Kaltschmidt, E., and Wittmann, H. G. (1970), *Anal. Biochem. 36*, 401.
Lowry, O. H., Rosebrough, N. J., Farr, L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mangiarotti, G., Apirion, D., Schlessinger, D., and Silengo, L. (1968), *Biochemistry 7*, 456.
Mizushima, S., and Nomura, M. (1970), *Nature (London) 226*, 1214.
Nashimoto, H., Held, W., Kaltschmidt, E., and Nomura, M. (1971), *J. Mol. Biol. 62*, 121.
Nashimoto, H., and Nomura, M. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1440.
Nomura, M. (1970), *Bacteriol. Rev. 34*, 228.
Nomura, M. (1973), *Science 179*, 864.
Nomura, M., Mizushima, S., Ozaki, M., Traub, P., and Lowry, C. V. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 49.
Osawa, S. (1968), *Annu. Rev. Biochem. 37*, 109.
Schaup, H. W., Green, M., and Kurland, C. G. (1970), *Mol. Gen. Genet. 109*, 193.
Schaup, H. W., Green, M., and Kurland, C. G. (1971), *Mol. Gen. Genet. 112*, 1.
Stanley, W. M., and Bock, R. M. (1965), *Biochemistry 4*, 1302.
Traub, P., Mizushima, S., Lowry, C. V., and Nomura, M. (1971), *Methods Enzymol. 20C*, 391.
Traub, P., and Nomura, M. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 577.
Traub, P., and Nomura, M. (1969a), *J. Mol. Biol. 40*, 391.
Traub, P., and Nomura, M. (1969b), *Cold Spring Harbor Symp. Quant. Biol. 34*, 63.
Wittmann, H. G., Stöffler, G., Hindennach, I., Kurland, C. G., Linkover, T., Randall-Hazelbauer, L., Birge, E. A., Nomura, M., Kaltschmidt, E., Mizushima, S., Traut, R. R., and Bickle, T. A. (1971), *Mol. Gen. Genet. 111*, 327.

# Topography of Mouse 2.5S Nerve Growth Factor. Reactivity of Tyrosine and Tryptophan†

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ABSTRACT: The chemical reactivity and degree of solvent exposure of the two tyrosine and three tryptophan residues of mouse 2.5S nerve growth factor (NGF) have been investigated. Spectrophotometric titrations and reaction with tetranitromethane indicate that both tyrosines are solvent available to a limited extent and that neither is required for activity. While the two tyrosines appear equivalent, the tryptophans exhibit a spectrum of reactivity. The reaction of these residues with *N*-bromosuccinimide is a kinetically ordered process. The most rapidly reacting residue is also solvent available by the criterion of *N*-methylnicotinamide chloride titration and is not required for activity. Reaction of the intermediate residue destroys both biological and immunological activity, while oxidation of the third residue leads to dissociation of the 2.5S dimer. Reaction of native NGF with dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide leads to modification of Trp-21 and Trp-99 and leaves Trp-76 unmodified. Modification of the rapidly oxidized residue with *N*-bromosuccinimide followed by reaction with dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide in 6 M guanidine hydrochloride reveals the modification of Trp-99 and Trp-76, identifying Trp-21 as the solvent available residue. Computer protein modeling studies show that this chemical description of the topography of the tyrosine and tryptophan residues of NGF is in extremely good agreement with topography of the corresponding residues in the three-dimensional structure of insulin. Thus the observed similarities in the function and mechanism of action of NGF and insulin may result from regions of similar primary structure dictating related three-dimensional conformation.

Nerve growth factor (NGF)[1] has been shown to be similar in primary structure to insulin and proinsulin (Frazier *et al.*, 1972). This relationship, presumably the result of evolution from a common precursor, is supported by similarities in the function and mechanism of action of the two proteins. For example, both NGF and insulin may be described as positive pleiotypic activators (Hershko *et al.*, 1971; Frazier *et al.*, 1972) and both have been suggested to exert this effect through a receptor on the surface membrane of their respective target cells (Cuatrecasas, 1969; Bradshaw *et al.*, 1972). While insulin affects many different cell types, the pleiotypic effects of NGF are limited to the differentiation and maintenance of adrenergic neurons, primarily those of the sympathetic nervous system (Levi-Montalcini and Angeletti, 1968). In addition, the conservation of a disulfide bridge in NGF, whose half-cystines represent identical residues in the sequence alignment of NGF with proinsulin (Frazier *et al.*, 1972), suggest that the functional similarities of NGF and insulin may reflect regions of similar three-dimensional structures as well.

Since the three-dimensional structure of insulin has been

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[1] Abbreviations used are: NGF, nerve growth factor; $C(NO_2)_4$, tetranitromethane; SucNBr, *N*-bromosuccinimide; NMN, *N*-methylnicotinamide chloride; $(HO)(NO_2)Bzl$, hydroxynitrobenzyl-; *N*-Ac-Trp-$NH_2$, *N*-acetyl-L-tryptophanamide.

determined by X-ray crystallographic means (Adams *et al.*, 1969; Blundell *et al.*, 1971) and the tertiary structure of NGF is at this time unknown, a comparison of conformation of the two proteins must necessarily employ indirect methods. Chemical probes have been used to compare the conformation of proteins in solution to the corresponding crystallographic structures determined by X-ray analysis. In this study the solution conformation of NGF has been compared with the crystal structure of insulin (Adams *et al.*, 1969; Blundell *et al.*, 1971) through the use of chemical probes of tyrosine and tryptophan residues. Since the crystal structure of proinsulin has not yet been reported, structural comparisons can be made only for residues which lie within the regions of the NGF sequence which correspond to the A and B chains of insulin. However, most of the identities of the sequence alignment are clustered in the A and B chain regions of the comparison (Frazier *et al.*, 1972). Both insulin (Frank and Veros, 1970) and NGF (Angeletti *et al.*, 1971) exist as dimers in solution and thus the unit of structure taken for comparison is the monomer of each protein.

The results of these experiments are consistent with the existence of regions of similar three-dimensional structure in NGF and insulin. At the same time, they provide further information on the role of tyrosine and tryptophan residues in the formation of the postulated NGF–receptor complex through which the biological activity of NGF is apparently expressed (Bradshaw *et al.*, 1972).[2]

## Experimental Section

### *Materials*

NGF was prepared from submaxillary glands of adult male Swiss ICR mice by the procedure of Bocchini and Angeletti (1969). Chicken egg-white lysozyme and trypsin were purchased from Worthington Biochem. Corp.

L-Tryptophan (A grade), *N*-acetyl-L-tryptophan (A grade), and 2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent) were obtained from Calbiochem. *N*-Bromosuccinimide (recrystallized from $H_2O$ before use), and *N*-acetyl-L-tryptophanamide were purchased from Sigma Chemical Co. Reagents for amino acid analysis and *p*-toluenesulfonic acid were from Pierce Chemical Co. Tetranitromethane and low ultraviolet absorbance guanidine hydrochloride were from Mann Research Laboratories. Carrier-free $Na^{125}I$ and [$^{14}$C]-iodoacetic acid were obtained from New England Nuclear.

*N*-Methylnicotinamide chloride was synthesized by the method of Karrer *et al.* (1936), and dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide was prepared from Koshland's reagent by the procedure of Horton and Tucker (1970).

### *Methods*

Ultraviolet and visible spectra were recorded on a Cary 15 spectrophotometer and optical rotatory dispersion (ORD) spectra were recorded with a Cary 60 spectropolarimeter in 0.1- or 1-cm cells at protein concentrations of 0.05–1 mg/ml. Optical rotation values were expressed as reduced mean residue rotation [$m'$], as calculated from $[3/(n^2 + 2)][M_0\alpha_{obsd}/dC]$, where $\alpha_{obsd}$ is the observed rotation; $M_0$, the mean residue molecular weight; $d$, the length of the cell in decimeters; $C$, the concentration in g/100 ml; and $n$, the index of refraction of the solvent at 230 nm.

Spectrophotometric titrations were performed according to the procedures suggested by Wetlaufer (1962). Absorbance values were taken from complete ultraviolet spectra (360–240 nm) recorded with a Cary 15 spectrophotometer immediately after adjusting the pH of the solution. NGF solutions were prepared in 0.02 M borate buffer–0.2 M NaCl with or without 6 M guanidine hydrochloride or in 0.01 M Tris–0.1 M NaCl.

Reaction of NGF with $C(NO_2)_4$ was performed at a protein concentration of 0.8–1 mg/ml in 0.1 M Tris-HCl buffer (pH 8.0) for 40 min. Nitro-NGF was separated from nitroform and $C(NO_2)_4$ on a 0.9 × 40 cm column of Sephadex G-25 (fine) or G-100 (medium) equilibrated with and developed in 0.05 M ammonium bicarbonate (pH 8.0). 3-Nitrotyrosine was quantitated by amino acid analysis after hydrolysis *in vacuo* at 110° in 6 N HCl with phenol for 24 hr (Sokolovsky *et al.*, 1966).

Oxidation of NGF and *N*-acetyl-L-tryptophanamide with *N*-bromosuccinimide was carried out according to the procedures outlined by Spande and Witkop (1967). Aliquots of an aqueous solution of SucNBr (1–5 mg/ml) were added with stirring to a solution of NGF (0.5–1.0 mg/ml) at room temperature in 0.1 M sodium acetate (pH 4.0), with or without 8 M urea. After each addition, the ultraviolet spectrum of the sample was recorded on the Cary 15 spectrophotometer. The number of residues of tryptophan oxidized per NGF monomer ($N$) was calculated by the formula of Spande and Witkop (1967): $N = (-\Delta A_{280} \times 1.31 \times M)/(A_{280_i} \times af \times 5500)$, where the molecular weight ($M$) of the NGF monomer calculated from the amino acid sequence, is 13,259 (Angeletti *et al.*, 1973). The term af, the reciprocal of the absorbance of a 1-mg/ml solution of NGF at 280 nm in a 1-cm path-length cell ($\epsilon_{280,1\text{ cm}}^{1\text{ mg/ml}}$), was calculated by determination of the protein concentration by amino acid analysis of NGF solutions of known $A_{280\text{ nm}}$ (in 0.1 N acetic acid). Five separate determinations yielded a value for the $\epsilon_{280,1\text{ cm}}^{1\text{ mg/ml}}$ of 1.64 (±0.03) which gives a value for af equal to 0.610. The empirical factor (1.31), which corrects for the absorbance of oxindole at 280 nm, was determined by oxidation of *N*-acetyl-L-tryptophanamide in 0.1 M acetate (pH 4.0) with or without 8 M urea, and is in agreement with the value of Spande and Witkop (1967).

Kinetic studies of the SucNBr oxidation of NGF and *N*-acetyl-L-tryptophanamide in the presence and absence of 8 M urea were performed in a Durrum-Gibson stopped-flow apparatus. Data was stored on a Hewlett-Packard 3960 FM tape recorder and analyzed on a PDP-12/40 laboratory computer (Digital Equipment Corp.) with the aid of the kinetic display and simulation programs of Bates and Frieden (1973). To study the kinetics of oxidation of native NGF, a 5.98 × $10^{-5}$ M solution of NGF in 0.2 M sodium acetate (pH 4.0) was placed in one drive syringe of the Durrum stopped-flow apparatus and a 5.38 × $10^{-4}$ M solution of SucNBr in water (9-fold molar excess) was placed in the other. The decrease in absorbance at 280 nm was followed over total times ranging from 0.5 sec to 1 min. For kinetic studies of the oxidation of the denatured protein, NGF (8 mg) was incubated for 72 hr in 10 ml of 6 M guanidine hydrochloride–0.2 M acetate buffer (pH 4.0) to effect complete denaturation. Long incubations in urea were avoided to prevent carbamylation. Immediately before the experiment, the sample was passed down a Sephadex G-25 column equilibrated in 8 M urea–0.2 M acetate (pH 4.0) and recovered in this solvent. This NGF solution (3.35 × $10^{-5}$ M) was placed in one drive syringe and a solution of SucNBr in 8 M urea in water (3.02 × $10^{-4}$ M, 9-fold molar excess) placed in the other drive syringe of the stopped-

[2] A preliminary report of this work has been presented (Angeletti *et al.*, 1972).

flow apparatus. The decrease in absorbance at 280 nm was followed for time intervals of 10 sec to 10 min. It was observed that SucNBr is unstable in 6 M guanidine-HCl solutions and thus this solvent cannot be used for kinetic studies. The mechanism of breakdown probably involves bromination of guanidine, the bromoguanidine being unable to oxidize tryptophan while *N*-bromourea remains an effective, but slow, oxidant.

Titrations of NGF and tryptophan model compounds with *N*-methylnicotinamide chloride were performed according to the procedure of Deranleau *et al.* (1969) and Deranleau and Schwyzer (1970) in 0.1 M acetate buffer (pH 4.0 or 5.0).

NGF was reacted with dimethyl-(2-hydroxy-5-nitrobenzyl)-sulfonium bromide as follows. NGF (26.5 mg; 2 $\mu$mol) was dissolved in 0.1 M sodium acetate (pH 5.0) to yield a final concentration of 1.27 mg/ml. To this solution was added a 15-fold molar excess of the (HO)($NO_2$)Bzl reagent as 4.07 ml of a 7.4 $\times$ $10^{-3}$ M solution in pH 5.0 buffer. The reaction proceeded with stirring in the dark for 2 hr at room temperature. The modification was terminated by passing the reaction mixture on a column of Sephadex G-25 (fine) in 0.1 N acetic acid. The modified protein was recovered, adjusted to pH 8.0, incubated for 3 hr to remove alkaline-labile (HO)($NO_2$)Bzl groups, and dialyzed against 0.1 N acetic acid for 18 hr. The number of (HO)($NO_2$)Bzl groups incorporated was determined from the absorbance at 415 nm of an aliquot adjusted to pH >12 (Barman and Koshland, 1967). The protein concentration was determined by amino acid analysis.

S-Carboxymethylation and S-aminoethylation of NGF derivatives and tryptic digestion was performed as previously described (Angeletti *et al.*, 1973). Peptide separations of tryptic digests of *S*-aminoethyl-(HO)($NO_2$)Bzl-NGF were done on columns of Sephadex G-25 fine in 0.05 M ammonium bicarbonate at pH 8.8 and further purified on SP-Sephadex columns in volatile buffers at 55° as described by Walsh *et al.* (1970). Tryptic peptides of nitro-NGF were purified on columns of Dowex 50-X8 as described (Angeletti *et al.*, 1973). Tryptophan content of NGF derivatives was determined on the short column (0.9 $\times$ 8 cm) of the Spinco Model 120C amino acid analyzer after hydrolysis in 3 N *p*-toluenesulfonic acid for 24 and 48 hr as described by Liu and Chang (1971). All other amino acid analyses were performed by the method of Moore and Stein (1963) on a Spinco Model 120C amino acid analyzer. Sedimentation velocity analyses were performed on a Spinco Model E ultracentrifuge equipped with a uv scanner attachment.

Biological assays of NGF derivatives were performed with a modification of the method described by Levi-Montalcini and Angeletti (1968). Immunological reactivity of NGF derivatives was assayed with a double-antibody radioimmunoassay. To 100–300 $\mu$l of unknown or standard NGF solutions was added 100 $\mu$l of an [$^{125}$I]NGF solution containing 30,000–50,000 cpm, and 100 $\mu$l of a $10^{-3}$ dilution of rabbit anti-NGF immune serum. All tubes were normalized to a volume of 0.50 ml with 0.1 M sodium phosphate (pH 7.4) containing 1.0 mg/ml of bovine serum albumin which was also used for diluting antiserum and NGF samples. Tubes were incubated 4 hr at room temperature, 25 $\mu$l of a dilution of goat anti-rabbit immunoglobin was added, and incubation was continued overnight at 4°. The tubes were then centrifuged on a Sorvall GLC-1 centrifuge (top speed) at 4°, washed once with 3 ml of buffer, and counted in a Packard Auto-Gamma counter. The optimal dilution of the goat antiserum was determined by titrating the assay mixture described above with no unlabeled NGF added. The standard curve covered the range of 1–50 ng of NGF. NGF was iodinated by the method of Hunter and Greenwood (1962) and purified by chromatography on Sephadex G-25 fine.

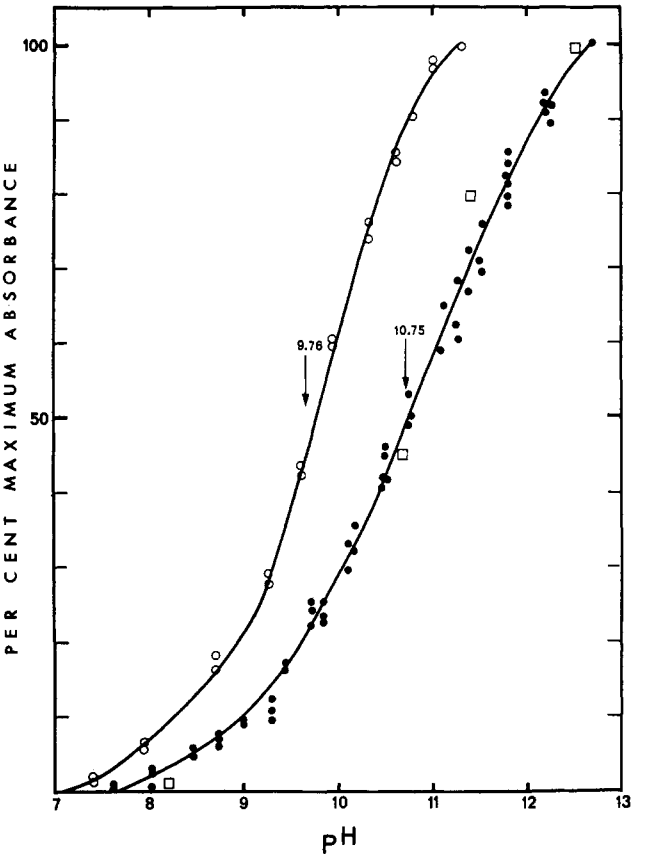


FIGURE 1: Spectrophotometric titration of the tyrosine residues of NGF. Solid symbols represent absorbance values at 255, 290, 295, and 305 nm from two experiments, one in 0.1 M NaCl, the other in 0.2 M NaCl. The open squares are from the ORD data shown in Figure 2. Open symbols represent absorbance values at 295 and 305 nm from a titration of NGF in 6 M guanidine hydrochloride. Arrows mark the midpoints of the two titration curves (50% of the total change).

## Results

*Estimation of the Apparent Dissociation Constants of the Tyrosyl Residues of NGF by Spectrophotometric Titration.* The apparent p*K* values of the two tyrosyl residues of the NGF monomer were determined using the increase in tyrosine absorbance which occurs as a result of the phenolic ionization at basic pH. Data taken from complete ultraviolet spectra recorded as a function of pH in the range 7–13 are shown in Figure 1. The filled symbols represent absorbance values taken from the spectra at 255, 290, 295, and 305 nm from two separate titrations of different preparations of NGF. The fact that points from four different wavelengths fall on the same titration curve as well as the maintenance of an isosbestic point at 275 nm throughout the course of a given titration indicate that the changes in absorption are a true reflection of the ionization of the two tyrosyl residues in the NGF monomer (Wetlaufer, 1962). The open symbols in Figure 1 are from a titration performed in 6 M guanidine hydrochloride followed at 295 and 305 nm. While both curves reach the same absolute extent, the maximum value is reached in the denaturing solvent at pH 11, whereas a pH in excess of 12.5 is required to fully ionize both tyrosines in the native molecule.

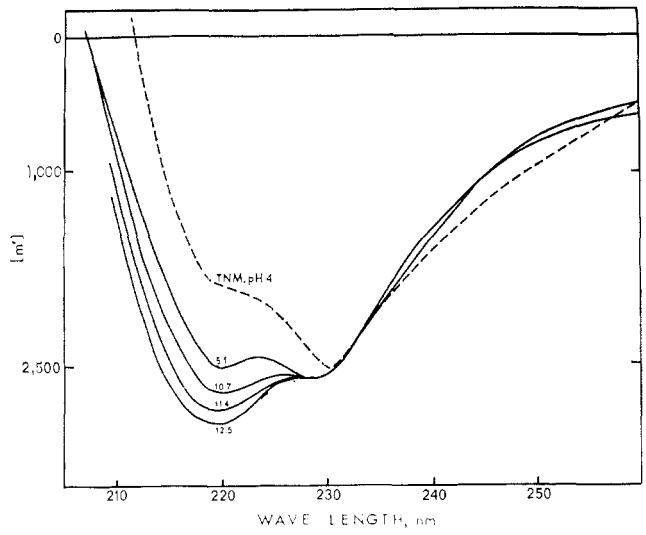


FIGURE 2: The ORD spectrum of NGF as a function of pH. The protein solution was the same used in the titrations shown in Figure 1. Spectra are labeled with the pH at which they were recorded. The units of $[m']$ (reduced mean residue rotation) are (deg cm$^2$)/dmol. The dashed spectrum is that of NGF whose two tyrosyl residues have been converted to 3-nitrotyrosine by reaction with $C(NO_2)_4$.

The midpoint, or apparent p$K$ of the tyrosyl ionization in native NGF is approximately 10.75 while in the denatured protein, this value is observed to be 1 pH unit lower (approximately 9.8), in agreement with the p$K$ estimated for an unperturbed phenolic side chain (Tanford, 1962). No apparent inflections are seen in the native titration curve, suggesting that the two tyrosyl residues have apparent p$K$ values which do not differ from the average (~10.8) by more than about 0.8 pH unit (Wetlaufer, 1962). At pH values greater than 12 a time-dependent increase in ultraviolet absorbance was noted. The difference spectrum generated by this increase was not that of ionizing tyrosine, but was similar to that of ionized cysteine (Wetlaufer, 1962). Furthermore, unlike the spectral changes which occurred on brief exposure to alkaline pH, this slow increase in absorbance was not reversible. A similar time dependent spectral increase, accompanied by disulfide-bond cleavage, has been noted for insulin at pH values greater than 12 (Frank *et al.*, 1972).

Figure 2 shows the ORD spectrum of NGF as a function of pH. These spectra were determined on the same solutions used for the spectrophotometric titration described above. The spectrum at pH 5.10 was the same whether measured with a freshly prepared solution of NGF at pH 5.10 or with a solution which had been used for a spectrophotometric titration and readjusted to pH 5.10. The intensification of the 220-nm trough, when plotted as per cent of maximum change as a function of pH, obeys the same titration curve as the spectrophotometrically observed tyrosyl ionization. These points from the ORD spectra are indicated as open squares in Figure 1. This result indicates that either the ionization of a phenolic chromophore is directly responsible for the dispersion observed at 220 nm or that such an ionization mediates the perturbation of a structure which gives rise to this feature of the ORD spectrum.

*Modification of NGF with Tetranitromethane.* Modification of NGF with a 50-fold molar excess of tetranitromethane results in the conversion of greater than 97% of the tyrosyl residues to 3-nitrotyrosine. When nitrated NGF is chromatographed on Sephadex G-100 (2 × 95 cm, 0.05 M ammonium bicarbonate, pH 8.0), a protein peak, accounting for 60% of the nitro-NGF applied, elutes at the position of native NGF and a second protein peak, containing 40% of the applied material, appears at a position corresponding to material with a larger Stokes radius, indicating polymerization has occurred (Boesel and Carpenter, 1970). The two protein peaks, nitro-NGF and poly(nitro-NGF), were analyzed for tyrosine and 3-nitrotyrosine. As shown in Table I, tyrosine was not present in either derivative. The expected amount of 3-nitrotyrosine was present in the unpolymerized material, but the polymerized protein contained only one-third (0.65 residue/molecule NGF monomer) as much 3-nitrotyrosine (Table I). In view of the report that tryptophan can be modified by $C(NO_2)_4$ (Cuatrecasas *et al.*, 1968), aliquots of each protein peak were hydrolyzed with 3 N *p*-toluenesulfonic acid and analyzed for tryptophan. As shown in Table I, there was no modification of tryptophan in the unpolymerized nitro-NGF, while more than one residue (40%) of the tryptophan was destroyed in the polymerized derivative. The apparent loss of 0.3 residue of tryptophan in the unpolymerized nitro-NGF pool can be attributed to contamination with polymerized material.

TABLE I: Tyrosine, 3-Nitrotyrosine, and Tryptophan Content of Native NGF and Nitrated Derivatives.

| | Nitro-NGF | Poly-(nitro-NGF) | Native |
|---|---|---|---|
| Tyrosine (residues) | 0 | 0 | 2 |
| 3-Nitrotyrosine (residues) | 1.8 | 0.65 | 0 |
| Tryptophan (residues) | 2.7 | 1.7 | 3 |

Biological assay of both forms of nitro-NGF revealed the unpolymerized derivative to be fully active, while, interestingly, the activity of the polymerized protein was only slightly less. The ORD spectrum of nitro-NGF is shown as the dashed curve in Figure 2. Nitration of tyrosine reduced the magnitude of the 220-nm trough which is also reflected by the shift of the intersection of the far-uv limb of the curve with the zero line to longer wavelength (211.5 nm *vs.* 207 nm).

In order to further demonstrate the homogeneity of the nitrated derivative, a column tryptic fingerprint of 1.5 μmol of fully modified nitro-NGF was prepared. The elution profile was identical to that obtained for the purification of the soluble tryptic peptides of *S*-carboxymethyl-NGF previously reported (Angeletti *et al.*, 1973). All peptide pools were characterized by amino acid analysis. The peptides containing nitrotyrosine, which appeared in comparable yields in the same position in the elution profile as the corresponding tyrosyl peptides from unmodified *S*-carboxymethyl-NGF, contained no free tyrosine and stoichiometric amounts of 3-nitrotyrosine. All other soluble tryptic peptides were identical with those recovered from unnitrated NGF.

*N-Bromosuccinimide: Equilibrium Oxidation.* As a means of obtaining information about the relative availability of the three tryptophan residues of NGF, equilibrium oxidations with *N*-bromosuccinimide were performed. The addition of SucNBr to NGF solutions resulted in a progressive decrease in 280-nm absorbance which was complete at a ninefold molar excess of SucNBr to NGF or 3.0 mol of SucNBr/mol of tryptophan. This data is shown in Figure 3 (solid lines). The oxidation of NGF in 8 M urea (pH 4.0) (Figure 3; dotted line)

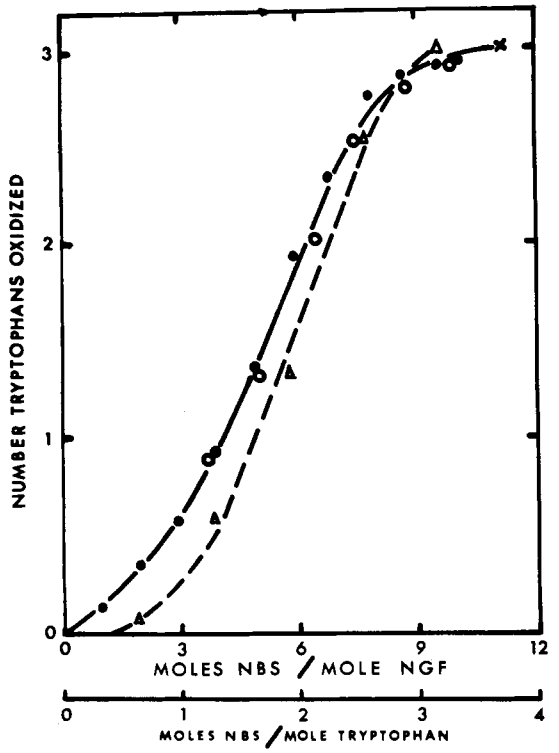


FIGURE 3: Equilibrium titration of the three tryptophans of NGF with *N*-bromosuccinimide. The solid curve represents the oxidation of the native protein at pH 4.0 and the dashed curve the oxidation of NGF in 8 M urea (pH 4.0).

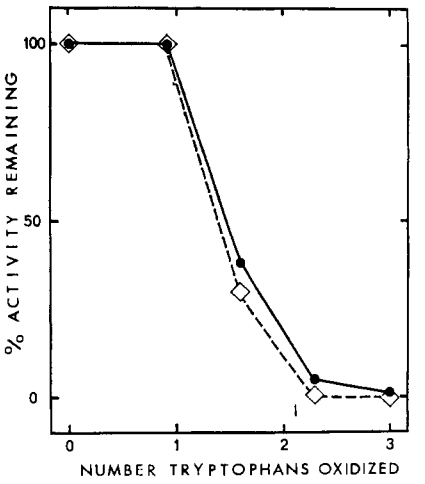


FIGURE 4: The immunological reactivity (closed symbols, solid line) and the biological activity (open symbols, dashed line) of NGF reacted with SucNBr to the extent of 0 (native) 0.9, 1.6, 2.3, 2.9, and 3.0 residues of tryptophan oxidized per monomer.

proceeds to the same extent at the same molar excess of SucNBr as the reaction in buffer alone.

To examine the effects of progressive oxidation of tryptophan residues on the biological and immunological reactivity as well as the conformation and physical properties of the NGF molecule, 1.5 μmol of NGF was oxidized by the addition of SucNBr. Aliquots of the reaction were removed at degrees of oxidation corresponding to the destruction of 0, 0.9, 1.6, 2.3, 2.9, and 3.0 residues of tryptophan per monomer as determined spectrophotometrically. The biological and immunological activity of these derivatives is shown as per cent of native (0 oxidized tryptophan) in Figure 4. Both biological activity and immunological cross-reactivity decay in the same manner with increasing tryptophan oxidation. Neither activity is affected by oxidation of a total of one residue of tryptophan, but oxidation of a second equivalent of tryptophan causes nearly complete loss of biological and immunological competence.

Since amino acids other than tryptophan may react with SucNBr (Spande and Witkop, 1967), the amino acid composition of each of the oxidized derivatives was determined. These results are summarized in Figure 5. The figure is divided into two parts: that corresponding to the oxidation occurring from 0 to 9 mol of SucNBr per NGF monomer during which 2.9 residues of tryptophan are oxidized and that corresponding to the reaction in which the last 0.1 tryptophan is oxidized. The oxidation of the last remaining fraction of tryptophan requires the addition of a slight excess of SucNBr since the criterion for complete tryptophan oxidation is that no further decrease in 280-nm absorbance be observed with the addition of SucNBr. Thus changes in the protein which occur between 2.9 and 3.0 residues of tryptophan oxidized are ascribed largely to side reactions of the residual SucNBr which was not consumed by reaction with tryptophan. Tyrosine, which is destroyed to the extent of 0.9 residue during the oxidation of 2.9 residues of tryptophan is destroyed to the extent of another 0.5 residue in the last phase of the modification (Figure 5). In a similar manner histidine is unaffected during the first phase of the oxidation, but 0.4 residue is destroyed upon the addition of the excess SucNBr. Interestingly, methionine is unaffected throughout the oxidation.

Possible perturbations of the hydrodynamic properties

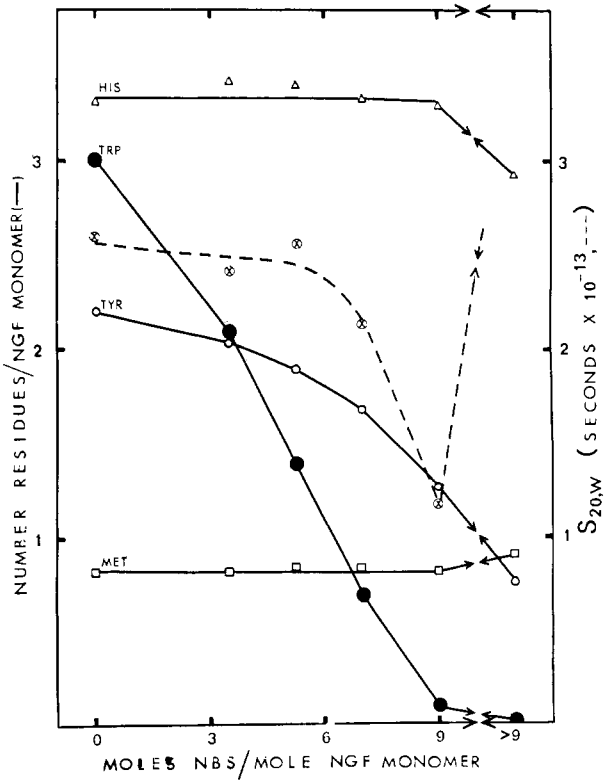


FIGURE 5: Content of SucNBr-reactive residues and $s_{20,w}$ as a function of increasing oxidation of the NGF monomer. Tryptophan (●), histidine (△), tyrosine (○), and methionine (□) are traced with solid lines and the $s_{20,w}$ (⊗) of the oxidized derivatives is traced by the dashed line. The graph is broken (→ ←) at the point where side reactions due to excess (greater than 9 mol/monomer) SucNBr become significant.

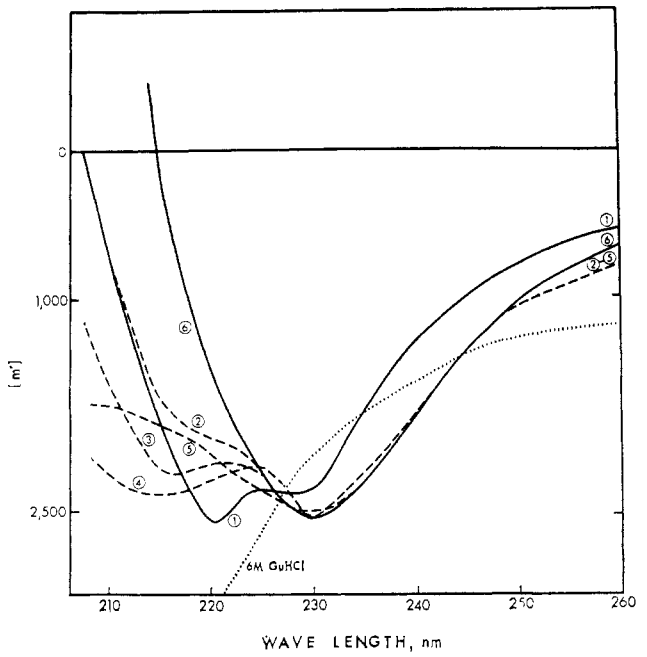


FIGURE 6: The ORD spectra of the SucNBr oxidized derivatives of NGF. Spectrum 1 is that of unoxidized NGF. Spectra labeled 2–6 are those of NGF reacted to the extent of 0.9, 1.6, 2.3, 2.9, and 3.0 residues of tryptophan oxidized, respectively. The dotted spectrum is that of unmodified NGF which was denatured in 6 M guanidine hydrochloride (6 M guanidine-HCl). The units of [$m'$] (reduced mean residue rotation) are (deg cm$^2$)/dmol.

(and thus aggregation state) resulting from oxidation were examined by performing sedimentation velocity studies in the ultracentrifuge. In the range of 0–2 tryptophans oxidized (0–6 mol of SucNBr/mol of NGF monomer) the $s_{20,w}$ remains at the native value of about 2.5 S. From 2–2.9 residues tryptophan oxidized, the sedimentation coefficient is reduced to 1.2 indicating probable dissociation of the NGF dimer. However, further addition of SucNBr results in extensive formation of polydisperse aggregates.

To determine whether these changes in aggregation state of the molecule are linked to changes in secondary structure, the ORD spectra of the oxidized derivatives were measured. These spectra, along with the ORD spectrum obtained with unmodified NGF in 6 M guanidine hydrochloride (pH 4) (incubated 120 hr before the spectrum was recorded), are shown in Figure 6. The spectrum designated 1 is that of unoxidized NGF, 2–5 are those of 0.9, 1.6, 2.3, and 2.9 residues of tryptophan oxidized, respectively, and curve 6 is the spectrum of the fully oxidized 3.0 derivative. Clearly, none of the spectra of the oxidized derivatives resembles the denatured state produced by guanidine hydrochloride (dotted line). It should be noted that the ORD spectrum of the 0.9 oxidized tryptophan derivative, which is dimeric and fully active, most closely resembles the native spectrum. With increasing oxidation to 1.6 and 2.3 residues oxidized (curves 3 and 4 in Figure 6) a major shift occurs in the far-ultraviolet portion of the spectrum as the ascending limb of the curve shifts to lower wavelength. As oxidation of the third residue of tryptophan approaches completion, this trend is reversed until finally at 3.0 residues (curve 6) the ORD spectrum has the appearance of a single featureless cotton effect with the negative trough centered at 230 nm.

*Kinetics of N-Bromosuccinimide Modification.* The decrease in the extinction of tryptophan at 280 nm which accompanies the conversion to oxindole on reaction with SucNBr provides

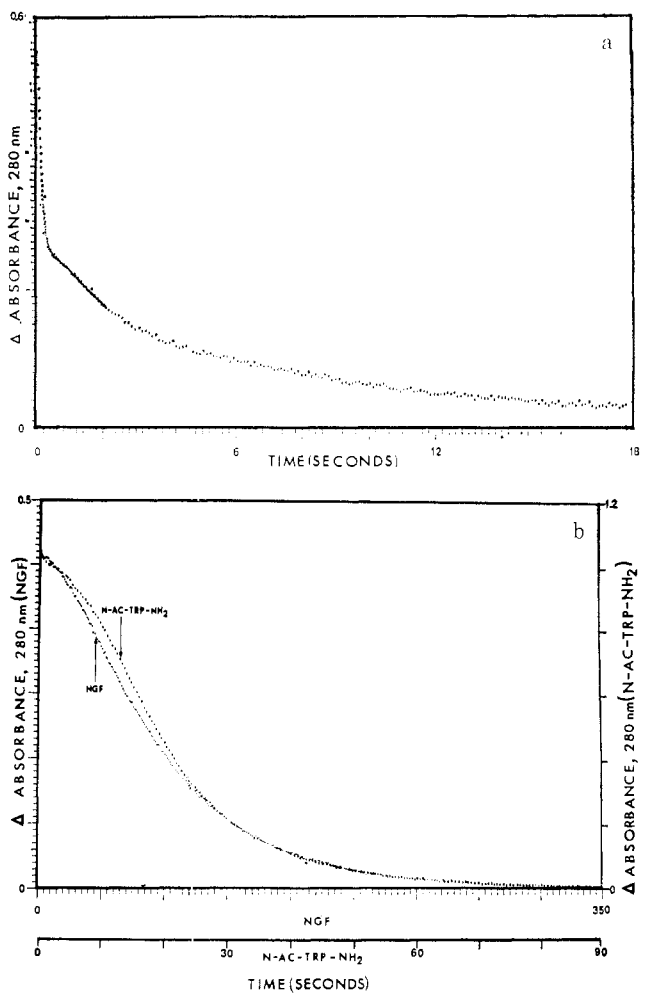


FIGURE 7: (a) Stopped-flow oxidation of native NGF with a ninefold molar excess of SucNBr, directly reproduced from the computer plot of the data. The absorbance of the solution at 280 nm remaining after oxidation of 3.0 tryptophans was subtracted from all points so that the plot appears to go to zero absorbance. The curve appears dotted because the computer samples the data at discrete intervals. (b) Stopped-flow oxidation of NGF and *N*-acetyl-L-tryptophanamide in 8 M urea. NGF was oxidized with a ninefold molar excess of SucNBr and the model compound with a twofold excess. The left absorbance and upper time axes refer to the NGF plot and the right absorbance and lower time axes refer to the model compound plot.

a direct and rapid assay easily measureable as a function of time with a stopped-flow instrument. For the study of the kinetics of oxidation of native NGF, the mode of mixing was NGF in acetate buffer with SucNBr (ninefold molar excess) in water. The decrease in absorbance at 280 nm which occurred on rapid mixing of equal volumes of each solution was followed over total time intervals ranging from 0.5 sec to 1 min. As late as 3 min no increase in absorbance, indicating over oxidation, was noted, and preliminary equilibrium experiments utilizing the Cary 15 confirmed that mixing of equal volumes of the NGF and SucNBr solutions used in the kinetic experiment, resulted in the oxidation of 2.98 residues of tryptophan. Figure 7a is a direct reproduction of the stopped-flow data plotted by the computer on a Versatec printer. The ordinate is the change in absorbance at 280 nm and the abscissa is the time scale of the experiment in seconds. Two experiments with the same solutions, one followed for a total of 2 sec at small increments of time and one followed for a total of 18 sec at larger increments, are shown superimposed. It is apparent that the decay of absorbance at 280 nm exhibits three distinct phases. A very

TABLE II: Second-Order Rate Constants for the Reaction of NGF and *N*-Acetyl-L-tryptophanamide with *N*-Bromosuccinimide in 0.1 M Acetate (pH 4.0).

| | $k$ (mol$^{-1}$ sec$^{-1}$) | $k^{NGF}/k^{N\text{-}Ac\text{-}TrpNH_2}$ | $k^{NGF}/k_3^{NGF}$ |
|---|---|---|---|
| NGF | | | |
| $k_1^{NGF}$ (fast) | $2.0 \times 10^6$ | 5.0 | 2040 |
| $k_2^{NGF}$ (medium) | $3.3 \times 10^4$ | 0.082 | 34 |
| $k_3^{NGF}$ (slow) | $9.8 \times 10^2$ | 0.0024 | 1 |
| N-Ac-Trp-$NH_2$ | $4.0 \times 10^5$ | | |

TABLE III: Binding Constants and Extinction Coefficients for the Complex of *N*-Methylnicotinamide Chloride with Lysozyme and NGF.[a]

| Protein | pH | $k$ | $\epsilon$ |
|---|---|---|---|
| Lysozyme | 5.0 | 2.8 | 1100 |
| Lysozyme | 4.0 | 1.7 | 1080 |
| NGF | 5.0 | 2.6 | 1450 |
| NGF | 4.0 | 1.8 | 1525 |

[a] All measurements were made at room temperature in 0.1 M sodium acetate as described by Deranleau *et al.* (1969).

rapid phase occurs during the first 200 msec of the reaction, an intermediate phase predominates for the next 2–3 sec and the reaction proceeds to completion in a very slow phase lasting some 25 sec. The value of the absorbance at 280 nm reached at this time is constant and corresponds to the oxidation of 2.98 tryptophan residues, in exact agreement with the static measurement.

The fact that three distinct phases can be seen in the data plot suggests that the three tryptophan residues are modified at unique rates, all differing by at least one or more orders of magnitude. Using the simulation system for the PDP-12 of Bates and Frieden (1973), the reaction shown in Figure 7a was simulated by fitting computer-generated curves derived for the sum of three simultaneously occurring irreversible second order reactions with the real data curve. The second-order rate constants obtained by this procedure for the fast ($k_1$(NGF)), medium ($k_2$(NGF)), and slowly reacting ($k_3$(NGF)) tryptophans are shown in Table II. Thus, as seen in the last column, the rapidly oxidized tryptophan residue reacts 60 times faster than the intermediate residue which reacts 34 times faster than the most slowly oxidized tryptophan.

In order to compare the rates of reaction of the tryptophans of NGF with a model system in which conformational constraints were absent, the second-order rate constant for the SucNBr oxidation of the compound *N*-acetyl-L-tryptophanamide was determined in 0.1 M acetate (pH 4.0). This value was found to be $4.0 \times 10^5$ mol$^{-1}$ sec$^{-1}$, in reasonable agreement with that observed for the rapidly reacting tryptophan of NGF, $k_1^{NGF} = 2 \times 10^6$ mol$^{-1}$ sec$^{-1}$. Thus the rapidly oxidized tryptophan of NGF reacts at least as rapidly, or slightly faster than the model compound *N*-acetyl-L-tryptophanamide, indicating that it is probably freely available to the solvent. The somewhat slower rates of reaction of the second and third tryptophans suggest that they are concealed to different degrees in the protein interior.

To determine if the slower rates of oxidation are due solely to structural constraints, the kinetics of the oxidation of the tryptophans of NGF in a denaturing solvent, 8 M urea, were studied in the stopped-flow apparatus. The mode of mixing was NGF in 8 M urea with SucNBr (ninefold molar excess) in 8 M urea. The resulting plot of absorbance at 280 nm *vs.* time over a 350-sec time course is shown in Figure 7b. The plot for the oxidation of *N*-acetyl-L-tryptophanamide under the same conditions is also seen in Figure 7b, plotted on a 90-sec time axis. The absorbance and time axes have been adjusted to compensate for the difference in tryptophan concentrations. The reaction of SucNBr with tryptophan in urea is known to proceed by a different mechanism than in simple aqueous buffer (Spande and Witkop, 1967). This is evidenced here by the long time course of the reaction and the initial lag which appears in both the protein and model compound curves. Both sets of data in Figure 7b, when simulated as simple second-order mechanisms (ignoring the initial lag), yielded second-order rate constants of approximately $10^2$ mol$^{-1}$ sec$^{-1}$. The significance of this result is the oxidation of the NGF by SucNBr is not triphasic in 8 M urea, but follows virtually the same course as the reaction of the model compound, thus indicating that the conformational constraints on the reaction of the second and third tryptophan have been obliterated.

*Titration of NGF with N-Methylnicotinamide Chloride.* The kinetics of SucNBr oxidation suggest the existence of a completely unhindered, solvent-available tryptophan in the NGF molecule. To further explore this possibility, NGF solutions (2.5–3 mg/ml) in 0.1 M acetate buffer (pH 4.0) were titrated with *N*-methylnicotinamide chloride by the method of Deranleau *et al.* (1969). This reagent reacts with indole moieties to form a noncovalent, yellow complex with charge-transfer properties. The geometric requirement for complex formation is that the donor (indole) and the acceptor (*N*-methylnicotinamide) be able to interact in a coplanar face-to-face manner. Distortion of this interaction rapidly lowers the extinction of the complex so that the reagent is a sensitive probe for the indole side chain of tryptophan residues which are completely exposed to solvent (Deranleau *et al.*, 1969; Deranleau and Schwyzer, 1970).

Data from the titration of NGF with *N*-methylnicotinamide chloride (three experiments, filled symbols and line) is presented in Figure 8 in the form of a Scatchard plot: $A/[P_0][X_0]$ *vs.* $A/[P_0]$, where $A$ is the absorbance at 350 nm, $[P_0]$ is the corrected protein (donor) concentration, and $[X_0]$ is the corrected concentration of *N*-methylnicotinamide chloride (acceptor). The slope of the plot is the negative of the binding constant, $k$, while the intercept of the plot with the abscissa yields the extinction ($\epsilon$) of the complex directly. These values for NGF are: $k = 1.83$ and $\epsilon = 1525$ at pH 4.0 (Figure 8) and $k = 2.6$ and $\epsilon = 1450$ at pH 5.0. By comparison, lysozyme, which has been shown to have one titratable tryptophan (Bradshaw and Deranleau, 1970), gives values of $k = 1.7$ and $\epsilon = 1080$ at pH 4.0 and $k = 2.8$ and $\epsilon = 1100$ at pH 5.0. These values are summarized in Table III. The latter values (pH 5.0) are in excellent agreement with those reported previously (Deranleau *et al.*, 1969). The decrease in binding constant with increasing acidity is not observed in model compounds[3] and has been tentatively ascribed to the apparent increase in positive charge of these basic proteins in this pH range.[4]

[3] R. A. Bradshaw, D. A. Deranleau, L. Hinman, L. Rothchild, and S. Chandler, manuscript in preparation.

[4] R. A. Bradshaw, S. Chandler, and W. A. Frazier, unpublished observations.

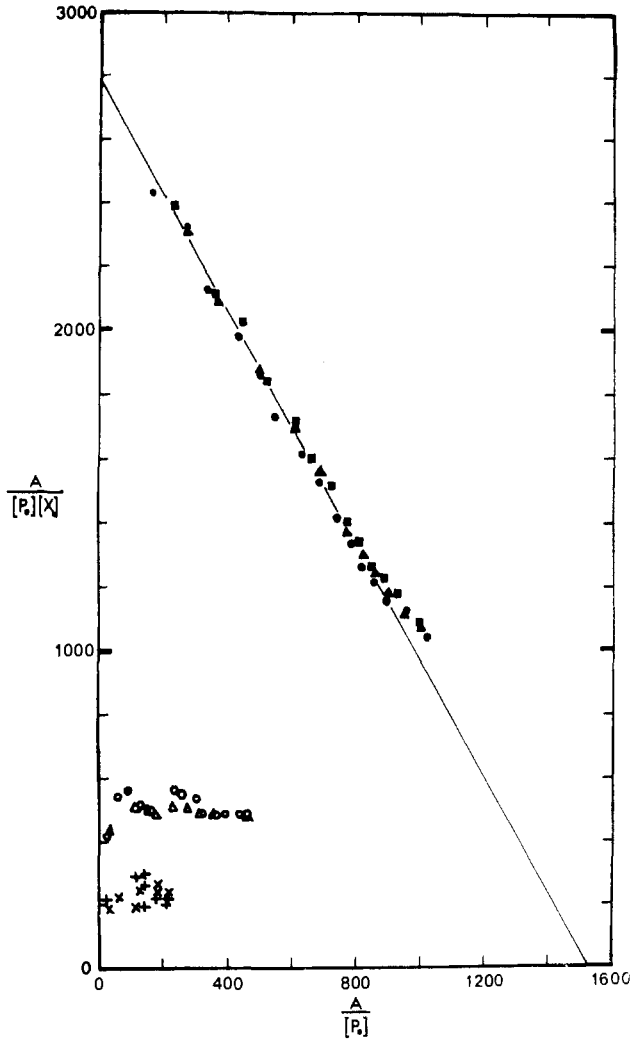


FIGURE 8: A Scatchard plot of the binding of *N*-methylnicotinamide chloride to native and SucNBr-oxidized NGF. *A* is absorbance at 350 nm, $[P_0]$ is the corrected protein (donor) concentration and $[X_0]$ is the corrected *N*-methylnicotinamide chloride (acceptor) concentration. The closed symbols (●, ■, ▲) and solid line are data from three titrations of the native protein at pH 4.0. Open symbols (○, △) are data from two titrations of NGF with one residue of tryptophan oxidized by SucNBr. Symbols in the extreme lower left are titrations of NGF with two tryptophans (+) and three tryptophans (×) oxidized.

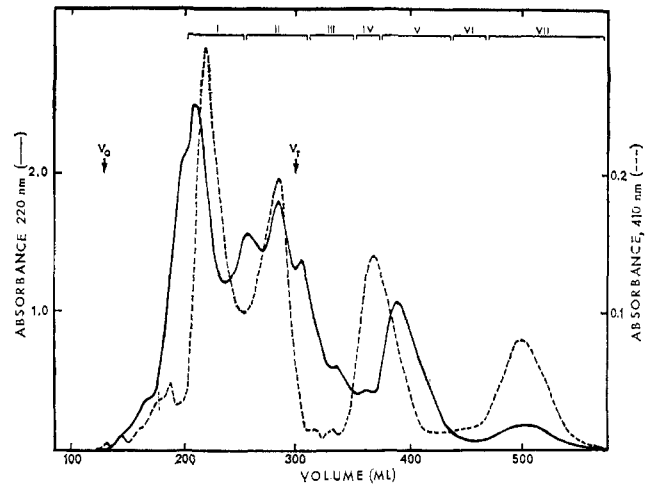


FIGURE 9: Sephadex G-25 (fine) elution profile of the tryptic peptides of *S*-aminoethyl-(HO)($NO_2$)Bzl-NGF. The column was 2.0 × 95 cm and was developed with 0.05 M ammonium bicarbonate adjusted to pH 8.8. The solid profile is the absorbance at 220 nm and the dotted profile the absorbance at 410 nm. Pools were made as indicated by horizontal bars and Roman numerals. $V_0$ marks the void volume of the column and $V_t$ marks the total column volume.

These data clearly support the existence of a single fully available tryptophan residue in NGF. In order to determine if the residue which is fully available by the criterion of *N*-methylnicotinamide titration is the same tryptophan which rapidly reacts with SucNBr, *N*-methylnicotinamide titrations of NGF derivatives oxidized by SucNBr were performed. The open symbols in the lower left of Figure 8 are the data points from two experiments in which NGF reacted to the extent of one tryptophan residue oxidized was titrated with *N*-methylnicotinamide chloride. Clearly, the loss of the most SucNBr-reactive tryptophan nearly obliterates complex formation with NMN. As indicated by the cluster of symbols (+ and x) in the extreme lower left of Figure 8, oxidation of the second (+) and third (x) tryptophan residues does not greatly reduce further the amount of complex formed. Thus a single tryptophan residue is rapidly oxidized by SucNBr and is the major locus for the complex formed with NMN.

*Reaction of NGF with Dimethyl-(2-hydroxy-5-nitrobenzyl)-sulfonium Bromide.* MODIFICATION OF NATIVE NGF. To directly identify which of the three tryptophans in the amino acid sequence of NGF is most available to solvent, the covalent probe dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide was employed. This water-soluble sulfonium salt of 2-hydroxy-5-nitrobenzyl bromide has been shown to be specific for tryptophan to which it can add one or two hydroxynitrobenzyl groups (Horton and Tucker, 1970).

NGF (2 μmol) was reacted with a 15-fold molar excess of the sulfonium bromide reagent. The (HO)($NO_2$)Bzl–NGF formed (1.3 (HO)($NO_2$)Bzl groups/NGF monomer) was reduced, S-aminoethylated and digested with trypsin. The soluble tryptic peptides (less than 1% of the (HO)($NO_2$)Bzl group was found in the insoluble fraction remaining at pH 8.8) were fractionated on a 2 × 95 cm column of Sephadex G-25 (fine, 0.05 M ammonium bicarbonate adjusted to pH 8.8). The elution profile of this column is shown in Figure 9. Seven pools were made as indicated by the horizonal bars. Four of these contained (HO)($NO_2$)Bzl groups (as judged by absorption at 410 nm) suggesting at least four modified peptides—two major peaks with the majority of peptide material (as judged by the absorption at 220 nm) and two major peaks at elution volumes greater than the column volume ($V_T$). Pools III–VII were immediately identified by amino acid composition as follows. Pool III contained primarily the peptide (residues 96–100) containing Trp-99 in unmodified form. Pool IV contained mostly the modified form of this peptide having one (HO)($NO_2$)Bzl group per mole of Trp-99. Pool V consisted of the peptide (75–80) containing unmodified Trp-76 and more of the mono-(HO)($NO_2$)Bzl-Trp-99 peptide. Pool VI had negligible amounts of peptide material. Pool VII contained the peptide (96–100) encompassing Trp-99, containing 2 mol of (HO)($NO_2$)Bzl/mol of peptide.

The peptides labeled with (HO)($NO_2$)Bzl groups in pools I and II were obviously impure since this region of the chromatogram contained all other soluble tryptic peptides as well. Pool I was fractionated on SP-Sephadex to yield two pure (HO)($NO_2$)Bzl peptides (as well as several unlabeled peptides) resulting from different tryptic cleavages. Both labeled peptides were derived from the region containing mono-(HO)-

$(NO_2)$Bzl-Trp-21. Pool II was purified in an identical manner and found to contain four pure (HO)$(NO_2)$Bzl-peptides. Two of these, obtained in low yield, were identical with those of pool I and the other two represented the same peptide sequence but contained [(HO)$(NO_2)$Bzl]$_2$-Trp-21. The recoveries of the peptides containing tryptophan residues in the modified and unmodified form are summarized in Table IV. As can be seen, 76% of Trp-21 and 61% of Trp-99 were found as the (HO)$(NO_2)$Bzl derivative, corresponding to a total number of modified tryptophan residues of 1.37, in excellent agreement with the number 1.3 determined at the start of the experiment. Clearly this reagent does not greatly distinguish between Trp-21 and Trp-99, although Trp-21 appears slightly more reactive. Trp-76, however, is probably essentially unmodified as indicated by the recovery of 86% of this residue in a peptide containing no (HO)$(NO_2)$Bzl groups. Apparently neither Trp-21 nor Trp-99 is directly required for biological activity as the derivative prepared in these studies as well as derivatives containing up to four (HO)$(NO_2)$Bzl groups per NGF monomer retain full biological potency.

MODIFICATION OF NGF PARTIALLY OXIDIZED WITH SucNBr. In view of the slight preferential modification of Trp-21 over Trp-99 with the (HO)$(NO_2)$Bzl reagent, a more conclusive identification of the solvent-exposed tryptophan residue was undertaken. NGF (1 $\mu$mol) was oxidized with SucNBr to the extent of 1.03 mol of tryptophan oxidized per monomer, thus destroying the rapidly reacting tryptophan (*vide supra*). The modified protein (1-OX-NGF) was reduced and S-aminoethylated and then reacted in 6 M guanidine hydrochloride at pH 3 with a 100-fold molar excess of dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide. The (HO)$(NO_2)$Bzl-labeled protein (1-OX-(HO)$(NO_2)$Bzl-NGF) was recovered and found to contain 3 mol of (HO)$(NO_2)$Bzl/mol of protein, suggesting the two unmodified tryptophan residues were not quantitatively converted to the disubstituted (HO)$(NO_2)$Bzl derivative. This derivative was digested with trypsin and the soluble tryptic peptides (containing 98% of the (HO)$(NO_2)$Bzl groups) were chromatographed on a Sephadex G-25 (fine) column as before. The elution profile of the column differed markedly from that seen in Figure 9. Less than 15% of the (HO)$(NO_2)$Bzl group was found in the early part of the chromatogram with the bulk of the unlabeled peptide material in the region of the peptides containing Trp-21. This region was not further characterized. The remainder of the chromatogram, eluting at volumes greater than the column volume, consisted entirely of mono- and dihydroxynitrobenzyl derivatives of peptides 75–80 (Trp-76) and 96–100 (Trp-99). The recoveries of the peptides corresponding to these two tryptophan positions from 1-OX-(HO)$(NO_2)$Bzl-NGF are listed in the lower half of Table IV. Modification of Trp-21 was estimated at less than 20% from the distribution of (HO)$(NO_2)$Bzl groups in the G-25 elution profile. Two significant differences in the pattern of modification between the reaction with native NGF and with oxidized NGF in the denaturing solvent are immediately obvious. First, there is at least a fourfold reduction in the amount of Trp-21 modified by (HO)$(NO_2)$Bzl, due undoubtedly to its prior modification by SucNBr. Second, Trp-76, 86% of which was recovered unmodified in the native experiment, is recovered in virtually identical yield (83%) in the (HO)$(NO_2)$Bzl-modified form in the oxidized NGF. This result is due to the fact that the (HO)$(NO_2)$Bzl modification was performed in guanidine-HCl, thus disrupting the elements of protein structure which had previously protected this residue from modification. The yield of modified Trp-99 is similar in both experiments. Partial oxidation of Trp-99 cannot be ruled out, however, since no unmodified version of this peptide (96–100) was recovered.

TABLE IV: Modification of the Tryptophan Residues of Native and Oxidized NGF with Dimethyl-(2-hydroxy-5-nitrobenzyl)-sulfonium Bromide.

| Derivative | Position in Sequence | % TRP Recovered[a] Modified | Unmodified | Total |
|---|---|---|---|---|
| (HO)$(NO_2)$Bzl-NGF | 21 | 76 | 0 | 76 |
| | 76 | 0 | 86 | 86 |
| | 99 | 61 | 34 | 95 |
| 1-OX-(HO)$(NO_2)$Bzl-NGF | 21 | <20 | | |
| | 76 | 83 | 0 | 83 |
| | 99 | 60 | 0 | 60 |

[a] Per cent recoveries were calculated from amino acid analyses and were based on the amount of peptide material applied to the G-25 Sephadex column.

## Discussion

The experiments reported here were performed for two reasons: to establish in as much detail as possible the relative distribution of the tyrosine and tryptophan residues of NGF with regard to the topography of the molecule in solution, and to assess the relationship of these residues to the biological activity of NGF.

Both the spectrophotometric titration and nitration of the two tyrosines of NGF indicate that these residues probably lie on or near the surface, but in a state in which the phenolic hydroxyl and the 3 position of the phenol ring are hindered from free interaction with solvent.

A comparison of the results of these experiments with comparable studies of insulin reveals several interesting correlations. In their thorough investigation of the ionization behavior of the tyrosine residues of insulin and proinsulin, Frank *et al.* (1972) found homogeneous titration curves for both proteins with a midpoint (apparent p$K$) of 10.5–10.6 at a salt concentration of 0.1 M (or 0.6 M). Lower ionic strength or zinc shifted the titration curve to higher pH. The average apparent p$K$ for the tyrosines of NGF observed in these studies is 10.7–10.8 at a salt concentration of 0.1 or 0.2 M, a value quite comparable to that of the insulin tyrosines. Morris *et al.* (1970) investigated the modification of insulin with $C(NO_2)_4$ at pH 7.4. They found that reaction of insulin with a 48-fold molar excess of $C(NO_2)_4$ for 50 min resulted in the complete modification of two tyrosine residues, A-14 and A-19. The two tyrosines of NGF are quantitatively modified by $C(NO_2)_4$ at pH 8.0, 50-fold molar excess for 40 min. One of these, Tyr-79, is a conserved residue, identical in position with tyrosine-A-19 in the sequence alignment of NGF and insulin (Table V). Interestingly, the nitration of this tyrosine residue in both proteins has little effect on their biological activity. The similarity in the state of these identical residues in NGF and insulin as revealed by their ionization behavior and reactivity to $C(NO_2)_4$ may be the result of their location in a region of similar three-dimensional structure.

A model of the insulin monomer (molecule 1) constructed from the coordinates determined from X-ray analyses (Adams *et al.*, 1969; Blundell *et al.*, 1971) was displayed by the macro-

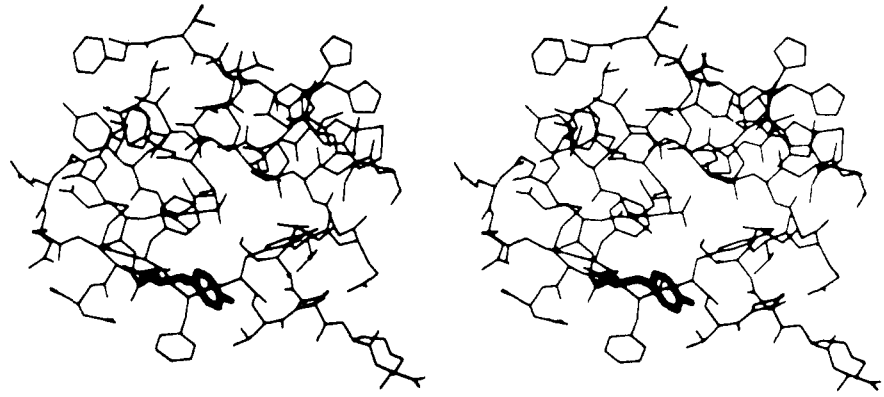

FIGURE 10: Stereorepresentation of the insulin monomer (molecule 1) with Tyr-A-19 intensified. Coordinates of the molecule are those described by Blundell *et al.* (1971). (Personal communication to Computer Systems Laboratory, Washington University, St. Louis, Mo.). This residue is conserved in the sequence alignment of NGF and insulin and is equivalent to NGF-Tyr-79 (Frazier *et al.*, 1972). This and succeeding figures are drawn to give the correct isomer when viewed with an optical superposition device.

modular protein modeling computer system (Computer Systems Laboratory, Washington University). Figure 10 is a stereopair (drawn by computer and photographed directly from the display scope) illustrating the three-dimensional structure of the insulin monomer. Tyrosine-A-19 (NGF-Tyr-79) is intensified. The hydroxyl group of this residue is seen to lie near the A-1 glycine and is thought to form a hydrogen bond to the A-1 peptide carbonyl (Blundell *et al.*, 1971), thus explaining its high p*K*. The site of the second tyrosine of NGF, Tyr-52, is located in the region corresponding to the C-peptide of proinsulin for which crystal structure data is unavailable.

While nitration of tyrosine has no effect on biological activity, it had been previously observed that modification of tryptophan led to a dramatic loss of biological and immunological activity (Angeletti, 1970). Thus, for the study of the structure–function relationships and topography of NGF, tryptophan represented a readily modified class of residues which appeared to be important for biological activity.

The SucNBr oxidation of the tryptophan residues of NGF presents several interesting facets. The equilibrium SucNBr titration of NGF gave no evidence for differential reactivity of the tryptophans especially in view of the fact that the same experiment performed in 8 M urea required exactly the same molar excess of SucNBr for complete oxidation. This result suggests a lack of significant side reaction of SucNBr with NGF. The sharp loss of biological and immunological reactivity between one and two residues of tryptophan oxidized and the apparent dissociation of the NGF dimer only after two residues have been oxidized both suggest that the oxidation proceeds by selective attack of specific tryptophans. A reaction of this type which appears ordered may be ordered in a strict sense, that is, oxidation of the first residue exposes the second whose oxidation exposes the third, or it may be kinetically ordered by virtue of widely different rate constants for the modification of the first, second, and third residues. The stopped-flow oxidation data (Figure 7a), which clearly exhibits three phases of oxidation, cannot distinguish between these possible mechanisms. Simulation of the data, however, cannot be achieved in a satisfactory manner with a strictly ordered mechanism, and a set of three simultaneous but kinetically ordered reactions corresponding to the oxidation of all tryptophans starting at time zero each with its characteristic rate constant, must be invoked. Thus, at a point in the reaction where the decrease in absorbance at 280 nm corresponds to 1.00 tryptophan oxidized, approximately 98.3% will be the fast residue ($k_1^{NGF} = 2 \times 10^6$ M$^{-1}$ cm$^{-1}$), 1.6% will be the intermediate residue ($k_2^{NGF} = 3.3 \times 10^4$ M$^{-1}$ sec$^{-1}$), and less than 0.05% will be the slow residue ($k_3^{NGF} = 9.8 \times 10^2$ M$^{-1}$ sec$^{-1}$).

The evidence obtained from the oxidation studies for a unique solvent-available tryptophan is further supported by the results of the NMN charge-transfer titrations. Since NMN can only form a charge-transfer complex with tryptophan when the entire face of the indole side chain is available for a coplanar interaction, this demonstration of complex formation is excellent evidence for a fully exposed tryptophan residue.

The observed extinction coefficient of the complex (1450–1525) with NGF is larger than that observed for lysozyme by about 400 (Table III) (Deranleau *et al.*, 1969). This increase could be due to complex formation at one or both tyrosyl residues (Deranleau and Schwyzer, 1970) or a second poorly formed tryptophan complex. It has been noted[5] that the tyrosine–NMN complex has virtually negligible extinction at 400 nm while the tryptophan complex retains significant absorbance at this wavelength. Titrations of *N*-acetyl-L-tryptophanamide yield a value for the ratio $\epsilon_{400}/\epsilon_{350}$ of 0.352. This value for the NGF–NMN complex is 0.350. If tyrosine were contributing the added extinction, this ratio would be on the order of 0.25–0.26. Thus the extinction of the complex above and beyond that of the full tryptophan site is more likely due to a weak indole–NMN interaction at a second tryptophan, presumably at Trp-99. This would explain the residual complex remaining after SucNBr oxidation of the first tryptophan (Figure 8, open symbols) which is destroyed

TABLE V: Tyrosine and Tryptophan Residues of NGF and the Corresponding Residues in the A and B Chains of Porcine Insulin.

| NGF Residue[a] | Insulin Residue[b] |
|---|---|
| Tyr-79 | Tyr-A-19 |
| Trp-21 | Phe-B-25 |
| Trp-76 | Leu-A-16 |

[a] Angeletti and Bradshaw (1971). [b] Chance *et al.* (1968).

[5] D. A. Deranleau, personal communication.

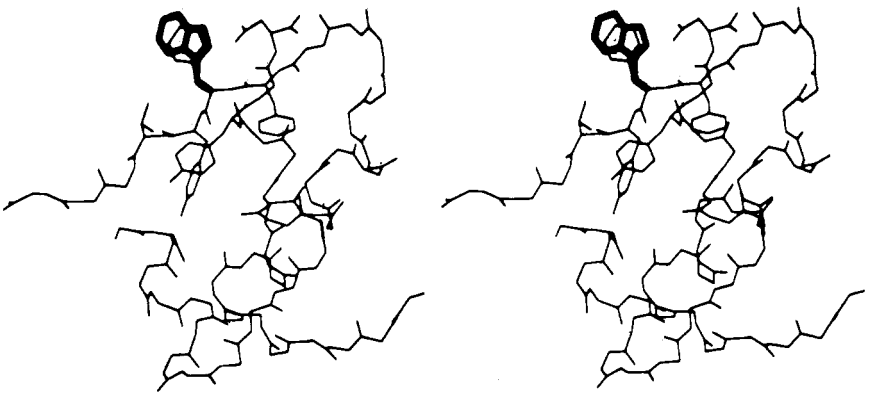

FIGURE 11: Stereorepresentation of the insulin monomer with a tryptophan (intensified) superimposed on insulin Phe-B-25 (structural data as in Figure 10). The indole ring has been aligned coplanar with the phenyl ring.

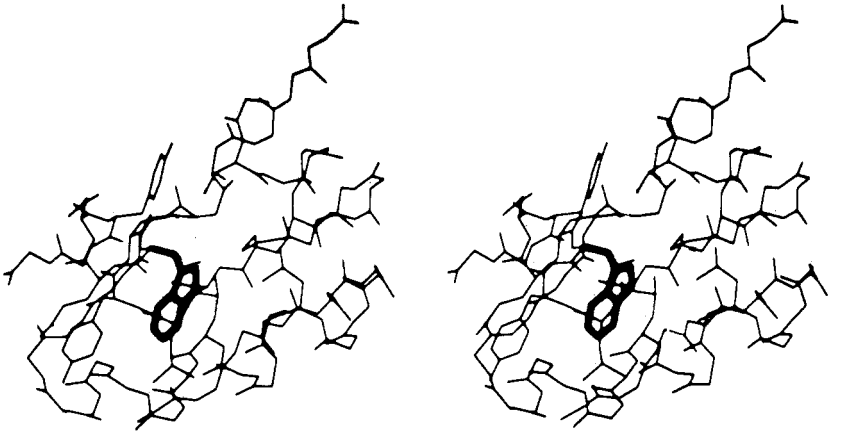

FIGURE 12: Stereorepresentation of the insulin monomer with a tryptophan (intensified) inserted by superposition of its $\alpha$, $\beta$, $\gamma$, and two $\delta$ atoms on the $\alpha$ through two $\delta$ carbons of insulin Leu-A-16 (structural data as in Figure 10).

upon oxidation of the second tryptophan (+ symbols, Figure 8).

The partial exposure of a second tryptophan is also indicated by the modification of native NGF with dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide. In this experiment, 76% of Trp-21 was modified while 61% of Trp-99 was also reacted. The reaction of this reagent clearly does not exhibit the same relative rates as SucNBr modification. One reason for the difference may be the pronounced hydrophobic nature of this reagent (Loudon and Koshland, 1970; Barman, 1972) which would tend to increase the reactivity of tryptophan residues that retain some access to the solvent and reside in hydrophobic environments.

Since it had been established that the residue which reacted most rapidly with SucNBr was the same residue which contributed the fully unhindered site for NMN complex formation (*vide supra*), this tryptophan was identified in a differential modification experiment. As seen in the lower half of Table IV, the pattern of (HO)($NO_2$)Bzl modification obtained after oxidation of the rapidly reacting tryptophan identifies Trp-21 as the fully exposed residue and Trp-76 as the one which is completely buried.

The description of the relative topography of the tryptophan residues of NGF which can be constructed from this series of experiments is quite precise. Trp-21 is rapidly oxidized by SucNBr ($k_1^{NGF} = 2 \times 10^6$ mol$^{-1}$ sec$^{-1}$), provides an unhindered site for NMN charge-transfer complex formation and is modified to the greatest extent by dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide. Trp-21 is apparently not important for biological activity and thus does not interact with the proposed NGF receptor (Bradshaw *et al.*, 1972). The residue oxidized at an intermediate rate by SucNBr ($k_2^{NGF} = 3.3 \times 10^4$ mol$^{-1}$ sec$^{-1}$) is presumably Trp-99 which is also modified by the sulfonium bromide reagent. The extensive (61%) reaction at this site before reaction at Trp-21 is complete may indicate that this residue lies partially exposed in a hydrophobic pocket. This idea is further supported by the observation that although SucNBr oxidation of this residue destroys biological activity, its substitution with the hydrophobic (HO)($NO_2$)Bzl group leaves activity unimpaired. Trp-76 is virtually unreactive to the (HO)($NO_2$)Bzl reagent in the native protein but reacts readily with this reagent in 6 M guanidine hydrochloride, thus implicating this residue as the probable site of slowest SucNBr oxidation ($k_3^{NGF} = 9.8 \times 10^2$ mol$^{-1}$ sec$^{-1}$).

Trp-21 and Trp-76 are contained within the two segments of the NGF sequence (1–26 and 62–81) which correspond to the B and A chains of insulin, respectively (Table V) (Frazier *et al.*, 1972). Thus, the chemical description of the location of these residues in NGF can be compared with the location of the corresponding residues in the insulin model. Since there are no tryptophan residues in insulin, the locations of Trp-21 and Trp-76 must be compared with their corresponding residues in the sequence alignment, Phe-B-25 and Leu-A-16, both of which are bulky, hydrophobic residues.

The data presented above indicate that NGF-Trp-21 is fully solvent available by several criteria. Figure 11 shows a tryptophan residue superimposed on the corresponding in-

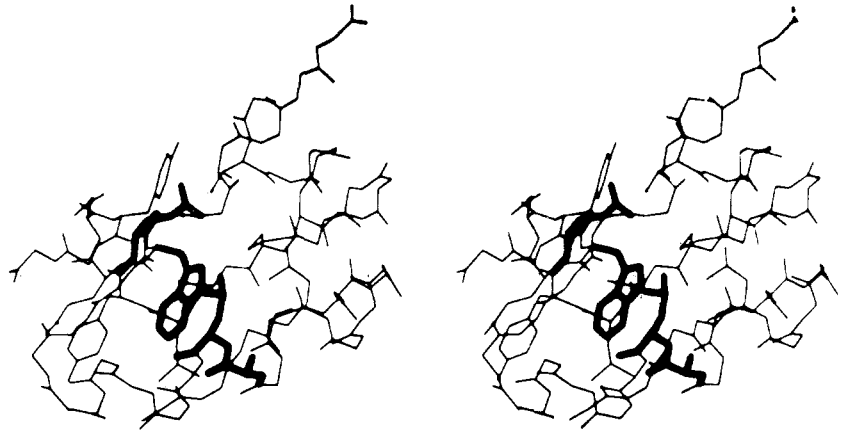

FIGURE 13: Stereorepresentation of the insulin monomer with the residues, which are deleted in the NGF sequence, intensified (B-14, B-15, and A-15). The substituted Trp-76 of NGF (for insulin Leu-A-16) is also intensified (structural data as in Figure 10).

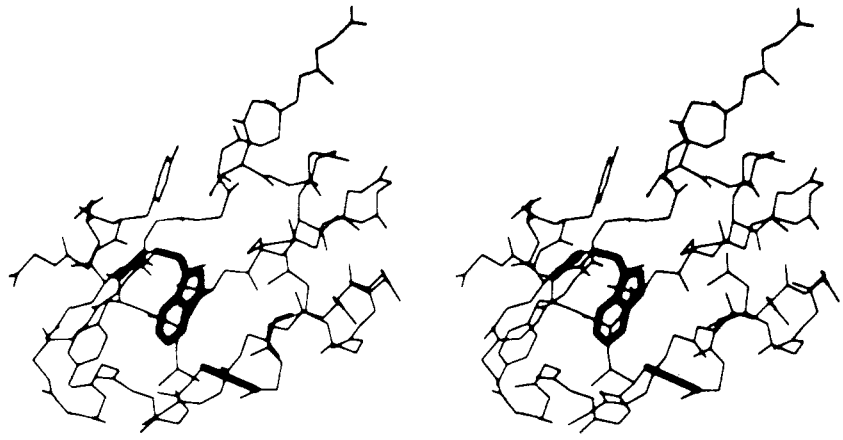

FIGURE 14: Stereorepresentation of the insulin monomer with the deleted residues of insulin replaced by straight bonds (intensified). No other alterations of any residues or chain conformation have been done (structural data as in Figure 10).

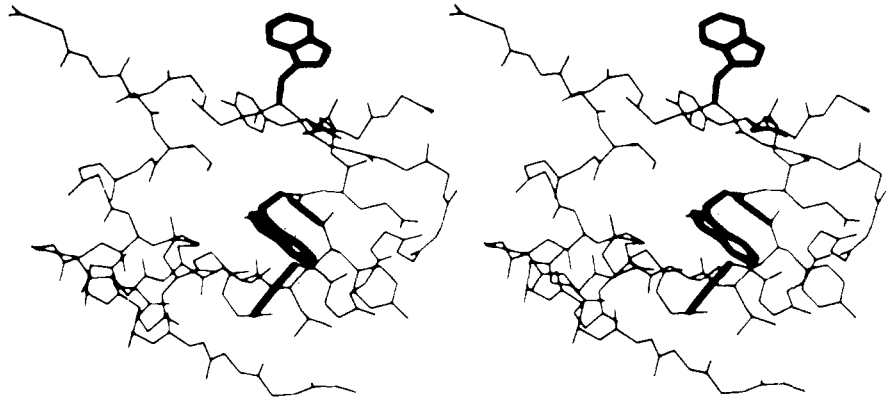

FIGURE 15: Stereorepresentation of the insulin monomer where Trp-21 and Trp-76 of NGF have been substituted for their corresponding insulin residues, Phe-B-25 and Leu-A-16, respectively (intensified). Also intensified are the straight bonds replacing the deleted residues, B-14, B-15, and A-15 (structural data as in Figure 10).

sulin residue, Phe-B-25 with the indole ring oriented coplanar with the phenyl ring. Clearly, the indole moiety is fully solvent available and there is no hindrance by nearby groups to the introduction of this tryptophan side chain into the insulin structure. The side chain to the right of the indole ring is that of Asn-A-21 which is replaced in NGF with the smaller residue Thr-81. The residue to the lower left of the indole is Thr-B-27, replaced in NGF with the smaller Gly-23.

NGF-Trp-76 is the least reactive of the three tryptophans (*vide supra*) and as such probably lies in the interior of the NGF molecule. The corresponding residue in insulin is Leu-A-16. Figure 12 is a stereodrawing of the insulin monomer with a tryptophan residue inserted by superposition of its $\alpha$, $\beta$, $\gamma$, and two $\delta$ atoms with the $\alpha$ through two $\delta$ carbons of Leu-A-16. The indole moiety is seen to lie in the center of the molecule completely enclosed within the pocket formed by the A and B chains. All of the NGF residues which correspond to those surrounding this central core are of the same

size or smaller than the insulin residues. The only serious steric encroachment of the indole side chain is by the side chain of Leu-B-15. Interestingly, this residue, and two others, Ala-B-14 and Glu-A-15, which surround the core of the molecule, are deleted in the NGF sequence (Frazier *et al.*, 1972). These residues, along with the replaced tryptophan, are shown intensified in Figure 13. In Figure 14, these residues have been replaced by intensified straight bonds. No attempt has been made to rearrange the insulin chains to account for the deletions, however, minimal distortion of the chain segments to close the deletions yields a plausible structure that allows the indole ring an orientation free of unfavorable steric interactions yet still completely enclosed within the hydrophobic interior of the molecule.

Thus, the predictions, in terms of chemical reactivity, concerning the relative positioning of NGF-Trp-21 and Trp-76, are completely compatible with the location of the corresponding residues in insulin. As can be seen in Figure 15, which summarizes the deletions and tryptophan replacements described, the topography of NGF elucidated in these studies is entirely consistent with a conservation of a similar three-dimensional structure for the two proteins. Furthermore, the introduction of tryptophan side chains into the insulin structure, by means of computer modeling, provides some speculative clues as to the rationale of the evolutionary events which led to the present structure of NGF.

## Acknowledgments

The authors express their appreciation to Ms. Linda Boyd for excellent technical assistance throughout this work and to Ms. Carmelita Lowry for her help with the ultracentrifugal analyses. They also thank Dr. David J. Bates for his assistance with the stopped-flow kinetic analyses and the computer calculation and simulation of these data, and Drs. John Fritsch and C. David Barry for their guidance and assistance in preparing the stereorepresentations on the macromodular system of the Computer Systems Laboratory. The stereophotographic reproductions of the computer display were kindly done by David Shupe. Finally, the authors acknowledge the helpful suggestions of Dr. Carl Frieden concerning the kinetic experiments, Dr. James Ferrendelli on the radioimmunoassay, and Dr. David A. Deranleau with the charge-transfer titrations.

## References

Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, B., and Sheats, S. (1969), *Nature (London) 224*, 491.

Angeletti, R. H. (1970), *Biochim. Biophys. Acta 214*, 478.

Angeletti, R. H., Bradshaw, R. A., and Wade, R. D. (1971), *Biochemistry 10*, 463.

Angeletti, R. H., Frazier, W. A., and Bradshaw, R. A. (1972), *8th Meeting Fed. Eur. Biochem. Soc., Amsterdam*, 462.

Angeletti, R. H., Mercanti, D., and Bradshaw, R. A. (1973), *Biochemistry 12*, 90.

Barman, T. E. (1972), *Biochim. Biophys. Acta 258*, 297.

Barman, T. E., and Koshland, D. E. (1967), *J. Biol. Chem. 242*, 5771.

Bates, D. J., and Frieden, C. (1973), Biomedical Computer Laboratory Monograph No. 181, St. Louis, Mo., Washington University.

Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., Mercola, D. A., and Vijayan, M. (1971), *Nature (London) 231*, 506.

Bocchini, V., and Angeletti, P. U. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 787.

Boesel, R. W., and Carpenter, F. H. (1970), *Biochem. Biophys. Res. Commun. 38*, 678.

Bradshaw, R. A., and Deranleau, D. A. (1970), *Biochemistry 9*, 3310.

Bradshaw, R. A., Frazier, W. A., and Angeletti, R. H. (1972), *in* Chemistry and Biology of Peptides, Meienhofer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, pp 423–439.

Chance, R. E., Ellis, R. M., and Bromer, W. W. (1968), *Science 161*, 165.

Cuatrecasas, P. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 450.

Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B. (1968), *J. Biol. Chem. 243*, 4787.

Deranleau, D. A., Bradshaw, R. A., and Schwyzer, R. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 885.

Deranleau, D. A., and Schwyzer, R. (1970), *Biochemistry 9*, 126.

Frank, B. H., and Veros, A. J. (1970), *Biochem. Biophys. Res. Commun. 38*, 284.

Frank, B. H., Veros, A. J., and Pekar, A. H. (1972), *Biochemistry 11*, 4926.

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

Hershko, A., Mamont, P., Sheilds, R., and Tomkins, G. (1971), *Nature (London), New Biol. 232*, 206.

Horton, R. H., and Tucker, W. P. (1970), *J. Biol. Chem. 245*, 3397.

Hunter, W. M., and Greenwood, F. C. (1962), *Nature (London) 194*, 495.

Karrer, P., Schwarzenbach, G., Benz, F., and Solmssen, V. (1936), *Helv. Chim. Acta 19*, 811.

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

Liu, T.-Y., and Chang, Y. H. (1971), *J. Biol. Chem. 246*, 2842.

Loudon, G. M., and Koshland, D. E. (1970), *J. Biol. Chem. 245*, 2247.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.

Morris, J. W. S., Mercola, D. A., and Arquilla, E. R. (1970), *Biochemistry 9*, 3930.

Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.

Spande, T. F., and Witkop, B. (1967), *Methods Enzymol. 11*, 498.

Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.

Walsh, K. A., MacDonald, R. M., and Bradshaw, R. A. (1970), *Anal. Biochem. 35*, 193.

Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.