form of experimental lead encephalopathy in the young. Oral administration is feasible from the time of birth. The clinical significance of these studies is that a higher incidence of lead exposure in some hyperactive children relative to "normals" has been reported (*16*). It is possible that increases in brain norepinephrine and increased motor activity may be an early response to low-level lead exposure during early developmental periods and not a lowering of brain dopamine as previously postulated by Sauerhoff and Michaelson (*6*). It is worthy to note that similar findings of hyperactivity and increased brain levels of norepinephrine have been reported in lead-exposed mice (*8, 9, 12*). The experimental design as described provides a means to study the biological effects of lead exposure on neonatal developing rats without debilitating histopathologies (*1*) or excessive depression in body weight (*1–6*), thereby eliminating factors such as malnutrition, which could conceivably confound the results. This permits better-controlled investigations into the relationship between behavioral sequelae of lead poisoning and its neurochemical mechanisms.

MARIANNE GOLTER
I. ARTHUR MICHAELSON
*Department of Environmental Health, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267*

### References and Notes

1. A. Pentschew and F. Garro, *Acta Neuropathol.* **6**, 266 (1966).
2. J. A. Thomas, F. D. Dallenbach, M. Thomas, *Virchows Arch. Abt. A Pathol. Anat.* **352**, 61 (1971).
3. I. A. Michaelson, *Toxicol. Appl. Pharmacol.* **26**, 539 (1973).
4. ——— and M. W. Sauerhoff, *ibid.* **28**, 88 (1974).
5. M. W. Sauerhoff and I. A. Michaelson, *Pharmacologist* **15**, 164 (1973).
6. ———, *Science* **182**, 1022 (1973).
7. A. Anton and D. Sayer, *J. Pharmacol. Exp. Ther.* **138**, 360 (1962).
8. E. K. Silbergeld and A. M. Goldberg, *Life Sci.* **13**, 1275 (1973).
9. ———, *Exp. Neurol.* **42**, 146 (1974).
10. B. J. Culliton, *Science* **184**, 644 (1974).
11. I. A. Michaelson, R. D. Greenland, W. Roth, *Pharmacologist* **16**, 250 (1974).
12. E. K. Silbergeld and A. M. Goldberg, *ibid.*, p. 249.
13. J. H. Gordon and M. K. Shellenberger, *Neuropharmacology* **13**, 129 (1974); T. H. Svensson and B. Waldeck, *Psychopharmacologia* **18**, 357 (1970).
14. M. A. Geyer, D. S. Segal, A. J. Mandell, *Physiol. Behav.* **8**, 653 (1972).
15. A. Randrup and J. S. Kruger, *J. Pharm. Pharmacol.* **18**, 752 (1966).
16. O. David, J. Clark, K. Voeller, *Lancet* **1972-II**, 900 (1972).
17. M.G. is enrolled in the neurobiology program of the University of Michigan, Ann Arbor, and is a recipient of the Grass Foundation summer training grant in neurochemistry. We thank Drs. Silbergeld and Goldberg for sharing their experimental findings with us.

30 July 1974; revised 23 September 1974

# Secretion of a Nerve Growth Factor by Primary Chick Fibroblast Cultures

Abstract. *Normal primary chick embryo fibroblast cultures produce a nerve growth–promoting factor which cross-reacts with monospecific antibody to pure male mouse submaxillary gland nerve growth factor (NGF). When taken together with the earlier demonstration that mouse $L_2$ cells and 3T3 cells also produce an NGF-like protein, these findings suggest that secretion of this factor may be a general property of fibroblasts.*

Cultured mouse $L_2$ cells, 3T3 cells, and SV 3T3 (simian virus 40 transformed) cells secrete a protein which, according to biological activity and immunological criteria, is indistinguishable from pure male mouse submaxillary gland nerve growth factor (NGF) (*1*). These findings suggest that secretion of NGF, or a protein closely similar to it, may be a general property of fibroblasts. However, $L_2$ cells are malignant fibroblasts, 3T3 cells are aneuploid, and SV 3T3 cells are transformed fibroblasts. We have now turned to primary normal chick embryo fibroblast cultures, and we find that these also secrete a biologically active NGF which reacts with antibody to pure mouse NGF.

Male mouse submaxillary gland NGF was isolated as previously described (*1*). The protein was pure as judged by gel electrophoresis in three solvent systems and by immunoelectrophoresis (*1*). Dorsal root ganglia of chick embryos 10 to 14 days old were used to estimate the neurite outgrowth–producing effect of culture supernatants by minor modifications of the methods originally described by Levi-Montalcini *et al.* (*2*) and Hier *et al.* (*3*). Ganglia were placed on collagen-coated cover slips in petri dishes; the nutrient culture medium contained 90 percent Eagle minimal essential medium with Earle balanced salt solution (MEM, Gibco) plus 10 percent heat-inactivated fetal calf serum (Gibco). Preparations were examined microscopically after incubation for 18 to 24 hours in a humidified atmosphere containing 5 percent $CO_2$ at 37°C. A highly sensitive immunoassay employing $T_4$ bacteriophage was used to measure concentrations of NGF as low as 1 ng/ml. The details of this immunoassay, as well as the preparation of antisera to mouse NGF, have been described elsewhere (*1*). The basic feature of this assay is that when NGF is covalently coupled to bacteriophage, the infectivity of the virus (for *Escherichia coli*) can be blocked by antibody to NGF. Free, uncoupled NGF competes with the phage-NGF conjugate in the antibody reaction, and this forms the basis for the immunoassay. [See (*4*) for other references to this method.]

The preparation of monolayer fibroblast cultures of cells from decapitated 12-day chick embryos has been described (*5*). Cells dispersed by trypsinization were plated at an initial density of approximately $5 \times 10^4$ cells per square centimeter in 32-ounce (~ 95-ml) glass bottles (monolayer surface, 110 $cm^2$). After 5 days growth in Eagle basal medium (BME) supplemented with 3 percent calf serum, the cells (confluent) were washed twice with sterile saline (100 ml per wash), drained, and fed with serum-free BME. *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (0.01*M*) was added in order to maintain the *p*H at approximately 7.4.

Examination of stained cells [May-Grünwald Giemsa stain (*6*)] from such cultures has revealed that 90 to 95 percent of the cells in the monolayer are fibroblasts, as determined by cell morphology, the presence of collagen fibers in electron microscope thin sections, and nuclear size and shape. The remaining cells are multinucleate muscle cells or pycnotic cells of unknown type. Transfer to serum-free medium for 24 to 48 hours eliminates virtually all cells of nonfibroblast morphology (*7*). Removal of serum arrests growth and net protein and RNA synthesis; the cells do not divide or make DNA (*8*).

To study the production of fibroblast NGF as a function of time, cultures were incubated at 37°C in serum-free BME; at intervals the culture fluid was removed, centrifuged to remove cells (2000*g* for 10 minutes), dialyzed exhaustively against 0.01*M* ammonium acetate at 4°C, and lyophilized. For ganglion assays, the dry powder from 60 ml of culture fluid was redissolved in MEM at 100 times the original concentration and dialyzed thoroughly against MEM at 4°C. One part (0.05 ml) of

this solution was added to 0.05 ml of 90 percent MEM plus 10 percent fetal calf serum, and the resulting solution was placed on ganglia. For immunoassay, the lyophilized residue from 20 ml of culture fluid was dissolved in 0.5 ml of 0.12*M* phosphate buffer, *p*H 7.3, and 0.4 ml of this solution was analyzed by the bacteriophage method (*1*).

Figure 1 presents the results of a sensory ganglion bioassay of a 50-fold concentrated supernatant removed from cells after 120 hours in culture. As shown in Fig. 1, this culture fluid, like mouse NGF, stimulates extensive neurite outgrowth from the ganglion. Serum-free BME elicits no such response from the ganglion cells.

Table 1 summarizes results of NGF phage immunoassays of media in continuous contact with the monolayer for the times indicated. The concentrations of immunoreactive material presented in Table 1 are based on a standard immunoassay for mouse NGF (*1*). Thus depending on the degree of cross-reactivity of the chick fibroblast culture material with antibody to mouse NGF, the values shown are minimal concentrations. The amount of material detected immunologically was greatest at 120 hours, after which the concentration apparently fell somewhat, perhaps because of degradation.

The results of Fig. 1 and Table 1 demonstrate that primary cultures of chick fibroblasts produce a nerve growth–promoting factor which is immunochemically similar to mouse submaxillary gland NGF. Although 90 to 95 percent of the cell population of these cultures are fibroblasts, it could be argued that the nonfibroblast cells are the source of the NGF-like factor. However, after 24 to 48 hours in serum-free medium the nonfibroblast cells have virtually disappeared, and yet (Table 1) the amount of material reacting with antibody to NGF more than doubles between 36 and 120 hours in culture. When taken together with earlier findings that $L_2$ and 3T3 fibroblasts also synthesize a factor which is closely similar to NGF (*1*), the results indicate that secretion of this protein is a property not confined to mouse cells and that its production may be a general characteristic of fibroblasts.

Table 1. Immunoassay of chick fibroblast culture supernatants as a function of time in culture. The NGF values refer to the amounts (± standard error of the mean) of immunoreactive material per 120 ml of original culture medium, based on a standard assay for mouse NGF.

| Time (hours) | NGF (ng) |
|---|---|
| 0 | 0 |
| 36 | 17.4 ± 1.7 |
| 96 | 25.8 ± 2.7 |
| 120 | 38.5 ± 4.0 |
| 144 | 29.3 ± 3.0 |

In 1961, Levi-Montalcini and Angeletti (*9*) demonstrated that experimentally induced mouse granulation tissue (rich in fibroblasts) displayed NGF-like activity in vitro. Moreover, Hendry and Iverson (*10*) have demonstrated that removal of mouse submaxillary glands only temporarily reduces the serum concentration of NGF. This indicates that cells outside the salivary glands must be capable of producing NGF. The results discussed here provide at least plausible explanations for these two observations. Finally, since a prominent feature of granulation tissue is the fibroblast, we wonder whether NGF might play a role in the biological function of this tissue.

MICHAEL YOUNG
*Departments of Medicine* and *Biological Chemistry, Harvard Medical School*, and *Massachusetts General Hospital, Boston 02114*

JOËL OGER
*Department of Neurology, Harvard Medical School*, and *Massachusetts General Hospital*

MURIEL H. BLANCHARD
*Departments of Medicine* and *Biological Chemistry, Harvard Medical School*, and *Massachusetts General Hospital*

HOVAN ASDOURIAN
HAROLD AMOS
*Departments of Microbiology* and *Molecular Genetics, Harvard Medical School*

BARRY G. W. ARNASON
*Department of Neurology, Harvard Medical School*, and *Massachusetts General Hospital*

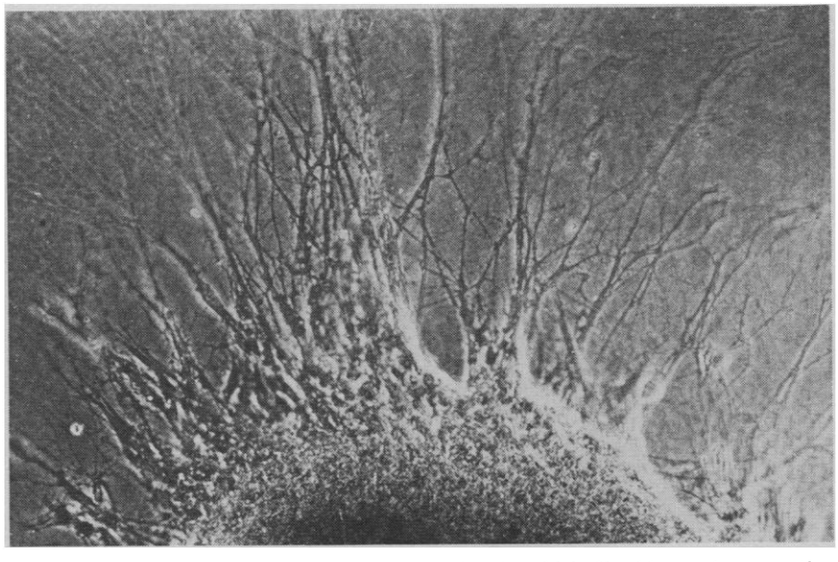

Fig. 1. Stimulation of ganglionic neurite extension by chick fibroblast culture medium. Chick embryo ganglion (10 day) was treated with a 50-fold concentrated fibroblast culture supernatant for 18 hours, as described in the text and in (*3*). Phase-contrast photomicrograph (× 235).

**References and Notes**

1. J. Oger, B. G. W. Arnason, N. Pantazis, J. Lehrich, M. Young, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1554 (1974).
2. R. Levi-Montalcini, H. Meyer, V. Hamburger, *Cancer Res.* **14**, 49 (1954).
3. D. B. Hier, B. G. W. Arnason, M. Young, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2268 (1972).
4. E. Hurwitz, F. M. Dietrich, M. Sela, *Eur. J. Biochem.* **17**, 273 (1970).
5. H. Amos and M. O. Moore, *Exp. Cell Res.* **32**, 1 (1963).
6. W. Jacobson and M. Webb, *ibid.* **3**, 163 (1952).
7. J. Cummins, J. André, H. Amos, in preparation.
8. R. Soeiro and H. Amos, *Science* **154**, 662 (1966).
9. R. Levi-Montalcini and P. U. Angeletti, in *Regional Neurochemistry*, S. S. Kety and J. Elkes, Eds. (Pergamon, Oxford, 1961), p. 362.
10. I. A. Hendry and L. L. Iverson, *Nature (Lond.)* **243**, 500 (1973).
11. Supported by NIH grants AM-09404 (to M.Y.), NS-06021 (to B.G.W.A.), and AI-00957 (to H.A.), and by ACS grant VC-105G (to H.A.). J.O. was supported by a joint fellowship from Harvard Medical School and Institut National de la Santé et de la Recherche Médicale (INSERM), France. We thank G. Geltemeyer for technical assistance and F. K. Suda for help in preparation of the manuscript.

25 April 1974; revised 9 August 1974