STUDIES ON EASTERN EQUINE ENCEPHALOMYELITIS

III. Intraocular Infection with Fixed Virus in the Guinea Pig

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Plate 41

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In the preceding paper (1) it was shown that a recently isolated strain of equine encephalomyelitis virus, when injected into the eye, sometimes spread along the optic pathways. This type of spread was, however, inconstant. But with a fixed strain of virus (2, 3) an entirely constant behavior was observed which was capable of systematic study.

For the study of virus activity within the central nervous system, inoculation into the eyeball presents certain great advantages due to the anatomical peculiarities of this organ, which make it the most readily accessible portion of the central nervous system.

Anatomy.—In general, the peripheral and central nervous systems differ fundamentally in the type of accompanying stroma. Within the brain or spinal cord there are glial cells which form the supportive tissue, but no true connective tissue stroma. Small quantities of connective tissue accompany the blood vessels but are shut off from the true nerve parenchyma by the pia-glial membrane. On the other hand, in the cranial or spinal nerves, including the sensory ganglia, there is a rich stroma of fibroblasts and associated cells, collagen, and reticulin, with a true lymphatic system. The junction between the central and the peripheral portions of the craniospinal nerves has recently been well described by Tarlov (4). The importance of this stroma in relation to certain problems of the so called hemato-encephalic barrier has been emphasized in recent publications (5, 6).

The optic nerve is not a peripheral nerve at all, in the sense applied to the vagus, hypoglossal, or any of the spinal nerves, for example. Instead, the optic nerve is embryologically and anatomically a tract of the brain substance proper, having all the characteristics of the central nervous system.

It is well known, of course, that the optic pathway in the lower vertebrates is chiefly though not entirely crossed, the decussation taking place in the optic chiasm. The principal connections of the right eye, for example, are with the left lateral geniculate body, and pretectal nucleus of the thalamus, and the left superior colliculus. The converse obtains with the left eye. From the lateral geniculate body a projection tract goes to the striate cortex of the same side.

Method.—In the experiments herein reported, a 27 gauge needle was inserted from the inner canthus of the eye directly through the sclera into the posterior chamber of the eye. Passage through the anterior chamber was avoided. The injection mass (0.03 to 0.05 cc.) was placed in contact with the ganglion cells of the retina and the overlying fibers of the optic nerve.

The virus was thus placed in the vitreous humor, in contact with the cells and fibers of the central nervous system with essentially no trauma to the latter.

Strains of Virus.—For the most part a strain of Eastern equine encephalomyelitis virus that had been modified by serial intracerebral passage in pigeons was employed. This strain was developed by Traub and TenBroeck (2), and has been further studied in detail by Traub (3). In the present experiments the 112th to 117th pigeon passages were utilized. In addition certain comparative experiments were carried out with an unmodified strain of the Eastern virus, isolated from a horse in 1937, of which the 2nd to 5th intracerebral guinea pig passages were utilized. All titer figures refer to tenfold dilutions of a 10 per cent suspension of pigeon (or guinea pig) brain, which is designated as 10^0 dilution.

Sensitivity of the Intraocular Route

To determine the relative sensitivity of the intraocular as compared with the direct intracerebral route, comparative titrations were performed using the same amount of inoculum for each route. Both the fixed strain and the unmodified strain were used. The results of two such titrations are given in Table I. In some experiments fatalities have resulted from a 10^{-3} dilution of the fixed strain, and as high as 10^{-6} of the unmodified strain, injected intraocularly.

With the fixed strain there is a definite difference between the intraocular and the sub- or intracutaneous routes. Traub (3) and TenBroeck and Traub (2) have already found that guinea pigs are quite insusceptible to subcutaneous inoculation, and the present work fully confirms their results. With the dosage used (0.05 cc.) the undiluted virus suspension regularly failed to produce encephalitis following an intracutaneous injection, although the intracerebral titer was 10^{-6} or 10^{-7} (with the same dosage).

With the unmodified virus, on the other hand, repeated experi-

ments with the same quantity of inoculum did not reveal any significant difference between the intraocular and intracutaneous routes of administration.

With the fixed virus, it is clear that the susceptibility of the guinea pig to intraocular inoculation is midway between the intra- or subcutaneous and the intracerebral routes. This difference does not obtain with the unmodified virus.

TABLE I

Titration of Intraocular and Intracerebral Inoculations in Guinea Pigs

Dilution	Fixed strain (pigeon passage)		Unmodified strain	
	Intraocular inoculation	Intracerebral inoculation	Intraocular inoculation	Intracerebral inoculation
10^{-8}	N.T.	N.T.	0, 0	0, 0
10^{-7}	"	2, 0, 0	0, 0, 0	5, 9, 0
10^{-6}	"	3, 0, 0	0, 0, 0	3, 4, 6
10^{-5}	"	2, 3, 3	4, 6, 0	3, 3, 4
10^{-4}	0, 0, 0	2, 2	N.T.	N.T.
10^{-3}	0, 0, 0	N.T.	"	"
10^{-2}	3, 4, 0	"	"	"
10^{-1}	3, 3, 5	"	"	"

N.T. = not tested.

0 = guinea pig survived.

2, 3, 4 = guinea pig died in 2, 3, or 4 days after the inoculation.

Action of the Virus within the Eyeball

When a 10^{-1} dilution of the fixed virus is injected into the eye, the animal invariably dies, usually from 72 to 96 hours after the injection. With this mode of inoculation it is easy to determine the minimum length of time the virus must act in order to produce a fatal infection. In a series of experiments the injected eyeball was surgically removed under deep ether anesthesia at different intervals following the inoculation. The eyeball can easily be removed intact, hemorrhage is negligible, and the animal always makes an uneventful recovery. A small quantity of pus may occasionally form in the eye socket but never causes symptoms of any sort.

The results of three such extirpation experiments following the injection of fixed virus are given in Table II. It is seen that when the

eye was removed in less than 6 hours, only 2 out of 17 animals died. When removal was performed 10 to 13 hours after inoculation, 8 out of 14 succumbed. All controls died. The period of 10 to 13 hours seems to be the average minimum time in which the virus must act within the eyeball in order to produce a fatal infection.

TABLE II

Guinea Pig Survival after Removal of Eyeball at Different Intervals after Injection with Fixed Strain of Virus

Experiment No.	Inoculated eye removed after					Controls
	1 hr.	3 hrs.	6 hrs.	10 hrs.	13 hrs.	
1	5, 0	0, 0*	0*, 0*	3, 3	N.T.	3, 3
2	0, 0*	0, 0, 0	4, 0, 0	3, 3, 0	3, 0	3, 6
3	N.T.	N.T.	0, 0, 0	3, 0, 0*	3, 3, 0, 0	3, 3

* In subsequent tests of immunity to intracerebral inoculation of 3×10^3 to 10^4 minimal lethal doses, the starred animals survived. Those not starred were not immune.

Other abbreviations as in Table I.

TABLE II*a*

Identical Procedure with Unmodified Strain

Experiment No.	Eye removed after			Control
	½ hr.	1 hr.	3 hrs.	
1	6, 8	5, 0	4, 8	5, 0
2	5, 0	4, 5	5, 6	4, 0*
3	4, 0	5, 6	0, 0	4, 5, 5

* This animal showed marked signs of encephalitis, but recovered. Later histological examination showed healing encephalitis.

Survivors were not tested for immunity.

In the foregoing experiments the survivors were tested for immunity by the intracerebral injection of 0.1 cc. of a 10^{-3} dilution of virus, whose virulence by intracerebral tests in mice was constantly 10^{-6} or 10^{-7}. The animals that survived the immunity test are designated by a star in Table II. There is no constancy in the induction of immunity. One animal whose eye was removed 1 hour after inoculation was immune. Other animals, with removal after 13 hours, were not immune. The reasons for this variability are not clear.

Extirpation experiments were also carried out with the unmodified strain, as shown in Table II *a*. The fairly definite time interval required with the fixed strain is not necessary for the fresh virus. This property is undoubtedly correlated with the relative abilities of the two strains to invade the blood stream. The unmodified strain is readily found in the blood stream, but the fixed strain is recovered only rarely, or not at all (3).

Course of the Virus after Inoculation into the Eye

It was desirable to try to trace the course of the virus by examining different portions of the brain for virus content at different intervals of time after inoculation.

For this purpose 6 animals were inoculated into the right eye and sacrificed at different periods of time. The brains were removed aseptically and subdivided in accordance with the known optic pathways. The method of sectioning the brain was as follows: The cerebellum, medulla, and inferior colliculi were first cut off and discarded. With the brain ventral surface upward, gentle traction was applied to the optic chiasm, resulting in separation of the optic tracts from the underlying tissue. The chiasm and tracts were then cut away. The brain was turned dorsal side upward, and with small sharp scissors the lateral ventricle was entered from the medial surface of the hemisphere. The posterior neocortex of one side was then cut away, following the line of the rhinal fissure ventrally. The portion so removed included the entire area striata, and portions of the temporal, parietal, and occipital areas. It is impossible to differentiate these areas macroscopically. The procedure was repeated for the opposite side. Then the corpus callosum was cut through, and the two hippocampi and the hippocampal commissure gently peeled forward, exposing the thalamus and superior colliculi. The thalamus was separated from the hemispheres by incisions along the striae terminales. The thalamus and midbrain were then divided in the midline, and each half used separately. The entire olfactory portion of the brain, together with the basal ganglia and anterior neocortex, was left and utilized as a single portion. Throughout the dissection special care was taken to avoid contamination of one part by another.

This method of division gave 6 portions of the brain to be tested: the optic chiasm and tracts; the right thalamus plus superior colliculus; the left thalamus plus superior colliculus; the right and the left posterior neocortex; and the remainder of the hemispheres. The chiasm was ground up in 0.5 cc. of saline; the other portions were also ground in saline in approximately 10 per cent dilution. After light centrifugation, the supernatant fluid was injected intracerebrally into 3 to 4 week old mice, the dose being 0.05 cc.

The optic pathway was thus divided into three successive neurones: the optic chiasm and tracts, constituted by the axones of the ganglion cells of the retina; the lateral geniculate body and superior colliculus, where the optic tract terminates; and the visual cortex, which receives fibers from the lateral geniculate body. The thalamus and neocortex were tested bilaterally.

The results of this experiment are recorded in Table III. A minute trace of virus was detected in the contralateral geniculate body (and midbrain) 13 hours after the injection, although the optic chiasm did not show virus. At 24 hours there was abundant virus in the optic chiasm and the contralateral geniculate body and midbrain, but nowhere else. At 36 hours virus was present not only in these

TABLE III

Spread of Fixed Virus after Inoculation into the Right Eye.
Virus Content of Brain Portions, Tested by Intracerebral Inoculation into Mice

Guinea pig	Time of sacrifice	Optic chiasm	Left geniculate and midbrain	Left occipital cortex	Right geniculate and midbrain	Right occipital cortex	Remainder of brain
	hrs.						
1	10	0, 0, x	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
2	13	0, 0, 0	8, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
3	24	3, 3, 4	2, 3, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
4	36	2, 2, 3	2, 2, 3	2, 2, 0	0, 0, x	0, 0, 0	0, 0, 0
5	48	2, 2, 2	2, 2, 2	2, 2, 3	3, 4, 5	2, 4, 0	2, 4, 4

x = death occurred within a few hours of the inoculation, and was not due to virus action.

two regions, but also in the visual cortex of the contralateral side; other portions of the brain were still virus-free. At 48 hours, however, virus was widely disseminated throughout the entire brain.

The results of this experiment are entirely consistent with the view that the virus, inoculated into the eye, spread along the optic pathway to the thalamic centers (and midbrain), and from the thalamus progressed by the projection path to the cortex. These are the regions which first contain virus. The contralateral optic pathway was predominantly affected, since in the guinea pig the great majority of the optic fibers decussate.

To study further the time relationships involved, the foregoing

experiment was repeated, but only the optic chiasm and the left thalamus and midbrain were tested for virus content. Again inoculation was into the right eye. The results are given in Table IV. Again minute quantities of virus were detectable as early as 12 and 17 hours after inoculation, but significant quantities were first present at 20 to 24 hours. An important feature to be noted is the pres-

TABLE IV

Relation between Virus Content of Optic Chiasm and Tracts and of the Secondary Optic Centers, Following Inoculation into the Right Eye. Tested by Intracerebral Inoculation into Mice

Time	Experiment No.	Guinea pig	Optic chiasm	Left geniculate and midbrain
hrs.				
12	2	1	0, 0, 0	12, 0, 0
14	1	2	0, 0, 0	0, 0, 0
	2	3	0, 0, 0	0, 0, 0
		4	0, 0, 0	0, 0, 0
17	1	5	8, 0, 0	0, 0, 0
	2	6	0, 0, 0	0, 0, 0
		7	0, 0, 0	0, 0, 0
20	1	8	0, 0, 0	0, 0, 0
	2	9	2, 3, 0	0, 0, 0
		10	0, 0, 0	0, 0, 0
24	1	11	2, 2, x	2, 3, 4
	2	12	0, 0, 0	0, 0, 0
		13	2, 2, 0	0, 0, x

ence of virus in fairly large quantities in the optic chiasm and tracts before virus was apparent in the thalamus or midbrain (guinea pigs 9 and 13).

Pathology

The histopathology of the brain after infection with the fixed strain of virus has been found by Traub (3) to be substantially the same as with the unmodified virus. The first paper of this series (7) has

described in considerable detail the pathology of the natural virus disease in the guinea pig. Further study with the fixed strain reveals certain differences. After intracerebral inoculation the inflammatory reaction is vastly more pronounced, and the hippocampal degeneration is much more slight than after similar inoculation with the natural virus. The intensity of the inflammatory reaction is especially surprising in view of the considerably shorter duration of the disease (generally 2 to 3 days rather than 3 to 5).

In the present study, however, there are three principal points of interest in the pathology: the reaction occurring within the eye, the nature of the first lesions to appear within the brain, and the distribution of these lesions.

Intraocular Pathology.—Sections of eyes removed at different intervals, cut serially at 7 microns with short ribbons mounted every 50 sections, showed the development of the process. At about 13 hours there is a very slight reaction consisting of serum exudation, and a few mononuclear cells within the eyeball. In the connective tissue coats of the eye there may be numerous polymorphonuclear leucocytes. Within the eyeball, however, in uncomplicated cases, there is surprisingly little pathological change, even when the eye is removed at the death of the animal. A few leucocytes are present in the vitreous humor, and occasionally scattered leucocytes penetrate the outer layers of the retina. But there is no necrosis of tissue, and none of the focal reaction that is so prominent in the brain substance elsewhere following intraocular injection.

Sometimes there is a pronounced accumulation of polymorphonuclear leucocytes within the eyeball, and then the invasion of the surface of the retina by leucocytes is more intense, and necrotic ganglion cells may rarely be seen. This, however, is an entirely non-specific reaction. This histological picture has been duplicated by injection of normal brain emulsion diluted to the same degree as the virus suspensions used. Furthermore, when large numbers of leucocytes are present, intracellular bacteria may usually be seen in the exudate. Eyes that are sterile by bacteriologic culture always show an insignificant reaction to the virus (Figs. 1 and 2).

It may be mentioned that the slight degree of intraocular contamination sometimes becoming noticeable 24 or more hours after inoculation, has never had the slightest effect on the action of the virus.

Nature of the Cerebral Lesions.—In contrast to the lack of reaction in the eye, the histological picture in the higher optic centers is identical with that already described for the unmodified, natural virus. In 9 animals, sacrificed at intervals of from 24 to 52 hours, the entire thalamus and superior colliculus was sectioned serially at 10 microns, and every 15th section examined. In 5 other cases, from

48 to 65 hours, the entire brain was sectioned. Hematoxylin and eosin, phloxin and methylene blue, and thionin were the stains used.

The earliest change, first found at 24 hours, is a mild perivascular reaction, consisting of mobilized glial cells and mononuclear leucocytes (Fig. 3). This speedily develops into the typical inflammatory focus already described for the natural strain. As early as 36 hours after inoculation the typical focus may be very intense (Fig. 4). The earliest lesions are always found in the contralateral geniculate body or superior colliculus, followed 12 to 15 hours later by similar lesions in the same centers of the side of the inoculation. The series of cases studied, therefore, has double opportunities for observations of early lesions, first on the contralateral, then on the ipsilateral side.

Since the virus appears to spread along the nerve paths, it might be thought that a terminal nerve center might show neuronal necrosis as the first sign of disease. This is very definitely not the case. A closely graded series of cases supports the finding already published (7), that a vascular and interstitial reaction is the first sign of disturbance. These data further support the conclusion previously expressed (1), that there is no difference between lesions caused by blood-borne and those caused by nerve-borne virus.

The later lesions present no new features beyond what has already been described (7).

Distribution of Lesions.—The relatively crude method of gross dissection of the brain and testing of each portion for virus content, indicated that a spread of virus along the optic pathway was likely. The method of topographical analysis of the lesions (1) entirely supports the previous evidence. As early as 24 hours after right-sided injection there may be definite histopathology in the left superior colliculus or lateral geniculate body, rapidly growing more severe. The first lesions are sharply focal and rather rare, but they rapidly become more numerous and more intense until a maximum is reached at about 48 hours. Early lesions on the right side may be detected at 38 hours. At 48 hours the process has extended fairly widely into adjacent thalamic nuclei, so that large portions of this subcortical center are affected. The optic chiasm and optic tracts first show lesions at 36 to 38 hours, which is significantly longer than the time required to produce injury at the terminus of these tracts. The basal meninges begin to show inflammatory changes at about the same time as the optic tracts.

Pathologic changes in the left visual cortex are present to a very slight degree at 48 hours, but rapidly become more severe. At 53 to 55 hours there may be slight changes in the right posterior neocortex, but the temporal and occipital areas are more involved than the striate area.

At 48 to 53 hours there are numerous demonstrable lesions in areas which are functionally independent of the visual pathway. Such lesions may be present in various portions of the olfactory pathway, especially the tuberculum olfactorium and the amygdala, but also the septum; in the basal ganglia; in scattered unrelated areas of the anterior neocortex; in the hypothalamus, midbrain, and medulla.

DISCUSSION

Susceptibility to intraocular inoculation of fixed virus, as has been pointed out, lies intermediate between the intracerebral and the sub- or intracutaneous routes. The latter is fatal only with massive doses. The intracerebral route is always fatal in high dilutions. The intraocular route is fatal only in low dilutions (10^{-2} or 10^{-3}). The mechanisms involved appear to be somewhat different in the different methods.

The simplest explanation would seem to be as follows. Virus introduced into the eye infects the superficial ganglion cells of the retina. Once the cell body is infected, the entire neurone speedily becomes involved. That is, the virus then infects the cell processes making up the optic nerve. In the case of intraocular injection this takes place entirely within the central nervous system.

Such a spread of virus is quite different from that following a peripheral inoculation; with virus injected, say, into the thigh, any possible passage up the local nerve must infect the terminus of the nerve first, with subsequent passage toward the cell body. The entire metabolism of the neurone is controlled by the cell body. Passage of virus away from the cell body (centrifugal spread) seems to be in a different category from centripetal spread, at least, so far as this virus is concerned. The mechanism by which the virus, once it has infected the cell body, can "travel" along the axone, still remains completely unexplained. The multiplication of virus occurs with ex-

traordinary rapidity, and over a significant distance. Any attempted explanation at present would be sheer speculation.

The time interval of 10 to 13 hours required for virus to act within the eye is capable of different interpretations. This interval may represent the time required for the virus to work its way into the cell and begin its action. Or, it may include the time actually necessary for the virus to pass the length of the optic nerve and tract, and reach the geniculate body. There are no grounds as yet for a definite decision.

In Table II, Experiment 1, one guinea pig died although the eye was removed after an hour. This instance is similar to the behavior of the natural virus (Table II *a*) and may be an indication that in rare instances the fixed virus may act like the unmodified strain.

Some of the other animals whose eyes were removed and which yet survived, were immune to subsequent intracerebral inoculation. Undoubtedly a small amount of the virus escaped into the blood stream, not enough to produce fatal infection, but sufficient to immunize.

There are certain points of interest in the histological studies. Within the eye there is a negligible reaction although there the virus acts first and longest. On the other hand, elsewhere in the central nervous system the histological reaction is typical and in complete accordance with the descriptions previously given. This behavior of the eye appears strictly comparable to the behavior of brain tissue toward intracerebral inoculation. In the latter case, the site of injection of the virus shows merely a non-specific reaction to injury, although elsewhere in the brain there is well marked encephalitis. Furthermore, in another connection, guinea pigs have been infected by injection of virus into the cistern, so that the inoculum directly entered the cerebrospinal fluid. Here too the contact of virus and brain tissue caused only a mild and completely insignificant reaction. Characteristic pathology, found within the parenchyma, was not present at the surface of the brain in contact with the virus.

Experimental juxtaposition of virus and nerve tissue does not call forth the same reaction produced by the virus after its natural mode of spread. An analogy may be drawn with the data of vital staining

where trypan blue, for example, placed in direct contact with the brain, acts entirely differently from the same dye brought to the brain by the blood stream. These facts, and their relation to the "blood-brain barrier," are elsewhere discussed (6).

SUMMARY

The behavior of a fixed strain of Eastern equine encephalomyelitis virus was studied in guinea pigs after intraocular inoculation. Such inoculation concerns the central and not the peripheral nervous system.

The susceptibility to intraocular injection lies midway between the highly virulent intracerebral and the quite avirulent peripheral routes. The virus must act for 10 to 13 hours in order to induce a fatal infection. Removal of the inoculated eyeball before this interval almost always prevents fatality although it may allow immunity to develop. The virus, at suitable intervals after injection into the eye, may be recovered from successive and appropriate optic centers before it is demonstrable in non-optic portions. Approximately 24 hours are required for the virus to reach a significant concentration in the contralateral geniculate body, 36 hours in the contralateral visual cortex. Significant amounts of virus may be present in the optic chiasm and tract prior to involvement of the higher centers.

Virus placed in contact with the retina produces an insignificant, essentially non-specific reaction comparable to that produced at the site of direct intracerebral inoculation. In the retina there is no ganglion cell necrosis unless there is a complicating intraocular infection. In the cerebral visual centers the first reaction is inflammatory and interstitial, and may appear in the lateral geniculate body as early as 24 hours after injection. Neuronal necrosis is not the primary action of the virus on the nervous system in these experiments. The distribution of lesions in the brain is in excellent agreement with the method of direct testing for virus content, and is far more accurate than the latter.

The virus in its primary distribution through the nervous system follows the nerve pathways of the optic system. This occurs within the central nervous system, where presumably there is first an in-

volvement of the nerve cell body and then a spread along the cell process or axone.

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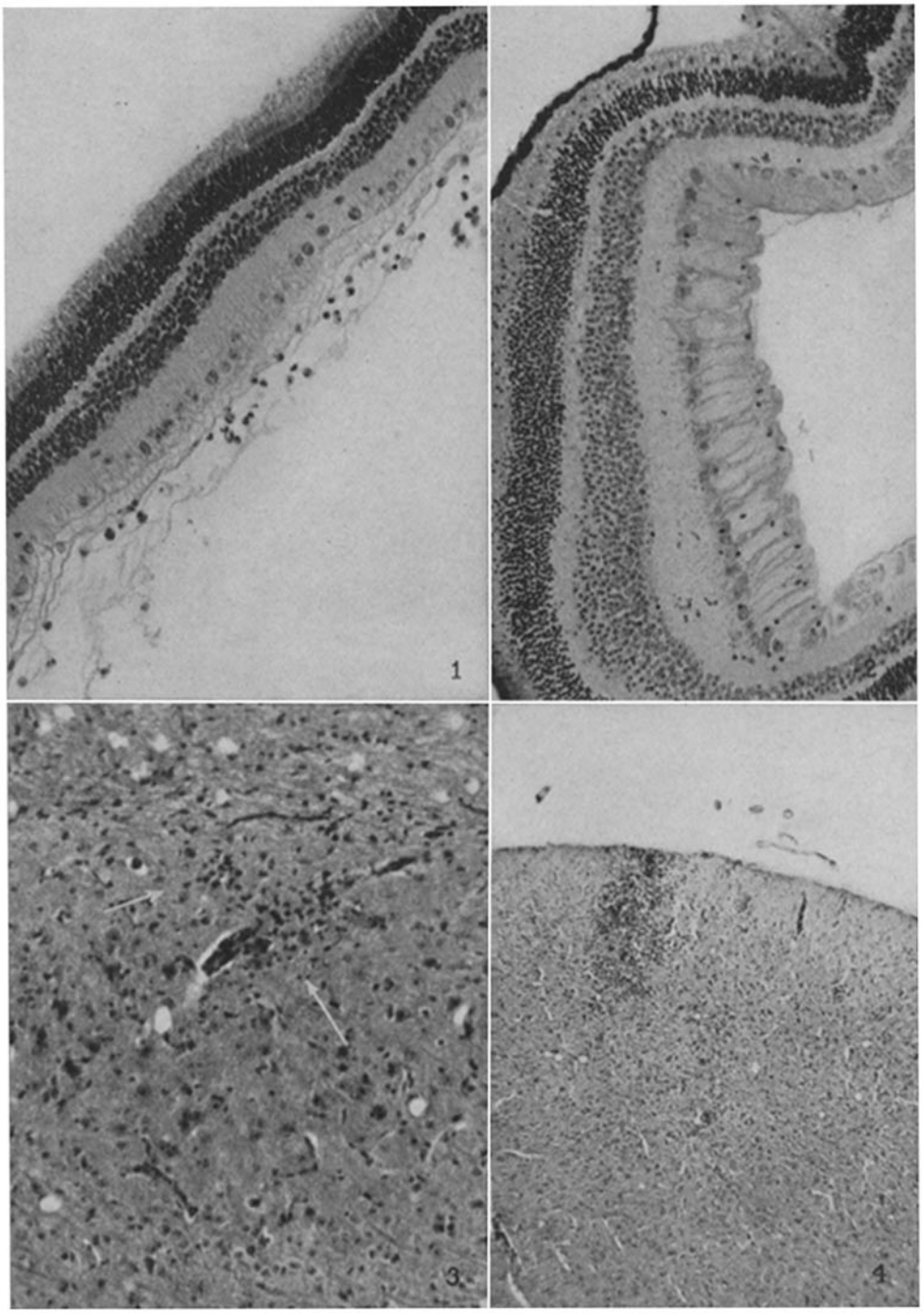
EXPLANATION OF PLATE 41

FIGS. 1 and 2. Retina of injected eye of guinea pig. Eye removed at death, 66 hours after inoculation. In Fig. 1 are a few leucocytes on the surface of the retina; in Fig. 2, within its substance. The ganglion cells, as well as other elements, are intact. Phloxin-methylene blue. × 152.

FIG. 3. Lateral geniculate body, contralateral to the injected eye. 24 hours after inoculation. The area indicated by the arrows shows a sparse infiltration with leucocytes and blood mononuclears, and some glial proliferation, all in relation to the blood vessel. There is no ganglion cell necrosis. Hematoxylin-eosin. × 152.

FIG. 4. Superior colliculus, contralateral to the injected eye. 36 hours after inoculation. There is a very intense, circumscribed focus consisting chiefly of polymorphonuclear leucocytes. Apart from the inflammatory area, there is no neuronal necrosis. Hematoxylin-eosin. × 56.



Photographed by J. A. Carlile

(King: Eastern equine encephalomyelitis. III)