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Nerve growth factor stimulates the development of substance P in sensory ganglia

(dorsal root ganglia/amputation)

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ABSTRACT **The development of the putative neurotransmitter substance P (SP) in rat dorsal root ganglion (DRG) was defined *in vivo*. The sixth cervical DRG of newborn rats contained 70 pg of SP, and the ganglionic content increased 5.5-fold during the first 5 weeks of life. Forelimb amputation partially prevented the normal developmental increase of SP in the sixth cervical DRG destined to innervate that limb. Conversely, treatment with nerve growth factor (NGF) increased both ganglionic SP and total ganglion protein. Moreover, NGF administration prevented the failure of SP development that followed amputation, suggesting that NGF may mediate the limb–DRG interaction. However, treatment with antiserum to NGF failed to significantly inhibit development of ganglion SP. Consequently, neonatal ganglia may remain responsive to NGF, without requiring the protein for survival. SP appears to be an excellent index of the maturation of neurons in dorsal root ganglia.**

The study of dorsal root ganglia has led to the elucidation of a number of principles governing neuronal ontogeny (1, 2). For example, normal ganglion maturation is dependent on the integrity of the peripheral field of innervation. Limb amputation in the embryo prevents normal growth of the innervating dorsal root ganglion (DRG) (3, 4). Although the factors mediating this effect are undefined, several lines of evidence suggest that the trophic protein nerve growth factor (NGF) may be involved. NGF stimulates growth of the embryonic DRG (2, 5) and is transported in a retrograde manner from limb to innervating ganglion (6). However, it remains to be demonstrated that the embryonic limb elaborates NGF. Moreover, the effects of limb tissue or NGF or both on the *biochemical* development of DRG neurons are virtually unknown.

The lack of a biochemical marker specific for peripheral sensory neurons has limited studies of DRG development. However, recent work suggests that substance P (SP) may be an excellent marker. SP is an undecapeptide that is widely, but unequally, distributed throughout the central and peripheral nervous systems (7–10). It is highly concentrated in some neuronal perikarya of the DRG and in neuronal processes within the dorsal horn of the spinal cord (7, 10–12). This distribution suggests that SP may act as a transmitter in primary sensory neurons, a hypothesis that has recently gained strong physiological and biochemical support (10–14). The high concentration, intraneuronal localization, and putative transmitter function of SP in spinal ganglion may make it an excellent index of ganglion development. Moreover, a reliable and highly sensitive radioimmunoassay for SP is available (15).

The present study defines the normal development of SP-like immunoreactivity (henceforth called SP) in the rat DRG and demonstrates that alterations in ganglion development are reflected by changes in ganglion SP content. In addition, the effects of limb amputation and NGF treatment on SP development are defined.

MATERIALS AND METHODS

Experimental Animals. Pregnant Sprague–Dawley rats (Charles River) were housed in clear plastic and wire cages and were exposed to 540–810 lux of cool-white fluorescent illumination from 5 a.m. to 7 p.m. daily. Ralston Purina Lab Chow and water were offered ad lib. Litter sizes were adjusted when necessary so that each litter had between 12 and 14 pups.

Surgical Procedures. *Amputation.* Amputations were performed during the first day of life. Pups were placed in an ether jar until they became unresponsive to painful stimuli. A single suture was placed as proximally as possible around the right forelimb of each pup and tightened until the limb was severed. Wounds were closed with collodion. Sham-operated animals were anesthetized but had no surgery performed.

Dissection of the DRG. Animals were placed in an ether jar until cessation of pulse and respiration. The spinal cord and paraspinal tissue were removed *en bloc* from the second cervical to the second lumbar vertebra. The cervical spine laminae were removed and the spinal ganglia were exposed. The sixth cervical (C_6) DRG on each side was removed for study.

Preparation of Antiserum to SP. Anti-SP was prepared by using a minor modification of the procedure of Powell *et al.* (15). Synthetic SP (Peninsula Laboratories) was conjugated to bovine serum albumin by means of carbodiimide, dialyzed against distilled water, and lyophilized. Guinea pigs were injected intradermally at monthly intervals with 0.15 mg of the SP-albumin complex in Freund's adjuvant. All animals developed antibodies to SP. The anti-SP used in the radioimmunoassay bound 10^4 cpm of ^{125}I-labeled SP (4 ng) at a dilution of 1:100,000. The antibody exhibited less than 1% crossreactivity at up to 1000-fold molar excess of eledoisin, methionine-enkephalin, or leucine-enkephalin.

Preparation of NGF. β-NGF was prepared from adult male mouse salivary glands by the method of Mobley *et al.* (16).

Preparation of Antiserum to NGF. Anti-NGF was prepared in rabbits as described (17).

Radioimmunoassay for SP. The procedure of Powell *et al.* (15) was used with minor modifications. The lowest detectable quantity of SP was approximately 15 pg. Tissue was prepared by a modification of the procedure of Chang and Leeman (18). Recovery of SP was approximately 70% by this procedure.

Protein Determination. Total soluble protein was measured by the method of Lowry *et al.* (19).

Statistics. Data were analyzed by Student's *t* test and, where applicable, by the paired *t* test.

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Abbreviations: SP, substance P; DRG, dorsal root ganglion; SCG, superior cervical ganglia; C_6, sixth cervical; NGF, nerve growth factor.

RESULTS

Development of SP and Protein in the DRG. To define the normal developmental profile for SP, the C_6 ganglia from rats of different ages were examined (Fig. 1). SP increased over a 5.5-fold range during the first 5 weeks of life, rising from 70 pg per ganglion at birth. SP increased most rapidly during the first 8 postnatal days, and tended to plateau by 5 weeks. However, SP continued to rise gradually thereafter, increasing by 150 pg (37%) between ages 34 and 340 days (Fig. 1). In contrast, total ganglion protein increased approximately 3-fold during the first 5 weeks of life, resulting in a 70% rise in specific SP content.

Effects of Limb Amputation. To determine whether ganglion SP content, as well as ganglion size, fails to increase normally after extirpation of target structures, unilateral forelimb amputation was performed in neonates. Two weeks later SP was measured in the ipsilateral C_6 DRG, the contralateral control DRG, and ganglia of sham-operated animals. There was no difference in SP content between sham-operated control ganglia and those contralateral to amputation. However, SP failed to increase normally in ganglia deprived of the normal field of innervation (Fig. 2). After amputation, only 58% of the normal developmental increase occurred in ipsilateral ganglia: The peptide increased by approximately 130 pg (185%) on the amputated side, compared to 225 pg (320%) in control ganglia.

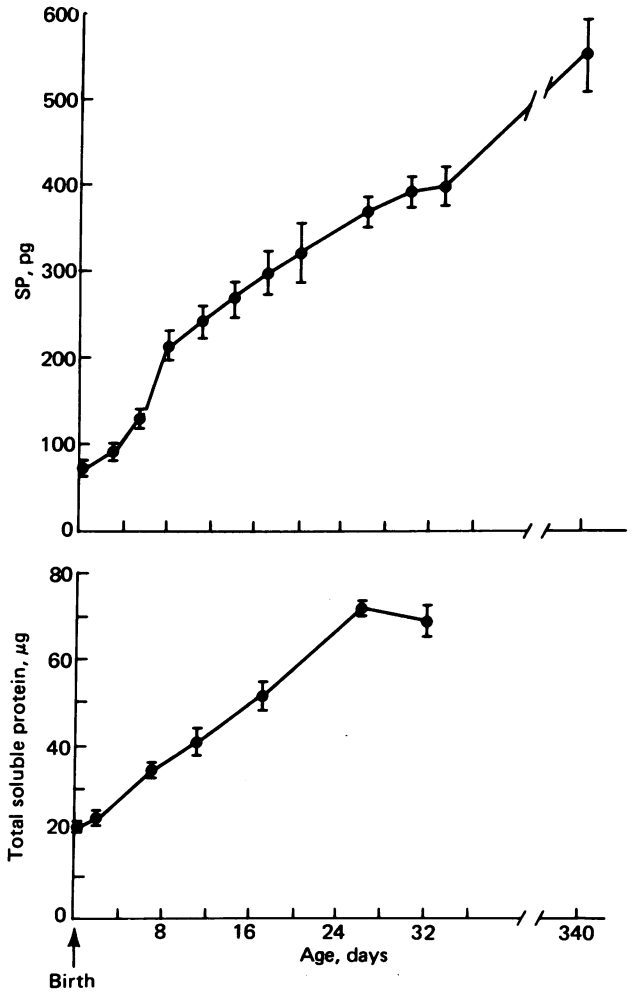

FIG. 1. Postnatal development of SP and total protein in dorsal root ganglia. The C_6 DRG was removed from animals at different ages and examined for SP content and total soluble protein. Each point represents seven or eight animals. SP is expressed as mean pg per ganglion (± SEM); protein is expressed as mean μg per ganglion (± SEM).

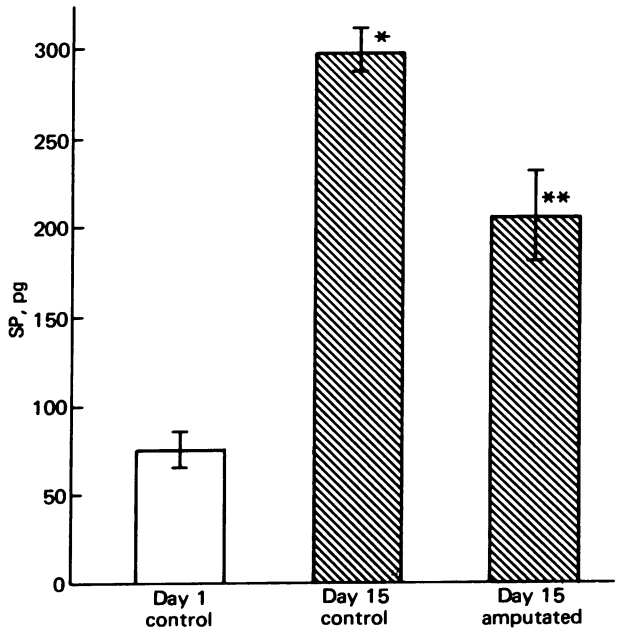

FIG. 2. Effects of forelimb amputation on SP in the C_6 DRG. Unilateral forelimb amputation was performed on eight animals on the first day of life. Two weeks later the C_6 ganglia on the side of amputation and on the contralateral control side were examined for SP content, which is expressed as mean pg per ganglion (± SEM). * Differs from day 1 control at $P < 0.001$. ** Differs from day 1 control at $P < 0.001$ and from day 15 control at $P < 0.01$.

Effects of NGF Treatment. Considerable evidence indicates that NGF regulates DRG development in the embryo (2, 5, 6). However, the role of the protein in postnatal ganglion maturation remains to be defined. To determine whether NGF stimulates postnatal DRG development, neonates were treated with the factor and C_6 ganglion SP was assayed 2 days later. NGF treatment caused a dramatic increase in SP content to 163% of the saline-treated control values (Fig. 3). The actual developmental rise in the NGF group of 62 pg was 6-fold greater than the 11-pg increase in the saline-treated controls

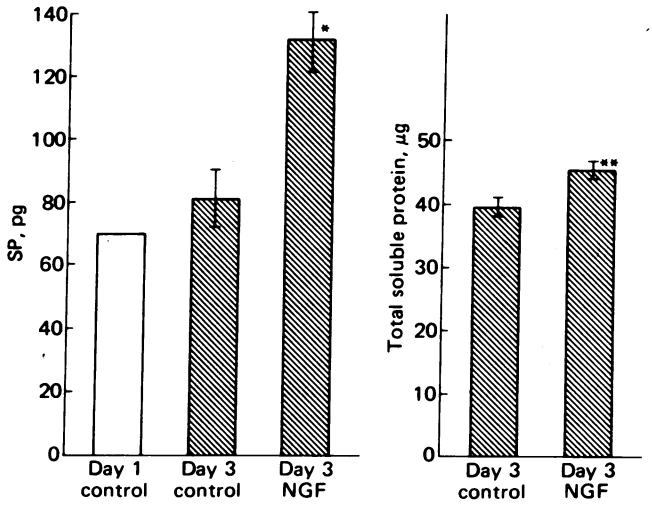

FIG. 3. Effects of NGF on SP and total protein in the C_6 DRG. Neonates were injected subcutaneously with 100 μl of either NGF (10^3 units) in saline (eight animals) or saline (eight animals). Two days later the C_6 dorsal root ganglia were examined for SP content and total protein. SP is expressed as mean pg per ganglion ± SEM; protein is expressed as mean μg per ganglion (± SEM). * Differs from respective control at $P < 0.001$. ** Differs from respective control at $P < 0.025$.

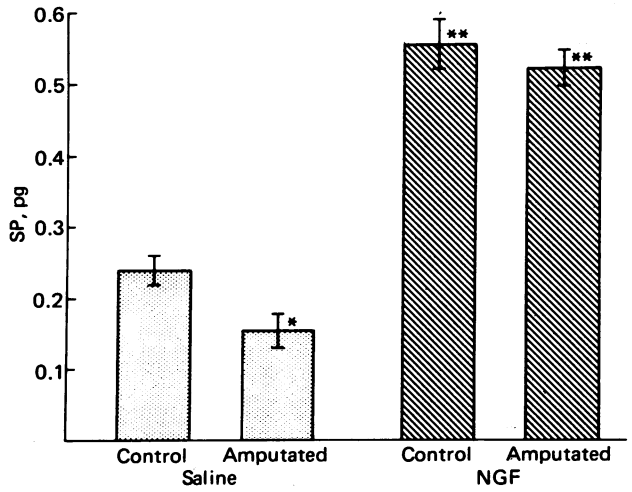

FIG. 4. Effects of combined amputation and NGF treatment. Unilateral forelimb amputation was performed in neonates (16 animals). Animals were then injected subcutaneously with 100 μl of either NGF (10^3 units) in saline (8 animals) or saline (8 animals) daily for 9 days. The C_6 ganglia on both the amputated side and the contralateral control side were examined for content of SP, which is expressed as mean pg per ganglion (± SEM). * Differs from saline control at $P < 0.01$. ** Differs from saline control at $P < 0.001$.

(Fig. 3). In contrast, total ganglion protein increased to only 115% with NGF administration, resulting in a significant elevation of specific SP content from 1.03 to 1.45 pg/μg of protein.

Effects of Combined Amputation and NGF Treatment. To determine whether NGF affects the response to limb extirpation, rats were treated with the factor after surgery. Subsequent to unilateral forelimb amputation, one group of rats was treated daily with saline and another group received NGF. Nine days postoperatively the four sets of ganglia were assayed for SP, the ganglia contralateral to amputation serving as controls in each group.

As expected, amputation reduced ipsilateral ganglion SP content to 65% in the saline-treated rats (Fig. 4). In contrast, NGF treatment prevented the amputation-induced decrease in ipsilateral ganglion SP: in the NGF-treated group there was no significant difference between ipsilateral and contralateral ganglion SP. However, NGF treatment significantly increased SP on both the control and amputated sides, compared to ganglia of saline-treated rats, reflecting the growth response to the protein. In the NGF-treated group, control ganglia contained 2.3-fold more SP than did saline-treated control ganglia, whereas ganglia on the amputated side contained 3.4-fold more SP than did experimental ganglia from the saline group.

Effects of Anti-NGF. To determine whether endogenous NGF is essential for normal postnatal DRG development, newborn pups received daily injections of anti-NGF. Seven days thereafter C_6 dorsal root ganglia were assayed for SP, and the superior cervical sympathetic ganglia (SCG) were assayed for tyrosine hydroxylase activity. The inhibitory effect of anti-NGF treatment on the development of SCG enzyme activity is well documented (20, 21), and we used this effect to ensure adequate anti-NGF treatment.

Anti-NGF administration lowered SP in the DRG by 15%, but this decrease did not attain statistical significance (Fig. 5). Moreover, SP did increase significantly over the 7-day anti-NGF treatment period, although the rise was only 75% of that in the controls. In contrast, anti-NGF completely abolished the developmental increase in SCG tyrosine hydroxylase activity.

DISCUSSION

The study of relatively simple well-defined systems has defined a number of mechanisms governing neuronal ontogeny. However, investigation of one such system, the DRG, has been hampered by lack of a suitable biochemical index of neuronal maturation. The present studies indicate that SP is a specific and convenient biochemical marker for growth and development of DRG neurons *in vivo*.

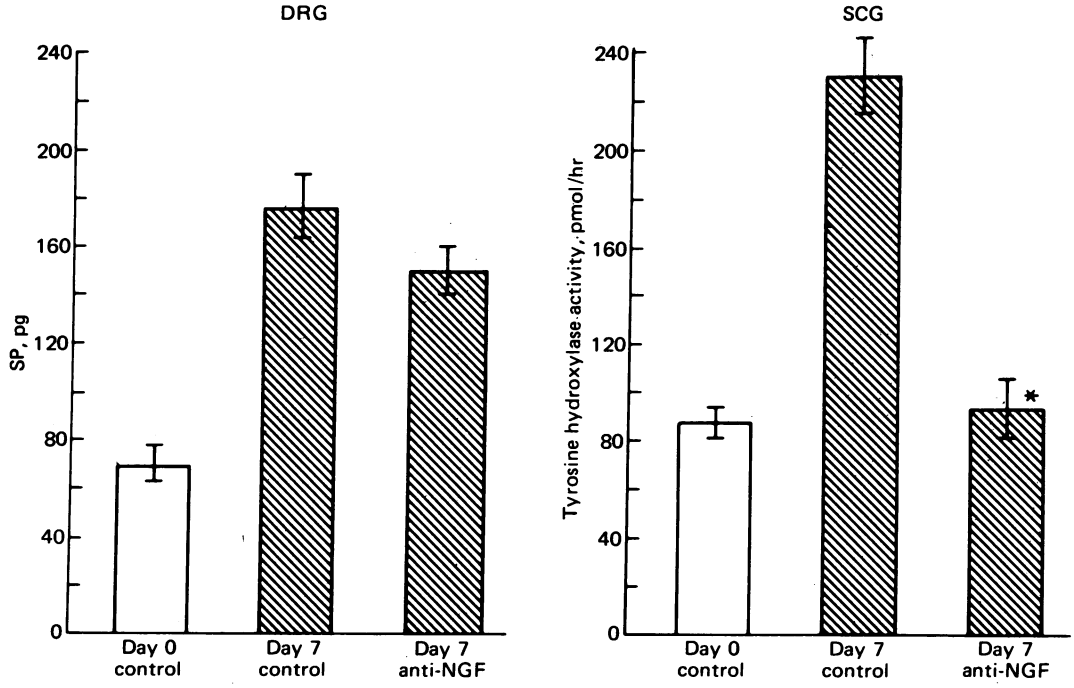

FIG. 5. Effects of anti-NGF on content of SP in the C_6 DRG and on content of tyrosine hydroxylase in the SCG. Neonates were injected subcutaneously with 100 μl of either anti-NGF (approximately 750 units) (eight animals) or saline (eight animals) daily for 7 days. SP is expressed as mean pg per ganglion (± SEM); tyrosine hydroxylase is expressed as mean pmol per ganglion per hr (± SEM). * Differs from respective control at $P < 0.001$.

SP content in the C_6 DRG increased over a 5.5-fold range during the first 5 weeks of life. Moreover, this represented a developmental rise in *specific* SP content, because total DRG protein increased only 3-fold. These observations are consistent with the well-documented localization of SP to sensory neurons in DRG (10–12) and suggest that the increase of SP faithfully reflects neuronal maturation. Consequently, the development of the putative transmitter in DRG is analogous to development of transmitter enzymes in sympathetic ganglia (22, 23). However, SP is not present in all DRG neurons (11), suggesting that its developmental accretion reflects maturation of only a subpopulation of ganglion neurons. Consequently, the increase in specific activity may reflect either the selective survival and development of SP-containing neurons or an actual increase in SP content in each individual neuron.

The response of SP development to a number of experimental manipulations supports the contention that it reflects sensory neuron development and also provides a number of insights regarding regulatory interactions. It is well documented that target structures regulate survival and development of motor (24, 25), parasympathetic (26), and sensory neurons (1, 3), to cite only some examples. In the case of the DRG, limb amputation results in decreased ganglion volume and a reduction in neuron numbers (1). In our studies, limb extirpation prevented the normal development of ipsilateral ganglion SP, suggesting that this index can be used to quantitate abnormal as well as normal development. The failure of SP to increase normally may have reflected direct damage to peripheral nerve fibers as well as loss of the field of innervation. Regardless of the mechanism involved, it was possible to discern that a reduced but significant degree of development occurred even after amputation, suggesting that targets are not the sole determinant of DRG maturation. Finally, the contralateral DRG, possessing a normal field of innervation, developed normal SP levels after amputation, suggesting that limb–ganglion interactions do not occur through humoral mechanisms.

One clearly defined factor that does influence DRG development prenatally is NGF (2, 5). However, previous work has failed to indicate whether NGF regulates sensory neuron maturation postnatally (27–29). The dramatic increase in SP elicited by treatment of neonates with NGF in our studies indicates that postnatal sensory neurons are responsive to the protein. Moreover, NGF increased specific SP content in the DRG, indicating that NGF specifically affects sensory neurons, and suggesting that the trophic molecule may selectively regulate SP content. It may be tentatively concluded that postnatal sensory neurons retain NGF receptors, the stimulation of which results in the increase of SP content. Consequently, NGF may continue to play a role in sensory development after birth.

The endogenous source of DRG-stimulating NGF has yet to be defined. It may be relevant that NGF treatment prevented abnormal DRG development consequent to limb amputation. It is possible, then, that limb extirpation deprived the ipsilateral ganglion of a source of NGF, and that, consequently, NGF mediates the limb–DRG interaction. This contention is consistent with the observation that DRG neurons can transport NGF from periphery to perikaryon in a retrograde manner (30).

Whereas the foregoing experiments indicate that SP in the developing DRG can respond to NGF, they do not necessarily indicate that endogenous NGF *normally* plays a critical regulatory role. Anti-NGF administration did not prevent development of SP in the DRG, whereas development of tyrosine hydroxylase in the SCG was completely inhibited. This result is subject to a number of interpretations. Postnatal sympathetic neurons may simply require greater quantities of NGF for development, and higher doses of anti-NGF may prevent DRG maturation. Alternatively, NGF may, in some manner, be transferred to DRG neurons in an antibody-resistant form. The possibility remains, of course, that although postnatal sensory neurons are sensitive to NGF, they do not require the factor for normal development.

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1. Hamburger, V. & Levi-Montalcini, R. (1949) *J. Exp. Zool.* **111,** 457–501.
2. Levi-Montalcini, R., Myer, H. & Hamburger, V. (1954) *Cancer Res.* **14,** 49–57.
3. Hamburger, V. (1934) *J. Exp. Zool.* **68,** 449–494.
4. Shorey, M. L. (1909) *J. Exp. Zool.* **7,** 25–63.
5. Cohen, S. (1960) *Proc. Natl. Acad. Sci. USA* **46,** 302–311.
6. Brunso-Bechtold, J. & Hamburger, V. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1494–1496.
7. Perrow, B. (1953) *Acta. Physiol. Scand.* Suppl. **29,** 105, 1–90.
8. Hökfelt, T., Myerson, B., Nilsson, G., Perrow, B. & Sachs, C. (1976) *Brain Res.* **104,** 181–186.
9. Kanazawa, I. & Jessell, T. (1976) *Brain Res.* **117,** 362–367.
10. Takahashi, T. & Otsuka, M. (1975) *Brain Res.* **87,** 1–11.
11. Hökfelt, T., Kellerth, J.-O., Nilsson, G. & Perrow, B. (1975) *Science* **190,** 889–890.
12. Hökfelt, T., Kellerth, J.-O., Nilsson, G. & Perrow, B. (1975) *Brain Res.* **100,** 235–252.
13. Konishi, S. & Otsuka, M. (1974) *Nature (London)* **252,** 734–735.
14. Otsuka, M. & Konishi, S. (1976) *Cold Spring Harbor Symp. Quant. Biol.* **40,** 135–143.
15. Powell, D., Leeman, S., Tregear, G., Niall, H. & Potts, J. (1973) *Nature (London) New Biol.* **241,** 252–254.
16. Mobley, N., Schenker, A. & Shooter, E. (1976) *Biochemistry* **15,** 5543–5551.
17. Levi-Montalcini, R. & Booker, B. (1960) *Proc. Natl. Acad. Sci. USA* **46,** 373–384.
18. Chang, M. & Leeman, S. (1970) *J. Biol. Chem.* **245,** 4784–4790.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
20. Hendry, I. A. & Iverson, L. L. (1971) *Brain Res.* **29,** 159–162.
21. Angeletti, P., Levi-Montalcini, R., Kettler, R. & Thoenen, H. (1972) *Brain Res.* **44,** 197–206.
22. Black, I. B., Hendry, I. A. & Iversen, L. L. (1971) *Nature (London)* **231,** 27–29.
23. Black, I. B., Hendry, I. A. & Iversen, L. L. (1972) *J. Neurochem.* **19,** 1367–1377.
24. Hamburger, V. (1958) *Am. J. Anat.* **102,** 365–409.
25. Prestige, M. C. (1967) *J. Embryol. Exp. Morphol.* **18,** 359–387.
26. Landmesser, L. & Pilar, G. (1974) *J. Physiol. (London)* **241,** 715–736.
27. Herrup, K. & Shooter, E. (1975) *J. Cell Biol.* **67,** 118–125.
28. Burnham, P., Raiborn, C. & Varon, S. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 3556–3560.
29. Varon, S., Raiborn, L. & Tyszku, E. (1973) *Brain Res.* **54,** 51–63.
30. Stoeckel, K., Schwab, M. & Thoenen, H. (1975) *Brain Res.* **89,** 1–14.