DEMYELINIZATION INDUCED IN LIVING RABBITS BY MEANS OF A LIPOLYTIC ENZYME PREPARATION*

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PLATES 15 TO 17

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Demyelinization characterizes the lesions of many forms of disseminated encephalomyelitis, notably of multiple sclerosis and Schilder's disease. To learn more about the pathogenesis of this disease process, attempts have been made to reproduce it by means of a lipolytic enzyme preparation injected intracerebrally and intravascularly into rabbits; additional studies have been made of the effects of the enzyme preparation on brain tissues *in vitro*.

Materials

A purified preparation of lipase, made from fresh hog pancreas by the Delta Chemical Works, was used. Each 100 mg. of the enzyme preparation in mixture with 10 cc. of olive oil produced enough fatty acid upon incubation during 2 hours at 37°C. to neutralize approximately 30 cc. of 0.1 N NaOH solution; purified trypsin (Difco) and chymotrypsin (Worthington Biochemical Laboratory) were used for control purposes. These products were not lipolytic when tested as described above, but on tests with azocoll (1), showed proteolytic activity that was markedly greater than that manifested by the lipase preparation in comparative tests.

Effects of the Lipolytic Enzyme Preparation Injected Intracerebrally in Rabbits

Suspensions of 5 to 8 mg. of the enzyme preparation in either 0.125 or 0.25 cc. of 0.9 per cent saline were injected intracerebrally in 15 adult albino rabbits weighing 2.5 to 3.5 kilos. Single injections were made into the region of the right basal ganglion and thalamus through a 20 gauge needle, with the rabbits under ether anesthesia. Three animals died immediately following injection and 4 developed convulsions and died after a latent period of 3 to 8 hours; 7 were killed with ether after intervals of 2 to 10 days, while the remaining animal was sacrificed after 6 months. The brains of all rabbits were fixed in 10 per cent formalin or formal-bromide solution and sectioned. Tissues were stained by the Weil Loyez, sudan III, Masson trichrome, Hortega silver carbonate, and Cajal methods.

The findings in the 8 rabbits that survived the injection are summarized in Table I. It can be seen that 3 (Nos. 1, 4, and 6) showed either paresis of the foreleg or tilting and tremor of the head, while 1 (No. 3) displayed both paresis

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of the foreleg and tilting of the head. These signs became manifest as a rule within 6 hours following the injection and persisted until the animals were sacrificed. On gross examination the sites of recent intracerebral injection were marked by petechiae, while those of longer duration were evident as lusterless gray areas that proved devoid of myelin on microscopic examination. Areas

TABLE I

Effects of Pancreatic Lipase Injected Intracerebrally in Rabbits

Rabbit	Interval between injection and sacrifice of animals	Pancreatic lipase injected	Neurological signs	Results of gross and miscroscopic examination of brain
No.	*days*	*mg.*		
1	2	6	Tilting of head	Microscopic focus of demyelinization in right basal ganglion
2	3	5	Lethargy	No lesion found
3	5	7	Paresis of left foreleg and tilting of head	Grayish discoloration 1 cm. across in right thalamus. This was devoid of myelin (Figs. 1 and 2)
4	5	5	Tremor of head	Lusterless area, 0.5 cm. across, in right thalamus—histologically an area of demyelinization with marked astrocytosis (Fig. 3)
5	7	7	None	No lesion found
6	10	8	Paresis of left foreleg	A pale gray area, 0.4 cm. across, in right basal ganglion—largely devoid of myelin in Loyez preparation
7	10	5	None	No lesion found
8	190	5	None	A pale yellow area, 0.4 cm. across, in right hippocampus—histologically an area of demyelinization with moderate gliosis

of demyelinization visible in the gross and confirmed by microscopic examination were present in the brains of 4 rabbits (Nos. 3, 4, 6, and 8). In addition, an area of demyelinization not detected on gross examination was found histologically in the brain of a 5th animal (No. 1). Neither the injection site nor areas of demyelinization were found on thorough gross and microscopic examination of the brains of the remaining 3 rabbits; whether the material had been injected into a cerebral ventricle in these cases was not ascertained.

A description of the histologic changes will be given further on, and also the results of control experiments.

Effects of Pancreatic Lipase Injected Intravascularly in Rabbits

To see whether demyelinization also followed the intravascular injection of lipase, 2 adult hybrid albino rabbits were given multiple injections in the ear veins of a filtrate of a 2.5 per cent solution of the pancreatic lipase in 0.9 per cent saline, and 3 other adult hybrid rabbits were given a single injection of the same filtrate in the internal carotid artery while under ether anesthesia. (The filtrate exhibited undiminished lipolytic activity for olive oil when tested concurrently with an unfiltered aliquot of the same material.)

TABLE II

Effects of Pancreatic Lipase Injected Intravascularly in Rabbits

Rabbit	No. of Injections	Pancreatic lipase injected	Injection period	Interval between injection and sacrifice of animal	Results of microscopic examination of the central nervous system*
No.		*mg.*	*days*	*days*	
9	10	680	32	2	Focal areas of demyelinization 1 to 2 mm. across in the basal ganglia and cerebellar white matter (Fig. 4)
10	6	1150	120	5	Foci of demyelinization 1 to 2 mm. across in the cerebral gray matter.
11	1	200	—	5	Areas of demyelinization 1 to 3 mm. across in the corpus callosum, cerebral gray matter, and brain stem (Fig. 5)
12	1	200	—	7	Areas of demyelinization 1 to 3 mm. across in the cerebral gray matter
13	1	500	—	7	Foci of demyelinization 1 to 3 mm. across in the corpus callosum, cerebral gray matter, and brain stem (Fig. 6)

* Rabbit 9 was lethargic when sacrificed; the rest seemed healthy.

Lesions were not seen on gross examination of the nervous system in any of the animals.

Four of the 5 rabbits injected intravascularly with the lipase in quantities up to 1150 mg., as indicated in Table II, manifested no ill effects, and they were sacrificed with ether 5 to 120 days after first injection. Small focal areas of demyelinization—to be described in detail in the next section—were found in the cerebrum, brain stem, and cerebellum of each of these animals (Nos. 10 to 13), but no lesions were seen in their spinal cords sectioned at the cervical, thoracic, and lumbar levels. The 5th rabbit (No. 9) became lethargic after receiving a total of 680 mg. of the enzyme, given intravenously in amounts of 50 or 100 mg. in 10 injections during a period of 32 days. The lethargy was noted during the final injection and persisted until the animal was sacrificed 48 hours later. At necropsy, microscopic focal areas of demyelinization up to

2 mm. across were found in the basal ganglia and in the cerebellar white matter; in addition the liver showed extensive acute central necrosis. The other viscera of this animal and those of the 4 rabbits already mentioned were free from gross and microscopic lesions.

In subsidiary tests serum specimens from 2 of the rabbits (Nos. 9 and 10), procured at the time of sacrifice, were examined for the presence of substances capable of inactivating lipase *in vitro*. 5 cc. of each serum specimen was incubated for 3 hours at 37° C. with 50 mg. of pancreatic lipase; serums from 2 normal rabbits were used as controls. The experimental and control mixtures developed equal amounts of acid when incubated with olive oil as previously described.

Characteristics of the Induced Demyelinization

In one instance the demyelinization was evident in Loyez preparations in an animal killed 48 hours after the intracerebral injection of pancreatic lipase (rabbit 1), and in another case the demyelinization was present 6 months after an intracerebral injection (rabbit 8). The areas of demyelinization measured up to 1 cm. across at the sites where the lipase had been injected intracerebrally. (Fig. 1.) They were sharply delineated and solitary in most instances, but occasionally were accompanied by smaller focal areas which were separated from the larger lesions by areas of intact myelin. (Fig. 1.) The central portions of these lesions were completely devoid of stainable myelin and this seemed the more noteworthy since other neuronal structures within the area were generally well preserved, as Cajal and Nissl preparations showed. At the periphery of the lesions, the myelin sheaths often stained poorly and were swollen and vacuolated. (Fig. 2.)

The changes following the intravascular injection of lipase were histologically similar to those following the intracerebral injections, though the lesions were smaller; again they were characterized by central zones of complete demyelinization usually surrounded by peripheral areas in which the myelin sheaths were swollen and pale staining. (Figs. 4 to 6.) Occasionally the areas of demyelinization in rabbits that had received intravascular injections were clearly perivascular in distribution (Fig. 6), but in no instance did the blood vessels contain thrombi or exhibit altered walls.

An astrocytic gliosis regularly accompanied the demyelinization in the older lesions. It was more marked in those induced by the intracerebral injection of lipase and was most conspicuous in lesions more than 5 days old. (Fig. 3.) The paucity of inflammatory cells in and about the areas of demyelinization was noteworthy. Leukocytes were present in moderate numbers at the sites of intracerebral injection when these were less than 2 days old, but these elements were not to be found in older lesions or in those induced by the intravascular administration of the enzyme. Gitter cells were present in great con-

centration about the areas of recent intracerebral injection but were generally less numerous in older lesions (Fig. 2) and were few in number in lesions induced by the intravascular injection. Small hemorrhages and some edema were present at the sites of recent intracerebral injection.

Effects of Proteolytic Enzymes Injected Intracerebrally in Rabbits: Other Control Observations

To learn whether similar changes could be produced by proteolytic enzymes, 6 rabbits were injected intracerebrally with a suspension containing 5 mg. of trypsin in 0.125 cc. of 0.9 per cent saline, and 6 with 3 to 5 mg. of chymotrypsin in 0.125 cc. of 0.9 per cent saline. After periods of 3 to 10 days the brains of these animals were examined.

Notable on gross and microscopic examination was the presence at the site where both trypsin and chymotrypsin had been injected of discrete focal areas 1 to 10 mm. across of necrosis, sometimes with hemorrhage, and always with marked gitter cell and leukocytic infiltration. Masson, Loyez, Cajal, and Hortega silver carbonate preparations showed a destruction of all cerebral tissue with necrosis and absence of nerve cells, axon processes, and myelin sheaths. It was noteworthy that the myelin immediately surrounding these lesions was always well preserved. The intracerebral injections of greater quantities of either proteolytic enzyme regularly resulted in massive fatal ntracerebral hemorrhage; smaller quantities (0.6 and 0.12 mg.), by contrast, produced either smaller but similar lesions or gave none at all. In no instance was focal demyelinization produced which resembled that following injection of the lipase preparation.

To test further the possibility that constituents of the pancreatic preparation other than lipase might be responsible for the demyelinization, a solution containing 32 mg. of the purified preparation per cc. in 0.9 per cent saline was heated to 100°C. for 8 minutes. Tests made with olive oil in the usual way showed that the heated material had no lipolytic activity. 0.25 cc. of the heated solution was then injected intracerebrally into 4 rabbits, and an equal amount of an unheated aliquot which had proved highly lipolytic in the control assay, was likewise injected into 6 other rabbits.

The 4 rabbits injected with the heated material survived without neurological signs and were killed with ether after 8 days; microscopic examination of the brains showed at the injection sites a few phagocytic gitter cells and some areas of perivascular lymphocytic infiltration, but the myelin sheaths were everywhere intact. All the animals injected with the unheated material, by contrast, manifested signs of disease of the central nervous system, notably convulsions and paresis of the forelegs, which became apparent within 4 to 40 hours after the injections and were progressive until death of the animals. In all 6 cases marked edema was present at the sites of injection, along with other manifestations of acute inflammation, as determined histologically; the 2 ani-

mals that survived more than 40 hours showed in addition areas of demyelinization.

In a further control experiment designed to ascertain whether the demyelinization might have been produced by fatty acids or other lipid fractions as released by the action of the pancreatic enzyme preparation on tissues of the host animal, suspensions of fatty connective tissue acted upon by pancreatic lipase were injected intracerebrally into rabbits. 2 gm. of adipose tissue, removed aseptically from the groin of a rabbit, was ground with sterile sea sand and incubated with 100 mg. of pancreatic lipase in 5 cc. of 0.9 per cent saline for 12 hours at 37°C. After centrifugation of the incubated mixture at medium speed for 5 minutes the supernatant liquid was heated at 100°C. for 8 minutes, and 0.25 cc. portions were then injected intracerebrally into each of 4 rabbits.

The animals survived without neurological manifestations and were sacrificed with ether after 8 days. Histologic preparations of sections of the brains taken through the injection sites showed small masses of amorphous foreign material around about which there was a slight infiltration of phagocytes. The myelin sheaths bordering these regions were intact.

Effects of Pancreatic Lipase on Brain Tissue in Vitro

In a first experiment designed to learn whether the pancreatic lipase preparation was capable of breaking down the fat of brain, 100 mg. was incubated for 2 hours at 37°C. with 2 gm. of rabbit brain tissue prepared as a homogenized 25 per cent suspension in 0.9 per cent saline, in a Waring blendor. Enough fatty acid was produced to neutralize 6.3 cc. of 0.1 N NaOH solution. Several similar experiments gave comparable results.

The incubation of rabbit brain tissue with the proteolytic enzymes in control experiments regularly failed to produce any measurable amounts of acid.

To see whether the *in vitro* effect was accompanied by histologic changes, discs of fresh spinal cord approximately 1 cm. in thickness were procured from 6 rats and incubated for 12 hours at 37°C. in a 0.5 per cent solution of the pancreatic lipase preparation in 0.9 per cent saline, to which penicillin (50 units/ml.) had been added. Other segments of the rat spinal cords were incubated for 12 hours at 37°C. with a 0.5 per cent solution of pancreatic lipase which had been heated at 100°C. for 10 minutes, and still other segments were incubated in a similar manner with 0.5 per cent solutions of trypsin and chymotrypsin. All segments were fixed in 10 per cent formalin after incubation and these, along with specimens that had been fixed while fresh, were stained by the Loyez method for myelin.

In the specimens that had been incubated with the solution of lipase the myelin at the periphery of the discs stained poorly in Loyez preparations, and the same was true of the myelin in the spinal nerve roots. In both situations the myelin sheaths were swollen and appeared as large irregular globules which were pale gray and granular, like ground glass; these changes were not

present in the myelin of the specimens that had been incubated with trypsin and chymotrypsin or with heated lipase. These findings have added interest in light of those of Brickner and others (2–4) in which similar *in vitro* techniques were used to detect the presence of lipolytic enzyme in the plasma and serum of patients with multiple sclerosis and other diseases.

SUMMARY AND COMMENT

Purified lipase, injected intracerebrally and intravascularly in rabbits, gave rise to focal areas of demyelinization in the central nervous system in 10 of 13 animals so treated. In one instance the lesions became manifest within 48 hours and in another they persisted for 6 months; they were not infrequently accompanied by paresis and by tilting or tremor of the head. They were characterized by a focal loss of myelin and moderate gliosis with little or no neuronal destruction or inflammatory reaction, in these respects resembling the plaques of multiple sclerosis. The intracerebral injection of trypsin and chymotrypsin in control animals failed to produce the characteristic demyelinization, but by contrast caused focal areas of necrosis in which all the cerebral tissues were involved. Furthermore, demyelinization did not result when heat-inactivated pancreatic lipase was injected intracerebrally, and similarly negative results were obtained when an incubated mixture comprised of fatty connective tissue that had been acted upon by the pancreatic preparation and then heated to inactivate the lipase, was injected into the brains of rabbits.

In supplementary experiments the pancreatic lipase preparation and fresh rabbit brain, incubated together *in vitro*, were found to form acid, presumably owing to the break-down of brain lipids to fatty acids; trypsin and chymotrypsin mixed with brain in control experiments failed to form acid. When incubated with segments of the spinal cord of experimental animals, the lipolytic enzyme brought about a loss of stainable myelin in peripheral areas and in the spinal nerve roots; again trypsin and chymotrypsin had no such effect in control experiments.

The findings as a whole show that an enzyme preparation with lipolytic activity has the ability to destroy myelin in living animals, and *in vitro* as well, and to produce lesions remarkably similar to those of multiple sclerosis. They have additional interest in light of the demonstration that a lipolytic enzyme is regularly present in the reactive histiocytes of guinea pigs with experimental encephalomyelitis (5).

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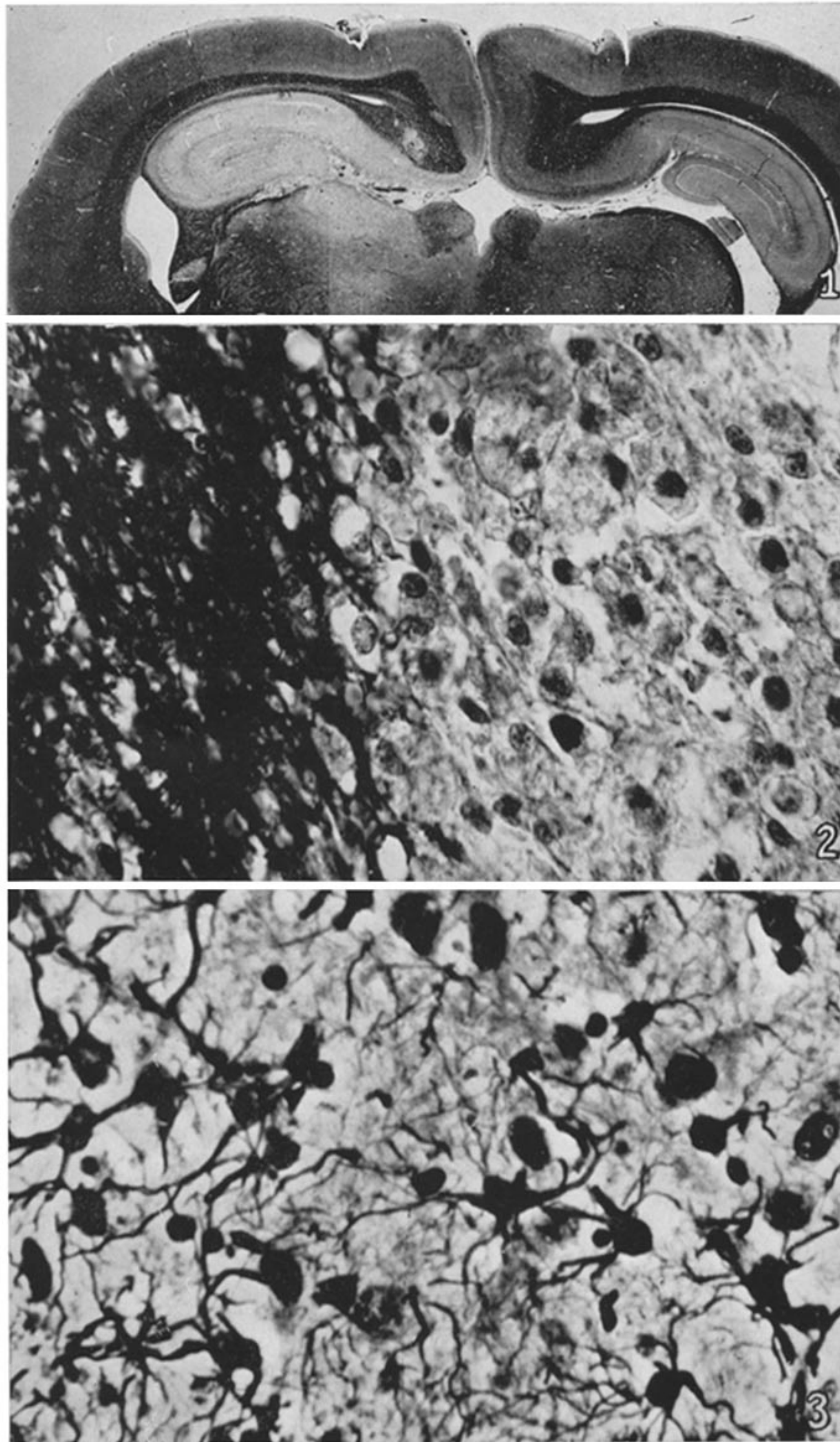
EXPLANATION OF PLATES

PLATE 15

FIG. 1. Extensive demyelinization throughout the thalamus and hippocampus in the brain of a rabbit that had received an intracerebral injection of 7 mg. of pancreatic lipase in 0.25 cc. of 0.9 per cent saline solution 5 days before being sacrificed. Loyez preparation. × 9.

FIG. 2. A sharply delineated area of demyelinization containing many gitter cells in the fornix of the rabbit of Fig. 1. Loyez preparation. × 500.

FIG. 3. Marked astrocytic proliferation in the brain of a rabbit that had received an intracerebral injection of 5 mg. of pancreatic lipase in 0.9 per cent saline 5 days before being sacrificed. Hortega preparation. × 500.

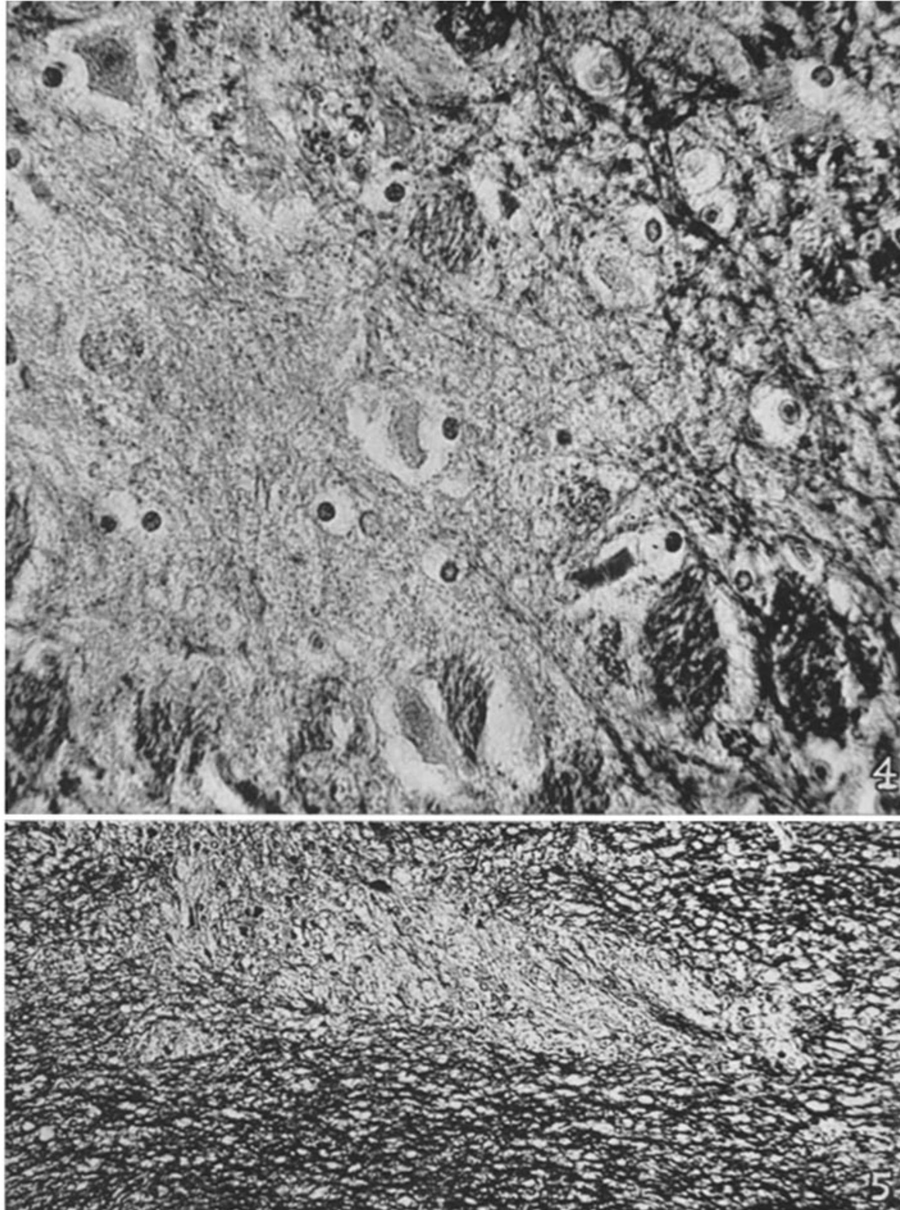


(Vogel: Demyelinization induced by lipase)

PLATE 16

FIG. 4. A focal area of demyelinization associated with atrophy of nerve cells in the basal ganglion of a rabbit that had received 680 mg. of pancreatic lipase intravenously in 10 injections during a period of 32 days. Loyez preparation. $\times$ 550.

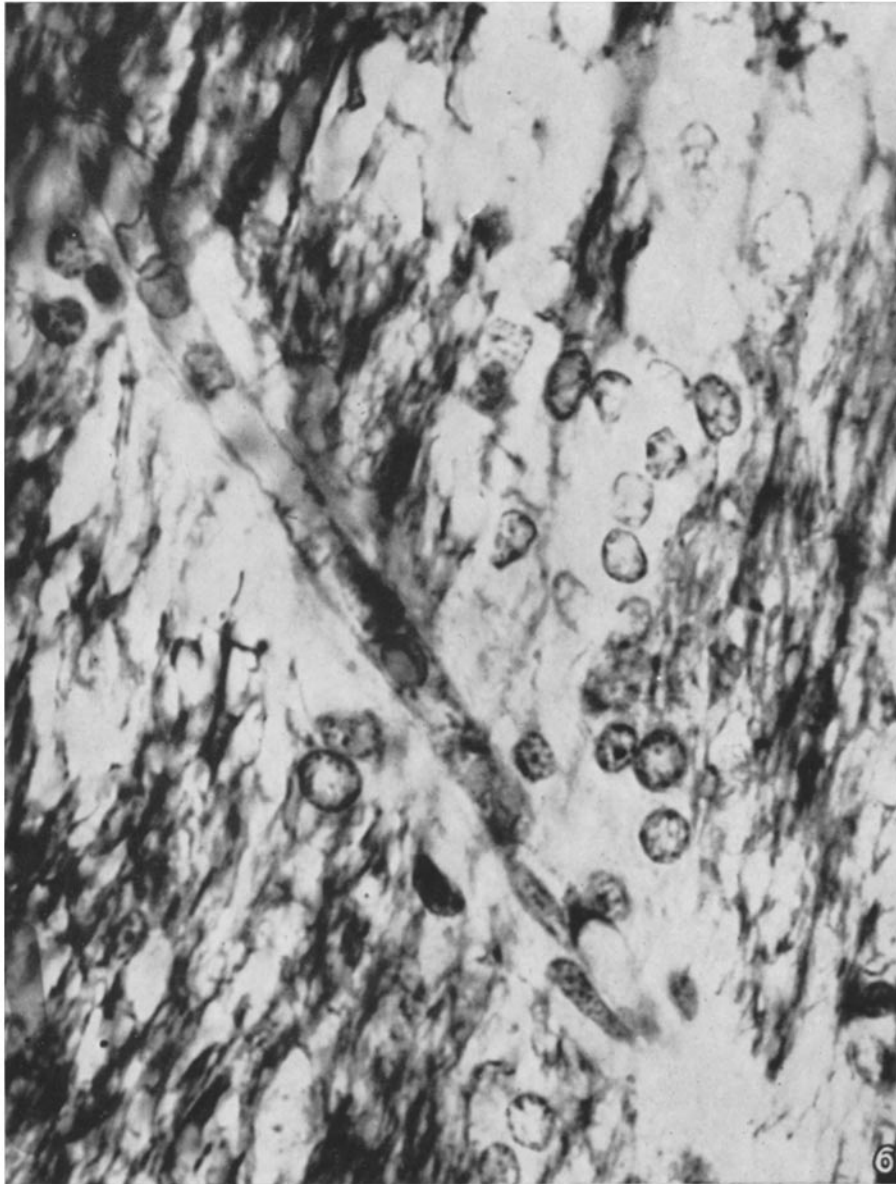
FIG. 5. An area of demyelinization infiltrated by microglial cells in the corpus callosum of a rabbit that received a single injection in the carotid artery of 200 mg. of pancreatic lipase in a 2.5 per cent solution in 0.9 per cent saline 5 days before being sacrificed. Loyez preparation. $\times$ 120.



(Vogel: Demyelinization induced by lipase)

Plate 17

Fig. 6. A perithelial area of demyelinization about a capillary in the corpus callosum of a rabbit that had received a single injection in the carotid artery of 500 mg. of pancreatic lipase as a 2.5 per cent solution in 0.9 per cent saline 7 days before being sacrificed. The lesion shows early astrocytic gliosis and contains several gitter cells. Loyez preparation. × 1000.



(Vogel: Demyelinization induced by lipase)