Cohen, S. (1960), *Proc. Natl. Acad. Sci. U.S.A. 46,* 302.
Frazier, W. A., Angeletti, R. H., and Bradshaw, R. A. (1972), *Science 176,* 482.
Greene, L. A., Shooter, E. M., and Varon, S. (1969), *Biochemistry 8,* 3735.
Holyer, R. H., Hubbard, C. D., Kettle, S. F. A., and Wilkins, R. G. (1966), *Inorg. Chem. 5,* 622.
Levi-Montalcini, R. (1966), *Harvey Lect. 60,* 217.
Levi-Montalcini, R., and Hamburger, V. (1953), *J. Exp. Zool. 123,* 233.
Moore, J. B., Mobley, W. C., and Shooter, E. M. (1974), *Biochemistry 13,* 833.
Pattison, S. E., and Dunn, M. F. (1975), *Biochemistry 14,* 2733.
Perez-Polo, J. R., Bamburg, J. R., DeJong, W. W. W., Straus, D., Baker, M., and Shooter, E. M. (1972), in Nerve Growth Factor and its Antiserum, E. Zaimis, Ed., London, Anthlone Press, p 19.
Schenkein, I. (1972), *Handb. Neurochem. 5B,* 503.
Sillén, L. G., and Martell, A. D., Ed. (1964), *Chem. Soc., Spec. Publ. 17.*
Smith, A. P. (1969), Doctoral Thesis, Stanford University.
Smith, A. P., Varon, S., and Shooter, E. M. (1968), *Biochemistry 7,* 3259.
Varon, S., Nomura, J., and Shooter, E. M. (1967), *Biochemistry 6,* 2202.

On the Mechanism of Divalent Metal Ion Chelator Induced Activation of the 7S Nerve Growth Factor Esteropeptidase. Thermodynamics and Kinetics of Activation†

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ABSTRACT: The 7S nerve growth factor protein (7S NGF) is a multisubunit zinc metalloprotein containing a masked trypsin-like esteropeptidase activity. Reaction of the native 7S NGF oligomer with divalent metal ion chelators effects an approximately sevenfold activation of the esteropeptidase activity via the sequestering and dissociation of the 7S NGF-bound zinc ion (Pattison, S. E., and Dunn, M. F. (1975), *Biochemistry 14,* 2733; Pattison, S. E., and Dunn, M. F. (1976), *Biochemistry,* preceding paper in this issue). In this study, investigation of the relationship between chelator concentration and the extent of activation, as measured by the steady-state rate of hydrolysis of α-*N*-benzoyl-D,L-arginine-*p*-nitroanilide, has demonstrated that (a) the chelator-induced activation is a freely reversible process, (b) activated 7S NGF undergoes a slow loss of reversibility when incubated with chelator over long time-periods, (c) the affinity constant of 7S NGF for zinc ion is $\sim 10^{10.5} \pm 10^{0.5}$ M^{-1}, (d) chelator activation depends only on the ability of the chelator to sequester zinc ion, and (e) the activation process does not involve dissociation of the 7S oligomer to smaller subunit aggregates under conditions of low ionic strength.

The nerve growth factor protein (NGF)[1] promotes growth and differentiation of the noradrenergic neurons of the superior cervical ganglia and the sensory neurons of the dorsal root ganglia (see reviews by Levi-Montalcini, 1966; and Schenkein, 1972). The 140 000-dalton nerve growth factor (7S NGF) is an oligomeric protein consisting of three distinct classes of subunits, α, β, and γ (Smith et al., 1968). The β subunit contains the growth-promoting activity, while γ is a potent trypsin-like esteropeptidase which is inhibited while bound in the oligomer (Greene et al., 1969). The existence of a stable oligomer (7S NGF) that appears to be no more biologically active in eliciting neurite outgrowth than one of its subunits (β) in the in vitro organ culture assay (Varon et al., 1967) has raised questions concerning the functional significance of the α and γ subunits. The objective of our studies has been to investigate the physical and enzymatic interactions between the γ enzyme and the α and β subunits in 7S NGF in order to define the structural and functional properties of the oligomeric protein.

Our previous studies have established the following: (1) 7S NGF is a zinc metalloprotein, (2) zinc ion is a potent and specific inhibitor of both the native 7S NGF esteropeptidase and the γ-esteropeptidase ($K_i \simeq 8 \times 10^{-7}$ M) (Pattison and Dunn, 1975), and (3) zinc ion interacts directly with chelators during 7S NGF esteropeptidase activation (Pattison and Dunn, 1976).

In this paper, we establish (1) chelator activation of the 7S NGF esteropeptidase is a reversible phenomenon that appears to involve direct interaction of the chelator with zinc ion (and not subunit dissociation), (2) the affinity of 7S NGF for zinc ion is several orders of magnitude higher than the affinity of any of the isolated subunits for zinc ion, and (3) both ther-

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[1] Abbreviations used are: NGF, nerve growth factor; BAPNA α-*N*-benzoyl-D,L-arginine-*p*-nitroanilide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; CDTA, (*trans*-1,2-diaminecyclohexane)tetraacetic acid; OP, *o*-phenanthroline; NTA, nitrilotriacetic acid; BP, 2,2′-bipyridyl; MDA, *N*-methyliminodiacetic acid.

modynamic and kinetic evidence strongly indicate that chelator-induced activation is dependent only on the chelating ability of the chelator.

Materials and Methods

The 7S NGF was isolated from the submaxillary glands of adult Swiss-Webster male mice (>35 g) according to the method of Varon et al. (1967). The individual subunits were isolated from 7S NGF via the method of Smith et al. (1968). All protein manipulations were carried out at 5 °C. Protein concentrations were determined as previously described (Smith, 1969; Pattison and Dunn, 1975).

The chromophoric substrate, α-*N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) (Sigma) was used to determine the esteropeptidase activity of both 7S NGF and the γ subunit by the procedure of Pattison and Dunn (1975). For each assay, the rate of *p*-nitroaniline production in 0.05 M Tris buffer (Sigma), pH 7.40, was monitored at 410 nm with either a Beckman DB-GT or a Varian 635 spectrophotometer at 25 ± 0.2 °C. The kinetic experiments were initiated by addition of either the esteropeptidase or BAPNA to a Tris-HCl buffered solution of the other assay components. The specific details of each assay are given in the appropriate figure captions, see Results.

Structural studies via gel filtration were carried out using both Bio-Gel P-100 and P-4 columns (each 1 × 50 cm). Control elution patterns were obtained by applying native 7S NGF to the same columns equilibrated with 0.05 M Tris buffer, pH 7.4. The same column was then reequilibrated with 0.05 M Tris buffer containing 1 mM EDTA, and 7S NGF, preincubated with 1 mM EDTA, was eluted again. The gel filtration was run at room temperature with a pressure head of ~45 cm.

Sedimentation velocity centrifugation was carried out using a Beckman Model E analytical ultracentrifuge equipped with an AN-D rotor and photoelectric scanner attachment. All runs were made at 59 780 rpm, 20 °C in 0.05 M Tris buffer, pH 7.4. The protein concentrations were approximately 0.4 mg/ml.

Results

Thermodynamics of Chelator Activation. The concentration dependence of the chelator-induced activation of the 7S NGF esteropeptidase activity, measured as the rate of hydrolysis of α-*N*-benzoylarginine-*p*-nitroanilide (BAPNA), is shown in Figure 1. These data show that at low chelator concentrations, increases in chelator concentration bring about nearly proportional increases in esteropeptidase activity for all chelators but EDTA, CDTA, and terpyridine. EDTA, CDTA, and terpyridine yield sigmoidal dependencies. At higher chelator concentrations, the extent of activation saturates for all chelators. When the various chelators, each at a concentration capable of maximally activating the 7S NGF esteropeptidase, are incubated with a standardized 7S NGF solution, the same final activated rate is achieved in all cases. (Since different preparations of 7S NGF were used for the various isotherms presented in Figure 1, the different saturated activities observed for different chelators reflect slight variations in the concentrations of native 7S NGF employed.)

It is clear in Figure 1 that the various chelator activation isotherms show varying degrees of sigmoidicity. The degree of sigmoidicity has been quantitated via a "Hill plot" (Table I). (In this regard, use of the Hill coefficient, *n*, is not intended to convey a mechanistic significance.)

Markedly sigmoidal activation occurs both with a tridentate heterocyclic chelator (terpyridine) and with hexadentate carboxylic acid chelators (EDTA, CDTA) (Figure 1d). Within

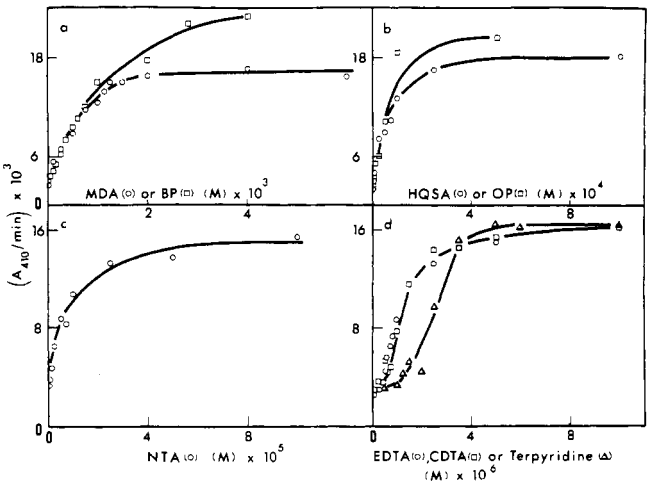

FIGURE 1: The dependence of the extent of 7S NGF esteropeptidase activation on the concentration of selected divalent metal ion chelators. A concentration of 0.2 ± 0.05 μM 7S NGF was incubated in 0.05 M Tris buffer, pH 7.4, with a selected chelator under the following conditions: (1) for all chelators except EDTA, the chelator–7S NGF mixture was incubated at room temperature for at least 1 h prior to initiation of the esteropeptidase assay, (2) the EDTA–7S NGF mixture was incubated at 5 °C for 24 h prior to initiation of the esteropeptidase assay. The steady-state esteropeptidase activity was assayed by measuring the hydrolysis rate (v) for the artificial amide substrate, BAPNA. The assay was initiated by addition of a 10-μl aliquot of substrate to give a final BAPNA concentration of 1 mM. The divalent metal ion chelators investigated are: (a) 2,2′-bipyridyl (BP, □) and *N*-methyliminodiacetic acid (MDA, ○), (b) *o*-phenanthroline (OP, □) and 8-hydroxyquinoline-5-sulfonic acid (HQSA, ○), (c) nitrilotriacetic acid (NTA, ○), (d) EDTA (○) CDTA (□), and 2,2′,2″-terpyridine (△).

their respective structural classes (either heterocyclic or carboxylic acid), these sigmoidal-activating chelators have the highest ligand per chelator ratio of the various chelators that were studied. These chelators also elicit activation at relatively low chelator concentrations in comparison to the nonsigmoidal-activating chelators.

Reversibility of Chelator Activation. Under normal circumstances, removal of small-molecular-weight effectors via dialysis may restore a protein to its native state. However, a reversal of chelator activation could not be achieved by this method for the following reasons: (1) removal of chelator from 7S NGF by dialysis also removes the zinc ions that are endogenous to native 7S NGF; (2) the time span necessary for dialysis allows irreversible protein changes to occur (see below). As activation involves divalent zinc ion chelation (Pattison and Dunn, 1976), the addition of excess divalent metal ions to a chelator-activated 7S NGF assay mixture should reverse the activation by lowering the effective concentration of chelator. The validity of this approach is based on the finding that, of a number of common divalent metal ions tested (Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}), only zinc ion, at concentrations below 0.25 mM, produces significant inhibition of the 7S NGF esteropeptidase (see Pattison and Dunn, 1975).

Due both to the relative stability of the Cu(II) oxidation state and to the relatively high affinity of Cu(II) for divalent metal ion chelators, this ion was chosen for a detailed investigation of activation reversal brought about by added metal ion. The concentration dependence of the Cu(II)-mediated reversal adheres to a sigmoidal isotherm with a Hill coefficient of 2.7 ± 0.9 for EDTA (Figure 2). Thus, both the concentration dependence of EDTA activation and the concentration dependence of the reversal of the activation by Cu(II) show, within experimental error, the same sigmoidicity. Note that

TABLE I: Equilibrium Constants for the Interactions between Chelator, 7S NGF, and Zinc Ion.[a]

Chelator[d]	Chelator–Zn^{2+} Interaction K_{Che}[b]	Chelator-7S NGF Interaction [Che] at $V_i + \frac{1}{2}(V_{max} - V_i)$ (μM)	7S NGF–Zn^{2+} Interaction			Hill Coefficient
			K_{calcd}[a]	ΔG_0[c]	K_{7S}[a]	n[e]
o-Phenanthroline (3)	10^{16} M^{-3}	60	10^{5} M^{-2}	−14.4	10^{11}	1.4
8-Hydroxyquinoline-5-sulfonic acid (3)	10^{13} M^{-2}	70	10^{2} M^{-1}	−14.4	10^{11}	1.2
2,2′-Bipyridine (3)	$10^{13.4}$ M^{-3}	700	$10^{2.5}$ M^{-2}	−14.4	10^{11}	1.2
N-Methyliminodiacetic acid (2)	10^{10} M^{-2}	400	10^{0} M^{-1}	−13.1	10^{10}	1.0
Nitrilotriacetic acid (1)	10^{8} M^{-1}	10	10^{-2} M^{0}	−13.1	10^{10}	1.2
trans-1,2-Diaminecyclohexanetetraacetic acid (1)	$10^{14.3}$ M^{-1}	1	10^{-1} M^{0}	−20.0	10^{15}	2.2
(Ethylenediamine)tetraacetic acid (1)	$10^{-13.1}$ M^{-1}	1	10^{-1} M^{0}	−18.5	10^{14}	2.4
2,2′,2″-Terpyridine (2)	10^{12} M^{-2}	2	10^{5} M^{-1}	−9.2	10^{7}	2.6

[a] Values calculated assuming a mechanism as discussed in the text (eq 1-5) and K_{7S} is given as M^{-1}. Note that equilibrium constants have been rounded to the closest order of magnitude in most instances. [b] Values are taken from Sillén and Martell (1964) assuming a normal zinc ion-chelator binding stoichiometry. [c] ΔG_0 is given in kcal/mol. [d] The most stable zinc ion-chelator binding stoichiometry (x) (Sillén and Martell, 1964) is given in parentheses for each chelator. [e] Values taken from Hill plots for the data presented in Figure 1.

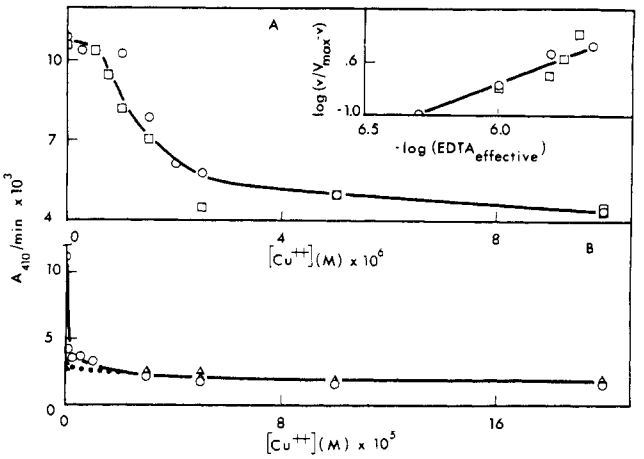

FIGURE 2: The Cu(II) concentration dependence of both EDTA-treated and native 7S NGF esteropeptidase activities. The effects of Cu(II) treatment on the 7S NGF esteropeptidase activity were determined under the following three sets of conditions: (1) a 0.05 M Tris, pH 7.4, buffered solution containing approximately 0.2 μM 7S NGF and 2.5 μM EDTA was first incubated for a minimum of 45 min at room temperature and then incubated for 10 min with various concentrations of Cu(II) (points designated by ○ in A and B); (2) following incubation of the same 7S NGF-EDTA mixture as in (1), the solution was incubated with 10 μM Cu(II) for 15 min. Then EDTA (final concentration, 10 μM) was added and the mixture was incubated for an additional 10 min with various concentrations of Cu(II) (points designated by □ in A); (3) a concentration of ~0.2 μM 7S NGF was incubated for 45 min in 0.05 M Tris buffer, pH 7.4, and then incubated for 10 min with various concentrations of Cu(II) (points designated by △ in B). A 10-μl aliquot of the BAPNA substrate was added in each experiment (final concentration 1 mM) to initiate the esteropeptidase assay. The esteropeptidase activity corresponds to the BAPNA hydrolysis rate (v, the rate of change of A_{410} with time). The inset to this figure shows plots of log $[v/(V_{max} - v)]$ vs. the log of the effective EDTA concentration for the data presented in Figure 3A. The effective EDTA concentration is approximated as the difference between the total EDTA concentration and the Cu(II) concentration.

only a slight excess of copper ion over EDTA is necessary to produce full reversal of the chelator-induced activation. The cycle of activation and reversal may be repeated on the same enzyme mixture with reproducible results (Fig. 2A). Once reversal is achieved, divalent copper ion elicits no further effect on the residual esteropeptidase activity over an incubation period of 48 h. Therefore, by all criteria examined, divalent copper ion causes a true reversal of EDTA-mediated activation.

During these experiments, it was noted that the ability of copper ion to effect reversal gradually diminished as the length of contact time between chelator and 7S NGF increased. In fact, after 24 h of incubation at room temperature, it was found that the addition of copper ion no longer brings about a reversal of chelator-induced activation. However, loss of reversibility has no effect on the esteropeptidase activity of activated 7S NGF.

The kinetics of the loss of reversibility were investigated by incubating 7S NGF with EDTA for various times followed by the addition of excess amounts of copper ion. This loss of reversibility fits a first-order rate law with a rate constant of 2.5×10^{-3} min^{-1} at 25.0 ± 0.2 ° C. Once 7S NGF has been incubated for 17 h, subsequent incubation of the assay mixture with Cu(II) for 30 h does not diminish the elevated esteropeptidase activity.[2]

Several concentration variables were found to have little or no effect on the rate constant for this slow step: (1) A 50% decrease in the 7S NGF concentration causes no significant change in the rate constant, although the initial rate is decreased by a similar proportion. (2) A twofold increase in the esteropeptidase activity brought about by the addition of γ enzyme to the assay mixture has no significant effect on the first-order rate constant. (3) A change in chelator concentration (i.e., three concentrations of EDTA, 2.5, 10, 25 μM, or two concentrations of 8-hydroxyquinoline-5-sulfonic acid, 0.1, 0.25 mM) produces no significant change in the rate constant.

Molecular Weight Changes Associated with Chelator Action. The EDTA system was chosen to investigate the effects of chelator concentration on the NGF aggregation state on the basis of chelator solubility and effectiveness in eliciting esteropeptidase activation. Since under saturating conditions (Figure 1) all the chelators used produce the same extent of activation (i.e., they yield the same final V_{max} value), it is assumed here and in the following discussion that the same final state is attained in each instance. Although the experiments have been limited to studies of the EDTA interaction with 7S NGF, the molecular weight data will be treated as a general chelator phenomenon.

Gel filtration of relatively small amounts of native 7S NGF (2 ml of ~4 μM) through a previously calibrated P-100 Bio-

[2] Use of the term "irreversible" here is both defined and limited by this observation.

Gel column at room temperature gave an elution profile with the protein peak eluting in the void volume. The shape of the trailing edge of this peak suggests the presence of trace amounts of molecular weight species that are smaller than the 7S NGF oligomer. The esteropeptidase activity of each fraction was found to be approximately proportional to the protein content of the fraction as measured by the 280-nm absorbance, Specific activity determinations for the main peak indicated that this protein possesses a specific esteropeptidase activity characteristic of native 7S NGF.

This same P-100 column was also equilibrated with a 1 mM EDTA solution. After incubation of 7S NGF with 1 mM EDTA for 30 min, the protein–EDTA mixture was applied to the column and eluted with buffer containing 1 mM EDTA. As with the control experiment, elution through the column required about 2 h. Thus, the 7S NGF is exposed to EDTA for only a short time relative to the time-course of the irreversible activation process. The elution pattern for this EDTA-equilibrated P-100 column was found to be indistinguishable from the profile of the control experiment. Again, the esteropeptidase activity was found to be proportional to the protein concentration as determined by 280-nm absorbance measurements. However, the esteropeptidase activity of the high-molecular-weight species was elevated approximately fivefold relative to the control.

A second set of experiments was carried out to compare the effects of EDTA on the zinc content of EDTA-treated 7S NGF on gel filtration through a P-4 Bio-Gel column. The untreated sample showed a zinc elution profile that was nearly coincident with the protein elution profile. However, after incubation with 1 mM EDTA for 30 min and elution over the same column equilibrated with 1 mM EDTA, the elution patterns showed that 7S NGF and zinc ion are well separated. Again, the esteropeptidase activities of the resulting protein fractions correspond respectively to native and to activated 7S NGF.

More sensitive molecular weight studies were undertaken via sedimentation velocity centrifugation in 0.05 M Tris-HCl buffer at pH 7.40. By use of the photoelectric scanner attachment, it was possible to carry out velocity sedimentation studies under the same conditions of concentration as the protein concentrations employed for the esteropeptidase activity assays. (A concentration of 1 mM EDTA produces maximum activation for protein concentrations up to at least 0.45 mg/ml.) In agreement with the findings of Smith et al. (1968), the data indicate that at protein concentrations of approximately 0.4 mg/ml, the native 7S species is in equilibrium with small amounts of one or more smaller species in the absence of EDTA. This conclusion is based on the observation of a slight skewing of the protein solution–solvent interface in the sedimentation profile that becomes increasingly prominent with increasing EDTA concentration. However, at an EDTA concentration sufficient to cause maximum esteropeptidase activation (i.e., 1 mM), the sedimentation pattern is essentially identical with that of native 7S NGF in the absence of EDTA. At higher EDTA concentrations (0.01 to 0.1 M) dissociation to lower molecular weight species is observed.[3]

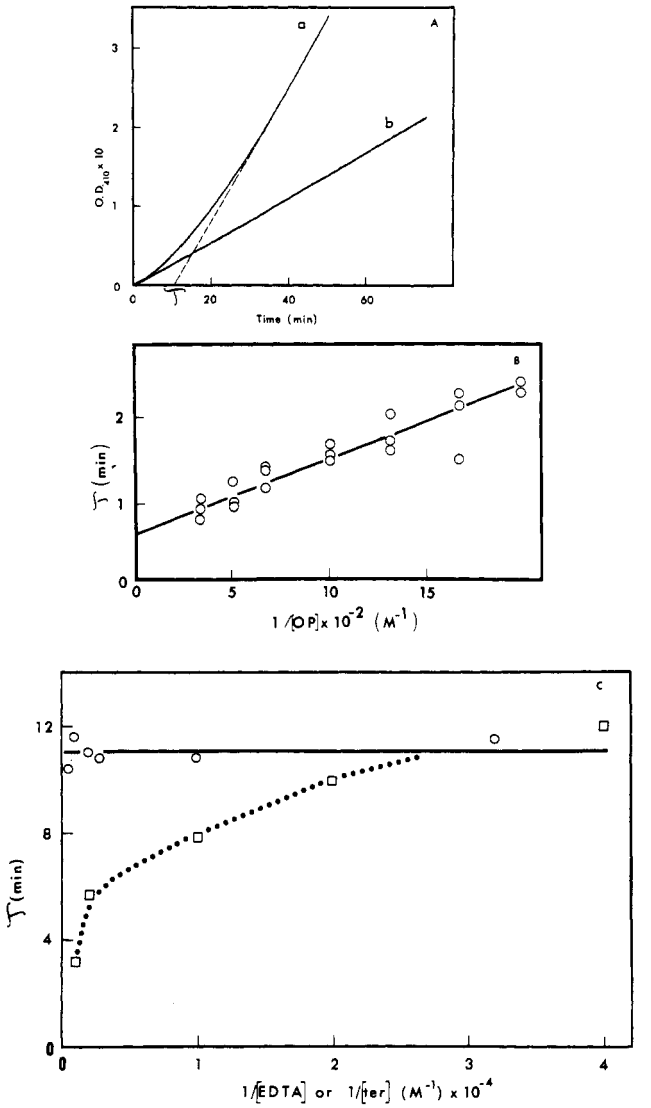

FIGURE 3: (A) Comparison of the effects of chelator on the 7S NGF-catalyzed hydrolysis of BAPNA. Trace a shows the initial time course for the hydrolysis of BAPNA by ~0.2 μM 7S NGF in the presence of 0.1 mM EDTA. The τ value for chelator activation is the time point obtained on extrapolation of the final steady state esteropeptidase rate (- - -) back to zero reaction (i.e., the abscissa intercept). Trace b shows the steady-state rate of BAPNA hydrolysis by 7S NGF in the absence of added chelator. (Data taken from Pattison and Dunn, 1975). The dependence of τ on the reciprocal of the *o*-phenanthroline concentration is shown in B. The dependence of τ on 1/(EDTA) (—) is compared with the dependence of τ on 1/(ter) (· · ·) in C. The values of τ were obtained as shown in Figure 3A. Procedure: A 1-ml sample containing ~0.2 μM 7S NGF in 0.05 M Tris buffer, pH 7.4, was incubated at room temperature for 1 h prior to the addition of BAPNA (final concentration, 1 mM). Then an aliquot of chelator was added, the solution was mixed, and the time course of the absorbance changes at 410 nm was monitored.

Kinetics of Chelator Activation of the 7S NGF Esteropeptidase. In order to simplify the kinetics of the activation process, the measurement of the rate of activation has been limited to conditions that render the kinetics quasi-irreversible. Thus, these measurements are restricted to chelator concentrations that exceed that necessary to produce maximum activation. Under these quasi-irreversible conditions, the rate of change of the steady-state esteropeptidase activity provides a measure of the rate of the activation process (Pattison and Dunn, 1975, 1976). The time value, τ, obtained from extrapolation of the final steady-state rate back to the abscissa intercept, as illustrated in Figure 3A, provides a convenient

[3] Sucrose density gradient studies (A. M.-J. Au and M. F. Dunn, unpublished results) show that the extensive dissociation observed at high EDTA concentrations (0.01–0.1 M) is primarily due to the high ionic strengths of these solutions rather than to a specific effect of the chelator. The sucrose density gradient studies at lower EDTA concentrations (0.25 mM) indicate that EDTA-activated 7S NGF has a slightly lower apparent sedimentation coefficient than native 7S NGF. However, gel isoelectric focusing studies on the high-molecular-weight fraction from the gradient show the presence of all three subunit classes in the activated species.

measure of the rate of activation. The relationship of τ to the specific rate constant(s) involved in the process of activation is given in the Appendix. Evidence for the validity of the proposed kinetic scheme on which this derivation is based is presented in the following paragraphs.

Investigation of the concentration dependence of τ (Figure 3C) has demonstrated that for carboxylic acid chelators (e.g., MDA, NTA, EDTA, and CDTA) activation is zero-order in chelator, and that, within the limits of experimental error, the carboxylic acid chelators all produce activation with identical τ values ($\sim$11 min).

However, for heterocyclic chelators (e.g., BP, OP, HQSA, and terpyridine) τ exhibits a dependence on chelator concentration that is between zero- and first-order (Figure 3B,C). The kinetics of heterocyclic chelator-induced activation have been analyzed in detail for both *o*-phenanthroline and terpyridine. Because the solubility of terpyridine is lower than the solubility of *o*-phenanthroline, it was not possible to study both chelators over the same concentration range. Since terpyridine is a more effective activator than *o*-phenanthroline, it was possible to examine the concentration dependence of the terpyridine activation kinetics at relatively lower concentrations of terpyridine but under conditions where the chelator still produces maximum activation (i.e., the quasi-irreversible condition still applies). Figure 3C shows that at low terpyridine concentrations τ approaches a limiting value that is approximately the same as that found for charged carboxylic acid chelators. At higher concentrations, τ decreases for both heterocyclic chelators (Figure 3B,C). In the concentration range examined with *o*-phenanthroline, a plot of τ vs. the reciprocal of the chelator concentration shows a linear dependence that approaches a limiting value of approximately 0.6 min when extrapolated to infinite chelator concentration (Figure 3B). Thus, for heterocyclic chelators, the activation rate is dependent on the chelator concentration, and τ approaches a minimum (limiting) value at high chelator concentrations. Note also that at lower heterocyclic chelator concentrations τ approaches the value obtained for carboxylic acid chelators ($\sim$11 min, Figure 3C).

Discussion

The phenomenon of 7S NGF esteropeptidase activation by divalent metal ion chelators occurs with chelators of widely varying structure and ligand number (Figure 1). The fact that activation to the same final state occurs independent of the structure and charge properties of the chelator makes it apparent that the primary chelator–7S NGF interaction does not involve a stereospecific, small-molecule binding site on the protein. Thus, the activation process is primarily dependent on chelating ability, and the specificity of action (see Figure 1) derives from this property.

The molecular weight studies via both velocity sedimentation and gel filtration clearly establish: (1) that 7S NGF does not undergo gross changes in molecular weight during the activation process,[3,4] and (2) that the action of the chelator involves removal of zinc ion from the protein.

Proof of the true equilibrium nature of the activation isotherms is obtained from the demonstration that the activation process is reversible. Figure 2 shows that reversal can be achieved by decreasing the effective concentration of chelator via the addition of exogenous Cu(II) ion. That this is a true reversal is established as judged by the following criteria: (1) EDTA activation is sigmoidal as is the concentration dependence of the reversal process. (2) The cycle of activation and activation reversal does not show hysteresis. (3) As reported in our previous work (Pattison and Dunn, 1975), the divalent metal ions used to effect activation reversal do not inhibit the *native* 7S NGF esteropeptidase activity. Once activation via the action of a chelator has been achieved, there occurs a slow, irreversible process that involves loss of the ability of cupric ion to effect the reversal of chelator activation on a time scale of hours. Since the rate of this process is independent of chelator structure and concentration (at concentrations above that necessary to elicit full activation), and since the activity of 7S NGF which has not been treated with chelator undergoes no significant change in activity on this time scale, it appears that the loss of reversibility must depend upon prior esteropeptidase activation.

The affinity of the 7S NGF oligomer for zinc ion can be estimated from the thermodynamics of the chelator-induced activation if it is assumed that: (1) 7S NGF-Zn exists predominantly as the 7S oligomer at concentrations that approximate the assay conditions.[4] (2) Only the reactant and product species exist in significant concentrations at equilibrium, e.g., ternary complexes involving 7S NGF-Zn-Che are assumed to be relatively insignificant species compared with 7S NGF_a and 7S NGF-Zn at equilibrium; (3) the zinc ion sites of 7S NGF are independent and equivalent. (*It will be shown* that this assumption gives rise to inconsistent results when applied to the sigmoidal-activating chelators.)

According to these assumptions, the overall reaction is described by eq 1 and 2:

$$x\text{Che} + \text{7S NGF-Zn} \overset{K_{\text{calcd}}}{\rightleftharpoons} \text{Che}_x\text{Zn} + \text{7S NGF}_a \quad (1)$$

$$K_{\text{calcd}} = \frac{(\text{Che}_x\text{Zn})\ (\text{7S NGF}_a)}{(\text{Che})^x\ (\text{7S NGF-Zn})} \quad (2)$$

where 7S NGF_a is the activated (zinc ion free) protein and 7S NGF-Zn is the native zinc metalloprotein.

The ratio of 7S NGF_a to 7S NGF-Zn upon chelator-induced activation has been approximated by the relative increase in esteropeptidase activity over the basal rate. The values for K_{calcd} have been estimated by noting that when the esteropeptidase activity is equal to $V_i + \frac{1}{2}(V_{max} - V_i)$, then (7S NGF-Zn) = (7S NGF_a) = (Che_xZn) = $\frac{1}{2}$(7S NGF-Zn)$_0$ and that (Che) = (Che)$_0$ − (Che_xZn). The subscript designations are as follows: i, the rate of BAPNA hydrolysis observed in the absence of chelator; a, the activated 7S NGF esteropeptidase; 0, initial concentration. The stoichiometry of chelator binding (x) is a function of the chelator structure and varies from one to three for the chelators that have been studied (See Table I). The chelator–Zn ion binding stoichiometries for the various chelators (x) are assumed to be that of the most stable zinc ion complex (Sillén and Martell, 1964). Equilibrium constants, K_{calcd} for the reaction shown in eq 1, also are listed in Table I.

Note that eq 2 may be expressed as the two-component reaction sequence:

$$x\text{Che} + \text{Zn(H}_2\text{O)}_6 \overset{K_{\text{Che}}}{\rightleftharpoons} \text{Che}_x\text{Zn} + 6\text{H}_2\text{O} \quad (3)$$

[4] The molecular form(s) of NGF have not been rigorously defined for the standard assay conditions. Thus, the 7S NGF-Zn oligomer is assumed to be the predominant species, since (a) only $\sim$10% of the esteropeptidase is active (Greene et al., 1969), (b) 7S NGF-Zn is the only inhibited form of the esteropeptidase known to be present under the conditions of the assay (Greene et al., 1969), and (c) 7S NGF-Zn is the most stable aggregate form of the α, β, and γ subunits (Smith et al., 1968). However, our unpublished gel-filtration studies (A. M.-J. Au and M. F. Dunn) show that substrate binding does not grossly alter the molecular weight of the EDTA-activated 7S NGF oligomer.

$$7S\ NGF\text{-}Zn \overset{1/K_{7S}}{\rightleftharpoons} 7S\ NGF_a + Zn(H_2O)_6 \qquad (4)$$

$$K_{calcd} = K_{Che}/K_{7S} \qquad (5)$$

With a knowledge of the chelator affinity constant for zinc ion, K_{Che}, reexpression of the overall equilibrium process as eq 3-5 allows calculation of the 7S NGF affinity for zinc ion, K_{7S}. The zinc ion-chelator affinity constants (K_{Che}) given in the literature (Sillén and Martell, 1964) are, to a good approximation, valid under the conditions employed in these assays. Although the same temperature was employed for all the measurements, the ionic strength conditions under which the literature K_{Che} affinity constants were calculated deviate from the experimental conditions by as much as 0.05 M. Calculations (according to the Debye-Hückel approximation, Rossotti and Rossotti, 1961) indicate that such a deviation will cause a perturbation in K_{Che} that is less than the experimental error (ca. a factor of 2) associated with the estimation of the overall equilibrium constant, K_{calcd}. The assay buffer anions interact so weakly with zinc ion (Sillén and Martell, 1964) that no correction is necessary. The chelator-zinc ion affinity constants (corrected to the pH conditions of the assay) are listed in Table I.

The 7S NGF-zinc ion affinity constants (K_{7S} values) have been calculated from eq 5 and are listed in Table I. For those chelators that show an approximately hyperbolic activation isotherm, $K_{7S} = 10^{10.5} \pm 10^{0.5}\ M^{-1}$. Note that this value is relatively constant for the first five chelators listed in Table I. This series of chelators is characterized by: (1) zinc ion-chelator affinity constants which cover a 10^8-fold range, (2) structures that vary from neutral heterocyclic chelators to charged aliphatic chelators, and (3) stoichiometries of chelation that vary from 1 to 3 chelators/zinc ion. The consistency of the calculated zinc ion-7S NGF apparent affinity constants strongly suggests that the "hyperbolic" activating chelators all operate via the same mechanism.

The three chelators that show a clear sigmoidal concentration dependence of activation (2,2′,2″-terpyridine, CDTA, EDTA) give values for K_{7S}, calculated via eq 1-5, which differ significantly from the values calculated for the "hyperbolic" activating chelators. This lack of consistency implies that the equilibrium expression of eq 1 is not applicable to these systems. The following considerations suggest that the binding mechanism proposed in eq 1 should not hold for "sigmoidal" activating chelators: (1) the sigmoidal isotherm implies that the zinc ion sites on 7S NGF are not independent and equivalent, (2) the high affinities of EDTA and CDTA for divalent metal ions probably render invalid the estimation of K_{calcd} via the approximations used above, (3) the terpyridine spectral studies (Pattison and Dunn, 1976) raise the possibility that stable 7S NGF-Zn^{2+}-chelator ternary complexes may be significant for at least this chelator, and (4) in the case of terpyridine the spectral changes that accompany the terpyridine-7S NGF reaction (Pattison and Dunn, 1976) yield a sigmoidal isotherm when the concentration variable plotted is either *free* or *total* chelator. Therefore, the sigmoidicity in the isotherm can not be due to the presence of contaminating metal ions. Because of the complex nature of the sigmoidal activation isotherm, no alternative calculation to determine the zinc ion-7S NGF affinity for these systems is proposed.

The kinetics of chelator-induced activation provide evidence as to the mechanism of the chelator action. As detailed in the Results (Figure 3), the apparent rate constant ($1/\tau$) for activation by carboxylic acid chelators is independent of chelator concentration, while $1/\tau$ values for heterocyclic chelators increase with increasing chelator concentration. Therefore, the kinetics of the overall activation process are consistent with a mechanism involving two parallel pathways as illustrated in eq 6:

$$\begin{array}{ccc} x\text{Che} + 7S\ NGF\text{-}Zn & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} & 7S\ NGF_a + (\text{Che})_x\text{Zn} \\ k_{-2} \Big\uparrow k_2 & & k_3 \Big\downarrow k_{-3} \\ (x-1)\text{Che} + \text{Che}(7S\ NGF\text{-}Zn) & & \\ 7S\ NGF_a + S & \xrightarrow{k_4} & 7S\ NGF_a + P \end{array} \qquad (6)$$

One pathway (k_1, k_{-1}) involves dissociation of the 7S NGF-bound zinc ion(s) to give the activated esteropeptidase (7S NGF_a) via a process which is zero order in chelator concentration. The second pathway involves the reversible formation of a complex between 7S NGF and chelator (step k_2, k_{-2}) that then breaks down in a rate-limiting step (k_3, k_{-3}) to give the activated species. Note that this kinetic scheme takes into account only those species that influence the kinetics of the overall activation process. Hence, species involving the rapid preequilibrium complexation of the 7S NGF-bound zinc ions by chelators such as terpyridine and HQSA (Pattison and Dunn, 1976) have not been included.

The mathematical treatment of this model is based on a more general transient kinetic model as given by Gutfreund (1965). The detailed derivation of the rate equations is presented in the Appendix. The expression derived for this mechanism relates the chelator concentration to an experimentally derived quantity, τ.

$$\tau = \frac{k_2(\text{Che})/k_{-2} + 1}{k_1 + k_3k_2(\text{Che})/k_{-2}} \qquad (7)$$

The value, τ, is defined as the time value obtained from extrapolation of the final steady-state rate back to the abscissa (Figure 3A). As τ is given by a complex expression involving rate constants and concentrations, two limiting cases are considered: (1) if only the reaction via k_1 is significant, i.e., if $(\text{Che})k_2/k_{-2} < 1$ and $k_3k_2(\text{Che})/k_{-2} < k_1$, then the equation simplifies to $\tau \simeq 1/k_1$; (2) if the reaction via k_3 dominates at high chelator concentrations, i.e., if $k_3k_2(\text{Che})/k_{-2} > k_1$, then $\tau \simeq 1/k_3 + k_{-2}/[k_3k_2(\text{Che})]$. Such a model predicts that the activation rate will show a dependence on the chelator concentration that saturates at high chelator concentration with a rate constant that approaches k_3. In this instance, the concentration dependence of τ will be a direct function of K_D (i.e., k_{-2}/k_2) for a particular chelator.

Case I applies to those carboxylic acid chelators for which τ shows no significant chelator concentration dependence (Figure 3C). The τ values for these chelators (~11 min) give a calculated activation rate constant (k_1) of $\sim 1.5 \times 10^{-3}\ s^{-1}$. In terms of this model, the constant, K_D, for carboxylic acid chelator-7S NGF binding is high enough that activation via the second process (k_3) is insignificant over the concentration range of interest. However, for heterocyclic chelators at high chelator concentrations (Figure 3B) the pathway via k_3 dominates. Figure 3B yields a K_D for heterocyclic chelator binding to 7S NGF of ~1 mM and an activation rate constant, k_3, of $\sim 1.6 \times 10^{-2}\ s^{-1}$. Another heterocyclic chelator, terpyridine (Figure 3C), has been studied in a concentration range where neither limiting case is fully applicable. However, at low terpyridine concentrations, τ approaches a value of 11 min, while at high concentrations the second activation process (k_3) becomes important. Again, the calculated K_D value is 1 mM. Note that the magnitude of K_D rules out the possibility that

the binding step (k_2, k_{-2}) and the rapid process that occurs when terpyridine (or HQSA) is mixed with native 7S NGF (Pattison and Dunn, 1976) are the same process. As the activation process via k_3 appears to be restricted to heterocyclic chelators, the possibility is raised that this process may be less dependent on affinity for divalent metal ion than on the hydrophobic and/or planar aromatic character of these compounds.

Greene et al. (1969) postulated that the increase in specific esteropeptidase activity observed when 7S NGF is subjected to high dilution acrues from the dissociation of 7S NGF. In contrast, the gel filtration and velocity sedimentation experiments strongly indicate that chelator-induced activation does not involve dissociation of 7S NGF to lower molecular weight species at low chelator concentrations.[3,4] Nevertheless, significant dissociation occurs at high EDTA concentrations (Pattison and Dunn, 1975). This dissociation appears to be an ionic strength effect.[3] Gel filtration experiments show the presence of an activated, associated species at concentrations as low as 0.1 mg/ml (~1 μM). These results provide clear evidence that strong interactions between 7S NGF subunits remain in the absence of divalent metal ions. Thus, both these experiments and the kinetic experiments described above imply a mode of activation that does not involve subunit dissociation.

In view of the reported sequence homology between proinsulin and the β subunit or 2.5S NGF (Frazier et al. 1972), the possibility is raised that zinc ion plays a role for NGF that is similar to the role(s) played by zinc ion in the structure and function of insulin. It has been postulated that zinc ion acts to control the conversion of proinsulin to insulin and that zinc ion plays an important rolé in the storage and release of the hormone from the β cells of the pancreas (Blundell et al., 1972). A similar function for zinc ion in relation to the β subunit would imply that regulation of the γ-esteropeptidase activity by zinc ion is important in controlling the conversion of a putative "pro-NGF" to the active growth factor. Furthermore, since zinc ion binds more tightly to the 7S oligomer than to any of the 7S NGF subunits, removal of zinc ion must decrease the stability of the oligomer toward dissociation. Therefore, it is possible that zinc ion also plays a role in regulating the dissociative release of the active growth factor from its storage cells (by analogy to the role postulated for the interaction of zinc ion and insulin in the pancreas).

On the other hand, there are some striking differences between the relationship of zinc ion to 7S NGF and the relationship of zinc ion to the insulin hexamer. First, zinc ion appears to be bound with a much higher affinity in 7S NGF ($K_D \simeq 10^{-11}$ M) than in the insulin hexamer ($K_D \simeq 1$ μM, Blundell et al. 1972). Second, the subunits of zinc-free 7S NGF interact much more strongly than do the subunits of zinc-free insulin (which dissociates completely in the absence of zinc ion at protein concentrations of ~1 μM, Blundell et al. 1972). Finally, zinc ion inhibits the 7S NGF esteropeptidase activity, a phenomenon which has no analogy to the insulin hexamer.

These differences in the interaction between zinc ion and 7S NGF vs. zinc ion and the insulin hexamer necessitate consideration of other functional models for the zinc ion of 7S NGF. In contrast to the above intracellular model, it is important to bear in mind that given (1) the high affinity of 7S NGF for zinc ion, and (2) the high affinity of the 7S NGF subunits for each other (in the absence of zinc ion), the high level of zinc ion in serum (~10 μM; Vallee, 1962) suggests that nerve growth factor may exist in vivo as the 7S oligomer in serum.

In conclusion, this paper and our previous work (Pattison and Dunn, 1975, 1976) have established that zinc ion binds both tightly and specifically to 7S NGF and, therefore, zinc ion serves both a structural and functional role in the 7S complex. Since the 7S NGF oligomer exhibits an affinity for zinc ion which is 10^5-fold greater than that of its component subunits, we conclude that the functional significance of zinc ion in 7S NGF is directly related to the functional significance of the 7S NGF oligomer.

Appendix

As given in the text, the simplest mechanism for chelator activation that is consistent with the kinetics of the reaction between chelator and 7S NGF is as follows:

$$x\text{Che} + \text{7S NGF–Zn} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{7S NGF}_a + (\text{Che})_x\text{Zn}$$

$$k_2 \uparrow\downarrow k_{-2} \qquad\qquad k_3 /\!/ k_{-3}$$

$$(x-1)\text{Che} + \text{Che(7S NGF–Zn)}$$

$$\text{7S NGF}_a + \text{S} \xrightarrow{k_4} \text{7S NGF}_a + \text{P}$$

Under the experimental conditions, (Che) $\gg$ (7S NGF–Zn), the activation process is a quasi-irreversible process. Also, the step (k_2, k_{-2}) is assumed to be a rapid preequilibrium. Since the reaction of aquo zinc ion occurs much faster than the activation steps (k_1, k_3) (Cotton and Wilkinson, 1966), the sequestering of zinc ion by chelator on dissociation from 7S NGF will have a negligible effect on the overall activation rate. Therefore, on elimination of excess (Che) from the mechanism, the following simplified kinetic scheme is obtained:

$$\text{Che} + \text{7S NGF–Zn} \xrightarrow{k_1} \text{7S NGF}_a + \text{Zn} + \text{Che}$$

$$k_2 \uparrow\downarrow k_{-2} \qquad \nearrow k_3$$

$$\text{Che(7S NGF–Zn)}$$

$$\text{7S NGF}_a + \text{S} \xrightarrow{k_4} \text{7S NGF}_a + \text{P}$$

If (Che) $\gg$ (7S NGF–Zn), k_2(Che) may be set approximately equal to k_2'. This mechanism then fits the following mathematical model (see Gutfreund, 1965):

$$\text{A} \xrightarrow{k_1} \text{C}$$

$$k_2' \uparrow\downarrow k_{-2} \qquad \nearrow k_3$$

$$\text{B}$$

$$\text{S} + \text{C} \xrightarrow{k_4} \text{P} + \text{C}$$

When A and B are assumed to be in rapid equilibrium,

$$B = A(k_2'/k_{-2})$$

and when A_0 is equal to the initial concentration of reactant A, the following differential equations describe this kinetic scheme:

$$dP/dt = k_4 C$$

and taking the second derivative,

$$\frac{d^2P}{dt^2} = k_4 \frac{dC}{dt}$$

The change in the concentration of C with time is defined by the reaction mechanism as

$$\frac{dC}{dt} = k_1 \frac{(A_0 - C)}{(k_2'/k_{-2} + 1)} + \frac{k_2'k_3}{k_{-2}} \frac{(A_0 - C)}{(k_2'/k_{-2} + 1)}$$

On substitution,

$$\frac{d^2P}{dt^2} = (k_4k_1) \frac{(A_0 - C)}{(k_2'/k_{-2} + 1)} + \frac{(k_4k_2'k_3}{(k_{-2})} \frac{(A_0 - C)}{(k_2'/k_{-2} + 1)}$$

and

$$\frac{d^2P}{dt^2} = (k_4k_1) \frac{[A_0 - (dP/dt)(1/k_4)]}{(k_2'/k_{-2} + 1)} + \frac{(k_4k_2'k_3)[A_0 - (dP/dt)(1/k_4)]}{(k_{-2})(k_2'/k_{-2} + 1)}$$

Upon rearrangement, the equation takes the following form:

$$\frac{d^2P}{dt^2} + \frac{dP}{dt}\left[\frac{k_1}{(k_2'/k_{-2} + 1)} + \frac{(k_3k_2'/k_{-2})}{(k_2'/k_{-2} + 1)}\right] = \frac{(k_4k_1A_0)}{(k_2'/k_{-2} + 1)} + \frac{(k_2'k_3k_4A_0/k_{-2})}{(k_2'/k_{-2} + 1)}$$

This differential equation is of the following form:

$$\frac{d^2P}{dt^2} + dP/dt \cdot W = X$$

where W and X are constants. The integrated form of this differential equation is as follows,

$$P = Xt/W + Ye^{-Wt} + Z$$

with the integration constants defined as

$$Y = X/W^2, \qquad Z = P_0 - (X/W^2) = -X/W^2$$

when the initial product concentration, P_0, is equal to zero. Then, by substitution

$$P = A_0k_4t + \frac{A_0k_4e^{-[(k_1+k_2'k_3/k_{-2})/(k_2'/k_{-2}+1)]t}}{\frac{(k_1 + k_2'k_3/k_{-2})}{(k_2'/k_{-2} + 1)}} - \frac{A_0k_4}{\frac{(k_1 + k_2'k_3/k_{-2})}{(k_2'/k_{-2} + 1)}}$$

By definition, τ is the time point at which the steady-state rate is extrapolated back to zero product (zero on the t axis). For this mechanism, the final steady-state rate is approached when the exponential term becomes infinitely small, that is, when

$$P = A_0k_4t - \frac{A_0k_4}{\frac{(k_1 + k_2'k_3/k_{-2})}{(k_2'/k_{-2} + 1)}}$$

Extrapolation of this linear equation to $P = 0$ yields

$$A_ok_4t = \frac{A_0k_4}{\frac{(k_1 + k_2'k_3/k_{-2})}{(k_2'/k_{-2} + 1)}}$$

Hence, the time point (which is defined as τ) is equal to

$$t \equiv \tau = \frac{(k_2'/k_{-2} + 1)}{(k_1 + k_2'k_3/k_{-2})}$$

Finally, substitution of k_2(Che) for k_2' yields

$$\tau = \frac{k_2(\text{Che})/k_{-2} + 1}{k_1 + (\text{Che})k_2k_3/k_{-2}}$$

References

Barcroft, J., and Hill, A. V. (1909), *J. Physiol. 39,* 411.

Blundell, R., Dodson, G., Hodgkin, D., and Mercola, D. (1972), *Adv. Protein Chem. 26,* 279.

Cotton, F. A., and Wilkinson, G. (1966), Advanced Inorganic Chemistry, New York, N.Y., Wiley, p 158.

Frazier, W. A., Angeletti, R. H., and Bradshaw, R. A. (1972), *Science 176,* 482.

Greene, L. A., Shooter, E. M., and Varon, S. (1969), *Biochemistry 8,* 3735.

Gutfreund, H. (1965), An Introduction to the Study of Enzymes, New York, N.Y., Wiley, p 56.

Levi-Montalcini, R. (1966), *Harvey Lect. 60,* 217.

Pattison, S. E., and Dunn, M. F. (1975), *Biochemistry 14,* 2733.

Pattison, S. E., and Dunn, M. F. (1976), in preparation.

Rossotti, F. J. C., and Rossotti, H. (1961), The Determination of Stability Constants, New York, N.Y., McGraw-Hill Book Co.

Schenkein, I. (1972), *Handb. Neurochem. 5B,* 503.

Sillén, L. G., and Martell, A. D., Eds. (1964), Stability Constants of Metal Ion Complexes, Special Publication 17, London, The Chemical Society.

Smith, A. P. (1969), Doctoral Thesis, Stanford University.

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

Vallee, B. L. (1962), *Miner. Metab. 2B,* 443.

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