The Effects of Nerve Growth Factor Antiserum on Ambystoma

The nerve growth factor (NGF), a protein isolated from certain tumors[1], snake venom[2], and mouse salivary glands[3], evokes growth of sensory and sympathetic neurons in chick embryos[4,5], new-born mice[6], young zebrafish[7], and growth of the spinal ganglia in the salamander *Ambystoma*[8]. When NGF is injected along with Freund's adjuvant into adult rabbits, a specific antiserum can be produced[3]. When this antiserum is injected into new-born mammals it causes extensive destruction of the sympathetic ganglia[9]. Much cytological damage to nucleus, mitochondria and ribosomes can be seen within the cells[10]. The following experiment was undertaken to determine the effects of the antiserum on *Ambystoma*.

Eggs of *A. maculatum* were raised in the laboratory. Treatment was begun when the embryos had reached Harrison stage 39–40 (see HAMBURGER[11]). The animals were injected 3 times a week for a period of 2 weeks with Abbott's bovine antiserum (Abbott Laboratories, N. Chicago, Illinois), the strength of which was 52,000 anti-units/ml.

The jelly coat was removed from the embryos which were placed on their sides and injected with 0.1 µl into the epaxial muscle mass. Injections were performed under a dissecting microscope by means of a microinjection apparatus on a micromanipulator as described previously[7]. Motile embryos were anaesthetized prior to the injection with a 1:1000 solution of MS-222 (ethyl m-aminobenzoate methanesulfonate, Eastman Organic Chemicals). Control embryos received similar injections of saline solution. The animals were maintained at 20 °C and had reached stage 43 by the end of the experiment. They were fixed in Bouin's fluid, dehydrated, embedded in paraffin, sectioned serially at 10 µ, and stained with Delafield's hematoxylin and eosin.

Analysis was performed on spinal ganglion No. 3, the first brachial ganglion, on the right side (No. 3R). Paper reconstructions were made by projecting the slides in a microprojector to a magnification of × 160, and tracing the outline of the ganglion on paper for each section in which the ganglion appeared. The outlines of all sections were cut out and weighed to get the relative mass of the ganglion. The percentage of error in this method is relatively small as previously determined[7]. Cell counts were made on the same ganglion. Neuroblast nuclei were counted in every section, and the raw counts were corrected by the Abercrombie method (as described by MARRABLE[12]) to compensate for errors resulting from counting the same nucleus in adjacent sections. To estimate cell size, the long diameters of the largest oval-shaped nuclei in the ganglion were measured by a calibrated ocular micrometer. To get an estimate of cell size distribution, the number of cells with a nuclear diameter of 5 U (6.25 µ) or less, relative to the total number of cells was determined in a section through the middle of the ganglion.

A total of 13 experimental and 11 control animals survived to the end of the experiment. The mean weight of paper reconstructions (in hundredths of a gram) of ganglia of experimental animals was 47.5 ± 3.5 (standard error), whereas the value for controls was 68.6 ± 3.5. The '*t*'-value for the difference between these means was 4.28, indicating significance beyond the 0.005 level.

The mean cell counts of the experimental animals was 208 ± 8.7, whereas that of the controls was 292 ± 12.9. The '*t*'-value for this difference was 5.38, indicating significance beyond the 0.005 level.

The nuclear diameters of the largest ganglion cells of both experimentals and controls was 18–20 µ. However, the percentage of cells smaller than 6.25 µ was 11.5% in the experimentals and 5.7% in the controls, indicating a doubling in percentage of these 'small' cells. In terms of absolute numbers, in a section through the middle of the ganglion, experimentals averaged 6.8 ± 0.59 'small' cells (out of a total number of 59.3), whereas controls averaged 3.8 ± 0.41 'small' cells (out of a total of 66.5 cells). The '*t*'-value of 4.21 indicated significance beyond 0.005. These data are summarized in the Table.

It has been demonstrated that anti-NGF caused a reduction in size of the spinal ganglia of *A. maculatum*. The size decrease was due primarily to a decrease in cel number, though the cytological appearance of the cells was normal. This type of response has been seen in the coeliac ganglion of mammals[13] and the spinal ganglion of the zebrafish[7]. The effects of antiserum on neonatal mammals are seen only in the sympathetic ganglia[9]. The spinal ganglia at this time are essentially mature, and are apparently refractory to the antiserum. However, in the zebrafish, antiserum treatments at the time when the spinal ganglia are actively growing will reduce their size[7]. It has been shown previously that during the period investigated, the spinal ganglia of *Ambyostoma* are actively growing, primarily by an increase in cell number[8]. This process was apparently inhibited by the antiserum. At the stages investigated the sympathetic ganglia were so rudimentary that nothing could be determined about them.

Comparison of spinal ganglion No. 3R from antiserum-treated and control *Ambystoma*

	Experimentals	Controls
Wt. of reconstructions	47.5 ± 3.5	68.6 ± 3.5 [a]
Cell counts	208 ± 8.7	292 ± 12.9 [b]
No. cells/section under 6.25 µ	6.8 ± 0.59	3.8 ± 0.41 [c]
% cells under 6.25 µ	11.5%	5.7%
Diameter of largest cells	18–20 µ	18–20 µ

[a] '*t*'-value 4.28; significant beyond the 0.005 level. [b] '*t*'-value 5.38; significant beyond the 0.005 level. [c] '*t*'-value 4.21; significant beyond the 0.005 level.

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Although there was no difference in the maximum size of neurons in the ganglia of experimentals and controls, there apparently was a change in cell size distribution, with the antiserum-treated ganglia having a greater proportion of smaller cells than the controls. Perhaps the antiserum interfered to some extent with the growth processes of the neuroblasts. It is also possible that the increased number of small cells was due to degenerative processes taking place in some neuroblasts, since many of these small cells were pyknotic.

The NGF and its antiserum operate extensively over phylogenetic lines. NGF has been detected in fishes[14], amphibians[15], and birds[16], as well as reptiles and mammals, and NGF has comparable effects in these various classes of vertebrates. Similarly, antiserum produced by a cow against mouse NGF will cross-react with snake venom NGF, as seen by complement fixation[17]. BURDMAN and GOLDSTEIN[18] found partial cross-reactivity of goat anti-mouse NGF with NGF from children with neuroblastoma, a tumor of neural crest origin. Furthermore, it has been shown that bovine antiserum against mouse NGF will inactivate NGF from the axial regions of the chick, tadpole, and goldfish, indicating immunological similarity of the molecules[15]. This extensive cross-reactivity of the antiserum is sufficient to cause biological effects across phylogenetic lines, extending now to the amphibia as well as the other classes of vertebrates.

Résumé. Des injections d'un sérum spécifique contre le facteur de croissance nerveuse (anti-NGF) provoquent une réduction de la taille des ganglions rachidiens de la salamandre *Ambystoma* par suite de la réduction de la masse cellulaire du ganglion. Il y a aussi un plus grand nombre de «petites» cellules (d'un diamètre nucléaire de moins de 6,25 μ) dans les ganglions des animaux utilisés.

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Effect of a Growth-Promoting Factor from Calf Muscles on the Weight Gain of Hypophysectomized Rats

SALMON and DAUGHADAY[1] proposed the hypothesis that the action of growth hormone on cartilage may be mediated by a component of serum called sulfation factor. Hypophysectomy causes a marked decrease in the sulfation activity of serum; this activity can by restored by injections of growth hormone, but not by adding the hormone to the serum in vitro. In a study of various tissues it was found that tissue extracts exhibit different degrees of sulfation activity. High activities were measured in extracts of rat skeletal muscles[2]. Previous reports on sulfation activity were based on results obtained in vitro. Here we report a method for extraction and separation of the active material from calf muscles. We also investigated the effect of the separated material on the growth hormone action in hypophysectomized rats.

The material used was 500 g of fresh calf meat obtained directly from the slaughterhouse. The muscles were incubated in 2000 ml of sterilized distilled water for 3 h at 37 °C. After incubation the muscles were discarded and the fluid filtered through a sterile Zeiss EKS filter. The filtrate was passed through a Dowex-50W-X2(H) resin, mesh size 100–200, pH 6.0, which was prepared according to the method originally described by BOUCHER et al.[3] for the extraction of angiotensin from plasma. The active material was eluted from the column as angiotensin is eluted using this method, and the eluate was lyophylized. The residue was dissolved in sterile distilled water, filtered again through a sterile Zeiss EKS filter, and diluted to 25 ml with water. This solution was then passed through a Sephadex G-25 (Pharmacia, superfine grade) column. The size of the column was 100×2.5 cm, and the Sephadex was equilibrated with 0.05 *M* *Tris*mabuffer (Sigma), pH 7.4. Three separate chromatographies were performed by applying a volume of 6 ml on the column in 2 instances and 4.45 ml of the solution containing the active material in one instance. In each separation the column was eluted at a flow rate of 30 ml/h. The volume of one fraction was 10 ml. The total amount of the gel filtrated solution (22.45 ml) corresponded to 329 g of muscles. The biological activities of the incubation fluid, the solution obtained after Dowex and the Sephadex fractions were tested in vitro by measuring the incorporation of ^{35}S-labelled sulfate in the pelvis of chick embryos[2]. The results obtained by separation on Sephadex G-25, and the sulfation activity determinations are shown in the Figure.

The Figure shows 3 different separations. The separation is fairly well reproduced as can be seen from the curves. The corresponding fractions from 3 separations were pooled, and the biological activity of the pooled fractions was tested. The bars show the biological activity. 2 of the 3 peaks of the biological activity are well expressed – one corresponding to fraction 34, and one to fraction 40. In a preliminary experiment these 2 fractions, together with human growth hormone (HGH), were injected into hypophysectomized rats. Each group consisted of 5 rats; the animals were injected each day for 6 days. The

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