THE DISTRIBUTION OF MATERIAL FOLLOWING INTRACEREBRAL INOCULATION INTO MACACUS RHESUS MONKEYS AND ITS POSSIBLE INFLUENCE UPON THE RESULTS OF NEUTRALIZATION TESTS IN EXPERIMENTAL POLIOMYELITIS *

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Intracerebral inoculations of monkeys with poliomyelitis virus have been employed extensively since Flexner and Lewis [1] in 1909 demonstrated that this route of inoculation is an effective method of transmitting the disease to these animals. Because monkeys have been most consistently infected with virus in this manner, the intracerebral inoculation has been considered the most reliable means of determining infectivity of the virus, especially after it has been subjected to treatment with an inhibitory reagent such as immune serum. However, despite the widespread use of injections into the monkey brain, there is little knowledge concerning the fate of the inoculum subsequent to its deposition into the brain substance.

Many investigators [2-5] have studied the diffusion of material throughout the central nervous system by the injection of dyes or of India ink; but as far as we could ascertain, no experiments have been conducted with a view toward determining to what degree and extent material introduced into an area of the brain is eventually distributed, and what bearing the resultant distribution may have upon the ultimate infectivity of infectious material thus deposited.

Hurst,[6] in a study on the pathogenesis of experimental poliomyelitis, as a preliminary step injected India ink into the cisterna magna of a monkey in order to follow the course of diffusion. Two days later the ink had penetrated into the meninges along the whole length of the cord, over large areas of the brain stem, cerebellum, base of the cerebrum, along certain of the fissures, about half way up the lateral surfaces of the hemispheres, the choroid plexuses and the lateral ventricles. The nervous substance itself was not

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discolored, except for slight staining in the floor of the fourth ventricle.

When virus was inoculated intrathecally the earliest lesions were usually situated in the floor of the fourth ventricle. This suggested to Hurst that the penetration of the virus through the nervous tissue may occur at the ependyma of the fourth ventricle where, under experimental conditions, the virus is regurgitated at operation; but the cerebrospinal fluid does not necessarily participate in its spread through the nervous system.

Experimental

The work to be presented here is an outgrowth of a series of experiments conducted on the neutralizing action of immune serum upon the virus of poliomyelitis.* During the course of this study numerous discrepancies in the results were observed, and an effort was made to determine what possible factors might be responsible for such variations. Among other things, the inoculation procedure and its effect upon the results were investigated.

Experiment I

To follow the course of distribution of substances from the site of inoculation, material containing India ink was introduced into the brains of *Macacus rhesus* monkeys in the manner usually employed in our previous experiments.

Technique: The customary serum-virus mixture used in our neutralization tests was prepared. This consisted of 1.5 cc. of a Berkefeld N filtered 5 per cent suspension of poliomyelitic monkey spinal cords, mixed with 1.5 cc. of human convalescent serum. To this mixture, 1 cc. of India ink was added. After thorough mixing, the material was injected into the right frontal lobes of 4 monkeys, each receiving 0.25 cc., 0.5 cc., 1 cc., and 2 cc., respectively, with a tuberculin syringe carrying a ¾ inch, 26 gauge needle, which was inserted through a trephined opening made in the frontal bone approximately 1 cm. to the lateral right of the midline and 1 cm. anterior to the coronal suture. After 2 hours the animals were chloroformed and their brains and cords examined at autopsy. The results of this experiment are summarized in Table I.

* The details of these experiments and a review of this subject, which entail some length, will be published elsewhere.

Experiment II

A second series of 4 monkeys was inoculated in a manner similar to those in Experiment I. In this group 2 monkeys received 1 cc. and 0.25 cc., respectively, of the serum-virus-ink mixture with a ¾ inch, 26 gauge needle, and 2 others were injected with 1 cc. and

TABLE I

The Diffusion of Material Inoculated Intracerebrally (Right Frontal Lobe) Throughout the Central Nervous System, as Evidenced by the Distribution of India Ink Contained in the Inoculum

Monkey No.	Amount of mixture injected	Appearance of the central nervous system 2 hours after injection
	cc.	
1	0.25	There was evidence of seepage of the material from the brain substance into the subarachnoid space above the site of inoculation. Slight hemorrhage at the site of inoculation was also noted. No India ink was observed on the spinal cord (Figs. 1 & 2)
2	0.5	When the monkey was chloroformed, leakage was observed to be still taking place externally at the site of injection. On flapping back the scalp India ink was found to be deposited around the trephined opening and the surrounding area (Fig. 3). The material had diffused over the surfaces of the brain, cerebellum and spinal cord (Figs. 4 & 5). Sections at the site of inoculation showed that the injection had probably been made directly into the lateral ventricle (Figs. 5 & 6)
3	1.0	This monkey had been used in a neutralization test 2 months previously and therefore, as is often observed, had a sterile abscess in the right frontal lobe at the previous site of inoculation. On examination it was noted that although some India ink had seeped into the subarachnoid space, the bulk of the inoculum was found to be confined to the necrotic cavity on the right side (Fig. 7)
4	2.0	The entire surface of the brain and cord of this animal was covered with India ink, indicating the extensive seepage of the inoculum from the site of inoculation into the cerebrospinal fluid (Figs. 8 & 9)

0.25 cc., respectively, using a ½ inch, 26 gauge needle. After 2 hours the animals were sacrificed and their brains and cords examined at autopsy. The findings are summarized in Table II.

From the results of these experiments it was evident that the distribution of material following intracerebral deposition varied

to some extent. In most cases, however, little of the material remained at the site of inoculation but rapidly entered the cerebrospinal fluid either by seeping backward through the path of inoculation into the subarachnoid space or via the ventricles into the spinal canal. Except for areas reached by the needle no carbon

TABLE II

Comparison of the Diffusion of Material Through the Central Nervous System after Intracerebral Inoculation with Needles of Two Sizes

Monkey No.	Amount of mixture injected	Size of needle	Appearance of the central nervous system 2 hours after injection
	cc.		
1	1.0	½ inch	The entire surface of the right frontal lobe (the side inoculated) up to the fissure centralis was covered with India ink (Fig. 10). No ink was observed on the opposite hemisphere or on the spinal cord. Sections examined at the site of inoculation indicated that the inoculum had been deposited in the brain substance at the site of inoculation below the cortex (Fig. 11), but some seeped backward and entered the subarachnoid space
2	0.25	½ inch	The general appearance of the brain and cord of this animal was similar to the one above, except that there was less distention of the brain tissue at the site of inoculation (Fig. 12)
3	1.0	¾ inch	The entire cerebral hemisphere opposite to the side inoculated was completely covered with India ink (Fig. 13). Ink was also present in all of the ventricles, the base of the brain and the spinal cord (Fig. 14). The photograph clearly shows the path of the needle
4	0.25	¾ inch	No ink was observed on the surface of the brain, but it was abundant on the surface of the spinal cord (Fig. 15). A section of the brain revealed a considerable quantity of ink in the lateral ventricle (Fig. 16)

particles were found deposited in the nervous substance itself, but the India ink adhered to the surfaces of the brain or cord. There was some suggestion that with a shorter needle and a smaller amount of material the inoculum did not as readily diffuse through the cerebrospinal fluid and reach the spinal cord. On that basis,

therefore, neutralization tests were performed to compare the results of inoculation of a large and small volume of material, using needles of 7/8 inch and 1/4 inch length. The 7/8 inch needle was employed in order that the inoculum might reach the lateral ventricle, and the 1/4 inch needle in order that the material might be deposited into the cerebral cortex. The volumes selected were 1 cc., the usual amount inoculated in our neutralization tests, and 0.25 cc., the injection of which in previous experiments had indicated a tendency toward greater regularity.

Experiment III

Technique: A set of 10 duplicate test tubes, each containing 1.5 cc. of a 5 per cent virus filtrate and 1.5 cc. of pooled human convalescent serum, was incubated for 2 hours at 37° C. and kept in the refrigerator overnight. From each of 5 of these tubes 2 monkeys were inoculated intracerebrally with 1 cc. and 0.25 cc., respectively, using a 7/8 inch needle. From each of the remaining 5 tubes 2 monkeys respectively received 1 cc. and 0.25 cc. with a 1/4 inch needle. Immediately before injection 0.25 cc. of sterile India ink was added to each tube in order that the dispersion of the inoculum could be followed in those animals that developed poliomyelitis. The results are summarized in Table III.

The variable manner in which material inoculated intracerebrally diffuses is again illustrated by this experiment. The extent of the distribution is apparently not entirely governed by the amount inoculated or the length of the needle employed. It is interesting to note, however, that none of the monkeys receiving material with the 1/4 inch needle developed poliomyelitis, while 4 of the 10 monkeys inoculated with similar mixtures, but with 7/8 inch needles, became infected.

It has been observed [7-10] that upon dilution of a neutral mixture of virus and immune serum a subsequent disruption of the virus-serum union, the so-called dilution phenomenon, takes place and the mixture again becomes infective. The results of the above experiments suggested the possibility that if some quantity of the inoculum escapes from the area of inoculation into the cerebrospinal fluid, the dilution phenomenon may occur within the animal body and thus account for the occasional infectivity of an otherwise apparently inactivated mixture.

TABLE III

A Comparison of the Effects of the Volume of Inoculum and the Length of the Needle Used to Inoculate Neutral Serum-Virus Mixtures

⅞ inch needle				¼ inch needle			
Test tube No.	Monkey No.	Amount inoculated	Result	Test tube No.	Monkey No.	Amount inoculated	Result
		cc.				*cc.*	
1	1	1.0	Remained well	6	11	1.0	Dead, 6 days. Colitis. Cord Sections showed no evidence of poliomyelitis. India ink in subarachnoid space over the site of inoculation and over the spinal cord
	2	0.25	Paralyzed, 18 days. Ink found at site of inoculation and in lateral ventricle. None on surfaces of brain or cord		12	0.25	Remained well
2	3	1.0	Remained well	7	13	1.0	Remained well
	4	0.25	Remained well		14	0.25	Remained well
3	5	1.0	Dead next day of unknown cause. India ink at site of inoculation, and subarachnoid space above it	8	15	1.0	Remained well
	6	0.25	Paralyzed, 17 days. Ink found at site of inoculation and in lateral ventricle below it		16	0.25	Remained well
4	7	1.0	Paralyzed, 12 days. Ink found in subarachnoid space over both cerebral hemispheres, also at the base of the brain and throughout the spinal cord	9	17	1.0	Remained well
	8	0.25	Paralyzed, 9 days. Ink confined to site of inoculation only **		18	0.25	Remained well
5	9	1.0	Remained well	10	19	1.0	Remained well
	10	0.25	Remained well		20	0.25	Remained well
Controls *		1.0	Paralyzed, 4 days. Ink in all the ventricles, base of the brain and spinal cord	Controls *		1.0	Paralyzed, 10 days. Ink in the subarachnoid space, base of the brain and site of inoculation
		0.25	Paralyzed, 5 days. Ink at site of inoculation and lateral ventricle			0.25	Paralyzed, 10 days. Ink at site of inoculation only **

* The controls received a similar mixture of normal monkey serum, 5 per cent virus filtrate and India ink.

** These animals had been inoculated 2 months previously with serum-virus mixtures and survived the tests. The India ink was confined mostly to the cavitation of the sterile brain abscesses often seen in monkeys examined a few weeks after intracerebral injection (see Fig. 7).

Experiment IV

To determine whether or not direct admixture with the cerebrospinal fluid would prove this point, a group of 5 monkeys was injected with 1 cc. of mixtures of virus and serum, prepared as described above, but without India ink. The inoculations were made below the dura and into the subarachnoid space above the right cerebral hemisphere. This was accomplished by surgical trephining of the frontal bone at the usual site of inoculation. The area exposed was made large enough so that sufficient assurance could be had that the brain substance was not touched upon subdural insertion of the needle. All of these animals remained well during an observation period of 2 months, whereas a group of 4 controls inoculated similarly, but receiving a mixture of the virus and normal monkey serum, all developed poliomyelitis within the usual incubation period.

Of another group of 4 monkeys, each inoculated with 1 cc. into the cisterna magna, none showed evidence of infection while the four controls became paralyzed.

Experiment V

Since during the process of an intracerebral inoculation the brain is traumatized, we decided to investigate the combined effect of direct inoculation into the spinal fluid and simultaneous brain trauma. Accordingly, a group of 12 monkeys was inoculated with 1 cc. of a neutral serum-virus mixture (4 cc. of undiluted pooled human convalescent serum and 4 cc. of 5 per cent virus suspension) intracisternally. In 6 of these monkeys, immediately following inoculation, a sterile ⅞ inch needle was pushed into the brain at the usual site of inoculation and then withdrawn. The other 6 monkeys were treated in a similar manner with a ¼ inch needle. None of the 12 animals developed poliomyelitis, while the control in each group became paralyzed.

Experiment VI

Having no indication from the above experiments that the dilution phenomenon took place *in vivo*, we attempted to determine whether it would occur *in vitro*. Therefore, monkeys were injected from 3 tubes containing mixtures which consisted of equal quantities (4 cc.) of 5 per cent virus filtrate and human con-

valescent serum, 0.5 cc. of which was diluted, after the usual incubation time, with 2 cc. of normal monkey spinal fluid, giving a dilution ratio of 1:5. Twelve monkeys, 4 injected from each tube, received intracerebrally 1 cc. each of these mixtures prior to dilution with the spinal fluid, and 6 monkeys, 2 injected from each tube, received 1 cc. each of the mixtures following dilution. None of these animals developed the disease. Two controls receiving the virus and normal monkey serum and 2 others injected with virus, normal monkey serum and spinal fluid, all became infected.

Discussion

Our experiments indicate that the manner in which material, following intracerebral inoculation, is distributed, resembles in many respects that noted after intrathecal inoculation, as described by Hurst.[6] While certain variations in the course and extent of the diffusion were observed, some admixture of the material with the cerebrospinal fluid occurred in almost every instance. Generally, little of the material was found in the brain substance at the site of inoculation, except in monkeys that had received intracerebral inoculations in other experiments, in which case most of the ink was usually confined in the necrotic area of the previous site of inoculation. When larger amounts or longer needles were used, it appeared that the material more readily found its way into the cerebrospinal fluid. It may be stated, however, that the results are uncertain in so far as the final deposition of the material is concerned, but, in any event, some seepage into the cerebrospinal fluid is to be expected.

What, if any, correlation exists between the diffusion of material and its ultimate effect upon the infectivity of the neutral virus-serum mixtures cannot be answered from the data at hand. From the results of Experiment III, it appears that intracerebral inoculations with a ¼ inch needle tend towards greater regularity, since monkeys receiving the same mixtures were infected when the ⅞ inch needle was used. At first, we were inclined to attribute the consistent infectivity of the neutral mixtures inoculated with a short needle to the fact that less seepage might have taken place. However, admixture with the cerebrospinal fluid seems to have no bearing upon the infectivity of these mixtures since, as observed in Experiments IV and V, direct subarachnoid or cisternal introduc-

tion or intracisternal inoculation with simultaneous brain trauma caused no reactivation of the inactive mixtures.

We sought an explanation for irregularities on the ground that the dilution phenomenon may occur. Although this has been reported to take place with a mixture of immune serum and poliomyelitis virus by Schultz and his collaborators,[11] we were unable to demonstrate, in our experiments, that the dilution phenomenon ensued either *in vitro* or within the animal body.

It is, therefore, difficult to state what factors may account for unexpected infections when a presumably neutral mixture of serum and virus is injected into a group of monkeys. So far as this work has been carried, there is some indication that the use of a ¼ inch needle or direct intracisternal inoculation may be an improvement over the usual intracerebral method for performing neutralization tests, but further studies on a larger scale are necessary to verify this.

Summary

1. The distribution of material throughout the central nervous system of *Macacus rhesus* monkeys, subsequent to intracerebral inoculation, was studied by the injection of India ink. The experiments indicated that certain variations in the degree and extent of diffusion occurred, but in most instances the inoculum rapidly entered the cerebrospinal fluid either via the subarachnoid space or ventricles. Except for the site of inoculation, no carbon particles were found in the brain substance itself. The India ink was deposited on the surfaces of the brain or cord.

2. It appeared that when material was inoculated in larger amounts or with longer needles, it more readily entered the cerebrospinal fluid.

3. In an experiment where apparently neutral mixtures of poliomyelitis virus and immune serum were inoculated intracerebrally into monkeys with ¼ inch needles and ⅞ inch needles, none of the 10 monkeys injected with the shorter needles developed poliomyelitis, while 4 of 10 monkeys receiving the mixtures with ⅞ inch needles succumbed to the disease.

4. Direct inoculation of serum-virus mixtures into the subarachnoid space or cisterna magna did not render these neutral mixtures active, nor were these mixtures infective when inoculated

intracisternally accompanied by brain trauma, although controls similarly inoculated were uniformly infected.

5. Experiments devised to demonstrate the occurrence of the dilution phenomenon *in vivo* or *in vitro* were negative.

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DESCRIPTION OF PLATES

PLATE 45

FIG. 1. Dorsal view of the brain of Monkey No. 21. India ink is deposited on the surface of the frontal lobe.

FIG. 2. Ventral view of the brain of Monkey No. 21.

FIG. 3. Monkey No. 300 with skull exposed showing external leakage following injection. India ink is deposited in the area surrounding the trephined opening.

FIG. 4. Ventral view of brain and spinal cord of Monkey No. 300. India ink is deposited over the surfaces of the brain, cerebellum and spinal cord.

FIG. 5. Dorsal view of brain and spinal cord of Monkey No. 300 with section through the site of inoculation showing India ink deposited in the lateral ventricle and on the surfaces of the brain and spinal cord.

FIG. 6. Brain of Monkey No. 300 sectioned through another plane at the site of inoculation. Note the path of the needle and ink in the lateral ventricle.

FIG. 7. Brain of Monkey No. 180 sectioned through the site of inoculation. The India ink is confined to the necrotic cavity present as a result of an inoculation given 2 months previously.

FIG. 8. Dorsal view of brain and spinal cord of Monkey No. 88. The surfaces are heavily coated with India ink.

FIG. 9. Ventral view of the brain and spinal cord of Monkey No. 88 showing considerable deposit of India ink.

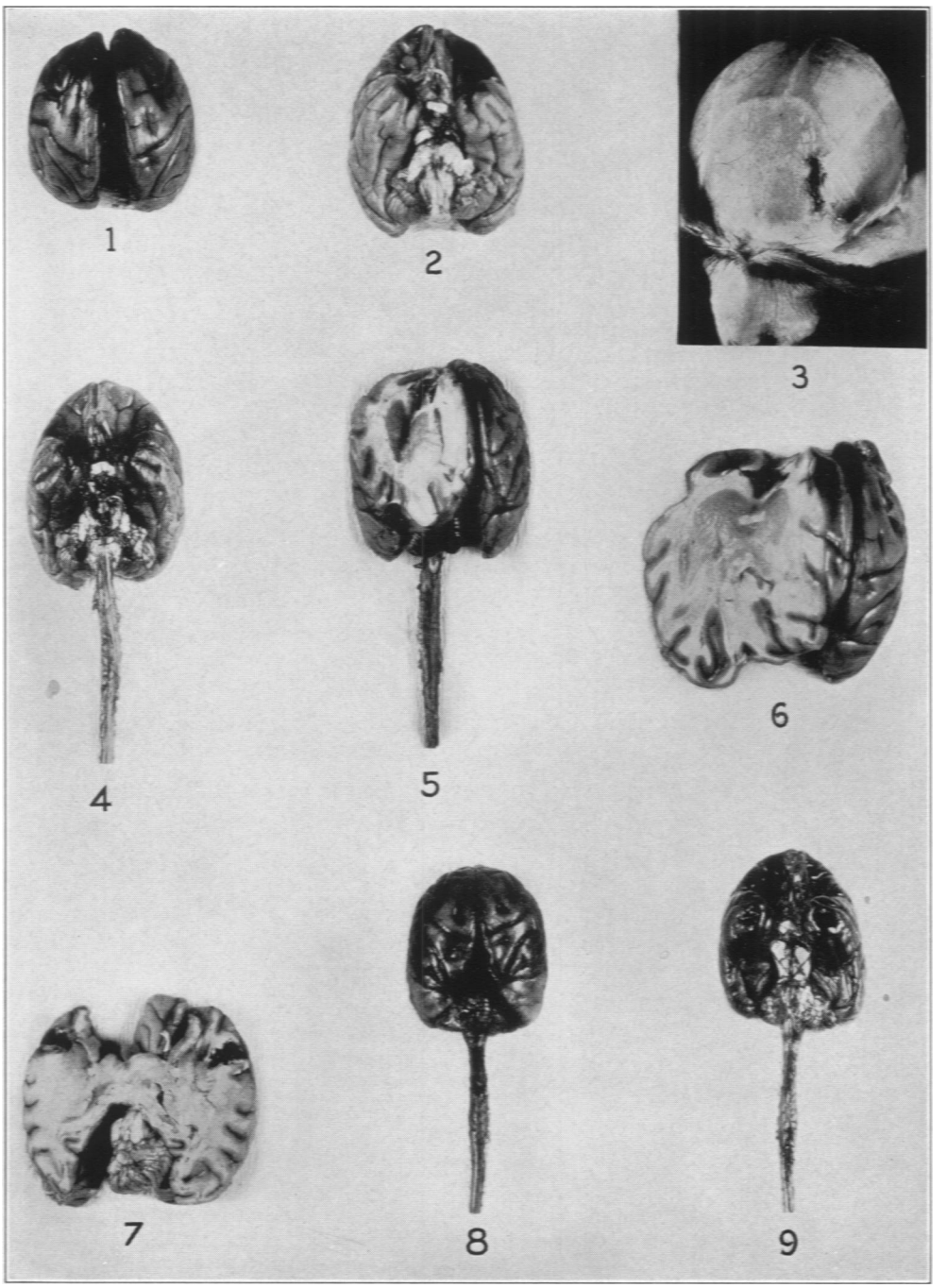

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PLATE 46

FIG. 10. Dorsal view of brain of Monkey No. 295. India ink is deposited on the entire surface of the right frontal lobe up to the fissure centralis.

FIG. 11. Sagittal section through the right hemisphere at the site of inoculation of the brain of Monkey No. 295. The area into which the inoculum has been deposited is distended.

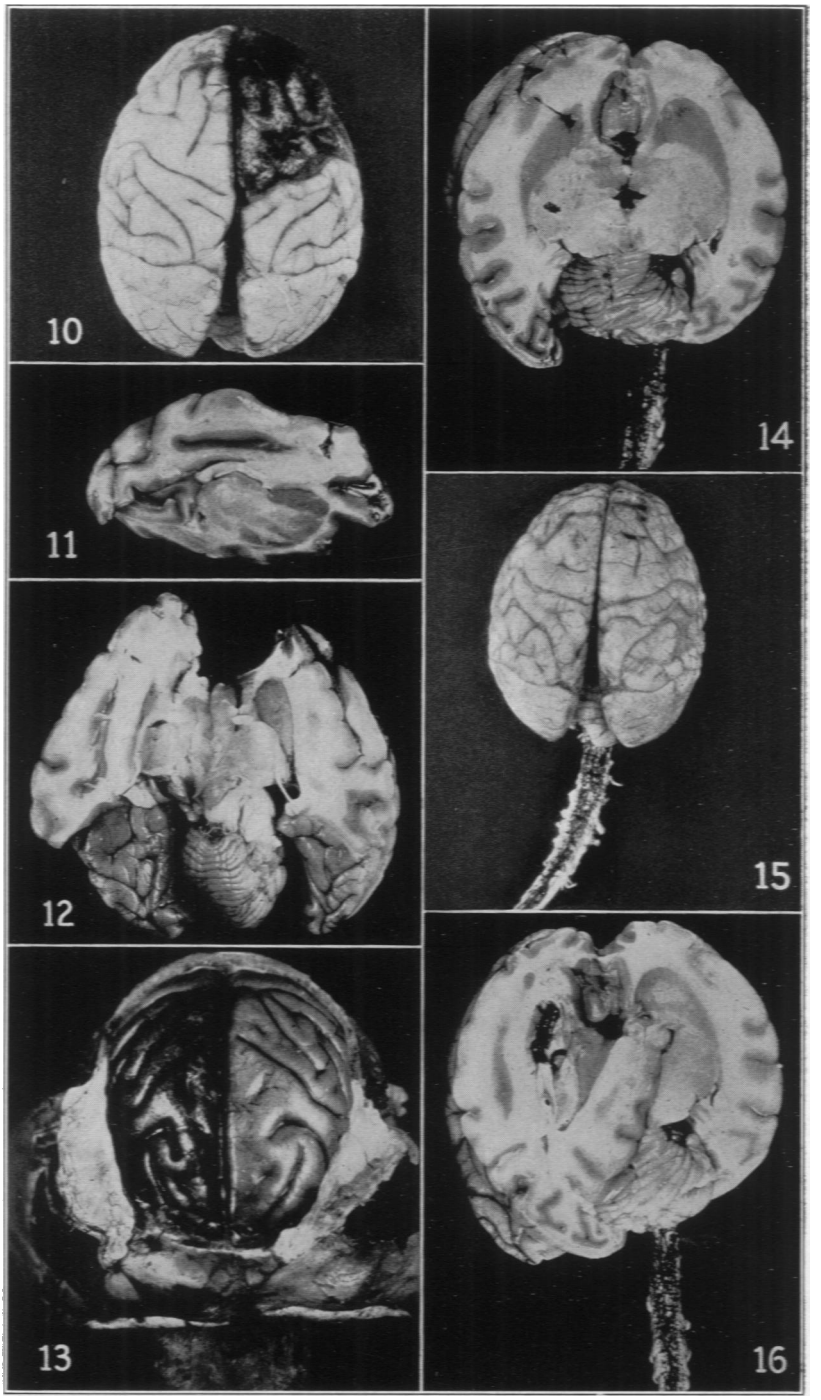
FIG. 12. Brain of Monkey No. 137 sectioned through the site of inoculation. Note the India ink on the surface and in the area inoculated. Distention is also present here but to a lesser degree than that noted in Monkey No. 295.

FIG. 13. Dorsal view of the brain of Monkey No. 225 *in situ* with dura removed showing deposit of India ink over the entire surface of the cerebral hemisphere opposite the side inoculated.

FIG. 14. Brain of Monkey No. 225 sectioned through the site of inoculation. India ink is present in the ventricles and on the spinal cord. The path of the needle is made clearly visible by the deposit of India ink.

FIG. 15. Brain and spinal cord of Monkey No. 207. No India ink is evident on the surface of the brain but it is abundant on the spinal cord.

FIG. 16. Brain of Monkey No. 207 sectioned through the site of inoculation. A large amount of India ink is seen in the lateral ventricle and on the spinal cord.



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