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Effects of Nerve Growth Factor from the venom of *Vipera russelli* on sensory and sympathetic ganglia from the embryonic chick in culture

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SUMMARY

Sensory and sympathetic ganglia of embryonic chick were cultured in media in which no growth of fibres occurred, and then transferred to culture conditions favouring the growth of fibres. The effects on fibre outgrowth of having NGF in neither, one or both media were examined quantitatively. It was concluded that the main effect of NGF, under these conditions, is to maintain the viability of the neurons. Subsidiary experiments supporting this view are also reported.

INTRODUCTION

Nerve Growth Factor (NGF) is a collective name given to a family of proteins and glycoproteins that specifically stimulate the growth of fibres from embryonic sensory and sympathetic neurons *in vitro*. The biochemical effects and the gross biological effects produced by these substances both *in vivo* and *in vitro* have been described in some detail (for recent reviews see Levi-Montalcini & Angeletti, 1968; Zaimis, 1972). However, in spite of intensive research, the mode of action of these substances is still poorly understood at the biological level, and not at all at the molecular level.

An early suggestion was that NGF acts by accelerating the differentiation of early neuroblasts in the receptive ganglia (Levi-Montalcini & Hamburger, 1951). Consistently, electron microscopic studies of explants of sensory ganglia showed that changes occur very rapidly in the presence of NGF. In particular, after only 4 h incubation, neurofilaments and neurotubules were reported to be prominent in the neurons (Levi-Montalcini & Angeletti, 1968).

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It might be supposed that if the main effect of NGF is on the differentiation of receptive neuroblasts, pretreatment of ganglia with NGF would lead to the growth of fibres on subsequent culture, irrespective of whether the media contained NGF or not. Such evidence that there is in the literature is not, apparently, consistent with this expectation. Sympathetic and sensory ganglia treated for periods of up to 24 h with impure NGF derived from a tumour failed to produce fibres when cultured in a medium not containing NGF (Cohen & Levi-Montalcini, 1957). More recently, it has been reported that if ganglia cultured in the presence of NGF were transferred to a medium not containing NGF all fibre growth immediately ceased (Angeletti, 1969).

We report a quantitative investigation of the effects of pretreatment of embryonic chick sensory and sympathetic ganglia with NGF on the subsequent outgrowth of nerve fibres *in vitro*. Detailed histological examination was made of some of the ganglia in order to compare the development of the neurons *in vitro* and *in vivo*. We have also investigated the response of sensory ganglia as a function of embryonic age. A preliminary account of some of the experiments has already been published (Vernon *et al.* 1969).

MATERIALS AND METHODS

Nerve Growth Factor

The material used was obtained from the venom of *Vipera russelli*. In some experiments the material was prepared by the method of Banks *et al.* (1968); in others the method described by Pearce *et al.* (1972) was used. The biological activities of samples prepared by the two different methods were indistinguishable: only the second method, however, gives a material which is homogeneous to a wide range of electrophoretic techniques and to isoelectric focusing in acrylamide gel. The active entity is a glycoprotein with a molecular weight of about 37000, which is chemically stable and resistant to the action of trypsin and certain other proteolytic enzymes (Pearce, 1972; Pearce *et al.* 1972).

Assay

The biological activities of solutions of NGF were determined using a modification of the tissue culture method first described by Levi-Montalcini, Meyer & Hamburger (1954). Sensory ganglia were removed from 8-day chick embryos and cultured in hanging drops on collagen-coated coverslips for 24 h (Pearce, Banthorpe, Cook & Vernon, 1973). The cultures were then fixed and stained in haematoxylin. The extent of fibre outgrowth was assessed on an arbitrary scale ranging, in half integers, from 0 (control medium, no added NGF) to 5 (maximum response to NGF). A standard set of slides was prepared thus defining the scale (Lamont, 1968). The response of ganglia that had been pretreated in various ways and then cultured in media containing a fixed amount of NGF

(sufficient to produce a strong response) was assessed in terms of this defined arbitrary scale.

The effect of subjective bias in using the arbitrary scale was investigated by allowing three observers to assess (blindly and independently) the scores from one complete experiment with sensory ganglia. The results, given in Table 1, show that there were no important differences between the observers in their assessments.

Experiments on the pretreatment of sensory ganglia in vitro

Sensory ganglia were dissected from 8-day-old chick embryos and incubated in rings (0·5 × 2·3 cm) containing either 1 ml of control medium (C) (medium 199:chicken serum:buffered saline; 2:1:1, v/v) or 1 ml of medium containing NGF (N) (as C but containing 0·025 μg ml^{-1} of NGF) for periods of either 24 h (1C, 1N) or 48 h (2C, 2N). The rings were waxed on to glass coverslips, and since the ganglia do not readily adhere to a glass surface and were discouraged from settling down, no growth of nerve fibres occurred under these conditions. The cultures were placed in covered Petri dishes in a desiccator containing water and maintained at 37·5 °C. Each ring contained about 30 ganglia. After 24 h, half the ganglia were removed from each ring, washed with medium 199 and then cultured for 24 h on collagen-coated coverslips in either a control medium (C) or in medium containing added NGF (N). After fixation they were stained with haematoxylin and scored for fibre outgrowth. The remainder of the ganglia were removed from the rings after a total of 48 h of incubation and treated similarly.

This procedure led to groups of ganglia which had been subjected to eight different treatments. The treatments were designated by appropriate symbols; for example, 2N → C, means that the ganglia were incubated under conditions where they could not grow fibres for 48 h in a medium containing NGF and then transferred to culture conditions for 24 h where fibre growth could occur, in a medium which did not contain NGF.

In one series of experiments some of the ganglia were removed at the end of the final treatment for histological examination. These ganglia were fixed in formol-saline, cleared in cedarwood oil and then embedded in paraffin wax. Transverse sections (5 μm) were cut and stained with silver (Holmes, 1943). Sensory ganglia taken immediately after dissection from 8-, 9- and 10-day embryos were treated similarly. Representative areas from both the mediodorsal and ventro-lateral regions of the ganglia (Levi-Montalcini & Levi, 1943) were projected on to graph paper at a final magnification of *ca.* 800 using a drawing mirror attached to a microscope. Relative cell areas were then estimated by weighing pieces of the graph paper cut out along the drawn outlines of recognizable neurons. Relative cell densities were estimated by counting the number of neuron nuclei present in projected areas of 5·5 cm^2.

Experiments on the pretreatment of sympathetic ganglia in vitro

Chains of 4–8 sympathetic ganglia were dissected from 8-day embryos and treated as described above. Before being cultured in the second medium the chains were cut up into individual ganglia.

Experiments on the pretreatment of sensory ganglia in vivo

A solution of NGF (1·2 mg in 3 ml phosphate-buffered saline, pH 7·2) was sterilized by filtration through a Millipore filter. The eggs to be used were candled and the positions of the embryos determined. A small square was marked on each shell in such a position that solution injected there would enter the yolk-sac. The marked area was swabbed with 70 % ethanol and pierced with a sterile needle. The solution containing NGF (0·25 ml) was injected through the hole into the yolk-sac and the hole then sealed with transparent tape.

The first injections were given to 6-day embryos; one or two injections were given on subsequent days. After 1–4 days of treatment, three ganglia from the thoracic region with attached spinal cord were dissected from each embryo, fixed in formol-saline and prepared for histological examination. In all cases the embryos were dissected 24 h after the final treatment. Corresponding ganglia were also removed from untreated embryos as controls.

The remaining ganglia of both the injected and untreated embryos were dissected out and cultured in hanging drops on coverslips coated with collagen in either a control medium (C) or in a medium containing NGF. After 24 h they were fixed, stained and examined for fibre outgrowth.

Experiments on the responsive period of sensory ganglia

Sensory ganglia were dissected from embryos of age 5, 6, 7, 12, 14, 15 and 16 days and cultured on collagen in either a control medium or in a medium containing a fixed amount of NGF. After 30 h of culture they were fixed and stained as detailed above.

The ganglia used in this experiment were mostly taken from the lumbar and thoracic regions, since these are the easiest to dissect from older embryos.

RESULTS

Pretreatment of sensory ganglia in vitro

In the first experiment the concentration of NGF in medium N was sufficient to give a fibre outgrowth, corresponding to a score of 3·5 on the arbitrary scale, from ganglia cultured immediately after dissection. The fibre outgrowths obtained with the eight different treatments were independently assessed by three observers; the results are given in Table 1. Two groups are distinguished, depending on whether the ganglia were cultured in the first medium for 24 h (group I) or 48 h (group II). Each group, therefore, contains four treatments.

Table 1. *Pretreatment of sensory ganglia: scores by three observers*

Group	Treatment	Mean scores: Observer 1	Observer 2	Observer 3	Total no. of scores
I	C → C	0·97	0·88	0·68	51
	C → N	2·38	2·38	1·94	48
	N → C	1·43	1·30	1·16	45
	N → N	3·32	3·46	3·37	60
II	2C → C	0·57	0·50	0·43	60
	2C → N	1·42	1·28	1·19	57
	2N → C	1·10	0·84	0·76	57
	2N → N	3·50	3·72	3·66	54

Table 2. *Analysis of variance of data in Table 1*

	Degrees of freedom	Sums of squares	Variance estimate	Variance ratio	*P**
Groups (G)	1	24·22	24·22	121·1	<0·001
Treatments (T)	3	535·72	178·57	897·9	<0·001
Observers (O)	2	2·99	1·495	7·45	<0·001
G × T	3	7·23	2·41	12·05	<0·001
G × O	2	0·21	0·105	<1	NS
T × O	6	2·76	0·46	2·29	NS
G × T × O	6	0·62	0·103	<1	NS
Residue	414	83·05	0·20		
Total	437	656·80			

* NS = not significant.

Inspection of the data suggests that although the mean scores given by the observers are not the same (in particular, observer 3 has, on the whole, scored lower than observer 1), the order of effectiveness of the various treatments is the same for all three observers. To test this the data were subjected to an analysis of variance (Table 2). The result shows that the three observers were indeed scoring differently. However, the fact that the interactions (G × O), (T × O) and (G × T × O) are not significant confirms what was evident by inspection, namely that the relative effectiveness of the treatments was assessed by all three observers in the same way. Since it is this which is important, and not the absolute values of scores, we conclude that the method of assessment adopted is appropriate for the present purpose.

The analysis of variance was repeated using only the scores obtained by the main observer (Table 3). Both analyses show that the interaction between groups and treatments (G × T) is significant. This can be interpreted to mean that the effectiveness of a treatment depends on the group or, in physical terms, that the

Table 3. *Analysis of variance of data in Table 1 (main observer only)*

	Degrees of freedom	Sums of squares	Variance estimate	Variance ratio	*P*
Groups (G)	1	8·28	8·28	39·4	<0·001
Treatments (T)	3	153·20	51·09	243·3	<0·001
G × T	3	2·92	0·97	4·6	<0·01
Residual	136	28·80	0·21		
Total	143	193·20			

Table 4. *Pretreatment of sensory and sympathetic ganglia in vitro: sets of significantly different treatments*

Each numbered set differs significantly from the one below at the level $P < 0{\cdot}002$. The probabilities were calculated by using Student's *t*-test. For the experiment with sympathetic ganglia, tests were carried out between group means over all treatments and between treatment means over both groups. For the experiments with sensory ganglia the *t*-tests were applied to the group means within each treatment and to the treatment means within each group.

Sensory ganglia						Sympathetic ganglia		
Expt. I			Expt. II					
Set	Treatment	Mean score	Set	Treatment	Mean score	Set	Treatment	Mean score
1	2N → N	3·6	1	2N → N	4·6	1	1N → N	2·3
	1N → N	3·4		1N → N	4·5		2N → N	2·1
2	1C → N	2·2	2	1N → C	2·4	2	1C → N	1·3
				1C → N	2·1		1N → C	1·2
3	1N → C	1·3	—	—	—		2N → C	1·1
	2C → N	1·3	3	2N → C	1·5		2C → N	1·0
				2C → N	1·1			
4	2N → C	0·9	—	—	—	3	1C → C	0·3
	1C → C	0·8	4	1C → C	0·4		2C → C	0·3
	2C → C	0·5		2C → C	0·4			
Mean NGF activity		3·5			4·0			2·5

effect of transfer of the ganglia from one medium to another depends on the time of culture in the first medium. Since the interaction (G × T) is significant, Student's *t*-tests were applied, both to the groups within each treatment and the treatments within each group. In this way sets of treatments were established such that each set differs significantly, in terms of final fibre outgrowth, from each other set. The result is shown in Table 4.

Table 5. *Mean cell areas of sensory neurons after various conditions of culture*

Treatment	Mean area ± S.E.		
	Ventro-lateral region		Medio-dorsal region
1C → C	4·77 ± 0·025		2·74 ± 0·007
1C → N		5·19 ± 0·027	
1N → N		5·80 ± 0·014	
1N → C	4·99 ± 0·007		2·98 ± 0·012
2C → C	5·80 ± 0·025		3·11 ± 0·014
2C → N		6·01 ± 0·027	
2N → N		5·89 ± 0·015	
2N → C	6·11 ± 0·028		2·71 ± 0·018
8-day uncultured	4·75 ± 0·019		3·14 ± 0·01
9-day uncultured	6·34 ± 0·032		3·44 ± 0·01
10-day uncultured	8·93 ± 0·069		5·02 ± 0·06

The experiment was repeated using a higher concentration of NGF (mean score, 4·0). The final result, also given in Table 4, is similar but not identical to that given by the first experiment.

Experiment on the pretreatment of sympathetic ganglia in vitro

In this experiment the concentration of NGF was, inadvertently, somewhat lower than in the experiments on sensory ganglia and produced a mean score of 2·5 under standard conditions. Analysis of variance showed that the interaction between groups and treatments was, under these conditions, not significant. Consequently, *t*-tests were carried out between group means over all treatments and between treatment means over both groups. The resulting sets of significantly different treatments are included in Table 4.

Histological examination of sensory ganglia given various pretreatments in vitro

The areas of recognizable neurons from both the ventro-lateral and medio-dorsal areas of sensory ganglia taken from 8-day embryos and subjected to various conditions of culture are given in Table 5. The values are the means and standard errors, in arbitrary units, of 100 measurements on the ventro-lateral region and thirty measurements on the medio-dorsal region. After certain treatments (1C → N, 1N → N, 2N → N, 2C → N) the two regions were no longer distinguishable; the single values for these treatments refer, therefore, to the ganglia taken as uniform structures. The conditions of the various treatments are the same as those stated for the second experiment on sensory ganglia given above, i.e. fibre growth was possible in the second medium but not in the first; NGF concentration was equivalent to a score of 4·0 on the arbitrary scale.

Table 6. *Mean numbers of neuron nuclei per unit area in sensory ganglia after various conditions of culture*

The values are the numbers of neuron nuclei in projected areas of 5·5 cm^2 at × 800 magnification. The numbers in parentheses refer to the numbers of areas examined in each case.

	Mean number ± S.E.	
Treatment	Ventro-lateral region	Medio-dorsal region
1C → C	19·4 ± 0·26 (30)	29·0 ± 2·55 (11)
1C → N	15·3 ± 0·69 (14)	
1N → N	16·0 ± 0·16 (80)	
1N → C	16·7 ± 0·29 (14)	17·6 ± 1·54 (9)
2C → C	16·7 ± 0·42 (20)	24·3 ± 0·81 (6)
2C → N	15·7 ± 0·57 (19)	
2N → N	16·3 ± 0·29 (20)	
2N → C	20·4 ± 0·76 (29)	20·5 ± 0·30 (16)
8-day uncultured	17·7 ± 0·67 (20)	29·3 ± 0·97 (14)
9-day uncultured	15·8 ± 0·60 (20)	23·3 ± 1·29 (10)
10-day uncultured	14·9 ± 0·24 (20)	19·6 ± 0·55 (8)

Table 7. *Fibre outgrowth of sensory ganglia from untreated embryos and from embryos injected with NGF*

		Mean scores			
		Injected embryos		Untreated embryos	
No. of injections*	Age of embryo (days)	Control medium	Medium +NGF	Control medium	Medium +NGF
1	7	0·9	2·3	1·0	2·7
2	8	1·1	2·2	0·8	3·0
3	9	0·9	3·0	0·8	2·8
4	10	0·8	3·1	0·7	3·0
5	9†	1·1	3·4	0·9	3·1

* Each injection contained 0·1 mg NGF. All injections started with 6-day embryos.

† This embryo received injections on day 6 (1 injection), day 7 (2 injections) and day 8 (2 injections).

The numbers of neuron nuclei per unit area were also recorded; the data are given in Table 6. In those cases where the ventro-lateral and medio-dorsal regions could be distinguished, it appeared that in those ganglia which had been cultured in the presence of NGF more nerve fibres were present in the medio-dorsal region than in the corresponding region of ganglia which had been cultured only in a control medium. It did not prove practicable, however, to assess this effect quantitatively.

Table 8. *Response to NGF of sensory ganglia from embryos of differing age*

Age (days)	Control	Medium +NGF
5	0·8 ± 0·08	2·0 ± 0·09
6	0·6 ± 0·08	2·2 ± 0·14
7	0·95 ± 0·11	3·6 ± 0·10
12	1·45 ± 0·09	2·7 ± 0·13
14	0·7 ± 0·12	2·5 ± 0·14
15	1·1 ± 0·14	2·1 ± 0·15
16	1·45 ± 0·13	2·3 ± 0·17

Scores are given as mean ± S.E. on an arbitrary scale from 0 to 5.

Pretreatment of sensory ganglia in vivo

Qualitative examination of sections of sensory ganglia taken from embryos which had been given up to four injections of a NGF solution revealed no obvious differences from controls. In particular, the sizes of the cells in the medio-dorsal regions appeared to be the same. After five injections, however, the medio-dorsal region could no longer be distinguished from the ventro-lateral region and the neurons appeared to be more intensely stained with the silver stain than in controls. Many more nerve fibres were also observed within the ganglia. The mean scores for fibre outgrowth from ganglia from injected and untreated embryos cultured *in vitro* are given in Table 7.

Response to NGF of sensory ganglia from embryos of differing age

Table 8 records the responses of sensory ganglia taken from 5- to 16-day embryos to culture in the presence of a fixed amount of NGF. Between 20 and 35 ganglia were cultured and scored for each age group in both control medium and in medium containing NGF. Although considerable variation occurred in the response of ganglia in each age group, particularly from older embryos, the presence of NGF increased the outgrowth of fibres in all cases.

DISCUSSION

The biological effects of NGF on sensory and sympathetic ganglia have been discussed in terms of two basically different theories, namely (*a*) that NGF, under appropriate conditions, promotes the differentiation of more primitive cells into neurons, and (*b*) that NGF is required, both *in vivo* and *in vitro*, for the continued viability of already differentiated neurons (for a summary see Levi-Montalcini & Angeletti, 1968). In order to devise a distinguishing test it is necessary to enquire, in so far as an 'either–or' situation obtains, what these two theories would entail. We take it that the first requires that NGF is necessary

for the transformation of a cell which is not a neuron into one which is, and that this process has an 'all-or-nothing' character in the sense that once the transformation is complete, the cell no longer needs NGF. The second requires that sensory and sympathetic neurons depend on an external source of NGF for their continued viability and that, in the absence of NGF, the cell loses the capacity to grow a fibre and eventually dies. If this is so, then the extent of fibre outgrowth can be used as an index of viability. There is considerable evidence that this is justified: (*a*) in a recent study of neurons obtained by dispersion of embryonic chick sensory ganglia (Banthorpe, Pearce & Vernon, 1974) it was found that the main effect of NGF was to maintain viability in the sense defined above; (*b*) examination of the conventional hanging-drop cultures of explants of embryonic sensory and sympathetic ganglia shows that NGF promotes the growth of fibres from a much larger proportion of neurons as compared with control cultures (Lamont, 1968), and that under the latter conditions a considerable number of cells die (Weis, 1970).

We have therefore attempted to distinguish between the two theories by the device of culturing ganglia with and without NGF under conditions where fibre growth does not readily occur (i.e. on glass) and then transferring them to conditions, again either with or without NGF, where fibre growth can occur (i.e. on collagen). It should be emphasized at the outset that the method of assessing the effectiveness of the various treatments (assigning the final outgrowth of fibres a score on an arbitrary scale by visual inspection) can never give results which are better than semi-quantitative. For this reason, we subjected the large amount of data obtained in each experiment to statistical analysis and constructed sets of treatments which could be shown to differ significantly from each other ($P < 0{\cdot}002$). Any further interpretation of the numbers in Table 4 or of the differences between them is not possible.

The simplest experiment to interpret is that with sympathetic ganglia. The treatments divide into three sets. In the first, NGF was present in both media and the final score obtained (in the case of 2N → N, after 3 days in culture) did not differ significantly from the score obtained after culture for 24 h of freshly dissected ganglia (N). The second set, with significantly lower scores, consists of all those treatments in which only one of the media contained NGF. The third set, with very low scores, consists of the two treatments in which neither medium contained NGF. These results argue against the differentiation theory. If new neurons, capable of producing fibres, were being produced under the influence of NGF then one would expect 2N → N to be better than N and 2N → C to be about as effective as 1N → N and better than 2C → N. We do not imply, of course, that differentiation does not occur with sympathetic ganglia of this age either *in vitro* or *in vivo*, but simply that if it does, the present experiments provide no evidence that NGF is involved. On the other hand, the results are wholly consistent with the viability theory. When NGF is present throughout, the viability of the neuron is maintained and,

given the appropriate conditions of culture, fibres are produced. In the total absence of NGF the neurons lose viability and, for the most part, become incapable of producing fibres. When NGF is present in one but not both of the media an intermediate situation prevails.

The results obtained with sensory ganglia are, in essentials, the same. When NGF is present in both media, fibre growth is maximal and indistinguishable from that given by freshly dissected ganglia cultured in a medium containing the same concentration of NGF. When neither medium contains NGF, very little fibre outgrowth occurs; when one of the media contains NGF an intermediate response is observed. For exactly the same reasons as those given above, the results are consistent with the viability theory and not with the differentiation theory.

In detail, however, the results obtained with sensory and sympathetic ganglia differ. Inspection of Table 4 shows that the intermediate set of treatments found for sympathetic ganglia has, in Expt. I with sensory ganglia, split so that the treatment 1C → N has moved up to an intermediate higher set on its own and the treatment 2N → C has moved down to the lowest set containing the two treatments in which neither medium contained NGF. In order to explain this, it is necessary to introduce a subsidiary hypothesis, namely that in treatments where NGF is present in only one medium it is more important, in terms of final fibre outgrowth, that it should be present in the second rather than the first. Hence 1C → N is better than 1N → C and 2C → N is better than 2N → C, as observed. This hypothesis is wholly reasonable, since it entails that when the viability of a cell depends on some extrinsic factor the dependence will be the greater when the cell is exerting its full metabolic activity; in this case, when it is growing a fibre. It might further be supposed that this differential effect could be partially offset by increasing the concentration of NGF in the first medium. Exactly this was done in Expt. II with sensory ganglia and, consistent with expectation, the treatments 1N → C and 2N → C each moved upwards by one set.

In both experiments with sensory ganglia it was found that 1C → N was better than 2C → N and 1N → C was better than 2N → C. The explanation for the first finding is obvious: in a medium not containing NGF there is a progressive loss of viability. Consequently, the longer the period of culture under these conditions the smaller the final outgrowth of fibres. The second finding is not so easy to explain. There is, however, some evidence that NGF is progressively lost, presumably by some degradative process, from media containing a suspension of cells obtained by dispersal of 8-day chick embryonic sensory ganglia (Banthorpe *et al.* 1974). If this is so, the longer period of culture in the first medium would result in a lower final concentration of NGF. The differential effect postulated above would then lead to an increased need for NGF in the second medium and hence treatment 1N → C would be better than treatment 2N → C. Thus all the data in Table 4 can be satisfactorily explained

in terms of the viability theory. It should be noted that the detailed differences in behaviour between the sympathetic and sensory ganglia have not been explained. They may arise because the two kinds of neurons differ in their responsiveness to NGF, but whatever the explanation it is not important to the present argument.

Implicit in the above analysis is the assumption that fibre growth is a good criterion of neuron viability. The assumption appears to be justified, since it leads to a complete and self-consistent analysis. However, an alternative criterion might have been chosen – for example, neuron size. This approach has already been used by Weis (1970). He found that the neurons of the ventro-lateral region of sensory ganglia did not increase in size on culture of the ganglia for a period of 20 h, irrespective of whether NGF was present in the culture medium or not, although *in vivo*, a clear increase in size (*ca.* × 1·3) could be demonstrated over the same time period. On the other hand, with the smaller medio-dorsal cells an increase in size on culture similar to that observed *in vivo* was observed if sufficient NGF was present in the culture medium. Weis reported that in all cases in culture a considerable proportion of the neurons failed to survive.

Our own results, obtained by histological examination of some of the sensory ganglia involved in Expt. II are, on the whole, consistent with those reported by Weis. Inspection of the data in Table 5 shows that for the neurons of the ventro-lateral region the effect of NGF in the cultures involving two media is negligible. For example, the mean values for neuron size after the treatments 2C → C and 2N → N are not significantly different. This is, of course, in sharp contrast to the findings with fibre outgrowth detailed above. With the initially smaller neurons of the medio-dorsal region one clear-cut effect of NGF is observed, namely that in the cultures involving two media, whenever NGF is present in the second medium and irrespective of whether it is present in the first, the neurons increase in size so as to be indistinguishable from those of the ventro-lateral region. This particular result is inconsistent with the differentiation theory, since if any of the changes in the medio-dorsal region are to be associated with differentiation induced by NGF there is no reason for their failure to occur in the first medium.

On the whole, the measurements of cell size are not particularly informative and one might conclude that cell size is not a good criterion for cell viability. The measurements of cell numbers (Table 6) are even less informative: the only firm conclusion which emerges is that the medio-dorsal region has a higher cell density than the ventro-lateral region. Since the measurements simply involved counting nuclei, and no attempt was made to determine whether these nuclei belonged to living or dead cells, it is perhaps not too surprising that they yield no information on cell viability.

It has been claimed that the responsiveness of embryonic sensory ganglia to NGF in culture decreases with embryonic age, and that with embryos older than about 14 days it becomes undetectable. For example, Levi-Montalcini (1965)

states '*in vitro*, as *in vivo*, the growth response is maximal in ganglia from 7 to 9 days of incubation, decreases in the following 3 days, and is no longer detectable after the 14th day of incubation'. Winick & Greenberg (1965), on the other hand, found a constant response within the 7- to 13-day period, but no response at all before or after this period. Our results are different. We find (Table 8) a clear response to NGF from sensory ganglia taken over the age range 5–16 days, although, in agreement with Levi-Montalcini, the response appears to be maximal at 7 days. The origin of the difference between our results and those previously reported is obscure. It may arise from differing methods of culture; we cultured in a liquid medium on collagen, whereas the other workers used a semi-solid medium (plasma clot). However, our results show that a response to NGF in culture can be observed with sensory ganglia over a wide range of embryonic ages and this can, of course, be taken as support for the viability theory.

We found it difficult to observe any effects on sensory ganglia of injection of NGF into embryos, largely because of the high mortality rate in repeatedly injected embryos. Even with those that survived, the problem of access remains. None the less, with embryos that did survive five injections with NGF, the sensory ganglia were clearly different from those of untreated embryos of the same age, in that the cells of the medio-dorsal region had increased in size so that this region was no longer distinguishable from the ventro-lateral region. However, these ganglia and those from other injected embryos showed exactly the same response in culture as ganglia from uninjected embryos (Table 7). This result also supports the view that the main effect of NGF, at least in culture, is the maintenance of neuron viability.

The results described above are consistent with the results obtained by studying cell suspensions made by dispersion of sensory ganglia from the embryonic chick (Banthorpe *et al.* 1974). It was found that NGF did not promote differentiation from more primitive cells into neurons, but was required for the maintenance and survival of the neurons.

It must of course be emphasized that most of our experiments were carried out with ganglia taken from 8-day chick embryos, and that extension of the conclusions to other conditions and, in particular, to what happens *in vivo* is necessarily speculative. Nevertheless, the notion that the main biological effect of NGF is a maintenance one is at least not inconsistent with results obtained from the *in vivo* studies. For example, we have shown that in mice the continued presence of NGF is necessary in order to maintain the hypertrophic and hyperplastic response invoked by injection of NGF into neonate animals, although the existence of these responses requires some subsidiary hypothesis. Furthermore, it has recently been suggested (Hendry & Iversen, 1973) that neurons of the sympathetic ganglia are maintained *in vivo* by the production of NGF at the end-organs. The mechanism by which NGF could exert this maintenance effect is, unfortunately, completely unknown.

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