Sulfenyl Halides as Modifying Reagents for Polypeptides and Proteins. I. Modification of Tryptophan Residues*

Ernesto Scoffone, Angelo Fontana, and Raniero Rocchi

ABSTRACT: Sulfenyl halides have been found to be specific, mild reagents for modification of tryptophan and cysteine residues of polypeptides and proteins in acidic media, such as acetic acid or formic acid. With hydrolysis-resistant halides, like 2-nitro- (NPS-Cl) and 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl), the reaction can be carried out in aqueous solvents such as 30–50% acetic acid. Tryptophan is smoothly converted by reaction with sulfenyl halides into a derivative with a thioether function in the 2 position of the indole nucleus, and cysteine to an unsymmetrical disulfide. The high specificity of the reaction toward tryptophan and cysteine was established after quantitative recovery on the amino acid analyzer of the other amino acids, after 15-hr reaction with NPS-Cl in acetic acid. As a further check, ribonuclease A, a protein containing neither tryptophan nor cysteine, was allowed to react with 20 equiv of NPS-Cl in 50% acetic acid. The protein was recovered unchanged and fully active toward RNA. The reaction with NPS-Cl introduces a chromophore into tryptophan-containing proteins, with an absorption peak at 365 mμ and molar absorptivity of ε 4000. This chromophore provides a convenient quantitative measure of the extent of reaction.

In order to test the effectiveness of the method several tryptophan peptides, β^{1-24}-corticotropin, and gramicidin A were modified by reaction with sulfenyl halides. Lysozyme, trypsin, and α-chymotrypsin were allowed to react with 20 equiv of NPS-Cl in 50% acetic acid, and the number of modified tryptophan residues per mole of enzyme was determined spectrophotometrically. As the conversion of tryptophan into a derivative with a chromophoric group covalently bound is quantitative, this procedure appears to offer considerable promise as an analytical tool for the determination of the tryptophan content in polypeptides and proteins.

Modification of tryptophan in proteins can be achieved through iodination (Ramachandran, 1956), photooxidation (Weil *et al.*, 1953), and ozonization (Scoffone *et al.*, 1963; Previero and Bordignon, 1964; Previero *et al.*, 1964). The use of *N*-bromosuccinimide represents a convenient spectrophotometric method for determining the tryptophan content in proteins (Patchornik *et al.*, 1958) and provides a means of cleaving the tryptophyl peptide bond (Patchornik *et al.*, 1958, 1960; Ramachandran and Witkop, 1964). Moreover it was employed in the preparation of oxidized proteins, in which some or all of the tryptophan residues were modified (Hayashi *et al.*, 1965). Recently Koshland *et al.* (1964) and Horton and Koshland (1965) have reported the use of 2-hydroxy-5-nitrobenzyl bromide as a selective reagent for tryptophan in proteins at acidic pH. The precise nature of this reaction is not yet known. The only other amino acid affected by this reagent is cysteine.

Previous work from this laboratory examined the reaction, in glacial acetic acid or 99% formic acid, of 2-nitro- (NPS-Cl)[1] and 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl) with tryptophan, and demonstrated the introduction of a thioether function into the 2 position of the indole nucleus (Fontana *et al.*, 1966a,b). Furthermore it was briefly reported (Scoffone *et al.*, 1966) that sulfenyl halides were useful modifying reagents for tryptophan-containing polypeptides and proteins. The results reported in this paper indicate that sulfenyl halides are highly specific reagents for tryptophan and cysteine, and since reaction conditions are very mild, selective modification is achieved readily.

Cysteine is converted into an unsymmetrical disulfide, from which cysteine can be reversibly obtained by treatment with reducing agents (*e.g.*, β-mercaptoethanol, thioglycolic acid, or sodium borohydride). Results on the reaction of sulfenyl halides with the cysteinyl residue in polypeptides and proteins are reported in the following paper (Fontana *et al.*, 1968).

The reaction can be easily quantitated by spectrophotometry since the nitrophenylsulfenyl groups, expecially NPS, absorb radiation in the visible part of the spectrum. The present study establishes optimal condi-

* From the Institute of Organic Chemistry, University of Padova, and Centro Nazionale di Chimica delle Macromolecole, del C. N. R., Sez. VIII, Padova, Italy. *Received September 26, 1967.* A preliminary account of this work has appeared (Scoffone *et al.*, 1966) and was presented at the 8th European Peptide Symposium, Noordwijk, The Netherlands (Scoffone *et al.*, 1967).

[1] Abbreviations used: NPS, 2-nitrophenylsulfenyl; pNPS, 4-nitrophenylsulfenyl; DNPS, 2,4-dinitrophenylsulfenyl; PS, phenylsulfenyl; Z, carbobenzoxy; DCCI, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; RNase, bovine pancreatic ribonuclease; RNase A, the principal chromatographic component of RNase. The amino acids, peptides and peptide derivatives have the L configuration. The abbreviations are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature, *Biochemistry 5*, 1445 (1966).

tions for the reaction of tryptophan residues in proteins and examines methods for their quantitative determination.

The reactions of NPS-Cl with lysozyme, trypsin, and α-chymotrypsin, and the quantitative conversion of tryptophan residue into 2-NPS-tryptophan appear to offer considerable promise as an analytical tool for the determination of the tryptophan content in polypeptides and proteins.

Materials

NPS-Cl (Hubacher, 1943) and DNPS-Cl (Kharasch *et al.*, 1950) were obtained from Fluka AG, Basle (Switzerland), and recrystallized from anhydrous ethyl ether (mp 75–76°) and anhydrous chloroform–ethyl ether (mp 94–95°), respectively; pNPS-Cl (Zincke and Lenhardt, 1913) and PS-Cl (Montanari, 1956) were prepared accordingly to the literature.

A standard solution containing Lys, His, NH_3, Arg, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys-, Val, Met, Ile, Leu, Tyr, and Phe (amino acid calibration mixture, type 1, for the amino acid analyzer) each at a concentration of 2.5 μmoles/ml was obtained from Beckman Instruments, Spinco Division, Palo Alto, Calif. The protected tryptophan peptides used in this work, Z-Trp-Gly-OEt, Z-Trp-Met-OMe, Z-Trp-Phe-OMe, Z-Thr-Trp-OMe, Z-Pro-Trp-OMe, Z-Leu-Trp-OMe, and Z-Ala-Trp-Gly-OEt, have already been described (Veronese *et al.*, 1967).

*Bis-N-carbobenzoxy-*L*-cystinyl-*L*-tryptophan Methyl Ester.* To an ice-cold suspension of 2.55 g (5 mmoles) of *N*-carbobenzoxy-L-cystine (Bergmann and Zervas, 1932), 2.55 g (10 mmoles) of L-tryptophan methyl ester hydrochloride (Boissonnas *et al.*, 1958), and 1.4 ml of triethylamine in 100 ml of ethyl acetate, 2.06 g (10 mmoles) of DCCI was added with stirring. The reaction mixture was shaken for 1 hr at 0° and for an additional 4 hr at room temperature. The precipitate was removed by filtration, and the solution was washed with 1 N HCl, 5% sodium carbonate, and water. The solution was dried over sodium sulfate, concentrated *in vacuo* to a small volume, and the product was precipitated by addition of ethyl ether and petroleum ether (bp 30–60°). After drying *in vacuo* the yield was 3.6 g (79%) (mp 98–100°) single Ehrlich reagent positive spot in thin-layer chromatography, $[\alpha]_D^{20}$ −50.0° (*c* 1, MeOH).

Anal. Calcd for $C_{46}H_{48}N_6O_{10}S_2$ (908.0): C, 60.79; H, 5.28; N, 9.25; S, 7.06. Found: C, 60.57; H, 5.26; N, 9.25; S, 7.23.

Gramicidin A (CCD/II/Sch; 340–380) was a gift of Dr. E. Gross (National Institute of Health, Bethesda, Maryland) and it was a 95% valine–gramicidin A, and 5% isoleucine–gramicidin A mixture (Sarges and Witkop, 1965). The β^{1-24}-corticotropin was a gift of Dr. W. Rittel, Ciba AG, Basle (Switzerland).

Bovine pancreatic RNase (five-times recrystallized) was purchased from Sigma Chemical Co., St. Louis. It was further purified by chromatography on Amberlite IRC-50, XE-64, in 0.2 M sodium phosphate buffer (pH 6.47) (Hirs *et al.*, 1953). The fraction containing RNase A was deionized on Sephadex G-25 (Pharmacia, Uppsala, Sweden) using 5% acetic acid, lyophilized, and stored at 4°. Yeast ribonucleic acid (RNA) in the form of sodium salt was purchased from BDH Laboratory Chemical Division and further purified by exhaustive dialysis against NaCl (0.1 M) and water (Wellner *et al.*, 1963). Egg white lysozyme, trypsin, and α-chymotrypsin were all obtained from Worthington Biochemical Corp. and used without further purification.

Methods

Instrumentation. A Beckman Model DU spectrophotometer was used for absorbance measurements at single wavelengths; continuous spectra were obtained with a Beckman Model DB spectrophotometer connected to a Sargent recorder. The rotatory powers were measured with a Perkin-Elmer Model 141 polarimeter at 22–24°; concentrations are given in g/100 ml of solvent. The melting points were determined by a Tottoli's apparatus (Büchi) and are not corrected. Amino acid analyses were carried out in the laboratory of Dr. E. Wünsch (Max Planck Institut für Eiweiß und Lederforschung, Munich, Germany) with a Beckman–Spinco Model 120B amino acid analyzer, according to the standard procedure of Spackman *et al.* (1958).

Recovery of Amino Acids after Reaction with NPS-Cl as Determined by Amino Acid Analysis (Table I). The standard amino acid mixture (1 ml) was lyophilized and dissolved in 2.5 ml of glacial acetic acid or 99% formic acid. To 1 ml of this solution, containing 1 μmole of each amino acid, 20 μmoles of NPS-Cl in acetic acid was added, the mixture was shaken for 20 min, then filtered and lyophilized. The residue was dissolved in pH 2.2 citrate buffer and placed on the amino acid analyzer.

Quantitative Determination of NPS-Cl after Reaction with Amino Acids (Table I). The amino acid (0.25 mmole) was dissolved by gentle heating in 10 ml of glacial acetic acid. After cooling, NPS-Cl (0.315 mmole), in the same solvent, (10 ml) was added and the total volume was brought to 25 ml. After 15 hr at room temperature, 5 ml was removed, poured into 10 ml of 5% KI solution, and 50 ml of water was added. The free iodine was then titrated with $Na_2S_2O_3$ (0.01 N), and the per cent of the amino acid recovery was calculated on the basis of the consumption of the sulfenyl halide. A blank was run under the same conditions with no addition of amino acid.

Kinetic Measurements of the Reaction of NPS-Cl and DNPS-Cl with Tryptophan Derivatives (Figure 1). The tryptophan derivative (0.5 mmole) was dissolved in 30 ml of glacial acetic acid. To the thermostatted solution at 21 ± 0.1°, 1 mmole of NPS-Cl or DNPS-Cl in 20 ml of glacial acetic acid (also thermostatted) was added. At various times, 5 ml was removed and analyzed as described previously.

Sulfenylation of Tryptophan Peptides (Table II). PROCEDURE A. To a solution of 10 mmoles of the tryptophan derivative in 40–60 ml of glacial acetic acid or 99% formic acid, 10 mmoles of the sulfenyl chloride was added under stirring at room temperature. After shaking for 4 hr, the product was precipitated by addition of water and then collected and dried under high vacuum

TABLE I: Recoveries of Amino Acids after Treatment with NPS-Cl in 99% Formic Acid or in Glacial Acetic Acid as Determined by Amino Acid Analysis or by Titration of the Unreacted NPS-Cl.

Amino Acid	Recov (μmoles)[a] Control, Not Treated	NPS-Cl Treated	% Recov[b]
Lysine	1.01	1.02	
Histidine	0.97	1.00	98
Arginine	0.96	0.97	98
Aspartic acid	1.02	1.00	
Threonine	0.98	0.95	100
Serine	1.00	0.98	
Glutamic acid	1.01	0.99	100
Proline	1.01	0.98	99
Glycine	1.00	1.00	
Alanine	1.02	1.00	100
Cystine	0.98	0.96	99
Valine	1.01	1.00	100
Methionine	1.00	0.94	
Methionine sulfoxides		0.06	
Isoleucine	1.02	1.00	99
Leucine	1.02	1.00	100
Tyrosine	0.96	0.99	
Phenylalanine	1.00	0.98	100
Tryptophan			3

[a] The reaction of the type I amino acid calibration mixture was carried out as described in the text, and the chromatographic analysis was performed according to Spackman *et al.* (1958). The reported values are mean values of two chromatographic analyses. [b] The reaction and the quantitative determination of NPS-Cl were performed as described in the text. The per cent recovery of the amino acid was determined from the consumption of NPS-Cl.

over P_2O_5. Recrystallization may be effected from ethanol or ethyl acetate–petroleum ether.

PROCEDURE B. The reaction was carried out as described under procedure A using 50–70 ml of chloroform as solvent. After standing for 4 hr at room temperature, the solution was washed with aqueous sodium hydrogen carbonate and with water. After drying over sodium sulfate, the solution was evaporated to dryness and the residual oil was recrystallized from ethanol–petroleum ether or ethyl ether–petroleum ether.

NPS-gramicidin A. To a solution of 24 mg of gramicidin A in 1.5 ml of glacial acetic acid, 10 mg of NPS-Cl was added, and the mixture was allowed to stand at room temperature for 1 hr. The product was then precipitated with 20 ml of ethyl ether–petroleum ether (1:3), separated by centrifugation, and washed several times with ethyl ether–petroleum ether (1:3). After drying under high vacuum over P_2O_5, the yield was 23 mg; single yellow hypochlorite–starch–KI test (Porn and Dutcher, 1956) positive, Ehrlich-negative spot (R_F 0.95) in thin-layer chromatography using chloroform–glacial acetic acid (1:2) as solvent. In the same solvent system gramicidin A had R_F 0.35.

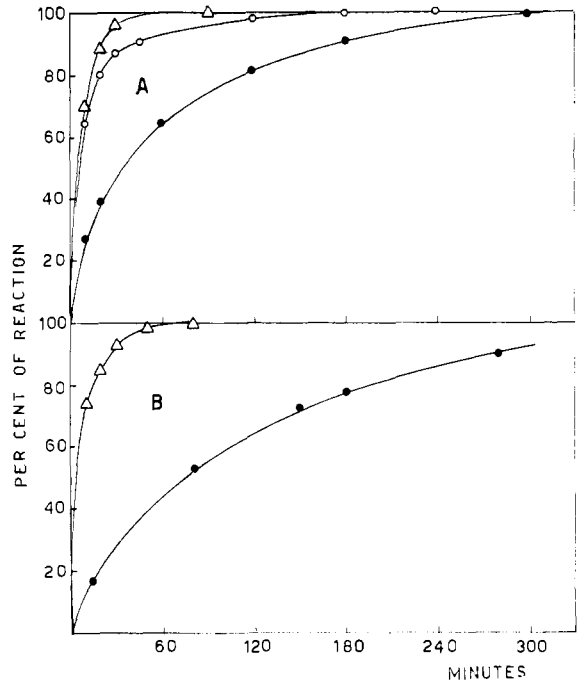


FIGURE 1: Treatment of NPS-Cl (A) and DNPS-Cl (B) with tryptophan derivatives in glacial acetic acid at 21 ± 0.1°. (A) (●) H-Trp-OH, (△) Z-Leu-Trp-OMe, and (○) Z-(Cys)$_2$-Trp-OMe. (B) (●) H-Trp-OH and (△) Z-Leu-Trp-OMe.

Anal. Calcd for $C_{129}H_{154}N_{25}O_{26}S_4$ (2506): S, 5.17. Found: S, 5.10.

NPS-β^{1-24}-corticotropin. To a solution of 25 mg of β^{1-24}-corticotropin, $6CH_3COOH \cdot 14H_2O$ (Schwyzer and Kappeler, 1963), in 2 ml of glacial acetic acid, 10 mg of NPS-Cl was added. After 1 hr at room temperature, the product was precipitated with ethyl ether, separated by centrifugation, and washed several times with ether. The yellow product was then further redissolved in acetic acid and the separation procedure was repeated. After drying *in vacuo* over P_2O_5, the yield was 18 mg; single yellow, ninhydrin-positive, Ehrlich-negative component on paper electrophoresis: 27°, 3 hr, 20 V/cm, pH 1.9, water–acetic acid (75:25, v/v).

Sulfenylation of Proteins. To a solution of 1 μmole of the protein in 2 ml of 30% acetic acid, various amounts of NPS-Cl or pNPS-Cl dissolved in 1 ml of glacial acetic acid were added at room temperature under stirring. After 1 hr, the modified protein was precipitated at −5° with acetone–1 N HCl (39:1), separated by centrifugation, and washed several times with acetone and ethyl ether. The product was dried *in vacuo* (10^{-2} torr) over P_2O_5. The extent of modification was calculated by dissolving the samples in 80% acetic acid and by reading the optical density at 365 mμ, as described below.

By the procedure reported above, no side products arising from the excess of reagent are present in the samples of the NPS protein. For a control, two samples (20 mg) of NPS protein were purified by gel filtration on a column (0.9 × 40 cm) of Sephadex G-25, using 0.2 M

TABLE II: Analytical Data and Specific Rotations of Tryptophan Peptides Treated with Sulfenyl Halides.

No.	Compound	Procedure[a]	Yield (%)[b]	Mp (°C) (not cor)	$[\alpha]_D$, deg (c 0.5, MeOH)	R_F[c]	Formula (mol wt)	Calcd (%) C	Calcd (%) H	Calcd (%) N	Calcd (%) S	Found (%) C	Found (%) H	Found (%) N	Found (%) S
1	Z-Leu-Trp(NPS)-OMe	A	77	93–95	−22.6	0.73	$C_{32}H_{34}N_4O_7S$ (618.6)	62.07	5.50	9.05	5.17	61.24	5.73	8.99	5.25
2	Z-Leu-Trp-(pNPS)-OMe	B	79	150–151	−17.6	0.95	$C_{32}H_{34}N_4O_7S$ (618.6)	62.07	5.50	9.05	5.17	62.18	5.38	9.09	5.22
3	Z-Leu-Trp-(DNPS)-OMe	A	83	100–102	−25.6	0.66	$C_{32}H_{33}N_5O_9S$ (663.6)	57.86	4.97	10.54	4.82	57.61	5.08	10.44	4.82
4	Z-Leu-Trp(PS)-OMe	A	83	136–137	−13.6	0.35	$C_{32}H_{37}N_3O_5S$ (573.6)	66.94	6.45	7.32	5.57	66.88	6.36	7.20	5.68
5	Z-Trp(NPS)-Gly-OEt	B	81	165–167	+7.6	0.35	$C_{29}H_{28}N_4O_7S$ (576.5)	60.36	4.85	9.71	5.55	60.05	4.82	9.60	5.61
6	Z-Trp(NPS)-Phe-OMe	B	76	75–77	+18.8	0.40	$C_{35}H_{32}N_4O_7S$ (652.6)	64.35	4.90	8.58	4.90	64.08	4.83	8.46	4.66
7	Z-Trp(NPS)-Met-OMe	A	71	73–75	+2.8	0.39	$C_{31}H_{32}N_4O_7S_2$ (636.6)	58.43	5.02	8.79	10.05	58.74	5.19	8.67	9.70
8	Z-Pro-Trp(NPS)-OMe	A	72	77–80	−43.4	0.45	$C_{31}H_{30}N_4O_7S$ (602.6)	61.73	4.98	9.29	5.31	62.11	4.92	9.07	5.42
9	Z-Pro-Trp-(DNPS)-OMe	B	69	98 dec	−36.8	0.45	$C_{31}H_{29}N_5O_9S$ (647.5)	57.44	4.47	10.81	4.94	57.73	4.67	10.56	4.87
10	Z-Thr-Trp(NPS)-OMe[d]	A	70	104–106	+6.0	0.27	$C_{30}H_{30}N_4O_8S$ (606.5)	59.35	4.94	9.23	5.27	59.27	4.77	9.14	4.92
11	Z-$(Cys)_2$-Trp(NPS) OMe	A	87	105 dec	−33.0	0.95	$C_{53}H_{54}N_8O_{14}S_4$ (1214.3)	57.31	4.44	9.22	10.56	57.74	4.48	9.19	10.45
12	Z-Ala-Trp(NPS)-Gly-OEt	A	69	206–208	+7.0[e]	0.24	$C_{32}H_{31}N_5O_8S$ (645.6)	59.47	4.80	10.84	4.95	59.62	5.15	10.71	4.74

[a] See Experimental Section. [b] Yields on analytically pure compounds. [c] The R_F value was determined by thin-layer chromatography (SiO_2) using the following solvent mixture: chloroform–benzene–glacial acetic acid (85:10:5). The compounds were detected using the hypochlorite–starch–KI test (Porn and Dutcher, 1956). [d] The residue of threonine is DL. [e] c 0.5, DMF.

acetic acid as eluent, at a flow rate of 0.2 ml/min. The effluent containing the protein was lyophilized, and the extent of reaction was further calculated by the method reported above. The number of modified residues was the same, within experimental error, before and after purification on Sephadex G-25.

Estimation of the Extent of Modification in Proteins Treated with NPS-Cl. The NPS protein (about 0.1 μmole) was dissolved in 80% acetic acid (5 ml) and the covalently bound chromophore was determined spectrophotometrically (ε 4000 at 365 mμ; *cf.* Table III). The protein concentration was calculated on the basis of the dry weight and assuming for NPS lysozyme (fully modified) a molecular weight of 15,285, for NPS-trypsin (fully modified) 24,400, and for NPS-α-chymotrypsin 25,200-26,200, depending upon the extent of the modification. The dry weight of the NPS protein was assumed equal to the dry weight, determined spectrophotometrically, of the protein treated in the same fashion but without NPS-Cl. A molar extinction at 280 mμ of 39,000 for lysozyme, 50,000 for α-chymotrypsin, and 35,000 for trypsin was assumed.

This method of calculation was checked by comparing the concentration of 2-NPS-tryptophan residues obtained spectrophotometrically with the amino acid content in the NPS protein hydrolysate (6 N HCl, 105°, 24 hr) obtained from an aliquot of the solution. Using this method, in the case of NPS lysozyme, the number of 2-NPS-tryptophan residues per mole (5.9) compared favorably with the value (6.2) determined by the method used routinely.

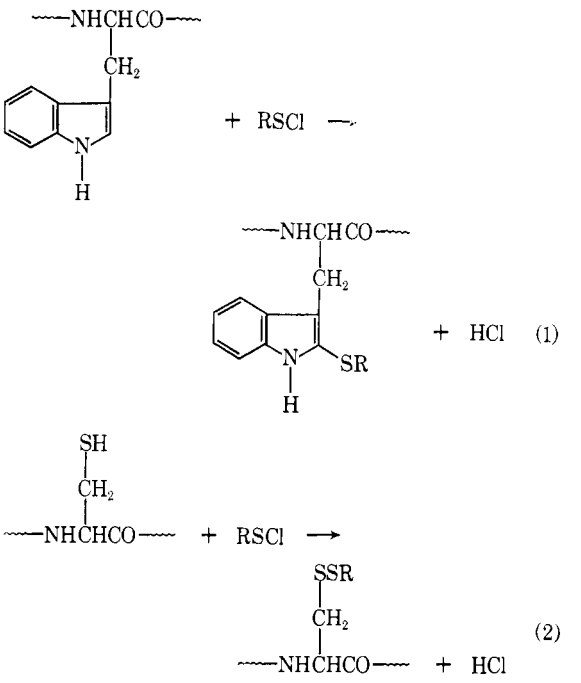
Assay of Enzymic Activity of RNase. The assay was based on the method of Kunitz (1946), following the decrease of absorbancy at 300 mμ. Changes in optical density were determined by using an expanded scale of the recorder which was coupled to the spectrophotometer. Activity was calculated from the slopes of initial velocities.

Results

Specificity of the Reaction. Sulfenyl halides are treated with tryptophan (Wieland and Sarges, 1962; Fontana *et al.*, 1966a,b) and cysteine, as shown in eq 1 and 2. The reaction of sulfenyl halides with cysteine and its application to proteins are reported in the following paper (Fontana *et al.*, 1968).

To establish the specificity of the reaction, an amino acid calibration mixture, in which no cysteine and tryptophan were present, was allowed to react with NPS-Cl (1:1 molar ratio for each amino acid) in glacial acetic acid or 99% formic acid for 15 hr at 22–24°. As shown in Table I virtually quantitative recovery of all tested amino acids was obtained, as determined by chromatographic analysis.

A further check of the selectivity of this reaction with amino acids was carried out by titration of the unreacted sulfenyl halide using the Orr and Kharasch (1953) method, slightly modified (see Experimental Section). This procedure involves titration with thiosulfate of iodine, quantitatively released by the sulfenyl halide from potassium iodide.



After 15-hr reaction in glacial acetic acid, titration of the sulfenyl halide reveals no appreciable loss of the reagent, except when tryptophan is present. In this case a stoichiometric consumption of the reagent was found (Table I).

However the method of titration fails in the presence of cysteine and methionine, since oxidation of these amino acids takes place by iodine. It was seen that in aqueous acetic acid, on standing at 22–24° for 15 min, cysteine is quantitatively converted into cystine whereas methionine is oxidized to methionine sulfoxide to the extent of about 50%. This reaction agrees with the results of Higuchi and Gensch (1966) on the oxidation of thioethers by iodine.

Two possible interfering reactions of sulfenyl halides with cystine and methionine were causes for concern on the basis of the data of Moore and Porter (1958, 1960). The authors stated that sulfenyl halides in acetic acid can react with organic disulfides and monosulfides according to eq 3 and 4. The extent of these reactions is

$$\mathrm{ArSCl} + \mathrm{RSSR} \longrightarrow \mathrm{ArSSR} + \mathrm{RSCl} \qquad (3)$$

$$\mathrm{ArSCl} + \mathrm{RSR} \longrightarrow \mathrm{ArSSR} + \mathrm{RCl} \qquad (4)$$

strictly related to the structure of the organic sulfide.

The practically quantitative recovery of cystine and methionine as determined by amino acid analysis (Table I) shows that under the reported conditions both amino acids are not involved in such reactions. The only observed modification is the oxidation of methionine to methionine sulfoxide, but only to a small extent (Table I).

The data are further supported by chromatographic analysis of the reaction mixture obtained by treating the dipeptides, *N*-carbobenzoxy-L-tryptophanyl-L-methionine methyl ester and bis-*N*-carbobenzoxy-L-cystinyl-

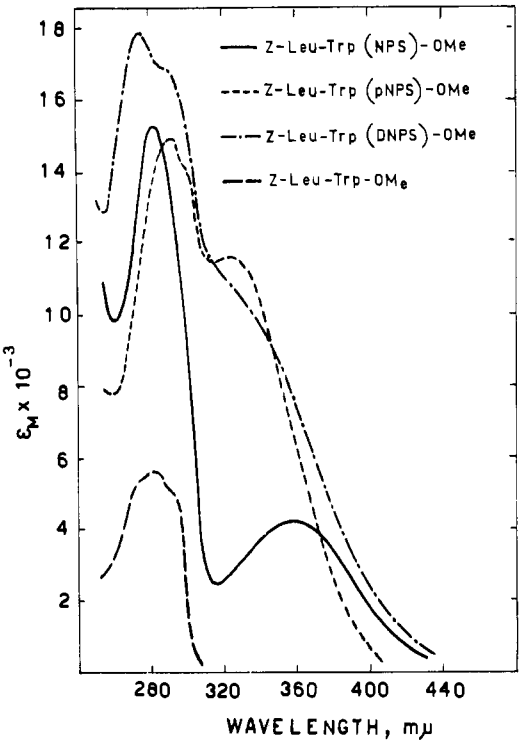


FIGURE 2: Absorption spectra of *N*-carbobenzoxy-L-leucyl-L-tryptophan methyl ester treated with NPS-Cl, pNPS-Cl, and DNPS-Cl. All samples were dissolved in 80% acetic acid.

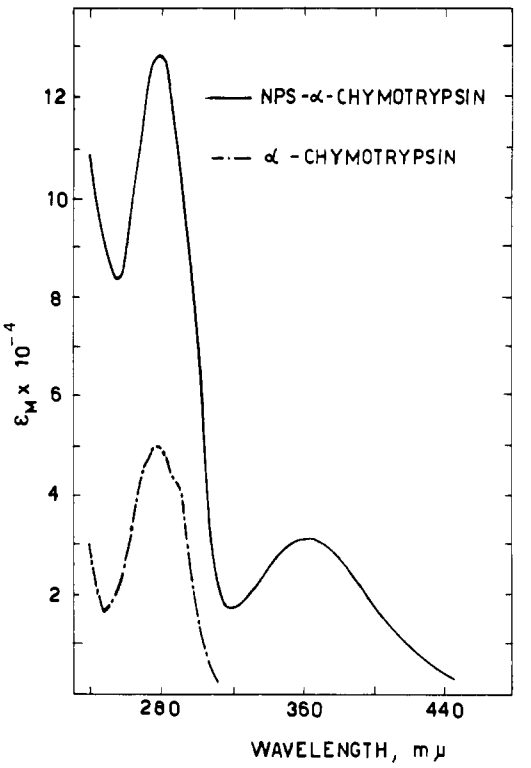


FIGURE 4: Absorption spectra of α-chymotrypsin (– · · –) and fully modified NPS-α-chymotrypsin (——) in 80% acetic acid. The NPS derivative was prepared by treating the enzyme with NPS-Cl (1:20 molar ratio) in 50% acetic acid, as described in the text.

L-tryptophan methyl ester, with NPS-Cl in glacial acetic acid. The corresponding 2-NPS-tryptophan peptides were obtained as the only products (Table II).

A disulfide-exchange reaction occurs at alkaline pH as indicated in eq 5, but not in neutral and acidic solutions (Sanger, 1953). Benesch and Benesch (1958) observed an extensive exchange reaction in strongly acidic solution (9.5 M HCl); furthermore under the same conditions the sulfenyl halides catalyzed the reaction.

$$\text{RSSR} + \text{R'SSR'} \longrightarrow \text{RSSR'} + \text{R'SSR} \qquad (5)$$

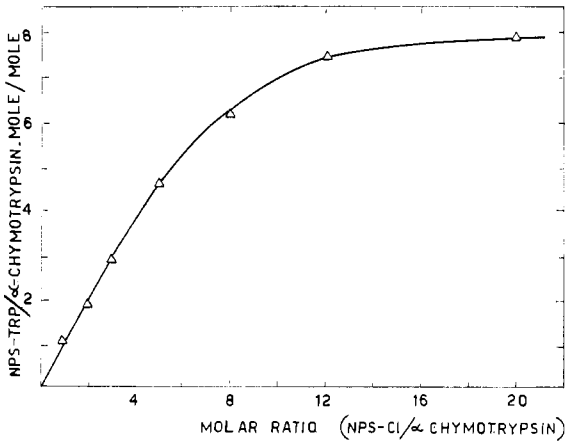


FIGURE 3: Sulfenylation of α-chymotrypsin: formation of NPS-tryptophan residues with increasing amounts of NPS-Cl. The reaction was performed in 50% acetic acid and the degree of sulfenylation was determined spectrophotometrically at 365 mμ as described in the text.

In order to exclude the disulfide reaction and to confirm the results obtained with amino acids, RNase A, a protein free from tryptophan and cysteine, was selected as a control. The enzyme was allowed to react in 50% aqueous acetic acid with NPS-Cl (1:20 molar ratio) as described in the Experimental Section. After acidic precipitation and further purification on Sephadex G-25, the enzyme was recovered fully active toward RNA and with the ultraviolet spectrum unchanged. This experiment confirms that sulfenyl halides in acidic solution are unreactive toward amino acids different from tryptophan and cysteine, and that the experimental conditions are not denaturing the enzyme.

Sulfenylation of Tryptophan Peptides and Proteins. The reaction between sulfenyl halides and compounds with indole residues was also carried out with several tryptophan-containing peptides. Besides acetic acid, formic acid, and chloroform many other organic solvents (such as dimethylformamide, dioxane, ethyl ether, etc.) were used. High yields of the corresponding modified peptides were obtained (Table II) with small peptides as well with β^{1-24}-corticotropin, which contains one tryptophan residue (Schwyzer and Kappeler, 1963), and with gramicidin A, a pentadecapeptide containing four tryptophan residues (Sarges and Witkop, 1965).

The reaction of sulfenyl halides with tryptophan and its peptides can be followed conveniently by titration of

TABLE III: Wavelengths of Maximum Absorption (λ_{max}) and Molar Absorptivities (ϵ) of Tryptophan Peptides Treated with Sulfenyl Halides.

No.	Compound	Solvent	λ_{max}	$\epsilon \times 10^{-4}$	λ_{max}	$\epsilon \times 10^{-3}$
1	H-Trp(NPS)-OH · H_2O[a]	HAc	280	1.67	362	3.75
2	Z-Leu-Trp(NPS)-OMe	MeOH	280	1.67	365	4.4
		HAc	280	1.55	362	4.1
3	Z-Leu-Trp(pNPS)-OMe	MeOH	290	1.70	323	12.6
		HAc	290	1.50	328	11.7
4	Z-Leu-Trp(DNPS)-OMe	MeOH	278	1.73		
		HAc	278	1.80		
5	Z-Leu-Trp(PS)-OMe	MeOH	292	1.77		
		HAc	290	1.55		
6	Z-Ala-Trp(NPS)-Gly·OEt	MeOH	280	1.71	362	4.45
		HAc	280	1.61	363	4.05
7	H-Phe-Val-Gln-Trp(NPS)-Leu-OH · $2H_2O$[b]	HAc	280	1.50	365	3.95
8	NPS-gramicidin A[c]	HAc	282	6.05	363	15.8
9	NPS-β^{1-24}-corticotropin[d]	HAc[e]	280	1.69	363	4.1

[a] Fontana *et al.* (1966b). [b] This pentapeptide corresponds to the 22–26 sequence of glucagon (Wünsch *et al.*, 1967). [c] Gramicidin A contains four tryptophanyl residues per mole (Sarges and Witkop, 1965). For NPS-gramicidin A a molecular weight of 2506 was assumed. [d] β^{1-24}-Corticotropin contains two tyrosyl (ϵ 1300 at 280 mμ) and one tryptophanyl residue per mole (Schwyzer and Kappeler, 1963). For NPS-β^{1-24}-corticotropin a molecular weight of 3650 was assumed. [e] HAc, 10%; the other HAc values are 80%.

the sulfenyl halide. Figure 1 shows the rates of reaction of various tryptophan derivatives with NPS-Cl and DNPS-Cl (1:2 molar ratio). A faster reaction was observed with tryptophan-containing peptides, as compared to the free amino acid (*cf.* also Scoffone *et al.*, 1966; Wünsch *et al.*, 1967).

The reaction of different nitrophenylsulfenyl halides with tryptophan residue results in characteristic absorption spectra (Figure 2 and Table III).

In order to compare the absorption maxima and the molar absorptivities of the sulfenylated tryptophan linked in a peptide bond, the absorption spectra of some model compounds were recorded.

The protected peptide *N*-carbobenzoxy-L-leucyl-L-tryptophan methyl ester was allowed to react with NPS-Cl, pNPS-Cl, DNPS-Cl, and PS-Cl. The PS derivative of this peptide shows an absorption maximum at 290 mμ and a molar absorptivity of 15,500 in 80% acetic acid, while the NPS, pNPS, and DNPS derivatives are yellow compounds, which absorb in the visible part of the spectrum. The NPS derivatives absorb at 360–365 mμ with ϵ 4000–4500 and pNPS derivatives absorb at 328 mμ with 11,700 (Table III and Figure 2).

2-Sulfenyltryptophan derivatives are not stable under the conditions commonly used for the hydrolysis of polypeptides and proteins, therefore amino acid analysis of an acid or alkaline hydrolysate cannot be employed for quantitative determination of the extent of modification. A detailed account of the conversion of 2-sulfenyltryptophan into 2-hydroxytryptophan by acidic hydrolysis will be reported in a separate communication.

In a previous note (Scoffone *et al.*, 1966) we described the reaction of sulfenyl halides with proteins using glacial acetic acid or 99% formic acid as solvents. Since many proteins are unstable under these conditions (Josefsson and Edman, 1957; Smillie and Neurath, 1959), an aqueous solvent system would be preferable operationally.

We have shown that the sulfenylation of tryptophan can be accomplished in partly aqueous solvents as 30–50% acetic acid, 30–50% dioxane, or 30–50% dimethylformamide, provided that hydrolysis-resistant sulfenyl halides, such as NPS-Cl or DNPS-Cl (Kharasch *et al.*, 1955; Di Nunno *et al.*, 1966) are used. In the case of the easily hydrolyzable sulfenyl halides, such as pNPS-Cl, an aqueous solvent can still be employed, provided that an excess of reagent dissolved in anhydrous solvent is used and added dropwise under stirring to the aqueous solution of the protein.

In order to find the best reagent:protein molar ratio, α-chymotrypsin, a protein containing eight tryptophan residues (Hartley, 1964) but no free SH groups, was treated with different amounts of NPS-Cl in 50% aqueous acetic acid. Since the proteins are transparent in the region where the NO_2 chromophore shows absorption maximum, the extent of reaction was determined by measuring the absorption at 365 mμ of samples of the NPS protein dissolved in 80% acetic acid, assuming ϵ 4000 for the 2-NPS-tryptophan residue (Table III). Maximum extent of reaction, corresponding to 7.8 modified residues/mole, was achieved by treatment of the enzyme with a 20-fold M excess of NPS-Cl (Figure 3). The absorption spectra of α-chymotrypsin and its NPS derivative are shown in Figure 4.

It should be noted that, when sulfenyl halides not carrying chromophoric groups are used, the quantitative relationship existing between the moles of covalently bound groups and disappearance of tryptophan can be obtained by the well-known *p*-dimethylaminobenzaldehyde reaction (Spies and Chambers, 1949). The quantitation of the extent of modification using this reaction will be discussed separately.

The quantitative conversion of tryptophan into 2-NPS-tryptophan determined spectrophotometrically was also obtained by treatment of lysozyme and trypsin with 20 equiv of NPS-Cl in 50% acetic acid (Table IV).

TABLE IV: Numbers of the Modified Tryptophan Residues in Various Proteins Treated with NPS-Cl as Determined by Absorption at 365 mμ.[a]

Protein	NPS-tryptophan (mole/mole)	Tryptophan Content
Lysozyme	6.2	6[b]
α-Chymotrypsin	7.8	8[c]
Trypsin	4.1	4[d]
Ribonuclease	0.1[e]	0[f]

[a] The reaction and the quantitative determination of NPS-tryptophan were performed as described in the text. For the NPS-tryptophan residue, ε 4000 at 365 mμ was assumed. [b] Canfield (1963). [c] Hartley (1964). [d] Walsh *et al.* (1964). [e] After purification on Sephadex G-25, the sample of RNase treated with NPS-Cl does not absorb at 365 mμ. [f] Smyth *et al.* (1963).

Discussion

The selective or preferential modification of the side-chains groups of amino acids is one of the most commonly used procedures for understanding the essential structural prerequisites for the biological function of proteins. 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 great attention was focused on chemical methods suitable for modification of such a residue.

The sulfenyl halides offer a broad new class of reagents which are highly specific and selective toward tryptophan and cysteine residues of proteins in acidic solvents. The reaction of cysteine with sulfenyl halides is examined in the following paper (Fontana *et al.*, 1968).

The selectivity of the reaction can be explained by a combination of properties. It is known that sulfenyl halides react as electrophilic reagents (Buess and Kharasch, 1950) with aromatic groups in the presence of Lewis acids, producing aromatic monosulfides. With more reactive aromatic systems, *e.g.*, resorcinol or *p,p'*-dimethylaniline, no catalyst is needed and reaction occurs readily. The indole ring behaves as a very reactive aromatic nucleus toward these reagents. The reactions of indole and substituted indoles toward sulfenyl halides were previously reported (Wieland and Sarges, 1962; Fontana *et al.*, 1966a,b). The lack of reactivity of other side-chain aromatic groups like phenylalanine and tyrosine in proteins can be explained in terms of less nucleophilicity toward sulfenyl halides than indole rings. The acidic solvent conditions are necessary for the selectivity of the reaction, since protonation of amino groups inhibits sulfenamide formation.

The sulfenylation reaction increases the number of procedures available for the modification of tryptophan, providing greater experimental latitude in the study of the biological function of tryptophan-containing enzymes.

By this additional technique the tryptophan residues in those proteins which lack sulfhydryl groups can be modified by using different sulfenyl halides, leading to a change in size, polarity, or other physicochemical properties. Such a large spectrum of possibilities offers a new approach to study the interactions of tryptophan with its environment in the protein molecule. The effect of the modification of tryptophan residues on the functional properties of enzymes will be reported elsewhere.

By using nitrophenylsulfenyl chorides, a chomophore which absorbs in the visible region of the spectrum is generated in a protein. Such a group, if optically active, might further reveal characteristic extrinsic Cotton effects as additional probes of protein conformation. We have recently applied this reaction to the modification of polytryptophan and structural studies by optical methods on these polymers will be reported.

The quantitative conversion of tryptophan into its NPS derivative is achieved by using a very low molar ratio of reagent to protein. This is shown in Figure 3 for α-chymotrypsin and NPS-Cl.

Among the useful information on the involvement of tryptophan in enzymic processes, the analytical utility of this reaction is another major application. The quantitative reaction with NPS-Cl of tryptophan in α-chymotrypsin, lysozyme, and trypsin (Table IV) appears to offer a new rapid and convenient spectrophotometric method for determining the tryptophan content of a protein.

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