Specific Cleavage of Cystine Peptides by Cyanide*

N. CATSIMPOOAS AND JOHN L. WOOD
From the Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee 38103

SUMMARY

The mechanism of cyanide cleavage of peptide bonds involving the cystine amino group has been shown to involve scission of the disulfide bond to yield a sulfhydryl and a thioacyano group. In pH ranges below 8, the latter cyclizes to produce an acyliminothiazolidine moiety on the peptide chain. This product is unstable and hydrolyzes spontaneously to effect cleavage of the peptide bond on the cystine amino group. Increasing the pH favors disulfide scission but decreases the rate of ring closure. Above pH 10, thioacyanate ion is eliminated from the primary reaction products. Since the disulfide scission is unsymmetrical, reoxidation of the newly formed sulfhydryl groups is necessary to convert all cysteine residues to iminothiazolidine rings at the NH2-terminal ends of peptide chains.

The cleavage of the peptide bond at the cystine amino group as a result of scission of the disulfide group by cyanide has been reported previously (1). The mechanism of the reaction has now been investigated further with model compounds. These studies indicate that the initial scission of the disulfide ring by cyanide yields a cysteinyl and a thioacyanoloalanine moiety (2). In high pH ranges, the latter eliminates thiocyanate ion to form a dehydroalanine residue; in the pH range near 7, ring closure to an acyliminothiazolidine formyl group on the amino acid that had been on the amino side of the cystine yields cysteinylglycine, and by reaction of cystinyldiglycine with cyanide. The compound was crystallized from 95% ethanol with a melting point of 228°. Commercial nitrogen was used.

Analytical Procedures—Thiocyanate ion was determined by the calorimetric method of Epstein (14). The latter method was applied to the reaction mixtures of both oxidized glutathione and N,N′-diacetylcystine with cyanide. Iminothiazolidine-4-carboxylic acid was prepared by the procedure of Schöberl and Hamm (3), and was isolated in crystalline form from 70% ethanol (4). The procedure of von Behringer and Zillikens (8) was followed for the preparation of 3-acetyl-2-iminothiazolidine-4-carboxylic acid. The compound was recrystallized from 95% ethanol with a melting point of 224–226°. Analytical values for carbon, hydrogen, and nitrogen agreed with the theoretical values. The infrared spectrum of the compound (KBr pellet) had a band at 1650 cm⁻¹ which was assigned to the tertiary amine bond and a band at 3400 cm⁻¹ representing the imino group. The 707 cm⁻¹ band characteristic of the iminothiazolidine ring was also present. The ultraviolet absorption spectrum showed a peak at 263 μ. The compound did not couple with diazotized sulfanilic acid-sodium carbonate reagent. On hydrolysis, it yielded iminothiazolidinecarboxylic acid. Iminothiazolidinecarboxylic acid was found to be unchanged after heating in a sealed tube with constant boiling HCl at 105° for 16 hours. The acetyl compound could be located on paper chromatograms with the potassium chromate-silver nitrate reagent of Knight and Young (6). Rf values obtained with the solvent systems n-butyl alcohol-acetic acid-water (60:15:25, v/v) or 0.5% urea in 77% ethanol were 0.82 and 0.75, respectively. Whatman No. 1 filter paper and descending chromatography were used.

N,N′-Diaceetylcytystine was prepared as described by Greenstein and Winitz (7). Its ethyl ester was obtained by the method of Hollander and du Vigneaud (8). The 2-iminothiazolidine-4-formylglycine was prepared by the reaction of cyanogen bromide with cysteinylglycine, and by reaction of cysteinylglycine with cystine. The compound was crystallized from 95% ethanol and had a melting point of 228°. Commercial nitrogen was purified according to the method of Fieser (9). Dinitrophenyl derivatives of amino acids were prepared by the method described by Smith (10).

Analytical Procedures—Thiocyanate ion was determined by the calorimetric method of Goldstein (11). The methods of Ellman (12) and Grunert and Phillips (13) were used for the determination of sulfhydryl groups. Cyanide ion was determined by the calorimetric method of Epstein (14).

Quantitative determination of 2-iminothiazolidine-4-carboxylic acid was accomplished by the diazotized sulfanilic acid method (15), and by a modification of the spectrophotometric method of Gawron and Fernando (16). The latter method was applied to the reaction mixtures of both oxidized glutathione and N,N′-diacetylcysteine with cyanide. Iminothiazolidine moieties were calculated from the absorption of the solution at 236 μ. Absorption readings were converted to iminothiazolidine molarity.

EXPERIMENTAL PROCEDURE

Materials—Reduced glutathione was obtained from Mann. It was oxidized in 0.2 M phosphate buffer, pH 7, by oxygen in the presence of a trace of copper ion. Synthetic oxytocin (Syntocinon) was supplied by Dr. R. T. Birchers of Sandoz. Crystalline ribonuclease was obtained from Worthington. 2-(3-Amino-1-carboxypropyl)pyridine was prepared by the method of Gawron and Fernando (16). The latter method was applied to the reaction mixtures of both oxidized glutathione and N,N′-diacetylcysteine with cyanide. Iminothiazolidine moieties were calculated from the absorption of the solution at 236 μ. Absorption readings were converted to iminothiazolidine molarity.

* This work was conducted under Contract AT-(40-1)-1637 with the Atomic Energy Commission.
by means of the equation

\[ \text{MTT} = \frac{A_t - (A_{GSH} + A_{GSSG} + A_{CN})}{E_{IT}} \]

where IT represents iminothiazolidine moieties. Molar extinc-
tion coefficients are given in Table I. The absorbance con-
tributed by reduced glutathione, \( A_{GSH} \), at time \( t \) was calculated
from its concentration determined by the sulfhydryl value of
the solution. The absorbance contributed by cyanide was
virtually constant. Subsequently, the absorbance of the oxi-
dized compound, \( A_{GSSG} \), at the time \( t \) was calculated by differ-
ence.

Cyanide ion was removed from the reaction mixtures before
analysis by acidification to pH 5 and aeration with nitrogen
until the test for cyanide was negative.

\textbf{Methods—} Incubations with cyanide were carried out in a test
tube stoppered with a perforable stopper. The reaction was
dissolved in deoxygenated 0.2 M phosphate buffer of predeter-
mined pH, and solid sodium cyanide was added. The container
was flushed with nitrogen, with care being taken to avoid aera-
tion of hydrogen cyanide. The mixture was incubated in a
constant temperature water bath. Aliquots were removed with a
hypodermic needle for analysis and pH determinations.

Reaction mixtures were subjected to descending paper chroma-
tography with Whatman No. 1 filter paper. Amino acids and
peptides were visualized with 0.2% ninhydrin in acetone and
with 0.5% isatin in acetone. Iminothiazolidine moieties did
not react with ninhydrin but could be detected by spraying the
paper with diazotized sulfanilic acid-10% sodium carbonate
solution (2:1). If the concentration of the compound was
insufficient to produce a red-purple spot with warming, it could
be visualized by inspection under ultraviolet light. Pyrrolidone-
carboxylic acid was detected with the xylose-aniline reagent (17).

\textbf{RESULTS}

\textbf{Reaction of Oxidized Glutathione with Cyanide}

\textbf{Effect of pH—} Fig. 1 shows the rate of formation of sulfhydryl
groups resulting from the reduction of the disulfide groups of
GSSG with cyanide at 37° and four pH values: 6.3, 7.0, 7.4, and
8.0. Sulphydryl group values represent GSH. The reaction
was complete at pH 8 in 6 hours, but at pH 6.3 it reached a
maximum with only 70% of the possible sulphydryl groups formed
in 30 hours. Further incubation did not increase this value.
Fig. 2 shows the rate of formation of free iminothiazolidine
rings and hence peptide bond cleavage. Only at pH 8 was
peptide bond cleavage complete in 25 hours. In 25 hours at
pH 7.4, 7.0, and 6.3, there was 0.55, 0.33, and 0.1 mole of
iminothiazolidine formed, respectively, per mole of GSH. At
pH 7.4, the cleavage was complete in 72 hours. At pH 7.0,
only 67% of the iminothiazolidine was free in 72 hours.

Incubation mixtures were found to contain no thioceyanate ion,
and increasing the pH to 10 followed by incubation for 24 hours
released no detectable thioceyanate ion.

The 72-hour reaction mixtures were investigated by means of
paper chromatography. GSH, glutamic acid, and 2-imino-
thiazolidine-4-formylglycine were found. Pyrrolidonecarboxylic
acid was also detected. Dinitrophenyl derivatives were prepared
and subjected to two-dimensional chromatography. Develop-
ment in the first direction was performed with 0.75 M phosphate
buffer, pH 6, and in the second, with toluene-pyridine-ammonia
(5:1.5:0.5). A pattern was obtained which was identical with
one prepared from a mixture of the dinitrophenyl derivatives
of GSH, GSSG (if the reaction was incomplete), glutamic acid,
and iminothiazolidineformylglycine. The spots were eluted
with water for quantitative estimation in the spectrophotometer
at 360 mμ. It was found that quantitative yields of glutamic acid
and iminothiazolidineformylglycine had been produced.

\textbf{Effect of Temperature—} The reaction GSSG with cyanide at
pH 8 was carried out at 55° as well as 37°. Fig. 3 shows the
temperature dependence of the rates of disulfide scission and
peptide cleavage. The maximum sulphydryl group value, 92% of
the theoretical amount, was reached in 20 min at 55°, but
required 6 hours at 37°. On the other hand, iminothiazolidine
formation, i.e. peptide cleavage, required 200 min at 55° and
24 hours at 37°.

\textbf{Effect of Cyanide Concentration—} Fig. 4 shows that, within a
250-min period, scission of the disulfide group was complete only
at 10-fold excess of cyanide ion, while the cleavage of the peptide
bond to produce iminothiazolidine was complete only at 100-fold
excess of cyanide when the reaction was carried out at 55°, pH
8, for 250 min. The cleavage of the peptide link was always
slower than scission of the disulfide.
Effect of Cyanide on Cystine Peptides

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Effect of pH on the formation of iminothiazolidine by the action of cyanide on GSSG. Data are from the same system as recorded in Fig. 1. • represents values obtained at pH 6.3; ○, at 7.0; □, at 7.4; △, at 8.0. The iminothiazolidine values, IT, were obtained by the spectrophotometric technique described under "Methods." Values at pH 8.0 were also obtained by colorimetric analysis.

Effect of Buffer Concentration and 8 M Urea—In all of the experiments described above, the concentration of phosphate buffer was 0.2 M. When the incubation of GSSG, 25 × 10⁻⁴ M, and sodium cyanide, 25 × 10⁻⁴ M, was carried out at pH 8.0 and 55° in 0.02 M phosphate buffer, an acceleration of the rate of disulfide scission was noted, but the time of complete reaction was the same (4 hours) as obtained with 100-fold excess of cyanide. The rate of peptide cleavage was faster in the more dilute buffer. After 4 hours, the amount of iminothiazolidine released was 0.6 mole per mole of initial GSSG in 0.02 M phosphate buffer and was 0.55 mole per mole in 0.2 M buffer. Disulfide scission was little affected by making the incubation mixture 8 M in urea. However, the rate of peptide cleavage was markedly affected. Fig. 5 shows iminothiazolidine formation to be complete in 20 hours in the presence of 8 M urea but only 85% complete when the urea was omitted.

Reaction of N, N'-Diacetylcysteine with Cyanide—N, N'-Diacetylcysteine was incubated with 10-fold and 100-fold excess of cyanide in 0.2 M phosphate buffer, pH 7, at 55°. Sulphhydryl and iminothiazolidine formation was followed by the colorimetric and spectrophotometric procedures described under "Methods." Fig. 6 shows the acetyl cleavage to be complete in 4 hours when 100-fold excess of cyanide was used but much slower when the concentration of cyanide was decreased by a factor of 10.

No thiocyanate ion was detected in the reaction mixture, even after removal of cyanide and incubation at pH 10. N-Acetylcysteine and 2-iminothiazolidine-4-carboxylic acid were identified in the reaction mixture by paper chromatography.

In an earlier experiment, N, N'-diacetylcysteine ethyl ester (20 μmoles) was incubated with sodium cyanide (1 mmole) in 0.2 M phosphate buffer, pH 7, at 37° for 24 hours. The solution was kept in a test tube under nitrogen, and the tube was opened at intervals to remove aliquots. A maximum sulphhdydryl value of 0.48 mole of -SH per mole of disulfide was reached in 120 min. Thereafter, the sulphhdydryl content diminished, probably because of oxidation. 2-Iminothiazolidine-4-carboxylic acid was identified in the reaction mixture by paper chromatography. At the end of the incubation period, 0.2 mole of thiocyanate ion per mole of disulfide was found. When an aliquot of the reaction mixture was analyzed, the concentration of 2-iminothiazolidine-4-carboxylic acid was 0.55 mole per mole of disulfide.
mixture was incubated at pH 10, no additional thiocyanate was produced.

Decayation of 3-Acetyl-3-iminothiazolidine-4-carboxylic Acid—
3-Acetyl-2-iminothiazolidine-4-carboxylic acid (25 μmoles) was
incubated with 0.2 M phosphate buffer, pH 7, at 37°. The abso-
sorption of the acetyl compound at 263 μm was recorded at
various time intervals. Aliquots were removed from the incu-
bation mixture for determination of the free iminothiazolidine ring
by reaction with diazotized sulfanilic acid. The acetylated
iminothiazolidine ring does not react with this reagent. Fig. 7
shows that the decrease in absorption at 263 μm, which is char-
acteristic of the acetylated compound, was proportional to the
rate of formation of the free iminothiazolidine ring. In 44 hours,
0.93 mole of iminothiazolidine was produced per mole of acetyl
derivative. Paper chromatography of the reaction mixture
confirmed the presence of 2-iminothiazolidine-4-carboxylic acid.
When the acetylminothiazolidine-4-carboxylic acid was incu-
bated at pH 7 and 37° with a 100-fold excess of cyanide, no
acceleration in the rate of deacylation was observed.

Reaction of Oxytocin with Cyanide—Oxytocin (0.24 pmole)
was dissolved in 10 ml of 0.2 M phosphate buffer, pH 7, and 192
μmoles of sodium cyanide were added. The mixture was incu-
bated under nitrogen at 55° for 72 hours. The course of the
reaction was followed spectrophotometrically at 235 μm. In-
creases in the absorbance in this region are contributed both by
the formation of sulfhydryl groups and by the release of imino-
thiazolidine groups. Maximum absorption was reached in 48
hours. After 72 hours, the excess cyanide was removed by aera-
tion at pH 5. The sulfhydryl content was determined by the
method of Ellman to be 0.96 mole of —SH per mole of oxytocin.
Chromatographic separation with n-butyl alcohol-pyridine-
water followed by ninhydrin treatment revealed the presence
of two peptides, Rf 0.10 and 0.42. Electrophoresis on S and S
No. 598 filter paper with 0.025 M Veronal buffer, pH 8.6, as the
supporting medium was carried out at a current of 5 ma (12
volts per cm) for 3 hours. When the paper was dipped in 0.5%
ninhydrin in methanol, two distinctive bands appeared 4 cm and
6 cm from the origin. No unchanged oxytocin was detected.
The peptides were converted to dinitrophenyl derivatives. Pa-
per chromatography in benzene-glacial acetic acid-concentrated
HCl-pentane (5:5:5:1) separated two peptides with Rf values
of 0.42 and 0.92. With 0.75 M phosphate buffer, pH 6, as the
developing medium, the Rf values were 0.90 and 0.56. Larger
quantities of the dinitrophenyl peptides were separated by gel
filtration on a Sephadex G-25 column. One dinitrophenyl pep-
tide, A, was eluted with 0.2 N acetic acid, and the other, B, by
0.2 N sodium hydroxide. The dinitrophenyl peptides were

![Graph](http://www.jbc.org/)
hydrolyzed with constant boiling HCl in a sealed tube heated
and hence was derived from the amino terminus. Similar treat-
ment of the original peptides before dinitrophenylation confirmed
the asparaginyl residue at position 6 with the half-cystinyl residue
carboxyl terminus of oxytocin by cleavage of the bond binding
thiazolidine-4-carboxylic acid, and was thus derived from the
leucine, glycine, and the dinitrophenyl derivative of 2-imino-
dimension paper chromatography in butanol-acetic acid-water
at 105° for 21 hours. The hydrolysates were subjected to two-
followed by ethanol-ammonia. Peptide A contained proline,
arginine, leucine, lysine, cysteine, and the dinitrophenyl derivative of
arginine was dissolved in 10 ml of oxygen-free water, and 1 mmole
of sodium cyanide was added. The pH was immediately ad-
justed to 8.0 with 0.2
mixture was acidified to pH 5 with acetic acid, and the cyanide
was removed by aeration with nitrogen. The mixture was then
lyophilized, and the residue was redissolved in a minimal amount
of water. The sulfhydryl content at the end of 21 hours of incubation
was 0.1 µmole; none was found after 48 hours of incubation.
Thiocyanate ion was not present in the reaction mixture. When
cyanide was removed from an aliquot of the reaction mixture and the solution was incubated for 16 hours at pH 10, no thio-
cyanate ion was detected. Acid hydrolysis followed by paper chromatography showed the presence of iminothiazolidinecarboxylic acid. In a similar experiment, the pH of the reaction mixture was adjusted to 7.0 and similar results were obtained.
In earlier experiments, ribonuclease was incubated with a 100 fold excess of cyanide in phosphate buffers at pH 7 or 8. However, some unreacted ribonuclease was detected in the reac-
tion mixtures. Addition of urea to 8 M did not carry the reaction to completion.

The peptides resulting from the complete reaction of ribo-
nuclease with cyanide were separated by paper electrophoresis followed by paper chromatography. Separation in the first
direction was carried out in pyridine-acetate buffer, pH 6.4. A current of 30 ma (22 volts per cm) was applied for 4 hours. The
chromatogram was developed in the second dimension with n-butyl alcohol-acetic acid-water (12:3:5). The peptides were
located with ninhydrin, isatin, or diazotized sulfanilic acid. Nine peptides were found. No unreacted ribonuclease was present. The peptide areas were cut from several peptide maps and eluted with water. Hydrolysis of the peptides was carried out in a sealed tube with constant boiling HCl for 12 hours at 110°. Each peptide was then subjected to two-dimensional paper chromatography with n-butyl alcohol-acetic acid-water as the first solvent and 70% tert-butyl alcohol as the second. Eight of the peptides contained 2-iminothiazolidine-4-carboxylic acid. The qualitative amino acid content of the separated peptides is shown in Table II.

**TABLE II**

Amino acid composition of isolated ribonuclease peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (1-25)*</td>
<td>(Ala, Arg, Asp, Glu, His, Lys, Met, Phe, Ser, Thr, Tyr)</td>
</tr>
<tr>
<td>II (26-39)</td>
<td>(Arg, Asp, Glu, Leu, Lys, Met, Ser, Thr, iminothiazolidinecarboxylic acid)</td>
</tr>
<tr>
<td>III (40-57)</td>
<td>(Ala, Asp, Glu, His, Lys, Phe, Pro, Ser, Thr, Val, iminothiazolidinecarboxylic acid)</td>
</tr>
<tr>
<td>IV (58-64)</td>
<td>(Ala, Asp, Glu, Lys, Ser, Val, iminothiazolidinecarboxylic acid)</td>
</tr>
<tr>
<td>V (65-71)</td>
<td>(Asp, Glu, Gly, Lys, Thr, iminothiazolidinecarboxylic acid)</td>
</tr>
<tr>
<td>VI (72-83)</td>
<td>(Asp, Glu, His, Met, Ser, Thr, Tyr, iminothiazolidinecarboxylic acid)</td>
</tr>
<tr>
<td>VII (84-94)</td>
<td>(Ala, Asp, Glu, Gly, Lys, Pro, Ser, Thr, Tyr, iminothiazolidinecarboxylic acid)</td>
</tr>
<tr>
<td>VIII (95-100)</td>
<td>(Ala, Asp, Glu, His, Ile, Lys, Thr, Tyr, Val, iminothiazolidinecarboxylic acid)</td>
</tr>
<tr>
<td>IX (110-124)</td>
<td>(Ala, Asp, Glu, Gly, His, Phe, Pro, Tyr, Ser, Thr, Val, iminothiazolidinecarboxylic acid)</td>
</tr>
</tbody>
</table>

* The figures denote the residue numbers in the peptide chain of ribonuclease and show the portion of the molecule from which each peptide was derived.

**DISCUSSION**

Oxidized glutathione was considered to be a simple model to study the factors affecting cleavage of the peptide bond by the action of cyanide because both the amino and carboxyl groups of cystine are covered by different amino acids, namely, glutamic acid and glycine. There was a possibility that the usefulness of the model might be limited by the presence of a γ-glutamyl-cystine peptide bond. This limitation was considered unim-
portant when it was found that the rate of sulfhydryl scission and peptide cleavage for isoglutathione and glutathione were not significantly different at 37° and pH 7.

The cleavage of the disulfide bond of cystine by cyanide is dependent upon pH, temperature, and concentration of cyanide as shown previously by Gawron and Fernando (16) and Gawron, Mahboob, and Fernando (18). The reaction is bimolecular and reversible and can be formulated as follows.

\[ \text{RS-SR} + \text{CN}^- \rightarrow \text{RS}^- + \text{RSCN} \]  \hspace{1cm} (1)

The thiocyanate derivative of cystine cyclizes irreversibly to form 2-iminothiazolidine 4-carboxylic acid as shown by Schöberl, Kawohl, and Hamm (19).

\[ \text{HOOC-C} = \text{CH-COOH} \rightarrow \text{S} \quad \text{NH} \quad \text{NH}_2 \]  \hspace{1cm} (2)

By analogy with the above equations, the reaction of oxidized glutathione with cyanide can be illustrated as follows.

\[ \text{GSSG + CN}^- \rightarrow \text{GS}^- + \text{GSCN} \]  \hspace{1cm} (3)

When the amino group of the cysteine formed is not free but participates in a peptide bond, cyclization in an alkaline environment (pH 10) is not favored because the thiocyanate group is readily eliminated. Aldridge (20) observed that when GSCN was synthesized from reduced glutathione and cyanogen chloride at pH 7.4, cyanide ion was released by the action of excess sulfhydryl compound. This suggested that the thiocyanate derivative has an appreciable half-life. We observed the release of thiocyanate ion when N,N'-diacetylcystine, cystine ethyl
ester, glutathione, or more complex molecules were treated with cyanide in unbuffered, highly alkaline solution. A pH 8, we found that neither cystine, diacetylcysteine, glutathione, nor oxytocin reacted with cyanide to produce free thiocyanate ion, but that some thiocyanate was obtained from \( N_{1},N^{'-}\text{diacetyl-cystine} \) ethyl ester and also from bovine serum albumin (21). At lower pH, the thiocyanate group is not eliminated and, as will be discussed below, the lower pH favors iminothiazolidine ring formation.

The amount of sulfhydryl groups in the glutathione reaction mixtures was determined by colorimetry. In most of the experiments, when adequate precautions were taken to avoid oxidation, the sulfhydryl yield was 90 to 95% of the theoretical. At pH 6.3 only 70% of the expected sulfhydryl content was found. In these experiments, reoxidation of the sulfhydryl groups to disulfide did not occur appreciably during the incubation with cyanide since the maximum yield of iminothiazolidine ring observed was never more than 1 mole per mole of GSSG.

The pH effects observed with glutathione are in agreement with previous observations on the reaction of cyanide with cystine (18). It was shown that the rate of reaction between hydrocyanic acid and cystine dipolar ion was quite slow compared with the reaction between cyanide ion and cystine dianion.

The pH effect can be attributed to increased ionization of hydrogen cyanide with increase in pH and also to increased formation of glutathione anions.

The spectrophotometric measurements described under "Experimental Procedure" permitted estimation of the iminothiazolidine moiety and hence the rate of peptide cleavage. The rate of change in absorption at 235 nm, which is due mainly to the iminothiazolidine moiety, was determined by calorimetry. In most of the experiments, when adequate precautions were taken to avoid oxidation, the sulfhydryl yield was 90 to 95% of the theoretical. At pH 6.3 only 70% of the expected sulfhydryl content was found. In these experiments, reoxidation of the sulfhydryl groups to disulfide did not occur appreciably during the incubation with cyanide since the maximum yield of iminothiazolidine ring observed was never more than 1 mole per mole of GSSG.

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stable mercaptide (the mercaptide of lower pKₐ) and the corresponding thiocyanate are formed as predominant products. However, this concept is based on the reversibility of Equation 7 and the establishment of an equilibrium. With cystine and its derivatives, cyclization of the thiocyanate group drives the reaction to the right (Equation 7) and the effect would obtain only if the rate of cyclization were very much slower than the rate of disulfide scission.

In oxytocin, the disulfide bond can probably be polarized by a close approach of the phenol group of the neighboring tyrosine to permit hydrogen bonding. This would account for the observation that the cyanide attacked the sulfur atom farther from the tyrosine. Thus, only two peptides should be present in the reaction mixture, Cys-Tyr-Ile-Gln-Asn and iminothiazolidineformyl-Pro-Leu-GlyNH₂. The peptide resulting from alternate attack by cyanide on the disulfide would be iminothiazolidineformyl-Pro-Ile-Gln-Asn, but this was not found. It would have been present also if the initial scission of the disulfide link had been followed by reoxidation of the sulfhydryl groups formed. This did not occur since, under these conditions, 0.96 mole of —SH was found per mole of oxytocin. The hydrolysis of the dinitrophenyl derivatives of the two isolated peptides clearly indicated that cysteine was present only in the peptide containing tyrosine, isoelucine, glutamine, and asparagine. Thiol-disulfide interchange was also excluded by the same reasoning.

In contrast to the above findings, the reaction of cyanide with ribonuclease was accompanied by reoxidation of the sulfhydryl groups formed. Reduced ribonuclease has been observed to undergo reoxidation of the sulfhydryl groups by oxygen with great ease (24). Subsequent attack by cyanide on newly formed disulfides would eventually convert all cysteine residues to iminothiazolidines. This is what was observed when ribonuclease was allowed to react to completion with cyanide at pH 7 and 37°C. Only nine peptides are theoretically possible and these were found. The peptide containing the NH₂-terminal amino acid contained no iminothiazolidine group as would be expected. The qualitative amino acid composition of the isolated peptides shows agreement with expectations on the basis of the known structure of ribonuclease.

The procedures utilized on the simpler peptides are not directly applicable to the degradation of proteins. All protein disulfide bonds are not susceptible to attack by simple nucleophilic reagents. For example, only two of the three disulfide bonds of insulin are cleaved by sulfite in the absence of a denaturing agent (26). The action of sulfite on bovine serum albumin cleaves only four to five disulfide bonds (27), and this behavior was observed similarly for the action of cyanide (21). Cyanide cleavage of a particular disulfide bond can be asymmetrical. This can be obviated by oxidation of newly formed sulfhydryl groups which, in the presence of cyanide, would eventually convert all sulfhydryl to iminothiazolidine derivatives. Since the cyanide needs to be present in great excess to carry the reaction of Equation 1 to the right, it becomes difficult to oxidize sulfhydryl group without producing cyanate and thus forming other derivatives (28). However, it seems likely that if the disulfide bonds of a protein can be allowed to react with cyanide in the pH range 6 to 8, the subsequent reactions observed with the simple peptides should occur.

REFERENCES

1 For a discussion of the reactivity of disulfide groups in proteins with nucleophilic reagents such as sulfite and cyanide, see a review on sulfur in proteins by Cecil and McPhee (23).

2 It is suggested that the reaction of cyanogen bromide with a fully reduced protein at pH 7 should initiate this sequence of reactions.
Specific Cleavage of Cystine Peptides by Cyanide
N. Catsimpoolas and John L. Wood


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