convex side than down the concave side of the bending axis.

Axial curvature by residual auxin in entirely defoliated and disbudded stems, turned as usual on the clinostat, has been observed in a few tests with tomatoes and seedling *Coleus blumei*. For all other such defoliated stems, curvatures in the growing internodes are producible by supplying indoleacetic acid in solution or emulsion form in place of the terminal bud. Solutions of the order of $10^{-4}M$ have produced curvatures in excess of 90° in 3 days, with the growth curvatures straightened in the immature tissues by return of the plant to the erect position for a few days. Substitution of a layer of lanolin containing 1 percent indoleacetic acid for the terminal bud of a similar straight axis of sunflower, geranium, or coleus, applied uniformly over the freshly cut surface at the tip, also produces a stem curvature that varies in degree with the rate of growth of the apical tissues, just as in an axis with leaves and terminal bud. Thus with a symmetrical supply of indoleacetic acid to the cut end of the stem, the growth is still asymmetrical without the normal action of gravity on the transport system.

The results of all these tests and observations are consistent with the hypothesis that gravity is a factor in maintaining an even distribution of one or more of the auxins within the growth zone of the plant's axis. In the absence of an effective gravitational force, more auxin enters one sector of a growing internode than other sectors. Differences in transport rate are probably more important than possible differences in auxin supply from the young leaves. The effect of gravity on the erect stem is to equalize any such differences in auxin content of the growing tissues, presumably by the same mechanism by which a nonvertical axis is caused to assume the erect position (*6*).

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References and Notes

1. H. E. Dolk, *Rec. Trav. Bot. Néerl.* **33**, 509 (1936).
2. H. Söding, *Die Wuchsstofflehre* (Thieme, Stuttgart, 1952), p. 120.
3. R. Snow, *New Phytologist* **52**, 194 (1953).
4. J. Sachs, *Verhandl. Physik.-med. Ges. Würzburg. n.s.* **2**, 253 (1872).
5. G. Kuse, *Mem. Coll. Sci. Univ. Kyoto Ser. B* **20**, 207 (1953); E. Niedergang-Kamien and F. Skoog, *Physiol. Plantarum* **9**, 60 (1956); J. R. Hay, *Plant Physiol.* **31**, 118 (1956).
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Dialyzable Cofactor in Nerve Growth Promoting Protein from Mouse Salivary Glands

Abstract. Cohen's method for preparing the nerve growth factor from mouse submaxillary glands was followed to the last ammonium sulfate fraction. Further purification was accomplished with carboxymethyl- and diethylaminoethyl- column chromatography. Three peaks were obtained for each column; the third peak obtained with diethylaminoethyl cellulose was the most active. Electrophoresis of this active fraction produced one anodal and two cathodal bands. Each band was inactive for nerve outgrowth. However combinations of the cathodal bands produced 3+ growth. One cathodal band was not dialyzable; the other was dialyzable and negative for ultraviolet absorption at 280 millimicrons.

Evidence for the existence of a nerve growth factor, specific for spinal (*1, 2*) and sympathetic ganglia (*3*), was first obtained from mouse sarcomas which were grown as intraembryonic grafts in chick embryos. Tumor fragments were transplanted to the flank of 2½-day-old chick embryos. After 9 to 14 days of incubation, the spinal and sympathetic ganglia adjacent to the grafts were found to be 1.5 to 6 times larger than comparable controls (*3, 4*). Neural centers in the spinal cord and parasympathetic centers failed to respond. Cell counts of comparable square areas and reconstructions of serial sections provided conclusive evidence that this increase in size of the ganglia was the result of neuron hypertrophy and hyperplasia (*2, 3*). This growth response was then also elicited from sarcoma fragments grown as allantoic grafts (*5*). It was postulated that a humoral agent was being transmitted to the embryo, possibly by way of the blood (*5*). In spinal or sympathetic ganglia grown as explants in hanging drop cultures supplemented with cell-free sarcoma derivatives (microsomal, nucleoprotein, protein fractions), a dense outgrowth of neurites appeared during 24 hours (*6*). These observations led to the concept of a nerve growth factor as an entity which has in part been characterized (*7*).

This nerve growth factor has since been shown to be present in a wide variety of biological materials (*8*) and has been isolated as a protein fraction from each of the following: mouse sarcoma 180, snake venoms, mouse submaxillary salivary glands (*9–12*) and axial structures of 8- to 9-day-old chick embryos (*8*). Cohen and Levi-Montalcini have shown that the submaxillary gland from the adult male mouse is by far the richest source (*9, 10*). Rabbit antiserum to this protein inhibited nerve outgrowth from ganglion explants in vitro and produced nearly total destruction of sympathetic ganglia in newborn mice, rats, rabbits, and cats (*13*).

Ultraviolet absorption ratios (for absorption at wavelengths of 280 and 260 mμ) for purified proteins from mouse sarcoma 180 (*11*), from snake venom (*12*), and from mouse submaxillary glands (*9*) were reported as 1:25, 1:30, and 1:53, respectively. The molecular weight of the protein fraction from sarcoma 180 was not determined. Molecular weights, as determined with the Spinco analytical ultracentrifuge, were of the order of 20,000 for the venom protein (*12*) and 44,000 for the mouse submaxillary gland protein (*9*). The purpose of the present study was to obtain further chemical information on the nature of the factor as isolated from the submaxillary gland of the adult male mouse.

We have followed the procedure of isolation and partial purification through the last ammonium sulfate precipitation as described previously (*9*). Briefly, the steps up to this point included homogenization, centrifugation, streptomycin sulfate precipitation, two ethanol precipitations, and ammonium sulfate precipitation in three steps. The fraction precipitating at 75 to 85 percent ammonium sulfate sat-

Table 1. Quantitative amino acid analysis of electrophoretic band A. Residues, on basis of arginine as 1 (corrected to nearest whole numbers, taking into account destruction during hydrolysis, and multiplied by 2). Total nitrogen (by micro-Kjeldahl method) added to column, 0.0934 mg. Total nitrogen recovered (not counting tryptophan and ammonia), 0.0858 mg. Total weight of residues, 8600.

Amino acids	Micro-moles	Resi-dues	N eluted (μg)
Arginine	0.159	1.00	8.90
Lysine	.339	2.12	9.54
Histidine	.165	1.03	6.84
Cysteic	.155	0.98	2.15
Aspartic	.617	3.95	8.53
Threonine	.307	1.95	4.27
Serine	.389	2.44	4.44
Glutamic	.473	2.99	7.23
Proline	.357	2.25	5.01
Glycine	.453	2.87	6.32
Alanine	.280	1.78	3.92
Valine	.276	1.75	3.86
Isoleucine	.184	1.10	2.56
Leucine	.487	3.04	6.80
Ø-Alanine	.136	0.85	1.90
Tyrosine	.173	1.05	2.44
Methionine sulfone	.065	0.50	1.15
Sulfoxide	.022		

uration was dissolved in distilled water. After centrifugation, this fraction was dialyzed against distilled water until free of sulfate.

Further purification was accomplished with carboxymethyl- (CM-) and diethylaminoethyl- (DEAE-) cellulose columns. Columns were prepared by the method of Peterson and Sober (*14*). Elutions after equilibration with 0.001*M* phosphate buffer (*p*H 7.4), were started with the same buffer and followed by a linear sodium chloride gradient in the same buffer. Each column gave three well defined peaks. Most of the biological activity from the CM- column was found in peak I. This material was lyophilized, taken up in 0.001*M* phosphate buffer (*p*H 7.4), and put on a DEAE- column. Peak III from this column contained most of the activity. The amount of protein recovered from each of the various steps was determined by the method of Lowry *et al.* Protein requirements for 3+ nerve outgrowth from ganglion explants in hanging-drop cultures were determined in micrograms per milliliter (*10*).

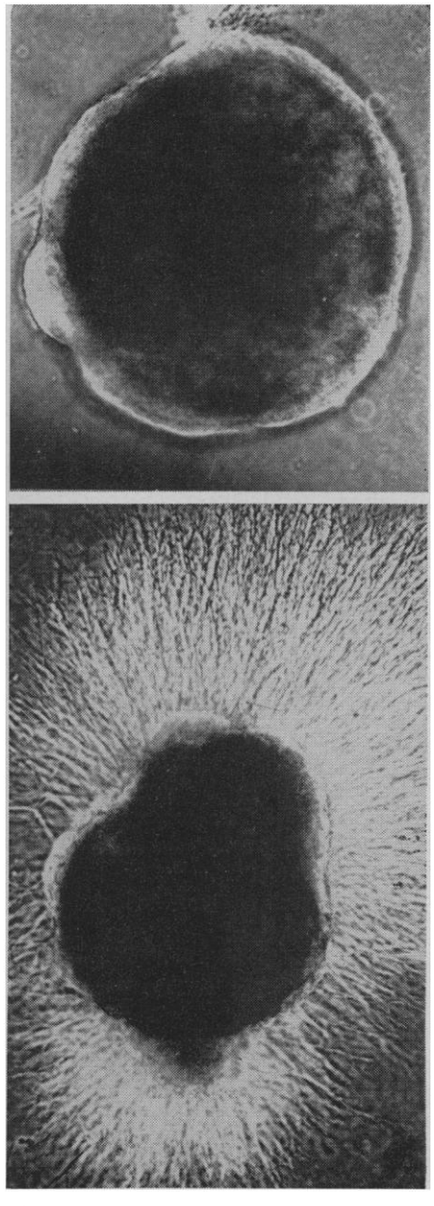

Fig. 1. Nerve outgrowth from spinal ganglion explants of 8-day-old chick embryos after 24 hours in haning drop cultures supplemented with electrophoretic band A or C, or A plus C combinations. (Top) Band C at 11.4 μg/ml to 0.0114 μg/ml dry weight, or band A at 1.69 μg/ml to 0.07 μg/ml. Lowry protein produced no outgrowth. (Bottom) Band A (0.07 μg/ml) combined with C (0.0114 μg/ml) produced 3+ growth.

From three runs, it was found that 132 to 152 (av. 143) mg dry weight of ammonium sulfate fractions contained 99.7 to 106.2 (av. 106.8) mg of protein; and produced 3+ growth at 0.28 to 0.33 μg/ml. Elution of ammonium sulfate fractions from CM- columns produced 60.2 to 72.9 (av. 70.6) mg dry weight from the peak I fractions; 55.4 to 72.7 (av. 64.3) mg protein; and 3+ growth at 0.225 to 0.300 μg/ml. Elution of CM-, peak I fractions from the DEAE- columns produced 19.9 to 26.2 (av. 24.0) mg dry weight from DEAE-, peak III fractions; 19.0 to 24.4 (av. 22.1) mg of protein; and 3+ growth at 0.05 to 0.11 μg/ml. From these observations, we judge that five- to sixfold purification was accomplished.

Paper electrophoresis patterns were prepared from samples of each of the fractions at 750 volts, 10 to 12 ma, in 0.01*M* phosphate buffer at *p*H 7.4 for 3 hours. As a result of further chromatography the number of components was reduced from six, as observed with the ammonium sulfate fraction, to three in the most active fraction, DEAE-, peak III. The electrophoretic bands resulting from DEAE-, peak III were designated as B, A, and C. Bands A and C were positively charged at this *p*H and moved toward the cathode, with C having the greater mobility. Band B was negatively charged. Five strips were run simultaneously. At the end of the run, one strip was stained with ninhydrin to locate the components. Corresponding areas of nonstained strips were cut out and eluted with distilled water. After protein determinations and suitable dilutions, bioassays (in vitro) of each band separately showed no significant activity. Combinations of bands were tried and it became clear that combinations of bands A and C produced excellent nerve outgrowth (Fig. 1).

Component A was nondialyzable, in contrast to C which was dialyzable. Ultraviolet-absorption for A gave a 280/260 ratio of 1:78. Band C was negative to ultraviolet-absorption at 280 mμ. A sample of A was hydrolyzed in 6*N* HCl in a sealed tube for 21 hours at 110°C. It was then subjected to quantitative amino acid analysis with a Technicon automatic analyzer (Table 1). The minimal molecular weight was estimated to be approximately 8600. End group analysis of A by the method of Sanger and of Edman gave arginine as the N- terminal group. The method of Akabori gave cysteine as the C-terminal end group Preliminary studies of C with the Technicon automatic analyzer indicate the absence of tyrosine, methionine, cysteine, and arginine. The analysis gave 13 amino acids with a total of 41 residues and a minimal molecular weight of approximately 4300 (*15*).

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References and Notes

1. E. D. Bueker, *Anat. Record* **100**, 735 (1948).
2. ———, *ibid.* **102**, 369 (1948).
3. R. Levi-Montalcini and V. Hamburger, *J. Exptl. Zool.* **116**, 321 (1951).
4. E. D. Bueker, *Cancer Res.* **17**, 190 (1957).
5. R. Levi-Montalcini and V. Hamburger, *J. Exptl. Zool.* **123**, 233 (1953).
6. S. Cohen, R. Levi-Montalcini, V. Hamburger, *Proc. Natl. Acad. Sci. U.S.* **40**, 1014 (1954).
7. R. Levi-Montalcini and P. U. Angeletti, *Proc. 4th Intern. Neurochem. Symp.* (1960), p. 362.
8. E. D. Bueker, I. Schenkein, J. Bane, *Cancer Res.* **20**, 1220 (1960).
9. S. Cohen, *Proc. Natl. Acad. Sci. U.S.* **46**, 302 (1960).
10. R. Levi-Montalcini and B. Booker, *ibid.* **46**, 373 (1960).
11. S. Cohen and R. Levi-Montalcini, *Cancer Res.* **17**, 15 (1957).
12. S. Cohen, *J. Biol. Chem.* **234**, 1129 (1959).
13. R. Levi-Montalcini and B. Booker, *Proc. Natl. Acad. Sci. U.S.* **46**, 384 (1960).
14. E. A. Petersen and A. A. Sober, *J. Am. Chem. Soc.* **78**, 751 (1956).
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