Chapter IV

NEURONAL REGENERATION IN VITRO

By

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Nerve regeneration is the restoration of morphological and physiological continuity in transected neurites. Whereas a very extensive literature exists on morphological, physiological and pathological aspects of regeneration *in vivo*, a surprisingly limited amount of work has been devoted to the analysis of the phenomena *in vitro*. This is perhaps due to the fact that the technique does not provide favorable conditions for analysis of this process. As is well-known, nerve fibers grow out from the explant in bundles of varying thickness or in small groups intermingled with migrating spindle cells. Even when the nerve fibers are separated at their origin they have the tendency to fasciculate and form complex plexuses in which the individuality of single neurites can hardly be traced. In this connection the observation of Lumsden ('51) on a dorsal root ganglion culture of a 9-day embryo in which one axon was followed for a very long distance deserves mentioning.

According to Levi and Meyer ('41) nerve cells lack the capacity for active migration *in vitro*. They remain packed in the explant, a condition very unfavorable for close inspection. More recent investigations (Murray and Stout, '47; Costero and Pomerat, '51) have demonstrated that nerve cells acutally can migrate *in vitro*. However, as stated by Costero and Pomerat ('51), "even though neurons are capable of active migration in tissue cultures, it is evident that such migration is not only slow but it occurs during limited periods . . . . [nerve cells] appear as the most static cells of the organism in moving picture records" (reference pp. 437-438).

The process of nerve regeneration *in vitro* and the reaction of nerve cells to microinjury of the cell body were studied by Levi and Meyer ('45) in the sensory ganglia of the chick embryo. Sensory ganglia undergo spontaneous dissociation after a few days *in vitro* and therefore were selected as particularly suitable material for the study of individual units. "The dissociation is chiefly to be referred to the very active migration of fibrocytes and of satellite cells. The ganglionic cells become well isolated from each other and can easily be distinguished by their form, by their great volume and by their typical refractiveness" (reference p. 145). Taking advantage of this condition, Levi and Meyer ('38; '45) studied in detail the effect of microsurgical injuries on individual neurons and on regenerative processes following transection of single nerve fibers or of fibers interconnected in plexuses. The first part of our report will deal with the results of their investigation.

REGENERATIVE PROCESSES AND REGENERATION PER PRIMAM

Experiments were performed on sensory neurons of 10- to 13-day chick embryos after 3 or 4 days of culture at low temperature in hanging drop (Levi and Meyer, '38). The method of growing nerve tissue at low temperature (30-32°) is particularly favorable for the study of nerve fibers because fibroblasts and spindle cells are almost completely prevented from migration whereas nerve fibers are unaffected or even enhanced in their growth. With the help of the micromanipulator, single nerve fibers were transected; to avoid the immediate union of the two stumps the plasma in which the fiber was embedded was also cut. The elastic retraction of the coagulum maintained a distance of a few microns between the two fragments. As a first result of the section minute granules were observed at the ends of both stumps. They increased 10 to 30 minutes later and at the same time the two cut ends of the fiber appeared swollen and turbid. From the proximal end, fine filaments emerged. A similar phenomenon, though less frequent, was observed at the central end of the peripheral stump. Under favorable conditions the filaments from the proximal stump

bridged the distance between the two cut ends of the fiber in about one hour (Fig. 18A, B, C). In such a case no regressive changes took place in the distal stump and *restitutio per primam* occurred before the onset of any sign of deterioration. When the establish-

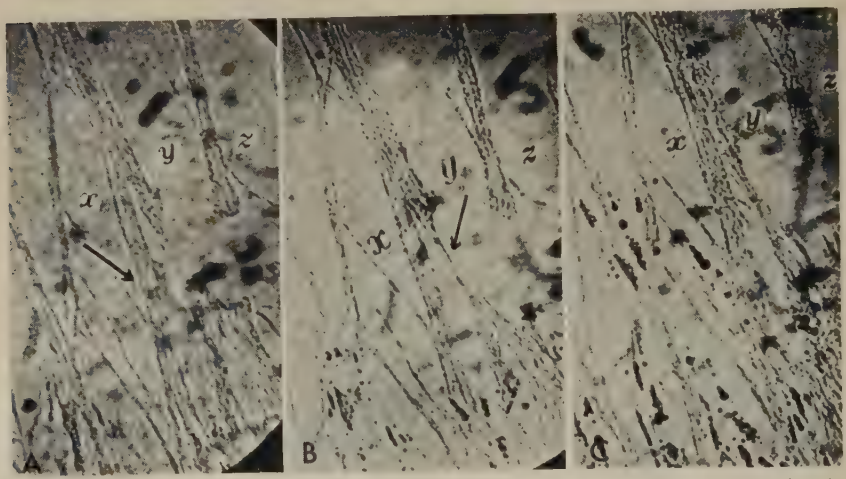


FIGURE 18. Regeneration *per primam* following transection of nerve bundles *in vitro*. A. Connection at x has been re-established 45 minutes after transection but gaps exist at y and z. B. A thin bundle connects the proximal and distal stumps at y, 3 hours after transection. C. The bundle at y has become thickened but there has been no regeneration at z, 19 hours after transection. Silver impregnation technique. Photomicrographs from Levi and Meyer ('45); courstesy, Wistar Institute Press.

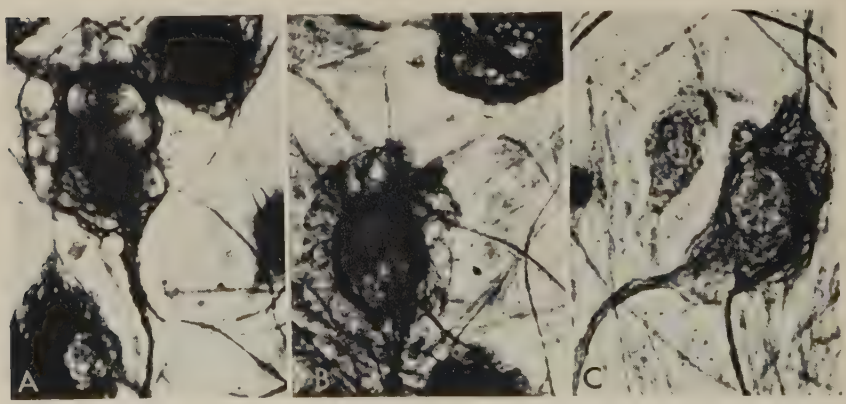


FIGURE 19. Sensory neurons showing different degrees of regressive changes with formation of vacuoles between the neurofibrils at A and B, following micropuncture of the cell body. A control cell is shown in C. Silver impregnation technique. Photomicrographs from Levi and Meyer ('45); courtesy, Wistar Institute Press.

ment of the connections took a longer time (3-4 hours) the peripheral stump showed regressive changes which, however, did not prevent the healing process. If a longer time was required the peripheral stump underwent irreversible regressive changes and it disintegrated even in those cases in which the proximal stump had eventually succeeded in bridging the gap.

A very interesting phenomenon was observed when the operation was performed on fibers growing in plexuses. In these instances the regression of the distal stump stopped at the point of its junction with an intact fiber. This result proved the material continuity between neurites of different neurons *in vitro*. We do not know if a similar continuity occurs in living organisms.

In another series of experiments Levi and Meyer ('37; '45) studied regressive processes following micropuncture of the nerve cell. As a result of the injury they observed a sudden flattening of the cell and the formation of vacuoles between the neurofibrils (Fig. 19A, B). The latter, on the contrary, showed a remarkable resistance to any traumatic injury; their disintegration was observed only as a result of very severe mechanical lesions.

It would seem that a reinvestigation of isolated nerve cells in the ganglionic explant should lead to new information on this important chapter of nerve cell physiology. By using the technique first introduced by Moscona[1] and subsequently modified by St. Amand and Tipton[2] for the isolation of neuroblasts of grasshoppers we have succeeded in growing different types of isolated neurons successfully *in vitro* (Fig. 20). We are planning to investigate the process of growth of single neurons under different experimental conditions and particularly under those to be discussed in the second part of this report.

EFFECT OF A DIFFUSIBLE AGENT OF MOUSE SARCOMAS

In most instances of nerve tissue explantation one deals with cells which had already produced nerve fibers at the time of the operation and were cut in the process of isolation and explantation

[1] *Exp. Cell Res., 3*:535; 1952.
[2] *Science, 119*:93; 1954.

in vitro. Strictly speaking, the subsequent fiber growth does not fit the usual definition of nerve regeneration since there is no peripheral stump. An extension of the use of the term, regeneration, to cover all phenomena of nerve growth *in vitro* seems, however, to be appropriate in view of the fact that we are dealing in most instances with a second outgrowth of fibers. From the large literature on this topic we have selected one particular instance, namely

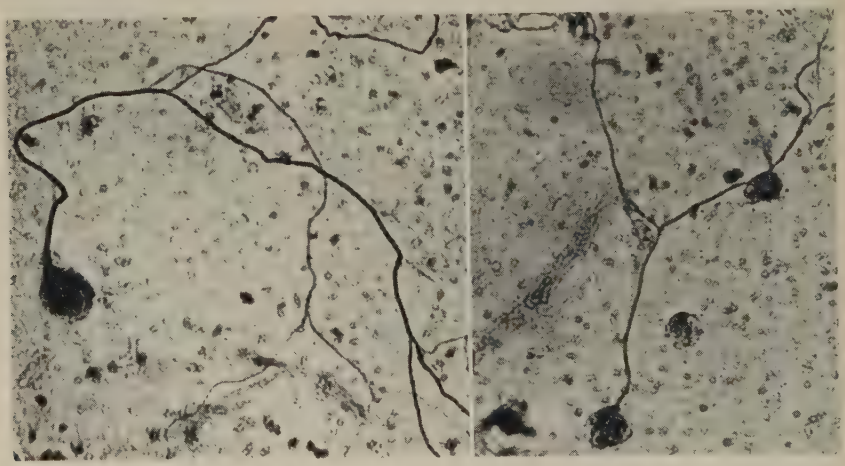


FIGURE 20. Isolation of sensory neurons of a 9-day embryo *in vitro*, 48 hours. Silver impregnation technique.

the effect of a diffusible agent on nerve growth. This choice is suggested for two reasons—to make use of our personal experience and because it is related to other instances of humoral agents affecting nerve regeneration which will be discussed in this conference.

Sensory and sympathetic ganglia from 6- to 13-day embryos were used in this investigation. It will be remembered that nerve fibers emerge from both types of ganglia in a very early stage of their embryonic life. Differentiation starts in the sensory ganglia at 3 days. At 6 days the ganglia are already connected by two rather large nerve roots to the spinal cord and the periphery. At 7 days well established reflex activity (Windle and Orr, '34) shows that not only morphological but also physiological maturation has been attained by sensory cells. Explantation after the sixth day of incubation requires the section of a large number of nerve fibers

collected in the proximal and distal roots. In all cases the two roots were exposed and cut in the immediate proximity of the ganglion. The ganglion was then explanted in hanging drop following the standard technique.

Sympathetic ganglia were explanted in a later stage, after the ninth day of incubation. At this stage the paravertebral chain is well differentiated and a solid strand of nerve fibers connects the ganglia.

Regeneration of nerve fibers from the sensory ganglia has been the object of extensive studies in the past (Levi and Meyer, '41). The process and the pattern of growth may be summarized. Several hours after explanation *in vitro* (10 to 15 hours depending on the stage of differentiation, the temperature and the medium) spindle cells begin to migrate from the entire circumference of the ganglion. The outgrowth of nerve fibers follows with some delay. It is not until 15 to 18 hours after the explantation *in vitro* that nerve fibers are found among the migrating cells. In the beginning they are few in number and usually restricted to the cut end of one of the two roots. In the following hours they increase in number and are found irregularly scattered all around the explant. They run as single fibers or join others and form nerve bundles or they creep along the surface of strands of cells and give origin to ribbonlike formations.

The mechanism of nerve elongation and the role played by the medium in determining the direction of growth of nerve fibers have been extensively investigated by Weiss ('34; '45; Weiss and Hiscoe, '48). The results of his investigations are too well known to be repeated here. In these as in many other problems of differentiation the method of tissue culture has provided invaluable information and settled long-lasting controversies. Even the often-mentioned shortcoming of this method—that it confronts us with an oversimplified version of more complex processes occurring *in vivo*—may represent a further asset if this method is used with discretion. It permits, in fact, direct exposure of the reacting system to the agent which one wishes to test. By comparing the results *in vitro* with the results *in vivo* one may hope to get a better insight into the phenomenon under investigation.

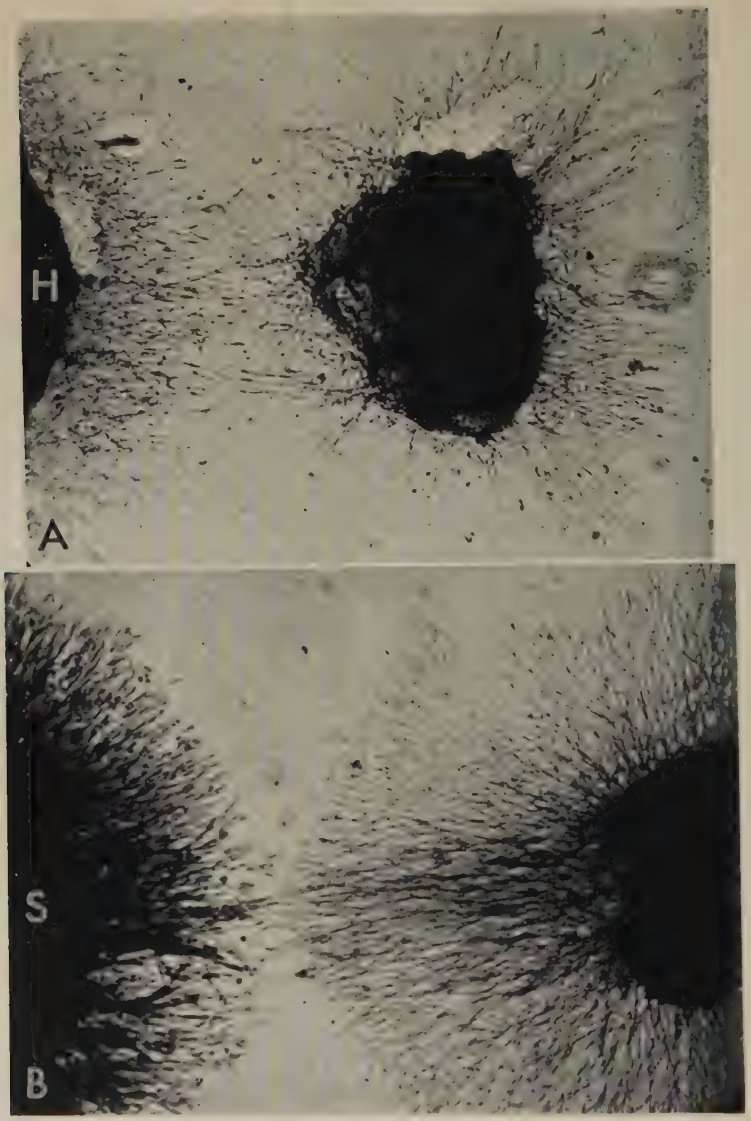


FIGURE 21. Thoracic sensory ganglion explants from a 7-day embryo cultivated 24 hours *in vitro*. A. combined with heart tissue (H); B. with sarcoma 37 (S). Silver impregnation of specimens.

These considerations suggested the use of the method of tissue culture to solve a problem which we were otherwise unable to

solve. Before stating the problem it will be necessary to mention the experiments which raised the problem. It was found that some mouse sarcomas,[1] when transplanted to the body wall (Bueker, '48) or onto the allantoic membrane of 3-4-day chick embryos released an agent (Levi-Montalcini, '52; Levi-Montalcini and Hamburger, '53) which affected the sensory and sympathetic systems of the host. Both systems underwent differentiation earlier than in control embryos; both were stimulated to excessive growth; due in part to increase in cell number and in part to increase in cell size. Nerve fibers were produced in numbers far exceeding the controls; they invaded the viscera and pierced the walls of blood vessels. Large blood vessels were obliterated by the aberrant nerves.

We were confronted with two major problems, the chemical nature of the agent and its mode of action. A number of experiments applying extract or homogenate of the tumor to the living embryo were unsuccessful. The question as to whether the tumor agent acted directly on the nerve cells or indirectly by changing the properties of the organs and making them "permeable" to nerve fibers remained unanswered. The possibility of testing the effect of the tumor *in vitro* was considered. Fragments of tumors (sarcomas 180 and 37) were explanted *in vitro* in close proximity to sensory or sympathetic ganglia of chick embryos (Levi-Montalcini, Meyer and Hamburger, '54). Both types of ganglia reacted by overproduction of nerve fibers, forming a dense halo around the ganglia (Fig. 21B). The effect was detectable 10 hours after the explantation and increased in the following 24 hours. Control ganglia showed fibroblasts but almost no nerve growth during the same period (Fig. 21A).

Detailed analysis of the effect *in vitro* led to the conclusion that it was due to the same agent which operated *in vivo*. The realization of the effect *in vitro* ruled out possible influences of the host embryo and gave support to the hypothesis that the nerve cells were the direct target of the tumor agent.

Our attention was then focused on the chemical nature of the

[1]A recent report (Bueker and Leeper, '54) indicates that a human sarcoma failed to stimulate neuronal growth in the chick. *Ed.*

agent. The following experiments were planned and performed in collaboration with Dr. Stanley Cohen (Levi-Montalcini, Cohen

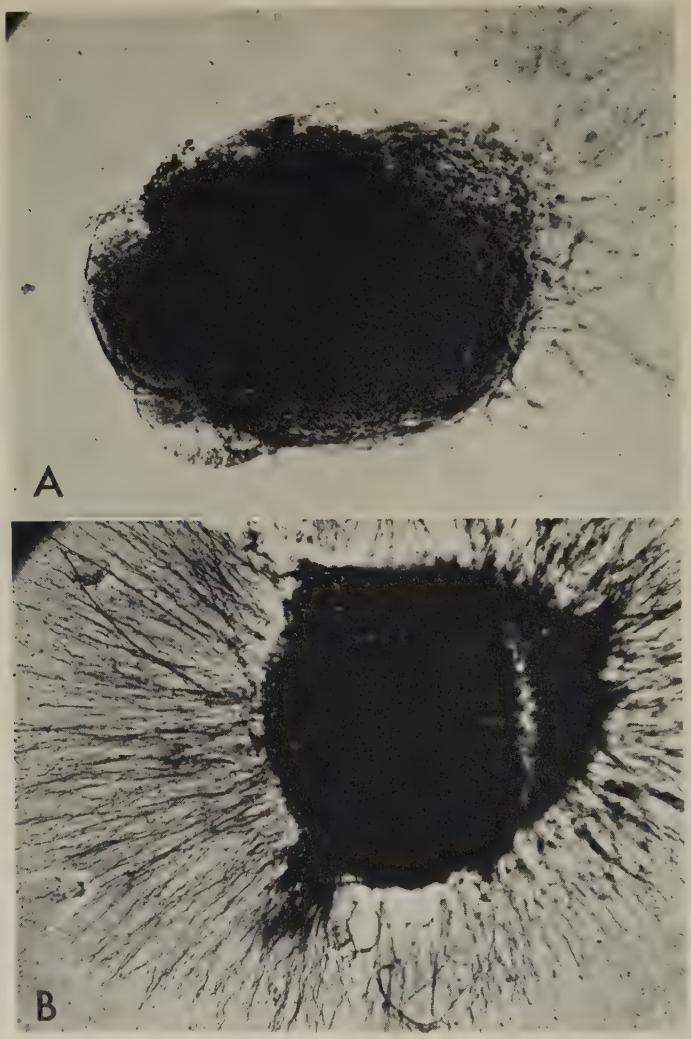
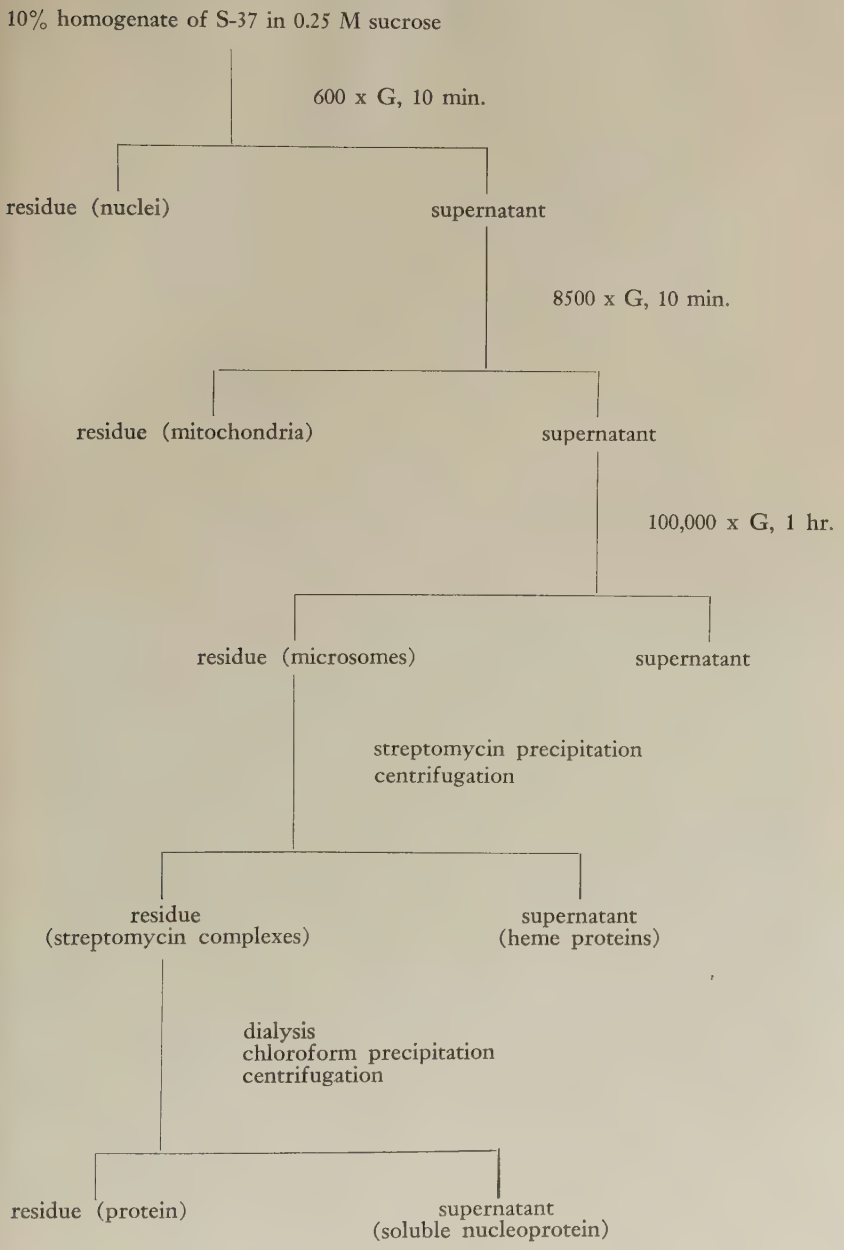


FIGURE 22. Lumbar ganglion explants of a 7-day embryo cultivated for 18 hours. A. in a standard medium; B. in a medium to which cell-free homogenate of sarcoma 37 was added. Silver impregnation of specimens.

and Hamburger, '54; Cohn, Levi-Montalcini and Hamburger, '54).

TABLE I

FRACTIONATION OF NERVE GROWTH PROMOTING FACTOR



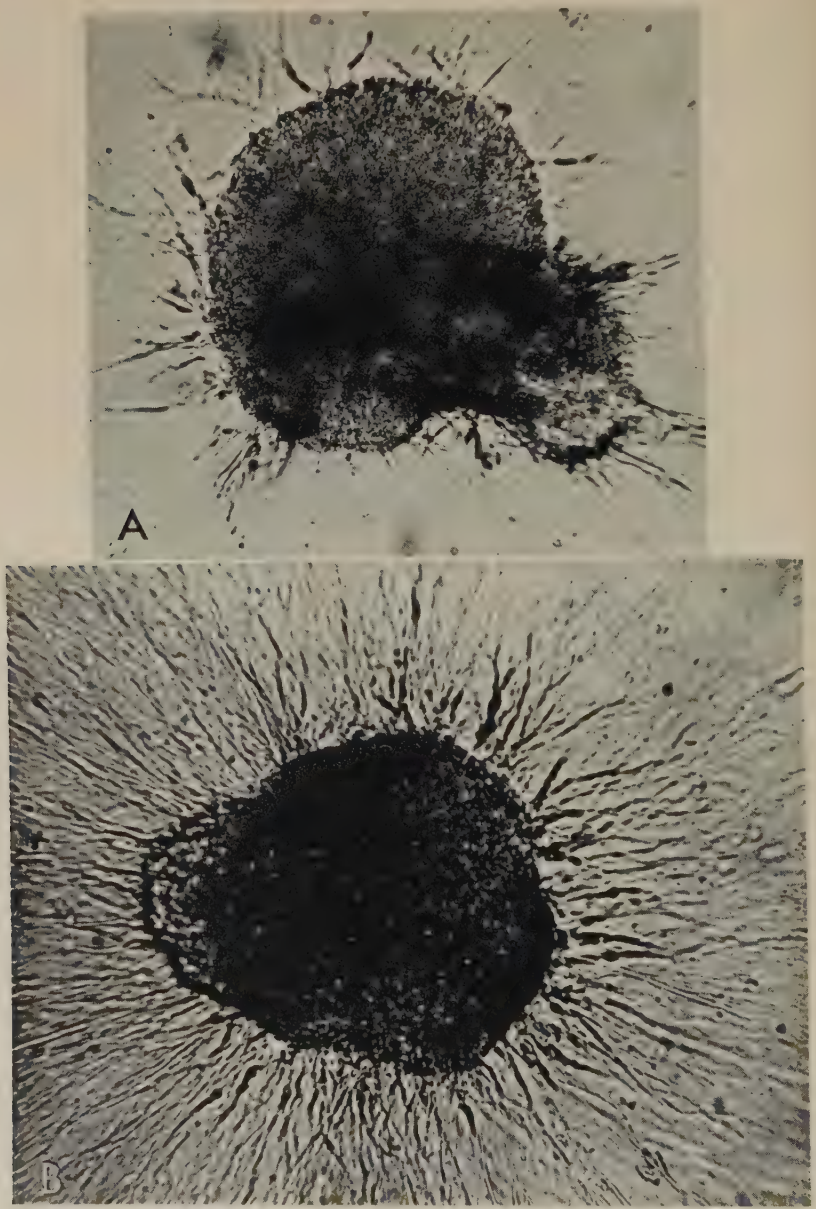


FIGURE 23. Sympathetic ganglion explants of a 10-day embryo cultivated for 18 hours. A. in a standard medium; B. in a medium to which the nucleoprotein fraction of the homogenate of sarcoma 37 was added. Photomicrographs of living cultures.

A cell-free homogenate of the tumor was prepared and a drop was added to the medium in which a sensory or sympathetic ganglion was explanted. The effect of the tumor extract was very much similar to the effect of a growing fragment of the tumor. Although control ganglia had few or no fibers after 15 to 20 hours *in vitro* (Fig. 22A), ganglia in the medium containing tumor extract showed a dense halo of nerve fibers (Fig. 22B). The effect could be detected as early as 6 hours after the beginning of the experiments.

Differential centrifugation of the homogenates in 0.25 M sucrose at pH 7.4 (Table I) gave evidence of the presence of the active tumor agent in the microsome fraction. Both nuclei and mitochondria fractions were inactive. The activity in the microsome fraction was retained after dialysis against water or saline; it was lost by heating at 75°C for five minutes.

A further purification of the microsome fraction was obtained by treating it with streptomycin sulfate (0.02M; pH 7.2). By this method the nucleoprotein content of the microsome fraction was precipitated but it retained all its activity. After removal of the streptomycin by dialysis, the precipitate was treated with chloroform (gently mixed for 10 minutes) and centrifuged at moderate speed (8500xG). A precipitate was thus obtained which contained some proteins (Table I). The lipids were dissolved in the chloroform, and the clear, slightly opalescent supernatant was fully effective (Fig. 23A, B). Analysis of this soluble material gave: proteins 66 per cent, RNA 27 per cent and DNA 0.2 per cent. It was still nondialysable and heat labile. This active fraction represented 4 per cent of the original tumor dry weight. The agent is, or is associated with, a ribonucleoprotein. A first step in the chemical analysis of the agent has thus been achieved but we are still far from its identification and no progress has been made in answering the second and equally important question: its mode of action. As so often in biological research, more questions have been raised than answered.