Biochemistry

Volume 13, Number 1 January 1, 1974

Cyanylation of Sulfhydryl Groups by 2-Nitro-5-thiocyanobenzoic Acid. High-Yield Modification and Cleavage of Peptides at Cysteine Residues†

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ABSTRACT: Peptides containing cysteine residues were quantitatively S-cyanylated using the reagent 2-nitro-5-thiocyanobenzoic acid in dilute solutions. With increasing concentrations of the reactants, the extent of cyanylation decreased, and identifiable side products were formed. Kinetic studies indicated that these side products resulted from a series of concentration-dependent consecutive competitive reactions, initiated by an interaction between the reagent and its released leaving group. Radioactively labeled β-thiocyanoalanine peptides obtained by the modification, were cleaved in high yields at the N-peptide bond of the modified residue, by incubations at 37–50° at pH 8, to form 2-iminothiazolidine derivatives. ^{14}C analyses performed during the reaction, indicated that the cleavage occurred either concerted with, or immediately subsequent to the cyclization. Increase of the pH accelerated the cleavage, but also caused the β-elimination of thiocyanate to a varying degree, depending on the respective structural properties of the β-thiocyanoalanine compounds. The presence of cystine during the cleavage reactions did not result in cyano exchange with the disulfide group, suggesting that by this modification method, cysteine peptide bonds could be selectively cleaved in the presence of cystine residues

In previous publications (Degani *et al.*, 1970; Degani and Patchornik, 1971) 2-nitro-5-thiocyanobenzoic acid (NTCB[1]) was introduced as a reagent for the selective cyanylation of thiol groups under very mild conditions. The reagent, in its readily prepared ^{14}C-labeled form, was shown to be useful for the reversible labeling of proteins at cysteine residues, with a [^{14}C]cyano group. The reagent was also suggested as a potential tool for the selective chemical cleavage of peptide chains at the N-peptide bonds of cysteine residues, in view of earlier reports (Wood and Catsimpoolas, 1963; Catsimpoolas and Wood, 1966) showing that β-thiocyanoalanine residues, when generated by excess cyanide treatment of cystine disulfide bonds, underwent cyclization to form 2-iminothiazolidine rings, with consequent cleavage of the N-peptide bond. (For a review, see Spande *et al.*, 1970.) We now present a study carried out to examine the modification of cysteine model peptides by NTCB, and the cleavage of the resulting β-thiocyanoalanine peptides.

Experimental Section

Materials

Glutathione was obtained from Calbiochem and DTNB from Aldrich. NTCB was prepared as its half-potassium salt as previously described (Degani and Patchornik, 1971). [^{14}C]NTCB was prepared using [^{14}C]KCN either analogously or by the previously described bromoacetyl-cellulose method (Degani *et al.*, 1970). The preparation of TNB used was described previously (Degani and Patchornik, 1971). [^{14}C]ITC was prepared as previously described (Degani *et al.*, 1970). *N,S*-Dicarbobenzoxyglutathione was prepared according to Sokolovsky *et al.* (1964a) and its purity was verified by the nuclear magnetic resonance (nmr)–titration method (Degani and Patchornik, 1972), using tetramethylammonium hydroxide. *N,S*-Dicarbobenzoxy-L-phenylalanyl-L-cysteinylglycine,

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[1] Abbreviations used are: NTCB, 2-nitro-5-thiocyanobenzoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, thionitrobenzoate; GSH, glutathione; GSSG, oxidized glutathione; GSCN, S-cyanoglutathione; GSTNB, mixed disulfide between glutathione and 5-mercapto-2-nitrobenzoic acid; ITC, 2-iminothiazolidine-4-carboxylic acid or its residue in peptide linkage; CySCN, β-thiocyanoalanyl residue; Z, *N*-carbobenzoxy; OBz, benzyl ester. NTCB, DTNB, TNB, and GSTNB are also referred to as ArSCN, ArSSAr, ArS^-, and GSSAr, respectively.

N,*N*-dicarbobenzoxy-L-cysteinyl-L-tyrosinamide, and *N*,*S*-dicarbobenzoxy-L-cysteinylglycine benzyl ester (the purity of all of which was established by thin-layer chromatography (tlc) and elemental analysis), were the kind gift of Dr. M. Wilchek of the Weizmann Institute of Science. Other materials used were commercial, reagent grade.

Methods

High-Voltage Paper Electrophoresis. Electrophoreses were run in Savant high-voltage electrophoresis apparatus Model LT-48A, at 3000 V (60 V/cm), using Whatman No. 1 paper for analytical runs and Whatman 3MM paper for preparative runs. A 90-min run at pH 3.5 (pyridine–acetate buffer) was used to separate any of the following compounds: ITC-Gly, GSCN, GSH, GSSG, glutamic acid, GSSNB, NTCB, and DTNB. All but ITC-Gly migrated toward the anode, in that order of increasing mobility. Thiocyanate was separated by a 20 min run at the same pH. Electrophoresis at pH 6.5 (pyridine–acetate buffer) for 30 min was used to separate *N*-carbobenzoxy compounds and pyrrolidonecarboxylic acid. ITC was separated by a 30 min run at pH 1.9 (formic–acetic acid).

For the location and identification of compounds on paper electropherograms, multiple parallel strips were examined by combinations of the following methods. Compounds containing free amino groups were detected by a ninhydrin dip (0.5% reagent in acetone containing 5% water). Compounds containing free thiol groups were identified by a DTNB spray (0.5% in 0.1 M sodium phosphate, pH 7.5). Compounds containing TNB moieties (*e.g.*, NTCB, DTNB, and GSTNB) were located by their uv-absorbing spots and identified by the yellow color formed upon spraying with Na_2S solution (5% in 2:3 water–ethanol). Compounds containing β-thiocyanoalanine residues were identified by the following procedure. The paper strip was exposed to the vapors of concentrated ammonium hydroxide solution (30% NH_3) in a closed chamber for 1 hr and then air-dried in a ventilated hood for 1 hr. Subsequent spraying with a ferric reagent (5% ferric nitrate in 0.5 M nitric acid) resulted in the appearance of a red spot. The same reagent was used to detect separated thiocyanate. Compounds containing β-iminothiazolidine rings were detected by diazotized sufanilic acid spray according to the procedure of Bradham *et al.* (1965). Compounds containing N–H groups (*e.g.*, *N*-carbobenzoxy derivatives and pyrrolidonecarboxylic acid) were detected by the hypochlorite–starch–iodide method according to the modified procedures of either Pan and Dutcher (1956) or Mazur *et al.* (1962). Pyrrolidonecarboxylic acid was also identified by spraying with 0.1% ethanolic 1,6-dichlorophenolindophenol. Radioactive compounds were located using a Packard radiochromatogram scanner, Model 7201.

Ninhydrin spots were estimated quantitatively by cutting into thin strips and eluting with 70% aqueous ethanol (3 ml, 45 min), followed by absorbancy measurement at 570 nm. GSTNB was quantitatively determined by eluting its uv-absorbing spot with β-mercaptoethanol solution, using the same procedure as previously described for NTCB and DTNB (Degani and Patchornik, 1971). For the quantitative estimation of radioactive compounds, the region corresponding to the radioactive peak was cut into 1-cm wide strips and counted separately in vials containing 10 ml of toluene scintillation solution, using a Packard Tri-Carb liquid scintillation spectrometer. The efficiency of the counting was controlled using parallelly chromatographed ^{14}C standards. Peptides were isolated from preparative paper electropherograms by elution with 30% acetic acid, and were stored in this solvent at −20°.

Amino Acid Analysis. Peptides were hydrolyzed with 6 N HCl at 110° for 22 hr in evacuated sealed tubes. Hydrolysates were analyzed using a Beckman amino acid analyzer Model 120B. [^{14}C]ITC was analyzed using the same instrument, combined to a Packard Tri-Carb flow analyzer Model 3022 which was inserted between the outlet of the 55-cm column of the amino acid analyzer and its ninhydrin mixing manifold.

Spectrophotometry. Measurements were made in glass cells of 1-cm optical path, using a Beckman Kintrac VII recording spectrophotometer. Kinetic measurements were made at 25°.

Modification of Cysteine Peptides. All buffers used in the modification experiments contained 1 mM EDTA and were freed of oxygen by bubbling a stream of nitrogen for 1 hr before use. *S*-Carbobenzoxy groups were quantitatively removed from blocked peptides prior to the modification, by the sodium methoxide method of Sokolovsky *et al.* (1964b), as assessed spectrophotometrically by DTNB titration (Ellman, 1959). NTCB and buffered peptide solutions were mixed at room temperature in the appropriate proportions as indicated. All concentrations mentioned in the Results refer to final concentrations of the reactants in the mixtures. Modification products were separated, identified, estimated, and isolated by methods above described. In the cyanylation experiments with GSH in concentrated mixtures (10 mM), the yields of the electrophoretically separated products GSCN, GSTNB, and GSSG were determined by two methods. (1) The paper was strained with ninhydrin and the resulting spots were estimated quantitatively as described above, using chromatographed GSSG standards for calibration, and assuming that all three compounds had the same specific ninhydrin color constant. (2) GSSG was determined as in method 1, GSTNB was determined from a parallel strip by eluting the uv-absorbing spot with mercaptoethanol as described above, and GSCN was determined by difference, based on the initial amount of GSH. The results obtained by both methods were in good agreement.

Cleavage of β-Thiocyanoalanine Peptides. Peptides were incubated under conditions indicated in the Results. Cleavage products were separated and analyzed by methods above described.

β-Elimination of Thiocyanate. Cysteine peptides were cyanylated in 0.7 mM solutions in 0.05 M phosphate buffer (pH 8.0) using a slight excess (10%) of NTCB. The extent of cyanylation ranged from 93 to 98%, as measured spectrophotometrically at 412 nm. Each solution was divided into three parts, two of which were adjusted to pH 9.0 and 10.0, respectively, by the addition of NaOH. All mixtures were incubated at 37°. Aliquots were taken at various time intervals and their thiocyanate content was measured colorimetrically by the method of Goldstein (1950). The effect of the TNB present on the colorimetric measurements was almost insignificant under the strongly acidic conditions of the method; this was corrected for, however, by including appropriate amounts of TNB in the thiocyanate standards used for calibration. When [^{14}C]NTCB was used for the cyanylation, [^{14}C]thiocyanate was also measured radiometrically after separating it from the reaction mixture by paper electrophoresis at pH 3.5. The results obtained by the colorimetric and radiometric methods were in good agreement. Yields of thiocyanate were calculated on the basis of the respective degree of cyanylation.

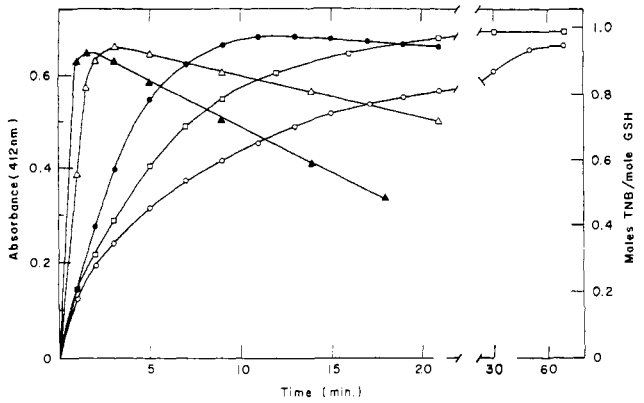
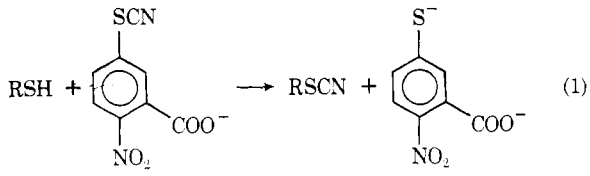


FIGURE 1: Release of TNB in the reactions of GSH with NTCB at pH 7.3. A_{412} was measured during the reaction of 0.051 mM GSH with NTCB in 0.1 M phosphate buffer (pH 7.3). The molar ratios of NTCB to GSH were 1:1 (○), 2:1 (□), 4:1 (●), 20:1 (△), and 50:1 (▲).

Results

Modification of Cysteine Peptides by NTCB

Kinetic Measurements. One of the useful properties of NTCB is the release of TNB which accompanies the cyanylation of thiol groups.



(1)

The rate and extent of the cyanylation can therefore be measured by following the appearance of optical absorption at 412 nm, using ϵ = 13,600 (Ellman, 1959; Degani and Patchornik, 1971). At this wavelength, NTCB is practically transparent. Figures 1 and 2 illustrate the change in A_{412}, measured on treatment of GSH with varying concentrations of NTCB at pH 7.3 and 8.0, respectively. The release of TNB was essentially quantitative, being faster at pH 8.0 than at pH 7.3. As seen in Figure 1, at high molar excess of NTCB, the initial sharp increase in A_{412} was followed by a gradual decrease in the absorbance. The loss of A_{412} was directly related to the initial molar excess of the reagent. Similar observations were made at pH 8.0 (not all shown in Figure 2). Control experiments in which pure TNB alone was treated with 50- and 100-fold molar excess of NTCB, indicated that the loss of absorbance at 412 nm was independent of the cyanylation reaction. This is illustrated in Figure 3, in which identical curves were obtained at pH 7.3 and 8.0. Other experiments, employing similar mixtures of TNB and NTCB, showed the presence of DTNB on pH 3.5 paper electrophoresis. The foregoing observations demonstrate the existence of equilibrium reaction 2:

$$\mathrm{ArSCN} + \mathrm{ArS^-} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{ArSSAr} + \mathrm{CN^-} \qquad (2)$$

When ArSCN is present in excess the equilibrium is shifted to the right, whereas by adding excess cyanide, the reverse reaction takes place. Both curves in Figure 3 represent pseudo-first-order rates, and by replotting and calculation on the basis of the concentrations of NTCB present, the second-order rate constant k_2 was found to be 9.0 M^{-1} min^{-1} for pH 7.3 and 8.0.

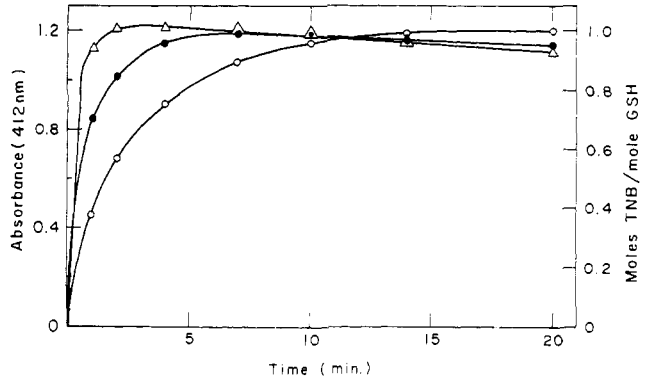


FIGURE 2: Reactions of GSH with NTCB at pH 0.8. 0.088 mM GSH in 0.1 M phosphate buffer (pH 8.0) was treated with NTCB in molar ratios of 1.5:1 (○), 3:1 (●), and 6:1 (△). Reactions were followed by measuring A_{412}.

To determine the equilibrium constant, K_{eq2}, equimolar 0.05 mM mixtures of NTCB and TNB in 0.1 M phosphte buffer solutions of pH 7.3 and 8.0 were incubated under nitrogen at 25°, and the A_{412} was measured at appropriate time intervals. Equilibria were reached within 9 hr, and from the equilibria concentrations of TNB (A_{412}), the equilibrium constant of reaction 2 was found to be 0.27 and 0.087 at pH 7.3 and 8.0, respectively. Similar values were obtained when the equilibrium was approached from the opposite direction, namely, by treating DTNB with cyanide under identical conditions. In this case the equilibria were attained more rapidly, in agreement with the values of the equilibrium constants, which are less than unity, and therefore imply that k_{-2} is larger than k_2.

To measure the second-order rate constants of the cyanylation of GSH by NTCB at pH 7.3 and 8.0, NTCB solutions were treated with an excess of GSH. Under these conditions reaction 2 was prevented and hence did not interfere with the

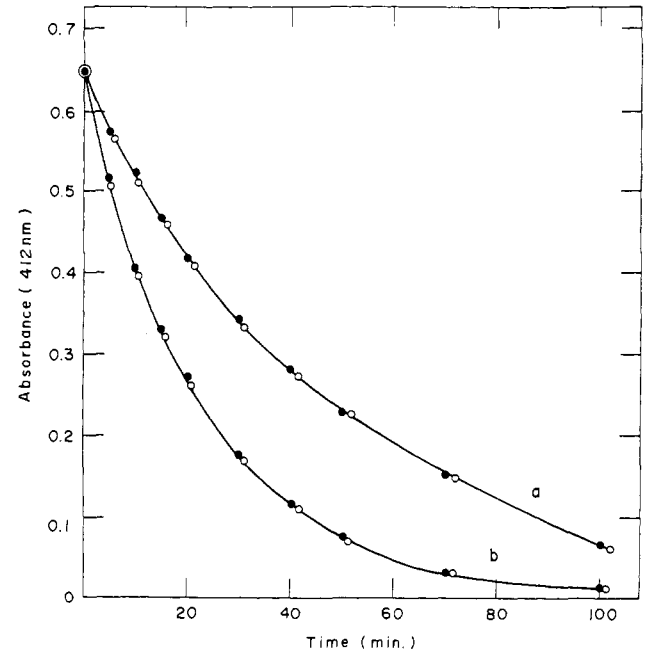


FIGURE 3: Reactions of TNB with NTCB. 0.048 mM TNB in 0.1 M phosphate buffer was treated with excess NTCB in molar ratios of 1:50 (a) and 1:100 (b) at pH 7.3 (●) and pH 8.0 (○). The reactions were followed by measuring the decrease in A_{412}.

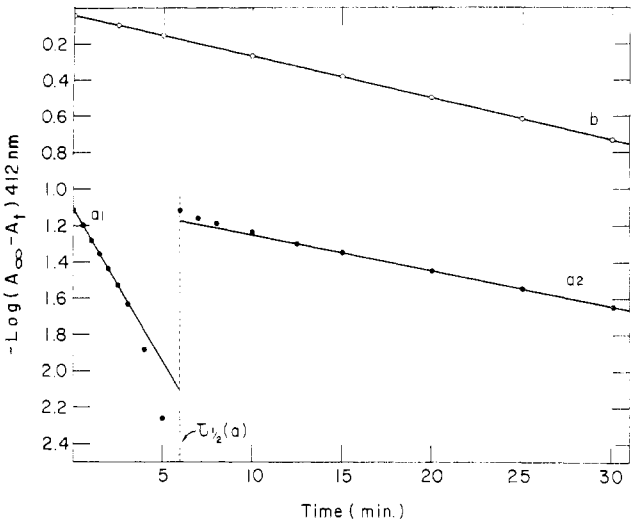


FIGURE 4: Rates of reactions of GSH with DTNB and with NTCB at pH 6.5. Rates were followed by measuring the increase of A_{412}. (a) Pseudo-first-order rate plots of the reactions occurring when 5.0 μM DTNB was treated wth 50 μM GSH in 0.04 M pyridine–acetate buffer (pH 6.5). $\tau_{1/2}$ (6 min) corresponds to the release of 1 mol of TNB/mol of DTNB (50% overall yield). For segment a_1, the value of A_{412} at $\tau_{1/2}$ was used as A_∞. In segment a_2, A_∞ was the absorbance corresponding to the release of 2 mol of TNB/mol of DTNB. (b) Pseudo-first-order rate plot of the reaction of 0.034 mM NTCB with 0.7 mM GSH in the same buffer as in (a).

measurements at 412 nm. Determination of the rates at 412 nm of the reaction of 0.05 mM NTCB with 0.5 mM GSH in 0.1 M phosphate buffers, yielded the values of 18.5 and 58.5 M^{-1} sec^{-1} for the second-order rate constants at pH 7.3 and 8.0, respectively.

The reaction of NTCB with GSH is considerably slower than that of DTNB. For example, whereas the half-life of the reaction of GSH with NTCB at pH 7.3 under the equimolar conditions shown for Figure 1 (lowest curve) was 6 min, the corresponding reaction with DTNB under identical conditions was completed within 30 sec. In order to obtain comparable second-order rate constants under pseudo-first-order conditions, the reaction with DTNB was slowed down to measurable rates by lowering the pH to 6.5 and using dilute solutions. When the reaction of 5.0 μM DTNB with tenfold excess of GSH in pH 6.5 pyridine–acetate buffer was followed at 412 nm, the rate was clearly biphasic. The expected 2 mol of TNB/mol of DTNB was released, but the rate dropped shortly after the release of the first mole, at t = 6.0 min, as is evident from Figure 4. The two linear parts of the pseudo-first-order plot a in Figure 4 show the two different rates. For segment a1 the absorbancy at 6.0 min (overall half-life) was taken as A_∞, and for segment a2 the absorbancy corresponding to the release of 2 mol of TNB was used. The two observed rates are those of the two separate reactions taking place under these conditions. In the first rapid step a mixed disulfide is formed (eq 3) which

$$\text{GSH} + \text{ArSSAr} \xrightarrow{k_3} \text{GSSAr} + \text{ArS}^- \qquad (3)$$

subsequently reacts at a slower rate with more GSH to form GSSG (eq 4). The deviations from linearity between t = 3 and 10 min are due to the fact that during this 7-min intermediary period both reactions were detected simultaneously.

$$\text{GSH} + \text{GSSAr} \xrightarrow{k_4} \text{GSSG} + \text{ArS}^- \qquad (4)$$

Figure 4a is thus a kinetic demonstration of the consecutivity of thiol oxidations by DTNB. The values calculated for the second-order rate constants k_3 and k_4 were 149 and 15.3 M^{-1} sec^{-1}, respectively. Curve b in Figure 4 is the pseudo-first-order plot of the rate of the reaction of GSH with NTCB in the same buffer, but at higher concentrations of reactants (0.034 mM NTCB and 0.7 mM GSH). The rate constant for this reaction was found to be 1.43 M^{-1} sec^{-1}. It might be noted that the complete linearity of plot b throughout the reaction does not imply anything about the occurrence or the rate of the possible subsequent reaction 5, since unlike the analogous re-

$$\text{GSH} + \text{GSCN} \longrightarrow \text{GSSG} + \text{CN}^- \qquad (5)$$

action 4 it would obviously be indistinguishable at 412 nm.

Thus the information obtained from Figure 4 is that GSH reacts with DTNB approximately 10 times faster than with its mixed disulfide, and approximately 100 times faster than with NTCB (at pH 6.5).

Product Analyses. The products obtained by NTCB modification of cysteine peptides were studied at two different concentration levels, namely, 10 and 0.1–1.0 mM, with different results.

The experiments with concentrated mixtures, originally intended for synthetic purposes, gave as a rule poor yields of 2-thiocyanoalanine derivatives. Thus when 10 mM GSH in 0.1 M Tris-acetate (pH 7.3) was mixed with the same final concentration of NTCB and allowed to incubate for 15 min, paper electrophoresis at pH 3.5 revealed the presence of three ninhydrin-positive compounds, none of which was the starting material GSH. Using qualitative and quantitative paper techniques described under Methods, the products were identified and quantitatively estimated as GSCN, GSSG, and GSTNB, in this order of increasing anionic mobility (which is in keeping with the respective order of their charge-to-mass ratios). The yields are shown in Table I (column a). In other experiments at the same concentration level, employing different salt buffers (*e.g.*, phosphate, pyridine-acetate), pH's (6.8–8.0), and molar ratios of reagent the results were essentially similar. Increasing the molar excess of NTCB to sixfold

TABLE I: Yields of Products Formed by the Reaction of GSH and NTCB under Various Conditions.[a]

Product	Conditions				
	a	b	c	d	e
GSCN	40–47	56–64	80–85	95–100	98–100
GSTNB	25–29	25–31	10–15		
GSSG	26–31	12–20	5		

[a] Yields are given in mol % and represent results of three or more experiments carried out under each set of conditions. Reaction mixtures were incubated for 15 min at room temperature in 0.1 M Tris-acetate buffers, prior to separation by paper electrophoresis at pH 3.5. Products were identified and quantitatively estimated as described under Methods. Compositions of reaction mixtures were as follows: (a) 10 mM GSH–10 mM NTCB (pH 7.3); (b) 10 mM GSH–60 mM NTCB (pH 8.0); (c) 1.0 mM GSH–1.3 mM NTCB or [^{14}C]NTCB (pH 8.0); (d) 0.5 mM GSH–0.65 mM [^{14}C]NTCB (pH 8.0); (e) 0.1 mM GSH–0.13 mM [^{14}C]NTCB (pH 8.0).

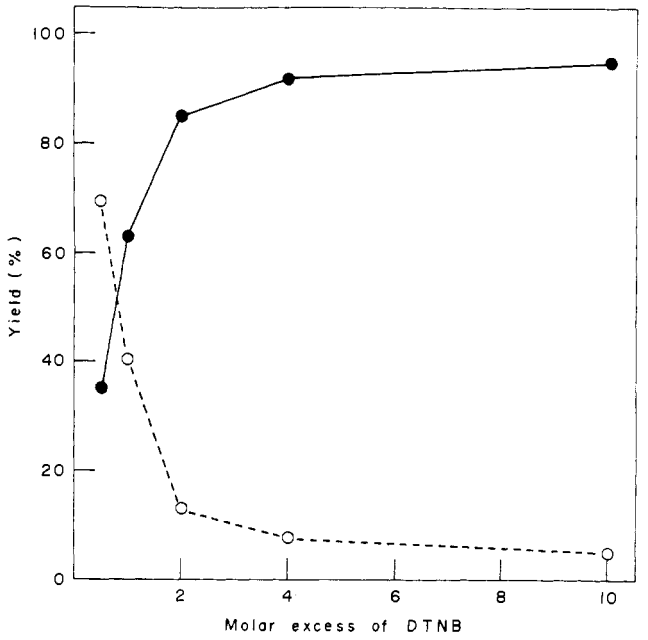


FIGURE 5: Yields of GSTNB and GSSG formed by the reaction of GSH with DTNB. 5.0 mM GSH in 0.1 M phosphate buffer (pH 8.0) was treated with DTNB in various molar proportions: (●—●) GSTNB; (○- - -○) GSSG. Products were separated by paper electrophoresis and quantitatively estimated as described under Methods.

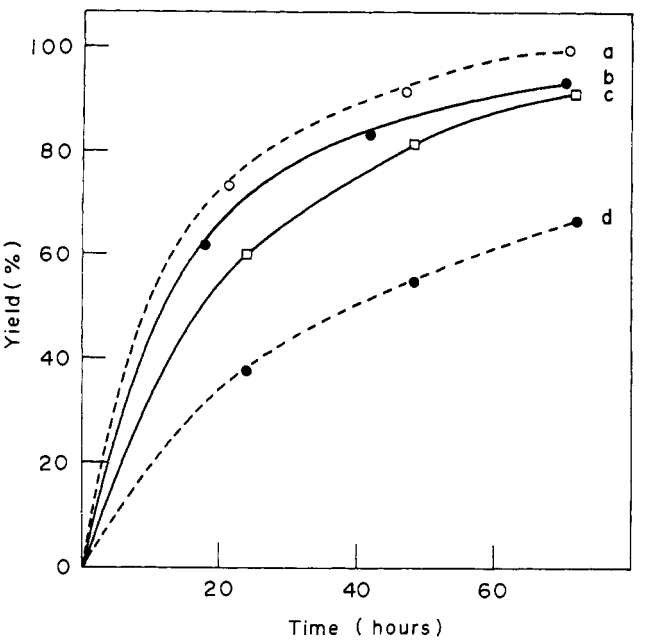


FIGURE 6: Yields of [^{14}C]ITC-Gly in the cleavages of ^{14}C-labeled β-thiocyanoalanine peptides. 0.1 mM solutions of peptides in 0.1 M Tris-acetate buffer (pH 8.0) were incubated at 37° (- - - - -) or 50° (—). (○) GSCN; (●) Z-GSCN; (□) Z-Phe-CySCN-Gly. [^{14}C]-ITC-Gly was separated by paper electrophoresis and determined as described under Methods.

increased the yields of GSCN slightly (column b). The yields of TNB in these experiments ranged from 30 to 50%, as measured spectrophotometrically with diluted aliquots.

The results changed markedly when dilute solutions were employed. When 0.10 mM GSH was treated with 0.13 mM [^{14}C]NTCB in Tris-acetate (pH 8.0), the reaction was completed within 15 min, releasing TNB quantitatively, as found by following A_{412}. [^{14}C]GSCN was separated from this mixture in 100% yield, based on its radioactivity. Similarily high yields (95–100%) were obtained when the concentrations of the reaction mixtures were increased up to 0.5 mM; 80–85% of either GSCN or [^{14}C]GSCN were isolated from 1.0 mM reaction mixtures by preparative paper electrophoreses (Table I, columns c–e).

The experiments with other cysteine peptides gave similar concentration-dependent results. Thus [^{14}C]Z-GSCN and [^{14}C]Z-Phe-CySCN-Gly were obtained in high yields (93–97%), only from dilute reaction mixtures (0.1–0.5 mM).

The purity of the ^{14}C-labeled β-thiocyanoalanine peptides obtained from these reactions was confirmed by their specific radioactivities, as related to their respective amino acid compositions. Under the conditions of total acid hydrolysis (6 N HCl, 100°, 22 hr), the β-thiocyanoalanine residue was found to be converted into a mixture of cystine, cysteine, and 2-iminothiazolidine-4-carboxylic acid (ITC), in varying proportions.

The products obtained from the reaction of GSH and DTNB at relatively high concentration (5 mM) were also analysed. As shown in Figure 5, mixtures of GSSG and GSTNB were always formed, but in this case the yields of the GSTNB increased sharply with increasing the molar excess of the reagent, as expected.

The dependence of the cyanylation yield on the concentration is examined in the Discussion section.

Cleavage of β-Thiocyanoalanine Peptides. When 1.0 mM GSCN solution in 0.1 M Tris-acetate buffer (pH 8.0) was incubated for 72 hr at 37° and the mixture subjected to pH 3.5 paper electrophoresis, spraying with diazotized sulfanilic acid revealed the presence of a product identical to ITC-Gly produced by cyanide treatment of GSSG under the conditions described by Catsimpoolas and Wood (1966). No ninhydrin-positive material was detected, including glutamic acid which would be expected to released along with ITC-Gly; however, pH 6.5 electrophoresis showed the presence of pyrrolidonecarboxylic acid. For further quantitative study of the cleavage reaction and its time course, [^{14}C]GSCN was incubated under similar conditions, and the formation of [^{14}C]-ITC-Gly was followed by periodic electrophoretic separations of aliquots of the reaction mixture. The results are shown in Figure 6 (curve a). At the end of the incubation the yield of [^{14}C]ITC-Gly was 100%, measured by its radioactivity. When [^{14}C]Z-GSCN was incubated under the same conditions, the rate and extent of the cleavage was lower than that of [^{14}C]-GSCN (curve d). The cleavage could be accelerated by increasing the temperature to 50°, resulting in a yield of 93% of [^{14}C]ITC-Gly after 70-hr incubation (curve b). Similar results were obtained when [^{14}C]Z-Phe-CySCN-Gly was incubated under the same conditions (curve c). At the end of the incubations a–c, the only radioactivity found was that of [^{14}C]ITC-Gly and no radioactivity corresponding to the positions of starting materials was detected. In contrast, in the separations performed at intermediate stages of the cleavage, a residual radioactivity was detected in the respective migration position of the starting material. This amount of radioactivity usually complemented that of [^{14}C]ITC-Gly, and together amounted to approximately full recovery of the initial radioactivity, within experimental error.

In order to test whether the β-thiocyanoalanine side chain in the residual intact peptides was present in the linear or the cyclized form, one of two duplicate aliquots from each reaction mixture was mixed with an equal volume of 0.14 M β-mercaptoethanol in 0.1 M phosphate buffer (pH 8.0), prior to the elec-

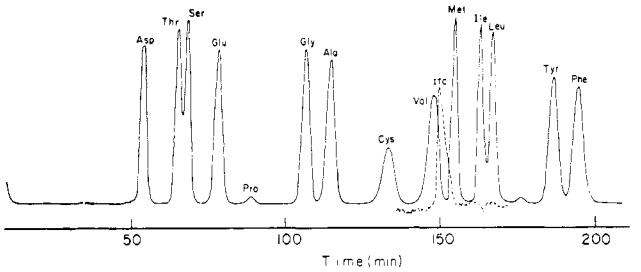


FIGURE 7: Amino acid analysis of amino acid calibration mixture containing [^{14}C]ITC. The analysis was performed using the 55-cm column of a Beckman amino acid analyzer Model 120B coupled to a recording Packard Tri-Carb flow analyzer Model 3022 as described in the text. A buffer system essentially identical to that described by Moore *et al.* (1958) was used, at a flow rate of 70 ml/hr. (—) A_{570}; (- - - -) radioactivity. The positions of the ninhydrin-positive amino acids were corrected for the 10-min delay caused by the passage of the effluent from the outlet of the column through the flow analyzer into the ninhydrin mixing manifold.

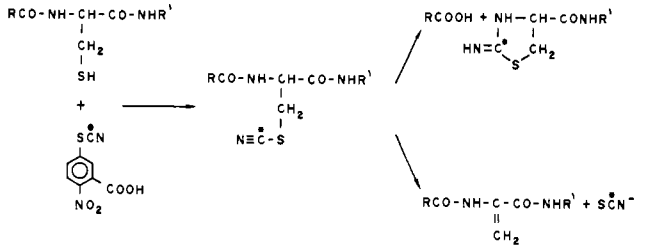


FIGURE 8: Cleavage and β-elimination in a cysteine residue modified by NTCB.

trophoresis. As a result of this treatment, the radioactivity corresponding to the starting material completely disappeared, whereas that of the [^{14}C]ITC-Gly remained unaffected. This showed that the β-thiocyanoalanine in the uncleaved starting material was present solely in its linear thiocyano form, and therefore lost its *S*-cyano group by the action of the excess mercaptan. The release of CN^- by excess thiol can only take place in the linear form of the β-thiocyanoalanine (Swan, 1958).

The cleavage was also tested under other conditions. Prolonged incubations of GSCN in various acidic buffers, including anhydrous trifluoroacetic acid, did not result in any observable cleavage. On the other hand, by incubating [^{14}C]-GSCN and [^{14}C]Z-GSCN (0.1 mM) at pH 10 (0.1 M Tris-acetate adjusted to pH 10 with NaOH) and 37° for 4 hr, [^{14}C]ITC-Gly was released in 85 and 73% yield, respectively, indicating that the cleavage is markedly accelerated by increase of pH. However, other experiments, described in the next section, showed that such conditions also resulted in β-elimination of thiocyanate from β-thiocyanoalanine peptides. Attempts to accelerate the cleavage at pH 8.0 by including an excess of the strongly nucleophilic hydroxylamine in the incubation mixtures, were unsuccessful and resulted in low yields of ITC-Gly. Thus GSCN, when incubated at 37° in 0.1 M Tris-acetate buffer (pH 8.0) containing 0.05 mM hydroxylammonium acetate, was partially converted into an unidentified ninhydrin-positive product which at pH 3.5 migrated to the anode about half as fast as the starting material. This compound could have been the addition product of hydroxylamine to the thiocyano group, since such addition would have resulted in a new positive charge on the peptide. Slow migrating radioactive products were also formed when *N*-carbobenzoxy-[^{14}C]-β-thiocyanoalanine peptides were treated under the same conditions.

The ^{14}C-labeled dipeptide ITC-Gly formed in the foregoing experiments was isolated by preparative paper electrophoresis, and its identity confirmed by its amino acid composition. Although ITC cannot be analyzed by the usual amino acid analysis owing to its very faint ninhydrin color, [^{14}C]ITC could be analyzed simultaneously with other amino acids present in acid hydrolysates, using an amino acid analyzer coupled to a radioactive flow analyzer equipped with a recorder. As shown in Figure 7, [^{14}C]ITC emerges from the 55-cm column of the amino acid analyzer between valine and methionine, and its analysis could be quantitated from the integrated peak area using known standards of [^{14}C]ITC for calibration. By this method, the one-to-one composition of [^{14}C]ITC-Gly was confirmed, after acid hydrolysis. The amount of glutamic acid found in the hydrolysate did not exceed the impurity level found in a control paper eluent. The recovery of [^{14}C]ITC under the conditions of acid hydrolysis was found to be 97%. This method of [^{14}C]ITC analysis could be useful in the characterization of peptides obtained by cleavage of ^{14}C-labeled proteins.

When [^{14}C]GSCN was incubated at pH 8 for 72 hr at 37° in the presence of an equimolar concentration of cystine, no detectable free [^{14}C]ITC was found among the products, and the formation of [^{14}C]ITC-Gly was quantitative was before. The same result was obtained when cystine was present during the cleavage of Z-GSCN at 50°. Since free β-thiocyanoalanine was previously shown to rapidly cyclize into ITC under these conditions (Aldrige, 1951; Degani *et al.*, 1970), the absence of free ITC in the products of these experiments showed that no cyano exchange had occurred between the β-thiocyanoalanyl residues and the cystine disulfide group. The results of these experiments support the suggestion that β-thiocyanoalanine peptide bonds could be selectively cleaved in the presence of cystine peptide bonds.

β-Elimination in β-Thiocyanoalanine Compounds. β-Thiocyanoalanine residues can undergo a β-elimination reaction to form thiocyanate and dehydroalanine residues (Figure 8). This reaction takes place under moderately alkaline conditions and has long been recognized as the primary cause for the formation of thiocyanate in cyanide-treated cystine-containing proteins, as well as the resulting formation of lanthionine by the re-addition of the released cysteine thiol to the dehydroalanine double bond (Cuthbertson and Phillips, 1945). Since both the β-elimination and the cyclization reactions in β-thiocyanoalanine residues are base catalyzed, the two reactions might be competitive under alkaline conditions, resulting in a low extent of peptide-bond cleavage. Experiments were therefore carried out with β-thiocyanoalanine compounds, in order to examine the extent of β-elimination in the pH range 8–10, in which cyclization and cleavage also take place. The results of these experiments are shown in Table II.

As can be seen, the compounds tested, which are listed in the Table in the order of their increasing observed readiness to liberate thiocyanate, showed considerable variability in their tendency to undergo β-elimination. Thus, whereas compound V liberated appreciable amounts of thiocyanate at as low a pH as pH 8, compound I (GSCN) released no detectable thiocyanate or [^{14}C]thiocyanate even at pH 10. Moreover, when [^{14}C]GSCN was treated with 0.01 N NaOH (pH 12) for 1 hr at room temperature, only trace amounts of [^{14}C]thiocyanate could be detected by paper electrophoresis. Most of the radioactivity was located in the cleavage product [^{14}C]ITC-Gly (78%), and in two unidentified radioactive products. Similar

TABLE II: Yields of Thiocyanate Eliminated from β-Thiocyanoalanine Peptides.[a]

Peptide	pH 8		pH 9		pH 10	
	4 hr	20 hr	4 hr	20 hr	4 hr	20 hr
Glu_{γ}-CySCN-Gly-OH (I)	0	0	0	0	0	0
Z-Glu_{γ}-CySCN-Gly-OH (II)	0	0	2	5	9	12
Z-Phe-CySCN-Gly-OH (III)	0	3	4	11	14	20
Z-CySCN-Tyr-NH_2 (IV)	2	5	13	17	29	35
Z-CySCN-Gly-OBz (V)	25	43	38	52	50	63

[a] Yields are given in mol %. Reaction mixtures were prepared as described under Methods and incubated at 37°. Thiocyanate was determined colorimetrically by the method of Goldstein (1950). When peptides were radioactively labeled, [^{14}C]thiocyanate was also determined by its radioactivity after a 20-min paper electrophoresis at pH 3.5.

behavior of GSCN was described by Swan (1959), who reported the finding of unexplainably low yields of thiocyanate at pH values above 12, along with unidentified products. It may be added that the only case where detectable amounts of thiocyanate were formed from GSCN in the present study, was when the compound was exposed on paper to the vapors of 30% ammonium hydroxide solution in a closed chamber for 1 hr. The apparent pH in this atmosphere, as shown by a wet pH-indicator paper, was about 13. This treatment, followed by the sensitive ferric reagent spray, formed the basis of the method for detecting β-thiocyanoalanine peptides on paper chromatograms, as described under Methods.

Discussion

Modification. The study has demonstrated the usefulness of NTCB as a modifying reagent for the rapid S-cyanylation of cysteine residues in peptides under mild conditions. The easily prepared [^{14}C]NTCB has proved to be a valuable variation of the reagent, since by introducing a radioactively labeled cyano group into the resulting β-thiocyanoalanine side chain it offers a sensitive tool for studying both the peptide bond cleavage and the β-elimination in β-thiocyanoalanine compounds.

The study has also revealed a remarkable dependence of the extent of cyanylation upon the concentration of the reactants. Whereas in dilute solutions of 0.1–0.5 mM, the extent of the modification was practically quantitative, increasing the concentrations of both thiol and NTCB lowered the degree of cyanylation. Thus in 10 mM solutions the yields seldom exceeded 60%. In the case of GSH, the by-products formed in concentrated mixtures were identified as GSTNB and GSSG (Table I).

The formation of GSTNB by treatment of GSH with NTCB cannot result from a direct reaction between both reactants, displacing CN^- instead of TNB, because the occurrence of this hypothetical side reaction cannot depend on the concentration. GSTNB must therefore be formed indirectly. A proposed sequence of reactions, leading to the formation of both GSTNB (*i.e.*, GSSAr) and GSSG, is shown in Figure 9. According to this scheme of consecutve competitive reactions, ArS^- formed by the primary reaction 1, displaces CN^- from still unreacted ArSCN to form ArSSAr (reaction 2). The aromatic disulfide then reacts with free GSH to form GSSAr

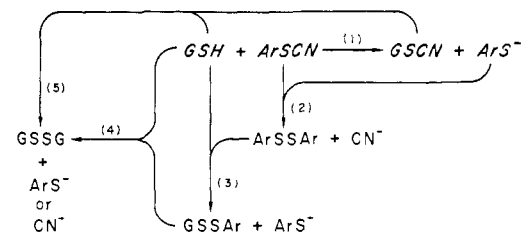


FIGURE 9: Reactions proposed to occur in concentrated mixtures of GSH and NTCB.

(reaction 3) which in turn reacts in reaction 4 with still unreacted GSH to form GSSG. The latter can also arise from GSCN and GSH through reaction 5. All the secondary reactions 2–5 were shown to exist independently. (For reactions 2–4 see Figures 3, 4, and 5; for reaction 5, see Aldridge, 1951.)

This system of reactions has some noteworthy properties. All nucleophiles can act as leaving groups and *vice versa*. The order of decreasing nucleophilicity is GSH > CN^- > ArS^-, which is also the order of increasingly good leaving groups. CN^- is thus both a moderate nucleophile and a moderate leaving group and its reactions (2 and 5) are therefore the only reversible reactions in the system. Kinetically, the rate constants of all reactions (including the reverse reactions and hence the equilibrium constants) will depend on the specific combination of a nucleophile and a leaving group, those reactions involving extreme combinations being the fastest ones. In addition, differences in electrophilicity contribute further kinetic effects. Thus, a sulfur substituent on a *p*-nitrophenyl ring is more electrophilic than the sulfur atom of the aliphatic glutathione side chain; k_3 is therefore larger than k_4 although both reactions 3 and 4 involve the same nucleophile (GSH) and the same leaving group (ArS^-). A similar difference in electrophilicity (or thiophilicity) between carbon and sulfur atom may account for the still lesser reactivity of ArSCN compared to either ArSSAr or GSSAr. Also, slight conjugation effects within the aromatic thiocyanate molecule (Campbell and Rogers, 1948; Bordwell and Boutan, 1956) would tend to somewhat stabilize it. On the other hand, the symmetry of ArSSAr may further contribute to the reactivity of this disulfide, which is the highest in the system.

In the pH range 7–8 the rate constants also depend on the pH. This applies to all the reactions except reaction 2, which is pH independent in this range (Figure 3). In contrast, the rate of its reverse reaction does depend on the pH, as implied by the pH dependence of K_{eq_2} (see Results). The difference is due to the fact that all the nucleophiles in the system react primarily in their anionic form, and their effective concentrations at any given pH will therefore depend on the pK_a's of their respective conjugate acids. The pK_a's of GSH and HCN are 8.56 (Friedman *et al.*, 1965) and 9.32 (Britton and Robinson, 1932), respectively, whereas that of ArSH is only about 5.1 (Ellman, 1958). At pH 7 and above ArSH is therefore essentially fully ionized, and hence in this region, the rate of reaction 2 does not depend on the pH.

Regarding the other reactions in the system, it can be assumed that rate constants involving the same ionizable nucleophile (*e.g.*, k_1, k_3, and k_4, all involving GSH) will be similarly affected by changes in pH. Therefore, on the basis of the kinetic data as summarized in Table III, it can be concluded that the following decreasing order in the magnitudes of the rate constants generally exists in the pH range studied: $k_3 > k_4 > k_1 > k_{-2} > k_2$. k_5 is not known but it is probably smaller than k_4 or k_1, judging by the difference in the leaving groups

TABLE III: Second-Order Rate Constants of Reactions Appearing in Figure 9 (M^{-1} min^{-1}, at 25°).[a]

pH	k_1	k_2	k_{-2}	k_3[c]	k_4[c]
8.0	3500	9.0	100	$>k_1$	$>k_1$
7.3	1100	9.0	33	$>k_1$	$>k_1$
6.5	77			8900	920

[a] Data summarized from Results. [b] Calculated from the experimental values of k_2 and K_{eq_2}. [c] At pH 7.3 and above, k_3 and k_4 were too large to be measured by common spectrophotometric techniques.

and by the conditions reported by Aldridge (1951) for reaction 5. Thus kinetically, the process occurring when equimolar amounts of GSH and ArSCN are mixed in concentrated mixtures can be described as follows. In an early stage of reaction 1, while ArSCN is still in excess of its product ArS^-, reaction 2 overcomes its unfavorable equilibrium constant (0.087 at pH 8.0) and produces some ArSSAr. Since k_3 is much larger than k_1 (by a factor of about 10^2), a relatively low concentration of ArSSAr is sufficient for reaction 3 to initiate a strong competition with reaction 1. ArSSAr is therefore a short-lived intermediate as long as GSH is still present. The rapid disappearance of ArSSAr continuously shifts the equilibrium of reaction 2 forward. Furthermore, all ArS^- used in reaction 2 is fully recovered in reaction 3 and is recycled, together with more ArS^- produced in reactions 4 and 1, back into reaction 2. This rapid chain of events, occurring concurrently with reaction 1 and triggered by the initial formation of ArS^-, is halted by the exhausting of the GSH supply, the entire process lasting less than 2 sec in 10 mM solutions at pH 8. Since GSH participates in more, and faster reactions than ArSCN, it will be consumed earlier than ArSCN, in spite of their initially equal concentrations. Thus the key factors which lower the yields of GSCN in concentrated reaction mixtures are the reversibility of reaction 2 and the large magnitude of k_3 (and to a lesser degree also k_4) compared to k_1. It should be noted that even if reaction 2 is avoided by adding the ArSCN very slowly to concentrated GSH, the product GSCN will be lost by reaction 5 (Aldridge, 1951).

Although the complete mathematical kinetic analysis of this complex system of consecutive competitive reversible reactions was not carried out, the critical effect of the concentration is apparent. Since all the secondary reactions are of the second order, their actual rates will largely depend upon the concentrations of both the reactants and the products of the primary reaction 1. In particular, the rate of reaction 2 which has the smallest rate constant and is the key step to reactions 3 and 4 will be greatly retarded by dilution of ArSCN and hence of ArS^-. It is therefore not surprising to find that upon sufficient dilution of both GSH and ArSCN, the contributions of all secondary reactions 2–5 diminish to the point of insignificance, and primary reaction 1 proceeds to completion (Table I).

From a practical point of view, the concentration range of 0.1–0.5 mM, in which the cyanylation proceeds smoothly and essentially quantitatively, is a convenient and commonly used concentration level in protein modifications. In experiments with sulfhydryl-containing proteins, carried out under these conditions, quantitative cyanylations were obtained, although in some cases, such as tobacco mosaic virus protein, denaturing agents were required to bring the cyanylation to completion. The present study indicates that caution must be exercised with regard to the following practical points. (1) In view of the greater reactivity of DTNB compared to NTCB, it is essential for obtaining quantitative cyanylations, that NTCB be free of any DTNB contaminant. The authors' recently improved method of NTCB synthesis has consistently afforded very pure peparations of the reagent, as assessed by chromatographic and electrophoretic procedures (Degani and Patchornik, 1971). (2) Undue excess of NTCB should be avoided even in dilute protein solutions to prevent the interference of the resulting consecutive reaction 2 to the spectrophotometric measurements of the rate and extent of the modification. (3) In cases of proteins requiring disulfide reduction by high concentrations of mercaptans, it is suggested that excess reducing agent be either separated or diluted prior to the modification, in order to avoid the possible formation of competitive DTNB upon addition of an overall excess of NTCB. If the constant presence of a mercaptan is required to maintain reducing conditions, 1,3-dithiols such as dithiothreitol would be preferable to monothiols, since the former were found to undergo rapid and quantitative intramolecular displacement reaction of the type 5, when treated with NTCB, and should therefore not interfere with the modification of the protein. It should be pointed out that under certain conditions similar cyclic displacements could also occur in proteins, in cases where cysteine residues are located in positions which sterically favor intramolecular reactions. An example is reduced bovine pancreatic ribonuclease, which was found to undergo intramolecular displacements of the types 4 and 5 when treated by DTNB and NTCB, respectively, in nondenaturing solvents (Y. Degani and A. Patchornik, unpublished data). Intramolecular displacement of type 4 was also reported for DTNB-treated lobster muscle glyceraldehyde-3-phosphate dehydrogenase (Wassarman and Major, 1969).

The foregoing discussion has pointed to implications resulting from the greater reactivity of DTNB relative to NTCB. This order of reactivity was found with all but one of the thiol compounds tested thus far, including proteins. The only exception found to date is papain, which reacts with NTCB faster than it does with DTNB (Y. Degani and A. Patchornik, unpublished data). Although the reason has not yet been established, the irregularity seems to be due to the fact that, unlike all other thiol compounds tested, the thiol of papain is part of an enzymic active site, and its reactivity would therefore be exceptionally sensitive to such factors as steric hindrance and electrostatic repulsion. The reversal in the reactivities of DTNB and NTCB might therefore be related to the differences in size and charge between the reagents. Should this prove to be the case, it would suggest that NTCB could be used in conjunction with DTNB for the detection and location of differentially accessible thiol groups in enzymes.

Unlike S-substituents formed by most sulfhydryl-specific reagents commonly in use, the CN group is both small and uncharged, and therefore less likely to induce conformational distortions. The importance of these structural features of the cyano group when used for probing the catalytic involvement of thiol groups in enzymes was also recently noted by Birchmeier *et al.* (1973). In several cases studied so far (Vanaman and Stark, 1970; Chung *et al.*, 1971; Birchmeier *et al.*, 1973; Y. Degani, F. M. Veronese, and E. L. Smith, in preparation), enzymic thiols which previously were considered to be essential or involved in catalysis were shown not to be so, as conversion to their *S*-cyano derivatives resulted in the restoration of previously lost activities. In contrast, papain—an enzyme in which the direct catalytic function of its thiol has been firmly established (Glazer and Smith, 1971)—is com-

pletely inactivated by S-cyanylation (Degani *et al.*, 1970). Inasmuch as S-cyanylation is apparently a more reliable probe for the essentiality of thiols in catalysis, than other known derivatizations, NTCB modification is not only a key step in a selective cleavage of proteins, but is also a most promising enzymological tool.

Cleavage. The study has confirmed that β-thiocyanoalanine peptides are cleaved at the *N*-acyl-β-thiocyanoalanine bond in high yields under mild conditions, employing 37–50° incubations at pH 8.0. The conditions used for cleavage were similar to those employed by Catsimpoolas and Wood (1966) for the cleavage of cystine peptides using excess cyanide. Whereas in the earlier studies β-thiocyanoalanine residues were cleaved *in situ* in the presence of excess cyanide, in the present case they could be used as starting materials and were thus proven to be the intermediates in the cyanide-promoted cleavage of cystine peptides. The presence of cyanide was shown to be unnecessary for the cleavage; the fission of the thiocyano group known to take place in other thiocyanates under moderately alkaline conditions (Bacon, 1961) does not occur under the mild conditions which induce the cyclization of β-thiocyanoalanine residues, and therefore no cyanide is required to repress it. Furthermore, whereas in the cyanide method cystine residues were only partially modified, and their peptide bonds randomly cleaved, the NTCB method makes it possible to cleave peptide chains that are fully and specifically S-cyanylated at cysteine residues.

The cleavage products of GSCN at pH 8 and 37° contained besides ITC-Gly only pyrrolidonecarboxylic acid and no glutamic acid. Wood and Catsimpoolas (1963) reported that glutamic acid was formed in 96% yield in cleavage of GSSG by cyanide treatment at pH 7, at the same temperature. Later, the formation of an unspecified mixture of glutamic acid and pyrrolidonecarboxylic acid was reported for the similar cleavage at pH 7.4 (Catsimpoolas and Wood, 1966). The formation of pyrrolidonecarboxylic acid indicates that in GSCN the α-amino group of the glutamic acid residue participates in the peptide-bond cleavage, by a 1–5 cyclic nucleophilic attack of the labilized γ-glutamyl peptide carbonyl function. This neighboring group effect, which would be expected to be pH dependent if the reactive species were the un-ionized amino group, appears to facilitate the cleavage of the peptide bond. This is supported by the finding that the cleavage of the amino-blocked derivative of the peptide, Z-GSCN, in which no such neighboring group effect is possible, was considerably slower than that of GSCN, and a higher temperature was required (50°) to obtain high yields of cleavage (Figure 6). The fact that Z-Phe-CySCN-Gly behaved similarly to Z-GSCN is in agreement with this conclusion.

Although the detailed mechanism of the cleavage reaction was not investigated in this study, some of the findings provided information on the nature of the reaction. The cleavage is base catalyzed. Increasing the pH from 8 to 10 markedly accelerated the cleavage of both GSCN and Z-GSCN. On the other hand, although incubations in acidic buffers, including the fairly strongly acidic conditions of anhydrous trifluoroacetic acid, did not induce cyclization or cleavage, the conditions of total acid hydrolysis of peptides (6 N, HCl, 100°, 22 hr) resulted in the partial conversion of the β-thiocyanoalanine residue into ITC, indicating that under very strongly acidic conditions the cyclization can be proton catalyzed. Thiocyanates are normally hydrolyzed by strong acids into the corresponding thiocarbamates (Bacon, 1961). The potential hydrolysis products of β-thiocyanoalanine residues are therefore *S*-carbamylcysteine residues, and these were previously shown to be converted into cysteine and cystine under acid hydrolysis conditions (Stark *et al.*, 1960; Stark, 1964). The presence of ITC together with cysteine and cystine in acid hydrolysates of β-thiocyanoalanine peptides suggests, therefore, that once the thiocyano group becomes protonated, both cyclization and hydrolysis take place. In this connection it should be pointed out that in view of the partial conversion of β-thiocyanoalanine residues into ITC under acid hydrolysis conditions, the content of ITC in the hydrolysates of non-separated mixtures of the cleavage products cannot be used as a measure for the quantitative estimation of the extent of the cleavage, as was previously suggested (Maclaren, 1968).

Separations and ^{14}C tests performed during the incubations at various stages of the cleavage process showed that the β-thiocyanoalanine side chain in the residual intact peptide was invariably present entirely in its linear, noncyclic form, since only from this form could [^{14}C]cyanide be displaced by the treatment of excess mercaptan. This suggests that the peptide-bond cleavage must occur either concerted with or immediately subsequent to the cyclization.

The presence of cystine during the cleavage of β-thiocyanoalanine peptides did not result in the formation of any free ITC, meaning that no cyano exchange took place between the two types of sulfur compounds. This supports the earlier suggestion that cyanylation with NTCB provides a tool for the selective cleavage of proteins at the N-peptide bonds of cysteinyl residues, in the presence of cystinyl residues. This option might be useful in cases of native proteins containing both types of residues, or in partially reduced proteins. In this respect the use of NTCB for the selective cyanylation of cysteine residues in proteins has a distinct advantage over the alternative indirect method involving treatment with excess cyanide of an initially formed cysteine–TNB mixed disulfide (Vanaman and Stark, 1970; Degani *et al.*, 1970). Whereas NTCB is an electrophilic reagent and hence inert toward disulfides, cyanide is nucleophilic and therefore attacks disulfide bonds. Although cyanide reacts with TNB mixed disulfides considerably faster than with cystine disulfides, it is possible that in proteins containing both types of disulfides, the conditions required to bring the fission of the mixed disulfides to completion, might also lead to some degree of nonspecific scission of cystine disulfide groups, resulting, to some extent, in nonspecific cleavage of peptide bonds. That this risk exists can be seen by comparing, for example, the conditions required to completely split the cysteine–TNB mixed disulfide in isocitrate dehydrogenase, as described by Chung *et al.* (1971) (50 mM KCN, 25 min, pH 7.2), with those reported by Klein and Kirsch (1969) to have caused the nonspecific incorporation of 0.1 mol of CN^- into the cystine disulfide bonds of 1 mol of papain (1 mM KCN, 4 hr, pH 6.8). The use of NTCB, besides being a simpler, single-step method, also eliminates that risk completely.

Preliminary experiments with ^{14}C-labeled S-cyanylated proteins have indicated that the presence of denaturing agents was required to bring about extensive cleavage. This effect of denaturing agents was also reported by Vanaman and Stark (1970). The effect may not be limited to proteins, in view of the observation made by Catsimpoolas and Wood (1966), that 8 M urea markedly accelerated the cyanide-promoted cleavage of GSSG.

A previous approach to the modification of cysteine residues, employing aromatic chloroformates, also resulted in cleavage at the N-peptide bond by cyclization of the modified side chain (Degani *et al.*, 1966). In this method cyclization took place by displacement of a leaving group to form a

2-ketothiazolidine ring, whereas in the present case cyclization proceeds by way of addition to the cyano triple bond, to form the 2-imino analog. The present method is superior to the former both in the selectivity of the modification and in the extent of the cleavage. First, NTCB is by far, a milder reagent than any of the chloroformates tried, and unlike the latter, has an absolute specificity for sulfhydryl groups. Secondly, unlike the *S*-aryloxycarbonyl derivatives formed in the previous modification, the β-thiocyano group is resistant to hydrolysis under the moderately alkaline conditions required for cyclization. In the absence of interference by hydrolysis, the cyclization of β-thiocyanoalanine residues should therefore proceed to completion, providing that β-elimination of thiocyanate does not occur.

β-Elimination. The base-catalyzed β-elimination of thiocyanate from β-thiocyanoalanine residues is competitive with the base-catalyzed cyclization and cleavage, and should therefore be avoided to ensure maximum extent of cleavage. The β-thiocyanoalanine compounds examined in this study showed marked variations in their tendency to undergo β-elimination reaction at the pH range 8–10 (Table II). Similar variations in the extent of thiocyanate formation can also be found in the reported studies on cyanide-treated cystine compounds including proteins. Thus, whereas the treatment of ribonuclease with excess cyanide at pH 8 for 48 hr at 37° resulted in no formation of thiocyanate (Catsimpoolas and Wood, 1966), bovine serum albumin released in a shorter incubation at the same pH, 4–5 mol of thiocyanate/mol of protein, compared to 18 mol at pH 12 and none at pH 7 (Catsimpoolas and Wood, 1964).

Although the β-elimination of thiocyanate from β-thiocyanoalanine residue is a competing reaction, the variations found among different β-thiocyanoalanine compounds closely resemble those found in another type of S-substituted cysteine derivatives, namely, S-2,4-dinitrophenylcysteine compounds, whose base-catalyzed β-elimination reaction is not subject to competition. In their study with model *S*-dinitrophenylcysteine compounds, Sokolovsky *et al.* (1964a) pointed to the effect of structural factors on the rates of the elimination of dinitrothiophenolate ion. Negatively charged carboxylate groups located in the vicinity of the C-α hydrogen of the modified cysteine residue, where the attack of the catalyzing hydroxyl ion is supposed to take place, retarded the rate of the elimination. On the other hand, the presence of an esterified amino acid adjacent to the cysteine residue greatly enhanced the elimination rate, presumably because of the electron-withdrawing effect of the ester group. The results shown in Table II indicate that the rate (and in the present case of competing β-elimination, also the extent) of release of thiocyanate from β-thiocyanoalanine derivatives, is dominated by similar structural factors. Whereas the negatively charged peptides I–III showed little or no tendency to undergo β-elimination, the ester V was much more reactive than the other neutral peptide IV. The enhancing effect of an ester group located at even closer proximity to the β-thiocyanoalanine side chain is evident from the observation made by Wood and Catsimpoolas (1963) who reported that cyanide-treated *N,N'*-diacetylcystine ethyl ester released 20% of thiocyanate at pH 7.

The outstanding inability of GSCN to undergo competing β-elimination at pH 8–12 is paralleled by the negative sluggishness of the uncompeting β-elimination of its *S*-dinitrophenyl analog, as the following example shows. Whereas the half-life of the reaction of *S*-dinitrophenylglutathione in 0.1 N NaOH was about 30 min (Sokolovsky *et al.*, 1964a), the same period of time was sufficient, in the case of *S*-dinitrophenylated ribonuclease, to completely eliminate all the dinitrothiophenolate groups from the octasubstituted protein molecule, under identical conditions (Sokolovsky and Patchornik, 1964). It therefore appears that contrary to a previous suggestion (Catsimpoolas and Wood, 1966), glutathione is not a good model for studying the present cleavage reaction at pH above neutrality (as opposed to its usefulness as a model for the modification step). In addition to the atypical role played by its α-amino group in facilitating the cleavage of the γ-glutamyl peptide bond, the binegatively charged molecule is also unusually reluctant to release its S substituents in a base-catalyzed β-elimination reaction which in this case would otherwise compete with the cleavage.

While both cyclization and β-elimination in the β-thiocyanoalanine side chain are based catalyzed, their relative rates differ considerably with pH. Under moderately alkaline conditions cyclization seems to be largely favored over elimination, wheras at higher pH's the relative rate of the elimination increases. From the results of the present study, as well as from previous studies on the cyanide-promoted cleavage of peptides and proteins (Catsimpoolas and Wood, 1966), the conclusion can be drawn that in general, the optimal pH for achieving efficient cleavage lies in the neighborhood of pH 8. The precise optimal pH value may vary somewhat from one protein to the other, depending on their respective structural properties. Since the use of [^{14}C]NTCB offers a convenient way to introduce a radioactive label into the β-thiocyanoalanine side chains of modified proteins, it should be possible, in individual cases, to experimentally find the exact optimal pH for cleavage, by preliminary incubations at the pH range 8–9 of minute quantities of the ^{14}C-labeled protein to be cleaved, and testing for the presence of [^{14}C]thiocyanate in the products.

Acknowledgment

The authors are grateful to Ms. Talia Miron for excellent technical assistance.

Added in Proof

After this paper was submitted for publication, Dr. George R. Stark kindly made available to us a manuscript by Jacobson *et al.* (1973) reporting the cleavage in high yield of proteins at cysteine residues by use of NTCB.

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Proton Magnetic Double-Resonance Study of Angiotensin II (Asn^1Val^5) in Aqueous Solution Employing Correlation Spectroscopy. Assignment of Peptide NH Resonances and Transfer of Saturation from Water†

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ABSTRACT: Complete assignment of the peptide NH resonances of angiotensin II (Asn^1Val^5) (AII′) in aqueous solution has been accomplished by double irradiation of α-CH absorptions. The Karplus equation was employed to calculate the dihedral angles ϕ of specific HNCH fragments from corresponding peptide NH–α-CH coupling constants. These data exclude the α helix, conventional β turn, γ turn, random coil, and the structure proposed by Weinkam and Jorgensen (*J. Amer. Chem. Soc. 93*, 7038 (1972)) as the preferred solution conformation of AII′ in water. Although the data are consistent with an order–disorder equilibrium, additional information is required for a definitive conformational analysis. The data suggest that the solution conformation of AII′ differs from its conformation at the receptor site. Transfer of saturation from the H_2O hydrogens indicates that the Arg^2 peptide NH hydrogen exchanges with the solvent at a significantly more rapid rate than do the other peptide NH hydrogens.

Angiotensin II (Asn^1Val^5) (AII′),[1] an octapeptide hormone with amino acid sequence $Asn^1Arg^2Val^3Tyr^4Val^5$-$His^6Pro^7Phe^8$, has a broad range of biological activity (Peach, 1972; Page and McCubbin, 1968; Fisher, 1971; Needleman *et al.*, 1972). Conformational analysis of AII′ by proton magnetic resonance (pmr) spectroscopy requires that resonances be identified with specific hydrogens of this peptide. An extensive pmr study of AII′ (Glickson *et al.*, 1972a, 1973) included the assignment of all the CH resonances observed in the spectrum of AII′ in D_2O solution.

Of particular significance to conformational studies are the NH resonances observed in spectra of AII′ in H_2O solution. The cis and trans Asn primary amide NH resonances and the four proton Arg guanidino NH peak were assigned by straightforward comparison with other compounds (Glickson *et al.*, 1972a, 1973). However, the intense absorption of the solvent

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[1] Abbreviations used are: AII′, angiotensin II (Asn^1Val^5); pmr, proton magnetic resonance.