STUDIES OF THE DEGENERATION AND REGENERATION OF AXIS CYLINDERS IN VITRO.*

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Plates 28 to 40.

The experiments of Harrison, 1 who cultivated the medullary cord of the embryonal frog in coagulated frog lymph, showed that from the neuroblasts of His hyaline threads grew out which increased in length by means of ameboid movements. Harrison has interpreted these threads as axis cylinders. Burrows observed the same phenomenon in cultures in chick plasma of the medullary cord of a chick embryo two days old, and succeeded in staining the newly formed axis cylinder. 2 Lewis and Lewis 3 have described the growth of sympathetic nerves from the intestines of chick embryos in saline solutions. 4

I began the experiments, the preliminary results of which are given in this publication, with the cultivation in coagulated chick plasma of the neural tube of chick embryos two to three days old, and in a few cases (5 out of 102 experiments) I observed that after ten to twenty-four hours there grew out from the transplanted piece slender filaments with a bulbular end which, by ameboid movements through the plasma, increased the length of the thread. Besides, there occurred in all the cultures a rich proliferation of the

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* Received for publication, November 2, 1912.


Since writing this paper, an article by Marinesco and Minea (Essai de culture des ganglions spinaux de mammifères in vitro, Anat. Anz., 1912, xlii, 161) has come to my attention. These authors have cultivated spinal ganglia of young cats and rabbits in plasma in large dishes. They observed growth of connective tissue cells and of axis cylinders, which were stained according to the method of Cajal. The newly formed nerve fibers are described in some cases as prolongations of the cell body ending in swellings, in other cases as pericellular networks and plexuses.
embryonal connective tissue cells, and this was probably the cause of the scant percentage of successful cultures, for we know that the growth of connective tissue prevents the development of tissues of a higher order.

I began, therefore, to cultivate the tissues of the central nervous system of chick embryos in a more advanced and differentiated stage and found that pieces of the cerebral cortex of chick embryos six to seven days old gave 100 per cent. of successful cultures as far as the growth of the axis cylinders is concerned.

The cultures were prepared in the ordinary way as described for cultivation of tissues in general by Carrel and Burrows. The embryo was taken out of the egg and placed in Ringer solution heated to 40°C. From the forebrain the covering membranes were removed by means of scissors and forceps, and then, with a cataract knife, very thin slices of the cortex, about one millimeter square, were taken out. These slices were put immediately at room temperature in a drop of plasma on a cover-glass, and the cover-glass was inverted over a hollow slide, without waiting for the coagulation of the plasma. In some cases the plasma was diluted one third or one fourth with distilled water. In other cases it was not diluted. In this way it was possible to prepare each culture in one half or one quarter of a minute. In some cases the tissues were placed on a piece of silk veil in the plasma, the silk being a support for the growing filaments. I shall now describe the phenomena of growth that occur in a culture of the brain of the chick embryo six to seven days old.

After incubating the cultures for ten, eighteen, and twenty-four hours, a number of thin, slender, light-breaking filaments consisting of hyaline protoplasm without granules or visible structure were seen growing out from the pieces. Most of these filaments were of the same thickness, about one half to one micron. Throughout their length they were curved and bent in several directions. After twenty-four hours, most of them reached a length of 250 to 300 microns, and a few even 460 microns. Several of these filaments ended in small swellings of the thread (figure 1). This bulb was sometimes regular and ovoid, and measured two to three microns in width. Some of the filaments ended in a point, and a few had
finger-like branches in which ameboid movements could be observed. These filaments might be present in small numbers in each culture, or they might grow so densely that they formed a real tissue around the fragment of brain. A few of them had branched by the twenty-fourth hour.

After forty-eight hours the fibers increased in length and thickness. Some of the fibers now reached a length of about one millimeter, and one of them was 1.1 millimeters. Some of the thickest fibers showed a plain fibrillation in their central parts, and further in their course swellings were seen. These swellings were of different size and shape. Most of them were slight and somewhat irregular, but in some cases they resembled very much the end bulb of the filament. They consisted always of hyaline protoplasm. Branching of the thickest fibers was frequently observed at this stage, and anastomoses between different branches occurred in some places (figure 2). After forty-eight hours another feature of interest was observed, that is, the occurrence of isolated cells lying free in the plasma near the border of the fragment of tissue. These cells were of two kinds. (1) Large cells, without any definite cell membrane, with many granules, and one large, clear nucleus. They sometimes have one or two short protoplasmatic, filamentary processes, and in a few cases I observed one or two of the above mentioned long protoplasmatic threads with bulbular ends growing out from these cells (figures 3 and 4). (2) Cells of another type which were mostly oblong, sometimes fusiform with sharp outlines. These cells had a very definite cell membrane, one nucleus, and powerful, straight, tapering outgrowths, three to five microns wide, in which granules were visible. The outgrowths never had bulbular endings and were easily distinguished from the above mentioned slender curved hyaline filaments (figure 5). There is no reason to doubt that the cells of this second type are connective tissue cells (glia (?), endothelial (?)), while the first type is similar in character to ganglion cells, and its outgrowths are very much like the filament described by Harrison in the cultures of embryonal frog, and in all probability are axis cylinders.

On the third day there was still some increase in size of these nerve fibers and the longest ones observed were 1.25 millimeters in length.
On the fourth day but little difference was seen. Granulation occurred in some of the fibers and their outlines became less definite. On the fifth and sixth days the fibers disappeared completely.

I continued to work with the brains of chick embryos of different ages. As far as the growth of nerve fibers was concerned a high percentage of the cultures was successful. A few cultures were discarded as not useful on account of technical errors.

### TABLE I.

<table>
<thead>
<tr>
<th>Age of the embryos</th>
<th>No. of animals employed</th>
<th>Total No. of cultures</th>
<th>No. of cultures in which axis cylinders were present</th>
<th>Percentage of growth of axis cylinders</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days</td>
<td>4</td>
<td>19</td>
<td>2</td>
<td>10.5</td>
</tr>
<tr>
<td>3 days</td>
<td>10</td>
<td>83</td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td>6–10 days</td>
<td>5</td>
<td>32</td>
<td>32</td>
<td>100.0</td>
</tr>
<tr>
<td>11–15 days</td>
<td>6</td>
<td>65</td>
<td>49</td>
<td>75.3</td>
</tr>
<tr>
<td>16–21 days</td>
<td>8</td>
<td>53</td>
<td>37</td>
<td>69.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>252</td>
<td>123</td>
<td></td>
</tr>
</tbody>
</table>

The growth of the axis cylinder from brains of chick embryo of a more advanced age showed practically the same characteristics as described formerly for the six to seven day old embryos. There were no striking differences in the rate of growth. In one of the cultures, there occurred a phenomenon which deserved closer attention. The culture was from the brain of a chick embryo eighteen days old (figure 6). On the third day there had developed a great number of thick and thin axis cylinders which branched and anastomosed, forming a network. The swellings of these fibers were larger than any observed earlier. They appeared like refractive homogeneous globes, four to five microns in diameter, lying in the course of the filament or at the anastomosis between two or three filaments. Sometimes they were oblong, and in shape were very much like bipolar ganglia cells. As they had no nucleus, however, I am sure they were not. I consider them as accumulations of protoplasm analogous to that in the end bulb, and like the latter they give rise to outgrowths of new fibers.

Having established these facts I tried in some few cases to cultivate the spinal cord of chick embryos and succeeded in observing...
axis cylinders sprouting out from the pieces of tissue (figure 7).

Next I began the cultivation of the cerebral nervous systems of young mammals. Rabbits, cats, and dogs were employed, and preparations were made from the brain, the cerebellum, the spinal cord, and the spinal ganglia. The animals were operated upon under ether anesthesia. The tissue was put directly from the animal into Ringer solution at 40° C., and was then cut into small pieces. The preparation of each culture took about half a minute. A little more time was required for the dissection and cutting of the spinal ganglia with their tough connective tissue envelopes.

<table>
<thead>
<tr>
<th>TABLE II.</th>
<th>Cultures of the Central Nervous System of a Rabbit Two Months Old.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals employed</td>
<td>Origin of cultures</td>
</tr>
<tr>
<td>2</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
</tr>
<tr>
<td></td>
<td>Spinal ganglia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE III.</th>
<th>Cultures of the Central Nervous System of a Rabbit Seven Months Old.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals employed</td>
<td>Origin of cultures</td>
</tr>
<tr>
<td>3</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
</tr>
<tr>
<td></td>
<td>Spinal ganglia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE IV.</th>
<th>Cultures of the Central Nervous System of a Cat Six Weeks Old.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals employed</td>
<td>Origin of cultures</td>
</tr>
<tr>
<td>3</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
</tr>
<tr>
<td></td>
<td>Spinal ganglia</td>
</tr>
</tbody>
</table>

The development of the axis cylinder in cultures from these young animals was slower than in those from embryonic nervous tissue. They were observed usually after thirty-six or forty-eight hours, and only a few were present in each culture. They showed,
however, the same main characteristics as those from embryonic
chicks. The filaments were curved, hyaline, ended usually in a
bulb, had branches, and were longitudinally striated when they had
reached a certain size. In some of them, especially in those pre-
pared from the gray substance of the spinal cord and from the spinal
ganglia, granulations were visible (figures 8 and 9).

The cultures prepared from the brain of these animals showed a
development of connective tissue cells only after three days, and,
fortunately for the axis cylinders, only a few connective tissue cells
appeared in each of the cultures, while the spinal ganglia gave rise
to a rich growth of connective tissue cells.

The cultures of the nervous system from rabbits were kept for
observation for only three days and a half, were then recorded as
positive or negative, and the negative ones were discarded. It is
possible, as was learned later, that the results obtained might have
been changed and might have shown a greater number of positive
cultures if they had been kept for observation for five or six days.
In adult animals it is necessary to observe the cultures for a long
time to be sure that they will not develop axis cylinders. If they
do develop, they live for only a very short time. I have never seen
axis cylinders which did not undergo degeneration after the seventh
day of the incubation of the culture at 40° C.

I had, however, not yet reached my goal, which was to observe
the development of axis cylinders in cultures of the cerebral nervous
system of mammals in such a large percentage of the cases that it
might be useful for the study of degeneration and regeneration. I
succeeded when I began to work with dogs. From the brain of a
dog three weeks old I observed a growth of axis cylinders. This
growth took place from the cerebrum, but still more abundantly
from the cerebellum. Of fifteen cultures prepared from the brain
of a dog three weeks old, eleven were positive, and most of these
showed an abundance of axis cylinders (figure 10). Out of ten
cultures prepared from the cerebellum of two such dogs, every one
showed a great number of axis cylinders; that is, 100 per cent. of
the cultures were successful.

After twenty-four hours the axis cylinders from the brain of
this dog had already appeared, and those from the cerebrum showed
Degeneration and Regeneration of Axis Cylinders in Vitro.

the characteristics mentioned above for the chick, cat, and rabbit. The growth from the cerebellum I will mention a little more particularly (figure 11). These nerve fibers have usually a big bulb at their ends and are bent in curves and angles. They are richly provided with swellings and form branches and anastomoses within the first twenty-four hours. After two or three days the fibers were rather thick (from two to three microns), and some of them showed a faint fibrillation. The swellings now have become very numerous and have a diameter of four to five microns; they are either globular or oblong and are quite homogeneous. After five days' incubation the length of these fibers was 0.9 of a millimeter.

On the fifth day these fibers showed the following degenerative changes: A faint granulation appeared first in the swellings and shortly afterward in the fibers. The outlines of the fibers became less definite, the fibers and the swellings became less refractive, and the edges of the globular swellings appeared as if they had been nibbled. The change was seen at the same time throughout the whole length of the fiber and increased during the next two or three days, so that on the seventh day after incubation all the fibers appeared degenerated.

EXPERIMENTAL PART.

I shall describe in detail one of the experiments in which I cut some of the nerve fibers in the cultures of the cerebellum of dogs three weeks old.

After twenty-four hours a large number of axis cylinders had developed, which increased in size during the following two days. On the fourth day I cut through the plasma and some of the nerve fibers contained in it close to the piece of tissue (figure 12). As the coagulated plasmatic jelly is under tension the cut in it produced a small gap by the retraction of the medium. This was immediately filled with fresh plasma from the same animal, which had been kept on ice, and which now coagulated. The cover-slip was again inverted on a hollow slide and incubated. It was observed from hour to hour until late in the evening. The cutting of the plasma had disturbed slightly the central part of the cut nerve fibers, as the retraction had taken place especially at this side. It was observed immedi-
Ragnvald Ingebirtsen.

ately after section that the central part of the nerve fibers had been a little crumpled and their outlines were more curved than before (figure 12). This was not true of the peripheral part in which no visible changes took place either at the time of the cutting or in the following nine hours. The next morning, however, that is, twenty hours after the cutting, changes of a different kind had occurred, degenerative as well as regenerative (figure 13). The cut fibers were thinner than on the previous day. This could be determined by comparing them with nearby fibers which were not cut, and which before section were of the same thickness. Moreover, the cut fibers had a faint granulation and the edge of the swellings appeared moth-eaten. The appearance of the central part of the cut fibers had not changed visibly. But in the gap that was filled with plasma there had grown out from the central part of the cut fibers three axis cylinders, of which the longest one, grown during the lapse of twenty hours since the cutting, had a length of 150 microns. There was nothing else extraordinary about them. They looked like the other young axis cylinders, had end bulbs and some swellings. Their growth was followed during the next two days, during which time one of them increased sixty-five microns in length, the others not quite so much (figure 14).

Meanwhile the degeneration in the peripheral part of the cut fibers continued slowly throughout its entire length (figures 14 and 15). The fibers became thinner and less refractive, and the granulations increased. At last, on the sixth day, they could hardly be seen. At this time the other fibers which had not been cut were partly granular but their outlines were still distinct (figure 15). The specimens were stained after three and six days.

The procedure which after several attempts was found to give the most successful preparation was the following:

1. Fixation in 2 per cent. formalin for 15 to 18 hours.
2. Staining in a very dilute solution of Held's molybdic acid hematoxylin (6 to 8 drops to 15 c.c. of water) for 12 hours.
3. Differentiation in a dilute solution of the Weigert borax-potassium-ferrocyanide solution (2 drops to 20 c.c. of water) for 12 to 20 hours.
4. Dehydration, xylol, Canada balsam.

The color of the fibers is greyish blue, and they are very distinct. The method seems to injure the fibers a little, for even when fixed
Degeneration and Regeneration of Axis Cylinders in Vitro.

and stained after three days’ incubation the stained specimens are plainly granular, while the living fibers show no granulation at all. I have, therefore, studied mainly the living specimens, and most of the illustrations given here are camera lucida drawings of living fibers. The camera lucida was employed because it is hardly possible to get a photograph of one nerve fiber in its whole length, as it curves and bends through many planes, and living fibers were drawn because these are of course of greater interest than those that are dead.

CONCLUSIONS.

1. The brains of chick embryos, of cats six weeks old, of rabbits two months old, and of dogs three weeks old, when cultivated in vitro, develop long filaments which, according to their growth and their anatomical and tinctorial characters, must be considered as true axis cylinders.

2. Similar structures develop from spinal ganglia of rabbits seven months old, and from the spinal cord of cats six weeks old, and of rabbits two months old.

3. When severed from their origin by section these threads undergo degenerative changes which do not appear after nine hours, but which are seen after twenty hours, and continue until in the course of the following two days the thread degenerates completely.

4. After twenty hours the development of new axis cylinders from the central part of the cut fibers is observed.

EXPLANATION OF PLATES.

PLATE 28.

Fig. 1. Culture of brain cortex twenty-four hours old from a chick embryo six days old. Culture on silk veil.

PLATE 29.

Fig. 2. Culture of brain cortex two days old from a chick embryo fourteen days old.

PLATE 30.

Fig. 3. Culture of brain cortex forty-eight hours old from a chick embryo six days old. Isolated cells, one of them with an axis cylinder.

PLATE 31.

Fig. 4. Culture of brain cortex forty-eight hours old from a chick embryo twelve days old. Isolated ganglion cell with axis cylinder.
FIG. 1.
(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders in Vitro.)
FIG. 2.

(Ingebrigten: Degeneration and Regeneration of Axis Cylinders in Vitro.)
Fig. 3.
(Ingebristsen: Degeneration and Regeneration of Axis Cylinders in Vitro.)
FIG. 4.
(Ingebrigsten: Degeneration and Regeneration of Axis Cylinders in Vió.)
FIG. 5.
Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders in Vitro.
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Fig. 8.
(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders in Vitro.)
Fig. 9.

Fig. 10.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders in Vitro.)
FIG. 11.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders in Vitro.)
FIG. 12.
(Ingebrigtseter: Degeneration and Regeneration of Axis Cylinders in Vitro.)
FIG. 13.
(Ingebritsen: Degeneration and Regeneration of Axis Cylinders in Vitro.)
FIG. 14.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders in Vitro.)
FIG. 15.

(Ingebrighten: Degeneration and Regeneration of Axis Cylinders in Vitro.)
Fig. 5. Connective tissue cells from a four day old culture of spinal ganglion of a cat six weeks old. Stained with hematoxylin.

Fig. 6. Culture of brain cortex three days old from a chick embryo eighteen days old.

Fig. 7. Culture of spinal medulla three days old from a chick embryo fourteen days old.

Fig. 8. Culture of spinal ganglion four days old from a rabbit seven months old.

Fig. 9. Culture of spinal ganglion two days old from a rabbit seven months old.

Fig. 10. Culture of brain cortex twenty-four hours old from a dog three weeks old.

Fig. 11. Culture of cerebellar cortex three days old from a dog three weeks old. Figures 12 to 15 are made from this culture in different stages.

Fig. 12. Culture of cerebellar cortex three days old from a dog three weeks old immediately after the cutting of the fibres. The gap is visible.

Fig. 13. Culture of cerebellar cortex four days old twenty hours after the cutting of the fibres which are slightly degenerated. New axis cylinders are seen in the gap.

Fig. 14. Culture of cerebellar cortex five days old forty-four hours after the cutting of the fibers which are still more degenerated. The new fibers in the gap have grown a little longer; one of them is slightly degenerated.

Fig. 15. Culture of cerebellar cortex five days old from a place just beyond the cut fibers. These axis cylinders are not degenerated.