soluble, retains its catalatic activity. This fact has made possible the identification of the catalase-anticatalase line in double-diffusion and immunoelectrophoresis plates.

Our data indicate that erythrocyte catalase proteins produced in five mutant mouse strains are quantitatively and immunologically indistinguishable from that in the normal, wild-type strain. The erythrocyte catalase activity, however, is almost absent in the acatalasemic mutant and is much lower than normal in the four hypocatalasemic mutants. These five mutants, therefore, all represent structural genetic mutations. This conclusion is based on the remarkable similarity in the results obtained in two distinct ways.

1) In all the agar-diffusion tests, the lysates from both the acatalasemic (Cs^b) and the hypocatalasemic (Cs^c) cells produce lines that have immunological identity with and are stained to the same degree as those from normal (Cs^a) lysates. In addition, immunologically identical lines due to catalase and its antibody degrade H_2O_2 if the lysate is from Cs^a or Cs^c, but not if the lysate is from Cs^b mice. After immunoelectrophoresis, lines in the same position and with equal staining show that Cs^a has more catalatic activity than Cs^c, and that Cs^b is acatalatic. Further, if the three antigens are diluted fourfold, all fail to produce precipitin lines. As expected, reciprocal tests of Cs^a and Cs^b lysates with antiserums produced against them in Cs^b and Cs^a mice, respectively, produce no precipitation lines.

2) The antigen titrations demonstrate that red-cell antigen of all four hypocatalasemic mutants attains about 90 percent precipitability, as normal red-cell antigen does, but reaches the region of antigen excess at a catalase concentration well below normal. Because the same antiserum was used in all cases, the implication is that all are behaving in identical immunological fashion, but that an equal number of the mutant catalase molecules, being subnormally active catalatically, reaches the threshold of antigen excess at a lower-than-normal level of catalase activity. Catalase protein in antigen prepared from acatalasemic red-blood cells is detected by its interference with the antigen titration curve of normal erythrocyte lysate. Active but unrelated catalase protein (from rat cells) does not affect the precipitin curve with the antiserum as Cs^b antigen does. Normal rabbit serum has no precipitability, and assorted heterologous proteins have no inhibitory activities.

In view of the foregoing considerations, coupled with the demonstration (*2*) that blood and tissue catalase of the mutant strains differ qualitatively from each other and from the wild-type (normal) strain, we are apparently dealing with a purely structural mutation in which the structural modifications involve the enzyme-activity site but not the antigenic site.

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Nerve Growth Factor of Very High Yield and Specific Activity

Abstract. *Nerve growth factor has been isolated from submaxillary glands of mature male mice at specific activities about a million times, and in yields of biological activity ten million times, greater than best previous results. The major improvement in the isolation is related to the separation of a highly active tosylarginine methyl esterase present in cruder preparations. The new nerve growth factor may be an entity different from the older one, although no gross differences in the qualitative aspects of their actions are apparent on superficial examination of chick ganglia influenced by them. The neurites which develop from a ganglion in the presence of nerve growth factor are of nearly equal length. The amount of nerve growth factor determines the number of neurites but not the extent of individual development. The amount of the new nerve growth factor which evokes the appearance of a hundred neurites from a single ganglion appears to be about ten molecules. Since each neurite seems to arise from a different neuron each molecule of nerve growth factor must affect several cells. This result can be rationalized by a catalytic mechanism or by indirect action of nerve growth factor through a hypothetical cell which produces a neurite evocator on contact with the molecule of nerve growth factor.*

Since very marked overgrowth of nerve fibers from chick embryonic spinal ganglia in the presence of implanted tumors was observed in vivo (*1*), it has become clear that a humoral agent is involved (*2*) and that the agent is protein or closely associated with protein (*3, 4*).

A test (*5*) for activity in vitro evolved into an assay method (*3, 6, 7*). The biological unit (bu) is that amount in one milliliter of medium which produces in 18 hours 3+ growth of neurites from spinal ganglion (of a 7 to 9-day chick embryo) suspended in 0.06 ml of medium. Scoring standards have been described (*6, 7*). The medium is one-third rooster plasma and two-thirds medium 199 containing the material of interest.

The richest known source of nerve growth factor (NGF) is the submaxillary gland of the mature male mouse. Materials having specific activities of 1×10^8 to 1.5×10^8 bu/g have been considered highly purified (*4, 6*). Electrophoresis of such material on paper separates it into three bands that stain with ninhydrin. No one of these is active by itself, but a combination of two is active (*8*). Important aspects of the action of NGF have been reviewed (*9*).

We have found that much more (10^7 times) NGF (in biological units) can be isolated from the crude homogenate than has been demonstrated in it by simple dilution; this purified material has a specific activity of 10^{15} bu/g. An enzyme highly effective in the hydrolysis of tosylarginine methyl ester is also found in the cruder extracts (*10*). The two activities are largely separable, and their separation may

have been crucial for our present findings.

The new NGF is produced as follows. Mature male Swiss mice are killed with chloroform. The submaxillary glands are removed to iced 0.1*M* phosphate buffer at *p*H 7.4 and homogenized as described by Cohen (*4*); all steps described by him are followed through the first precipitation with ethanol. The supernatant is then brought to 55 percent ethanol by the addition of iced absolute alcohol, and the solution is kept for 1 hour at −20°C. The precipitate is sedimented by centrifugation at 10,000*g* for 10 minutes. The pellet is then dissolved in water, solid ammonium sulfate is added to 35 percent saturation, and the *p*H is adjusted to 7.4. After an hour at 2° to 4°C, the precipitate is removed by centrifugation. Ammonium sulfate is added to the supernatant to 75 percent saturation. After an hour in the cold, this precipitate is collected by centrifugation and dissolved in a small volume of water. The solution is dialyzed in the cold until the outer fluid produces no precipitate on the addition of barium chloride solution. The dialyzed solution is placed on a carboxymethyl cellulose column (*4*) and eluted with water. The NGF is not retained by the column, and the fractions containing the first peak are combined, lyophilized, and dissolved in a small volume of water. This is preparation CM in Table 1.

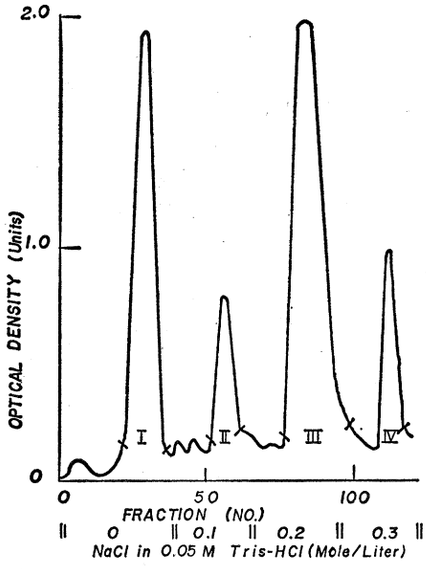

Fig. 1. Chromatography of CM material on DEAE–Sephadex A-25 column (170 × 2.8 cm). Eluting buffer was 0.05*M* tris-HCl, *p*H 7.4, with additional NaCl as indicated for stepwise elution at 60 ml/hour. Volume per fraction was 6 ml. Optical density units per tube equals the optical density at 278 nm times the volume of the solution in tube times the dilution. Roman numerals refer to peaks indicated DS I to IV.

Preparation CM is dialyzed against 0.05*M* tris(hydroxymethyl)aminomethane-HCl buffer at *p*H 7.5. This is also the suspending buffer for chromatography on diethylaminoethyl-Sephadex and the initial eluant for the column, which was prepared according to method A of the supplier (*11*). After the first peak, increasing concentrations of NaCl in tris buffer were used as eluants (Fig. 1). Each set of fractions as indicated was combined to give the samples marked DS I to IV, and the combined fractions were lyophilized. Analytical disk electrophoresis (*12*) was performed on each peak (Fig. 2). We determined protein concentrations by the method of Lowry *et al.* (*13*), which we calibrated with bovine serum albumin; or we determined protein concentrations from the optical densities of the solutions at 278 and 260 nm and the equation given by Layne (*14*).

Our best fractions were not homogeneous (Fig. 2). Comparison of Fig. 2 with the corresponding data in Table 1 indicates that disks 2, 3, and 4 as a group are high in enzymatic activity and that 5, 7, 10, and 12 as a group are associated with high concentrations of NGF. We do not know whether a particular disk or several materials represented by disks carry the activity.

The specific activity of DS II as NGF is 10^6 times that of any previously reported preparation, and the yield of activity is 10^7 times that apparent in the original homogenate (Table 1). Since making this observation, we have found that the relationship between dose and response (in cruder extracts, such as CM) is complex. High concentrations of the extracts inhibit outgrowth of neurites (*15*). This effect is generally attributed to toxic materials or inhibitors in the extracts which can be diluted out. Once a dilution is found which gives noticeable neurite growth, successive (often twofold) dilutions produce an increasing density of outgrowth, then a decrease. The decrease is naturally attributed to dilution of the "factor." We now find that successive tenfold dilutions reveal a second peak of activity (Table 2). The first peak is typical of older assays; the second is a new feature not previously reported because the needed high dilutions were not examined. The figures given in Table 1 for homogenates are based on the older assays.

The neurites grown in extracts at the two levels of dilution are not morphologically distinguishable. Fibroblasts are sometimes seen, but distinguishing their fibers from neurites is not difficult. At this time we see no way of explaining the peculiar response curve, without assuming that there are two NGF's which produce the same result, namely neurite development. We will refer to these as NGFA (responsible for activity at low dilutions) and NGFB (responsible for activity at high dilutions). A recent report (*16*) suggests that there are at least two NGF's, as well as an inhibitor, in the crude extracts.

The separation of the enzyme of fraction DS I from the nerve growth activity in DS II is probably important for the stability of NGF in the extracts. The small enzyme activity shown in Table 1 for DS II was demonstrated by lyophilization of DS II from a volume of 200 to 4 ml. When kept frozen this material retains its high activity, but when thawed and kept in the cold room (2° to 4°C) overnight 10^{-6} μg produces 3+ growth, whereas 10^{-10} μg of the original material promoted similar growth. After the material stood

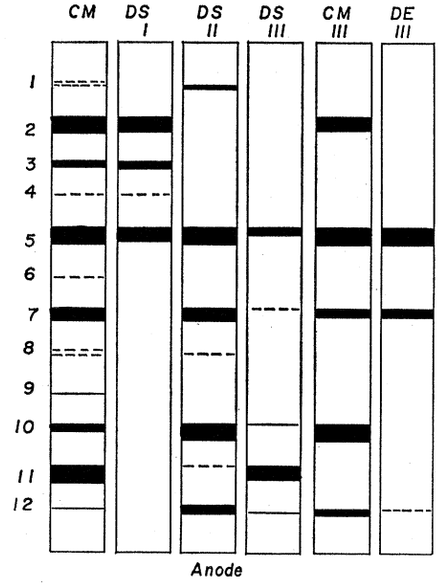

Fig. 2. Representation of disc electrophoresis of various preparations of nerve growth factor; CM, DS I, DS II, DS III are preparations described in the text; CM III is a standard Cohen (*4*) preparation, and DE III is the preparation described in reference *8*.

Table 1. Fractionation of submaxillary glands of mature male mice. Totals are calculated for 100 mice. The biological unit (bu) is the amount in 1 ml of solution, 0.04 ml of which added to 0.02 ml of rooster plasma and a spinal ganglion of a 7 to 9-day chicken embryo produces 3+ outgrowth of neurites in 18 hours (*3, 5*). The esterase unit (tu) catalyzes hydrolysis of 1 μmole of tosylarginine methyl ester per minute for 4 to 8 μmole in 10 ml of solution containing 40 μmole of ester and maintained at 37°C and *p*H 8.

Fraction	Total amount			Specific activities	
	Protein (mg)	NGF (bu)	Esterase (tu)	NGF (bu/μg)	Esterase (tu/μg)
Homogenate	2200	2.6×10^6	3×10^5	1.1	0.14
35 to 75 percent $(NH_4)_2SO_4$	320	8×10^5	2.2×10^5	2.5	.70
CM	180	6×10^5	2×10^5	3.0	1.1
DS I	64	*	1.8×10^5	*	3.1
DS II	19	2×10^{13}	6×10^2	1×10^9	0.003
DS III	78	5×10^4	2.6×10^4	0.6	.4
Cohen CM III (*3*)	3–4	2×10^5		67.	
DE III (*8*)	2–5	$2–3 \times 10^5$		150	

* Could not be determined because of clot lysis.

overnight at room temperature, the standard biological response required 10^{-3} to 10^{-2} μg. When equal quantities of DS I and DS II are mixed at room temperature the biological unit becomes 0.1 μg. These data indicate that the enzyme (or enzymes) can inactivate NGF.

Large amounts of NGF produce "stunted" growth. A great many neurites seem to start but grow to less than full length (*5*). This may result from the exhaustion of some component of the culture medium by the 5000 to 20,000 cells presumed capable of axonal growth in each ganglion. As the amount of NGF is reduced fewer but longer neurites are produced. At 4+ response a maximum length appears to be produced for the time of growth. Greater dilutions result in the appearance of fewer neurites, but these are as long as those observed at levels scored 4+. Since each neurite may be assumed to be the product of a single cell and neurites of intermediate length are not seen, the response of the neuron to NGF never appears partial. We therefore suggest that any neuron which responds to NGF responds completely by producing a complete axon.

Incubation of cultures beyond 18 to 48, or even 72, hours produces no additional neurites, although those already present elongate. Textbooks describe ganglionic neurons as producing in vivo a single neurite which may or may not bifurcate. Neurites developing in cultures also bifurcate. We think it is appropriate to suggest that each neuron can produce just one neurite under culture conditions as it can in vivo.

The very high specific activity of NGFB is of great interest. At 3+ response, achieved with 10^{-10} μg of NGFB per milliliter of test solution, the neurites are too numerous for convenient counting. At 10^{-11} μg about 100 neurites appear to grow from a single ganglion. At 10^{-12} μg fewer appear, greater in numbers than in control cultures, but of the full length seen at 3+ growth. The two dialyses in the method of preparation indicate that the NGF must have a molecular weight greater than 12,000 [the upper limit for permeation of cellophane casings (*17*)]. If we assume that the molecular weight is 20,000, the number of molecules which produces 100 neurites is $0.04 \times 10^{-11} \times 10^{-6}/20,000$ multiplied by Avagadro's number. This is 12 molecules. Use of any higher molecular weight will reduce this number. If the several protein disks in DS II (Fig. 2) are not NGF's, the number will be less. This calculation is taken as evidence that each molecule of NGF evokes more than one neurite. Each molecule must in some way affect a number of cells. If the release mechanism for neurite outgrowth is correct, NGF can affect several cells only if it does not become a part of the neuron affected. Thus a further mechanism must be postulated to allow a single molecule to act on several cells.

Table 2. Dose of NGF and response of ganglia. Chick embryonic ganglia (8 days of incubation) with 0.02 ml of medium 199, containing the indicated quantity of protein per milliliter, and 0.01 ml of rooster plasma were incubated for 16 to 18 hours and scored. The arbitrary scale of response is based on references *6* and *7*. The numbers are the average scores of two to four embryos at each dose.

Dose (μg)	Response
3000	No clot
300	No clot
30	1
3	1
0.3	Stunting (*5*)
0.03	3.5
3×10^{-3}	2.5
3×10^{-4}	1.8
3×10^{-5}	0.9
3×10^{-6}	1.9
3×10^{-7}	2.0
3×10^{-8}	1.9
3×10^{-9}	2.3
3×10^{-10}	1.0
3×10^{-11}	0.5
3×10^{-12}	.5
3×10^{-13}	.5
3×10^{-14}	0
3×10^{-15}	0
3×10^{-16}	0

Two possible mechanisms follow. (i) NGF may be similar to an enzyme, with the neuron analogous to substrate. The same NGF molecule may move from neuron to neuron causing each to produce a neurite. The NGF must be destroyed after an active life which is short compared to the 18-hour incubation time. Otherwise neurites with various lengths would be produced. (ii) NGF may enter as a molecule into some unidentified cell in the ganglion and there initiate [perhaps by derepression of a repressed operon (*18*)] the production of a neurite evocator which is the direct stimulator of neurite growth.

Four different batches of NGFB have been prepared, all with the same high specific activity. The dilute preparations are much more stable in the cold (2° to 4°C) than are CM preparations. The NGFB is stable for several months rather than for a few weeks as NGFA is. Varon and his coworkers (*19*) report a form of NGF with a high molecular weight whose specific activity is in the range found for Cohn fractions. We would liken it to NGFA. It is possible that NGFA and preparations with its activity carry NGFB bound to proteins which are not directly required for activity and appear to be homogeneous and therefore very pure. It would take a chemically undetectable "impurity" of NGFB to account for the specific activity of NGFA preparations. The carrier protein would be responsible for the apparent homogeneity.

The necessity for NGF in the intact mouse is indicated by the results of immunological studies. Antiserums to Cohen's NGF have been produced. When given to newborn mice, it selectively destroys sympathetic neurons almost completely (*20*). Levi-Montal-

cini and Angeletti (*21*) demonstrated that fluorescent forms of the antibodies are fixed in the cytoplasm of neurons. Sabatini and his co-workers (*22*), who used the electron microscope, describe "considerable damage to most of the neurons while neurolemma and endothelial cells remain intact" a few hours after injection of the antiserum. These findings are interpreted as cytotoxic antigen-antibody reactions in sympathetic neurons. The findings seem to require that NGF or some closely related hapten be present in the neurons. However, the antibodies produced against Cohen NGF in this laboratory or by Abbott (*23*) are complex as judged by the several precipitin lines that appear on Ouchterlony plates or by immunoelectrophoresis. We do not know whether there is an antibody to NGFB among these.

We are uncertain whether NGFA and NGFB are entirely different substances or differently bound forms of the same biologically active substance. However, it seems clear that the response by neurons is all-or-none, and that a single molecule may affect several neurons.

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Tetanus Toxin: Fine Structure Localization of Binding Sites in Striated Muscle

Abstract. *When striated muscle from mice poisoned with purified tetanus neurotoxin was incubated with tetanus antitoxin labeled with horseradish peroxidase and stained for peroxidase activity, an electron-opaque reaction product was found in the transverse and terminal sacs of the longitudinal elements of the sarcotubular system. Demonstration of toxin in this location suggests that it acts by interfering with contraction coupling or mechanisms of contraction-relaxation.*

Although many data (*1*) indicate that the central nervous system is a major site of toxin action, tetanus toxin binds to skeletal muscle and produces abnormalities of electrolyte flux within it (*2, 3*). Studies in which toxin labeled with fluorescein was used have shown marked selectivity for the central nervous system and striated muscle. To increase the resolution of binding studies, we used the technique of labeling proteins with horseradish peroxidase (*4*).

Mice poisoned with a minimum saturation dose (9 μg) of purified tetanus toxin (*5*) were killed when signs of generalized intoxication were well developed. Pieces of intercostal and leg muscles were excised, fixed in 10 percent phosphate-buffered formalin for 4 hours, and washed overnight in 0.7*M* phosphate buffer (*p*H 7.6) containing 5 percent sucrose. Frozen sections (40 μm) were cut in a cryostat and incubated 30 minutes in tetanus antitoxin labeled with horseradish peroxidase according to the method of Nakane and Pierce (*4*). The sections were washed with agitation for 30 minutes in phosphate-buffered saline, fixed with 4 percent glutaraldehyde, washed, and incubated for 10 minutes in 0.05*M* tris-HCl buffer (*p*H 7.6) containing 3′,3′-diaminobenzidine and 0.001 percent H_2O_2. After additional washing, the sections were treated for 90 minutes in 1.3 percent OsO_4 in buffered saline (*p*H 7.6) containing 5 percent sucrose. The sections were dehydrated and embedded in Epon 812. The embedded material was sectioned with a Porter-Blum microtome and examined in an RCA EMU-3F electron microscope. Unstained sections and sections stained in 1 percent uranyl acetate in ethanol were examined. Staining increased the contrast of muscle structures but left the histochemical reaction product unaltered. Control preparations consisted of normal skeletal muscle incubated with tetanus antitoxin labeled with peroxidase. Immunoelectrophoresis of the labeled antitoxin was also performed to ascertain whether the antitoxin was labeled and whether it retained its ability to react with the toxin. The precipitin line was found in its normal position, and staining with 3′,3′-diaminobenzidine revealed that the line contained peroxidase activity. Despite the rigorous treatment of the tissues, the major ultrastructural components of skeletal muscle were still recognizable. Myofibrillar fine structure was less disrupted than elements of the sarcotubular system. However, transverse and longitudinal components were easily identified.

In muscle from intoxicated mice, frequent but not ubiquitous accumulations of electron-opaque amorphous material characteristic of the histochemical reaction product were found free within the lumen and on the lumenal