Proc. Natl. Acad. Sci. USA
Vol. 75, No. 8, pp. 4042–4046, August 1978
Neurobiology

Nerve growth factor in mouse and rat serum: Correlation between bioassay and radioimmunoassay determinations

(immunoglobulins/affinity chromatography/serum proteins)

K. SUDA, Y. A. BARDE, AND H. THOENEN

Department of Pharmacology, Biocenter of the University, Basel, Switzerland

Communicated by Francis O. Schmitt, May 22, 1978

ABSTRACT **High levels of nerve growth factor (NGF) determined by competition radioimmunoassay do not agree with values obtained by bioassay. This discrepancy is illustrated here with rat and mouse serum as examples in which values up to 1000 ng/ml have been found by using competition radioimmunoassays. An explanation for the discordant results is presented: serum components bind NGF with an intermediate affinity ($K_d = 10^{-7}$ M) but with a very large capacity (up to 0.5 mg of NGF per ml of rat serum). The binding of ^{125}I-labeled NGF to serum components competes with the binding to the solid-phase antibodies ($K_d = 10^{-9}$ M) present in limiting amounts, according to the principle of competition radioimmunoassays. Thus, less radioactivity is recovered bound to the antibodies and this gives the erroneous impression that NGF is present. To overcome this difficulty, a two-site radioimmunoassay has been developed which utilizes nonlimiting numbers of antibody binding sites. This assay provides a reliable determination of NGF levels in serum and it can be shown that in rat and mouse serum (either sex) there is less than 5 ng of NGF per ml, in agreement with the results of the bioassay.**

Nerve growth factor (NGF) is a protein that supports the growth and maintenance of peripheral sympathetic neurons and the development of some sensory neurons during a brief period of development (1). The highest concentration of NGF occurs in the submaxillary gland of the adult male mouse, from which it has been isolated, characterized, and sequenced (2, 3). There is strong evidence that NGF is present in other mammals as well. When antibodies raised against purified mouse NGF are injected into newborn rats, kittens, or rabbits, there is an almost complete destruction of the peripheral sympathetic nervous system, comparable to that occurring in mice (4). However, it is not known if there is a preferential accumulation of NGF in certain tissues, particularly in target organs of the sympathetic neurons.

In order to measure NGF levels in these tissues conveniently, radioimmunoassays (RIA) have been developed. With these assays, apparently high levels of NGF have been detected in virtually every sample tested (5, 6). However, the interpretation of the results of these studies is difficult. First, the range of the reported values is impressively large: for instance, in mouse serum values from 2 to more than 1000 ng/ml have been reported (5, 6). Second, the use of impure antisera might have led, in some cases, to erroneous results. The presence of antibodies directed against mouse gamma globulins in commercially available anti-NGF horse antisera has been reported recently (7). Third, and most disturbing, the values obtained for mammalian samples by RIA cannot be confirmed by bioassay (7). The NGF bioassay was introduced more than 20 years ago (8) and, although it is a semiquantitative method, it is sensitive enough to detect the amounts of NGF predicted by RIA with a reasonable accuracy.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

This discrepancy between the results obtained for NGF determinations by RIA and by bioassay is the subject of the present paper. We report how the discrepancy can be explained and also how it can be resolved by a two-site RIA using purified immunoglobulins.

MATERIALS AND METHODS

Serum Samples. Swiss albino mice and Sprague–Dawley rats (Süddeutsche Versuchstierfarm, Tuttlingen, Germany) were anesthetized with ether and bled by heart puncture. The blood was allowed to clot overnight at 4° and the serum was obtained after two 20-min centrifugations at 20,000 × *g*.

Preparation of NGF and ^{125}I-Labeled NGF (^{125}I-NGF). NGF was isolated from adult male mouse submaxillary gland as described by Bocchini and Angeletti (9), with the exception that the Sephadex G-100 step was replaced by a 62% (wt/vol) ammonium sulfate precipitation. The purity of that preparation was >90% as judged by sodium dodecyl sulfate/15% polyacrylamide gel electrophoresis. The biological activity was 5–10 ng per biological unit by the plasma clot assay (see below). NGF was labeled with Na^{125}I by the chloramine-T method (10) with a minor modification (11). Its specific activity was 100 mCi/mg of protein and it was stored at 4° in 0.02 M sodium phosphate buffer, pH 7.6/1% (wt/vol) bovine serum albumin (Sigma Chemical Co.)/0.02% (wt/vol) sodium azide/0.15 M NaCl (1% albumin buffer).

NGF Antiserum and Purification of Immunoglobulins. The antiserum was prepared by injecting an adult sheep intradermally with 1 mg of NGF in 1 ml of distilled water mixed with an equal volume of Freund's complete adjuvant. A second injection (with 1 mg NGF in 1 ml of distilled water and 1 ml of Freund's incomplete adjuvant) was given 4 weeks later and the animal was bled 2 weeks after that. The serum was lyophilized and kept at 4° until used. Anti-NGF immunoglobulins were purified by passing the reconstituted serum over a NGF-Sepharose 4B affinity column as described by Stöckel *et al.* (12). Purified anti-NGF antibodies, eluted with 4.5 M $MgCl_2$, were dialyzed against 50 mM Tris·HCl, pH 7.2 (at 20°)/150 mM NaCl (Tris·HCl buffer) and stored in 45% saturated ammonium sulfate. Portions were dialyzed against Tris·HCl buffer, filtered (Millipore filter, 0.22 μm), and kept at 4° until used. Immunoglobulins were iodinated by the procedure described above to give a specific activity of 5–10 mCi/mg. They were stored at 4° in 1% albumin buffer.

One-Site RIA. Both one-site RIA and two-site RIA were based on the technique introduced by Catt and Tregear (13), which makes use of antibody adsorption to polystyrene tubes. The lyophilized antiserum was dissolved in double-distilled water (80 mg/ml) and diluted 1:1000 with 0.02 M sodium bi-

Abbreviations: NGF, nerve growth factor; ^{125}I-NGF, ^{125}I-labeled NGF; RIA, radioimmunoassay.

carbonate buffer (pH 9.6). The diluted antiserum (0.2 ml) was placed in polystyrene tubes (5 × 50 mm) and incubated overnight at room temperature. After two washes (0.5 ml each) with 1% albumin buffer, 0.1 ml of 1% albumin buffer containing 10,000 cpm of ^{125}I-NGF and varying amounts (0–10 ng) of NGF plus 0.1 ml of fetal calf serum (Gibco) or 0.1 ml of the sample to be determined (rat or mouse serum) were added to each tube and incubated for 3 hr at room temperature on a rotatory shaker. At the end of this period, the tubes were washed three times (0.5 ml) with 1% albumin buffer and assayed for radioactivity in a Packard γ counter with an efficiency of 65%. A standard curve obtained under these conditions is shown in Fig. 1.

Two-Site RIA. One microgram of purified anti-NGF immunoglobulin was diluted in 0.2 ml of 0.05 M Tris·HCl, pH 8.5 (at 20°), and placed in the bottom of each of a set of polystyrene tubes (10 × 55 mm). The tubes were incubated overnight at room temperature, washed three times with 1.5 ml of 50 mM Tris·HCl, pH 8.0 (at 20°)/75 mM NaCl/0.02% (wt/vol) sodium azide and 0.1% bovine serum albumin (0.1% albumin buffer). Each tube received 80 μl of 0.1% albumin buffer containing varying amounts of NGF (0–10 ng) and 20 μl of either culture medium [Dulbecco's modified Eagle's medium (Gibco)] containing 70 g of bovine serum albumin per liter or sample (rat or mouse serum). After 48-hr incubation at room temperature, the tubes were washed three times (1.5 ml) with 0.1% albumin buffer and reincubated at room temperature for 72 hr with ^{125}I-labeled anti-NGF immunoglobulins diluted in 0.1% albumin buffer (150,000–200,000 cpm per tube, 100 μl per tube). At the end of the second incubation, the tubes were washed three times (1.5 ml) with 0.1% albumin buffer and assayed for radioactivity. Standard curves are illustrated in Fig. 2. Reliable NGF determinations can be made between 1 and 100 ng/ml (38 pM–3.8 nM).

Long incubations at room temperature were found necessary to obtain sufficient sensitivity. The tubes were not placed on a shaker, so only a very small part of the polystyrene surface had to be coated with specific immunoglobulins. It was also found necessary to dilute the samples 1:4. Undiluted sera occasionally gave blank values significantly lower than those obtained in control buffer.

Binding of NGF to Serum Components. Two-microliter samples of rat serum diluted in 1 ml of 20 mM sodium phosphate, pH 7.6/0.5% (wt/vol) bovine serum albumin/1 M NaCl/0.1% (wt/vol) NaN_3 were incubated with various concentrations of ^{125}I-NGF for 15 hr at room temperature. Separation of bound NGF from free NGF was performed by rapidly passing 10 μl of the mixture over small Sepharose 6-B columns (bed volume, 0.6 ml in 1-ml disposable polypropylene syringes, 0.47 × 3.5 cm; flow rate, 18 ml/hr). The relative distribution of bound ^{125}I-NGF and free ^{125}I-NGF was estimated from the elution profile.

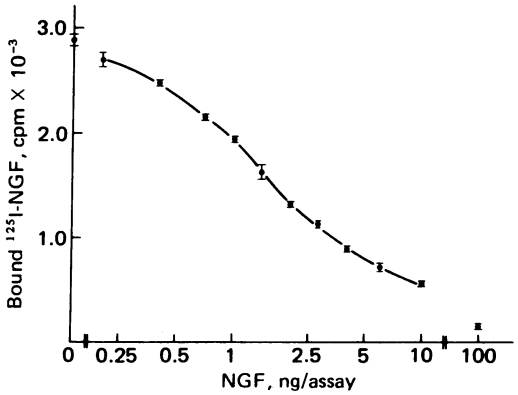

FIG. 1. One-site RIA standard curve. Polystyrene tubes were coated with diluted (1:1000) NGF antiserum, washed, and incubated with ^{125}I-NGF (10,000 cpm) and varying amounts of unlabeled NGF in a volume of 200 μl. After final washing, the tubes were assayed for radioactivity for 2 min in a gamma counter. Each point is the mean (±SD) of three determinations. The curve is fitted by eye.

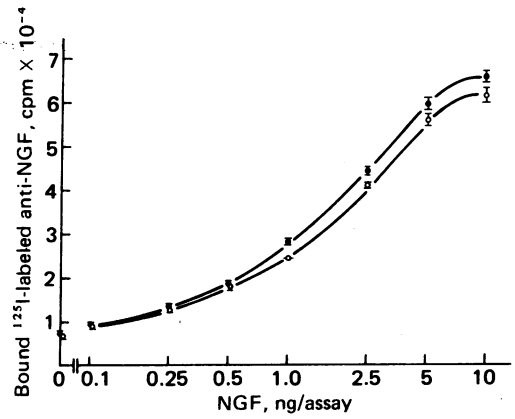

FIG. 2. Two-site RIA standard curve. Polystyrene tubes were coated with 1 μg of purified anti-NGF immunoglobulins, washed, incubated with varying amounts of unlabeled NGF and 20 μl of medium (●) or rat serum (○) in a volume of 100 μl, washed and reincubated with ^{125}I-labeled anti-NGF immunoglobulins (180,000 cpm) in a volume of 100 μl. After final washing, the tubes were assayed for 1 min in a gamma counter. Each point is the mean (±SD) of three determinations. The curves are fitted by eye.

NGF Bioassay. Bioassays were performed by using the plasma clot method (8, 14). Three or four dorsal root ganglia of 10-day-old chicken embryos were placed in microwell tissue culture plates (Falcon). Each well contained 75 μl of culture medium supplemented with 10% (vol/vol) fetal calf serum to which varying amounts of NGF were added (0–2 ng) and 25 μl of freshly thawed hen plasma. When samples were tested, they were substituted, undiluted, for the 75 μl of supplemented culture medium. After 20-hr incubation at 37° with 5% CO_2 in air in a humidified incubator (Assab), fiber outgrowth was judged according to the usual criteria (14).

RESULTS

NGF Determinations by One-Site RIA and Bioassay. Table 1 shows the results obtained by one-site RIA for mouse and rat serum. These values are in agreement with those reported by other investigators using one-site RIA (15). Some have found much higher levels, particularly for male mouse serum (5).

It can be seen that all the values were >5 ng/ml, a concentration of NGF that would be expected to give a strong response in the bioassay. It was therefore of interest to compare the biological response with the results of the RIA.

At a concentration of 2 ng/ml, NGF elicited a strong fiber

Table 1. Serum NGF as determined by one-site RIA

	NGF, ng/ml	
	Male	Female
Adult rat (>150 g)	38.6 ± 3.5	39.6 ± 9.5
Adult mouse (>25 g)	12.9 ± 1.7	8.9 ± 1.3

The values were read on a linearized standard curve obtained by a computed logit-logarithm transformation. They represent the mean ±SD from four different animals in each case.

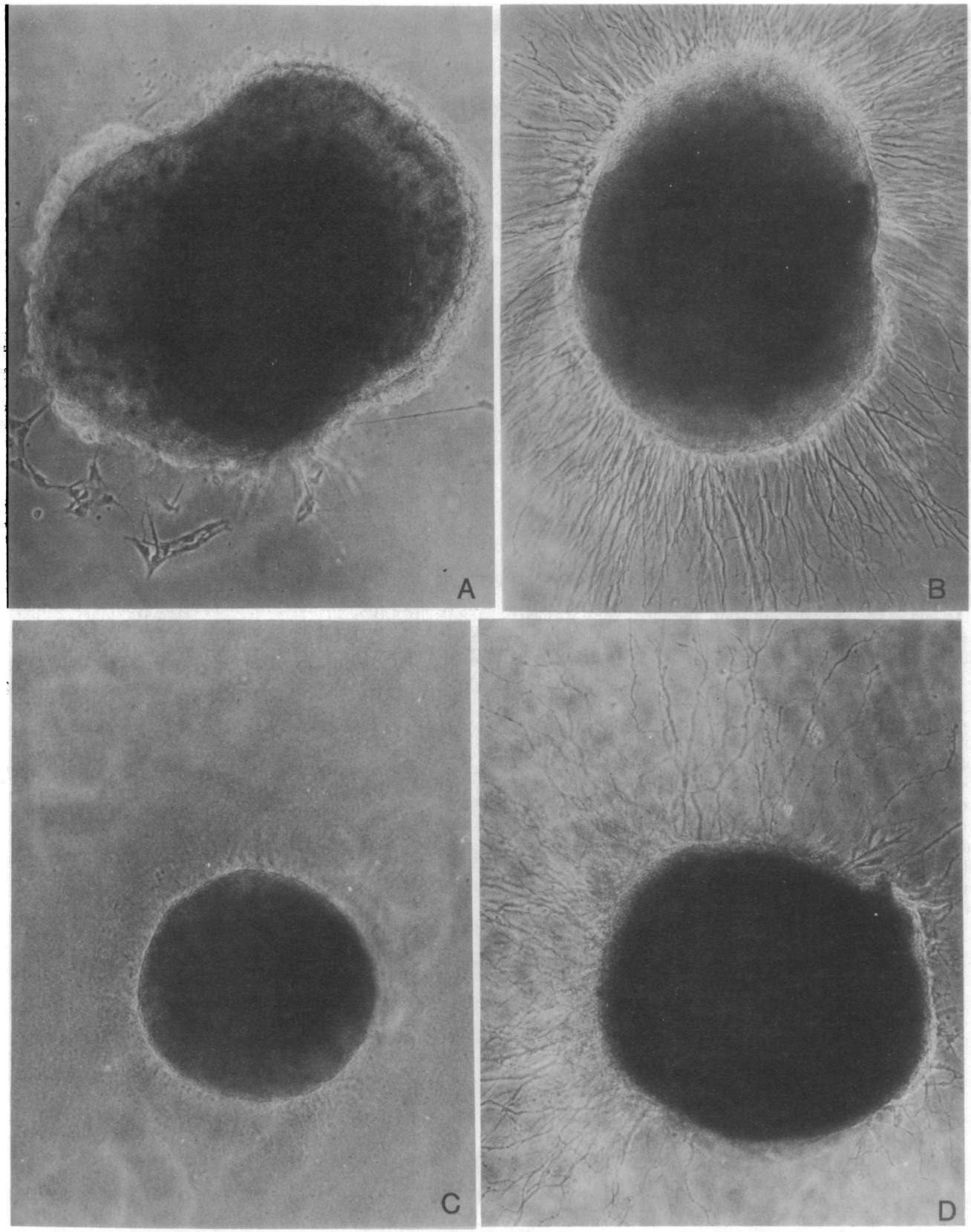

FIG. 3. NGF bioassay. (*A*) Chicken dorsal root ganglion grown for 20 hr in control medium without NGF. (*B*) Effect of NGF at 2 ng/ml added to the control medium. (*C*) Ganglion incubated in the presence of rat serum (substituted for the control medium). The NGF concentration predicted by one-site RIA was 30 ng/ml. (*D*) Same as *C*, with NGF (2 ng/ml) added to the rat serum sample.

outgrowth (Fig. 3*B*). In contrast, a rat serum sample with a NGF concentration of 30 ng/ml according to the one-site RIA did not give any detectable response (Fig. 3*C*). However, when NGF was added to this sample at 2 ng/ml, a distinct fiber outgrowth became apparent (Fig. 3*D*). This response was somewhat less than that of the control (Fig. 3*B*) and also was partially obscured by the cloudiness of the serum (used undiluted). It nevertheless was a positive response and could clearly be distinguished from the absence of fiber outgrowth with rat serum alone (Fig. 3*C*). Similar results were obtained with mouse sera from either sex. Ten different sera from both species were tested.

Determination of NGF Affinity for Serum Proteins and Anti-NGF Antibodies. In a competition RIA, any decrease in radioactivity bound to the solid-phase antibodies is interpreted as evidence of the presence of antigen—i.e., NGF. Hence, if serum components bound ^{125}I-NGF and prevented its binding to the solid-phase antibodies, the result would be an overestimation of NGF in serum. We therefore tested whether NGF binding components are present in sera. In Fig. 4, the elution patterns of ^{125}I-NGF in the presence of either 2 mg of unlabeled NGF or rat serum are compared. A considerable shift occurred, suggesting that NGF indeed binds to large molecules in serum. The respective affinities of ^{125}I-NGF for the serum components and the antibodies were therefore measured. Fig. 5 shows an estimation of the affinity for the rat serum proteins. From the Scatchard plot (16), a dissociation constant of approximately 10^{-7} M (2.65 μg/ml) and a remarkably high capacity (0.5 mg of NGF per ml of serum) can be estimated.

The affinity of the antibodies was more difficult to assess because of the multivalency of both the antigen and the antibody and the heterogeneity of the antibody population. An attempt was made to determine the overall affinity by using the data of the two-site RIA (see below) and assuming that, in the second phase of the assay, one molecule of antibody binds to one molecule of antigen. Fig. 6 shows a linear transformation of Fig. 2. An overall dissociation constant of approximately 10^{-9} M can be obtained.

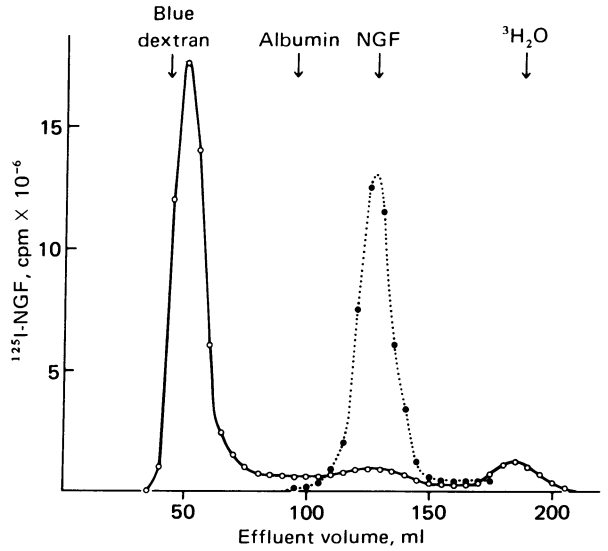

FIG. 4. Gel filtration pattern of ^{125}I-NGF in the absence (●···●) or presence (○—○) of rat serum. A Sephadex G-150 column (1.5 × 90 cm) was equilibrated with 0.1 M Tris·HCl, pH 8.0 (at 20°)/1 M NaCl and calibrated with blue dextran, bovine serum albumin, and ^3H_2O (arrows). The dotted line represents the elution pattern of ^{125}I-NGF in the presence of 2 mg of unlabeled NGF; the solid line, that of ^{125}I-NGF in the presence of 4 ml of rat serum.

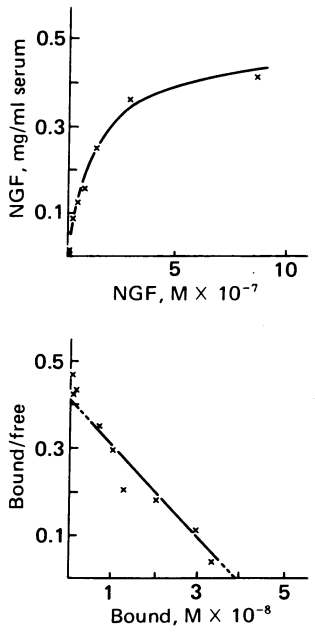

FIG. 5. Binding of ^{125}I-NGF to rat serum. Bound NGF was separated from free NGF by gel filtration. The Scatchard plot (*Lower*) shows, for the linear part of the graph, a K_d of 10^{-7} M and a maximum binding capacity of 0.5 mg of NGF per ml of rat serum (least squares analysis, $r^2 = 0.949$).

Two-Site RIA. Because, in one-site RIAs, the amount of specific binding sites is necessarily limiting, we have adopted a two-site RIA (6). Polystyrene tubes were first coated with 1 μg of purified immunoglobulins. Complete immune serum cannot be used for this purpose because the capacity of the polystyrene tubes for the binding of proteins is limited and a high capacity of solid-phase antibodies can be achieved only if all the adsorbed proteins are the specific antibodies. By using this assay, it was possible to measure NGF even in the presence of the large amounts of binding components in rat serum (Fig. 2, open circles). Although the NGF recovery was not complete (at least at high concentrations), it is clear that low concentrations of NGF can be detected. When adult rat and mouse sera were tested (male and female, six determinations for each case), no NGF was detected (lowest level of reliable detection, 5 ng/ml).

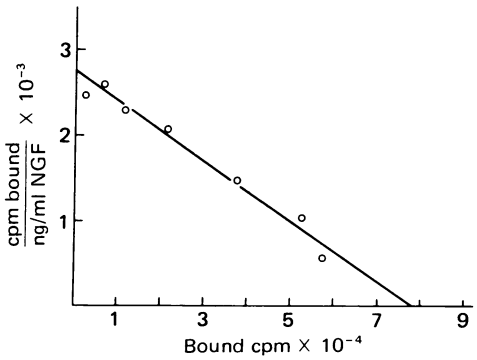

FIG. 6. Affinity of the antibodies for NGF. This graph is a linear transformation of the two-site RIA standard curve shown in Fig. 2 (filled circles) with the blank value subtracted. The straight line was obtained by the least squares analysis ($r^2 = 0.976$). By assuming a 1:1 relationship between NGF and antibody, a K_d of 10^{-9} M is obtained.

As a control to show that with this method NGF can be detected where its presence has been well established, a pair of adult male mouse submaxillary glands was homogenized in water (320 mg in 3.2 ml) and diluted 1:1600 in culture medium containing 1% (wt/vol) bovine serum albumin. A value of 80 μg per pair of salivary glands was obtained, which is in good agreement with the values obtained after purification and isolation of NGF from the same material (17).

DISCUSSION

The results of this study demonstrate that a RIA with solid-phase antibodies and competition of sample with radiolabeled NGF give values for rat and mouse serum that cannot be confirmed either by bioassay or by two-site RIA. From the two-site RIA it can be concluded that the concentration of NGF in mouse or rat serum is <5 ng/ml and from the bioassay, <2 ng/ml. It could be argued that the discrepancy between one-site RIA and bioassay is due to the presence of an inhibitor in the serum that prevents the immunologically crossreacting molecule to be active in the bioassay. This assumption is unlikely because low concentrations of NGF added to the inactive serum sample do produce fiber outgrowth and the two-site RIA does not detect the high levels predicted by the one-site RIA. Our explanation for this discrepancy is that serum components bind ^{125}I-NGF with a reasonably high affinity ($K_d = 10^{-7}$ M) and a very high capacity (0.5 mg of NGF per ml of rat serum). Thus, the binding of ^{125}I-NGF to solid-phase antibodies is impaired by competitive binding to serum components. Because the NGF standard curve is not made under the same conditions (fetal calf serum binds only small amounts of ^{125}I-NGF, according to gel filtration experiments, as illustrated in Fig. 4) less radioactivity is recovered in the sample tested. This is usually interpreted as evidence that NGF is present.

The binding of NGF to serum components is already well documented in the literature. When rabbit serum and unlabeled NGF are mixed and subjected to gel filtration, the bioactivity elutes at a higher molecular weight (18). The existence of this serum component introduces a complication in any NGF assay based upon the competition for a limited number of specific binding sites. This situation is not unique for NGF and can be compared with the determinations of cardiac glycoside concentrations by competition assay in the presence of serum albumin, to which they bind with low affinity but very high capacity. A general discussion of such problems can be found in ref. 19.

So far it has not been possible to identify the components responsible for the binding of NGF. There is probably more than one: when the experiment illustrated in Fig. 4 was performed using Sepharose 4B, ^{125}I-NGF eluted in two peaks corresponding to molecular weights of 500,000 and 150,000. Attempts to purify the factor by procedures used for the preparation of gamma globulins from rat serum (ammonium sulfate precipitation and chromatography on DEAE-cellulose) gave preparations without any NGF-binding activity. It is possible that the interaction between binding proteins and NGF is hydrophobic, because detergents (1% Triton-X and 0.5% Na deoxycholate) reduced the shift illustrated in Fig. 4. It is of interest to note that, when a one-site RIA is performed in the presence of detergents, the apparent level of NGF in serum samples is decreased by more than 70% (data not shown).

The physiological relevance of NGF binding to serum proteins and whether or not bound NGF is inactivated are not known. No conclusion can be reached from the bioassay data. High-affinity binding sites (2×10^{-11} M as recently suggested*) coupled to a large number of receptors might be enough to displace NGF from the binding proteins, which do not bind NGF irreversibly. In this respect, the bioassay is comparable to the two-site RIA. In both cases, small amounts of NGF can be detected even in the presence of a large serum binding capacity and this results from the presence of a sufficiently large number of high-affinity binding sites. There is no *a priori* reason to suppose that NGF-binding proteins are present exclusively in rat and mouse serum. Therefore, it cannot be predicted how these results will influence data concerning NGF distribution in various tissues. It can only be stressed that the NGF bioassay and the blockade of possible effects by specific anti-NGF antibodies should be systematically used to check values predicted by RIA.

The necessity to correlate RIA values with biological measurements also was emphasized in a recent publication (7) in which it was shown that commercially available antisera raised against 7S NGF contain significant amounts of antibodies directed against mouse gamma globulins. This might explain why, when such an antiserum is used, NGF levels largely >10 ng/ml in male mouse serum have been found by using a two-site RIA (4). Our failure to confirm this result might be due to the use of purified immunoglobulins in both phases of the assay.

We thank Dr. J. Rosenbusch for careful reading of the manuscript. This work was supported by the Swiss National Foundation (Grant 3.432.74) and a grant from the Sandoz Foundation for the Advancement of Medical-Biological Sciences.

1. Levi-Montalcini, R. & Angeletti, P. U. (1968) *Physiol. Rev.* **48**, 534–569.
2. Hogue-Angeletti, R., Mercanti, D. & Bradshaw, R. A. (1973) *Biochemistry* **12**, 90–100.
3. Hogue-Angeletti, R., Hermodson, M. A. & Bradshaw, R. A. (1973) *Biochemistry* **12**, 100–115.
4. Levi-Montalcini, R. & Angeletti, P. U. (1966) *Pharm. Rev.* **18**, 619–628.
5. Johnson, D. G., Gorden, P. & Kopin, I. J. (1972) *J. Neurochem.* **18**, 2355–2362.
6. Hendry, I. A. (1972) *Biochem. J.* **128**, 1265–1272.
7. Carstairs, J. R., Edwards, D. C., Pearce, L. C., Vernon, C. A. & Walter, S. J. (1977) *Eur. J. Biochem.* **77**, 311–317.
8. Levi-Montalcini, R., Meyer, H. & Hamburger, V. (1954) *Cancer Res.* **14**, 49–57.
9. Bocchini, V. & Angeletti, P. U. (1969) *Proc. Natl. Acad. Sci. USA* **64**, 787–794.
10. Greenwood, F. C., Hunter, W. M. & Glever, J. S. (1963) *Biochem. J.* **89**, 114–123.
11. Stöckel, K., Paravicini, U. & Thoenen, H. (1974) *Brain Res.* **76**, 431–421.
12. Stöckel, K., Gagnon, C., Guroff, G. & Thoenen, H. (1976) *J. Neurochem.* **26**, 1207–1211.
13. Catt, K. J. & Tregear, G. W. (1967) *Science* **158**, 1570–1572.
14. Fenton, E. L. (1970) *Exp. Cell Res.* **59**, 383–392.
15. Murphy, R. A., Saide, D. J., Blanchard, M. H. & Young, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2330–2333.
16. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672.
17. Varon, S., Nomura, J. & Shooter, E. M. (1967) *Biochemistry* **6**, 2202–2209.
18. Hogue-Angeletti, R. (1969) *Brain Res.* **12**, 234–237.
19. Parker, C. W. (1976) in *Radioimmunoassay of Biologically Active Compounds*, eds. Oster, A. G. & Weiss, L. (Prentice-Hall, Englewood Cliffs, NJ), pp. 165–179.

* Sutter, A., Riopelle, R. J., Harris-Warrick, R. M. & Shooter, E. M. (1977) *Society for Neuroscience*, Abstracts, Vol. 3, pp. 461.