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Interaction of Nerve Growth Factor with the Mouse-Brain Neurotubule Protein(s)

(tubulin/divalent cations/colchicine/GTP)

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ABSTRACT Addition of nerve growth factor to a 105,000 × *g* supernatant of mouse brain induces the formation of a precipitate whose main constituent is the microtubule protein(s) (tubulin). The binding of nerve growth factor to purified tubulin is not inhibited by colchicine and does not appear to depend on the presence of GTP or Mg^{++}. GTP, however, and divalent cations, exert a marked effect on the increased turbidity induced by interaction of nerve growth factor with tubulin. These findings are tentatively interpreted with the hypothesis that binding of the factor to tubulin and the induced aggregation is a sequential two-step process; the latter but not the former would be influenced by GTP or divalent cations.

The most striking effect of nerve growth factor (NGF) is stimulation of rapid neurite outgrowth (1). This effect is limited to embryonic sensory nerve cells during a restricted period of their development and sympathetic nerve cells during all developmental stages (1, 2). One of the earliest effects elicited by NGF is massive production of neurofilaments and neurotubules, filling the cytoplasm. This response is detectable as early as 2 hr (3, 4) after the beginning of incubation with NGF. A quantitative analysis of this effect has been reported (5).

The neurotubules or microtubules are formed by the assembly of a precursor, dimeric protein (molecular weight 110,000) called microtubule protein or tubulin (6–8). The dimer appears to be composed of two related but not identical subunits (9) and is characterized by its unique ability to bind colchicine, so that it is also referred to as colchicine-binding protein (10). Tubulin is ubiquitous within the cell cytoplasm in a soluble and in a membrane-bound form (11, 12).

We report here on a specific, high-affinity binding of NGF to this protein. This finding appears to be pertinent to the mechanism of action of NGF as well as to its still unknown receptor.

MATERIALS AND METHODS

Purification of NGF. NGF was prepared by the method of Bocchini and Angeletti (13), and its molar concentration was based on the assumption of a molecular weight of 28,000 (14). ^{125}I-Labeled NGF, a generous gift of Dr. Roberto Revoltella, had a specific activity of 10^6 cpm/μg of protein when counted on a Wallac gamma counter (LKB) GTL 300-500 equipped with a NaI crystal. The labeled NGF retained full biological activity, as determined by its effect *in vitro* on chick-embryo sensory ganglia.

Abbreviations: NGF, nerve growth factor; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Purification of Tubulin. The microtubule protein was purified by the method of Shelanski *et al.* (8) from adult mouse brain with a minor modification. The composition of the reassembly buffer was 10 mM KH_2PO_4–Na_2HPO_4, pH 6.5, instead of 100 mM MES; 1 mM EGTA, 1 mM GTP, and 0.5 mM $MgCl_2$ were also present to form the reassembly buffer as described by Weisenberg (6). Tubulin was generally 80–90% pure, as judged by densitometric scanning after sodium dodecyl sulfate gel electrophoresis (see also Fig. 1*d*). For binding studies, aliquots of tubulin in 8 M glycerol were diluted to 4 M with reassembly buffer, incubated for 20 min at 37°, and centrifuged at 100,000 × *g* for 60 min. The pellet was resuspended in the reassembly buffer, except where otherwise stated, and kept at 2° for at least 30 min to ensure depolymerization. Before use for binding studies this preparation was centrifuged at 3000 rpm for 30 min.

^{125}I-Labeled NGF and [^{3}H]Colchicine Binding to Tubulin. [^{3}H]Colchicine binding was measured by the filter method (6), with an incubation at 37° for 30 min. ^{125}I-Labeled NGF binding was measured by incubating aliquots of purified tubulin (generally 100–150 μg) for 30 min at 37° in 0.25 ml of the reassembly buffer also containing 0.1 M NaCl and 2 mg/ml of a mixture of immunoglobulins (in order to reduce adsorbtion of the NGF to the test tube) plus constant amounts of ^{125}I-labeled NGF and various concentration of unlabeled NGF. After incubation, the tubes were mixed with 0.5 mM vinblastine, further incubated for 15 min, and then centrifuged for 8 min in a Beckman 152 microfuge at room temperature. The counts in the pellet were taken as a measure of the extent of protein bound. Vinblastine facilitates precipitation of the tubulin–NGF complex, thus avoiding the need of high-speed centrifugation without interfering with the binding, once the complex is formed. Controls without tubulin, at every NGF concentration, were run in the same experiment in order to subtract the nonspecific contribution due to spontaneous precipitation or adsorption of NGF to the test tube, which never exceeded 5–8% of the total NGF bound.

Light Scattering Measurements. The interaction between NGF and tubulin was followed by measurement of the light scattering of tubulin at 400 nm after addition of NGF. Generally, to a solution of tubulin (50–100 μg) in the reassembly buffer, aliquots of NGF were added in a final volume of 0.25 ml. The solution was rapidly stirred, and the increase in absorbancy (*A*) was monitored with an MQ III Zeiss spectrophotometer. The spontaneous aggregation or polymerization of tubulin (8, 15) in the absence of NGF that occurs in the reassembly buffer never exceeded 5–7% of that induced by

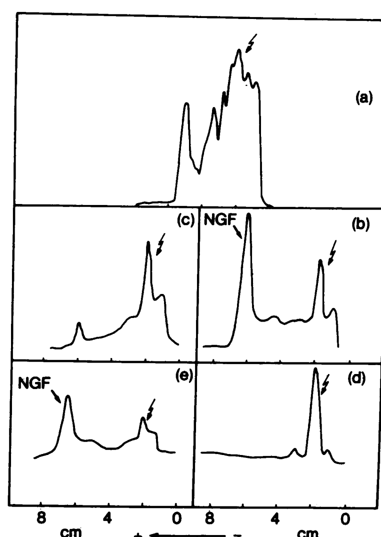

FIG. 1. Sodium dodecyl sulfate electrophoresis after NGF or vinblastine treatment of brain supernatants. Two mouse brains were homogenized with a 3:1 v/w of 10 mM MES (pH 6.5), 0.5 mM $MgCl_2$, 1.0 mM EGTA, and 1.0 mM GTP, and centrifuged at 105,000 × *g* for 90 min. The supernatant was divided into 0.25-ml aliquots (0.8 mg of total protein), and 0.04 ml of buffer containing 140 μg of NGF (*b*) or 0.03 ml of 10 mM vinblastine (*c*) was added. After standing for 30 min at 2°, the mixtures were centrifuged for 30 min at 105,000 × *g*. The pellets of NGF- and vinblastine-treated samples were resuspended in buffer and centrifuged again at 105,000 × *g* for 30 min. After centrifugation the sediments were dissolved in 0.1 ml of 1% sodium dodecyl sulfate–mercaptoethanol (see *Methods*). After centrifugation and removal of the pellet, 0.25 ml of the vinblastine-treated supernatant was dialyzed overnight and 140 μg of NGF was subsequently added. The sample (*e*) was then treated as described for *b* and *c*. (*a*) 105,000 × *g* total supernatant; (*b*) 0.025 ml of the pellet after NGF treatment; (*c*) 0.025 ml of the pellet after vinblastine precipitation; (*d*) 50 μg of purified tubulin (8); (*e*) 0.025 ml of the pellet after vinblastine treatment, dialysis of supernatant, addition of NGF, and recentrifugation. The *unlabeled arrows* indicate the tubulin peak.

the lowest amount of NGF tested. The contribution of this scattering was subtracted from the induced effect of NGF. Also the slight absorbancy of 0.1 mM colchicine (generally 0.06–0.08) was subtracted when this substance was present in the cuvette.

Sodium Dodecyl Sulfate Electrophoresis was performed by the method of Weber and Osborn (16). Gels were stained with Coomassie blue and densitometric tracings of the destained gels were made with a Joice-Loebl Chromoscan. Aminoacid analysis of the band corresponding to tubulin in the gels was performed as described (17).

Reagents. Bovine-serum albumin, cytochrome *c* from horse heart, beef hemoglobin, and ribonuclease A type III from bovine pancreas were from Sigma. The 14.3.2 protein was a generous gift of B. W. Moore and the S-100 protein was purified according to Moore (18). Vinblastine (Velbe) was a generous gift of Eli Lilly. [^{3}H]Colchicine (690 mCi/mmol) was a gift of Dr. Alfonso Grasso.

RESULTS

During a study on the interaction of NGF with the soluble proteins of brain we found that when NGF is added to a 105,000 × *g* supernatant of mouse brain, an almost instantaneous turbidity of the protein solution occurs. After standing for 30 min at 2°, the solution is centrifuged at 105,000 × *g* for 30 min and the resulting pellets, dissolved in the sodium dodecyl sulfate buffer for electrophoresis (see *Methods*), shows that NGF is precipitated mainly with one protein component among all proteins present (Fig. 1*a* and *b*). Thus, the band indicated by the arrow in Fig. 1*a*, which accounts for about 8–10% of the total proteins in the gel, is increased to 40–50% after precipitation with NGF, while most of the other proteins are left in the supernatant. This band has an apparent molecular weight of 52,000–53,000 when compared to standard proteins of known molecular weight (cytochrome *c*, chymotrypsinogen, bovine-serum albumin, and ovalbumin), and shows the same mobility of a purified preparation of tubulin (Fig. 1*d*) (see *Methods*). When the two bands (NGF-precipitated protein and purified tubulin) are cut out and analyzed, they show an overimposable aminoacid composition. The vinblastine-induced precipitate (Fig. 1*c*) of an identical 105,000 × *g* supernatant is very similar to that obtained with NGF. In addition, the concentration required by the alkaloid (1.0 mM) to enrich selectively the precipitate in the tubulin is much higher than the amount of NGF (15 μM) required to obtain an analogous precipitate. The similarity of the two effects is also indicated by the finding that when NGF is added to the supernatants previously treated with vinblastine, almost no precipitate is observed with the exception of a small peak migrating as tubulin (Fig. 1*e*). This peak probably represents the small portion of tubulin not precipitated by the vinblastine treatment.

These preliminary findings indicated that NGF interacts and precipitates mainly with the precursor proteins(s) of neurotubules among all the soluble proteins of brain. It remains to be established whether the other few compounds detectable in the NGF-induced precipitate (Fig. 1*b*) represent a group of proteins with the affinity and specific properties of interacting with NGF exhibited by tubulin and described in this paper.

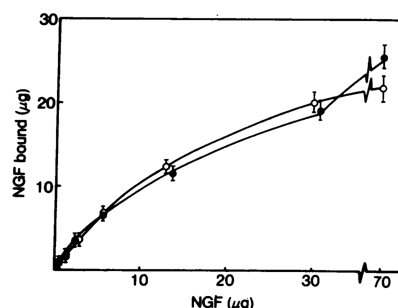

FIG. 2. Binding of NGF to tubulin in the presence and absence of colchicine. The assay was performed with 60 μg of purified tubulin plus various amounts of NGF (from 100 to 1.5 μg) and other substances (see *Methods*) in a final volume of 0.25 ml. At the end of incubation (30 min at 37°), complex was precipitated with 0.5 mM vinblastine. The concentrations of NGF on the *abscissa* refer to free NGF at the end of incubation, which is given by total NGF minus the fraction of the protein bound at the end of the experiment. (○) NGF alone; (●) plus 0.1 mM colchicine.

Tubulin from mouse brain was then purified and its interaction with NGF studied. Fig. 2 shows that NGF binds to this protein. At saturation there are, under these conditions, two moles of NGF bound (molecular weight 28,000) per mole

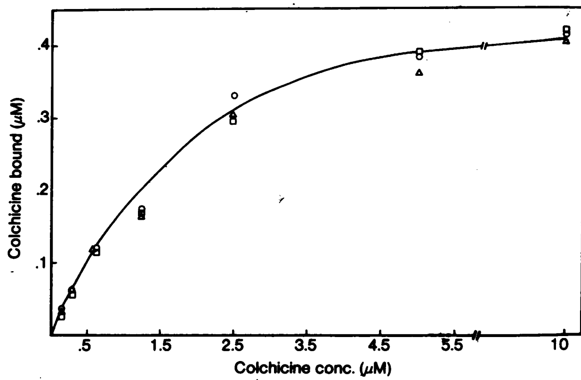

FIG. 3. Binding of colchicine in the presence and absence of NGF. Colchicine binding was performed by the filter method (6) with an incubation of 30 min at 37°. Each test tube contained, in a final volume of 1.0 ml, 200 μg of tubulin, 80 mM NaCl, colchicine ranging from 10 to 0.08 μM, plus constant amounts of [^{3}H]-colchicine (0.1 μCi). Colchicine alone (□); colchicine plus 20 μg (○) or 100 μg of NFG (Δ).

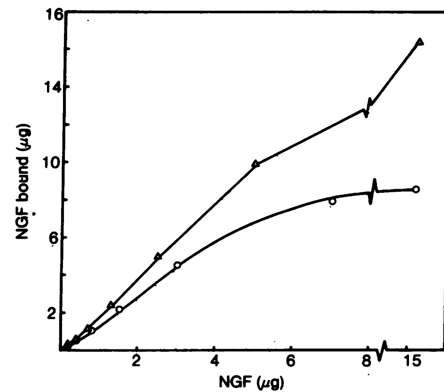

FIG. 4. Binding of NGF at 2° or 37°. Tubulin, dissolved in the reassembly buffer at 1.0 mg/ml, was divided in two samples. One was incubated for 90 min at 37°, the other was left at 2°. Each sample was then used for the binding assay in the presence of various concentrations of NGF plus ^{125}I-labeled NGF. Aliquots of the batch of tubulin previously incubated at 37° were added to the binding assay mixture (see *Methods*) and further incubated for 30 min at 37° (○). An analogous procedure was followed for the batch left at 2° except that binding with NGF was allowed to proceed at 2° for 20 hr (Δ). Each test tube contained 50 μg of tubulin. NGF concentrations on the *abscissa* are expressed as in Fig. 2.

of tubulin dimer (molecular weight 110,000). The binding assay we used, based on precipitation of the NGF–tubulin complex, does not allow direct measurement of the association constant of NGF for tubulin in solution. It is possible, however, to infer that the affinity of NGF for tubulin is quite high. Thus, under the conditions described, with initial concentrations of 20 μg for NGF and 60 μg for tubulin in 0.25 ml, 50% total NGF appears in the precipitate. Moreover, the complex formed does not dissociate significantly even after 10–15 hr of incubation at 25°.

The microtubule protein(s) bind 1 mole of colchicine specifically and with a relatively high association constant (1.8×10^6 liters/mole) (10). We investigated whether NGF interferes with the binding of colchicine with tubulin. NGF, at two different concentrations, does not inhibit the binding of colchicine (Fig. 3), which has an association constant for tubulin of 1.5×10^6 liters/mole both in the absence and presence of NGF. The noncompetitive binding of NGF and colchicine to tubulin was also confirmed in experiments where NGF binding was measured in the presence of constant amounts of colchicine at a concentration two orders of magnitude higher than its association constant for tubulin (Fig. 2). No significant inhibition of NGF binding by colchicine could be detected.

Both the stoichiometry and the apparent affinity of NGF for tubulin vary with the experimental conditions. Temperature, for instance, seems to play an important role (Fig. 4). At 2° more NGF is bound than at 37° after preincubation of tubulin for 90 min, while saturation seems to occur at higher NGF concentrations. Substances like GTP or Mg^{++}, which favor the spontaneous assembly of tubulin, do not interfere significantly with the binding of NGF either at 37 or at 2° (Fig. 5). When the experiments at 37° or 2° were performed with longer incubation times, the amount of NGF bound did not change, indicating that maximum binding was attained within the time of incubation used in these studies.

In order to further check the specificity of interaction between NGF and tubulin, binding experiments under the standard conditions (10 μg of NGF plus 100 μg of tubulin in 0.25 ml of reassembly buffer) were performed in the presence of a 20-fold excess over NGF of different proteins with a large range of isoelectric points. Under these conditions, cytochrome *c*, hemoglobin, ribonuclease A, bovine-serum albumin, and the brain-specific protein S-100 never reduced the extent of binding by more than 10–15%. Another brain-specific protein, 14.3.2, seemed to favor the binding since under the experimental conditions described, 25% more NGF appeared in the precipitate than in the presence of

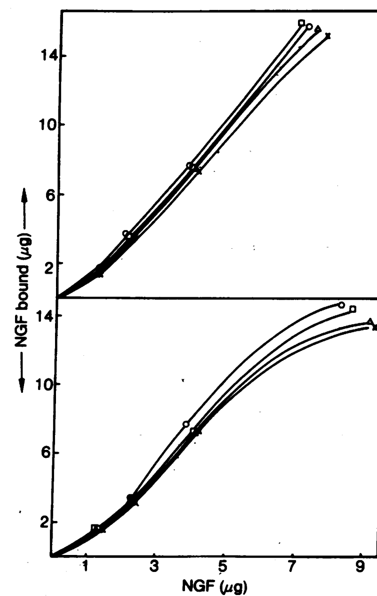

FIG. 5. Effect of GTP, Mg^{++}, or complete reassembly buffer on binding of NGF to tubulin. Binding, under standard conditions, at two different temperatures, [2° for 20 hr (*top*) or 37° for 30 min (*bottom*)] was performed in the presence of (○) 0.01 M KH_2PO_4–Na_2HPO_4; pH 6.4; (□) phosphate buffer plus 0.5 mM $MgCl_2$; (Δ) phosphate buffer plus 0.5 mM GTP; or (×) complete reassembly buffer. NGF concentrations on the *abscissa* are expressed as in Fig. 2.

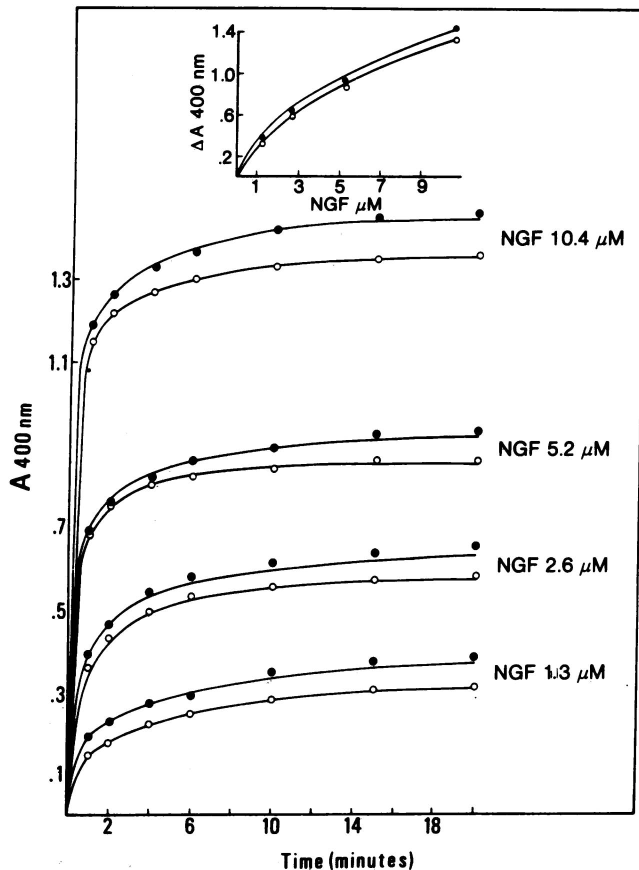

FIG. 6. Effect of NGF on light scattering of tubulin. In a final volume of 0.25 ml of complete reassembly buffer also containing 0.1 M NaCl, were present 120 μg of purified tubulin plus (●) or minus (○) 10^{-4} M colchicine. At zero time, NGF was added at the final concentration indicated in the figure, the mixture was rapidly stirred, and the increase in absorbancy (A) was monitored. The *inset* reports the change in absorbancy after 15 min of incubation at room temperature at the various concentrations of NGF.

tubulin alone. The apparent interaction of NGF and this protein merits further studies. Moreover, since the 14.3.2 protein exhibits on sodium dodecyl sulfate electrophoresis, a molecular weight of 48,000–50,000 (19), it could belong to those few proteins detectable in the NGF or vinblastine precipitate described above (Fig. 1*b* and *c*). A strong inhibition of binding is exerted by 1.0 M NaCl. On the other hand, if the NGF–tubulin complex is treated with 1.0 M NaCl and allowed to stand for 30 min at room temperature, no significant dissociation of the complex occurs. These findings suggest that the first, probably electrostatic, interactions between the two proteins is followed by a more complex binding that makes the complex stable even at high ionic strength. Alternatively, 1.0 M NaCl may induce a conformational change on tubulin or NGF or both, which markedly alters the respective sites of interaction.

The interaction of NGF with tubulin was followed by measurement of the change in light scattering occurring when NGF is added to a solution of tubulin in the reassembly buffer. This procedure has been used (8, 15) to follow the spontaneous assembly of tubulin. Fig. 6 shows that NGF, in a range of concentrations from 10^{-6} to 10^{-5} M, induces a strong increase in light scattering. The effect depends on the amount of NGF added (see also *inset* of Fig. 6) and appears to be

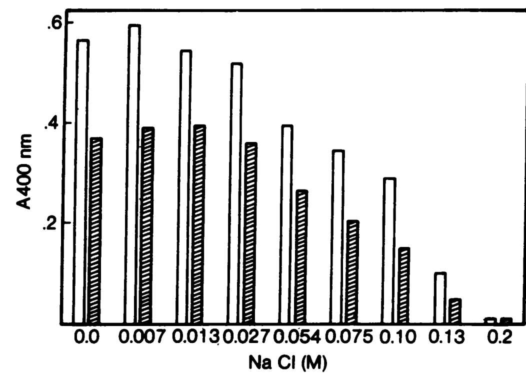

FIG. 7. Effect of GTP or NaCl on the increased turbidity induced by NGF. Light scattering was followed by the procedure described under *Methods* except that the buffer was 0.01 M MES instead of KH_2PO_4–Na_2HPO_4. Tubulin, after centrifugation from 8 M glycerol (8), was redissolved in complete reassembly buffer (*dashed bars*) or in MES alone (*empty bars*). Before addition of a constant amount of NGF (20 μg), NaCl, at the final concentration indicated on the *abscissa*, was added to tubulin.

quite fast since at the lowest concentration of NGF it is half maximum already after one minute at room temperature. Colchicine (10^{-4} M) does not exert any antagonizing effect, in agreement with the binding data reported above. The meaning of the slight but reproducible increase in light scattering when both NGF and colchicine are present together is not clear.

Vinblastine, which induces precipitation of the microtubule protein(s) (21), also induces an increase in light scattering. This alkaloid, however, at a 0.4 mM concentration, induces an increase in turbidity which is approximately half of that elicited by NGF at a molar concentration 400 times lower (1 μM). The two substances, added together to tubulin, do not sum their effects.

The action of NGF on tubulin aggregation or assembly is markedly favored by the addition of divalent cations (Table 1*a*) to tubulin before the addition of NGF. Among these, Ca^{++} is the most effective. GTP and a monovalent cation like Na^{++}, on the contrary, exert a marked inhibition on the NGF-induced turbidity (Table 1*b* and Fig. 7).

TABLE 1. *Effect of divalent cations, GTP, or complete reassembly buffer on NGF induced turbidity of tubulin*

Additions	$A_{400\ nm}$ Tubulin	$A_{400\ nm}$ Tubulin + NGF
(*a*) None	0.05	0.320
Ca^{++}	0.06	0.720
Mg^{++}	0.044	0.480
Ba^{++}	0.052	0.550
Sr^{++}	0.046	0.605
(*b*) None	0.08	0.285
Mg^{++}	0.08	0.520
EGTA	0.075	0.215
GTP	0.070	0.115
Mg^{++} + EGTA + GTP	0.090	0.445

Each cuvette contained in a final volume of 0.3 ml, 50 μg of tubulin in 0.01 M MES, pH 6.4, plus 0.3 mM of the cations to be tested (Table 1*a*) or 1.0 mM EGTA or GTP and 0.5 mM Mg^{++} (Table 1*b*). NGF (20 μg) was then added and the increase in light scattering monitored at 400 nm. The number refers to the absorbancy after 15 mm at room temperature.

DISCUSSION

The binding of NGF to tubulin, followed by the rapid increase of turbidity of the complex, resembles macroscopically the process occurring when the protein is incubated for a long time at 37° in the reassembly buffer (8, 15). In contrast with the spontaneous reassembly of tubulin, however, the turbidity induced by NGF interacting with this protein is very fast and occurs also at 0°. It remains to be investigated whether, once bound, NGF alters some of the peculiar properties of tubulin (GTP-GDP interconversion, conformational changes, etc.) and whether the NGF–tubulin aggregate assumes a somewhat ordered structure. In this connection, the apparent similarity of NGF to vinblastine deserves further study. The alkaloid *in vivo* induces the breakdown of microtubules with subsequent rearrangement of the tubulin subunits into hexagonally packed crystals (20) and *in vitro* induces aggregation of purified tubulin (21, 22). NGF interacts with tubulin present in brain supernatants (Fig. 1*b*) or in purified form (light-scattering measurements) in an apparently similar fashion although at molar concentrations at least two orders of magnitude lower than the alkaloid. Its effect on the target cells *in vivo*, however, is strikingly different from the alkaloid since it elicits a massive production of neurotubules and neurofilaments.

The data reported on the binding of NGF to tubulin and those on its effect on the assembly process (Figs. 4, 5, and Table 1) call for some comment. While NGF binding is not significantly affected by GTP or Mg^{++}, these substances, as well as other divalent cations, appear to exert a marked effect on the NGF-induced assembly of tubulin. One attractive explanation would be that the binding of NGF to tubulin and the induced assembly or aggregation is a sequential, two-step process and that the latter but not the former is affected by the presence of GTP or divalent cations. Colchicine, on the contrary, does not affect the binding or the turbidity induced by NGF, suggesting that the two substances have a different binding site(s) and mechanism of interaction with the microtubule protein(s).

This hypothesis would attribute an important role to the conformation and quaternary structure of tubulin interacting with NGF. Such a role is suggested by the dependence on temperature of the binding (Fig. 4). Since, at 2°, tubulin is prevented from polymerization and is maintained in solution essentially as a dimer, while at 37° it forms larger oligomers (8, 15), the differential binding of NGF at the two temperatures may reflect a different availability and/or degree of exposure of the binding sites on the tubulin molecule according to the size of the polymers. Since tubulin exists in the living cell in different conformational states (dimer $\rightleftharpoons$ polymer, cytoplasmic or membrane-bound), the possibility of modulating its interaction with NGF according to its conformation, which in turn depends on the ionic or metabolic environments, may have interesting functional implications.

It has been reported that NGF specifically binds to membrane preparations from the superior cervical ganglia (23) and to a line of neuroblastoma cells (24) or to an extract from the same cells with an apparent high affinity constant. It would be tempting to speculate that the same or a very similar protein(s) is responsible for these bindings and for the interaction with tubulin reported here. This hypothesis implies that the microtubule protein(s) is present in both the cytoplasm, which was the source of tubulin for our studies, and on the surface of the target cells of NGF. A colchicine-binding activity has been demonstrated in membranes (12) and in particulate fractions of mouse brains, the richest activity being in microsomes and nerve-ending subfractions (11). These studies support the idea that the binding described here and those on the NGF receptor could occur with the same or a very similar protein. As an alternative possibility, we may assume that NGF will interact with the microtubule protein(s) after the binding with its own receptor.

Both hypotheses postulate an intimate connection between the interaction of NGF with tubulin and its mechanism of action on the target cells. The findings reported here offer the possibility of verifying these two hypotheses and following, at the molecular level, the interaction of the microtubule protein(s) with a ligand (NGF) of great biological significance and potency.

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