EXPERIMENTS ON THE CULTIVATION OF THE MICROÖRGANISM CAUSING EPIDEMIC POLIOMYELITIS.*

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Plates 38 to 41.

Several attempts have been made to demonstrate under the microscope and to develop in artificial cultures the microörganism causing epidemic poliomyelitis, but hitherto unsuccessfully. Giersvold[1] cultivated certain micrococci from the cerebrospinal fluid, and Fox[2] a bacillus from the circulating blood of poliomyelitic patients, but both have now been discredited as causes of the disease. The discovery of the filterable nature of the causative agent independently by Flexner and Lewis[3] and Landsteiner and Levaditi[4] not only disposed finally of the claims made for the above mentioned bacteria, but also discouraged renewed efforts at cultivation. Flexner and Lewis[5] noted and Levaditi[6] confirmed a clouding of serum bouillon by an aqueous Berkefeld filtrate of the central nervous tissues of poliomyelitic monkeys, but the phenomenon proved to be due to protein precipitation and not to multiplication of a living parasite. Proescher[7] has recently stained certain coccus-like bodies in films prepared from the central nervous organs of monkeys infected experimentally with the virus of poliomyelitis. The precise nature of these bodies has still to be worked out; ap-

* Received for publication, June 20, 1913.

1 Giersvold, M., *Norsk Mag. f. Lægevidensk.*, 1905, iii, 1280.

2 Dixon, S. G., Fox, H., and Rucker, J. B., *Pennsylvania Department of Health, Laboratory Report,* March 2, 1911.

3 Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1909, liii, 2095.

4 Landsteiner, K., and Levaditi, C., *Compt. rend. Soc. de biol.*, 1909, lxvii, 592.

5 Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1910, liv, 45.

6 Levaditi, C., *Presse méd.*, 1910, xviii, 44.

7 Proescher, F., *New York Med. Jour.*, 1913, xcvii, 741.

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parently they have not yet been demonstrated in human nervous tissues, and similar bodies have been found in the nervous organs of animals experimentally infected with rabic materials. The comparatively large size of many of these coccoid bodies leaves it doubtful whether they can bear relation to filterable viruses.

The successful cultivation of spirochætæ, both parasitic and saprophytic, by Noguchi[8] provided the impulse for a renewed attempt at the cultivation of the microörganismal cause of poliomyelitis. The application by Noguchi of his method to the central nervous tissues derived both from human and from experimental (monkey) examples of poliomyelitis has yielded results of an entirely different order from those previously obtained. The results have already been described briefly in our Fifteenth Note[9] and they will be described more fully in this place.

NATURE AND SOURCES OF MATERIALS.

During the several years that epidemic poliomyelitis has prevailed in the eastern United States we have collected many specimens from affected human beings and monkeys. Portions of nervous and other organs were regularly set aside in 50 per cent. sterile glycerin in the refrigerator. Hence there were available for the cultivation tests both fresh specimens yielded by recent autopsies upon human beings and monkeys and material preserved for periods ranging from a few days to many months. The new experiments upon cultivation were begun during the summer of 1912, at which time many acute cases of poliomyelitis were being cared for in the Hospital of the Rockefeller Institute, where a number of autopsies came to be performed upon fatal cases. Thus the cultivation was carried out simultaneously upon fresh, sterile, or practically sterile (for ordinary bacteria) specimens derived from human beings and from monkeys experimentally inoculated, and upon glycerinated specimens from both sources.

The material subjected to cultivation should be taken as soon after the death of the person or animal as possible, and should be obtained when possible in an aseptic condition. While any part

[8] Noguchi, H., *Jour. Exper. Med.*, 1911, xiv, 99; 1912, xv, 90; xvi, 199, 211.
[9] Flexner, S., and Noguchi, H., *Jour. Am. Med. Assn.*, 1913, lx, 362.

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of the central nervous system is suitable, the brain is to be preferred for the reason that it can be obtained more readily than the spinal cord or the intervertebral ganglia in an aseptic state. The dura covering one of the hemispheres having been seared with a hot instrument is incised with sterile instruments and the underlying cortex scrupulously exposed. From this a piece of about two cubic centimeters extending into the white matter is excised and placed at once into a sterile dish. If desired, portions of the cerebellum may be obtained in a similar manner, and by removing the cerebral hemispheres and then lifting up the cerebellum the medulla may be exposed in an aseptic manner, so that portions may also be taken.

It is advisable to make the initial inoculation with fragments as well as with emulsions or with filtrates of the nervous tissues. The fragments are to be preferred since they require almost no manipulation. When, however, the original material is suspected or known to be contaminated, it is advisable to employ filtrates at the same time. The filtrates are prepared by grinding the nervous tissue in strength of about 5 per cent. with sand in distilled water or physiological salt solution. The resulting emulsion is shaken in the machine for about thirty minutes, after which the suspended matter is removed by centrifugalization and the supernatant fluid is pressed through a Berkefeld filter, N. or V.

METHOD OF CULTIVATION.

The culture medium consists essentially of human ascitic fluid, to which has been added a fragment of sterile fresh tissue. In contradistinction to the spirochætæ, for which sheep serum water supplies a suitable medium, the microörganism isolated from the nervous organs of cases of poliomyelitis demands ascitic fluid. In obtaining the initial culture, the exclusion of oxygen is necessary, but it is not, as in the case of *Treponema pallidum,* essential that the inoculated tubes should be placed in an anaerobic jar; it suffices to cover the liquid with a deep layer of sterile paraffin oil. In some instances, however, the initial growth can be obtained more certainly when the anaerobic jar is employed.

Test-tubes measuring 1.5 by 20 centimeters are chosen. Into

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each of a series a fragment of the usual size of sterile fresh kidney of the normal rabbit is placed. Next is added a fragment of corresponding size of the cerebrum or other part of the brain or spinal cord. Upon these are poured about fifteen cubic centimeters of sterile ascitic fluid and finally about four cubic centimeters of the sterile paraffin oil. The ascitic fluid must have been originally sterile, since sterilization by fractional heating or filtration renders it unsuitable. The control tubes consist of the ascitic fluid and kidney fragment without brain tissue and ascitic fluid plus brain tissue without the kidney fragment. It is best to prepare two sets of the cultures, one to be placed in the anaerobic jar and the other kept outside. The incubation is conducted at the temperature of the thermostat, namely, at 37° C.

The culture tubes in the anaerobic jars should not be disturbed for from seven to twelve days, while those not within the jar may be inspected daily. Any tube showing marked turbidity, coagulation, or gas production within one or two days may be set aside as grossly contaminated. The tubes which remain clear or show only slight turbidity may none the less be contaminated. To exclude contamination small quantities of the medium are removed with pipettes and stained for bacteria in the ordinary way and cultivated upon the usual solid or fluid media. Obvious bacteria in the films as well as any growth taking place in the ordinary media in the presence of air may be put down as contaminations and the tubes containing them discarded.

Gross contaminations having been excluded, the peculiar microorganism obtained from the poliomyelitic nervous tissues exhibits the following growth characters. At the expiration of about five days' incubation a faint opalescence appears about the fragments of tissue at the bottom of the tube. This opalescence can be gradually diffused through the tube by gentle shaking (agitation), in the course of which it is observed that the turbidity about the tissues was really greater than was at first apparent (figure 1, fluid culture). The control tubes when not grossly contaminated either remain perfectly clear or a slight granular precipitate of washed out particles of tissue forms about them. The two appearances are quite distinct and become more pronounced after another period of

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three to five days, during which time the opalescence first described extends into the upper portion of the medium, while in the controls the precipitate gathers more and more at the lower end of the tubes. At the expiration of ten or twelve days the diffuse opalescence of the medium in the tubes in which growth has been observed to take place begins to diminish as sedimentation sets in, during which minute, irregular particles form, heavy enough to fall slowly to the bottom of the tube.

The corresponding tubes contained in the anaerobic jar when examined on the seventh day show a similar opalescent growth but slighter than that just described. When replaced in the jar and returned to the thermostat the opalescence increases so that at the expiration of another period of five or six days it approximates that present at the end of one week in the tubes kept outside the jar.

Cultivation may also be performed in a solid medium, consisting of ascitic fluid and sterile rabbit tissue, to which a suitable quantity of 2 per cent. nutrient agar has been added in order to produce a solid mixture. The melted agar, cooled to a temperature of 40° C., is mixed with the ascitic fluid in the tube containing the fragment of tissue, also previously heated to 40° C. After cooling and solidification the medium should be sufficiently firm to maintain its stability. In this medium an initial growth, aside from contaminations, from poliomyelitic material has never been obtained, but once growth has been secured in the fluid medium it is possible, but not in all instances, to transmit it to the solid medium. Often successful transfer from the fluid to the solid medium is not accomplished until about the third generation of the growth has been obtained in the fluid medium. The changes indicative of growth in the solid medium begin about the fragment of tissue and appear first as a diffuse opalescence which gradually aggregates into visible grains of minute size that are recognized as colonies. Several days are usually required before this initial change in the medium becomes apparent. The rapidity with which it does become apparent is affected by the generation of the culture and hence by its adaptation to the medium. Gradually the opalescence rises in the medium within the tube, until it reaches within about three centimeters of the surface of the medium, at which a sharp line of demarcation

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appears. When the number of colonies developing is large the individuals are so minute as to give a diffuse grayish appearance to the medium, which requires a lens to bring out the separate colonies. When the number of colonies developing is smaller they may reach a diameter of about one third of a millimeter and hence become visible as such to the naked eye, to which they appear round in shape and grayish white in color. Under favorable conditions the opalescence indicating growth about the tissue fragment becomes apparent at the end of forty-eight hours and distinct after seventy-two to ninety-six hours, although several days may be required before the ascent of the growth to the line of demarcation, at which the presence of oxygen brings about a cessation of development (figure 2, solid culture).

The description just given relates to cultivation with fragments of fresh central nervous organs in the ascitic fluid and in the presence of sterile rabbit tissue. It is, however, possible to secure cultivation even in the absence of the rabbit tissue. For this purpose a somewhat larger fragment of the poliomyelitic brain is placed in the tubes containing the ascitic fluid, which is then covered with the layer of paraffin oil. After incubation at 37° C. the initial growth takes place, but with less constancy than when the rabbit tissue is also present. When it occurs the growth is usually so slight that the opalescence remains absent or nearly so, and it becomes necessary to transplant into a medium containing fresh rabbit tissue to establish its existence.

Just as it is possible to eliminate the fresh rabbit tissue so is it possible to dispense with the ascitic fluid, which may be substituted by an extract prepared from the brain tissue or even sheep serum water. The extract is prepared by grinding in a porcelain mill and then afterwards shaking for half an hour in a machine twenty grams of fresh normal brain of a *Macacus rhesus* monkey in 500 cubic centimeters of distilled water or Ringer solution. The emulsion is centrifugalized and the supernatant fluid passed through a Berkefeld filter, V. The clear sterile filtrate is used to replace the ascitic fluid, but in order to obtain growth in this fluid or in serum water it is necessary to employ a fragment of rabbit tissue. A successful result has never been obtained with either, in the absence of the

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rabbit tissue, from which it follows that they are inferior to the ascitic fluid as a medium of cultivation.

In several parallel experiments an emulsion of the brain tissue was compared with the brain fragment as the source of the culture and it was found that growth was obtained with greater difficulty and somewhat less frequency from the emulsion than from the whole tissue fragment. While growth was detected during the first week in the corresponding tubes of fragment and emulsion, a week later the growth may have disappeared from the emulsion tubes, from which it was concluded that certain constituents of the nervous tissue, liberated by the emulsion, exerted an injurious effect upon the developing microörganism. In support of this supposition it was found that when an emulsion or extract of the brain was added to an active culture prepared in the usual way the microorganisms previously readily demonstrable in stained preparations became indistinguishable. That they do not entirely disappear under these circumstances is shown by the fact that when transplanted into the ascitic fluid tissue medium a growth reappears.

The Berkefeld filtrates of emulsified tissue also give rise to cultures, but less constantly than the original nervous material, from which the filtrates were prepared. In cultivating with the filtered extract, the ascitic fluid rabbit tissue medium should always be employed, and two sets of tubes prepared, one to be placed in the anaerobic jar and the other to be kept outside. The initial appearance of growth is somewhat delayed, so that an average of ten days of incubation may be necessary before the characteristic alteration of the medium becomes apparent. In no instance was success attained with the filtrate in the absence of the rabbit tissue.

Glycerinated human and monkey poliomyelitic tissues were employed as fragments, and, in the case of monkeys, as filtrates for cultivation. The specimens had been kept in 50 per cent. glycerin at a temperature of 2 to 4° C. for periods varying from twenty-five days to one year. Portions of the brain or spinal cord so preserved were cut off with sterile scissors, washed thoroughly in sterile distilled water, and placed in the ascitic fluid tissue medium. The resulting growths, when they occurred, produced the characteristic appearances already noted. On the whole, the glycer-

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inated tissues gave somewhat more inconstant results than the fresh material.

Once the initial growth from any of the sources mentioned has been secured it is, as a rule, possible to obtain subcultures by transplanting about 0.2 of a cubic centimeter of the original culture per tube into the next series. Since the initial growth begins about the tissue at the bottom of the tube it is advisable to take the fluid for transplantation from this location. Subcultures are obtained more constantly in the fluid than in the solid medium. Hence growth will sometimes appear in the former and not in the latter. Once the microörganism has been adapted to the conditions of artificial cultivation, transplantation to the solid medium may be effected. The adaptation is indicated by a more rapid and abundant growth in the fluid medium, and its occurrence is promoted by the presence of fresh sterile tissue and is prejudiced by its absence. Among the monkey tissues was one specimen which had been glycerinated for nine months, and from which a growth was obtained with a Berkefeld filtrate.

In the course of the growth the sterile kidney turns first pink and then gray in color, but no obvious solution or hemolysis of the red corpuscles contained in the tissue occurs.

The addition of various carbohydrates and alcohols, such as glucose, saccharose, lactose, maltose, gelactose, levulose, mannite, glycogen, dextrin, inulin, dulcite, and arabinose, to the ascitic fluid or sheep serum water in proportion of 0.5 per cent. did not affect the cultivation, and the growths when pure had no apparent effect upon them, since neither acid nor gas are produced.

Litmus milk, to which fragments of the sterile fresh tissue have been added, affords a medium in which growth will take place without producing any alteration in the appearance of the medium.

NUMERICAL RESULTS OF CULTIVATION.

In a series of thirty-three experiments with the nervous tissue of monkeys, comprising thirteen specimens of brain tissue, two of brain emulsion, and eighteen filtrates of brain and spinal cord from twenty-four animals infected from two different strains of human poliomyelitic virus, an initial growth was obtained in nineteen in-

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stances, of which sixteen proved to be pure and three mixed cultures. Of these, pure subcultures were obtained thirteen times, namely, eight times from brain tissues, four times from filtrates, and once only from emulsion. In a second series in which human nervous tissues were directly employed, eight successful cultivations were secured. One was from fresh tissue, three were from recently glycerinated tissues, and four from tissues which had been glycerinated for many months. In two specimens of the last four, initial cultures but no subcultures were obtained. In all the others subcultures resulted and were maintained alive for an indefinite period.

MORPHOLOGICAL CHARACTERS.

Uncontaminated cultures of the microörganism from the various human and monkey sources already mentioned possess common morphological and staining characters. Fluid cultures viewed under the dark-field microscope exhibit among the innumerable dancing protein and other granules present minute bodies, globular in form, hanging together in short chains, pairs, and small masses, devoid of independent motility and distinguishable with difficulty as a special class among the indefinite granules present. Stained preparations, on the other hand, bring out unmistakable microorganisms grouped in the three ways stated and of very minute size. Staining may be accomplished with more or less difficulty in several ways, but the two methods which have thus far yielded the most satisfactory results are those of Giemsa and Gram.

The Giemsa stain may be applied in two ways according as a rapid or a somewhat slower but better result is desired. In the first, the film, either upon a slide or a cover-glass, is allowed to become air-dry, when it is fixed over the flame, and several drops of Giemsa solution are dropped upon it and diluted with about twenty drops of distilled water. After gentle mixing of the dye and water the preparation is held over the flame until a trace of vapor rises. Care is exercised to avoid boiling. The preparation is then washed in a stream of distilled water, pressed between blotters, and dried. This method does not yield uniform staining or constancy of shade, but is useful for rapid examinations. The second method is the one usually employed. The film having been air-dried is

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fixed in methyl alcohol for about an hour, washed in distilled water, and is then immersed in the solution containing one drop of the stain per cubic centimeter of distilled water, in which the film remains for from two hours to twelve to fourteen hours (over night). Gram's stain is applied in the usual way, using gentian violet as the dye and safranin as the counterstain. The microörganism behaves variably towards the Gram stain. When the medium in which it is grown contains peptone, it retains the stain more assiduously than when it is devoid of that substance. Then the intensity with which the Gram stain is retained varies with the age of the culture. The older the culture the more strongly the gentian violet is held. While the grouping is of course identical whichever stain is employed, the apparent size of the microörganism is affected by the dye. When the gentian violet is retained the individuals appear larger than they do with either the safranin or the Giemsa stain. Loeffler's alkaline methylene-blue, carbol-fuchsin, and carbol-gentian violet all stain the microörganism slightly or without affording sharp differentiation. The microscopical appearances of the microörganism vary somewhat according to whether the growth is in a fluid or solid medium and peptone is present or absent. Peptone and possibly other constituents of the bouillon render the microörganisms somewhat larger as well as enable them to retain more firmly the Gram stain.

Films prepared from the lower layer of the ascitic fluid culture about the sterile rabbit tissue and stained by Giemsa solution reveal the presence of a variable number of minute globoid bodies, arranged in pairs or short chains, or in small aggregated masses. In the fluid culture the pairs and chains predominate (figures 3, 4, and 5). According as the staining operation was carried out by the rapid or slower method, the globoid bodies assume a bluish or a violet color; rarely they present a red tint. Since the most uniform results are obtained by the slower method of staining, it may be considered that the violet coloration is the normal. The individual microörganisms average about 0.2 of a micron in diameter, the limits of visible bodies being 0.15 to 0.3 of a micron. Possibly still smaller individuals exist that exceed the limit of visibility. For purposes of comparison a group of chain forms has been compared by photography with *Streptococcus pyogenes* (figure 6).

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Considerable difficulties may be encountered in demonstrating the microörganism in initial cultures not older than four or five days. Either nothing whatever that can be recognized as microörganisms may be detected, or merely roundish bodies the nature of which it may be difficult to decide. When the same tubes are examined on the sixth or seventh day the unmistakable minute globoid bodies are usually detected. Although the original growth tends to appear slowly, the final multiplication takes place rapidly, and is completed from about the eighth to the twelfth day, after which the cultures remain stationary for a variable period. Kept in the thermostat the microörganisms may remain largely unchanged for many days, and in the refrigerator for many weeks.

Within the solid cultures the microörganism develops not as chains but as pairs and aggregated groups. The arrangement is very readily ascertained by compressing a fragment of the agar between two slides, which are afterwards separated. The resulting films having been stained in the usual manner by Giemsa or Gram solution, the minute particles of agar adhering to the glass will frequently exhibit colonies, of which the members are sufficiently separated to expose the grouping (figure 7). In the Gram-stained films the bodies are somewhat coarser than in the Giemsa-stained ones. In young agar cultures the Gram-stained bodies are partly purple and partly pink, while in older cultures almost all appear purple.

After a somewhat variable period, and generally from ten days to three or four weeks after the maximum growth has been obtained, certain metamorphoses or degenerations set in. Probably the enlarged and irregular stained bodies are to be viewed as degenerations, while the minute fragments just at the limit of visibility may possibly represent a metamorphosis into a minuter variety with which submicroscopic forms may be associated. When the conditions of culture are unfavorable, as, for example, when the brain extract or other medium without sterile tissue is employed, this supposed metamorphosis appears to arise earlier, and indeed within the first few days of growth. Subcultivation from one of the altered cultures or from their Berkefeld filtrates yields frequently but not uniformly new developments of the visible microörganisms.

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The important question, as to whether the cultures just mentioned contain actual ultramicroscopic forms of the microörganism, or whether the failure to detect the visible forms is due chiefly to dilution, must remain for the present undecided. What is certain, however, is that the globoid organisms are capable of passing though Berkefeld filters which exclude the usual test bacteria (*e. g., Micrococcus prodigiosus*). Cultures from the Berkefeld filtrates should always be made in the ascitic fluid tissue medium, as in the case of the initial growth.

The film preparations stained by the Giemsa stain and especially by the rapid method sometimes show the globoid organisms surrounded by a zone either faintly tinted or colorless, that may equal in width the diameter of the individual microörganisms. Single bodies, aggregated groups, or chains may be thus enveloped. The nature of the material composing the zone is unknown, as is also whether it represents an independent part of the organism (capsule?) or is derived from the culture medium.

INFECTION OF MONKEYS.

The microörganism described is present so constantly in the central nervous tissues of affected human beings and monkeys that a strong presumption exists to the effect that it bears some relationship to epidemic poliomyelitis. It is sufficiently obvious that whatever they may ultimately prove to be, as far as we now know the globoid bodies are not common pathogenic or even saprophytic microörganisms. Whether the bodies are limited to the central nervous organs or occur more widely distributed in the body of infected human beings and monkeys is still to be determined. The question which presses at the moment is whether the cultures are capable of setting up in monkeys clinical and pathological states corresponding with those characteristic of experimental poliomyelitis. Before proceeding to describe the inoculation experiments, it may be well to point out that apparently the globoid bodies are highly parasitic microörganisms. In view of this circumstance it may readily happen that they may quickly lose pathogenic properties in artificial cultures. There are of course several instances known among the bacteria and spirochætæ of which this circum-

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stance is true. We were therefore prepared to encounter difficulties in the way of infecting monkeys with cultures. It is necessary to recall the difficulties surrounding the original implantation of highly infected human poliomyelitic material upon monkeys in order to have presented emphatically the probable obstacles that stand in the way of the successful accomplishment of the infection of monkeys with the cultures.

Two series of inoculations were made. In one the cultures derived from the nervous tissues of monkeys and in the other from human beings directly were separately inoculated into *Macacus rhesus* monkeys. The inoculations were made either into the brain or into the sciatic nerve and peritoneal cavity simultaneously.

Before describing the several instances of successful inoculation, certain important points should be presented. It is known that the virus in an emulsion or filtrate prepared from the nervous tissues of infected monkeys survives in the thermostat and remains infectious for at least thirty days, and, also, that the filtrate soon becomes inactive after being transferred to a new fluid medium consisting of serum bouillon. Possibly the inactivity arises chiefly from the great dilution which the virus has undergone. Whence it follows that dilutions of the virus beyond the point at which infection through intracerebral inoculation of several cubic centimeters of suspension can be produced are easily secured. We have frequently had occasion to confirm this general fact, since second transfers into the fluid medium from a supposed culture derived from monkeys previously inoculated with the highly active M A virus have in many instances failed to cause infection even when injected intracerebrally. Dilution beyond the point of infectivity of the virus, even in the culture media, thus is readily accomplished.

Bearing these facts in mind it follows that successful inoculation with culture generations still more remote than the second dilution of the original virus is to be interpreted as representing, probably, an implantation of the artificially cultivated microörganism upon the susceptible monkeys. Moreover, from the monkeys in which experimental poliomyelitis has been thus produced the microörganism introduced can be recovered in culture and even implanted successfully again.

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The implantation upon monkeys of the virus contained in the original human nervous tissues is admittedly difficult. When filtrates are inoculated failure usually follows. When emulsions are employed, infection occurs in from about 50 to 100 per cent. of the instances, probably depending upon variation in the degree of infectivity of the original virus. The human strains of the virus are less infectious for monkeys than the adapted or so called monkey strains, a fact that is the more significant as respects the successful inoculation of cultures derived directly from human tissues.

Cultivated Human Strains.—Fluid and solid cultures in the third generation from four human sources, namely, D., Kr., Ko., and F., were mixed. Each culture had been obtained from recently glycerinated specimens of the central nervous system.

Experiment A.—Macacus rhesus. December 27, 1912. 0.5 c.c. of the mixture was injected into the left sciatic nerve and 0.8 c.c. into the left cerebral hemisphere.

December 30, 1912. Animal less active than normally; legs and arms weak.

December 31, 1912. Excitable; uses legs awkwardly. Etherized.

Autopsy.—At the site of intracerebral inoculation punctiform hemorrhages; the left lateral ventricle contains a small, dark clot; the cervical, dorsal, and lumbar spinal cord appear congested; the left sciatic nerve is slightly swollen at site of injection. Film preparations from the two inoculation sites show no ordinary bacteria. The microscopical examination of the medulla, cervical, dorsal, and lumbar levels of the spinal cord, and intervertebral ganglia from the cervical and lumbar regions shows in all typical and marked characteristic lesions of experimental poliomyelitis. The lesions consist of degeneration of the nerve cells, associated with neurophagocytosis, infiltration with mononuclear cells of the ground substance, and perivascular and meningeal infiltration (figures 8, 9, and 10).

Experiment B.—Macacus rhesus. December 31, 1912. A suspension of the medulla and spinal cord of monkey A was injected into left sciatic nerve and left cerebral hemisphere.

January 1 to January 4, 1913. Increased excitability.

January 6, 1913. Excitable; vision defective; ataxia.

January 7, 1913. Arms weak, right more than left.

January 8, 1913. 9.30 A.M., prostrate; respiratory paralysis. Death at 10 A.M.

Autopsy.—A small cyst with clear contents at the site of injection. Sciatic wound healed. Congestion of gray substance of spinal cord.

The microscopical examination exhibits marked lesions of experimental poliomyelitis in the spinal cord, medulla, and intervertebral ganglia. Portions of the nervous system were placed in 50 per cent. sterile glycerin.

Experiment C.—Macacus rhesus. May 14, 1913. Inoculated intracerebrally with emulsion of glycerinated spinal cord and medulla of monkey B.

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May 18, 1913. Excitable.
May 19, 1913. Arms weak.
May 20, 1913. Arms and legs paralyzed.
May 21, 1913. Etherized.

Autopsy.—A small cyst at the site of inoculation. At various levels of the spinal cord are visible lesions.

Microscopical examination shows marked and characteristic lesions of experimental poliomyelitis in the medulla, spinal cord, and intervertebral ganglia.

Cultures in the ascitic fluid tissue medium were made at autopsy from monkeys A, B, and C. From monkeys A and B the original microörganism injected was recovered in pure culture. The culture from monkey C was unsuccessful. Hence another portion of the original glycerinated material was inoculated as follows:

Experiment D.—Macacus rhesus. June 7, 1913. Intracerebral injection of emulsion of glycerinated brain and cord from monkey B.

June 12, 1913. Excitable; tremor; prostrate.
June 13, 1913. Etherized.

Autopsy.—A small cyst at the site of inoculation; visible lesions in the spinal cord.

Microscopical examination shows typical lesions of experimental poliomyelitis in the medulla, spinal cord, and intervertebral ganglia.

Cultures in the ascitic fluid tissue medium from this animal gave a pure growth of the microörganism originally injected into monkey A, the beginning of this series, more than six months previously. The successful culture in this instance indicates that the failure to recover the microörganism from monkey C was due to a technical fault.

Cultivated Monkey Strains.—These strains consisted of cultivations from the highly adapted M A virus which had been long propagated in monkeys from emulsions or filtrates of the nervous organs. Hence the cultures, while representing the same original virus, varied in precise generation according to the period at which they were isolated. All were far removed from the original human strain.

Experiment E.—Macacus rhesus. December 3, 1912. Two cultivated strains, one in the third and the other in the fifth generation, were combined, and of the mixture 1 c.c. was injected into each sciatic nerve, and 10 c.c. were injected into the peritoneal cavity.

December 6, 1912. Excitable; coat roughened.
December 9, 1912. Somewhat less excitable.

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December 23, 1912. Again excitable. The monkey remained excitable continuously, and on March 19, 1913, was found in convulsions, which recurred at frequent intervals, until on March 25 death occurred.

The microscopical examination reveals focal lesions in the medulla, consisting of minute hemorrhages, small aggregations of mononuclear cells, partly in the ground substance and partly about the small vessels, and a few small vascular thrombi. Small hemorrhages also occur in the crura cerebri, and in the cervical region of the spinal cord, in which slight perivascular infiltration with mononuclear cells is also present.

Cultures yielded the original microörganism.

The interpretation of experiment ~~D~~ E is not perfectly clear. The long intervening period between the inoculation and the development of convulsions and occurrence of death is not strictly paralleled by the effects of direct inoculation of either emulsion or filtrate of the nervous tissues. Moreover, thrombi do not occur in uncomplicated instances of experimental poliomyelitis. On the other hand the infiltrative lesions are similar to those present in experimental poliomyelitis.

Experiment F.—Macacus rhesus. December 27, 1912. Injected into left cerebral hemisphere 0.8 c.c. of cultivated virus, fifth generation.

January 1, 1913. Excitable; arms weak.

January 2, 1913. Excitability continues; arms and back partly paralyzed; tremor of head.

January 3, 1913. Legs weak.

January 4, 1913. Animal prostrate. Etherized.

Autopsy.—A small cyst at the site of injection. Spinal cord edematous. No visceral lesions.

Microscopical examination shows marked characteristic degenerative and infiltrative lesions of experimental poliomyelitis in the medulla, spinal cord, and intervertebral ganglia.

Cultures yielded the original microörganism.

Experiment G.—Macacus rhesus. January 27, 1913. Injected into left cerebral hemisphere 2.5 c.c. of culture in fifth generation from monkey E.

February 1, 1913. Excitable; movements uncertain; vision appears to be defective.

February 2, 1913. Neck and back paralyzed; unable to sit up; convulsions in course of which death occurs.

Autopsy.—February 3, 1913. The general viscera are apparently normal. A small cyst the size of an almond is present at the point of inoculation in left hemisphere. Gray matter of medulla and spinal cord is congested.

The microscopical examination of the medulla, spinal cord, and intervertebral ganglia shows typical lesions of experimental poliomyelitis.

Cultures yielded the original microörganism.

Experiment H.—Macacus cynomolgus. February 5, 1913. Injected into the left cerebral hemisphere 2.5 c.c. of a Berkefeld filtrate prepared from the site of inoculation of monkey G.

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February 6, 1913. Shows tendency to incline toward right side; does not use the right arm, which is spastic, the result probably of injury from the injection.

February 7, 1913. Death at 10 A.M.

Autopsy.—At the site of inoculation there is a cyst containing blood stained fluid communicating with the lateral ventricle.

Microscopical examination shows marked lesions in the intervertebral ganglia and lesions of the blood vessels adjacent to the site of inoculation resembling those characteristic of experimental poliomyelitis. The spinal cord also reveals fine lesions of the same nature. Hence it appears that within the short period of forty-eight hours the specific lesions developed throughout a wide area of the nervous system of this animal.

Experiment I.—Macacus rhesus. February 8, 1913. Repetition of experiment H. Injected into the left cerebral hemisphere 1 c.c. of filtrate from site of inoculation of monkey F.

February 14, 1913. Prostrate; legs, back, and arms paralyzed. Etherized.

Autopsy.—A small, blood-containing cyst at the site of inoculation. Spinal cord throughout shows gross lesions of the gray matter.

The microscopical examination confirms the character of these lesions. Spinal cord, intervertebral ganglia, and medulla all show marked degenerative and infiltrative lesions of poliomyelitis, and identical lesions occur in the crura cerebri and in the cerebral hemispheres.

Experiment J.—Macacus rhesus. February 13, 1913. Suspension of glycerinated brain and spinal cord of monkey F injected intracerebrally.

February 18, 1913. Death.

Autopsy.—A cyst the size of an almond containing clotted blood occurs at the site of inoculation. Throughout the spinal cord the gray matter is congested.

Microscopical examination shows marked characteristic lesions of experimental poliomyelitis.

Experiment K.—Macacus rhesus. May 14, 1913. Injected intracerebrally with an emulsion of glycerinated spinal cord and medulla obtained from monkey F.

May 18, 1913. Distinctly weak.

May 19, 1913. Ataxic.

May 20, 1913. Paralysis of arms, legs, and back.

May 21, 1913. Etherized.

Autopsy.—At the site of inoculation there is a small cyst containing blood. Visible lesions in the spinal cord.

Microscopic examination reveals marked characteristic poliomyelitic lesions of the medulla, spinal cord, and intervertebral ganglia.

Culture yielded microörganism identical with that inoculated in monkey F almost six months previously. This culture in the fourth generation was inoculated intracerebrally on June 11, into a *Macacus rhesus.* On June 16 the monkey was weak, slow, and ataxic; it was etherized on July 2. The microscope reveals typical lesions of the brain and spinal ganglia, or, in other words, evidences of the encephalitic type of poliomyelitis.

Experiment L.—Macacus rhesus. January 13, 1913. Injected into left cerebral hemisphere 1 c.c. of cultivated virus, sixth generation.

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January 16, 1913. Movements irregular; tends to move in circles.

January 17, 1913. Apparently normal.

February 6, 1913. Ataxic; excitable, arms and legs weak.

February 10, 1913. Weakness of arms and legs increased. Etherized.

Autopsy.—No lesion at the site of inoculation, and no gross changes in the spinal cord.

Microscopical examination shows fine infiltrative and degenerative lesions of the medulla, spinal cord, brain, and intervertebral ganglia.

Culture yielded microörganisms identical with that inoculated.

Experiment M.—Macacus rhesus. February 11, 1913. Injected emulsion of the spinal cord and brain of monkey K into the sciatic nerve and peritoneal cavity.

February 14, 1913. Excitable; convulsions.

February 15, 1913. Legs weak.

March 1, 1913. Excitability continues; movements slow and restricted.

March 5, 1913. Condition unchanged. Etherized.

Microscopical examination shows typical poliomyelitic lesions of the intervertebral ganglia in the lumbar and cervical regions, and similar but inconspicuous lesions of the spinal cord.

Experiment N.—Macacus rhesus. February 27, 1913. Inoculated into left hemisphere with 2 c.c. of monkey culture from two sources in the eighteenth and twentieth generation respectively.

March 1, 1913. Inclines slightly towards the right side.

March 2, 1913. Death.

Autopsy.—At the site of inoculation is a cyst the size of an almond containing clear fluid and communicating with the lateral ventricle; gray matter of the spinal cord is congested.

Microscopical examination of the cyst shows the presence of a Gram-positive coccus (mixed infection).

Experiment O.—Macacus cynomolgus. March 4, 1913. 2 c.c. of Berkefeld filtrate from the site of inoculation of monkey N are injected intracerebrally, and 100 c.c. intraperitoneally.

March 17, 1913. Left arm weak; legs somewhat spastic.

March 18, 1913. All extremities weak and spastic.

March 19, 1913. Head retracted; convulsions; unable to rise. Etherized.

Autopsy.—No lesion at the site of inoculation. A *placque jaune* 1.5 by 2 cm. occupies the surface of the cortex on the left side, posterior to the motor area. General viscera normal.

Microscopical examination reveals characteristic focal lesions of experimental poliomyelitis in the intervertebral ganglia, and vascular and interstitial lesions of the spinal cord, crura cerebri, and cerebral hemispheres.

Because of contamination with micrococci at the inoculation site in monkey N, a filtrate prepared from the local site was injected into monkey O, resulting in combined flaccid and spastic paralysis. However, the sections of the nervous tissues of monkey O by showing lesions of experimental poliomyelitis indicate the existence of that disease.

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The preceding experiments show unmistakably that the inoculation of the cultures is followed by the appearance of the clinical symptoms and pathological effects characteristic of experimental poliomyelitis in the monkey. The two methods of inoculation now chiefly practised with the usual suspensions or filtrates of nervous tissues were employed successfully with the cultures. The essential results are identical whether the cultures are inoculated into the brain or simultaneously into sciatic nerve and peritoneal cavity. The inoculation of the cultures produces in monkeys no direct effect. Immediately after injection the animals appear normal and they remain apparently in this condition for an incubation period which may be as brief as forty-eight hours, but commonly endures for one to several weeks, at the expiration of which the usual symptoms of excitement, tremor, weak and then paralyzed muscles succeed one another. Death may arise from prostration, convulsions, or paralysis of the respiration, although in most of the instances related it was artificially hastened by etherization.

The central nervous tissues of the monkeys may be employed for recultivation of the microörganism inoculated, or for transmitting the experimental affection to still other and probably an indefinite series of monkeys. This transmission can be accomplished either with emulsions or Berkefeld filtrates of the fresh or glycerinated organs. Since the experimental disease is accurately reproduced in this manner, it follows that not only the cultures but the nervous tissues infected with them possess the distinctive disease-producing properties of the virus of poliomyelitis as commonly understood.

The local effects of the cultures vary with the inoculation site and the dosage. Unless secondary infection arises from the outside, the intrasciatic and intraperitoneal inoculations cause no considerable reaction, while the intracerebral injection leads to the formation of a small cyst, in which the introduced microörganism may or may not be readily discoverable in film preparations. If relatively few of the microörganisms are inoculated, they tend to disappear from view; while if many are introduced, they are readily detected, either lying free or enclosed in emigrated polynuclear or mononuclear cells (figure 11). When pyogenic micrococci enter from the skin, the local lesion tends to be larger, and to contain

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many pus cells, and the two microörganisms may appear side by side, both without and within cells. When an excess of the mixed fluid and solid cultures is injected into the brain, the local effect is also more severe and many leucocytes may be called out by the positively chemotactic elements contained within the medium. The action of contaminated cultures is less readily interpreted than are the effects of pure cultures; but any doubt may be removed by preparing a Berkefeld filtrate from the local lesion and portions of the medulla, spinal cord, and intervertebral ganglia, which is to be employed for inoculation.

The disappearance of the microörganism from the inconspicuous local lesions probably results from a general diffusion throughout the nervous organs and restricted multiplication at the site of injection. The microörganism can indeed be recovered in cultures from any part of the central nervous system, and is best recovered from the intact cerebral hemisphere, where it usually exists in a pure state and is readily accessible under aseptic conditions.

RELATION OF THE MICROÖRGANISM TO NERVOUS TISSUES.

The microörganism the cultures of which have been described can be detected in film preparations and in sections prepared from the central nervous organs of human beings who have succumbed to poliomyelitis and of monkeys subjected to inoculation with the ordinary virus, or with cultures. A special technique which has been devised by Noguchi is required for the demonstration.

Staining Method for Film Impressions.—1. Cover-glasses of suitable size and thinness (No. 1 or 0) receive a succession of impressions from the selected piece of nervous tissue.

2. After becoming air-dry the cover-glass is placed, film side down, in a mixture which is freshly prepared at each operation, consisting of one part of Grübler's Giemsa solution, and two parts of Merck's methyl alcohol reagent, where it remains for two minutes. In order to prevent the film surface from contact with the bottom of the staining dish, it is well to support one end upon a drawn out glass capillary tube. The staining vessel may conveniently be a small Petri dish and cover.

3. Twenty parts of a 1 to 10,000 potassium hydrate solution are

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poured into the dish at the expiration of the two minutes, and the whole is thoroughly mixed by gentle agitation. At the expiration of one hour the cover-glass is removed.

4. The film side is washed in distilled water for a few seconds, after which it is differentiated in a solution of tannic acid, consisting of one or two drops of a 20 per cent. solution, added to forty cubic centimeters of distilled water. This operation requires several seconds and careful observation.

5. The cover-glass is now washed in distilled water for two minutes, dried in the air, and mounted in cedar oil.

Staining Method for Sections.—The nervous tissues having been hardened in Zenker's solution or sublimate alcohol are embedded in paraffin and the sections are mounted upon slides in small series.

1. The sections are treated with Lugol's solution and afterwards with a 0.5 per cent. solution of sodium hyposulphite, after which they are thoroughly washed in distilled water.

2. The sections are passed successively through 95 per cent. alcohol, absolute alcohol, chloroform, aceton, benzol, ether, absolute alcohol, 95 per cent. alcohol, and finally distilled water, in each of which reagents they remain about one hour.

3. The staining solution consists of three cubic centimeters of concentrated Giemsa solution to forty-five cubic centimeters of distilled water, in which the sections remain for twenty-four hours.

4. After brief washing in distilled water, the sections are rapidly passed in succession through aceton, aceton thirty parts plus xylol seventy parts, aceton fifty parts plus xylol fifty parts, aceton thirty parts plus xylol seventy parts, and finally pure xylol.

5. The cleared sections are mounted in cedar oil.

With these methods the minute microörganism has been demonstrated in the films in eighteen out of nineteen specimens of glycerinated human nervous tissues. It was detected regularly lying between cells and in some instances within the cytoplasm of cells, some of which were nerve cells (figures 12 and 13). The microörganism appears invariably in pairs or small aggregated groups and never in chains. The demonstration of the microorganism in sections is far more difficult, but it has been accomplished in a few instances in the interstices of the tissues and in the protoplasm of cells.

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The detection of the microörganism in film preparations and in sections has been accomplished also with nervous tissues derived from monkeys inoculated at one time with the ordinary virus, at another with cultures. In this case also it is more readily brought out in film preparations than in sections. The grouping is identical with that present in the specimens of human origin.

DISCUSSION.

From the facts presented it follows that by employing a specially devised method there has been cultivated from the central nervous tissues of human beings and monkeys the subjects of epidemic poliomyelitis a peculiar minute organism that has been caused to reproduce the symptoms and lesions of experimental poliomyelitis. The microörganism consists of globoid bodies measuring from 0.15 to 0.3 of a micron in diameter, and arranged in pairs, chains, and masses, according to the conditions of growth and multiplication. The chain formation takes place in a fluid medium, the other groupings in both solid and fluid media. Within the tissues of infected human beings and animals the chains do not appear.

No statement is ventured at present as to the place among living things to which the bodies belong. It is obvious that the cultural conditions are those that apply more particularly to the bacteria.

On the other hand, the microörganism is associated with the production of an acute disease in which suppuration does not form a prominent part. No special attention at the present time has been given to the solution of the question as to whether the microorganism actually belongs to the bacteria or to the protozoa. In the manner of evolution of the symptoms, and in the appearance of the lesions, the experimental disease caused by the inoculation of the cultures resembles that produced by the virus of poliomyelitis as ordinarily employed. The central nervous organs of monkeys infected with the cultures bear preservation and glycerinization as do the infected human tissues, or the monkey tissues infected directly from human tissues. Cultures to which glycerin is directly added survive in the refrigerator at least eight days.

The microörganism passes through Berkefeld filters and the

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filtrates yield upon recultivation the particular microörganism contained within the filtered culture. Moreover, Berkefeld filtrates prepared from the nervous tissues of infected human beings and monkeys yield also in culture the identical microörganism.

By employing a suitable staining method the microörganism has been detected in film preparations and sections prepared from human nervous tissues, and from the corresponding tissues of monkeys inoculated with the usual virus or with cultures or filtrates prepared from monkeys previously injected with cultures. From all the infected materials mentioned, irrespective of the manner of their origin, the microörganism has been recovered in cultures. As would be expected it is more uniformly recoverable from the original nervous tissues than from filtrates, and doubtless for the reason that in the former it exists in greater abundance.

To obtain the initial culture is difficult, and this irrespective of whether the tissues submitted to cultivation have come immediately from man or from monkeys previously inoculated with the ordinary virus or even with the cultures. Once the microörganism adapts itself to a parasitic state it is developed with greater difficulty under saprophytic conditions. Whenever the nervous tissues have been shown to be infectious, the microörganism has been recoverable, notwithstanding long preservation and glycerination. In other words, infectivity of the nervous organs and the presence of the microörganism exist together. It has indeed happened that a specimen of infected nervous tissue has at the first attempt not yielded the initial growth, although it has yielded it upon the second attempt. Persistence will usually lead to a successful cultivation, provided no technical fault is committed. An important factor in the technique of cultivation is the sample of ascitic fluid. Not all samples are suitable, and a preliminary test is necessary, using for the purpose a growing culture, in selecting samples for culture purposes. Once a suitable ascitic fluid is obtained it should be carefully husbanded in the refrigerator. Even with this precaution failure may still occur. In such an instance repetition, using the same materials but in two series, one of which is prepared for enclosure in the anaerobic jar, while the other is allowed to remain outside, may yield the desired result; or the

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result may come on a second trial that appears to be an exact repetition of the first.

Only the exceptional cultures possess the degree of pathogenicity sufficient to cause specific infection, and the production of experimental poliomyelitis. A pathogenic strain may be effective at different and even remote generations, and a non-pathogenic strain may lack pathogenicity even in the second generation. This important fact indicates strongly that the pathogenic effect is not due to mere mechanical carrying over into the cultures of an invisible parasite or virus with which the cultivated microörganism is accidentally associated. If such accidental association were the cause of the experimental disease produced by the cultures in monkeys, it would display itself preferably in the first generations and without reference to the strain of the visible microörganism. On the other hand in this fluctuation of pathogenicity the cultures imitate the action of the virus as contained in human materials, namely, nervous tissue, secretions from the nasopharynx, and intestinal washings, in which the virus, either known or believed to be present, may yet fail to be demonstrated by reason of the want of infectious power for monkeys or for the particular monkey inoculated in a given instance. Moreover, it is a common experience in bacteriology to find even among the ordinary bacteria lack or rapid loss of virulence among saprophytic cultures, while virulence is not only retained, but may be increased in rapid passages from animal to animal.

In view of these considerations it would appear that an etiological relationship has been shown to exist between the cultivated microorganism and epidemic poliomyelitis as it occurs in human beings or in monkeys. There remains merely a single other possibility, already indicated, namely, that two factors are present in the cultures, the one an invisible because ultramicroscopic organism, the other the globoid bodies described. On this basis it would have to be supposed that the former but hypothetical factor is the essential agent of infection. As against this supposition it may be urged that an instance of symbiosis of this nature is not known to animal pathology. Regarding the cultivated minute but visible microörganism itself, it may be held on the basis of the data presented that it fulfills the conditions hitherto demanded for the

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establishment of causal relation between an extraneous parasite and a specific disease. The microörganism exists in the infectious and diseased organs; it is not, as far as is known, a common saprophyte, or associated with any other pathological condition; it is capable of reproducing, on inoculation, the experimental disease in monkeys, from which animals it can be recovered in pure culture. And besides these classical requirements, the microörganism withstands preservation and glycerination as does the ordinary virus of poliomyelitis within the nervous organs. Finally, the anaerobic nature of the microörganism interposes no obstacle to its acceptance as the causative agent, since the living tissues are devoid of free oxygen and the virus of poliomyelitis has not yet been detected in the circulating blood or cerebrospinal fluid of human beings, in which the oxygen is less firmly bound; nor need it, even should the microörganism be found sometimes to survive in these fluids.

EXPLANATION OF PLATES.

PLATE 38.

FIG. 1. Fluid culture showing opalescence. Natural size.

FIG. 2. Solid culture showing colonies and line of demarcation. Natural size.

PLATE 39.

FIG. 3. Separate globoid bodies. × 1,000.

FIG. 4. Aggregated masses of globoid bodies. × 1,000.

FIG. 5. Chains and pairs of globoid bodies. × 1,000.

FIG. 6. Chains of globoid bodies compared with *Streptococcus pyogenes.* × 1,000.

FIG. 7. Agar fragment showing pairs of globoid bodies compared with *Streptococcus pyogenes.* × 1,000.

PLATE 40.

FIG. 8. Spinal cord showing degeneration of nerve cells and invasion of leucocytes (neurophagocytosis). × 144.

FIG. 9. Perivascular and interstitial mononuclear cell infiltration of medulla. × 98.

PLATE 41.

FIG. 10. Interstitial infiltration and degeneration and phagocytosis of nerve cells in intervertebral ganglion. × 160.

FIG. 11. Globoid bodies in site of inoculation taken up by leucocytes. × 1,000.

FIG. 12. Film preparation from central nervous tissue of monkey inoculated with human virus (not culture) showing pairs of globoid bodies. × 1,000.

FIG. 13. Section prepared from spinal cord of monkey inoculated with M A virus (not culture) showing intracellular pairs of globoid bodies. × 1,000.

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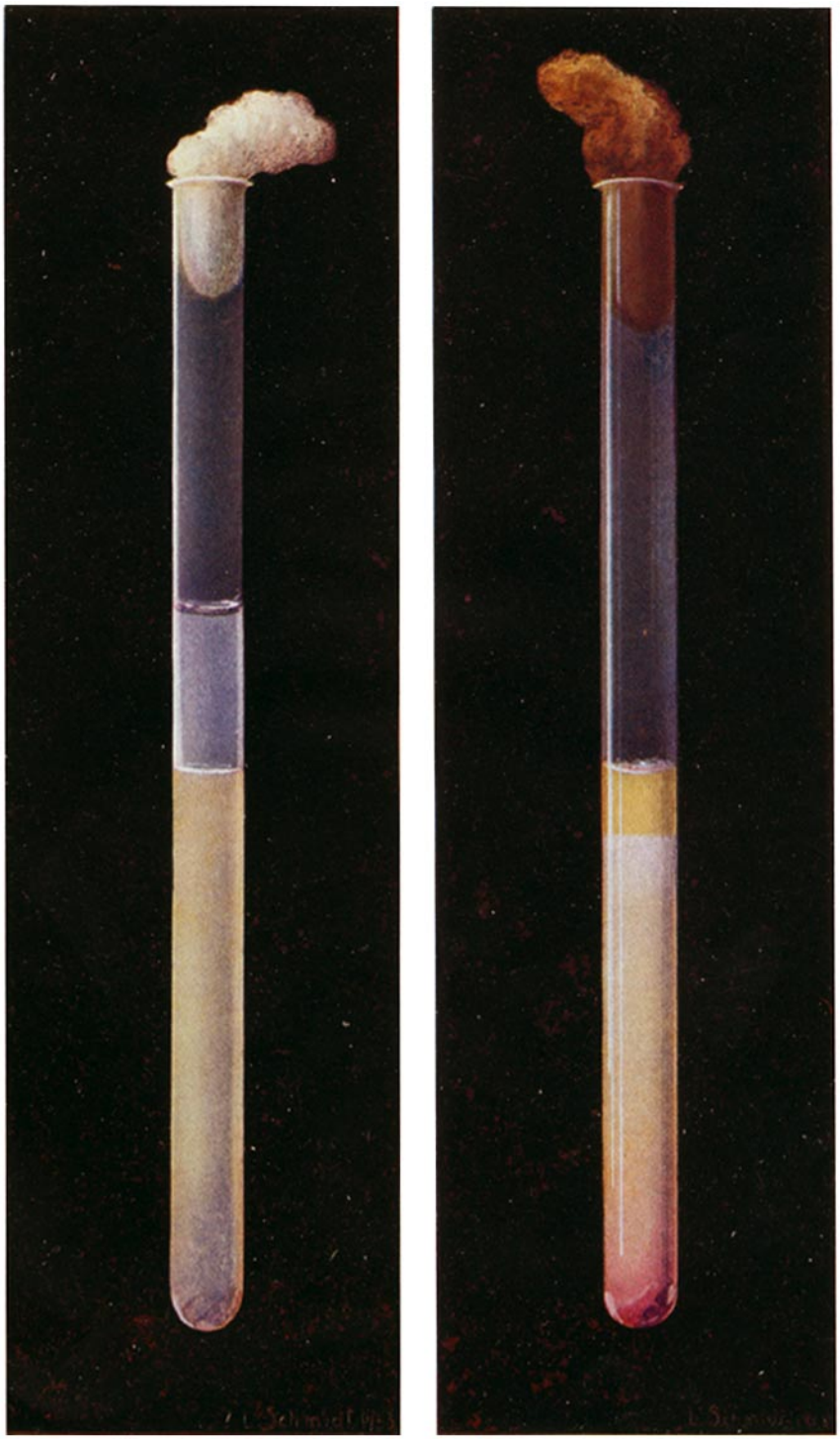

FIG. 1. FIG. 2.

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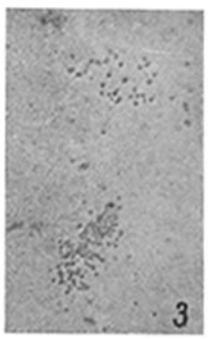

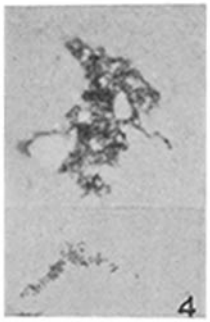

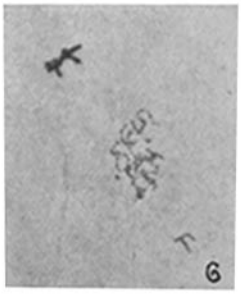

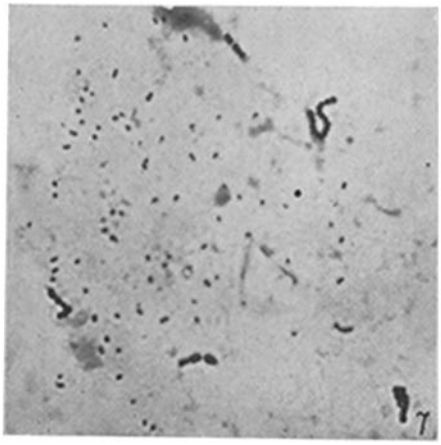

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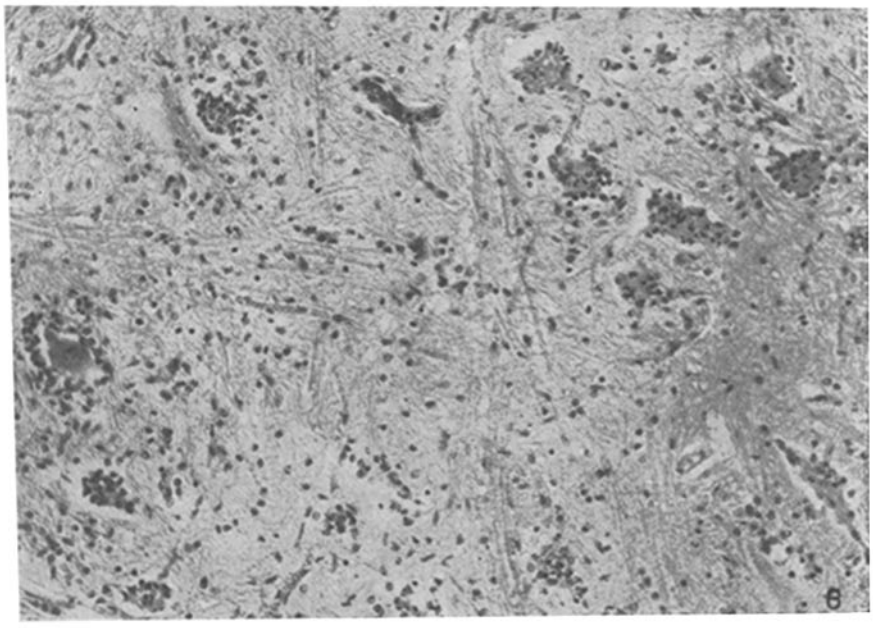

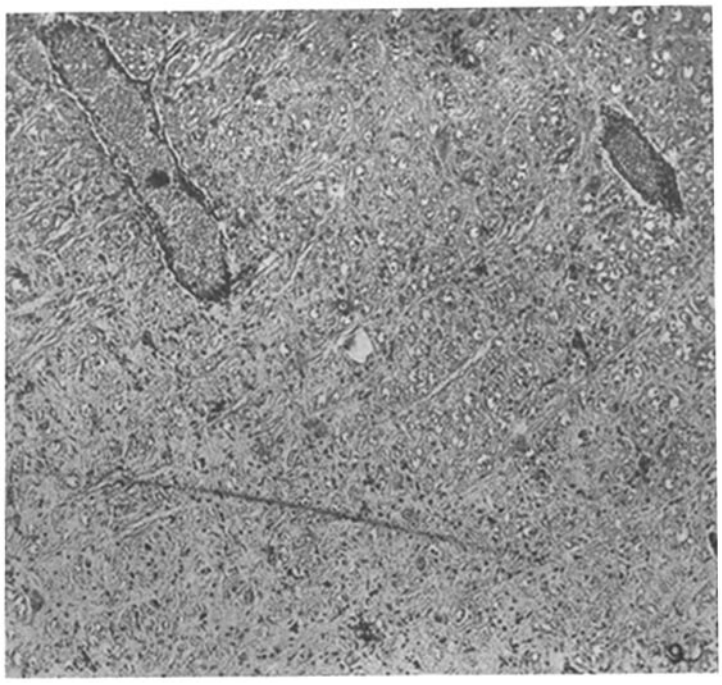

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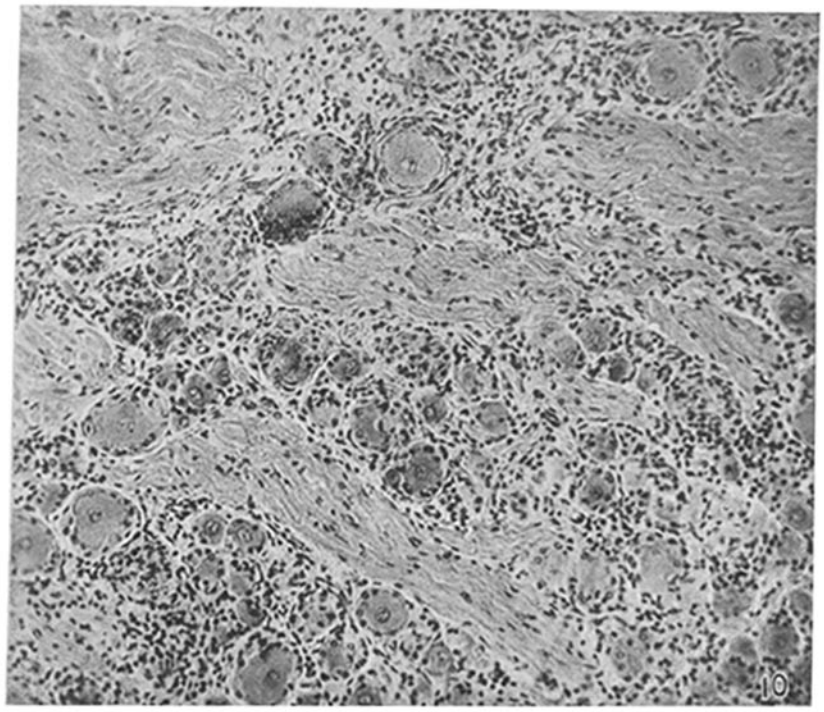

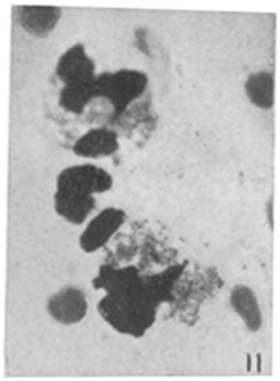

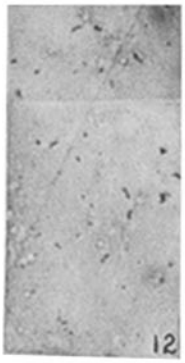

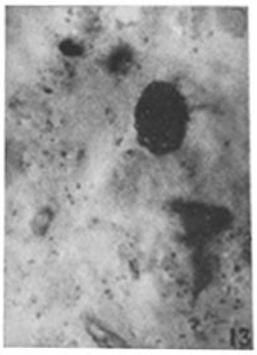

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