Developmental Changes in Tissue and Plasma Concentrations of the Biologically Active Species of Nerve Growth Factor in the Mouse, by using a Two-Site Radioimmunoassay

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A sensitive and specific radioimmunoassay has been developed for the biologically active β -subunit of the mouse submaxillary-gland nerve-growth-factor protein. This assay is based on the 'two-site' radioimmunoassay method of Addison & Hales (1971), and a detailed description of the experimental method is given. The assay is readily adaptable for the rapid assay of nerve growth factor in partially purified preparations, or can be used as a sensitive method for measuring the low tissue concentrations of this protein, measurement of concentrations as low as 0.25 ng/ml in plasma or tissue extracts being possible. This assay was used to determine tissue concentrations of nerve growth factor in adult mice and developmental changes in its content in various tissues. There were significant sex differences in the nerve-growth-factor content of submaxillary glands, plasma and sympathetic ganglia, with males having higher concentrations, whereas the concentrations in all other tissues measured showed no significant difference between male and female animals. The changes in plasma concentration of the factor during development parallel the changes observed in the submaxillary gland, and it was suggested that plasma nerve growth factor may be derived mainly from this source. Circulating nerve growth factor appears to be specifically concentrated in sympathetic ganglia. There is a relatively high concentration of the factor in neonatal ganglia and plasma, and it is suggested that this may be derived from maternal sources.

Studies of the biological role of mouse salivary-gland nerve growth factor have been hindered by the lack of an assay that is sensitive enough to detect the low tissue concentrations of this protein, and accurate enough to measure small changes in tissue concentrations. The biological assay of nerve growth factor (Levi-Montalcini *et al.*, 1954; Cohen, 1959; Fenton, 1970) involves the growth of 8-day-old chick-embryo sensory ganglia in tissue culture containing serial dilutions of the factor. Subjective estimates are then made to determine the concentration producing maximum fibre outgrowth, and this is defined as 1 biological unit (B.U.)/ml. This assay can measure a minimum of twofold changes in protein concentration (Cohen, 1959), and as both sides of the maximum response should be spanned (Fenton, 1970) it requires a relatively large amount of material for the set of serial dilutions.

Nerve growth factor can be purified in two forms from the mouse submaxillary gland: the 7S complex as described by Varon *et al.* (1967) and the 2.5S protein as described by Boccacini & Angeletti (1969). The 7S species dissociates readily into three subunits with minor changes of pH and *I* (Varon *et al.*, 1968). These subunits are called α , β and γ and the β -

subunit contains all of the biological activity. The amino acid sequence of the 2.5S species has been published (Angeletti *et al.*, 1971) and it appears to differ only in minor detail from that of the β -subunit (E. C. Shooter, personal communication).

All of the biologically active forms of nerve growth factor have been used to prepare antibodies, and three types of immunoassay have recently been described. A complement-fixation assay has been developed (Zanini & Angeletti, 1971; Angeletti & Vigneti, 1971), but this is time-consuming and requires relatively large amounts of antigen, as a full complement-fixation curve must be obtained for each determination. This assay was based on antibodies prepared against the 2.5S protein. The other two assays are both based on the principles of radioimmunoassay: the first (Johnson *et al.*, 1971) used antibodies prepared against the complete 7S aggregate. This assay has the characteristic advantages of radioimmunoassays in sensitivity and specificity, and requires only very small quantities of the antigen. However, it has the disadvantage that changes in the relative proportions of the inactive α - and γ -subunits might give misleading indications of the apparent biological activity. In addition, it is not known

whether nerve growth factor exists in the 7S form in any tissue other than the salivary gland.

The other radioimmunoassay is the one developed and reported in preliminary form from this laboratory (Hendry *et al.*, 1972). It was decided to use the β -subunit of the nerve growth factor of mouse salivary gland (referred to as the β -subunit) for the development of the assay, as it appeared to be the simplest peptide sequence that retains full biological activity. The present assay is based on the 'two-site' procedure of Addison & Hales (1971), which confers a high degree of specificity as two antigenic sites are required to detect full activity.

Experimental and Results

β -Subunit and antisera

These were kindly provided by Dr. D. C. Edwards of the Wellcome Research Laboratories, Beckenham, Kent, U.K. The β -subunit was prepared from mouse salivary glands by the method of Varon *et al.* (1967). It was electrophoretically homogeneous and yielded only one band on immunodiffusion against a rabbit antiserum to the β -subunit. In the tissue-culture bioassay of Fenton (1970) this preparation had an activity of 1 B.U./2ng of protein. Rabbit antiserum to the β -subunit (batch no. 61/9969) had an activity of 3250 B.U./ml in the tissue-culture assay.

Horse antiserum to the β -subunit (batch no. 8849/46) had an activity *in vitro* of 13000 B.U./ml. Both antisera were used in the present experiments; most of the results reported here, however, were obtained with the horse antiserum.

Preparation of immunoabsorbent paper

Aminocellulose paper was prepared from Whatman no. 1 filter paper by a modification (Addison & Hales, 1971) of the method of Gervich *et al.* (1961). Ten papers of 25cm diameter were dipped individually into a solution containing 3.5% (w/v) sodium *m*-nitrobenzoyloxymethylpyridinium chloride (BDH Chemicals Ltd., Poole, Dorset, U.K.) and 1.25% (w/v) sodium acetate in 80% (v/v) ethanol. They were air-dried and heated at 125°C for 40min; the ten papers were washed together on a Buchner funnel with benzene (2 litres) and then with water (20 litres). The nitro groups were reduced by soaking the papers in 600ml of 20% (w/v) sodium hydrosulphite at 60°C for 30 min. They were washed on a Buchner funnel with water (10 litres), 30% (v/v) acetic acid (2 litres) and again with water (10 litres), and then dried and stored in a vacuum desiccator.

A semi-purified IgG globulin fraction was prepared from 10ml of the antisera by precipitation with 18% (w/v) Na_2SO_4 at 20°C. The precipitated IgG fraction was dissolved in 5ml of 5mM-potassium

phosphate buffer, pH8.0, and dialysed against three changes (2 litres each) of this buffer and finally against 2 litres of a 0.2M-sodium borate buffer, pH8.0. This crude antibody preparation was linked to the paper by the method of Addison & Hales (1971). Aminocellulose papers were individually diazotized by placing a paper in a beaker containing 2ml of 1% NaNO_2 in 150ml of 2M-HCl at 0°C and leaving it for 30min. Solid urea was then added until the starch-iodide test was negative. The paper was washed by repeated decantation six times with water and finally twice with 0.2M-borate buffer, pH8.0. The paper was then incubated with approx. 15mg of the semi-purified IgG in 5ml of 0.2M-borate buffer, pH8.0, for 72h at 4°C in a covered glass Petri dish. The residual diazo groups were neutralized by the addition of 5ml of a saturated solution of glycine and incubation was continued for a further 24h. The paper was then washed by decantation with water (six times) and finally was washed twice with a 0.05M-veronal-NaOH buffer, pH8.6, containing 0.9% NaCl, 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, U.S.A.) and 0.1% NaN_3 .

The papers were then stored in this 'veronal buffer' at 4°C.

Preparation of labelled antibodies

Aminocellulose powder was a gift from Dr. G. M. Addison. It was prepared as described by Miles & Hales (1968). An immunoabsorbent was prepared with this material by the method of Miles & Hales (1968). The aminocellulose powder was converted into diazocellulose by reaction with NaNO_2 in 2M-HCl as described above. The diazocellulose powder was suspended at a concentration of 10mg/ml in 0.2M-borate buffer, pH8.0. To 1.0ml of this suspension 0.25ml of the borate buffer containing 2.5mg of β -subunit was added, giving a final protein concentration of 2mg/ml. After 72h the unchanged diazo groups were neutralized with 1ml of a saturated glycine solution and mixed for 24h. The suspension was centrifuged and the supernatant assayed for protein content; 64% of the added β -subunit was covalently linked to the cellulose by this procedure. This immunoabsorbent was washed six times by suspension in water, followed by centrifugation, and then suspended in 'veronal buffer'. A portion (1mg) of the immunoabsorbent was taken to bind the anti-(β -subunit) antibodies by mixing this suspension with daily changes of 1ml of the semi-purified IgG fraction for 3–4 days. The immunoabsorbent was then washed free from contaminating serum proteins, and a sample containing 25 μ g of protein was taken for iodination by the method of Greenwood *et al.* (1963). This was carried out by using 25 μ l of 0.1M-potassium phosphate buffer, pH7.4, containing the immunoabsorbent, to which was added successively 1 μ Ci of

carrier-free ^{125}I as sodium iodide in a minimum volume, $25\mu\text{g}$ of chloramine-T in $10\mu\text{l}$ of the phosphate buffer and, 20s later, $60\mu\text{g}$ of sodium metabisulphite in $10\mu\text{l}$ of the phosphate buffer. The reaction mixture was then diluted with a modified 'veronal buffer' containing 1% albumin and filtered through a Whatman no. 1 paper. The retained immunoabsorbent was washed with 200ml of 'veronal buffer' and 100ml of 1mM-HCl (pH3.0). The labelled antibodies were then rapidly eluted with four washes (4ml each) of 10mM-HCl at pH2 into 4ml of double-strength 'veronal buffer'. Between 10 and 25% of the original radioactivity was recovered in this eluate, yielding approx. 0.5–1.5 atoms of ^{125}I /antibody molecule. The labelled antibodies were stored either free at 4°C , or re-bound to the immunoabsorbent and stored at 4°C . The length of storage of the re-bound antibodies was limited only by the half-life of ^{125}I , which is 60 days. The antibodies could be washed again on the immunoabsorbent and eluted before each assay, thus ensuring the removal of any molecules damaged by radiochemical decomposition.

Radioimmunoassay procedure

Principle of the assay. The reaction is carried out in two stages (Scheme 1). The first stage requires the incubation of the sample containing nerve growth

factor with the immunoabsorbent paper. Any nerve growth factor in solution will bind to the paper, and then the paper can be washed free from other proteins. The paper is then incubated, in the second stage of the reaction, with the purified ^{125}I -labelled antibodies. Nerve growth factor bound to the paper will bind labelled antibody to a second antigenic site on the molecule of the factor, hence the term 'two-site' assay. The paper can then be washed free from unbound labelled antibody and the bound radioactivity measured.

Experimental procedure. The immunoabsorbent paper was cut into squares of side 0.5cm, and each square was incubated in 1ml of tissue extract or a standard solution of nerve growth factor in a glass specimen bottle ($2\text{cm}\times 2\text{cm}$). The first stage of the incubation was performed in a cold-room at 4°C , and the samples were continuously mixed at slow speed on a rotary mixer. The papers were then washed on Whatman no. 1 filter paper in a Buchner funnel by using six 10ml washes of 'veronal buffer' diluted 1:10 with water. The papers were then transferred to disposable polystyrene tubes ($3\text{cm}\times 0.5\text{cm}$ diam.) and incubated with 0.1ml of a solution containing ^{125}I -labelled antibodies diluted with 'veronal buffer' to a concentration of 400000d.p.m./ml. The second stage of the incubation was carried out at 4°C without mechanical mixing. The paper was washed as described above, air-dried and transferred to

Scheme 1. Protocol for the two-site radioimmunoassay

NGF, Nerve growth factor.

Fig. 1. Effects of incubation time on the bound radioactivity in the two stages of the assay procedure

(a) The first incubation time was kept constant at 3h and the time was varied for the second stage; ●, 5ng of nerve growth factor; ○, blank (no nerve growth factor). (b) The second incubation period was constant at 3h and the time of the first stage was varied; ●, 5ng of nerve growth factor; ○, blank. Values are means of quadruplicate determinations.

Fig. 2. Standard curves after two different incubation periods

(a) The incubation was for 3h in each stage of the reaction; (b) incubation was for 16h in the first stage of the reaction and 6h in the second stage. Amount of the β -subunit added is plotted against ^{125}I radioactivity (c.p.m.) bound on the papers. Values are means of quadruplicate determinations; S.E.M. were generally less than $\pm 5\%$ of the means.

scintillation vials containing 10ml of 0.4% 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (CIBA Ltd., Duxford, Cambs., U.K.) in toluene. The radioactivity was counted in a Packard model 3300 scintillation counter at an efficiency of approx. 50%.

Parameters affecting the assay. (a) Time of incubation. The times chosen for the two stages of the assay can be altered, depending on the sensitivity required. In the first stage of the incubation a long time is needed for the complete binding of free nerve

Table 1. Concentrations of the β -subunit of nerve growth factor in various organs of adult mouse

The results are expressed as the mean values $\pm$ S.E.M. for five animals in each group, except for the submaxillary gland, which is the mean value found for eight animals. The assay was performed by using a 16h incubation for the first stage and a 6h incubation in the second stage (see 'Radioimmunoassay procedure').

Tissue	Nerve growth factor β -subunit (ng/mg wet wt.)	
	Male	Female
Submaxillary gland	404.00 $\pm$ 50.300	51.00 $\pm$ 3.300
Diaphragm	0.41 $\pm$ 0.056	0.42 $\pm$ 0.049
Uterus	—	0.27 $\pm$ 0.011
Vas deferens	0.36 $\pm$ 0.041	—
Adrenal	0.25 $\pm$ 0.023	0.37 $\pm$ 0.065
Heart	0.28 $\pm$ 0.042	0.26 $\pm$ 0.061
Kidney	0.17 $\pm$ 0.023	0.19 $\pm$ 0.014
Spleen	0.18 $\pm$ 0.012	0.16 $\pm$ 0.018
Liver	0.10 $\pm$ 0.019	0.12 $\pm$ 0.016
Spinal cord	0.12 $\pm$ 0.009	0.13 $\pm$ 0.011
Brain	0.09 $\pm$ 0.021	0.08 $\pm$ 0.015

growth factor to the immunoabsorbent paper (Fig. 1b). Hence the most sensitive assays require an incubation period of 16–24h; if the incubation time is shortened the range of the assay is extended but the sensitivity is decreased.

In the second stage of the assay, longer incubation times lead to an increase in the total amount of labelled antibody bound (Fig. 1a). Here an optimum sample/blank ratio was obtained with incubation for 6h. Typical standard curves for short and long incubation times are shown in Fig. 2.

(b) Concentration of ^{125}I -labelled antibodies. There was a linear relationship between the amount of labelled antibody added and the amount bound to the paper for amounts of antibody between 2000 and 1320000 d.p.m. indicating that the system was not saturable in this range. It was found convenient to use 40000 d.p.m. of ^{125}I -labelled antibody in each incubation tube, to give high enough radioactivity for accurate counting.

(c) Antibody type. There is a marked difference in these parameters depending on the type of antiserum used. In earlier work (Hendry *et al.*, 1972), where we used a rabbit antiserum, much longer incubation times were required for saturation of the paper. In the present work the majority of results were obtained with horse antiserum, and much shorter incubation periods were required for saturation in the two stages of the assay. This probably reflects the differences in affinity of different antisera.

Application of assay to 7S species of nerve growth factor. The immunoassay was found to be equally effective in measuring the β -subunit whether the protein was present as the free subunit, or in combination with α - and γ -subunits in the 7S complex. Assays of the 7S species of nerve growth factor gave results that were in close agreement with parallel measurements of the biological activity of such preparations (Hendry *et al.*, 1972). This would suggest that the antigenic sites on the β -subunit molecule are not masked by combination with the other subunits in the 7S aggregate. There is, further, no evidence that the α - and γ -subunits have any immunological cross-reactivity in this assay.

Preparation of tissue extracts. Tissues were homogenized in 10vol. of a modified 'veronal buffer' containing 1% bovine serum albumin, and dilutions were also made in this buffer. As a routine samples were centrifuged for 10min at 2000g to remove cell debris before the first stage of the assay. This step, however, made no difference to the amount of nerve growth factor measured, but the washing of the papers

was made easier. Some samples from each type of tissue were incubated with added internal standards of the β -subunit and the values thus obtained were always as predicted from the standard curves. Plasma samples were diluted with 3vol. of 'veronal buffer'.

Tissue concentrations of the β -subunit

Adult mouse tissues. A range of adult mouse tissues was examined and these results are given in Table 1. In agreement with other reports, the highest concentration of the β -subunit was found in adult male submaxillary glands and the lowest concentration was in the brain. In adult animals there was a sevenfold difference in submaxillary gland concentration of the β -subunit between males and females. The only other tissue that showed a significant male-to-male difference was the superior cervical ganglion (Fig. 4); however, the concentration of nerve growth factor in plasma also showed a sex difference in adult animals (Fig. 3). All other tissues had low concentration of the

Fig. 3. Changes in the content of the β -subunit of nerve growth factor in mouse plasma with age

Values are means $\pm$ S.E.M. for four to six determinations; for animals less than 8 days old plasma obtained was pooled to give 250 μ l of plasma for each determination. $\circ$, Male animals; $\bullet$, female animals; $\bullet$, pooled plasma from male and female animals.

Fig. 4. Changes in the content of the β -subunit of nerve growth factor in mouse superior cervical ganglion with age. Values are means $\pm$ S.E.M. for six determinations; four ganglia were pooled for each determination. ○, Male animals; ●, female animals; ●, pooled ganglia from male and female animals.

Table 2. Changes with age of content of the β -subunit of nerve growth factor in mouse submaxillary gland

Results are expressed as mean values $\pm$ S.E.M. for six to eight animals. Assays were performed under the incubation conditions described in Table 1 and with suitable dilutions in 'veronal buffer' containing 1% bovine serum albumin.

Age (days)	Nerve growth factor β -subunit (ng/mg)		
	Male	Both sexes	Female
5		1.0 $\pm$ 0.14	
10		0.4 $\pm$ 0.10	
21	4.8 $\pm$ 0.35		2.2 $\pm$ 0.17
28	81.6 $\pm$ 17.20		10.8 $\pm$ 0.88
60+	404.0 $\pm$ 50.30		51.0 $\pm$ 3.30

β -subunit and did not show any significant sex difference.

Changes in the submaxillary-gland content of the β -subunit during development. The changes of concentration of β -subunit in the submaxillary glands (Table 2) agree well with previously reported work (Cohen, 1960) where biological activity was measured. There was very low activity in neonatal animals and no sex difference was detected during the first 2 weeks after birth, but after 3 weeks the sex difference becomes significant. Other work has shown that the post-pubertal changes in concentration of nerve growth factor in the submaxillary gland are related

to the concentrations of circulating testosterone (M. Lyon, I. A. Hendry & R. Short, unpublished work).

Changes in the plasma concentrations of the β -subunit during development. There was a decrease in concentration of β -subunit in the plasma during the first 2 weeks of life, followed by a progressive increase to adult values (Fig. 3). The plasma concentrations at 3 weeks of age and subsequently parallel the activity found in the submaxillary glands, with a marked sex difference. Thus plasma concentrations of the β -subunit appear to be related to the concentrations in the submaxillary gland.

Changes in the sympathetic-ganglia content of the β -subunit during development. There was no significant difference in the total β -subunit content of superior cervical sympathetic ganglia from animals of up to 3 weeks of age. After this time there was a slight increase in the content of the ganglia from male animals but no change in ganglia from females (Fig. 4). However, there was a fall in relative content of the β -subunit during development, owing to the increasing size and protein content of the ganglia (Black *et al.*, 1971) during the first 3 weeks of age. These changes in the β -subunit content of the ganglia therefore appear to follow the changing plasma concentrations of the β -subunit in the developing animal.

Discussion

The assay described here has several advantages over previous methods for measuring the activity of

nerve growth factor. It is as sensitive as any previous assay, with a lower limit for accurate measurement of only 250pg of the β -subunit; but if high sensitivity is not needed it may be performed much more rapidly than any other method, requiring only 6h for the complete assay procedure. The present assay is specific for the smallest known biologically active species of nerve growth factor, and because two antigenic sites are required it is more selective than other immunological assays. There is thus less chance of cross-reaction with other proteins or inactive fragments of the parent protein moiety. This may account for some of the discrepancies between these findings and other recently published results (Johnson *et al.*, 1971). The latter authors, who used a conventional radioimmunoassay, found only a tenfold increase from birth to adulthood in the concentration of the 7S species of the nerve growth factor of the mouse submaxillary gland. On the other hand I find approximately a 1000-fold increase in the β -subunit concentration in this gland during development. This compares favourably with the increase in the concentrations of nerve growth factor (as measured by its biological activity) of mouse submaxillary gland during development, reported by Cohen (1960). He found an increase of approximately 6000-fold in the biological activity of the factor in this gland during the same period. Thus the two-site assay for β -subunit would appear to yield results closer to the assays of the biological activity than does the immunoassay against the 7S species of nerve growth factor. However, some of the values obtained by my method from adult tissues are similar to those reported by Johnson *et al.* (1971). For example in submaxillary glands of male mice aged 4 weeks I find 40.8 B.U./mg and Johnson *et al.* (1971) reported 39.3 B.U./mg. In females of this age, however, I find 5.4 B.U./mg and Johnson *et al.* (1971) report 16.1 B.U./mg. My assay method thus gives a greater difference in content of the nerve growth factor in salivary gland when comparing immature with adult, or male with female, tissues. This could be due to the presence of a greater proportion of the inactive α - and γ -subunits in immature or adult female tissue; alternatively these differences might be related to differences in the strains of animals used in the two studies.

In other tissues from adult mice the results presented here are in general agreement with those of Johnson *et al.* (1971). I have found plasma concentrations of the β -subunit, however, that are 10–50 times lower than those reported by Johnson *et al.* (1971), and have also observed a definite male-to-female difference in the plasma concentration of this protein. This result may be obscured when an assay of the 7S species of nerve growth factor is used, owing to the possible presence of an excess of the inactive α - and γ -subunits of this complex in plasma.

The low plasma concentrations of nerve growth

factor that have been observed rule out the possibility that its content in mouse tissues can be attributed to contamination of the tissue samples with plasma. The changes in plasma concentrations of the factor after puberty appear to parallel those occurring in the submaxillary gland, suggesting that plasma nerve growth factor may have its origin in the latter tissue. In neonatal animals, however, relatively high plasma concentrations of nerve growth factor are present at a time when the submaxillary glands contain only very little of the protein. Since the concentration of the factor in neonatal plasma is similar to that of adult females, it is possible that in the neonatal animals it may be predominantly of maternal origin.

Since nerve growth factor has potent biological effects on the development of the sympathetic nervous system, the results obtained from assay of sympathetic ganglia are particularly noteworthy. Because of the small size of the superior cervical ganglia, it was not possible to weigh the fresh tissue accurately. The results obtained, however, indicate tissue concentrations of the β -subunit of approx. 1–2ng/mg, which is considerably higher than in most other tissues examined, and approximately 50 times higher than plasma concentrations. There was, further, a significant difference between the content of nerve growth factor of adult male and female ganglia, which paralleled the sex difference in concentration of plasma nerve growth factor. No sex-related differences in the content of nerve growth factor of adult tissues were found in organs other than the salivary gland and sympathetic ganglia (Table 1), suggesting that sympathetic ganglion may selectively take up and bind circulating factor. The relatively high content of the factor in sympathetic ganglia in neonatal animals would be consistent with this hypothesis, and with the suggestion that this protein plays an important role in the normal maturation of sympathetic neurons.

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References

- Addison, G. M. & Hales, C. N. (1971) *Horm. Metab. Res.* **3**, 59–60
- Angeletti, P. & Vignetti, E. (1971) *Brain Res.* **33**, 601–604
- Angeletti, R. H., Bradshaw, R. A. & Wade, R. C. (1971) *Biochemistry* **10**, 463–469
- Black, I. B., Hendry, I. A. & Iversen, L. L. (1971) *Brain Res.* **34**, 229–240

- Boccacini, V. & Angeletti, P. U. (1969) *Proc. Nat. Acad. Sci. U.S.* **64**, 787–794
- Cohen, S. (1959) *J. Biol. Chem.* **234**, 1129–1137
- Cohen, S. (1960) *Proc. Nat. Acad. Sci. U.S.* **46**, 302–311
- Fenton, E. C. (1970) *Exp. Cell Res.* **59**, 383–392
- Gervich, A. E., Kuzovleva, O. B. & Tumanova, A. E. (1961) *Biokhimiya* **26**, 803
- Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- Hendry, I. A., Addison, G. M. & Iversen, L. L. (1972) *Nerve Growth Factor and its Antiserum* (Zaimis, E., ed.), pp. 262–270, Athlone Press
- Johnson, P. G., Gordon, P. & Kopin, I. J. (1971) *J. Neurochem.* **18**, 2355–2362
- Levi-Montalcini, R., Meyer, H. & Hamberger, V. (1954) *Cancer Res.* **14**, 49–56
- Miles, L. E. M. & Hales, C. N. (1968) *Biochem. J.* **108**, 611–618
- Varon, S., Noumura, J. & Shooter, E. C. (1967) *Biochemistry* **6**, 2202–2209
- Varon, S., Noumura, J. & Shooter, E. C. (1968) *Biochemistry* **7**, 1296–1303
- Zanini, A. & Angeletti, P. U. (1971) *Biochim. Biophys. Acta* **229**, 724–729