# Selective Chemical Cleavage of Tryptophanyl Peptide Bonds by Oxidative Chlorination with *N*-Chlorosuccinimide†

Yoram Shechter,* Abraham Patchornik, and Yigal Burstein*

ABSTRACT: Tryptophanyl peptide bonds are selectively cleaved by *N*-chlorosuccinimide (NCS) under acidic conditions. All other peptide bonds are resistant to cleavage by this reagent. Optimal conditions for cleavage are: 2 equiv of NCS, pH 4–5, or 50–80% acetic acid for 30 min at room temperature. Under these conditions methionine residues are oxidized to methionine sulfoxides and cysteine to cystine. Other amino acids are not modified. The cleavage reaction was studied with several peptides containing tryptophan residues and yields of cleavage were in the range of 35–45%. The method was successfully applied to several proteins. In α-lactalbumin, Kunitz trypsin inhibitor, and apomyoglobin, selective cleavage of the expected tryptophanyl peptide bonds was obtained in 19–58% yield. The glucagon molecule was fragmented into two peptides in 32% yield.

Oxidative bromination has been widely used for the modification of amino acid side chains in protein. Several of these reagents have been successfully used for the chemical cleavage of tryptophanyl peptide bonds in peptides and proteins (Witkop, 1961; Spande and Witkop, 1967; Cohen, 1968; Stark, 1969; Spande et al., 1970; Fontana, 1972). The method most widely used for modification and cleavage of tryptophan peptides utilized NBS[1] as oxidant (Patchornik et al., 1960). NBS is an extremely reactive reagent, which can cause modification, as well as cleavage of peptide bonds, not only of tryptophan, but also of tyrosine (Schmir et al., 1959) and histidine residues (Schmir and Cohen, 1961; Shaltiel and Patchornik, 1963). In order to obtain a more selective cleavage, milder halogenating agents were needed. NBS in 8 M urea (Funatsu et al., 1964), BNPS-skatole (Omenn et al., 1970; Fontana, 1972), and TBC (Burstein and Patchornik, 1972) were introduced. Using these reagents, only tryptophanyl peptide bonds were cleaved; however, other amino acids, e.g., tyrosine, cysteine, cystine, and methionine, were modified as well. Oxidative iodination was also tried. *N*-Iodosuccinimide (Junek et al., 1969) ICl, Chloramine-T + KI, $I_2$, and $I_3^-$ (Alexander, 1974) cleaved tryptophanyl peptide bonds, but again, histidine, cysteine, cystine, tyrosine, and methionine were modified as well.

We wish to report here the use of a mild chlorinating agent *N*-chlorosuccinimide (NCS) for the selective cleavage of tryptophanyl peptide bonds in peptides and proteins.

## Experimental Procedures

*Materials.* Bovine trypsin was a product of Worthington, sperm-whale myoglobin was purchased from Sigma, glucagon from Calbiochem, and bovine α-lactalbumin was prepared according to Castellino and Hill (1970). *N*-Chlorosuccinimide was a product of Pfaltz and Bauer and was recrystallized from ethyl acetate; *N*-bromosuccinimide was purchased from Fluka and was crystallized from water; tryptophanyl, histidyl, and tyrosyl dipeptides and Cbz-tryptophanyl dipeptides were products of Miles-Yeda, Rehovot, Israel. Standard calibration mixture of amino acids type I (containing: lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine in equal concentrations) was a product of Spinco-Beckman, and to it 1 equiv of tryptophan was added.

*N*-Acetyl dipeptides and *N*-acetylmethionine were prepared by incubating the dipeptides or methionine with acetic anhydride–water–pyridine (1:1:0.8, v/v) for 1 h at 0 °C.

*Amino acids analyses* were performed with a Beckman Model 120C automatic amino acid analyzer. Hydrolysis of proteins was performed in evacuated tubes with 6 N HCl at 105 °C for 22 h.

*Protein concentrations* were determined by amino acid analyses of solutions of known absorptivity and then checked routinely by measurement of absorbancy at 280 nm.

*Ultraviolet absorption* spectra were obtained with a Beckman Acta V recording spectrophotometer.

*Amino acid sequence analyses* were performed with a Beckman sequencer Model 890C according to a modification (Hermodson et al., 1972) of the method of Edman and Begg (1967) and Beckman program No. 071872. Pth-amino acids were identified and determined quantitatively by gas–liquid chromatography, or by amino acid analysis after hydrolysis with hydroiodic acid (47%) for 20 h at 130 °C in evacuated sealed tubes. The sequencer chemicals were products of Beckman and Pierce.

*Reaction of Free Amino Acids with N-Chlorosuccinimide.* To a solution of free amino acids (0.25 μmol/0.25 ml in 80% acetic acid), NCS (100 μmol) in 50 μl of dimethylformamide was added. After 30 min at room temperature, excess *N*-acetylmethionine (200 μmol) was added. The solvent was evaporated under reduced pressure at 4 °C and the residue was redissolved in citrate buffer (pH 2.2) and analysed for its amino acids content.

*Determination of Yields of Cleavage in Dipeptides.* The method is illustrated by the following experiment: Cbz-Trp-Gly (5 μmol/ml) was dissolved in the appropriate solvent. To this

† From the Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel. *Received June 17, 1976.* This research was supported in part by Grant No. AM05098 from the National Institutes of Health, United States Public Health Service.

[1] Abbreviations used: AA, amino acid; NBS, *N*-bromosuccinimide; NCS, *N*-chlorosuccinimide; TBC, 2,4,6-tribromo-4-methylcyclohexadienone; Cbz, benzyloxycarbonyl; Bz, benzyl, BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromindolenine; Ac, acetyl; Abu, α-aminobutyric acid; Tris, tris(hydroxymethyl)aminomethane; Pth, phenylthiohydantoin.

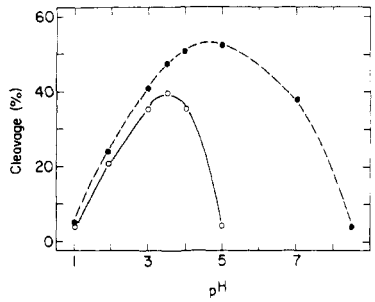



FIGURE 1: Effect of pH on the cleavage of Ac-Trp-Gly. The reaction mixture contained 5 μmol/ml Ac-Trp-Gly and 10 μmol/ml NCS (○) or NBS (●). The solvents were 0.1 M KCl-HCl buffer (pH 1-2) and 0.1 M acetic acid adjusted to pH 3-5 with 5 N NaOH or 0.1 M Tris-HCl (pH 7-8.5). After 30 min at room temperature, 30 equiv of *N*-acetylmethionine was added, and yields of cleavage were determined by automatic amino acid analysis of the released glycine.

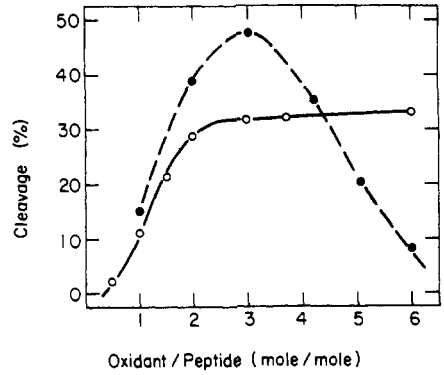



FIGURE 2: Cleavage of Ac-Trp-Gly as a function of added NCS (○) or NBS (●). The reaction mixture contained 5 μmol/ml of the peptide in 80% acetic acid, and to it the oxidant was added in dimethylformamide (500 μmol/ml) for 30 min at room temperature. Excess reagent was destroyed by *N*-acetylmethionine, and yields of cleavage were determined by automatic amino acid analysis of the released glycine.

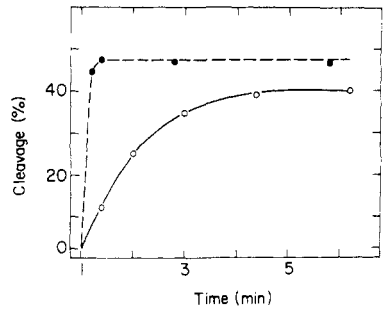



FIGURE 3: Release of glycine with time from Ac-Trp-Gly. The reaction mixture contained the peptide (5 μmol/ml) and 15 μmol/ml of NCS (○) or NBS (●) in 80% acetic acid. The reaction was stopped by addition of excess *N*-acetylmethionine; yields of cleavage were determined by automatic amino acid analysis of the released glycine.

stirred solution, at room temperature, a solution of NCS or NBS in dimethylformamide was added. The reaction was terminated by the addition of excess *N*-acetylmethionine. A sample (0.1 ml) of the reaction mixture was diluted tenfold with citrate buffer (pH 2.2) and analyzed by the amino acid analyzer.

*Cleavage of Tryptophanyl Peptide Bonds in Proteins.* To a solution of 1-2 μmol of the protein in 3.0 ml of 50% acetic acid, 50-100 μmol of NCS in dimethylformamide (300 μmol/ml) was added (tenfold excess of NCS over tryptophan residue). The reaction was allowed to proceed at room temperature with constant stirring for 40 min and then excess NCS was destroyed with methionine. The solvent was evaporated under reduced pressure at room temperature, redissolved in 0.8 ml of 0.1 M acetic acid, and loaded on a column of Sephadex G-25 (1.8 × 40 cm, unless otherwise indicated) which has been equilibrated with the same solvent. Fractions containing the protein peak were combined and lyophilized.

## Results

*Cleavage of Tryptophanyl Peptide Bonds.* When Ac-Trp-Gly was treated with NCS, under acidic conditions, glycine was liberated. In order to arrive at optimal conditions for cleavage, models of tryptophanyl dipeptides were treated with various molar ratios of NCS or NBS at different hydrogen ion concentrations, for varying times.

Figure 1 illustrates the pH dependency on the cleavage at room temperature. From this figure it is evident that optimal yields of cleavage with NCS can be obtained at pH 3-4 whereas with NBS higher yields were obtained at pH 4-5. Similar yields of cleavage were obtained in 50-80% acetic acid.

Two moles of NCS per mole of peptide brought about optimal cleavage (Figure 2). When the same peptide was treated with 2 mol of NCS per mole of peptide in 80% acetic acid at room temperature, we found that the cleavage reaction was relatively slow and reached its highest value after about 20 min; for comparison with NBS (3 mol) under the same reaction conditions, the reaction was complete after less than 2 min (Figure 3).

We therefore chose to perform the cleavage reaction in 80% acetic acid, with 2-3 equiv of NCS for 30 min at room temperature. By this procedure up to 45% cleavage was obtained with several tryptophanyl dipeptide models (Table I). The yields of cleavage are comparable with those obtained with NBS (Table I), or with BNPS-skatole according to Omenn et al. (1970) and with TBC according to Burstein and Patchornik (1972).

Since tryptophanyl (Patchornik et al., 1960), tyrosyl (Schmir et al., 1959), and histidyl (Shaltiel and Patchornik, 1963) peptide bonds are cleaved with NBS under acidic conditions, it was necessary to test the selectivity of the cleavage reaction by NCS. This was done by cleaving equimolar mixtures of three dipeptides (Table I). The extent of cleavage of each of the peptides in the mixture could be determined simultaneously since the dipeptides were chosen so that a different amino acid was liberated from each of the cleaved peptides in the mixture. As can be seen from this table, only tryptophanyl peptide bonds were cleaved by NCS, whereas tyrosyl and histidyl peptide bonds remained intact. Dipeptides containing tryptophan at their free amino terminus were also resistant to cleavage with NCS (Table I).

*Selectivity of Reagent.* To compare the oxidizing properties of NCS with those of NBS and TBC, a mixture of amino acids was reacted with these reagents in 80% acetic acid. As shown in Table II, after exposure to 120 equiv of NCS for 30 min at room temperature, tryptophan was completely absent, and methionine was oxidized quantitatively to methionine sulfoxide, while other amino acids were recovered quantitatively. By contrast, in the amino acid mixture oxidized with TBC, tryptophan, histidine, cystine, and tyrosine were completely absent and methionine was oxidized to its sulfoxide. With NBS, again tryptophan, histidine, cystine, and tyrosine were completely absent and methionine was oxidized to methionine

TABLE I: Yields of Cleavage of Tryptophanyl, Tyrosyl, and Histidyl Peptides.[a]

| Peptide Mixture | Peptide[b] | Cleavage Products | Yield of Cleavage (%)[c] NCS | NBS |
|---|---|---|---|---|
| 1 | Cbz-Trp-Gly | Gly | 42 | ND[d] |
| | Cbz-Trp-Val | Val | 45 | ND[d] |
| | Cbz-Trp-Leu | Leu | 43 | ND[d] |
| 2 | Ac-Trp-Gly | Gly | 40 | 45 |
| | Ac-Tyr-Ala | Ala | 0 | 45 |
| | Ac-His-Phe | Phe | 0 | 26 |
| 3 | Ac-Trp-Val | Val | 37 | 42 |
| | Ac-Tyr-Gly | Gly | 0 | 45 |
| | Ac-His-Ala | Ala | 0 | 24 |
| 4 | Trp-Gly | Gly | 0 | ND[d] |
| | Trp-Ala | Ala | 0 | ND[d] |
| | Trp-Val | Val | 0 | ND[d] |

[a] In 80% acetic acid, for 30 min at room temperature. [b] To an equimolar mixture of three peptides (5 μmol per ml per peptide), 9 equiv of NCS or NBS was added. [c] Yields of cleavage were determined by automatic amino acid analysis of the released amino acid. [d] Not determined.

TABLE II: Recovery of Amino Acids after Incubation with NCS, TBC, and NBS.[a]

| Amino Acid | Recovery (%) NCS | TBC | NBS[b] |
|---|---|---|---|
| Trp | 0 | 0 | 0 |
| Lys | 98 | 98 | 115 |
| His | 100 | 0 | 0 |
| Arg | 99 | 98 | 92 |
| Asp | 100 | 100 | 107 |
| Thr | 100 | 100 | 106 |
| Ser | 100 | 99 | 78 |
| Glu | 100 | 100 | 81 |
| Pro | 98 | 98 | 103 |
| Gly | 97 | 100 | 98 |
| Ala | 98 | 98 | 100 |
| Cys | 97 | 0 | 0 |
| Val | 100 | 100 | 99 |
| Met | 0 | 0 | 0 |
| Ile | 100 | 99 | 101 |
| Leu | 100 | 100 | 100[b] |
| Tyr | 98 | 1 | 0 |
| Phe | 100 | 101 | 81 |
| Methionine sulfoxide[c] | 100 | 100 | 0 |
| Methionine sulfone[c] | 0 | 0 | 100 |

[a] The reaction mixture contained 0.25 μmol of each of the 18 amino acids (see Experimental Procedures) and 30 μmol of the oxidant in 0.25 ml of 80% acetic acid and was incubated at room temperature for 30 min. Yields of recovery of the various amino acids were determined by automatic amino acid analysis. [b] Recoveries of amino acids are expressed on the basis of leucine (which was recovered in 82% yield). [c] Oxidation products of methionine.

sulfone; moreover other amino acids were partially degraded as well.

*Stoichiometry of the Reaction of NCS and NBS with Tryptophanyl Peptides.* The peptides Cbz-Trp-Gly, Cbz-Trp-Ala-OBz, and Ac-Trp-Gly were incubated with excess NCS or NBS in 50% acetic acid at room temperature for 30 min. No further oxidation took place during longer incubations. The reaction was terminated by the addition of methionine (10–20% excess over the oxidant) part of which was oxidized to methionine sulfoxide by unreacted oxidant. Determination of methionine sulfoxide or the remaining methionine enabled us to calculate the amount of oxidant which had been consumed by the tryptophanyl peptide. The results are presented in Table III. Both oxidants reacted with the tryptophanyl peptides. NCS was consumed at a ratio of 2 mol of oxidant per mol of peptide. NBS was consumed at a ratio of 3 mol of oxidant per mol of Ac-Trp-Gly, 4 mol of oxidant per mol of Cbz-Trp-Gly, and 5 mol of oxidant per mol of Cbz-Trp-Ala-OBz.

*Cleavage of Tryptophanyl Residues in α-Lactalbumin.* α-Lactalbumin of bovine milk (10 mg) was incubated with excess NCS (tenfold excess over each tryptophan residue) in 50% acetic acid for 1 h at room temperature. Excess NCS was destroyed by the addition of *N*-acetylmethionine, and the protein was purified by gel filtration on a Sephadex G-25 column (2 × 40 cm). The fractions corresponding to the protein peak were pooled and lyophilized, and samples (180 nmol) were subjected to three cycles of the automatic Edman degradation. The anilinothiazolinone-amino acids from each cycle were analyzed as described under Experimental Procedures. The results are presented in Table IV. The $NH_2$-terminal end of the protein was unblocked and glutamic acid (The $NH_2$-terminal residue) served as an internal standard for cycle 1. As a result of the cleavage of $Trp^{28}$, $Val^{29}$ was exposed as a new $NH_2$ terminal and was recovered in 21% yield. The cleavage of $Trp^{60}$ should have exposed $Cys^{61}$; however, this residue was not detected since it was probably connected with the protein via the disulfide bonds. The cleavage of both $Trp^{104}$ and $Trp^{118}$ yielded leucine residues which were recovered in 75% yield. The

TABLE III: Oxidation of Tryptophanyl Peptides with NCS and NBS.[a]

| Tube | Peptide | Oxidant (nmol) NCS | NBS | Met Analyzed (nmol) | Oxidant Consumed per Peptide (mol/mol) |
|---|---|---|---|---|---|
| 1 | Ac-Trp-Gly | 1200 | | 610 | 1.95[b] |
| 2 | Ac-Trp-Gly | | 1200 | 809 | 2.90 |
| 3 | Cbz-Trp-Gly | 1200 | | 637 | 2.08 |
| 4 | Cbz-Trp-Gly | | 1200 | 1023 | 3.92 |
| 5 | Cbz-Trp-Ala-OBz | 1200 | | 659 | 2.19 |
| 6 | Cbz-Trp-Ala-OBz | | 1200 | 1240 | 4.95 |
| 7 | Cbz-Trp-Ala-OBz | | | 1388 | |
| 8 | None[c] | 1200 | | 208 | |
| 9 | None[c] | | 1200 | 195 | |

[a] To the peptide solution (210 nmol/0.25 ml) in 50% acetic acid was added 6 μl of a solution of NCS or NBS (200 μmol/ml) in dimethylformamide. After incubation for 30 min at room temperature, a solution of methionine (1400 nmol/120 μl) was added. A sample was diluted with 0.1 M sodium citrate buffer (pH 2.2) and methionine was determined by automatic amino acid analysis. [b] The results of duplicate experiments varied at the most by 7%. [c] Acetic acid, 50% (0.25 ml).

results from cycle 2 could not be interpreted quantitatively since the internal standard was glutamine (residue 2), which was determined as glutamic acid, and glutamic acid was also "carried over" from the previous cycle. In cycle 3, $Leu^3$ served as the internal standard; α-aminobutyric acid (hydrolysis

TABLE IV: Cleavage of Tryptophanyl Peptide Bonds in α-Lactalbumin and Acetyl-α-lactalbumin.[a]

| Protein | Bond Cleaved | Cleavage Products Determined by Automatic Edman Degradation[b] Cycle 1 AA | Cycle 1 Yield[c] (%) | Cycle 2 AA | Cycle 2 Yield[c] (%) | Cycle 3 AA | Cycle 3 Yield[c] (%) |
|---|---|---|---|---|---|---|---|
| (a) α-Lactalbumin[d] | $Trp^{28}$—Val-Cys-Thr | Val | 21 | | | Abu | 17 |
| | $Trp^{60}$—Cys-Lys-Asx | | | | | Asp | 20 |
| | $Trp^{104}$—Leu-Ala-His | Leu | 75 | | | | |
| | $Trp^{118}$—Leu-Cys-Glu | Leu | | | | Glu | 57 |
| (*N*-terminal) | $H_2N$-$Glu^1$-Gln-Leu | Glu | 100 | | | Leu | 100 |
| (b) Ac-α-lactalbumin | $Trp^{28}$—Val-Cys-Thr | | | | | Abu | 21 |
| | $Trp^{60}$—Cys-Lys-Asx | | | Lys | (12–30) | Asp | 24 |
| | $Trp^{104}$—Leu-Ala-His | | | Ala | 36 | | |
| | $Trp^{118}$—Leu-Cys-Glu | | | | | Glu | 33 |
| (Lysozyme)[e] | $H_2N$-$Lys^1$-Val-Phe | | | Val | 100 | Phe | 100 |

[a] The protein (100–200 nmol) was cleaved with 25 equiv of NCS in 50% acetic acid for 30 min at room temperature. [b] Pth-amino acids were hydrolyzed to free amino acids with HI (47%). [c] Determined by automatic amino acid analysis; corrected for losses during hydrolysis. [d] According to Brew et al. (1970). [e] According to Canfield (1963).

product of Pth-$Thr^{31}$) was recovered in 17% yield, and aspartic acid (hydrolysis product of $Asx^{63}$) was recovered in 20% yield. Histidine could not be determined quantitatively and glutamic acid ($Glu^{121}$) was recovered in 57% yield. This value for glutamic acid seemed too high and probably originated from "carry-over" of Glu from the previous cycle. These results indicated that $Trp^{28}$ was cleaved in 17–21% yield, $Trp^{60}$ was cleaved in 20% yield, and $Trp^{104}$ and $Trp^{118}$ were cleaved in an overall yield of 75%.

In order to determine quantitatively the yield of cleavage of $Trp^{104}$ and $Trp^{118}$, and to avoid the carry-over of glutamic acid to cycle 3, α-lactalbumin was first acetylated, its $NH_2$-terminal end was blocked, and only then it was cleaved with NCS. Since the $NH_2$ terminal was blocked and could not serve as an internal standard a different protein molecule, hen egg white lysozyme, was added to the sequencer cup and served as the "internal standard". The results presented in Table IV for Ac-α-lactalbumin were interpreted in the following way. The results from cycle 1 were not interpreted quantitatively since lysine was not a reliable standard; however, the ratio of Leu:Val was 3.4:1 which is in a very good agreement with the results of the previous experiment (with α-lactalbumin alone). In cycle 3, cysteine was not detected, again probably since it was still hooked to the protein through the disulfide bond. $Ala^{105}$ was recovered in 36% yield and lysine in 12–30% yield. Here $Val^2$ (of lysozyme) served as the internal standard. In cycle 3 ($Phe^3$ of lysozyme was the internal standard), $Abu^{31}$, $Asp^{63}$, and $Glu^{121}$ were isolated in 21, 24, and 33% yield, respectively. From the two experiments one can conclude that in α-lactalbumin $Trp^{28}$, $Trp^{60}$, $Trp^{104}$, and $Trp^{118}$ were cleaved in 19 ± 2, 22 ± 2, 36 ± 3, and 33 ± 4% (mean ± standard deviation of four experiments), respectively.

*Cleavage of Tryptophanyl Residues in Kunitz Trypsin Inhibitor, Apomyoglobin, and Glucagon.* Kunitz trypsin inhibitor (20 mg) was incubated with 25 equiv of NCS in 50% acetic acid (2 ml) for 1 h at room temperature. The reaction was terminated by the addition of excess methionine, the solvent was dried under reduced pressure, and the product was purified by gel filtration on a Sephadex G-15 column (in 0.05 M ammonium bicarbonate). The protein peak (void volume) was pooled, lyophilized, and subjected to two cycles of automatic Edman degradation. This protein contains two tryptophans at positions 93 and 117; cleavage at these residues exposed $Ser^{94}$ and $Phe^{118}$ as new amino terminals in addition to $Asp^1$ which is the amino terminal residue of trypsin inhibitor; no other points of cleavage were detected. In Kunitz inhibitor $Asp^1$ served as an internal standard (100%) for determining the yields of cleavage which were 47% for $Trp^{93}$ and 58% for $Trp^{117}$.

Sperm whale apomyoglobin (5 mg) was incubated with 15 equiv of NCS in 50% acetic acid (0.7 ml) for 1 h at room temperature. Then, excess *N*-acetylmethionine was added, and extent of cleavage was determined by automatic sequence analysis of the released new amino terminals (one cycle). This protein contains two tryptophans at positions 7 and 14, cleavage at these residues exposed $Gln^8$ and $Ala^{15}$, and again the N-terminal residue ($Val^1$) served as the internal standard. Yields of cleavage of sperm whale apomyoglobin were 47% for $Trp^7$ and 42% for $Trp^{14}$. No other peptide bond was cleaved since $Val^1$, $Glu^8$ (hydrolysis product of $Gln^8$), and $Ala^{16}$ were the only detectable amino terminal residues.

When glucagon was incubated with 5 equiv of NCS in 80% acetic acid for 1 h at room temperature, cleavage of the tryptophanyl peptide bond occurred. The released COOH-terminal tetrapeptide was isolated by gel filtration and hydrolyzed. By amino acid analysis we found 1.0 ml of aspartic acid and 0.9, 0.95, and 1.02 mol of threonine, methionine, and leucine respectively. These were the only amino acids present, which was in agreement with the known sequence of this peptide (Bromer et al., 1957). The yield of isolation of this peptide was 32%.

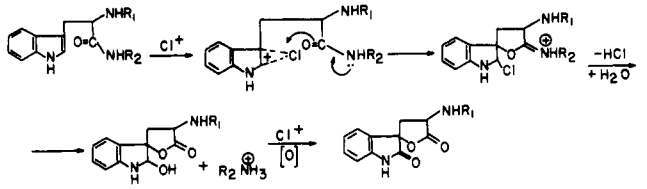
## Discussion

*N*-Chlorosuccinimide (NCS), under neutral conditions, is a relatively mild oxidant and was recently introduced for the selective oxidation of methionine residues in non-SH proteins. Its selectivity was demonstrated with several native proteins such as α-chymotrypsin, ribonuclease, α-lactalbumin (Shechter et al., 1975), and ovoinhibitor (Y. Shechter, Y. Burstein, and A. Gergler, unpublished data). In aqueous solutions under slightly acidic conditions (pH 2–5), NCS oxidizes the exposed indole rings of tryptophan side chains in proteins to oxindoles; however, the tryptophanyl peptide bonds of native proteins are generally not cleaved under these conditions. Again only methionine and cysteine could be oxidized to methionine sulfoxide and cystine, respectively; no other amino acid was modified. These findings proved promising since until now brominating (Patchornik et al., 1958; Ramachandran and Witkop, 1959; Lawson et al., 1960; Funatsu et al., 1964;

Omenn et al., 1970; Burstein and Patchornik, 1972) or iodinating (Junek et al., 1969; Alexander, 1974) reagents were the only tools which have been successfully used for the oxidative cleavage of tryptophanyl peptide bonds in peptides and proteins; however in addition to the cleavage reaction, other oxidation sensitive amino acids, e.g., tyrosine, histidine, cysteine, cystine, and methionine, were also modified.

We tried therefore to establish conditions for the selective cleavage of tryptophanyl peptide bonds by NCS. Using derivatives of tryptophan and tryptophanyl peptides as model compounds for the cleavage reaction, we found that, contrary to brominating reagents, only 2 equiv of oxidative chlorine was consumed during the cleavage (Figure 2 and Table III). Spectrophotometric monitoring of the oxidation of tryptophanyl peptides with NCS proved that the indole ring was oxidized to an oxindole derivative (Shechter et al., 1975) and since this requires 2 halogenating steps we believe that the oxidative cleavage of tryptophanyl peptides during chlorination probably occurs by the mechanism described in Scheme I. This

Scheme I



mechanism is analogous to that suggested by Patchornik et al. (1960). According to the mechanism, 2 equiv of active halogen ($Cl^+$, $Br^+$, or $I^+$) halogenate the indole nucleus and undergo spontaneous dehalogenation through a series of oxidation and hydrolysis reactions. These reactions led to the formation of an oxindole derivative which promotes the cleavage reaction. The pH profile of the NCS-cleavage reaction, as well as the optimal pH value and the yields of the cleavage reaction, supports this mechanism.

The modification-cleavage reaction with NCS was compared with those performed with TBC and NBS. Thioether side chains, such as of methionine and *S*-carboxymethylcysteine, are oxidized to sulfoxides. TBC reacts similarly; however, NBS oxidizes the thioethers to the sulfones. The sulfoxides could be reduced back to the corresponding thioethers with thioglycolic acids or with thiosulfate. If present, sulfhydryl groups must be protected, as in selective modification with other classes of reagent. No other amino acids were modified with NCS, however, TBC or NBS oxidized tyrosine, histidine, and cystine as well (Table II). Another indication that the oxidation potential of NCS is less than that of TBC or NBS is presented in Table III. The indole moiety of tryptophan consumed 2 equiv of NCS and 3 equiv of NBS or TBC (Burstein and Patchornik, 1972). NBS was also capable of brominating the OBz moiety in the carbobenzoxy and the benzyl ester protecting groups, while TBC or NCS were unable to do so under the reaction conditions.

The results of the cleavage reaction of the model compounds (Table I, Figure 3) indicated that the yields of cleavage with NCS were somewhat lower than those with NBS and the reaction was slower; however, the cleavage with NCS was very selective, and not a trace of cleavage of tyrosyl and histidyl peptide bonds was observed. Amino-terminal tryptophan residues resisted cleavage with NCS, as has already been shown by Wilchek and Witkop (1967) using NBS as an oxidant.

The chlorinative cleavage of simple tryptophanyl peptides with NCS can not necessarily be extrapolated to larger peptides and proteins that possess secondary structures (Green and Witkop, 1964). Indeed tryptophanyl peptide bonds of native proteins resisted cleavage in aqueous buffered solutions at acidic pH; only denatured protein molecules could be cleaved. We therefore performed the cleavage reaction in 50–80% acetic acid, which is an excellent solvent for most proteins and for NCS. In $\alpha$-lactalbumin, Kunitz trypsin inhibitor, myoglobin, and glucagon, all expected tryptophanyl peptide bonds were cleaved with NCS in 19–58% yield; no other peptide bonds were cleaved by NCS.

These studies indicate that, today, NCS is the most selective reagent for cleavage of tryptophanyl peptide bonds in proteins and, therefore, it may be of possible general utility for fragmentation of polypeptides and proteins, especially for sequence analysis.

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