ing a more rapid indication of toxic responses than is presently available through classical toxicological animal feeding studies. The method of tissue culture and the application of growth analyses, *i.e.*, DNA, protein, and P.C.V., offer a quantitative means of detecting cytotoxicity before morphological changes become evident. Tissue culture offers a screening procedure capable of indicating substances requiring additional testing by more intensive methods, and a packed cell volume assay appears to offer a rapid direct procedure. ABS was found to be growth-suppressive and evocative of cytopathological changes, at several concentrations. In addition, these reactions were produced when relatively non-toxic levels were in contact with the growing cells for an extended period: 6 days in this series of experiments. Again, non-toxic levels showed similar toxic patterns when added in increasing increments, step-wise fashion, daily.

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## Nerve Growth Factor Requirement for Development of Dissociated Embryonic Sensory and Sympathetic Ganglia in Culture. (29373)

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Nerve growth factor (NGF), a specific protein material extracted from mouse salivary glands, stimulates the growth of embryonic sensory and sympathetic nerve cells (1,2). Earlier, Nakai(3) experienced little success in attempting to grow enzymatically dissociated dorsal root ganglia in culture, but recently Levi-Montalcini and Angeletti(4) pointed out the requirement of NGF for survival and maintenance of dissociated embryonic sensory and sympathetic nerve cells in culture.

This paper concerns the finding that NGF under special culturing conditions enhanced the later development and organization of similar dissociated ganglia from single cell cultures into ganglia-like masses.

*Materials and methods. Tissue culture.* The entire lateral lines of dorsal root and sympathetic ganglia of 8-day-old chick embryos were dissected, cleaned, and teased apart with fine forceps. For dissociation of the ganglia, 0.15% Pancreatin (Wilzyme 600, Wilson Laboratories) was used. In early experiments the agitation was carried out at 37°C for 45 minutes (one fraction only). This resulted in complete dissociation of the tissue but yielded many supporting cells in culture. However, it was later found that agitation at 37°C using 3 $\times$ 20 minute fractions and plating only the third fraction after centrifugation produced cultures nearly completely free of supporting cells. Approximately 2.5 $\times$ $10^5$ cells were plated on plastic petri dishes (Falcon disposable) some of which contained uncoated glass coverslips. A total of 4 ml of growth medium was used per plate and the incubation carried out in a gas exchange incubator in a 5% $CO_2$ atmosphere at 37°C.

Various media were tested to determine optimal growth effects on the cells (Eagle, 199, 1066 and tryptose phosphate with various concentrations of different sera). The medium of choice consisted of 0.5% lactalbumin hydrolysate (Difco) dissolved in Earle's salt solution fortified with vitamins, glutamin, 0.053 M glucose and antibiotics. The

complete growth medium contained 20% calf serum.

NGF was prepared from mouse submaxillary salivary glands and purified by the procedures described by Cohen(1). The material assayed $2.0 \times 10^5$ units/mg and 0.5 $\mu$g/ml was added to the growth medium.

Crystalline zinc insulin was obtained from Eli Lilly and Co., and used at a concentration of 50 milliunits/ml of growth medium. The medium was changed 3 times a week at which time insulin and NGF were also added to appropriate plates.

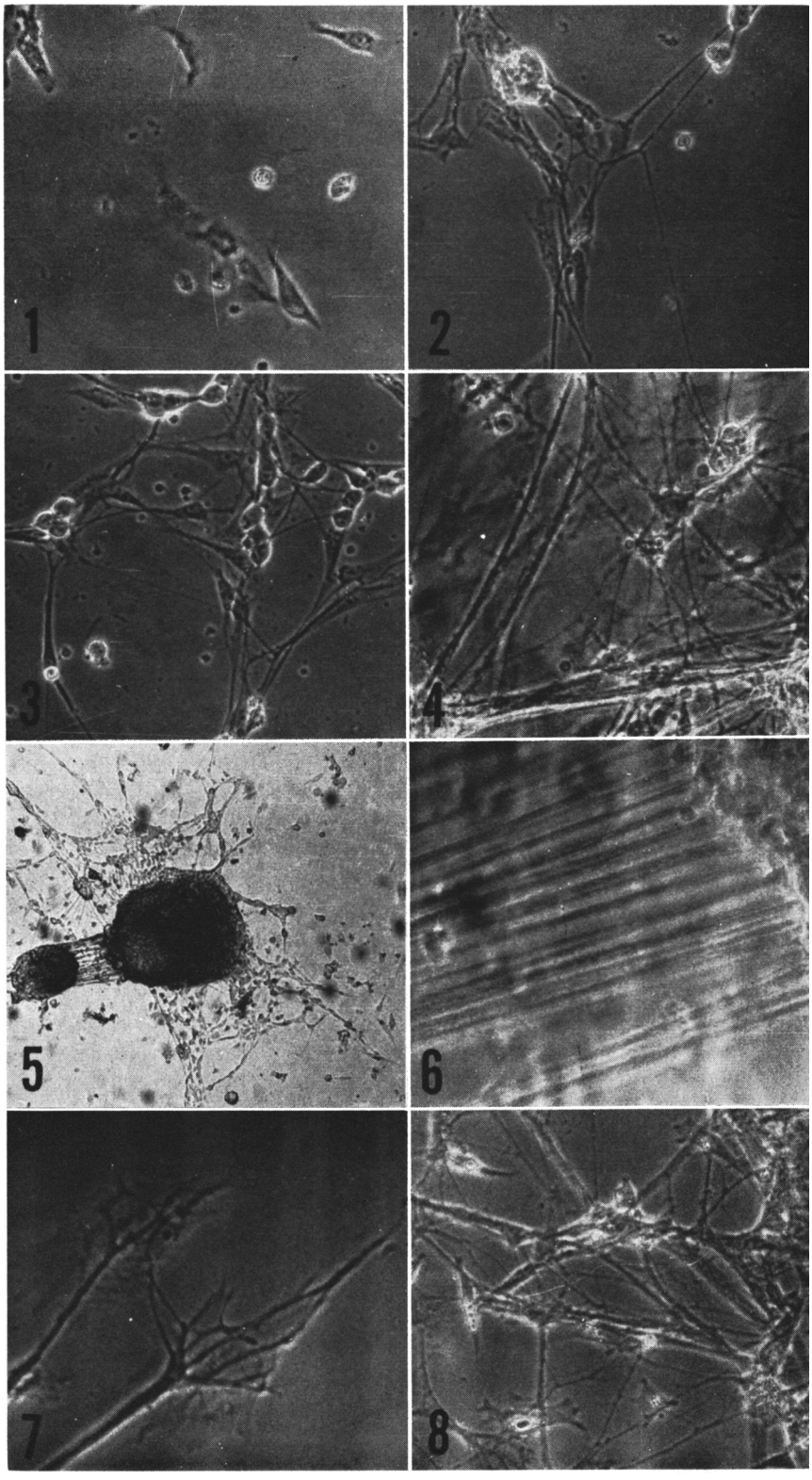
*Electron microscopy.* Coverslip cultures were prepared for study in the electron microscope by fixing, dehydrating and embedding the tissue while still attached to the glass. Fixation was in Palade's veronal buffered osmium tetroxide with addition of sucrose as suggested by Caulfield(5). Dehydration in a graded series of ethanol was followed by embedding in epon according to the method of Luft(6). The coverslip was covered with a 2 mm layer of epon and following polymerization the resin was separated from the glass by touching the glass to dry ice for a few seconds. The cells could then be observed in the plastic utilizing a dissecting microscope. Selected areas of the culture were cut from the resin sheet and mounted on blank epon blocks with epoxy glue. Ultrathin sections were cut and examined with the electron microscope.

*Results. Tissue culture.* In the absence of NGF approximately 10% of the plated cells attached and stretched within 48 hours (Fig. 1). All the culture dishes were carefully screened to remove any fragments which may have been plated. The cell nuclei and cytoplasmic granules were clearly defined, but no outgrowth of nerve fibers was observed. With passage of time no further development occurred in these cells. When 400 units of NGF was added at time of plating about 3 times as many cells attached and stretched within 24 hours. By the end of the second day small aggregations of stretched nerve cells were observed with clearly defined nerve processes (Fig. 2). A high glucose concentration in the medium was essential in order that this effect occur. Possibly to increase the glucose utilization by the nerve cells, insulin was added to the medium. This markedly enhanced the development of networks of the stretched nerve cells and the outgrowth of filamentous processes (Fig. 3). All subsequent studies reported here were done with a combination of insulin and a high glucose concentration in the growth medium.

At the end of the first week in culture the small aggregates of stretched nerve cells tended to form dense cell clusters and the fiber outgrowths developed into a thicker network (Fig. 4). The nerve cells forming clusters were no longer stretched but compact, small and somewhat rounded in appearance. Cell detail was not clear when observed with the phase microscope. Between the clusters there soon formed multi-fiber nerve trunks. It appeared from numerous daily observations that these clusters were being formed by migration of the nerve cells in different areas of the plate to form randomly a number of small ganglia-like masses. Small clusters in turn seemed to coalesce and develop into larger ganglia-like masses (Fig. 5). By the end of the third or fourth week nearly the entire nerve cell population appeared to complete its migration to form large ganglia-like masses some of which existed as a chain of 5 or 6 nerve cell structures with interconnecting fiber trunks and surrounding fibrous networks (Fig. 6). The terminals of fibers proliferating from a thin nerve trunk may be seen in Fig. 7. Only a few single stretched nerve cells were visible at this time. We are not certain whether any cell division occurred in the developmental and organizational stages described above.

Throughout the study supporting and satellite cells were not observed in any significant number. After approximately a month's growth, the fiber trunks between the ganglia-like structures became detached from the plastic or glass surface, swinging freely in the medium. This was quickly followed by degeneration of the ganglia-like structures. Only at this time did the few supporting cells vigorously multiply and eventually overgrow the plates.

The identical pattern of nerve cell development in culture occurred when the em-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bryonic chick sympathetic ganglia were used (Fig. 8).

Ten separate plating experiments were done all of which were positive for the NGF-stimulatory growth pattern effect. In the absence of NGF the results were always negative. Insulin with NGF always enhanced the development of the ganglia-like structures, but insulin alone was no different from the controls.

*Morphology.* Stained coverslip preparations were examined by light microscopy and ultrathin sections were studied with the electron microscope to gain an understanding of the morphology of the tissue and its relationship to organized nervous tissue from intact animals. The cells had many of the morphological characteristics associated with nerve tissue and neurons. The cell bodies took a strong stain for Nissl substance (gallocyanin/chrome alum) and electron micrographs revealed large compact masses of ribonucleoprotein (RNP) granules in an arrangement similar to that of Nissl substance in neurons.

The cell processes which could be observed by phase microscopy were even more striking when stained. Each organized cell mass was surrounded by a dense network of fibers which became oriented into a few long processes extending great distances into the media and often connecting 2 cell masses several millimeters apart. These cell processes took a dense silver stain (modified Bodian) and in such preparations many fine fibers became visible for the first time (Fig. 9, 10). The nerve processes resembled axons more than dendrites because of their great length and infrequent branching: their response to silver staining was typical of that for nerve fibers.

The ganglia-like structures observed with the electron microscope were made up of numerous cells and their processes in an interwoven pattern reminiscent of some organized nerve tissue or ganglia. There was no evidence of myelin formation. The interweaving fibers and smaller cells filled in the spaces between larger cells and seemed to form a supporting mechanism for the tissue.

Nuclei and cell bodies were randomly spaced among bundles of processes which ran in many directions. Thus, a section included cross sections, longitudinal sections, and tangential sections of bundles of cell processes (Fig. 11). The individual cell process was similar to an axon in ultrastructure. It was surrounded by a unit membrane and was filled with fine neurofilaments undulating in the longitudinal direction. There were occasional small elongate mitochondria and a few fine tubular elements. RNP particles were not present in the cell processes. Cell bodies contained dense masses of RNP particles just as were seen in ganglion cells of intact nervous tissue.

*Discussion.* NGF was required for the development of embryonic sensory and sympathetic nerve cells in culture. Peterson and Murray(7) have shown in tissue culture that only with intact embryonic dorsal root ganglia has progressive differentiation of this tissue been observed without NGF. The specialized morphology of the nerve cells and fiber network with terminal endings, and collateral connections was maintained in the presence of the specific growth factor. The extraordinary ability of the dissociated nerve cells to reaggregate and reorganize under appropriate culture conditions into organ-like structures is similar to what has been described by Moscona(8) with other cell types. Large areas of an electron micrograph of a newly-formed ganglionic structure were indistinguishable from similar pictures of intact chick sensory ganglia.

PLATE I. Phase-contrast microphotographs of cultures of dissociated sensory (Fig. 1-7) and sympathetic (Fig. 8) chick embryo ganglia.

FIG. 1. 2-Day culture without NGF or insulin. × 240.
FIG. 2. 2-Day culture with NGF. × 240.
FIG. 3. 2-Day culture with NGF and insulin. × 240.
FIG. 4. 8-Day culture with NGF and insulin. × 240.
FIG. 5. 22-Day culture with NGF and insulin. × 38.
FIG. 6. 27-Day culture with NGF and insulin. × 240.
FIG. 7. 7-Day culture with NGF and insulin. × 240.
FIG. 8. 6-Day culture with NGF and insulin. × 240.

The beneficial effect of having a high glucose concentration in nerve cell media has been recommended previously(9). Other workers have reported the influence of insulin on intact nerve tissue(10,11) and on organ cultures of various tissues(12).

*Summary.* Dissociated embryonic sensory and sympathetic nerve cells required NGF for development in culture from single cell stage to the formation of ganglia-like struc-

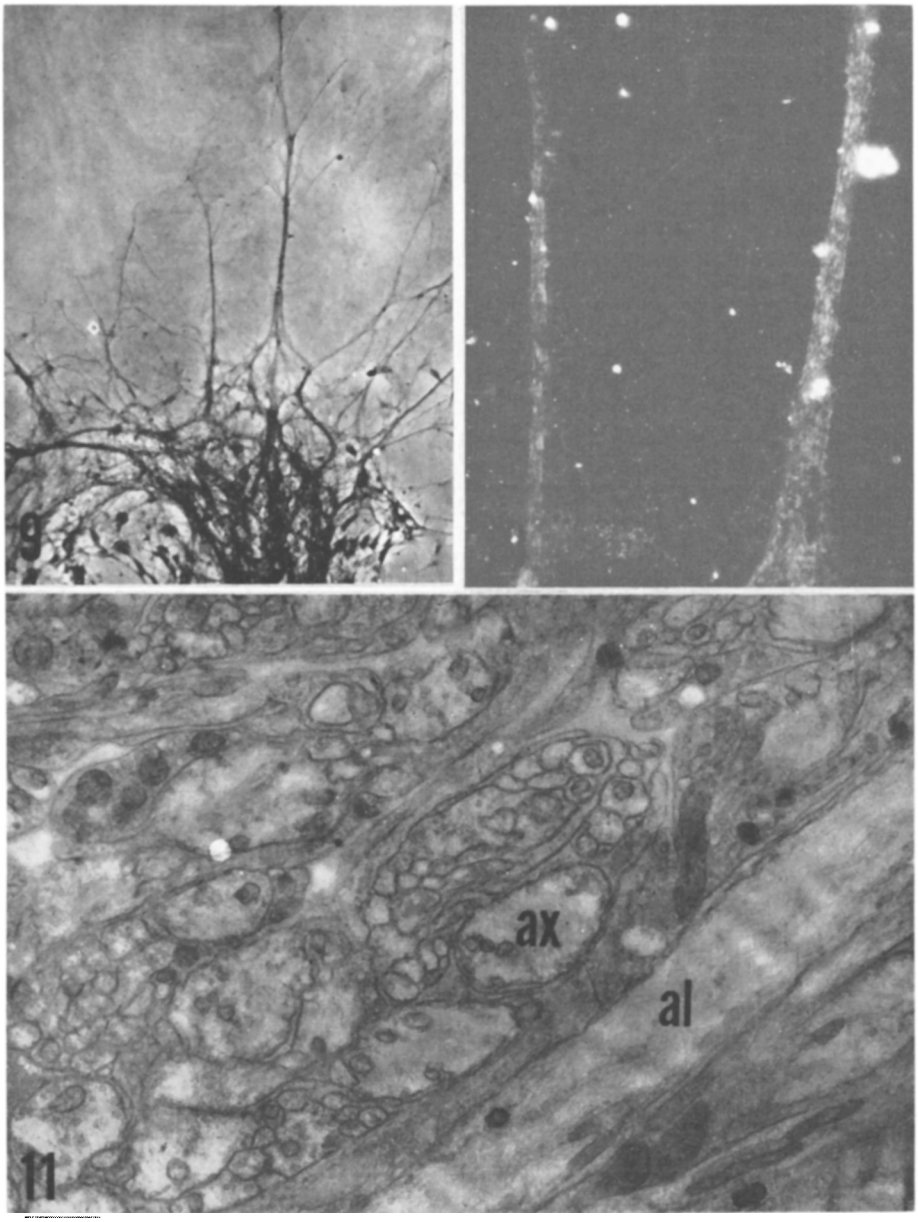


PLATE II. Fixed specimens of cultures of sensory nerve cells on uncoated glass coverslips.

FIG. 9. Fiber network outgrowth from ganglia-like mass of 15-day culture with NGF and insulin, silver stained. × 80.

FIG. 10. Dark-field illumination of individual fiber trunks of network in Fig. 9. × 320.

FIG. 11. Electron micrograph of a ganglia-like mass from an 8-day culture with NGF and insulin. *al* represents a longitudinal section and *ax* a cross section of undulating neurofibrillae within fibers characteristic of nerve axons. × 14,000.

tures. The further addition of insulin markedly enhanced the growth pattern. Nissl and silver stains as well as electron microscopy studies supported the contention that these cells and fiber outgrowths were nerve cells and axons and not supporting or satellite cells. However, functional activity has not been proven.

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## The Role of RNA in the Synthesis of Vaccinia Virus.* (29374)

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Recent studies have shown that several ribonucleic acid (RNA) containing animal viruses can replicate in spite of severe impairment of deoxyribonucleic acid (DNA)(1, 2) synthesis and DNA-dependent RNA synthesis(3). However, the multiplication of some DNA-containing animal viruses has been reported to be dependent upon the essential participation of RNA during the early stages of the infectious sequence(4,5,6,7). Agents such as ribonuclease or dichlororibofuranosylbenzimidazole(4,5) were found to inhibit the synthesis of DNA-containing viruses by specifically interfering with the metabolism of RNA within the infected cell. The present report supports this view. The pyrimidine anti-metabolite, 6-azauridine (6-AZU), was found to inhibit multiplication of vaccinia virus. Previous studies have indicated that the mode of action of this analogue is by interference with the conversion of orotic acid to uridine ribotide(8) and consequently with nucleic acid metabolism.

*Materials and methods.* The line of HeLa cells was originally obtained from Difco Laboratories, and routinely grown in Eagle's basal medium (EBM)(9) containing 10% calf serum (CaS). Except when stated, the basic experimental medium consisted of EBM and 2% CaS supplemented with thymidine ($10^{-4}$ M) and deoxycytidine ($10^{-4}$ M) in order to restrict the action of the analogue to RNA metabolism. To this medium was added either 6-AZU ($10^{-4}$ M) alone, or with uridine ($2 \times 10^{-4}$ M). Cultures were infected with approximately 15 plaque-forming units (PFU) of centrifugally purified HeLa-passaged vaccinia virus per cell. After an adsorption period of 2 hours, the cultures were washed with Hanks' balanced salt solution (BSS) and fresh medium was introduced. Virus growth was assayed by the liquid overlay plaque technique(10). The preparation of immune sera to the vaccinial antigens (heat-labile-heat-stable and nucleoprotein) and the experiments with fluores-

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