CONCERNING SURVIVAL AND VIRULENCE OF THE MICROORGANISM CULTIVATED FROM POLIOMYELITIC TISSUES.*

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Plates 12 to 17.

In previous reports[1] we described the cultural, morphological, and pathogenic properties of the minute microörganism cultivated from the nervous organs of human and experimental cases of poliomyelitis. We propose in the present paper to describe a strain of that microörganism which survived in a pathogenic state in cultures for a period of thirteen months.

The culture mentioned was obtained originally on November 14, 1912, from a monkey which had succumbed to an inoculation of the M A strain of the poliomyelitic virus,[2] and of which subcultures had proved pathogenic for monkeys in the third, seventh, and twentieth generations.[3] Two tubes of the culture in the ascitic fluid-agar-kidney medium in the second generation had been placed aside, one at room temperature, the other in the thermostat at 37° C., where they remained unmolested until December 20, 1913; that is, for something more than thirteen months. They were now transplanted into the solid medium, and from both sources growths of the minute microörganism were obtained. Hence the cultures survived a period of thirteen months under both conditions. Upon retesting for virulence six months later, that is, eighteen months after isolation, subcultures of this strain proved pathogenic.

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[1] Flexner, S., and Noguchi, H., *Jour. Am. Med. Assn.*, 1913, lx, 362; *Berl. klin. Wchnschr.*, 1913, l, 1693; *Jour. Exper. Med.*, 1913, xviii, 461.

[2] Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1909, liii, 1639.

[3] Flexner and Noguchi, *Jour. Exper. Med., loc. cit.*

MASS CULTURES IN FLUID MEDIUM.

From the solid subcultures fluid cultures were prepared by a special method to be described. The microörganism multiplies even more readily in a fluid medium consisting of a fragment of rabbit kidney and ascitic fluid overlaid with paraffin oil than in the solid medium. As has been previously explained, an initial culture has never been secured in the solid medium.[4] However, the growth in ascitic fluid is not abundant and hence it is not adapted to the preparation of cultures in mass.

To obviate this difficulty, as well as to eliminate when desired the ascitic fluid, Noguchi employed the double tube method which he devised to secure fluid cultures of spirochætæ.[5] In this method the tube carrying the solid medium, consisting of the kidney fragment, ascitic fluid, and agar, is superimposed upon a tube containing a kidney fragment and ascitic fluid alone or with bouillon, so as to bring the lower end of the agar medium in contact with the surface of the liquid by means of a narrow connecting tube. Thus prepared, the minute microörganism continues to multiply at the point of contact of the two media so that the growth passes into the liquid from which it settles to the bottom of the tube.

This method yields a richer growth than the ordinary ascitic fluid culture and is also suitable for obtaining cultures nearly devoid of ascitic fluid. But, on the other hand, the minute microörganisms begin quickly to degenerate in the medium when the bouillon is predominant. There is probably no actual multiplication of microörganisms, but merely an accumulation.

On this account it is necessary to retain the ascitic fluid. And in order to secure a more abundant growth still the method was modified as follows: Erlenmeyer or Florence flasks of approximately 100 cubic centimeter capacity are charged first with the solid medium. The fragment of kidney having been introduced into the flask, there is placed upon it about 0.5 of a cubic centimeter of a culture of the microörganism, after which fifteen cubic centimeters of a mixture of equal parts of ascitic fluid and 1 per cent. nutrient agar at 40° C.

[4] Flexner and Noguchi, *Jour. Exper. Med., loc. cit.*

[5] Noguchi, *Jour. Exper. Med.*, 1912, xvi, 211.

are carefully poured over the kidney, covering it with a solid layer one centimeter deep. After congelation fifty cubic centimeters of an equal mixture of sterile ascitic fluid and bouillon are introduced, and then a quantity of sterile paraffin oil sufficient to yield a layer of about one centimeter in height (figure 11). The incubation is conducted at 37° C.

The microörganism multiplies throughout the solid medium and, reaching the surface, grows into and within the ascitic fluid bouillon. The large surface of contact facilitates growth, so that at the expiration of about three days the fluid has become highly turbid from the contained microörganisms which have also begun to form a sediment on the surface of the agar medium. Viewed under the microscope the microörganisms are aggregated into small groups chiefly, although short chains are also present, and no degeneration whatever is detectable at this stage of development (figure 1).

ACTIVITY OF POLIOMYELITIC VIRUS.

The object of this communication is to record the fact that the microörganism isolated from poliomyelitic tissues may possess specific pathogenic properties after having been cultivated artificially for a period of a year or more, and after an almost indefinite degree of dilution of the original nervous tissues from which it was derived.

It will be recalled that the original cultures are prepared by inserting a fragment of the uncomminuted brain into the ascitic fluid-kidney medium contained in a deep test-tube.[6] When growth takes place, approximately 0.2 of a cubic centimeter is removed with a sterile pipette and transferred to a second deep tube of the medium in which it becomes diffused. The average content of the deep tubes is fifteen cubic centimeters, so that the fluid transferred into the second tube undergoes a seventy-five-fold dilution. From the second generation of the cultures so obtained successive fluid or solid cultures are prepared by a similar transfer of the medium.

The culture with which this paper deals in the second generation was in the solid medium, and from this the third generation, also

[6] Flexner and Noguchi, *Jour. Exper. Med., loc. cit.*

in solid medium, was prepared. From the third generation the combined solid and fluid cultures were prepared. Since the tubes carrying the third generation were of the same capacity as those of the second, the dilution of the materials carried over from the first generation was now approximately 1 : 5,625. The next, or fourth generation was made in the mixed medium so that the inoculated culture was retained by the congealed agar-ascitic fluid, except as it might diffuse into the overlying ascitic fluid-bouillon. If we assume that the diffusion was uniform, which of course it was not, the dilution brought about by the seventy-five cubic centimeters of combined solid and fluid media would now be approximately 1 : 843,750. From now on each successive culture yields such a rapidly diminishing content of any material originally carried over as soon to approach the infinite.

The minimal effective dose of the poliomyelitic virus represented by filtrates prepared from emulsions of nervous organs of 2.5 to 5 per cent. strength is about 0.001 of a cubic centimeter.[7] However, only occasional strains of the virus are as active as this. At an early period after the adaptation of the M A virus to monkeys it possessed this extreme degree of virulence. At the period at which the cultures were prepared the activity of the M A filtrate had diminished at least 100 times. In other words, the minimal effective dose had risen to 0.1 to 0.2 of a cubic centimeter. Since the quantity of brain tissue employed in the cultivations is about 5 per cent. of the volume of fluid (one gram of brain to fifteen cubic centimeters of ascitic fluid), the calculated potency of the fluid, irrespective of any increase of the virus and assuming that all diffused into the surrounding medium, would have been 0.2 of a cubic centimeter, which is the average quantity transferred to the tubes composing the second generation of cultures.

This consideration is affected by the period of survival of the poliomyelitic virus at 37° C. Incomplete observations only are directly available covering that point. Flexner and Lewis[8] observed that a Berkefeld filtrate mixed with rabbit serum and bouillon and inoculated at 37° C. was active at the expiration of ten days,

[7] Flexner, *Lancet*, 1912, ii, 1271; *Science*, 1912, xxxvi, 685.

[8] Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 45.

and Levaditi[9] noted a similar mixture to be still active after fifteen days. Hence it is doubtful whether the limits have been reached in the tests.

On the other hand, the limits have been reached in another series of tests which, in themselves, have an especial significance for us. Conceiving a possible interaction or symbiosis between nerve cells and the microbe of poliomyelitis, Levaditi[10] cultivated in normal monkey plasma *in vitro* fragments of the intervertebral ganglia of paralyzed monkeys. Successive transplantations having been made, the survival and increase of the virus were determined by inoculation. In one experiment the virus was still present at the fourth passage on the twenty-first day. In a second experiment it was present in the second passage on the thirteenth day, but not later. In the third and final experiment the first and second passages at seven and thirteen days respectively were effective, while later passages were devoid of infective power. The inoculation of the plasma alone in which the fragments grew was ineffective. Levaditi regards this failure as explained by the fact that the microbe multiplies only in association with living cells of tissue and not in the surrounding fluid. The point of these experiments that concerns us here relates to the period of survival of the virus in an effective state, the maximum being twenty-one days.

This result supports the observation reported by Flexner and Noguchi;[11] namely, that when culture tubes, set up with brain tissue, are tested for virulence as early as the second generation in the ascitic fluid medium, which is at the twentieth day or later after the original preparation, they are only exceptionally effective and then only when growth of the minute microörganism has taken place. The conclusions are therefore: (a) only exceptional strains of the culture are pathogenic for monkeys; (b) the virus diffusing into the fluid from the brain tissue soon deteriorates or undergoes dilution beyond the effective dose.

[9] Levaditi, C., *Presse méd.*, 1910, xviii, 44.
[10] Levaditi, *Compt. rend. Soc. de biol.*, 1913, lxxiv, 1179; 1913, lxxv, 202.
[11] Flexner and Noguchi, *Jour. Exper. Med., loc. cit.*

EXPERIMENTAL.

The fourth and subsequent generations of the culture, used for inoculation purposes, were prepared in the fluid medium in the manner for obtaining the abundant or mass growth of the microörganism. The strain was kept pure and ready for transfer to the fluid medium by implantation from time to time into the solid medium. The fluid is, of course, far more subject to contamination during manipulation than the solid culture; and contamination is more readily detected in the latter.

The inoculation of monkeys was performed not with a single dose but with several doses of the fluid culture. The reason was two-fold. Earlier studies had shown that only exceptional cultures of the microörganism are capable of infecting monkeys; and although the strain of microörganism now available had at the time of original isolation been effective, the retention of pathogenic power over the long period since its removal from the body was regarded as improbable. On the other hand, Flexner and Lewis[12] had observed that in process of immunization of monkeys with subcutaneous injections of the ordinary virus there sometimes supervened not increased resistance but paralysis. Hence successive inoculation offered two possible and opposite sets of effects: (1) infection might be induced, and (2) immunity might be secured. In the series of experiments to be described immediately the former was accomplished.

The culture in the second generation which survived thirteen months was subcultured into solid medium until June 2, or eighteen months after its isolation. It was in the ninth generation. The culture was now transplanted to the combined solid and fluid medium in flasks and subcultured in that medium until June 27, when it was employed for the inoculation of monkeys. At this time it had passed through more than twelve generations.

Experiment 1.—Macacus rhesus. June 27. 1 c.c. of the fluid culture was injected intraspinally by lumbar puncture. June 29. Lumbar puncture yielded turbid fluid containing red and white corpuscles and a few small masses of the injected microörganism staining indistinctly. No growth was obtained in cultures from the spinal fluid. July 3. No symptoms. 2 c.c. of the next generation of

[12] Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 1780; 1910, lv, 662.

fluid culture injected intraspinally. July 9. No symptoms. 4 c.c. of fluid culture injected intraspinally. July 20. No symptoms. A new series of fluid cultures had been prepared with a mixture of the stock solid cultures ranging from the fourth (made on Jan. 15) to the tenth (made on July 1), and of the fluid so prepared 4 c.c. were injected intraspinally. July 25. Legs spastic; animal ataxic. July 26. All four extremities paralyzed; animal prostrate; died the same day.

Autopsy.—The membranes about the spinal cord were edematous. The pial vessels at the base of the brain were congested. Section of the spinal cord at several levels showed gross lesions resembling those of poliomyelitis. A block of brain tissue was employed for cultivation tests. Portions of the spinal cord and medulla were put aside in 50 per cent. sterile glycerin.

Histology.—Sections from the spinal cord, medulla, and intervertebral ganglia were studied. The lesions are those of severe poliomyelitis. The meningeal and vascular infiltrations are pronounced (figures 2 and 3); the anterior gray matter of the spinal cord shows extensive necrosis and neurophagocytosis of nerve cells (figure 4); the medulla is the seat of focal infiltration of the vessels and nervous tissue (figure 5); the intervertebral ganglia exhibit nodular and diffuse cellular infiltration and necrosis of nerve cells. The accumulations in the interstitial tissue consist chiefly of mononuclear cells, while the necrotic nerve cells are invaded by polymorphonuclear neurophages (figures 6 and 7). A part of the ganglia show extensive diffuse necrosis of nerve cells and cellular infiltrations.

Experiment 2.—Macacus rhesus. The cultures inoculated were obtained from the same source as the preceding. June 27. 2 c.c. of the fluid culture injected into the peritoneal cavity. July 3. No symptoms. 4 c.c. of the culture injected into the peritoneal cavity. July 20. No symptoms. 10 c.c. of the mixed culture injected into the peritoneal cavity. July 30. No symptoms. 20 c.c. of the culture employed on July 20 injected into the peritoneal cavity. Aug. 3. Animal moves about slowly. Aug. 4. Prostrate. Aug. 5. Dead.

Autopsy.—The organs generally and the peritoneal cavity appeared normal. The spinal cord showed at several levels lesions resembling those of poliomyelitis. A block of brain tissue was removed for cultivation tests. Portions of the spinal cord and medulla were put aside in 50 per cent. glycerin.

Histology.—Sections from the spinal cord, medulla, and intervertebral ganglia were studied. All show typical poliomyelitic lesions. The meningeal infiltration is less than in experiment 1. The perivascular infiltrations in the medulla are especially pronounced (figure 8), and the sheaths of the larger vascular branches are edematous.

Experiment 3.—Control. When the first monkey of this series responded on July 26 with symptoms of poliomyelitis, a control inoculation was decided upon. This was carried out on July 30 at the same time that the last injection was given to experimental monkey 2 and with the culture employed for that inoculation. 4 c.c. of the fluid were injected intraspinally into a *Macacus rhesus.* No symptoms developed.

Experiment 4.—Two additional tests were made with the glycerinated specimens put aside from experiments 1 and 2. Emulsions of the spinal cord and medulla preserved in glycerin were injected intracerebrally into two *Macacus*

rhesus monkeys. The emulsion from monkey 2 was inoculated on Aug. 11; symptoms appeared on Aug. 15, progressed, and the animal was etherized on Aug. 24. The lesions in the spinal cord, medulla, and intervertebral ganglia were typical of poliomyelitis. The emulsion from monkey 1 was inoculated on Sept. 14; symptoms appeared on Sept. 24, progressed, and the animal was etherized on Sept. 26. The lesions in the nervous tissues are, in this instance, typical also. Some of the intervertebral ganglia show especially severe lesions in which, besides diffuse infiltration, the nerve cells in wide areas are hyaline and degenerated and the small periganglionic cells proliferated (figures 9 and 10).

The symptoms, lesions, and infectiousness of the glycerinated tissues, as represented by data given, are further indicative of the power possessed by the cultures to set up in monkeys experimental poliomyelitis.

Moreover, the microörganisms inoculated were recovered in the cultures prepared with the brain tissue of monkeys 1 and 2. The recovery was accomplished not readily but with difficulty, just as in the case of the brain tissue from human subjects or from monkeys infected with the ordinary virus. This point is significant; for it appears that the acquisition of parasitic properties by the microorganism unfits it for ready multiplication in artificial culture media.

DISCUSSION.

The data presented in the foregoing pages are believed to bear essentially on the question of the etiological relationship to epidemic poliomyelitis of the minute microörganism cultivated from poliomyelitic tissues.

Since the publication of our first full paper on this microörganism, several members of the staff of the Institute have made attempts to isolate it from nervous tissues and with a degree of success which attended the previous efforts. In other words, the cultivation was accomplished in some, but not in all instances, and success is determined by various circumstances; by the sample of ascitic fluid, the degree of anaerobiosis, the original sterility of the rabbit kidney, as well as by some other factor or factors at present unknown and uncontrollable. It is this last circumstance that makes the result uncertain; but it is one, fortunately, which experience tends to eliminate. Practice in the work of cultivation leads to greater assurance of success; and hence those who undertake the cultivation

should use many samples of ascitic fluid, employ different means of obtaining anaerobic conditions, and should not be easily discouraged by failure. In view of the difficulties mentioned, the fact should be emphasized that while this general method for the cultivation of refractory microörganisms devised by Noguchi has been employed extensively at the Institute, in no instance has a microörganism resembling that isolated from poliomyelitic tissues been obtained from any other source than that described.

It is significant and illuminating in this connection to find that when poliomyelitis has been incited in monkeys by means of cultures, the recovery of the microörganism is made with difficulty. This fact suggests the conclusion that the reacquisition of parasitic properties by the microörganism contained in cultures unfits it for ready multiplication in an artificial medium, which is, however, well suited to the growth of the same microörganism undergoing saprophytic development.

Perhaps this peculiarity of behavior may account for the wide fluctuation in pathogenic action shown by different cultures of the microörganism, since exceptional strains only are effective. Pathogenic power does not depend wholly on the culture generation and thus on distance from the parent stem. A non-virulent culture is already ineffective in the second generation, from which fact the side conclusion can be drawn that activity is not, probably, dependent on mechanical admixture of an invisible virus derived from the brain tissue employed in the cultivation.

The experiments reported in this paper not only confirm the earlier successful results, but extend them in a way to strengthen the evidence in favor of the etiological relationship of the minute microorganism to epidemic poliomyelitis. An experiment now to be described adds support from another side.

It had been established that the only certain way in which immunization to poliomyelitis may be experimentally secured in monkeys is by passing them through an attack of the disease. Inoculations of the poliomyelitic virus which are ineffective afford no protection to a subsequent effective inoculation. By carrying forward with great care successive injections of a virus, immunity may be established in some, but not in all instances. But the successive injections may

unexpectedly and for unknown reasons suddenly produce paralysis just when an immunity might have been inferred.[13]

And the same series of events have been observed with the cultivated microörganism. Successive injections of a culture wholly devoid of virulence yield neither infection nor immunity, and the monkeys so treated possess no neutralizing principles in the blood for the virus, and develop paralysis promptly after inoculation of an active sample. The following experiment illustrates this point.

Experiment 5.—Macacus rhesus. Successive subcutaneous injections of fluid cultures contained in the liquid portion of Noguchi's double tubes were given over a period of several months. The condition of the animal remained good throughout the treatment. Apr. 23. Withdrew blood in order to obtain serum for a neutralization test with ordinary virus. 1 c.c. of the serum failed to neutralize either 0.1 or 0.3 c.c. of the usual filtrate. June 12. Injected 0.2 c.c. of a filtrate of the ordinary virus intracerebrally. June 22. Excitable; ataxic; tremor. June 24. Arms paralyzed, legs weak. June 25. Dead.

Microscopical examination of the central nervous organs showed typical lesions of experimental poliomyelitis.

In contradistinction to the results of this experiment, which shows the lack of immunizing power of inert cultures of the minute microorganism, are experiments 1, 2, and 3, which establish the important fact that a culture may be ineffective at the first, and effective at a subsequent injection, although the material employed for the final injection may prove inactive in a fresh monkey. The deduction from the experiments seems to be that in the course of successive inoculation, under certain circumstances, the resistance of the monkeys is gradually suppressed, and that this undermining takes place more quickly when the cultures are introduced into the cerebrospinal membranes than when injected into the peritoneal cavity.

SUMMARY.

The minute microörganism cultivated from poliomyelitic tissues survives and maintains its pathogenicity in cultures for more than one year.

Upon inoculation into monkeys poliomyelitis may fail to appear upon the first injection and yet follow from the effects of successive injections of the culture.

[13] Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 1780; 1910, lv, 662; *Jour. Exper. Med.*, 1910, xii, 227. Flexner, *Jour. Am. Med. Assn.*, 1910, lv, 1105.

Inoculations of cultures into monkeys which fail to produce paralysis may fail also to induce resistance or immunity. In this respect the action of the cultures resembles that of the virus as contained in infected nervous tissues.

The lesions occurring in the spinal cord, medulla, and intervertebral ganglia of the monkeys which respond to the several inoculations of the cultures are identical with those present in the nervous organs of the animals responding to injection of the ordinary virus.

Glycerinated nervous tissues derived from the monkeys responding to several injections of the cultures transmit experimental poliomyelitis to monkeys upon intracerebral inoculation.

The microörganism inoculated may be recovered in cultures from the monkeys which develop poliomyelitis; but cultivation from the brain tissue is attended with the usual difficulties surrounding the obtaining of the initial growth.

The microörganism cultivated from poliomyelitic tissues is adapted with difficulty to saprophytic conditions of multiplication, but once adapted growth readily takes place upon suitable media. When, however, as a result of inoculation into monkeys, the parasitic propensities of the microörganism are restored, it again displays the marked fastidiousness to artificial conditions of multiplication present at the original isolation.

The experiments reported in this paper afford additional strong evidence in support of the view already expressed, that this microorganism bears an etiological relationship to epidemic poliomyelitis in the human subject and to experimental poliomyelitis in the monkey.

EXPLANATION OF PLATES.

PLATE 12.

FIG. 1. Sediment showing the minute microörganisms after three days' growth in mixed ascitic fluid and bouillon in a flask employed for mass cultivation. Giemsa stain. × 1,000.

FIG. 2. Spinal cord showing meningeal cellular infiltration extending into the anterior median fissure. Experiment 1. × 100.

PLATE 13.

FIG. 3. Spinal cord showing perivascular cellular infiltration in the meninges and white matter. Experiment 1. × 85.

FIG. 4. Anterior horn of spinal cord showing necrosis and neurophagocytosis of ganglion cells. Experiment 1. × 170.

PLATE 14.

FIG. 5. Medulla oblongata showing focal cellular invasion of the gray matter and perivascular cellular infiltration. Experiment 1. × 110.

FIG. 6. Intervertebral ganglion. Interstitial cellular infiltration. Experiment 1. × 250.

PLATE 15.

FIG. 7. Intervertebral ganglion. Two necrotic ganglion cells invaded with neurophagocytes. Experiment 1. × 200.

FIG. 8. Medulla oblongata. Perivascular cellular infiltration in floor of fourth ventricle. Experiment 2. × 106.

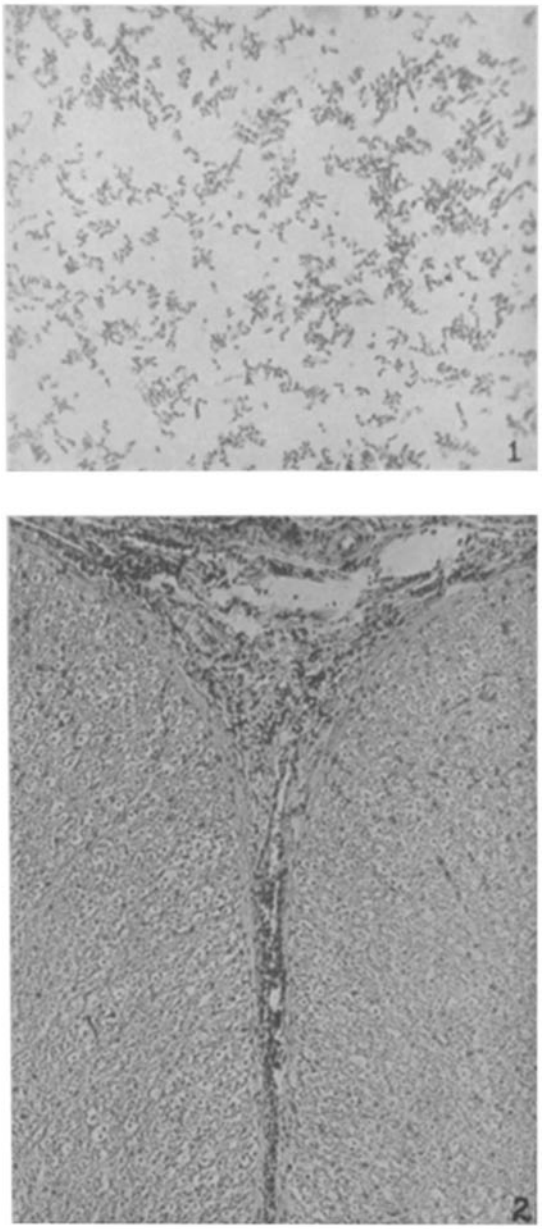
PLATE 16.

FIG. 9. Intervertebral ganglion. Diffuse cellular invasion of interstitial tissue and necrotic ganglion cells. Experiment 4. × 150.

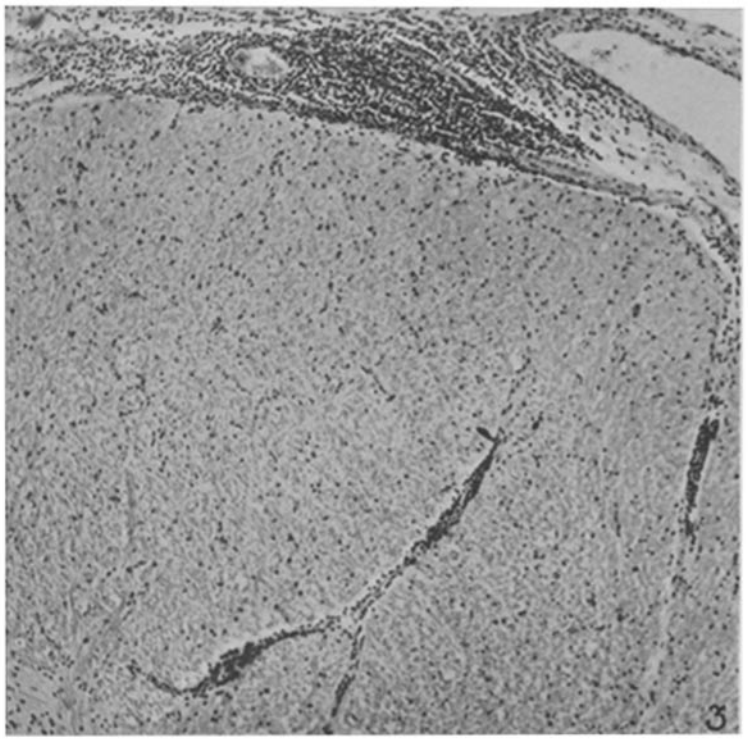
FIG. 10. Same as figure 9, showing several ganglion cells in which the peri-ganglionic cells have proliferated and neurophagocytes have invaded degenerated cells. × 250.

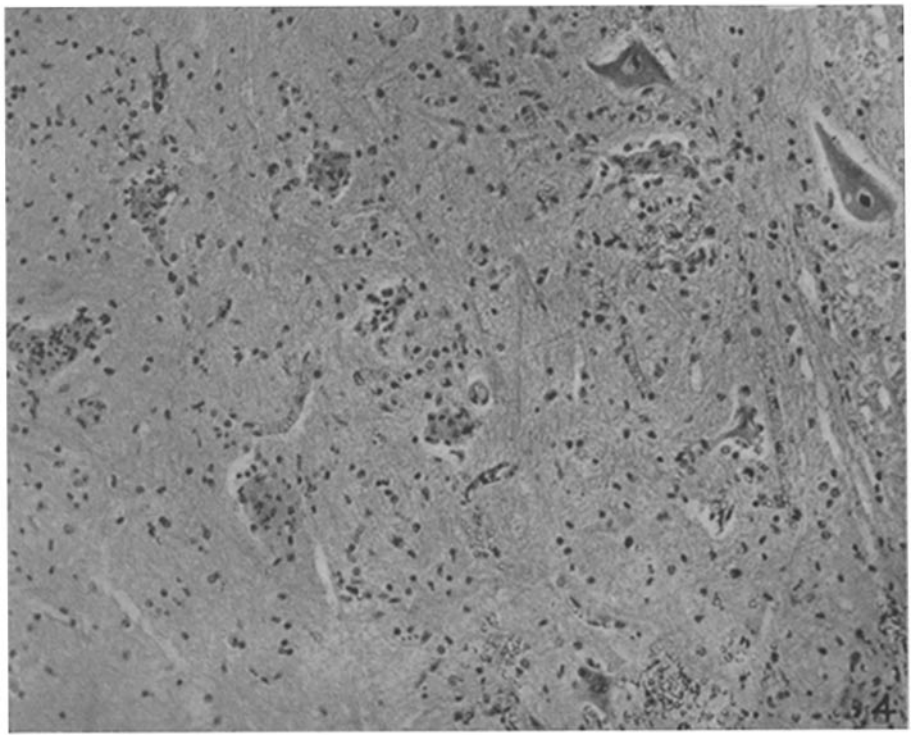
PLATE 17.

FIG. 11. Mass culture. 1, uninoculated control flask; 2, inoculated flask; a, layer of paraffin oil; b, layer of ascitic fluid-bouillon; c, layer of ascitic fluid and agar containing fragment of rabbit kidney.



(Flexner, Noguchi, and Amoss: Survival and Virulence of Poliomyelitic Microörganism.)





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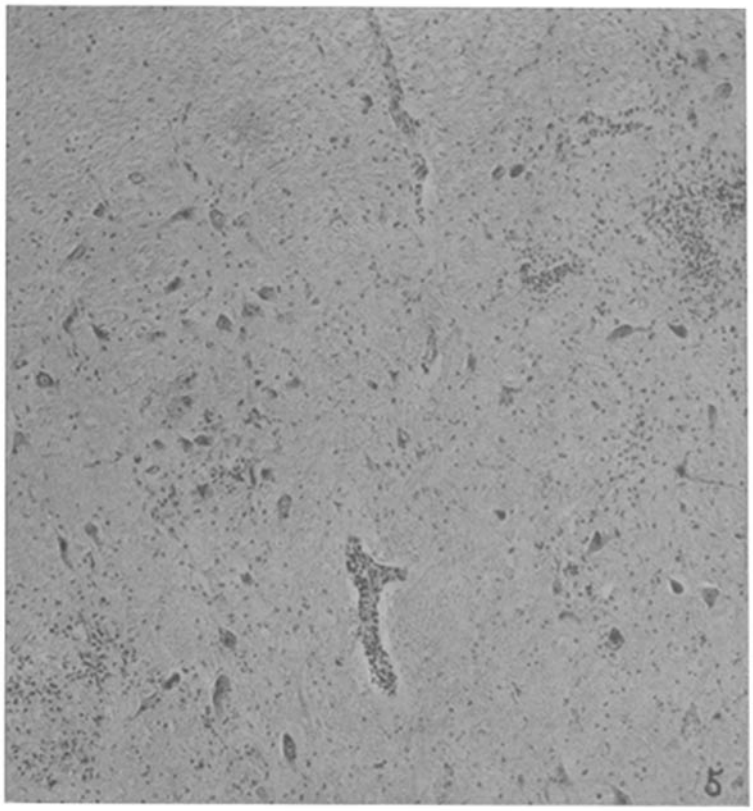

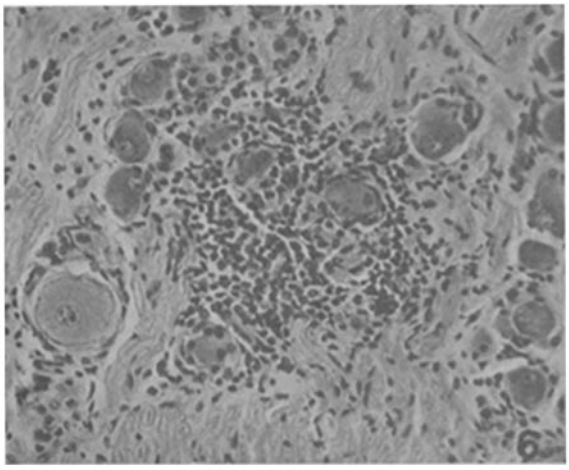

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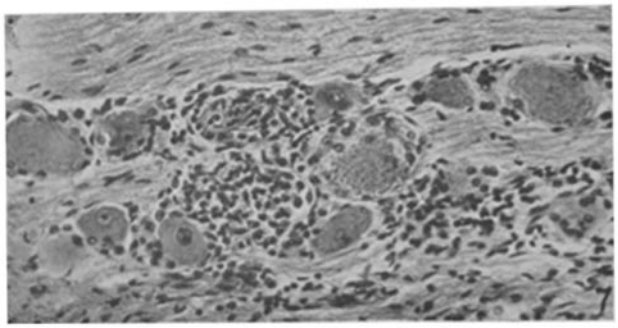

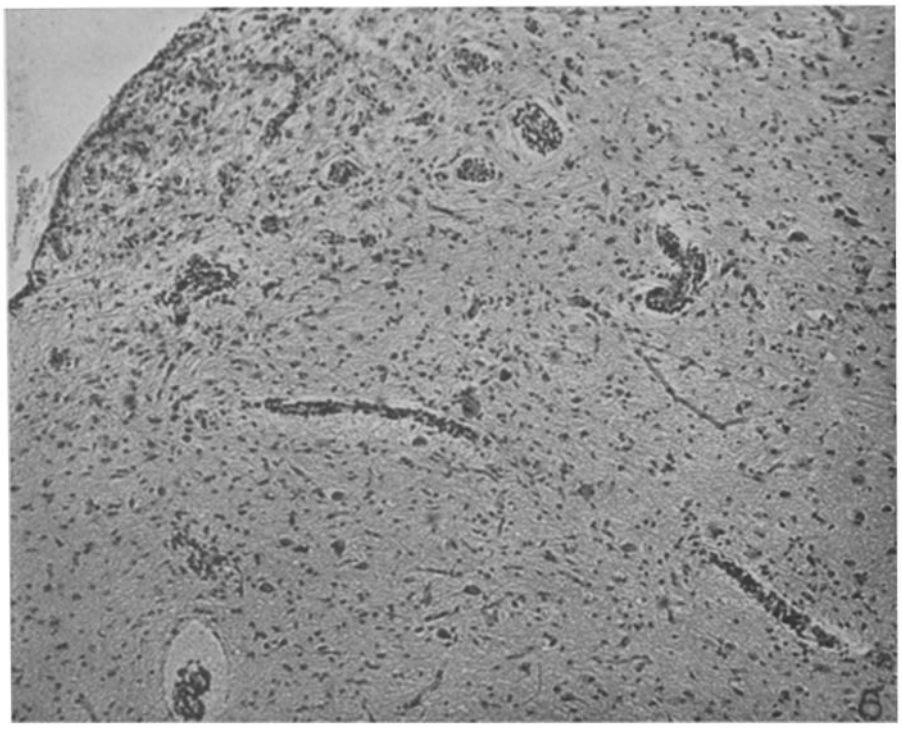

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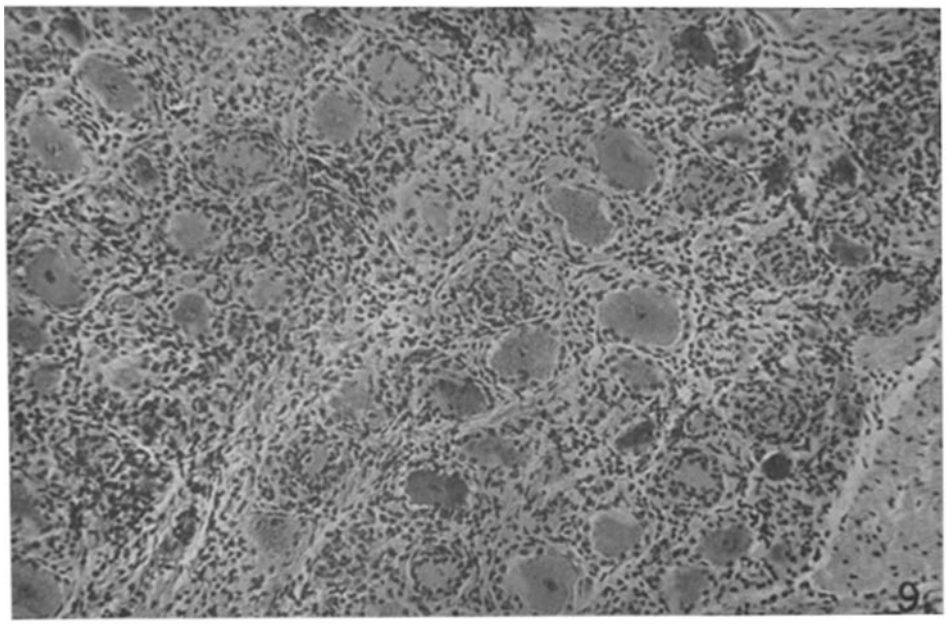

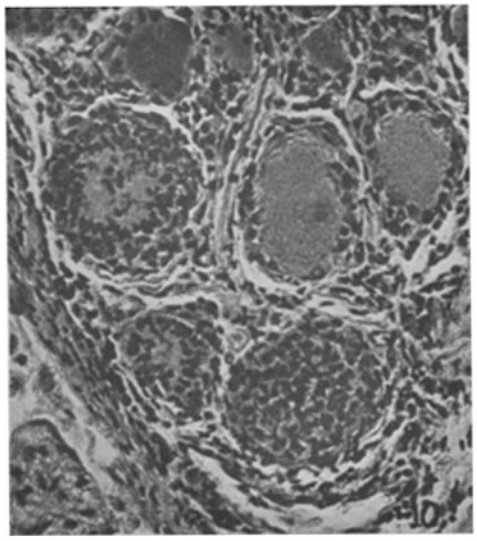

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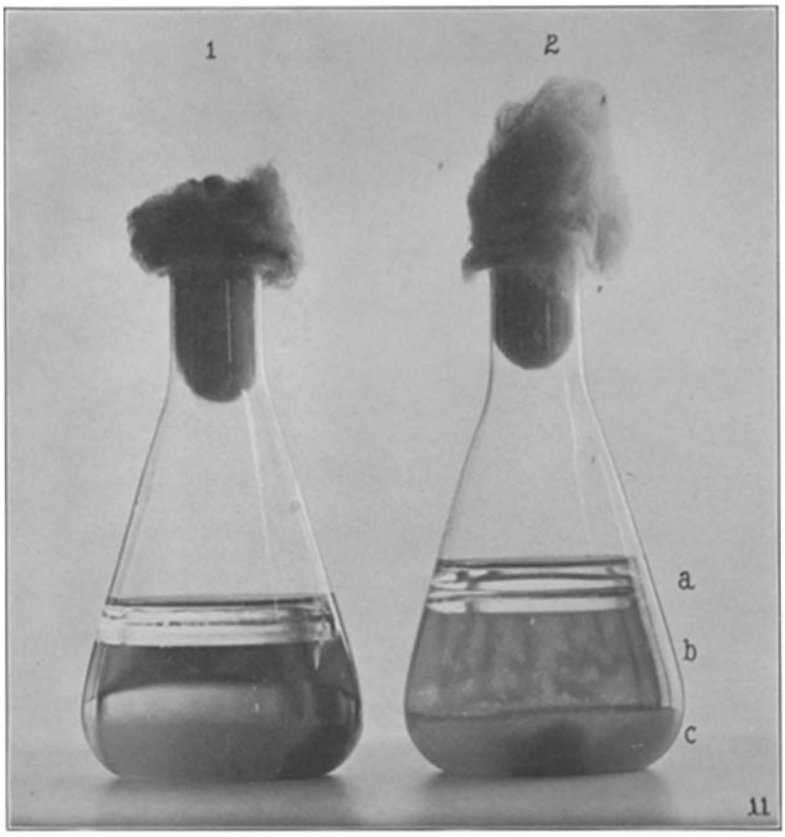

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