Subunit Interaction and Enzymatic Activity of Mouse 7S Nerve Growth Factor*

Lloyd A. Greene,† E. M. Shooter, and Silvio Varon

ABSTRACT: The 7S nerve growth factor preparation possesses a potent esterase activity on the substrate α-*N*-benzoyl-L-arginine ethyl ester. Upon dissociation of the parent molecule, this enzymatic activity is carried by the γ subunits alone. The 7S material differs from the isolated γ subunits when tested at neutral pH in that it exhibits an initial lag phase during substrate hydrolysis and expresses an enzymatic activity lower than would be expected by the stoichiometric contribution of the γ subunits to the 7S complex. The lowered activity is shown to be the result of a specific combination of the α and β subunits with the γ subunits to form the 7S species. The lag phase is shown to be generated by dilution of the 7S preparation for enzymatic assay and may be eliminated by preincubation of this material at low concentrations. A model is presented in which an active form of the γ subunit is released in dissociation–equilibrium with the enzymatically inactive, or very poorly active, parent 7S species. This hypothesis is supported by the effects produced on the observed enzymatic activity of the 7S complex by a number of different manipulations of the assay conditions.

Nerve growth factor (NGF)[1] is a term applied to a group of proteins which selectively stimulate the growth and possibly the differentiation of sympathetic and embryonic sensory ganglia. While these proteins apparently share the same biological activity, they often differ in physicochemical properties depending upon both their sources and the procedures used in their isolation. Nerve growth factors have been extensively studied in the past 15 years and have been the subject of several recent reviews (Levi-Montalcini, 1965; Varon, 1968; Levi-Montalcini and Angeletti, 1968; Shooter and Varon, 1969).

One source of NGF activity is the submaxillary gland of the adult male mouse (Cohen, 1960). Recent investigations (Varon *et al.*, 1967a) have revealed that, in crude extracts of the mouse gland, the NGF activity is associated exclusively with and may be purified as a protein characterized by a sedimentation coefficient of about 7 S, indicative of a molecular weight of approximately 140,000. It was further shown (Varon *et al.*, 1967b, 1968) that this protein could be reversibly dissociated, outside a pH range between 5 and 8, into three groups of subunits each of mol wt 25,000–30,000. Accompanying this dissociation was a likewise reversible three- to fourfold drop in biological activity. The three subunit groups, separable by ion-exchange chromatography, are designated as α, β, and γ. The NGF activity remaining after the dissociation of the 7S species is found exclusively with the β subunits. Both the α and γ subunits are heterogeneous and arise from the multiple forms of the 7S NGF (Smith *et al.*, 1968). After isolation, the three groups of subunits may be brought together again at neutral pH to reconstitute the original 7S NGF with restoration of a higher biological activity. The β subunits will also combine with either α or γ subunits to form complexes of mol wt 60,000 and 120,000, respectively, but without a recognizable enhancement of the biological activity.

Further investigation of the properties of the 7S NGF preparation showed (Greene *et al.*, 1968) that it possesses a potent enzymatic activity when tested with the substrate α-*N*-Bz-L-Arg-ethyl ester. On dissociation of the 7S complex and separation of the subunits, the enzymatic activity was found to reside exclusively with the γ subunits. The multiple forms of the γ subunit, γ^1, γ^2, and γ^3, are all active and possess identical specific activities. Several differences were noted in the enzymatic behavior of the isolated γ subunits and of the 7S NGF preparations. With the latter, α-*N*-Bz-L-Arg-ethyl ester hydrolysis was characterized by an initial "lag phase" followed by a maximal linear rate, whereas with the purified γ enzyme it occurred at an immediate linear rate. Differences in the specific activities of the two preparations indicated that the γ subunit was less active in the 7S complex than would be expected if the protein contributed by γ to the 7S species were free to fully express its enzymatic activity.

The kinetic and specific activity differences between the 7S and the γ preparations were further investigated. The experiments reported here indicate that the 7S complex itself has little, if any, enzymatic activity and that 7S NGF preparations owe their activity to dissociated forms of the γ enzyme, with which the 7S complex is in equilibrium. Thus, the interaction of the γ enzyme with both the α and the β subunits results in a nearly complete inhibition of its activity. Neither subunit alone has any detectable effect.

Materials and Methods

The 7S NGF was isolated from mouse submaxillary glands as previously described (Varon *et al.*, 1967a). The methods for

* From the Departments of Chemistry (L. A. G.) and Biology (S. V.), University of California, San Diego, La Jolla, California, and the Department of Genetics and Biochemistry and Lt. Joseph P. Kennedy, Jr. Laboratories for Molecular Medicine, Stanford University School of Medicine, Stanford, California (E. M. S.). *Received April 17, 1969.* This research was supported by U. S. Public Health Service research grants from the National Institutes of Neurological Diseases and Stroke (NB 07606 and 04270) and a National Science Foundation grant (GB 4430).

† National Science Foundation graduate trainee, 1968–1969.

[1] Abbreviation used is: NGF, nerve growth factor.

TABLE I: Comparison of the Specific Activities of 7S NGF, Isolated γ Subunits, and Trypsin on Various Substrates.[a]

	7S NGF	γ	Trypsin
α-*N*-Bz-L-Arg-ethyl ester (a)	32[b]	180	27
p-Toluenesulfonyl-L-Arg-methyl ester (b)	22[b]	78	37
α-*N*-Bz-L-Arg-amide (c)	0.019[b]	0.13	0.25
α-*N*-Bz-DL-Arg-β-napthyl-amide (c)	0.0008[b]	0.0100	0.030
α-*N*-Bz-DL-Arg-*p*-nitro-anilide (d)	2.8[b]	12.5	10.0
L-Lys-ethyl ester (e)	0.365[b]	1.27	27.7

[a] Specific activities are reported as micromoles of substrate hydrolyzed per minute per milligram of enzyme at 25°. Assays were performed according to the methods given by (a) see Materials and Methods (pH 7.0), (b) Hummel (1959), (c) Popov and Krutyakov (1964), (d) Erlanger *et al.* (1961), and (e) Werbin and Palm (1951). Reliable measurements required the use of different amounts of enzyme in the various assays. [b] Initial hydrolysis characterized by a lag phase.

TABLE II: The Effect of Preincubation with α and β Subunits on the Enzymatic Activity of the γ Enzyme.

	Rel Enzymatic Act.[a] Tested at		
	pH 7	pH 8	pH 9
γ	1	1	1
$\gamma + \beta$	0.99	1.06	1.01
$\gamma + \alpha$	1.10	1.13	1.10
$\gamma + \alpha + \beta$	0.20[b] (lag phase)	0.51[b] (lag phase)	1.05[b] (no lag phase)

[a] Enzymatic activities are given as ratios of the activity of each mixture to that of the γ enzyme alone at the various pH values. Mixtures of the γ enzyme (100 μg/ml) with either or both α or β subunits (200 μg/ml) were incubated at 0° for 1 hr in Tri-Cl buffer (I = 0.05) at the appropriate pH values. Aliquots (10 μl) were then assayed with 1.0 mM α-*N*-Bz-L-Arg-ethyl ester in same buffer. [b] Lag phase present.

the separation of the α, β, and γ subunits and the resolution of the individual γ species are given elsewhere (Smith *et al.*, 1968). In some experiments, 7S NGF and subunits were recycled on G-150 Sephadex and CM-cellulose, respectively. All materials were prepared and stored at 4°.

The enzymatic activity on α-*N*-Bz-L-Arg-ethyl ester (Calbiochem) was measured at 25° on a Zeiss PM-QII spectrophotometer. Unless otherwise specified, the assays were performed by adding 0.1 ml of the sample to be tested in the appropriate buffer to 2.9 ml of 1.0 mM α-*N*-Bz-L-Arg-ethyl ester in the same buffer and following the hydrolysis of the substrate by the absorbance change at 253 mμ (Schwert and Takenaka, 1955) against an enzyme-free blank. Activities are reported in micromoles of α-*N*-Bz-L-Arg-ethyl ester hydrolyzed per minute for the maximal linear part of the hydrolysis and specific activities computed on the basis of protein values obtained by the method of Lowry *et al.* (1951). The trypsin used for comparison of activities on various substrates was purchased from Worthington Biochemical Corp. For enzymatic assays on substrates other than α-*N*-Bz-L-Arg-ethyl ester, see legend of Table I.

Tris-Cl buffers used in the enzymatic assays were of ionic strength 0.05 at 25° unless otherwise stated, and were prepared according to the data given by Datta and Gryzbowski (1961).

Sucrose gradient sedimentation was carried out at 25° in the SW-65 rotor of the Spinco Model L-2 centrifuge.

Results

Behavior of the 7S NGF and γ Subunits on Various Substrates. The specific enzymatic activities of the 7S NGF and the γ subunits with regard to a number of different substrates are given in Table I. Activities for trypsin are also included for comparison. In each case, the hydrolysis with 7S NGF was characterized by an initial lag phase followed by a maximal linear rate while the γ enzyme, as well as trypsin, exhibited initial linear rates of hydrolysis. Also in each case, the ratio between 7S NGF and γ specific activities was found to be lower than the 0.4–0.5 value expected on the basis of the proportion of γ to total 7S protein (A. P. Smith, S. Varon, and E. M. Shooter, in preparation). Although the different conditions and enzyme concentrations used for the various assays, as will become clear in subsequent sections, preclude the possibility of strictly comparing the relative activity of the γ subunits and the 7S NGF with different substrates, it is evident that the divergence in kinetic behavior previously noted for the two species (Greene *et al.*, 1968) holds for all the substrates tested.

Subunit Interaction and Enzymatic Activity. The differences in enzymatic properties between the isolated γ protein and the 7S NGF preparation indicate that the former is functionally altered in some manner when it interacts with the α and β subunits to form the 7S complex. To examine the nature of this interaction, hydrolysis of α-*N*-Bz-L-Arg-ethyl ester by γ subunits was studied in the presence of either or both of the other subunits.

Mixtures of the γ enzyme with either α or β subunits, or both, were incubated in pH 7.0 Tris-Cl buffer (I = 0.05) at 0° for 1 hr and then a portion of each mixture was tested for enzymatic activity with α-*N*-Bz-L-Arg-ethyl ester as substrate in the same buffer. The incubation and assay in the presence of excess α or β subunits had very little effect on the observed activity of the γ subunits (Table II). The same results were obtained when purified γ^3 subunits were tested in the presence of β subunits. This form of the γ subunits has been shown to aggregate with β subunits to form a $\beta\gamma$ complex under conditions similar to those of the present incubation (Smith *et al.*, 1968). When all three subunits were incubated together and then assayed as described above, a dramatic decrease in the enzymatic activity of the mixture was observed, together with the appearance of a lag phase (Table II). Hence, the features characterizing the enzymatic behavior of 7S NGF were re-

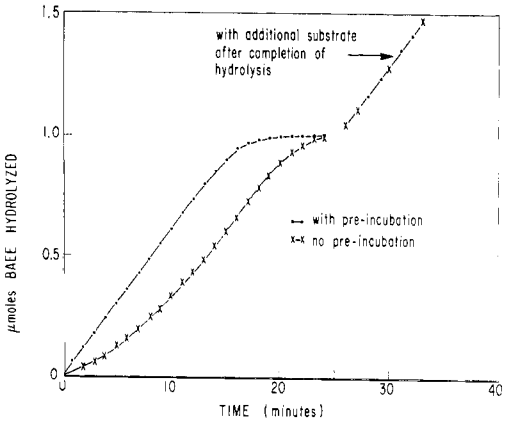


FIGURE 1: The effects of preincubation of 7S NGF on the kinetics of the hydrolysis of α-*N*-Bz-L-Arg-ethyl ester. Aliquots (10 μl) of 7S stock solution (200 μg/ml) were diluted into Tris-Cl buffer (pH 8.0, $I = 0.05$) containing 0.5 mM α-*N*-Bz-L-Arg-ethyl ester. Both samples were assayed immediately after addition of substrate. After substrate exhaustion, both mixtures received an additional 0.1 ml of 10 mM α-*N*-Bz-L-Arg-ethyl ester and were immediately examined for substrate hydrolysis.

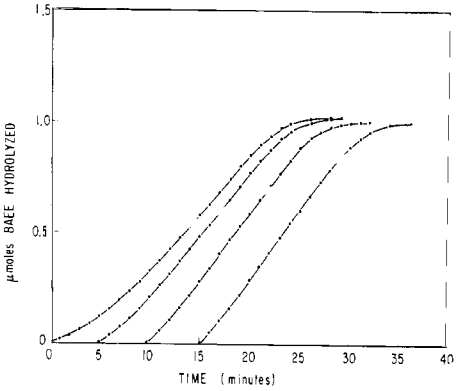


FIGURE 2: Effects of the length of preincubation on the lag phase. Mixing and preincubation of samples as in Figure 1. Length of preincubation given by the point on the abscissa at which each assay begins.

produced under conditions where it is known that aggregation of the subunits results in the formation of the 7S complex (Varon *et al.*, 1967b). A lower activity and a lag phase were also observed at pH 8, although to a lesser extent, but could not be detected at pH 9 (Table II). These observations will be discussed in a later section.

Origin of the Lag Phase. The occurrence of a lag phase in the hydrolysis of substrate by 7S NGF and by combinations of all three subunits suggests either that the enzymatic properties of the 7S complex are affected by the presence of the substrate and/or its hydrolysis products, or that a slow activation of the enzyme occurs due to the physical conditions of the assay itself. The esterase activity of the 7S NGF complex was tested at pH 7.0 at concentrations of α-*N*-Bz-L-Arg-ethyl ester from 0.125 to 1.0 mM. A double-reciprocal Lineweaver-Burk plot constructed from these data revealed no activation of the enzyme by the α-*N*-Bz-L-Arg-ethyl ester substrate. In addition, the presence of 1.0 mM concentrations of α-*N*-Bz-L-Arg, L-Arg, or ethyl alcohol during an assay had no effect on the kinetics of hydrolysis. Hence, interaction of 7S NGF with the products of α-*N*-Bz-L-Arg-ethyl ester hydrolysis also appears not to be responsible for the generation of the lag phase.

To assess the effect of the assay conditions on the 7S esterase activity, the preparation was allowed to preincubate in the appropriate buffer before addition of the substrate. To achieve this, a preparation of the 7S material was diluted from a stock solution (200 μg/ml in Tris-Cl buffer pH 8.0, $I = 0.05$) to a final concentration of 1 μg/ml in the same buffer. This mixture was incubated at 25° for 20 min, the approximate time necessary for complete hydrolysis of 1 μmole of α-*N*-Bz-L-Arg-ethyl ester at this concentration of enzyme. Following this incubation, substrate was added and its hydrolysis was observed. For comparison, an aliquot from the same stock solution of 7S NGF was diluted directly into substrate-containing buffer and immediately assayed. As shown in Figure 1, the two samples hydrolyzed α-*N*-Bz-L-Arg-ethyl ester at similar maximal rates but no lag phase was observed with the preincubated 7S preparation. After both assays had gone to completion, another equal aliquot of α-*N*-Bz-L-Arg-ethyl ester was added to the two reaction mixtures. Figure 1 shows that in both cases, hydrolysis of the new substrate proceeded without a lag phase and with similar linear rates.

The manner in which the elimination of the lag phase depended upon the time of preincubation, that is the interval between dilution and substrate addition, was examined next. The stock solution of 7S material was diluted into buffer as in the previous experiment, the α-*N*-Bz-L-Arg-ethyl ester substrate was added after no or various time intervals, and the completed mixtures was assayed at once. Figure 2 shows that the lag period decreased as the time of preincubation was increased. Thus, the lag period appears to reflect an activation phenomenon associated with the dilution of the 7S NGF preliminary to its enzymatic assay.

To test also whether this activation phenomenon was reversible, a dilute (1 μg/ml) 7S NGF solution, preincubated to eliminate the lag phase, was concentrated 75-fold with a UM-1 Diaflo membrane (Amicon Corp.) and then reassayed in the usual manner immediately after dilution to its original concentration. The typical lag phase appeared in the hydrolysis of substrate in this assay. If, however, this rediluted solution was preincubated without substrate for 20 min, the lag phase was once again eliminated. The linear maximal rates reached in both assays were the same. Thus, the activation of the 7S NGF preparation by dilution appears to be reversible.

Dissociation Equilibrium and the Enzymatic Activity of 7S NGF. One possible interpretation for the origin of the reversible activation of the 7S NGF preparation as seen in the lag phase was that a change in the state of aggregation of the molecule on dilution released a more active enzyme species. Since the enzymatic activity of the γ subunit is observed to be greatly reduced when the γ protein is incorporated into the 7S complex, then, conversely, dissociation of the 7S NGF complex should be accompanied by an increase in enzymatic activity. The lag phase would thus reflect the time required to achieve the new dissociation equilibrium between the 7S species and active subunits or other intermediate complexes. The initial level of enzymatic activity observable in the lag phase is so low that it appears likely that the 7S NGF species itself possesses little if any enzymatic activity. To test this interpretation, several factors which might be expected to change the degree of

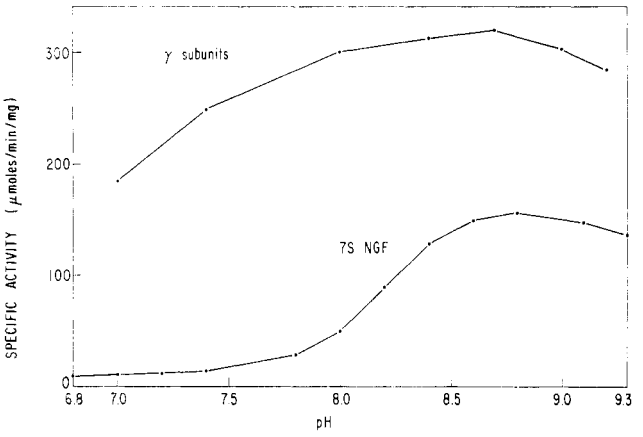


FIGURE 3: Effect of pH on the specific activities of 7S NGF and γ subunits. Enzymatic assays performed in Tris-Cl buffer of constant ionic strength 0.05. Concentrations of 7S NGF and γ subunits for all assays were 1.2 and 0.3 μg per ml, respectively.

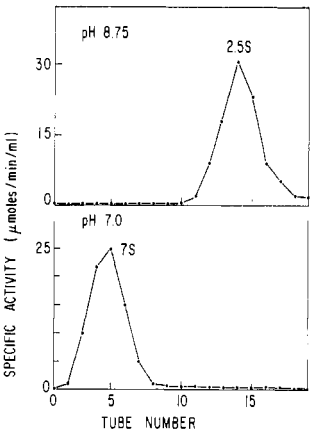


FIGURE 5: Effects of pH on the aggregation state of the γ subunit in a 7S NGF preparation. Stock 7S NGF was diluted into Tris-Cl buffer (I = 0.05), pH 7.0 or 8.75. Loads (2 μg of protein in 200 μl) were applied to 5–20% sucrose gradients in the same buffer. Centrifugation was carried out at 25° for 11.5 hr at 45,000 rpm. Contents were collected fractionally and assayed for enzymatic activity at pH 8.75.

association of the 7S NGF material were examined for their influence on its observable enzymatic activity.

Effects of pH on the Enzymatic Activity. The pH-induced dissociation of the 7S species has been studied (Varon *et al.*, 1968) at 4° and at concentrations near 10 mg/ml. As the pH was raised from 8 to 10, there was a progressive decrease in the $s_{20,w}$ of the main components from 7 to 4.5S along with the appearance of free 2.5S subunits. Complete dissociation into 2.5S subunits was apparently achieved by pH 9.3. The model presented for the enzymatic behavior of 7S NGF would, therefore, predict an increase in observed esterase activity of the preparation as its pH is increased. Increases in pH might also be expected to increase the rate at which the dissociation equilibrium is reached on dilution and therefore decrease the duration of the lag phase. Under conditions of complete dissociation, the lag phase should be absent.

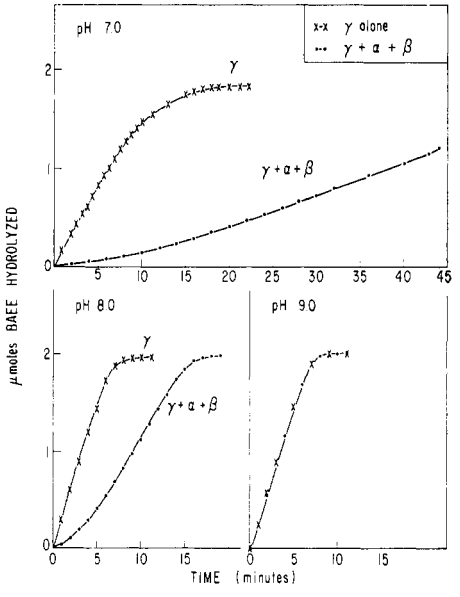


FIGURE 4: Effects of pH on the kinetics of hydrolysis of α-*N*-Bz-L-Arg-ethyl ester by isolated γ subunits mixed and incubated with α and β subunits. The γ subunits (100 μg/ml) were incubated alone or in the presence of excess α and β subunits (200 μg/ml) for 1 hr at 0° in the appropriate Tris-Cl buffer (I = 0.05). A 10-μl portion of each mixture was then tested for enzymatic activity with 2.0 ml of 1 mM α-*N*-Bz-Arg-ethyl ester in the same buffers.

In order to test these predictions, the rate of hydrolysis of α-*N*-Bz-L-Arg-ethyl ester by preparations containing either 7S NGF or γ subunits was studied in Tris-Cl buffer of constant ionic strength over a pH range from 6.8 to 9.3. As shown in Figure 3, the specific activity of the 7S NGF underwent a marked rise from 10 to 160 μmoles per min per mg over this pH range. In contrast, the specific activity of the γ enzyme increased only from 190 μmoles/min per mg at pH 7.0 to a broad maximum of about 320 μmoles/min per mg at pH 8–9. In addition, with increasing pH values the lag phase observable with 7S preparations became progressively shorter and was no longer detectable at pH values greater than 8.6. The effects of exposure to pH 9 could be reversed by dialysis against a lower pH buffer, with the return of both the lower specific activity and the lag phase. The maximal specific activity exhibited by 7S preparations at pH values higher than 8.6 was about 50% of that exhibited by the γ subunits at the same pH values (Figure 3). This value agrees reasonably well with the relative content of γ protein in the 7S complex and suggests that the γ subunits contained in the original 7S NGF were completely active at pH values near 9. These experiments are complemented and supported by those described in Table II where the enzymatic activity of a fixed amount of γ protein was decreased by preincubation with α and β subunits by fivefold at pH 7, twofold at pH 8, and not at all at pH 9. Figure 4 shows that, for these experiments, a lag phase occurred at pH 7, was shorter at pH 8, and was no longer detectable at pH 9.

The interpretation of these results in terms of the dissociation equilibrium model would be that, at pH values near 9 and in the concentration ranges used, most or all of the γ protein is in the form of free subunits. This was confirmed by sucrose density sedimentation of 7S material at pH 8.75 at comparable concentrations and temperatures. A scan of the gradient revealed esterase activity solely in the 2.5S subunit region (Figure

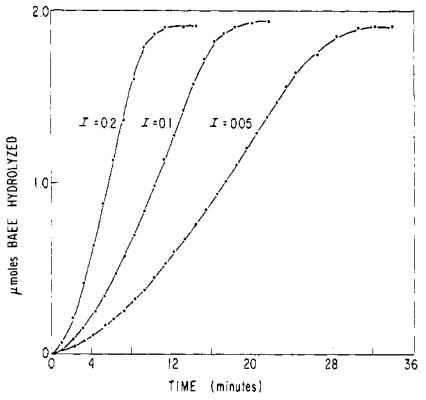


FIGURE 6: Influence of ionic strength on the observed kinetics of hydrolysis of α-*N*-Bz-L-Arg-ethyl ester with 7S NGF. Assays performed in pH 7.6 Tris-Cl buffers and with a 7S NGF concentration of 2.5 μg/ml.

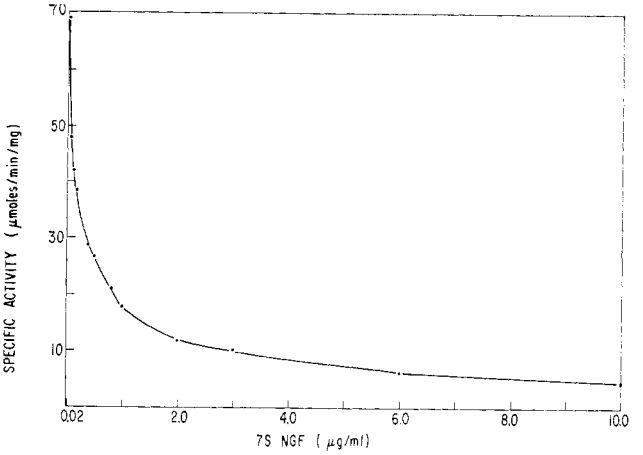


FIGURE 7: Specific enzymatic activity of a 7S NGF preparation tested at various concentrations. Assays performed in Tris-Cl buffer (I = 0.05), pH 7.0.

5a). A similar gradient run at pH 7.0 showed the enzymatic activity to sediment only in the 7S region (Figure 5b).

Influence of Ionic Strength on the Enzymatic Activity of the 7S NGF. Because of the relatively extreme electrochemical properties of two of the subunits of 7S NGF, changes in ionic strength may be expected to affect the rate and degree of dissociation of the 7S NGF complex and hence the lag phase and the enzymatic activity of the system. As shown in Figure 6, at pH 7.6 both of these effects were observed. As the ionic strength was varied from 0.05 to 0.2 the lag phase decreased and the apparent specific activity increased threefold as compared with a 10% rise with isolated γ subunits.

Effects of High Dilution on Enzymatic Activity of 7S NGF. High dilution should shift a dissociation equilibrium toward a less aggregated state, which in the proposed model would result in the release and, therefore, the activation of the γ enzyme. The specific activity of 7S NGF preparations was examined over a range of concentrations from 0.02 to 12 μg per ml at pH 7.0, ionic strength 0.05. The results, as shown in Figure 7, indicate that, under these conditions, the specific activity increased greatly as the concentration in the assay mixture was decreased. The specific activity of isolated γ subunits, when similarly tested over a range of 0.015–1 μg/ml, remained constant. The effect of dilution on the 7S NGF was reversible in that a curve similar to that of Figure 7 could be reproduced after reconcentration of a highly dilute preparation. Furthermore, when the 7S material was assayed at pH 9 over a range of 0.05–6 μg/ml, the specific activity remained the same at all concentrations, as would be expected under conditions already favoring complete dissociation.

Effects of Free α and β Subunits on the Enzymatic Activity of the 7S NGF. If conditions which favor dissociation of the 7S complex increase the observed enzymatic activity of the preparation, then conditions which shift the dissociation equilibrium toward the complex should decrease or possibly eliminate the observed esterase activity. If the dissociation ultimately involves the formation of free subunits, then the introduction of additional subunits into a 7S NGF preparation should, under given conditions, shift the dissociation equilibrium more in favor of the 7S complex and lower the enzymatic activity of the system.

Purified α and β subunits were added to a 7S NGF preparation of 2.5 μg/ml (pH 7.0). In each case, the mixtures were allowed to incubate for 3–6 hr at 25° so that the system could reach equilibrium before being assayed. When either the α or the β subunits were added separately, the observed activity of the system was decreased to limiting values of approximately 5 μmoles/min per mg of initial 7S material or about 50% of the starting activity (Figure 8). No additional decrease beyond this level was achieved with only one free subunit at concentrations up to 20 μg/ml. However, when both α and β subunits were added either simultaneously or successively at concentrations of 5 and 2 μg per ml, respectively, the original activity was decreased to 10% of its original value (Figure 8). Further addition of both isolated subunits was again ineffective in reducing this value.

Discussion

A number of observations reported elsewhere suggest that 7S NGF is in equilibrium with its dissociation products at neutral pH and at relatively high protein concentrations.

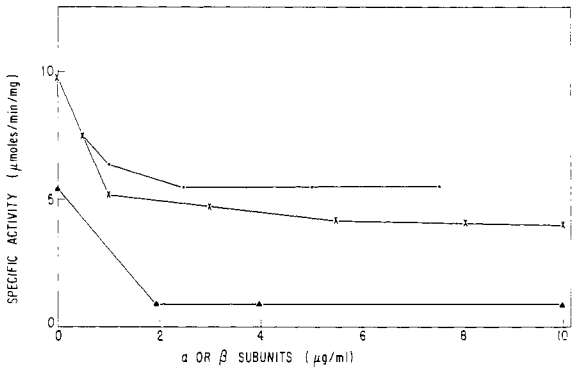


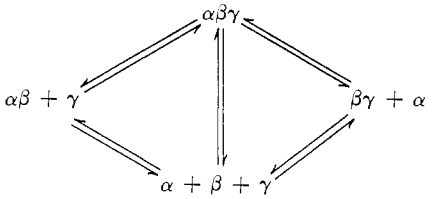
FIGURE 8: Influence of isolated α and β subunits on the apparent specific activity of 7S NGF. Assays performed in all cases in pH 7.0 (I = 0.05) Tris-Cl buffer with 7S NGF (2.5 μg/ml) preincubated and tested in the presence of (1) varying concentrations of β subunits (●—●—●), (2) varying concentrations of α subunits (×—×), and (3) 5 μg/ml of β and varying concentrations of α subunits (▲-▲-▲).

Firstly, good recovery of the 7S NGF protein on DEAE-cellulose chromatography is only achieved by elution at unusually fast flow rates, slower rates leaving a proportion of the α subunits bound to the resin (Varon *et al.*, 1967a,b; Shooter *et al.*, 1968). Secondly, the multiple forms of 7S NGF themselves appear to be in equilibrium with one another, as indicated by the failure to resolve them on electrophoresis or ion-exchange chromatography and by the distribution of subunits across the 7S NGF zones in either procedure (Smith *et al.*, 1968). Thirdly, the existence of a relatively rapid equilibrium between 7S NGF and its α subunits at neutral pH has been directly demonstrated (A. P. Smith, L. A. Greene, H. R. Fisk, S. Varon, and E. M. Shooter, in preparation). This equilibrium, at neutral pH and high protein concentrations, lies largely in favor of the 7S complex, as judged by sedimentation properties. It is not unreasonable to expect that changes in concentration, pH, or ionic strength might shift the equilibrium toward a greater formation of dissociation products.

Evidence is presented in this paper that the enzymatic properties of the 7S NGF preparation may be described in terms of an equilibrium association–dissociation model. In this model, the enzymatic activity of a 7S NGF preparation is associated with the free, or partially complexed, species of the γ protein in equilibrium with the 7S complex, while the latter has itself very low or no enzymatic activity. The effects on the apparent specific activity of 7S NGF by a number of conditions such as high pH, high ionic strength, high dilution, preincubation at high dilution, and incubation with free α and β subunits all tend to support this model. High pH and high ionic strength in dilute solution promote dissociation of the 7S complex and release fully active forms of the γ subunits. Conversely, the addition of excess free α and β subunits favors the aggregation of the γ protein into a 7S complex and decreases the measured enzymatic activity of the mixture.

The inhibition of an enzymatically active subunit by recombination with other nonidentical subunits has been described in other systems, for example, the tryptophan synthetase complex (Crawford and Ito, 1964). Such cases involve interaction of the active subunit with one other type of subunit. The 7S NGF system stands unique, at present, in that two nonidentical subunit types (α and β) are required for the inhibition of the activity of the third, the γ enzyme.

It is not clear at present by which steps the association–dissociation of 7S NGF takes place. Since the species $\alpha\beta$ and $\beta\gamma$[2] have been demonstrated to exist (Varon *et al.*, 1968), one may conceive of equilibrium steps as



The effects of isolated α and β subunits on the apparent specific activity of 7S NGF indicate that association–dissociation by at least one of these pathways may be occurring at neutral pH under conditions at which the enzymatic assays are performed. Although high dilution greatly increases the expressed esterase activity of the 7S preparation, it is difficult to judge the state of aggregation of the system since one is measuring the relative competition between the substrate and free subunits or subunit complexes for the active species of γ rather than the extent of the dissociation itself.

From the data presented here, it is not possible to distinguish whether or not the 7S complex is either very poorly active or totally inactive. The small amount of activity remaining at pH 7.0 after addition of excess α and β subunits may be attributable to the 7S complex itself, to a residual species still in equilibrium with the 7S NGF, or to a minor active contaminant in the purified materials.

Biological Relevance of the γ and the 7S Proteins. As an individual species, the γ enzyme belongs to the category of esteropeptidases, which has been the subject of several reports and was briefly reviewed in a preceding paper (Greene *et al.*, 1968). Gel filtration of male mouse submaxillary extracts resolves these esteropeptidases into three classes, the 7S complex containing the γ enzyme, an enzyme, or enzymes with mol wt 60,000–70,000, and a third class of proteins with mol wt 25,000–30,000 (S. Varon and L. A. Greene, unpublished). Two distinct species of the latter size have been isolated (Calissano and Angeletti, 1968). Furthermore, the γ enzyme itself occurs in four forms, recognizable and separable by net charge differences (Greene *et al.*, 1968). While all these enzymes have been found to act on certain esters, amides, and protein, there is no definite clue as to what their specific "physiological" substrate(s) may be, nor is it known to which cellular targets they may be directed. One intriguing possibility that they may be involved in control mechanisms for growth and differentiation is suggested by the reported association of such enzymatic activities with the mesenchymal growth factor (Attardi *et al.*, 1967) and the nerve growth factor (Greene *et al.*, 1968, and present paper, Schenkein *et al.*, 1968), by an NGF-like activity reported for thrombin, itself an esterase (Hoffman and McDougall, 1968), and by a recently described effect of submaxillary esteroproteases on the growth of cultured rat hepatoma cells (Grossman *et al.*, 1969).

The occurrence of the γ enzyme as a 7S complex in association with two other distinct subunits, β and α, raises a more specific question as to the interrelation among these three protein subunits and its relevance to biological phenomena. A good deal of evidence supports the specificity of the physical interaction among the α, β, and γ components of the 7S complex. NGF activity is recovered from the male gland crude extract exclusively in the 7S complex (Varon *et al.*, 1967a,b; Angeletti *et al.*, 1967). The same is true for crude extracts of female glands either before or after testosterone treatment (S. Varon and L. A. Greene, unpublished). No 7S complex formation could be obtained by mixing α and β subunits with either the 60,000–70,000 or the 25,000–30,000 molecular weight esteropeptidases fractionated out of a crude gland extract by gel filtration (S. Varon and L. A. Greene, unpublished), even though the latter class of proteins resemble the γ proteins in enzymatic abilities, molecular size and net charges. Thus, free forms of neither β nor γ subunits are observable in the crude extracts by the above criteria. The 7S protein occurring in the gland extract is a defined species by a number of physicochemical criteria. It can be purified in a stable form and exhibits a constant stoichiometric relationship among its

[2] Since the stoichiometry of these complexes has not yet been determined, 7S NGF will be designated as $\alpha\beta\gamma$ and the complexes of β with α and γ, respectively, as $\alpha\beta$ and $\beta\gamma$.

three subunits, the same as that shown to be necessary and sufficient for the regeneration of the 7S complex from its isolated subunits. Finally, the β protein is not replaceable as a 7S complex component by other basic proteins such as trypsin, ribonuclease, lysozyme, or protamine (Shooter and Varon, 1969).

The work reported here clearly establishes the occurrence of a functional, beside a physical, interaction among all three components of the 7S complex, in that the enzymatic activity of the γ protein is suppressed by aggregation with both, but not either, the α and the β subunits. There is evidence (S. Varon and L. A. Greene, unpublished) that the complex masks the γ protein not only with respect to substrates but also to a number of inhibitors. It is not inconceivable that a similar situation be also true for the β subunit carrying the nerve growth-promoting activity. The observation that a greater amount of β protein is needed in the isolated form than in the complex form to elicit the same response in the standard bioassay for NGF activity may indeed reflect such a protective effect of the complex on the active subunit, one of the various interpretations already suggested for this apparent "activation" (Varon *et al.*, 1967b, 1968). It will be important to establish what biological activity may be associated with the third obligatory partner in the 7S complex, the α subunit. The specific association in the same complex of at least two biological activities, the enzymatic activity of the γ protein and the nerve-growth promoting activity of the β protein, raises the question as to what role or roles such a complex may play in the organism. While a number of speculations are possible, the question cannot be answered at present.

References

Angeletti, P., Calissano, P., Chen, J. S., and Levi-Montalcini, R. (1967), *Biochim. Biophys. Acta 147*, 180.

Attardi, D. G., Schlesinger, M. J., and Schlesinger, S. (1967), *Science 156*, 1253.

Calissano, P., and Angeletti, P. U. (1968), *Biochim. Biophys. Acta 156*, 51.

Cohen, S. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 302.

Crawford, I. P., and Ito, J. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 390.

Datta, S. P., and Gryzbowski, A. (1961), Biochemists' Handbook, Long, C., Ed., Princeton, N. J., D. Van Nostrand, p 33.

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys. 95*, 271.

Greene, L. A., Shooter, E. M., and Varon, S. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 1383.

Grossman, A., Lele, K. P., Sheldon, J., Schenkein, I., and Levy, M. (1969), *Exptl. Cell Res. 54*, 260.

Hoffman, H., and McDougall, J. (1968), *Exptl. Cell Res. 51*, 485.

Hummel, B. C. W. (1959), *Can. J. Biochem. Physiol. 37*, 1393.

Levi-Montalcini, R. (1965), *Harvey Lectures Ser. 60*, 217.

Levi-Montalcini, R., and Angeletti, P. U. (1968), *Physiol. Rev. 48* (3), 534.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Popov, A. G., and Krutyakov, V. M. (1964), *Biochemistry USSR 29*, 751.

Schenkein, I., Levy, M., Bueker, E. D., and Tokarsky, E. (1968), *Science 159*, 640.

Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.

Shooter, E. M., and Varon, S. (1969), *in* Protein Metabolism of the Nervous System, Lajtha, A., Ed., New York, N. Y., Plenum (in press).

Shooter, E. M., Varon, S., and Nomura, J. (1968), *Chimia 22*, 144.

Smith, A. P., Varon, S., and Shooter, E. M. (1968), *Biochemistry 7*, 3259.

Varon, S. (1968), *J. Pediatr. Surg. 3*, 120.

Varon, S., Nomura, J., and Shooter, E. M. (1967a), *Biochemistry 6*, 2202.

Varon, S., Nomura, J., and Shooter, E. M. (1967b), *Proc. Natl. Acad. Sci. U. S. 57*, 1782.

Varon, S., Nomura, J., and Shooter, E. M. (1968), *Biochemistry 7*, 1296.

Werbin, H., and Palm, A. (1951), *J. Am. Chem. Soc. 73*, 1382.