Jencks, W. P. (1959), *J. Am. Chem. Soc. 81*, 475.
Levene, C. I. (1962), *J. Exptl. Med. 116*, 119.
Levene, C. I., and Gross, J. (1959), *J. Exptl. Med. 110*, 771.
Martin, G. R., Gross, J., Piez, K. A., and Lewis, M. S. (1961), *Biochim. Biophys. Acta 53*, 599.
Nagai, Y., Gross, J., and Piez, K. A. (1964), *Ann. N. Y. Acad. Sci. 121*, 494.
Orloff, S., and Gross, J. (1963), *J. Exptl. Med. 117*, 1009.
Piez, K. A. (1965), *Biochemistry 4*, 2590.
Piez, K. A., Eigner, E. A., and Lewis, M. S. (1963), *Biochemistry 2*, 58.
Rojkind, M., Blumenfeld, O. O., and Gallop, P. M. (1964), *Biochem. Biophys. Res. Commun. 17*, 320.
Schleyer, M. (1962), *Z. Physiol. Chem. 329*, 97.
Veis, A. (1964), The Macromolecular Chemistry of Gelatin, New York, N. Y., Academic.
Wood, G. C. (1963), *J. Pharm. Pharmacol. Suppl. 15*, 134T.
Wood, G. C. (1964), *Intern. Rev. Connective Tissue Res. 2*, 1.

The Reactivity toward *N*-Bromosuccinimide of Tryptophan in Enzymes, Zymogens, and Inhibited Enzymes*

T. F. Spande, N. M. Green, and B. Witkop

ABSTRACT: The oxidation of tryptophan by *N*-bromosuccinimide (NBS) in α-chymotrypsin, acetylchymotrypsin, diisopropylphosphoryl- (DIP) chymotrypsin, *N*-*p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone- (TPCK) inhibited chymotrypsin, and chymotrypsinogen A was investigated over a pH range of 4.0–7.0.

The native enzyme possessed an additional 2–3 moles of NBS-reactive tryptophan at pH 5.5–6.0 compared with the inhibited enzymes or the zymogen. Acetylchymotrypsin after deacylation behaved exactly like native chymotrypsin in its oxidizability by NBS. This suggests that the difference in reactivity of bound tryptophan is the result of a conformational change. Much smaller differences in reactivity were observed in the pH range 5.5–6.0 between trypsin, DIP-trypsin, and trypsinogen; however, a large difference was noticeable between trypsin and its complex with the inhibitor from beef pancreas, where at pH 5.0 two tryptophan equivalents in the complex were protected from oxidation by NBS. The nature of the pH effect in the reaction of NBS with proteins and the use of NBS in effecting selective modifications of proteins are discussed.

The use of *N*-bromosuccinimide (NBS)[1] as a specific reagent for the modification of tryptophan in proteins has led to two major applications: one is a rapid and convenient spectrophotometric method for determining the tryptophan content of a protein (Patchornik *et al.*, 1958); the other provides a means of cleaving the tryptophyl peptide bond (Patchornik *et al.*, 1958, 1960; Ramachandran and Witkop, 1959, 1964; Witkop, 1961).

The former application is based on the large decrease in absorbance at 280 mμ accompanying the transformation of the indole to the oxindole chromophore. For most tryptophan-containing proteins this oxidation is instantaneous and quantitative at acidic pH values. The second application utilizes an excess of reagent, also at low pH, for the cleavage of the tryptophyl peptide bond in yields ranging from 30 to 70%. In this cleavage the amide carbonyl of the tryptophan participates in the opening of the postulated labile indole bromonium intermediate. This procedure has been used to determine or audit the identity of the residues following tryptophan. The comparatively low yield of the cleavage reaction has so far limited the utility of this procedure to spot-checking of protein sequences.

Recently, NBS has seen a further application in the preparation of oxidized proteins, in which some or all tryptophan residues have been modified (Hayashi *et al.*, 1964, 1965; Davidson and Westley, 1965; Viswanatha and Lawson, 1961; Okada *et al.*, 1963; Lokshina *et al.*, 1962; Green, 1963). Such selective modifications have provided useful information on the involvement of tryptophan in enzymic processes and in the interaction of proteins with other proteins and small molecules.

Many of these oxidative modifications, as we have

* From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received January 27, 1966.*

[1] The following abbreviations were used in this paper: NBS, *N*-bromosuccinimide; DIP, diisopropylphosphoryl; TPCK, *N*-*p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone; PTI, pancreatic trypsin inhibitor.

now found out, can be carried out in the pH range 5–7, close to neutrality, in contrast to the older procedure which utilized acidic conditions in the pH range 3–4. Oxidations at higher pH values have the advantage that no cleavage occurs and that the differential reactivity of individual tryptophan residues (Okada *et al.*, 1963; Lokshina *et al.*, 1962) becomes more noticeable in analogy to oxidations with hydrogen peroxide in aqueous dioxane (Hachimori *et al.*, 1964, 1965).

Spectral and gas chromatographic studies on model compounds, *e.g.*, skatole, 3-indole-β-propionylglycine, demonstrated that indoles could be converted to oxindoles in good yield at pH 6, although a model more appropriate for bound tryptophan, *N*-acetyl-L-tryptophanamide, gave the oxindole (40% yield), accompanied by additional oxidation products (Green and Witkop, 1964).

Although the reaction between NBS and all the model indoles tested proceeded rapidly and seemed independent of pH over the range 3–10, a pronounced pH effect was observed in the reactivity of tryptophan toward NBS in many proteins, as reflected in the extent of oxidation observed at a given pH (Green and Witkop, 1964). In a series of proteolytic enzymes and proteins, it was found that at a pH >7, little or no oxidation of tryptophan occurred; at pH 5.5, some of the bound tryptophan could be oxidized, and at pH 4.0, usually all of the tryptophan present could be oxidized. A few proteins in their native state possessed tryptophan residues inert to NBS even at acid pH (Ramachandran and Witkop, 1959; Ramachandran, 1960; Green, 1963; Green and Witkop, 1964).

These observations suggested that some of the tryptophans in proteins may lie in an environment to which access by NBS is hindered or excluded. Tryptophan has been assigned an important role in determining and stabilizing the tertiary structure of a protein by its interaction with other hydrophobic residues and hence, such "buried" tryptophan residues could be anticipated on theoretical grounds (Kauzmann, 1959).

Small molecules as well as proteins are capable of protecting the tryptophan of a protein from NBS oxidation. All of the 16 reactive tryptophans of the protein, avidin, are rendered unreactive toward NBS by the specific binding of the small molecule, biotin (Green, 1963). This protection was correlated with a shift of the tryptophan absorption spectrum to the red, showing that the chromophores had moved from an aqueous to a nonpolar environment. Some of the tryptophans of trypsin are protected from NBS oxidation by interaction with the trypsin inhibitor from beef pancreas, a tryptophan-free protein whose molecular weight is roughly 0.25 of trypsin's (Spande and Witkop, 1965).

Thus, it seemed reasonable to expect that the NBS oxidation of a protein might show differences in the environment of its tryptophan residues and that even subtle conformational changes in a protein, such as have been demonstrated or postulated in the transformation of a zymogen to enzyme or an enzyme to an inactive enzyme, might be revealed in modified tryptophan reactivity (Biltonen *et al.*, 1965; Koshland, 1958).

Early studies with NBS on trypsin and trypsinogen at pH 4.0 indicated a difference in tryptophan reactivity between the enzyme and zymogen (Viswanatha *et al.*, 1960). A report exists (Wootton and Hess, 1962) indicating a slight difference in tryptophan oxidizability, even under conditions of denaturation, between chymotrypsin and DIP-chymotrypsin.

This study investigates the chemical reactivity of tryptophan toward NBS in two well-characterized enzyme systems, those of trypsin and chymotrypsin, examining particularly the role of pH.

Experimental Section

Biochemicals. The enzymes and related materials below were obtained from the Worthington Biochemical Corp., Freehold, N. J. Stock solutions, of the concentration indicated, were prepared in 0.002 M HCl and stored at 5° until used; trypsin, twice crystallized, lyophilized, TRL 6254, *c*, 5 mg/ml; trypsin, DFP treated, T-DIP-109, *c*, 5 mg/ml; trypsinogen, once crystallized, TG 6401, *c*, 8 mg/ml; pancreatic trypsin inhibitor compound, PIC-5556, *c*, 12 mg/ml of salt-containing material or 9 mg/ml of protein; a solution was also prepared in 0.2 M acetate (pH 5.0); α-chymotrypsin, chromatographically homogeneous, CDC-51, *c*, 6 mg/ml; α-chymotrypsin, DFP-treated, CD-DIP 204, *c*, 6 mg/ml; chymotrypsinogen A, chromatographically homogeneous, CGC-762, *c*, 5.3 mg/ml; α-chymotrypsin, acetylated at the reactive serine, was prepared following a published procedure (Bender *et al.*, 1964). G-25 (5.0 g) Sephadex (medium), equilibrated with 0.2 M acetate buffer (pH 3.5), was used in the separation procedure, and only the first 15 ml of eluate was taken. This solution, containing 3.5 mg/ml, was stored at 5° and used within 48 hr.

α-Chymotrypsin, inhibited by reaction of one of the histidine residues at the active site (Ong *et al.*, 1965) with the reagent *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), was prepared following the published procedure (Schoellmann and Shaw, 1963). A fivefold increase in enzyme concentration was used. A slight turbidity developed during the addition of the reagent to the enzyme solution. After dialysis and lyophilization, centrifugation (10,000 rpm; 20 min) was necessary to remove a small amount of water-insoluble material. A solution containing 4.3 mg/ml was prepared.

Chemicals. A sample of *p*-nitrophenyl acetate was obtained from Dr. G. W. A. Milne and after one crystallization from methyl alcohol melted at 79–80°.

The TPCK reagent, lot F-1465, was obtained from Cyclo Chemical Corp., Los Angeles, Calif., and had mp 101–102°, $[\alpha]_D^{20}$ +5.80°. The NBS used was obtained, as practical grade, from Eastman Organic Chemicals and was recrystallized once from glacial acetic acid. Aqueous solutions (0.01 M) could be stored for several days at 5° without deterioration.

N-Acetyl-DL-phenylalanine, CP, lot E1841, mp 150–151°, was obtained from Mann Research Laboratories,

New York, N. Y. A solution containing 30.9 mg in 10.0 ml of 0.117 M phosphate buffer (pH 7.0) was prepared.

A copolymer of L-glutamic acid and L-leucine was a generous gift from Dr. M. Sela, Weizmann Institute of Science, Rohovoth, Israel. A saturated solution of $(L\text{-Glu-}L\text{-Leu})_{190}$ (1:1.2) in 10.0 ml of 0.11 M acetate buffer (pH 5.5) containing 68 mg of copolymer was prepared. A drop of *n*-octyl alcohol (to control foaming) and centrifugation at 12,000 rpm for 30 min were necessary to obtain a clear solution.

Instrumentation. A Cary Model 11, self-recording spectrophotometer and 1-cm cells were used in the measurement of optical densities. A Radiometer pH-meter, type TTTlc, with a type GK 202 1B combination electrode, calibrated with appropriate reference buffers, was used to adjust the pH of the buffers and, when necessary, the pH of the system in the spectrophotometer cell.

Methods

The oxidation of tryptophan in enzymes with NBS proved to be very sensitive to environmental factors. After investigation of the variables, temperature, enzyme concentration, buffer species, and buffer concentration, the following conditions were chosen: 0.1 M acetate buffer, a temperature of 25° and enzyme concentrations of *ca.* 3×10^{-5} M.

Into a 48 × 10 × 10 mm ultraviolet cell were pipetted 1.0 ml of 0.20 M acetate or phosphate buffer of the desired pH and enough enzyme or derivative to yield an absorbance of 1.5–1.7 when diluted to 2.0 ml, and water was added to make up a total volume of 2.0 ml. In the reference cell was pipetted 1.0 ml each of 0.20 M buffer and water. The sample solution was stirred by a "flea" and a magnetic stirrer. A capillary and 2.0 N HCl or 1.0 N NaOH were found convenient for the adjustment of the pH when necessary. The spectrum was scanned from 400 to 250 mμ to establish the base line. In the case of TPCK-inhibited chymotrypsin and acetyl-chymotrypsin, the base line was observed to rise slightly from 400 to 320 mμ. In the case of trypsin, incubated with the synthetic polypeptide inhibitor, observable faint turbidities at pH 5.5 or 6.0 led to a rising base line of much steeper slope. In these cases, the base line was extrapolated from the 380- to 320-mμ region to correct the absorbance readings at 280 mμ for the contribution due to light scattering. Concentrations of the enzyme (mg/ml) were determined using the optical factor 0.695 for trypsin, trypsinogen, and DIP-trypsin (Laskowski and Laskowski, 1954) and the factor 0.810 for the trypsin–pancreatic inhibitor complex. An optical factor of 0.500 at 282 mμ was used for all of the chymotrypsin systems (Laskowski, 1961).

The sample cell was placed in a thermostated bath (25 ± 0.1°) and allowed to equilibrate, and the initial optical density at 280 or 282 mμ was determined. The cell was replaced in the bath and 10-μl portions of a 0.01 M NBS solution were added *with rapid stirring* to the contents of the cell, holding the tip of the Lang–Levy blow-out type micropipet in the "flea's" vortex. After *ca.* 30 sec, the absorbance was again determined and recorded for at least 20 sec. Addition of NBS was continued in this stepwise manner until the absorbance readings no longer decreased or (see Results) began to increase. The final absorbance was corrected for the volume increase due to the added reagent (usually 100–150 μl). The extinction of the oxidized tryptophan was calculated as the product of the corrected decrease in absorbance at 280 or 282 mμ and the empirical factor, 1.31 (Patchornik *et al.*, 1958).

This factor is based on oxidation studies with model tryptophan peptides which are converted by NBS to oxindoles at pH 4.0. On the basis of recent (T. F. Spande, N. M. Green, and B. Witkop, unpublished data) experiments, this factor may be used up to pH 7.0. Whether the oxidation of tryptophan in proteins leads to (ring brominated) oxindoles or dioxindoles does not significantly affect the calculation of the extent of oxidation of bound tryptophan, since the molar extinctions at 280 mμ of models containing these chromophores are nearly the same. Cleavage of trypsin (Viswanatha *et al.*, 1960) and chymotrypsin (Viswanatha and Lawson, 1961) with NBS at pH 4 was negligible. The higher pH and the absence of denaturation in our present investigation would minimize cleavage, *i.e.*, formation of dioxindoles, even further.

Using 5500 as the molar extinction coefficient for tryptophan at 280 mμ, and the sample volume of 2.0 ml, the micromoles of tryptophan oxidized equal $\Delta OD_{280}/2.10$. The micromoles of tryptophan present was determined from the micromoles of protein in the sample, multiplied by the number of tryptophan residues/mole; four for trypsin systems and eight for chymotrypsin systems (Walsh and Neurath, 1964). A molecular weight of 23,800 was assumed for trypsin, and 25,000 for α-chymotrypsin (Desnuelle, 1960). The extent of oxidation is defined as the ratio of the moles of tryptophan oxidized/the moles of tryptophan present.

Two runs were generally made at each of the following pH values: 6.0, 5.5, 5.0, 4.5, and 4.0. In some instances, oxidations were performed at the pH values 7.0, 3.5, and 3.0. The general equation is: % oxidation =

TABLE I: The Effect of Variables, Temperature, Buffer Species, and Buffer Concentration on the Extent of Tryptophan Oxidation with NBS.

Chymotrypsinogen A + NBS (pH 5.5, 25°)		Trypsin + NBS (0.1 M Phosphate, pH 6.0)	
Buffer (M)	% Oxidation	Temp (°C)	% Oxidation
Phosphate (0.1)	23	25.0	20
Acetate (0.1)	23	30.0	25
Acetate (0.05)	48	35.0	52
Acetate (0.03)	70	40.0	47

$[(\Delta OD/OD)100](1.31 \times \text{mol wt}/1000 \times V/(5.50fnV)$ where ΔOD = corrected decrease in optical density at 280 or 282 mμ; OD = initial optical density at 280 or 282 mμ; 1.31 = empirical factor (Patchornik *et al.*, 1958); 5.50 = millimolar extinction coefficient for tryptophan at 280 mμ; mol wt = molecular weight of protein titrated; V = sample volume, ml; f = optical factor for protein at 280 or 282 mμ; n = number of tryptophan residues/mole of protein. For chymotrypsin, this simplifies to: % oxidation = % decrease $OD_{282} \times 1.49$. This is also considered a very good approximation for its derivatives, as the change in molecular weight and the optical factor should nearly cancel one another in the general expression above. For trypsin, using the same expression: % oxidation = % decrease $OD_{280} \times 2.02$.

From the optical factors for trypsin (0.695) and the inhibitor (1.26) at 280 mμ (Laskowski and Laskowski, 1954), a factor of 0.869 was derived to correct the optical density of the complex at 280 mμ for the contribution due to trypsin alone. A molecular weight of 30,300 was assumed for the complex (Laskowski and Laskowski, 1954).

Results

The effect of the variables, temperature, buffer species, and buffer concentration on the extent of tryptophan oxidation with NBS is demonstrated in Table I. The effect of buffer concentration and temperature on the extent of oxidation of tryptophan in enzymes with NBS is very marked; decreasing the concentration of the buffer or increasing the temperature increases the extent of oxidation, at a given pH. Thus pH alone does not govern the extent of oxidation. The effect of enzyme concentration on the extent of oxidation seems less crucial. Changing from acetate to phosphate buffer seemed to have little effect on the extents of oxidation observed, although titrations in phosphate buffer required much more NBS to achieve the same extent of oxidation as in acetate.

Table II gives the extent of oxidation (expressed as a percentage) of tryptophan at a given pH with excess *N*-bromosuccinimide in α-chymotrypsin, chymotrypsinogen A, DIP-chymotrypsin, acetyl-α-chymotrypsin, and TPCK-inhibited α-chymotrypsin. Table III gives the results of NBS oxidation of α-chymotrypsin in the

TABLE II: Oxidation of α-Chymotrypsin and Derivatives with NBS.[a]

α-Chymotrypsin (mol wt 25,000) concn = 1.57–1.58 mg/2.0 ml							
pH	7.0	6.0	5.5	5.0	4.5	4.0	
Extent of oxidation (%)	37	49	49	81	92	95	
Moles of Try oxidized/mole of protein	3.0	3.9	3.9	6.5	7.3	7.5	
Moles of NBS/mole of Try	14	5.1	4.3	3.0	2.8	2.7	
Chymotrypsinogen A (mol wt 25,400) concn = 1.58–1.61 mg/2.0 ml							
pH		6.0	5.5	5.0	4.5	4.0	
Extent of oxidation (%)		11	23	72	95	101	
Moles of Try oxidized/mole of protein		0.9	1.9	5.8	7.5	8	
Moles of NBS/mole of Try		7.1	5.2	3.0	2.9	2.6	
DIP-chymotrypsin (mol wt 25,170) concn = 1.76–1.77 mg/2.0 ml							
pH		6.0	5.5	5.0	4.5	4.0	
Extent of oxidation (%)		12	11	64	90	95	
Moles of Try oxidized/mole of protein		1.0	0.9	5.0	7.1	7.5	
Moles of NBS/mole of Try		12	10	3.5	3.0	2.7	
Acetyl-α-chymotrypsin (mol wt 25,000) concn = 1.47–1.48 mg/2.0 ml							
pH		6.0	5.5	5.0	4.5	4.0	
Extent of oxidation (%)		19[b]	19	67	93	97	
Moles of Try oxidized/mole of protein		1.5	1.5	5.3	7.4	7.7	
Moles of NBS/mole of Try		10	8.9	3.5	2.8	2.6	
TPCK-inhibited-chymotrypsin (mol wt 25,300) concn = 1.72–1.76 mg/2.0 ml							
pH	6.0	5.5	5.0	4.5	4.0	3.5	3.0
Extent of oxidation	16	23	49	71	87	91	104
Moles of Try oxidized/mole of protein	1.2	1.9	3.9	5.7	7.0	7.2	8
Moles of NBS/mole of Try	6.9	5.4	4.8	4.3	3.3	3.2	2.6

[a] These oxidations were conducted at 25.0° in 0.1 M acetate buffers, with the exception of the pH 7.0 study, which used 0.1 M phosphate buffer. "Moles of NBS/mole of Try" refers to moles of NBS added/mole of tryptophan oxidized. [b] Concn = 0.68 mg/2.0 ml.

TABLE III: α-Chymotrypsin + Inhibitor + NBS.[a]

	N-Acetyl-DL-phenylalanine + α-Chymotrypsin		
Moles of inhibitor/ mole of chymotrypsin	380[b]	70	0
Extent of oxidation (%)	37–39	33	37

[a] These oxidations were conducted at 25.0° in 0.1 M phosphate buffer (pH 7.0). [b] 1.7 ml of 0.12 M phosphate buffer (pH 7.0) containing 3.09 mg of *N*-acetyl-DL-phenylalanine/ml + 300 μl of solution of α-chymotrypsin in 0.002 N HCl (6 mg/ml).

presence of the inhibitor, *N*-acetyl-DL-phenylalanine.

A demonstration that acetylation of the active serine residue in α-chymotrypsin is alone responsible for the diminished reactivity of the tryptophan residues in acetyl-α-chymotrypsin in comparison with α-chymotrypsin itself was provided in the following experiment. A sample of acetyl-α-chymotrypsin at pH 5.5 was titrated wih NBS until no further decrease in OD_{282} was observed. Another sample of acetyl-α-chymotrypsin in the same buffer was first adjusted to pH 8.6 using a capillary and 1.0 N NaOH (*ca.* 5 μl required), allowed to stir at 25° for 10 min [under these conditions, deacylation occurs (Bender *et al.*, 1964)], then the pH was readjusted to 5.5 using 2 N HCl. This sample was titrated with NBS as above. The results and a comparison with α-chymotrypsin are shown in Table IV.

TABLE IV: Acetyl-α-chymotrypsin + NBS before and after Deacylation.[a]

Protein Concn (M)	Extent of Oxidation of Acetyl-α-chymotrypsin before Deacylation	after Deacylation	Extent of Oxidation of α-Chymotrypsin
2.0×10^{-5}	20 (11)	44 (5.0)	
3.0×10^{-5}	17; 22 (10) (7.9)	52; 49 (4.1) (4.3)	49 (4.3)

[a] These oxidations were conducted at 25.0° in 0.1 M acetate buffer (pH 5.5). The values in parentheses are moles of NBS added/mole of tryptophan oxidized. The concentration of α-chymotrypsin is given in Table II.

Table V gives the results of the NBS oxidation of trypsin, DIP-trypsin, trypsinogen, trypsin inhibited with the pancreatic inhibitor, and trypsin partially inactivated with a synthetic polypeptide (Sela and Rigbi, 1964).

In the titration of α-chymotrypsin and all of its derivatives in the pH range 5.0–7.0, the end point of the titration was coincident with, or followed by, the development of a faint but slowly increasing turbidity. In some cases (particularly in phosphate buffers), several minutes elapsed before the turbidity became noticeable by the increase in optical density. This turbidity could also be produced, with either trypsin or chymotrypsin samples, by addition of an aliquot of NBS solution to an *unstirred* solution. This phenomenon has been reported (Green and Witkop, 1964) for hen's egg lysozyme at pH 7.0, though the present study indicates that α-chymotrypsin is no less prone to form a precipitate with NBS than is lysozyme.

Discussion

With either the chymotrypsin system or the trypsin system, the extent of oxidation increased as the pH was lowered, reaching values for complete oxidation at pH 3.0–4.0. The efficiency of NBS as an oxidizing agent also increased as the pH was lowered. At pH 6.0–7.0, 5–10 moles of NBS was required to oxidize each mole of tryptophan. As the pH was lowered, a limiting value of 2.6–2.7 moles of NBS/mole of tryptophan oxidized was reached for each protein, as reported in an earlier investigation (Viswanatha *et al.*, 1960) on trypsin and trypsinogen.

It is difficult to rationalize this apparent pH effect on the oxidizability of tryptophan in these two systems, particularly in view of the absence of any report of pH-induced conformational changes for either chymotrypsin or trypsin. Solvent perturbation studies which measure tryptophan exposure by the reversible interaction of a nonpolar solvent with the indole chromophore reveal no pH dependence on the degree of tryptophan exposure for lysozyme or chymotrypsinogen A (Williams *et al.*, 1965) in the pH range 2.5–7.0. The results of this method need not necessarily agree with those found using NBS, as the latter method would measure tryptophan exposure in terms of the momentary accessibility of the 2–3 double bond of the indole ring to NBS, whereas the effect of solvent upon the absorption spectrum depends on the environment of the entire indole chromophore. Furthermore, the reaction of a tryptophan residue with NBS would lead to irreversible modification of the indole ring, and to a major change of its hydrophobic character, which, in turn, would lead to irreversible changes in the protein's structure. The pH dependence of oxidation would not necessarily be comparable to any effect of pH on a native protein, but should instead be correlated with the effect of pH on a protein molecule containing some oxidized tryptophan residues. At present, no such data are available, but it is likely that a protein containing oxindole residues would be more sensitive to pH change and so show pH effects not observed with an unmodified protein.

The reduced efficiency of NBS at higher pH values is probably a reflection of the slower reaction of partially buried tryptophan. Under such circumstances, tyrosine,

TABLE V: Oxidation of Trypsin and Derivatives with NBS.[a]

Trypsin (mol wt 23,800) concn = 2.17 mg/2.0 ml								
pH	7.0	6.0	5.5	5.0	4.5	4.0	3.5	3.0
Extent of oxidation (%)	13	25	44	65	80	90	93	96
Moles of Try oxidized/mole of protein	0.5	1.0	1.8	2.7	3.2	3.6	3.7	3.8
Moles of NBS/mole of Try	11	8.7	7.2	4.2	3.5	3.1	3.0	2.6
DIP-trypsin (mol wt 23,900) concn = 2.14 mg/2.0 ml								
pH	7.0	6.0	5.5	5.0	4.5			
Extent of oxidation (%)	0.0	10	42	60	81			
Moles of Try oxidized/mole of protein		0.4	1.7	2.4	3.3			
Moles of NBS/mole of Try		28	11	5.6	3.1			
Trypsinogen (mol wt 24,400) concn = 2.40 mg/2.0 ml								
pH	7.0	6.0	5.5	5.0	4.5	4.0		
Extent of oxidation (%)	0.0	32	38	59	76	83		
Moles of Try oxidized/mole of protein		1.3	1.5	2.3	3.0	3.3		
Moles of NBS/mole of Try		9.5	8.1	5.2	3.4	3.1		
Trypsin–Pancreatic Inhibitor Complex (mol wt 30,300) concn = 2.08 mg of trypsin/2.0 ml								
pH	7.0	6.0	5.5	5.0	4.5	4.0	3.5	3.0
Extent of oxidation (%)	0.0	4.1	5.3	16	44	58	65	94
				55[b]				103[b]
Moles of Try oxidized/mole of protein		0.16	0.21	0.64	1.8	2.3	2.6	3.8
				2.2[b]				4.1[b]
Moles of NBS/mole of Try		13	15	8.6	3.1	2.8	2.9	2.9
								2.1[b]
Trypsin + (L-Glu-L-Leu)$_{190}$ (1:1.2)								
pH		6.0			5.5			
mg of copolymer/mg of trypsin	10.5	5.1		8.6	4.9[c]			
Extent of oxidation (%)	47	45		52	53			
Moles of Try oxidized/mole of protein	1.9	1.8		2.1	2.1			
Moles of NBS/mole of Try	8.3	7.1		6.9	4.6			

[a] These oxidations were conducted at 25.0° in 0.1 M acetate buffers with the exception of the pH 7.0 study which employed 0.1 M phosphate. Moles of NBS/mole of Try is NBS added/mole of tryptophan oxidized. [b] Urea (8 M) adjusted to the desired pH with HOAc. [c] Activity (22%) remains after incubation for 1 hr with inhibitor at 25.0°.

histidine, methionine, and cystine, which react relatively slowly, will begin to compete with tryptophan for NBS (Ramachandran, 1962; Schmir and Cohen, 1961). This effect may be further accentuated by the increase in concentration of the more reactive tyrosine phenolate ion as the pH is increased.

It seems likely from the available data on the inactivation of trypsin, chymotrypsin, lysozyme, α-amylase, and avidin (Spande and Witkop, 1966) that tryptophan residues in many proteins are attacked by NBS in a selective, sequential fashion rather than at random. This is reasonable, since the environment and consequently the reactivity of each tryptophan in the protein molecule is likely to be unique.

In the pH region between 5.5 and 6.0, there were marked differences in oxidizability between chymotrypsin and its inactive derivatives. Possibly (2–3) discrete residues of oxidizable tryptophan in the active enzyme were unreactive in chymotrypsinogen, DIP-chymotrypsin, acetylchymotrypsin, and TPCK-inhibited chymotrypsin. The differences between trypsin and its derivatives were much smaller. With the exception of the PTI complex, the only significant observation was the presence of 0.6 more mole of resistant tryptophan in DIP-trypsin at pH 6.0. This difference between the two enzyme systems is not surprising, since there are three or four more tryptophans in chymotrypsin (Keil *et al.*, 1963; Hartley, 1964) than in trypsin. The four residues present in trypsin significantly occupy positions homologous with four tryptophans in chymotrypsin (Walsh and Neurath, 1964).

The close correspondence (Table IV) between the titration data for acetylchymotrypsin, after brief exposure to mildly alkaline conditions, and native chymotrypsin supports the belief that the difference in oxidizability of tryptophan in acetylchymotrypsin and the native enzyme does indeed reflect a conformational change produced in the enzyme when the active site is modified, even reversibly. A primary steric role for the small acetyl group is unlikely.

The most significant effect on the oxidizability of tryptophan in an inhibited enzyme was observed with trypsin inhibited by the inhibitor from beef pancreas (Laskowski and Laskowski, 1954) recently proven identical with kallikrein inhibitor from beef lung (Anderer, 1965). The inhibitor, a tryptophan-free protein of molecular weight 6500, whose primary structure is nearly complete (Kassell *et al.*, 1965; Chauvet *et al.*, 1964; Dlouhá *et al.*, 1965), is thought to inactivate trypsin by a reversible, nonbonding interaction (Green, 1953). An irreversible reaction whereby a covalent bond is formed between the inhibitor and trypsin, with concomitant rupture of a peptide bond in the inhibitor, may also occur. Both mechanisms are operative in the interaction of trypsin with the inhibitor from soy bean (Finkenstadt and Laskowski, 1965).

At pH 5.0 to 6.0, where the enzyme–inhibitor complex is known to be stable (Laskowski and Laskowski, 1954), very little of the tryptophan in the complex could be oxidized with NBS. At pH 5.0, 65% of the tryptophan in trypsin could be oxidized, whereas only 16% was oxidizable in the complex (Table V), *i.e.*, a protection of two tryptophan equivalents from NBS oxidation. Oxidation increases with decreasing pH, probably a composite of both the increasing dissociation of the complex and the increasing accessibility of tryptophan in both complexed and dissociated trypsin.

It has been shown (Beaven and Green, 1952; Edelhoch and Steiner, 1965) that complex formation is accompanied by a red shift in the absorption spectrum of the tryptophan residues of trypsin. The present results show that this shift, in analogy to the avidin–biotin complex, can be correlated with a concomitant loss of chemical reactivity of some tryptophan residues. Not surprisingly the effect is more marked than with the low molecular weight inhibitors, because the opportunities for side chain interactions which may occlude tryptophan residues are considerably greater.

Since the inhibition is reversible and the action of NBS is irreversible, the inhibitor conceivably should offer little protection to the oxidizable tryptophan. However, in all these experiments, one is dealing with kinetic rather than equilibrium effects. The amount of NBS added is usually the limiting factor, so that, if the tryptophan does not react rapidly with it, NBS will be consumed in other reactions, as discussed above.

Synthetic polypeptides containing L-glutamic and another amino acid in *ca.* a 1:1 ratio are known to be effective inhibitors of trypsin (Sela and Rigbi, 1964), though they lack the specificity of the natural polypeptide inhibitors.

Trypsin, after incubation for 1 hr with five or ten times its weight of the copolymer from L-glutamic acid and L-leucine (see footnote to Table V), was found to react with NBS in a manner distinctly different from trypsin inhibited with the natural polypeptide. At pH 6.0, the oxidizability of tryptophan in trypsin inhibited with the synthetic polypeptide was actually enhanced appreciably; at pH 5.5, little difference was noted between trypsin and the inhibited enzyme, although the inhibition caused a loss of 78% of the activity of the enzyme. The enhanced tryptophan reactivity observed is probably the result of a partial reversible denaturation of the trypsin on interaction with the polyelectrolyte and exposure of buried residues. In these oxidations a faint turbidity developed when trypsin was incubated with the copolymer and persisted throughout the titration (see Methods). Although the results are somewhat less reliable, we feel the effect is a real one.

The addition of a large excess of the chymotrypsin inhibitor, *N*-acetyl-DL-phenylalanine (Green and Neurath, 1954), to chymotrypsin had little effect on the oxidizability of tryptophan at pH 6.0 or 7.0 (Table III). It has been reported that virtual substrates can alter the conformation of chymotrypsin at low pH values, as detected by optical rotatory dispersion measurements (Parker and Lumry, 1963). If such an effect were operative at the higher pH values, it is not reflected in the NBS oxidations.

There are now a number of examples known where spectral shifts in enzymes are brought about by combination with inhibitors or substrates (Fujioka and Imahori, 1962; Suelter and Melander, 1963; Yankeelov and Koshland, 1965; Moon *et al.*, 1965). These shifts provide some of the strongest evidence for a substrate-induced conformational change, often postulated as an important aspect of the catalytic mechanism (Koshland, 1958). The spectral method has the advantage over most other physical methods of following a conformational change in that it is concerned only with the environment of a few specific residues (*viz.*, those having aromatic side chains), whereas other techniques examine the average properties of the whole molecule. Thus, if the specific residues involved in a conformational change happen to be aromatic, there is a good chance that it can be detected. If the spectral shifts can be correlated with changes in chemical reactivity as here (the PTI–trypsin complex) and in the cases of avidin (Green, 1963) and lysozyme (Rupley and Hartdegen, 1964; Hayashi *et al.*, 1964), then it may be possible to locate the side chains responsible. In principle, this could be done, in applications using NBS, by the isolation and identification either of oxytryptophyl peptides or the unmodified tryptophyl peptides. The former approach has recently been successfully applied to lysozyme (Hayashi *et al.*, 1965).

References

Anderer, F. A. (1965), *Z. Naturforshung 20b*, 462, 499.

Beaven, G., and Green, N. M. (1952), *Advan. Protein Chem. 7*, 342.

Bender, M. L., Kézdy, F. J., and Gunter, C. R. (1964), *J. Am. Chem. Soc. 86*, 3715.

Biltonen, R., Lumry, R., Madison, V., and Parker, H. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1412.

Chauvet, J., Nouvel, G., and Acher, R. (1964), *Biochim. Biophys. Acta 92*, 200.

Davidson, B., and Westley, J. (1965), *J. Biol. Chem. 240*, 4463.

Desnuelle, P. (1960), *Enzymes 4*, 103, 124.

Dlouhá, V., Posṕišilová, D., Meloun, B., and Šorm, F. (1965), *Collection Czech. Chem. Commun. 30*, 1311.
Edelhoch, H., and Steiner, R. F. (1965), *J. Biol. Chem. 240*, 2877.
Finkenstadt, W. R., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, PC962.
Fujioka, H., and Imahori, K. (1962), *J. Biol. Chem. 237*, 2804.
Green, N. M. (1953), *J. Biol. Chem. 205*, 535.
Green, N. M. (1963), *Biochem. J. 89*, 599.
Green, N. M., and Neurath, H. (1954), *Proteins 2*, 1120.
Green, N. M., and Witkop, B. (1964), *Trans. N. Y. Acad. Sci. 26*, 659.
Hachimori, Y., Horinishi, H., Kurihara, K., and Shibata, K. (1964), *Biochim. Biophys. Acta 93*, 346.
Hachimori, Y., Kurihara, K., Horinishi, H., Matsushima, M., and Shibata, K. (1965), *Biochim. Biophys. Acta 105*, 167.
Hartley, B. S. (1964), *Nature 201*, 1284.
Hayashi, K., Imoto, T., Funatsu, G., and Funatsu, M. (1965), *J. Biochem. (Tokyo) 58*, 227.
Hayashi, K., Imoto, T., and Funatsu, M. (1964), *J. Biochem. (Tokyo) 55*, 516.
Kassell, B., Radicevic, M., Ansfield, M. J., and Laskowski, M., Sr. (1965), *Biochem. Biophys. Res. Commun. 18*, 255.
Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.
Keil, B., Prušik, A., and Šorm, F. (1963), *Biochim. Biophys. Acta 78*, 559.
Koshland, D. E. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 98.
Laskowski, M. (1961), Biochemist's Handbook, Princeton, N. J., Van Nostrand, p 304.
Laskowski, M., and Laskowski, M. (1954), *Advan. Protein Chem. 9*, 215.
Lokshina, L. A., Orekhovich, V. N., and Pandakova, V. N. (1962), *Dokl. Akad. Nauk. SSSR 142*, 471.
Moon, A. Y., Mercouroff, J., and Hess, G. P. (1965), *J. Biol. Chem. 240*, 717.
Okada, Y., Onoue, K., Nakashima, S., and Yamamura, Y. (1963), *J. Biochem. (Tokyo) 54*, 477.
Ong, E. B., Shaw, E., and Schoellman, G. (1965), *J. Biol. Chem. 240*, 694.
Parker, H., and Lumry, R. (1963), *J. Am. Chem. Soc. 85*, 483.
Patchornik, A., Lawson, W. B., Gross, E., and Witkop, B. (1960), *J. Am. Chem. Soc. 82*, 5923.
Patchornik, A., Lawson, W. B., and Witkop, B. (1958), *J. Am. Chem. Soc. 80*, 4747, 4748.
Ramachandran, L. K. (1960), *Biochim. Biophys. Acta 41*, 524.
Ramachandran, L. K. (1962), *J. Sci. Ind. Res. (India) 21C*, 111.
Ramachandran, L. K., and Witkop, B. (1959), *J. Am. Chem. Soc. 81*, 4028.
Ramachandran, L. K., and Witkop, B. (1964), *Metabolism 13*, 1016.
Rupley, J. A., and Hartdegen, F. (1964), *Biochim. Biophys. Acta 92*, 625.
Schmir, G. L., and Cohen, L. A. (1961), *J. Am. Chem. Soc. 83*, 723.
Schoellmann, G., and Shaw, E. (1963), *Biochemistry 2*, 252.
Sela, M., and Rigbi, M. (1964), *Biochemistry 3*, 629.
Spande, T. F., and Witkop, B. (1965), *Biochem. Biophys. Res. Commun. 21*, 131.
Spande, T. F., and Witkop, B. (1966), *Methods Enzymol.* 7 (in press).
Suelter, C. H., and Melander, W. (1963), *J. Biol. Chem. 238*, PC4180.
Viswanatha, T., and Lawson, W. B. (1961), *Arch. Biochem. Biophys. 93*, 128.
Viswanatha, T., Lawson, W. B., and Witkop, B. (1960), *Biochim. Biophys. Acta 40*, 216.
Walsh, K. A., and Neurath, H. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 884.
Williams, E. J., Herkovits, T. T., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, 3574.
Witkop, B. (1961), *Advan. Protein Chem. 16*, 221.
Wootton, J. F., and Hess, G. P. (1962), *J. Am. Chem. Soc. 84*, 440.
Yankeelov, J. A., and Koshland, D. E. (1965), *J. Biol. Chem. 240*, 1593.