural coronary arteries. These changes consisted of intimal and medial edema and vacuolization, and disruption of the endothelial lining. In other studies[9] it was shown that the administration of colloidal iron in conjunction with an intravenous injection of endotoxin resulted in the deposition of iron admixed with hyaline fibrinoid mate-

* These studies were supported by grants from the Minnesota Heart Association and the United States Public Health Service (H-2540, A-1536, and B-1688).
Received for publication, December 3, 1957.
† Part of this work was done during the tenure of a research fellowship from the Minnesota Pathological Society.
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rial in the walls of the coronary arteries. It was demonstrated also that a high molecular weight acid polymer, sodium polyanetholsulfonate (Liquoid), when given in conjunction with endotoxin, resulted in vascular lesions of the central nervous system. These lesions were characterized by the deposition of fibrinoid material in the capillaries of the choroid plexus and by the presence of thrombi in the cerebral vessels.[10]

These observations suggested that gram-negative bacterial endotoxin, if injected directly into the circulation of the central nervous system, might be associated with changes in the blood brain barrier leading to passage of colloidal materials through the capillaries and into the substance of the brain. We were also led to investigate the effects produced by the intravenous or intra-arterial administration of endotoxin upon the passage of other substances such as fluorescein into the substance of the central nervous system.

Materials and Methods

One hundred fifty-nine hybrid albino rabbits of both sexes weighing 1.0 to 1.5 kg. were used. They were fed Purina rabbit pellets and had free access to water. Of the total number, 97 were used in the studies in which trypan blue, Evans blue, or colloidal iron were given, and 62 were employed in the experiments in which fluorescein was administered. Thirty-three animals were used as controls in the experiments, and the remainder received either an intravenous or an intra-arterial injection of gram-negative endotoxin in conjunction with the administration of one or another of the above dyes.

In each instance, a single injection of 50 μg. of the lipopolysaccharide fraction of *Escherichia coli* in a volume of 1 ml. of sterile, pyrogen-free, isotonic saline solution was given. The fraction was generously supplied by Difco Laboratories, Detroit, Michigan. The intra-arterial injection of endotoxin was carried out by isolation and proximal ligation of one of the carotid arteries. Ligation was found to be necessary in order to prevent hemorrhage following administration of the toxin. The material was introduced directly into the carotid artery by means of a sterile syringe and a 20 or 23 gauge needle. Following withdrawal of the needle, the carotid artery was ligated distal to the site of administration. The intravenous injections of endotoxin were given into the marginal ear vein.

Following administration of the endotoxin, at intervals varying from 15 minutes to 12 hours, a single injection of 2 per cent trypan blue, 2 per cent Evans blue, saccharated iron oxide (Proferrin,® Merck Sharp & Dohme, containing 20 mg. available iron per ml.), or a combination of colloidal iron with one of the dyes, was given into the carotid artery

in 2 ml. amounts. Injection of these substances was made into the same artery as that used for the administration of endotoxin. Control animals were given 2 ml. of isotonic, pyrogen-free saline solution into the carotid artery which was ligated in the usual manner. This was followed by administration of the dyes at various time intervals. In each instance the carotid artery was then ligated distal to the site of the second injection. In the group of animals in which fluorescein was used, 2 ml. of a 2 per cent solution were injected into the marginal ear vein at intervals varying from 15 minutes to 24 hours after administration of toxin. Details of the time intervals and number of animals used in the various experiments are given in the text.

The animals died or were sacrificed by Nembutal® or ether anesthesia within two hours after injection of the dye. All animals given fluorescein were sacrificed 15 minutes after administration of the dye, and the brains were examined under ultraviolet light (Wood's light) for evidence of fluorescence. Necropsy examinations were performed on all animals, and the tissues were fixed in 10 per cent neutral formalin. Sections were prepared from the brain, heart, lung, liver, spleen, adrenal glands, skeletal muscle, and kidney. Staining was accomplished with hematoxylin and eosin, or eosin alone. In addition, many sections of the brains were stained by the periodic acid-Schiff method or by Perls's method for iron. In those animals which had received colloidal iron, gross evidence of staining was obtained by placing the brain in a mixture of potassium ferrocyanide and hydrochloric acid for 30 minutes.

Results

A violent reaction was observed commonly following injection of the dye substance in animals given endotoxin. This consisted of struggling, convulsive movements, and occasionally frank seizures accompanied by neck rigidity and arching of the back. The reactions were more violent in those animals whose brains subsequently showed gross evidence of staining. Marked pupillary constriction was observed almost invariably on the side of carotid injection. However, bilateral pupillary constriction was common in the animals whose brains revealed staining by the dye substance.

Staining of the skin, gums, and eyes was noted immediately following administration of the dye. The staining was limited commonly to the side of injection, the contralateral gums and skin remaining unstained. The entire head region occasionally stained diffusely, but this did not correlate well with staining of the brain. However, it was noted that extensive, diffuse staining of the brain was present only when both eyes stained equally well. A majority of the animals with diffuse

staining of the brain died within 5 to 15 minutes following administration of the dye. It was observed also that there appeared to be a markedly increased susceptibility to anesthesia, since a much lower dose of Nembutal® or ether proved to be lethal as compared with the control animals or those in which passage of dye into the brain did not occur.

In the animals receiving colloidal substances after intracarotid injection of endotoxin, the incidence of gross staining of the brain was

TABLE I

Incidence of Cerebral Lesions Following Intracarotid Arterial Administration of Endotoxin (50 μg.) and Colloid *

Interval between toxin and colloid	No. of animals	No. with gross staining of brains	Total (gross and microscopic)	Percentage stained
Controls (no toxin)	21	1	2	10%
15 minutes	4	0	1	25%
1 hour	8	1	4	50%
2 hours	12	4	6	50%
3 hours	8	4	7	87%
4 hours	20	12	13	65%
6 hours	12	7	8	66%
8 hours	8	4	5	63%
12 hours	4	0	1	25%

* Evans blue, trypan blue, colloidal iron, or combination of colloidal iron with Evans blue or trypan blue (2 ml. volume).

greater when the dyes were given 3 to 8 hours following the administration of toxin (Table I). More regular, diffuse staining was observed when the intervals between toxin and dye were 3 to 6 hours (Figs. 2, 4 and 5). At shorter or longer intervals, the staining was often spotty or irregularly distributed, although occasionally one hemisphere was diffusely stained (Fig. 3).

In the rabbits receiving fluorescein (Table II), the incidence of fluorescence was greatest when the fluorescein was administered at intervals of 8 to 12 hours after an intravenous injection of endotoxin, or 2 to 4 hours after an intracarotid injection. In these, fluorescence was noted to be of high intensity and was diffuse (Fig. 1). In other animals in which fluorescence was detected, it was usually of low intensity and was limited in distribution to scattered areas or to one hemisphere.

Microscopic examination of the brains from the rabbits which had received colloidal substances disclosed the presence of these materials within the lumens and walls of blood vessels, and within the cytoplasm of phagocytes and neurons (Figs. 6 to 12).

In animals which died or were sacrificed within 15 minutes after administration of the colloid, it was observed that the dye was localized predominantly in vessels, lying within the lumens and in the blood vessel walls. With increasing intervals of time between dye injection and death, the dyes were observed to be spread diffusely through the

TABLE II

Incidence of Fluorescence of Brain Following Administration of Endotoxin (50 μg.) and Fluorescein *

Interval and method of administration of endotoxin	No. of animals	No. with fluorescence of brain	Percentage with fluorescence
Controls			
(fluorescein alone)	12	0	0%
1 hour			
Intravenous	4	0	0%
Intracarotid	4	2	50%
2 hours			
Intravenous	4	0	0%
Intracarotid	4	2	50%
4 hours			
Intravenous	4	0	0%
Intracarotid	7	3	43%
8 hours			
Intravenous	3	3	100%
12 hours			
Intravenous	8	8	100%
18 hours			
Intravenous	7	1	14%
24 hours			
Intravenous	5	1	20%

* 2 cc. of 2 per cent solution given intravenously.

substance of the brain (Figs. 8 and 9). Many of the ganglion cells were stained, as were the microglia and astrocytes.

Within the lumens of vessels, the dye appeared as homogeneous, blue masses (Figs. 10 to 12), but when present within the vessel walls (Fig. 11) it was commonly granular. In many instances the granules appeared to lie within the cytoplasm of endothelial cells, but this was difficult to evaluate. Alternate sections stained by the periodic acid-Schiff method showed intensely staining deposits in the same sites occupied by the colloidal materials. This substance was similar to the hyaline fibrinoid material described in other papers.[9,10]

DISCUSSION

The results of the present study indicate that the permeability of the central nervous system vasculature may be altered to allow passage

of circulating colloidal materials into the brain substance by an intracarotid injection of gram-negative bacterial endotoxin. It is also shown that soluble substances such as fluorescein may pass the blood brain barrier when given intravenously in conjunction with an intravenous or an intracarotid injection of endotoxin.

The functional breakdown of the blood brain barrier may be attributed in part to the morphologic vascular alterations represented by localization of particulate dye, admixed with hyaline fibrinoid material, within the vessel walls. These vascular lesions bear a striking similarity to those of the generalized Shwartzman phenomenon.[9]

Previous studies[9] have shown that the intravenous injection of colloidal materials and endotoxin may result in the deposition of fibrinoid admixed with colloidal particles in the walls of the coronary arteries. This was similar to the deposits observed in the cerebral vessels in these studies. Examination of other tissues showed identical fibrinoid material, admixed with colloidal substances, in the coronary arteries, splenic sinusoids, and renal glomerular capillaries. These observations emphasize the morphologic similarity between the lesions observed in the central nervous system and those which occur in other organs in the generalized Shwartzman phenomenon. Likewise, the interval between injections of toxin and dye (3 to 8 hours) required for maximal staining or fluorescence of the brain, corresponds to that required in the intravenous injections of endotoxin and colloidal substances which regularly produce the generalized Shwartzman phenomenon.

It should be emphasized, however, that the development of the Shwartzman phenomenon, as evidenced grossly by bilateral renal cortical necrosis, requires pretreatment of the animal with colloidal material, followed by an intravenous injection of endotoxin.[11] In this respect, the method used in the present study differs from that used to produce the Shwartzman phenomenon. Investigations now in progress, utilizing heparin and nitrogen mustard, may provide further information regarding this point, since these substances inhibit the development of the generalized Shwartzman reaction.[12]

The mechanisms involved in the production of the alterations of the blood brain barrier are not known at present, but several factors should be considered. These include an intermediary action of adrenal gland secretions, shock, vasospasm of the cerebral vessels, and active uptake of the dye particles by certain vascular and extravascular cellular elements.

An intermediary action produced by secretions from the adrenal medulla in response to endotoxin is suggested by the results of preliminary studies in which certain sympathomimetic amines have been

shown to produce alterations in the permeability of the cerebral vessels similar to those described with endotoxin.[13] These studies suggest also that hypotension is not of primary importance, since breakdown of the blood brain barrier can be effected by the use of vasopressor amines which produce elevations in blood pressure. It is possible, however, that sudden changes in blood pressure at the arteriolar level may be important in altering capillary permeability. It is also possible that vasospasm may play a role, but this does not appear to be the primary factor in view of the relatively long interval between injections of toxin and dye required for optimal staining or fluorescence of the brain.

It has been proposed[14] that endotoxin and colloidal materials such as trypan blue, Thorotrast or colloidal iron, may have similar actions in producing a functional "blockade" of the reticuloendothelial system. Our observations of the intracellular position of dye particles that passed into the brain substance, and the presence of particulate dye within the cytoplasm of the vascular endothelial cells, suggest that endotoxin may allow passage of materials into cells which are not normally considered to have phagocytic properties.

Although the mechanisms involved in the breakdown of the blood brain barrier are poorly understood at this time, the results of the present study emphasize again the importance of gram-negative bacterial endotoxin in the production of alterations in the functional or structural integrity of the vascular system. This action of endotoxin, in increasing the permeability of the cerebral vessels, may provide more information concerning the anatomic location of the blood brain barrier. It may help to clarify the pathogenesis of many poorly understood human disorders and aid in an evaluation of the sites of action and the effects produced on the central nervous system by certain drugs.

Summary and Conclusion

Hybrid albino rabbits received a single intravenous or intracarotid arterial injection of 50 μg. of gram-negative bacterial endotoxin. At varying time intervals thereafter, one group of animals received an intracarotid injection of 2 ml. of trypan blue, Evans blue, colloidal iron, or a combination of iron and one of the blue dyes. Another group received an intravenous injection of 2 ml. of 2 per cent fluorescein.

In those receiving endotoxin and one of the colloidal substances, approximately 60 per cent showed diffuse gross staining of the brains when the interval between injections of toxin and dye was 4 hours. The incidence of breakdown of the blood brain barrier, as manifested by gross staining of the brain, decreased to 13 per cent when the

interval between toxin and dye was only 1 hour, and to 0 per cent when the interval was 12 hours. In the group receiving fluorescein, the greatest incidence of fluorescence of the brain occurred when the interval between intravenous injections of toxin and dye was 8 to 12 hours.

Microscopic examination disclosed the presence of dye particles in the lumens and walls of the vessels, and in phagocytes and neurons. The vascular lesions were characterized by the presence of subendothelial deposits of hyaline fibrinoid material, similar to that which characterizes the lesions of the generalized Shwartzman phenomenon.

The morphologic similarity of these lesions to those which occur in other organs in the Shwartzman phenomenon is discussed. Although the mechanisms concerned in alterations in the permeability of the cerebral vessels are not known, preliminary studies suggest participation by secretions from the adrenal gland.

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[*Illustrations follow*]

LEGENDS FOR FIGURES *

FIG. 1. Gross photograph of brains from 2 animals which had received a 2 ml. intravenous injection of 2 per cent solution of fluorescein. Photographed under ultraviolet light. The specimen on the right is from an animal which received a single intravenous injection of 50 μg. of endotoxin, followed in 12 hours by an intravenous injection of fluorescein. Note diffuse fluorescence of brain. The specimen on the left is from a control animal which received only fluorescein. Note absence of fluorescence.

FIG. 2. Gross photograph of a brain from an animal which received an intracarotid injection of 50 μg. of endotoxin, followed in 3 hours by an intracarotid injection of 2 ml. of a mixture composed of equal parts of 2 per cent trypan blue and colloidal iron. Specimen stained in a mixture of potassium ferrocyanide and hydrochloric acid. There is diffuse, regular staining of the vessels and substance of the brain.

FIG. 3. Brain from another animal which received 50 μg. of endotoxin in the carotid artery, followed in 8 hours by an intracarotid injection of 2 ml. of a 2 per cent solution of Evans blue dye. The left cerebral hemisphere shows diffuse staining, while the remainder of the brain is unstained. Formalin fixation.

FIG. 4. Basilar surface of brain from a rabbit which received an injection of 50 μg. of endotoxin in the left carotid artery, followed in 4 hours by an intracarotid injection of 2 ml. of a mixture of equal parts of 2 per cent trypan blue and colloidal iron. Note extensive penetration of blue color into the cerebral substance. Animal sacrificed 2 hours following administration of the dye. The brain was stained by placing it in a mixture of potassium ferrocyanide and hydrochloric acid for 30 minutes.

FIG. 5. Superior surface of brain from another animal treated in a manner identical to that described in Figure 4, showing penetration of dye into the cerebral hemispheres.

* The photographs for Figures 1 to 12 were made by Mr. Henry Morris.

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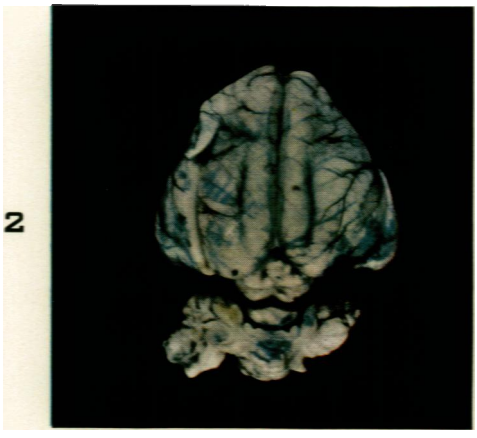

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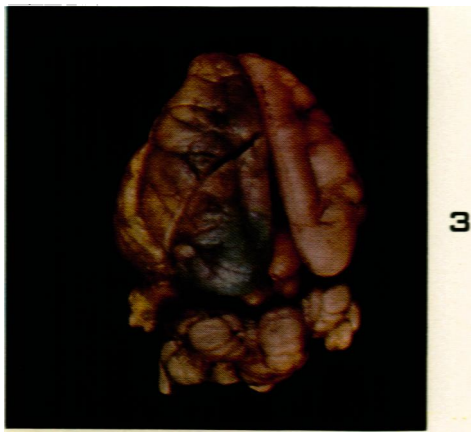

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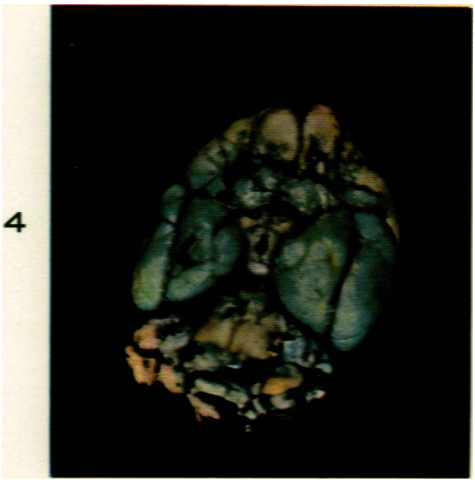

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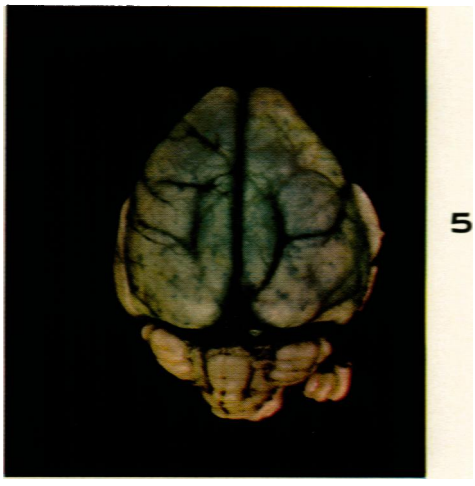

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FIG. 6. Microscopic section from the brain of a rabbit which received an intracarotid injection of 50 μg. of endotoxin, followed in 4 hours by an intracarotid injection of 2 ml. of a mixture of equal parts of colloidal iron and trypan blue. Sacrificed 2 hours after administration of the dye. Stained by Perls's method for iron, counterstained with hematoxylin and eosin. Numerous vessels containing iron-trypan blue both in their lumens and walls may be observed. Dye may also be observed in numerous phagocytic cells of the brain. × 125.

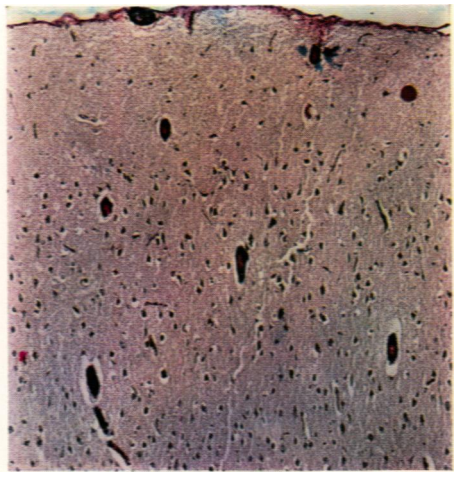
FIG. 7. Microscopic section from another animal treated in a fashion similar to that described in Figure 6. Sacrificed 15 minutes after administration of trypan blue-colloidal iron. Both the large and small vessels contain the blue dye material, but none appears to be within phagocytes. × 125.

FIGS. 8 and 9. Sections from the brains of 2 animals, each of which received an intracarotid injection of 50 μg. of endotoxin, followed in 4 hours by an intracarotid injection of 2 ml. of trypan blue. Sacrificed 2 hours following the injection of dye. Eosin stain only. Note diffuse blue background of brain. The photograph on the left shows 2 small vessels, both of which are intensely stained by the dye, and numerous phagocytic cells which contain dye. The photograph on the right is somewhat similar, but shows that numerous ganglion cells are also stained. × 125.

FIG. 10. Microscopic section from the brain of an animal which received 50 μg. of endotoxin in the left carotid artery, followed in 4 hours by an intracarotid injection of Evans blue dye. Sacrificed 15 minutes after the dye was administered. A large globule of dye is present within the lumen of the vessel. Hematoxylin and eosin stain. × 375.

FIG. 11. Section from the brain of a rabbit treated in a manner similar to that of the animal shown in Figure 10, but receiving 2 ml. of colloidal iron. Sacrificed 15 minutes after injection of the iron. Stained by Perls's method for iron. Note presence of globules of iron in the vessel lumen, and the granular particles in the wall of the vessel. × 375.

FIG. 12. Section from the brain of another animal which received an injection of 2 ml. of trypan blue in the left carotid artery 4 hours after an intracarotid injection of endotoxin. Sacrificed 15 minutes after the dye was introduced. Note presence of dye in the lumen and wall of the vessel. Eosin stain only. × 375.



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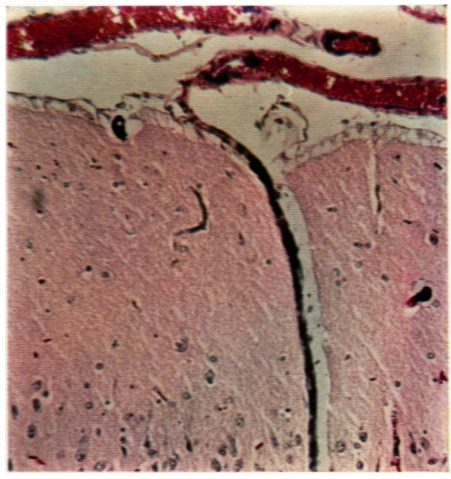

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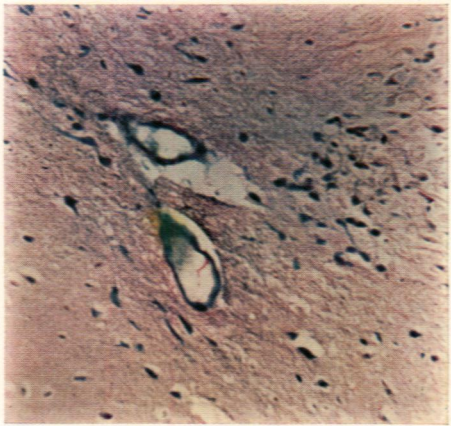

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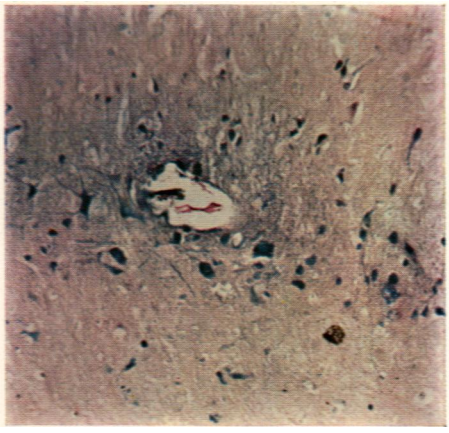

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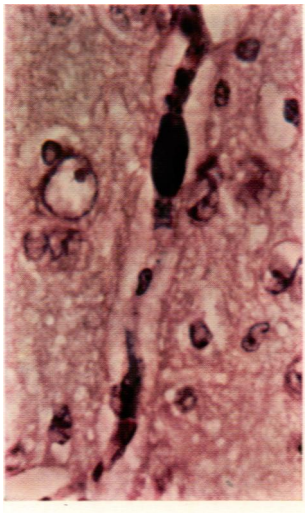

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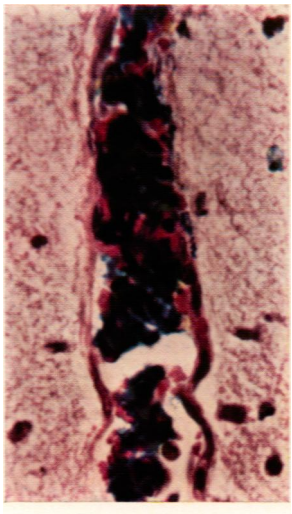

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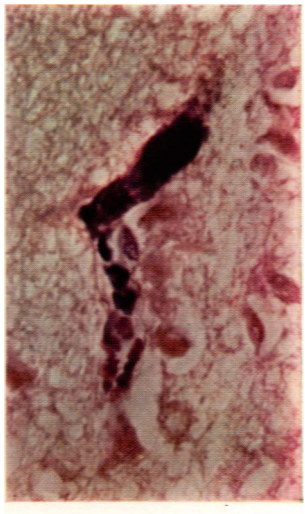

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