PEPTIDE CLEAVAGE AT THE CARBONYL SIDE OF CYSTEINE BY 2-CYANO-6-METHOXYBENZOTHIAZOLE WITH REDUCED GLUTATHIONE AS A MODEL

By

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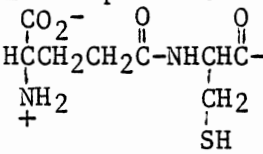
VI.	Effect of Various S-Acyl Groups on the Cleavage of Reduced Glutathione,  , by RC(O)Cl	18
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CHAPTER I

INTRODUCTION

An extremely wide variety of proteins are found in nature. In some cases the chemical and physical properties of proteins differ dramatically from one another. For example, the major proteins of hair and collagen fibrils, keratin and tropocollagen, are quite different in molecular weight and chemical composition from the proteins of low molecular weight, such as insulin, that act as hormones (1). The variations in chemical structure among other proteins are small or subtle, and yet these small differences may be associated with major differences in physiological function. For example, hemoglobin S differs from hemoglobin A by only a single amino acid residue (2), and yet the behavior of the two proteins in red blood cells is significant. To be sure, such differences occur in terms of the total number and chemical nature of amino acids in a protein and in the arrangement of the amino acids sequence which results in the folding of the polypeptide chain in space (3). Thus, a study of the sequencing and chemistry of amino acids should provide a foundation for a deeper understanding of the biological function of any organism.

Among the twenty amino acids in proteins, cysteine perhaps is one of the most interesting. Instead, it was discovered that cysteine may exhibit different biological functions by virtue of its remarkable chemical properties (4). For instance, cysteine has an SH group and

it is known that sulfhydryl groups dissociate near a neutral pH. Thus, the reactive species RS^- is frequently 500 times more nucleophilic than the corresponding oxygen analogue RO^- (5). Moreover, RS^- may also lose an electron to give a reactive free radical which may participate in chain reactions (6). Another interesting biological property of an SH group is that it can be oxidized to form disulfide bridges which are much less active than SH groups and can function as the cross-linking system to add stability to the protein structure. Interestingly, an SH group can also act as a reagent and react with the S-S group of a different disulfide (7) to generate a new -S-S- system and a new thiol.

Because the thiol group can undergo oxidation and reduction with ease and because it is an aggressive nucleophile, it has been observed that thiol groups are at the catalytic sites in many enzymes (8, 10). Thus, enzymes like phosphoglucomutase, ovalbumin, β-lactoglobulin, phosphorylase, oxidoreductases, transferases, hydrolases, lyases and isomerases possess SH as the catalytically functional group (9).

Nature may also employ the thiol group as the key in the regulation of cellular activities. Because stimulation of many SH-enzyme activities by glutathione also occurred intracellularly, Barron suggested in a review in 1951 (10) that it might be involved in "the regulatory mechanisms of cellular respiration." It was implied that possibly certain SH-enzymes were partly in an inactivated state due to the presence of natural inhibitors which inactivated the SH group.

Thus, the SH group may be involved in many fundamental biological functions (11) such as: 1) direct catalysis, 2) binding of substrates, 3) binding of regulators at allosteric sites or concerning allosteric regulation, and 4) structural cohesion in the interaction of protein

subunits. Considering these four basic functions of SH groups, one can extend the theory even further. Perhaps some protein hormones, drugs, vitamins and various disease conditions may activate or inhibit SH groups in enzymes or other vital proteins so that their physical conditions may be altered and thus the biological behavior of organisms is affected.

CHAPTER II

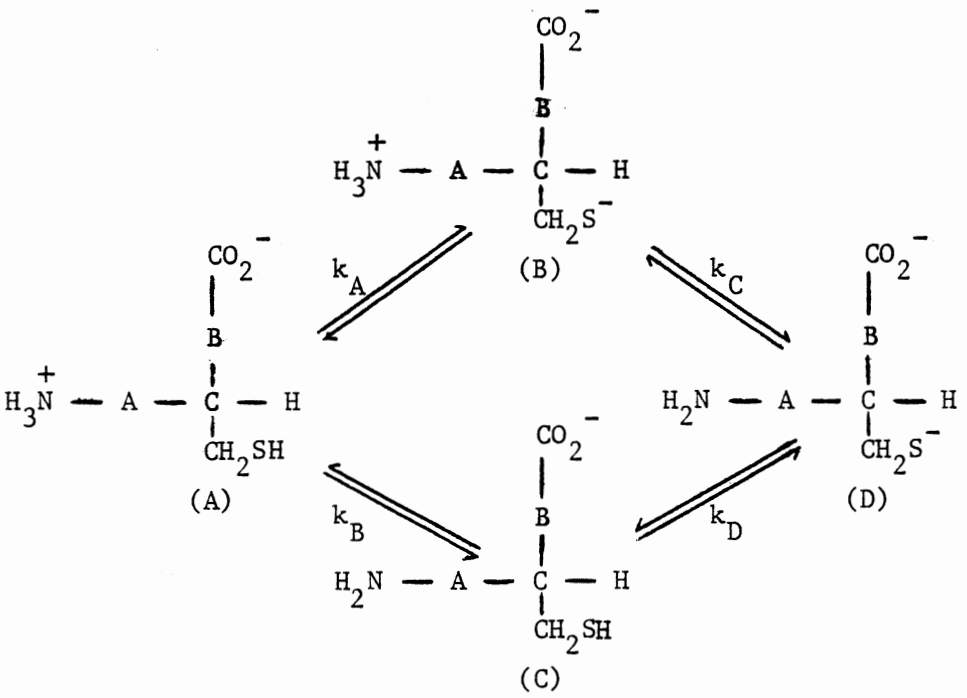
LITERATURE REVIEW

In view of the results to be discussed in this thesis, it is appropriate to establish an adequate background in several areas: 1) physical and chemical properties of thiols; 2) alkylation reactions; 3) other general thiol reactions, S-S cleavage reactions; and 4) cleavage of peptide bonds. Each of these areas will be treated separately.

General Physical Properties

As mentioned previously, thiol-containing compounds are frequently 500 times more reactive than the hydroxyl counterpart in nucleophilic displacement processes (5). The drastic difference can partially be explained by the ease of thiol deprotonation compared to deprotonation of the hydroxyl group near pH=7. As an example, the ionization pattern and constants for cysteine and reduced glutathione are illustrated in Figure 1 and Table I.

In comparing the pK_a of hydroxyl derivatives with the corresponding thiol compounds, the pK_a of the R-OH is frequently 15-16 while the pK_a of the R-SH group is near 9 (14). Therefore the number of RS^- anion species compared to RO^- species at neutral pH is, in general, much greater from RSH than from ROH, respectively. Moreover, it is usually conceded that RS^- is a much better nucleophile than the RO^- species (15).



Cysteine: A = Nothing

B = Nothing

Reduced glutathione: A = $HC(CO_2^-)CH_2CH_2C(O)NH-$

B = $-C(O)NH\,CH_2-$

Figure 1. Ionization Diagram for Cysteine and Reduced Glutathione

TABLE I

IONIZATION CONSTANTS OF CYSTEINE AND REDUCED GLUTATHIONE (25°C)*

Compound	Macroscopic Constants		Microscopic Constants				Ionic Strength
	pK_1	pK_2	pK_A	pK_B	pK_C	pK_D	
L-Cysteine	8.35	10.46	8.49	8.81	10.39	10.13	0.1
Glutathione	8.74	9.65	8.92	9.20	9.16	9.44	0.16

*Data measured by kinetic, titration, ultraviolet, Raman and calorimetric methods; the values are averaged from data in references (12-14).

Alkylation Reactions

Since with cysteine in water, the concentration of reactive species, i.e., sulfide ions, is highly pH dependent, near the neutral pH the rate of alkylation of the sulfide ion by an alkylating agent must also depend upon the pH of the media. Increasing the pH of the medium results in an increase in RS^- available for nucleophilic attack. Thus, the rate of reaction should increase at alkaline pH (16-18) values. However, success of the attack also depends upon the bond strength and solvation properties of the leaving group X in R'X, as illustrated in the general reactions (Table II). The greater the bond energy of R'X (Table III), the slower the reaction. It can be seen that the rate pattern in the halogen family in Table II does correlate with the bond strengths shown in Table III.

General Thiol Reactions

Thiols undergo a host of chemical reactions which are of biological interest (20). Such reactions which involve the thiol group are substitution, addition, elimination and/or oxidation to give products containing S-C, S-metal, S-S bonds or sulfonyl group (21). In this section, major types of reactions are summarized and, where possible, are illustrated via applications in reactions of biochemical interest. For example, a number of reagents react with cysteine for a variety of reasons such as to provide: (a) a radioactive marker, (b) a fluorescent marker, (c) a reversible masking group, (d) a nonreversible masking group, (e) a marker for photospectrometry, (f) an environmental reporter, and, perhaps the most widely used of all, (g) an

TABLE II

$RS^- + R'-X \rightarrow RS-R' + X^-$
RATE OF REACTION OF THIOLS AT DIFFERENT pH VALUES WITH HALOACETIC ACIDS (XCH_2CO_2H)

pH	Thiol (RS^-)	X	Relative Rate
5.6	cysteine	I	0.14
6.3	cysteine	I	0.28
7.0	cysteine	I	1.0
7.4	cysteine	I	1.3
8.4	cysteine	I	2.1
7.4	cysteine	Br	0.094
7.4	cysteine	Cl	0.07
7.4	cysteine	F	no reaction
7.0	thioglycolate	I	1.33
7.0	glutathione	I	0.056
7.0	ethane thiol	I	no reaction

TABLE III

BOND DISSOCIATION ENERGY OF C-X BONDS

Bond	E_D
CH_3-F	108 kcal/mole
CH_3-Cl	81 kcal/mole
CH_3-Br	67 kcal/mole
CH_3-I	53 kcal/mole

enzyme inhibitor. Several of the reagents are given in Table IV.

S-S Cleavage Reactions

In nature, cysteine is rarely found free in protein but rather in the oxidized form as cystine with disulfide bridges, apparently to maintain specific three dimensional structures. Chemical cleavage of the S-S bond is a quite common procedure in protein sequencing and other related studies (29). A variety of cleavage techniques are summarized in Table V.

Cleavage of Peptide Bonds

Protein research frequently requires the chemical cleavage of peptide bonds. One common reason for this has been for partial degradation of this type of complex biological molecules. In protein sequencing for example, chemical cleavage of specific peptide bonds can result in the generation of peptide fragments useful for establishing the original protein structure. A series of chemical cleavage reagents are listed in the following sections.

The Cyanogen Bromide Reaction

A highly specific cleavage at the carbonyl side of methionine in a peptide can be effected using BrCN (30). However, the polypeptide must be unfolded to expose methionyl residues (31). Bovine pancreatic ribonuclease was the first protein to be cleaved by cyanogen bromide (32), because it contained a relatively high amount of methionine units. Two of the four methionine units present in the molecule are adjacent in the peptide. Thus, cleavage occurred at this site and

TABLE IV

OTHER CYSTEINE REACTIONS

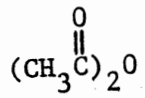
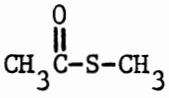
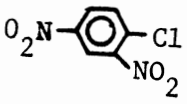
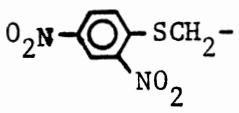
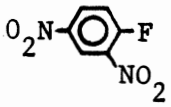
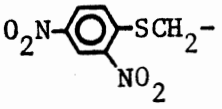
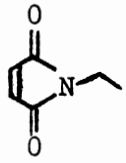
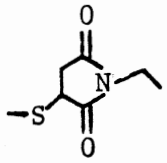
Classification/Reagent	Product	Comment
Acylation (22)		
 acetic anhydride		Remove with dilute ^{-}OH or NH_2OH.
Benzylation (23)		
 chlorodinitrobenzene		Quite specific.
 fluorodinitrobenzene		Reacts with lysine, histidine and tyrosine also.
Oxydation (24)		
 peracids	$-CH_2SO_3^-$	Used as a pretreatment before acid hydrolysis for cysteine and tryptophan.
Alkene Addition (25)		
 N-ethylmaleimide (NEM)		Reacts slightly with lysine.
$CH_2{=}CHC{\equiv}N$ acrylonitrile	$-CH_2S-CH_2CH_2C{\equiv}N$	Very selective at pH 8.0; other groups react at higher pH, such as lysine, arginine and histidine.

TABLE IV (Continued)

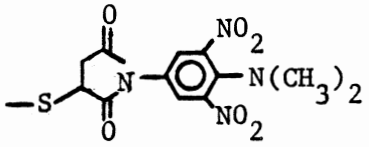
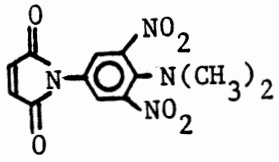
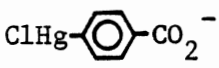
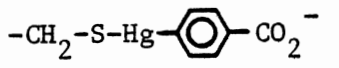
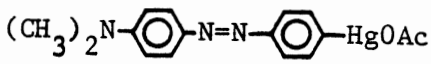
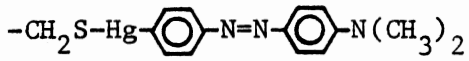
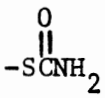
Classification/Reagent	Product	Comment
 N-(4-dimethylamino-3,5-dinitrophenylmaleide)		Allows colorimetric estimation of substitution; pK_a of dimethyl ammonium group depends on its environment used as a probe of -SH environment.
Mixed Disulfide Exchange (23)		
$^-O_2CCH_2SH$ thioglycolate	$-CH_2S-S-CH_2CO_2^-$	Readily reversible with thiols.
Metal Reactions		
 p-chloromercuribenzoate		Extent of reaction can be estimated spectrophotometrically.
 azobenzene mercurial		The pK_a of the dimethyl ammonium ion depends on its local environment.
Carbamylation (27)		
HO-C≡N potassium cyanate		Product is stable below pH 6.0.

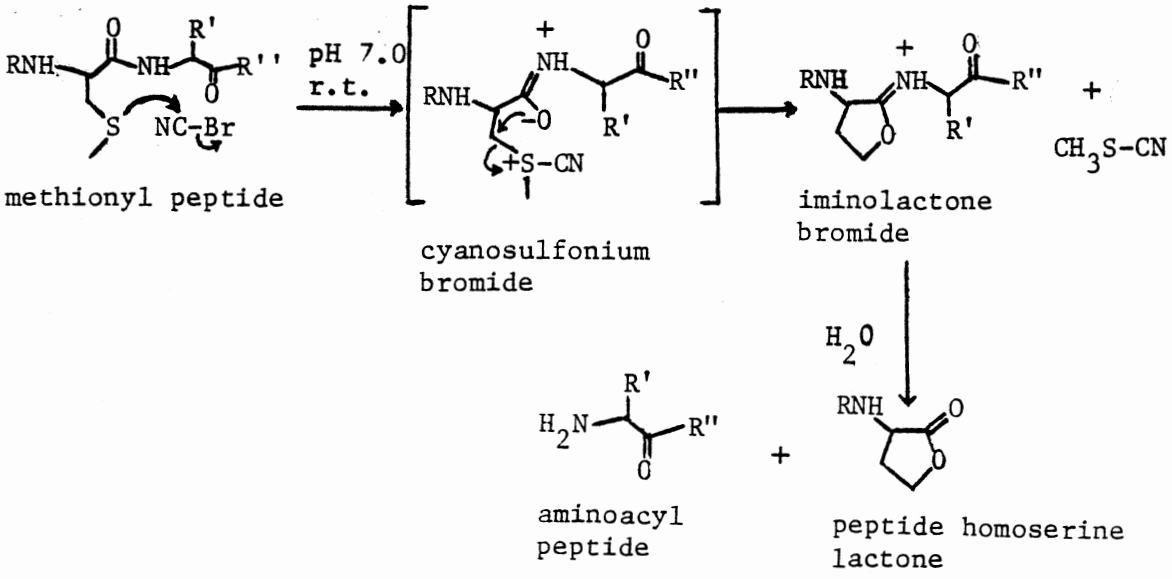
TABLE V

REAGENTS FOR CYSTINE CLEAVAGE

Reagent (R'H)	Product (RCH_2-SR')
$HSCH_2CH_2OH$ mercaptoethanol	RCH_2-S-S-CH_2CH_2OH* + RCH_2SH or $2RCH_2SH$ + $HOCH_2CH_2$-S-S-CH_2CH_2OH
RCO_3H peracids	$2RCH_2SO_3H$
$Na_2S_2O_3$ sodium thiol sulfate	RCH_2S-$SO_3^-Na^+$ + $RCH_2S^-Na^+$

*Product formed reversibly.

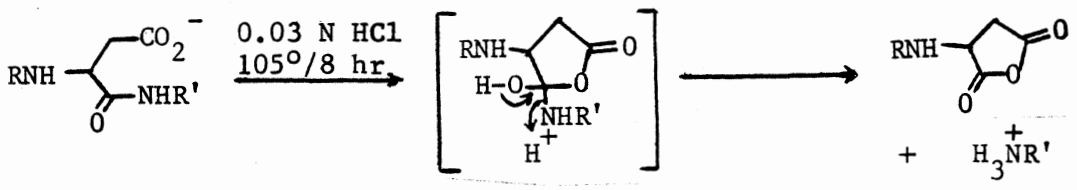
homoserine lactone was liberated. The reaction can be briefly outlined:



The use of BrCN is now quite general.

Cleavage at Aspartic Acid

With peptides which have aspartic acids, it is possible to bring about a fairly specific cleavage at the C(O)-N bond of the aspartic acid unit at a controlled pH level. The aspartic acid peptide bonds are preferentially cleaved at a rate at least 100 times greater than other bonds (33). A number of acids and buffers have been used to cleave several proteins. The one to be described will be with hydrochloric acid although in certain cases acetic acid alone or in combination with other acids has been used (34). Various concentrations of ribonuclease A or insulin were heated with 0.003 N HCl at 105°C for about 25 hr. and 80% cleavage occurred at the C(O)-N bond of aspartic acid (35). The proposed mechanism is outlined as follows:

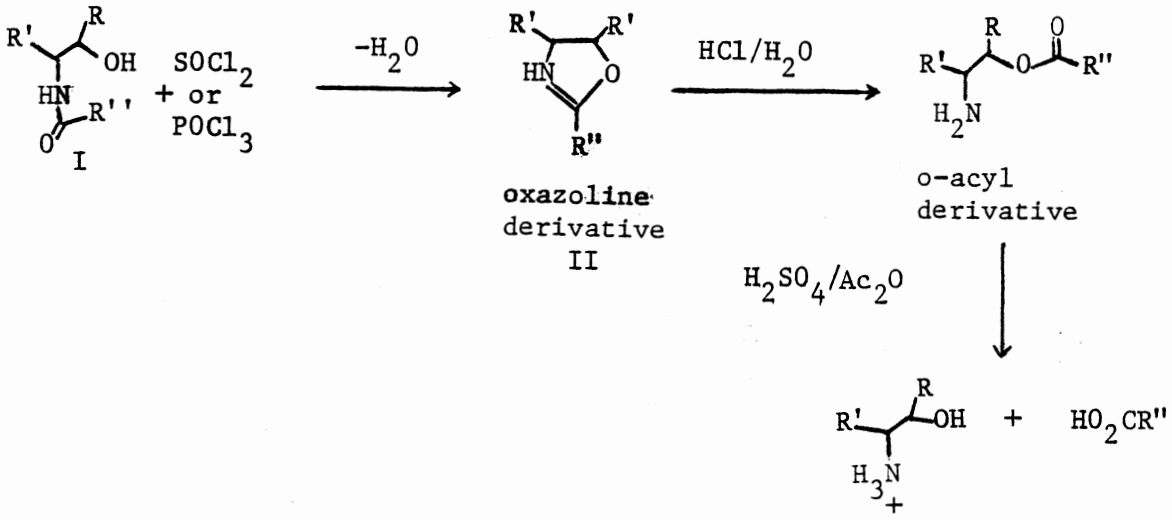


Cleavage of Seryl and Threonyl Residues

(N-O Acyl Rearrangement)

In peptides containing the above units, cleavage of the C(O)-N bond (N is part of serine or threonine unit) in I can be done at low temperatures (36). The process can be summarized as follows with II as the suspected intermediate.

Serine: R = H; Threonine: R = CH_3

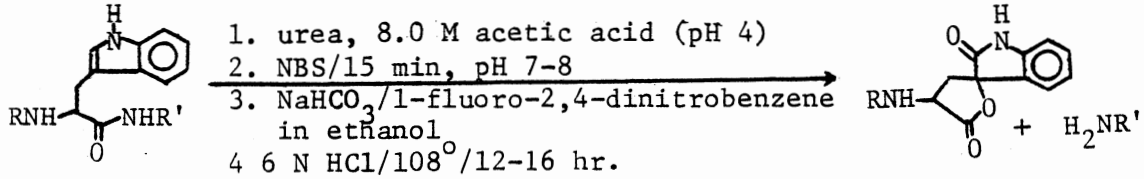


Many proteins have been treated by this method, and it has been found that the amount of cleavage varies from 60% to 90% (36) of the available threonyl or seryl units. Generally, threonine units undergo a smaller degree of cleavage than the serine units.

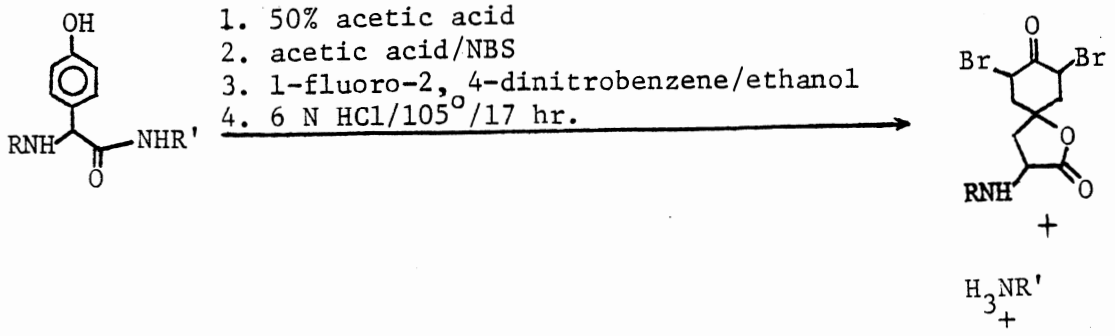
N-Bromosuccinimide (NBS) Cleavage on a Tryptophane-Containing Peptide, Tyrosine-Containing Peptide, and Histidine-Containing Peptide

Cleavage of peptide bonds with NBS is not as specific or efficient as with the cyanogen bromide (37-42). The amount of cleavage with the former is often 50-90% of the C(O)-N bonds of tryptophan, tyrosine or histidine in simple model systems and 10-50% in proteins (37). Such cleavages of tyrosine-containing peptides also depend upon the nature of the adjacent amino acid. The following data indicate the critical nature of the adjacent amino acid: tyrosine-glutamic (34%), tryptophan-serine (65%), tryptophan-valine (60%), tryptophan-lysine (55%), tyrosine-carboxymethylcysteine sulfone (40%), and tyrosine-proline (36%) (37). All of these data are for cleavages performed on the known primary structure of ribonuclease (37).

Trytophanyl Peptide



Tyrosyl Peptide

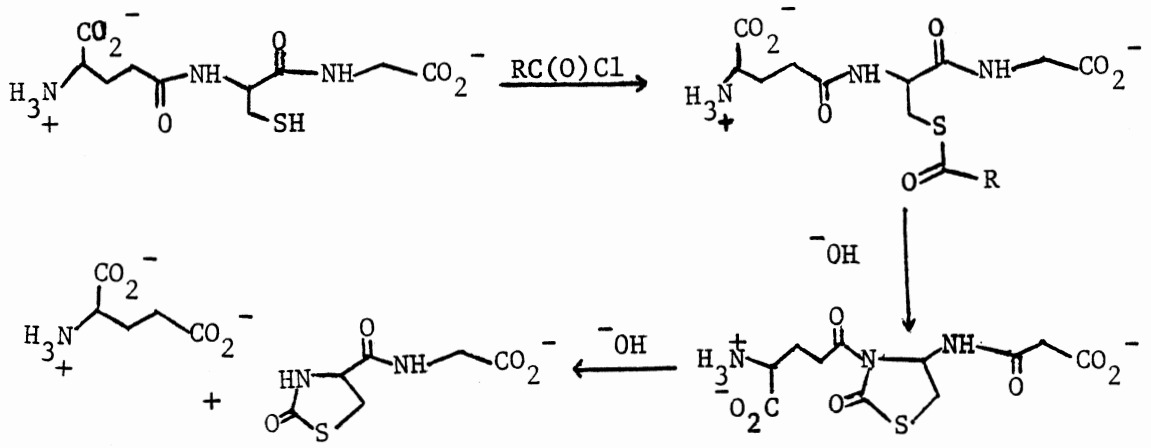


Histidine Peptide

Histidine Peptide —(1. pyridine acetate buffer; 3 x NBS/r.t.; 2. 85°-100°/1 hr.)→ 50% Cleavage

Peptide Cleavage at the H_2N Terminals of Cysteinyl Residues

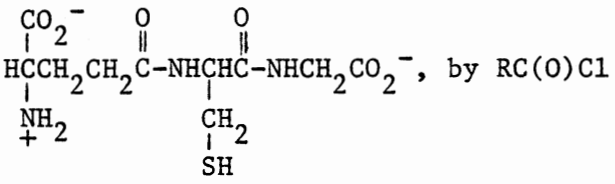
One of the reactions for peptide cleavage at the cysteinyl residues in reduced glutathione is based upon a reaction with an acyl chloride followed by a cyclization as illustrated (43).



Acid chlorides used are quite reactive but the reaction conditions are critical. This cleavage depends upon the abilities of thiol group to react spontaneously with the acid chlorides to give an S-acyl-containing system. The data in Table VI demonstrate that the cleavage rate is also related to the stability of the anion R^- which is liberated (43).

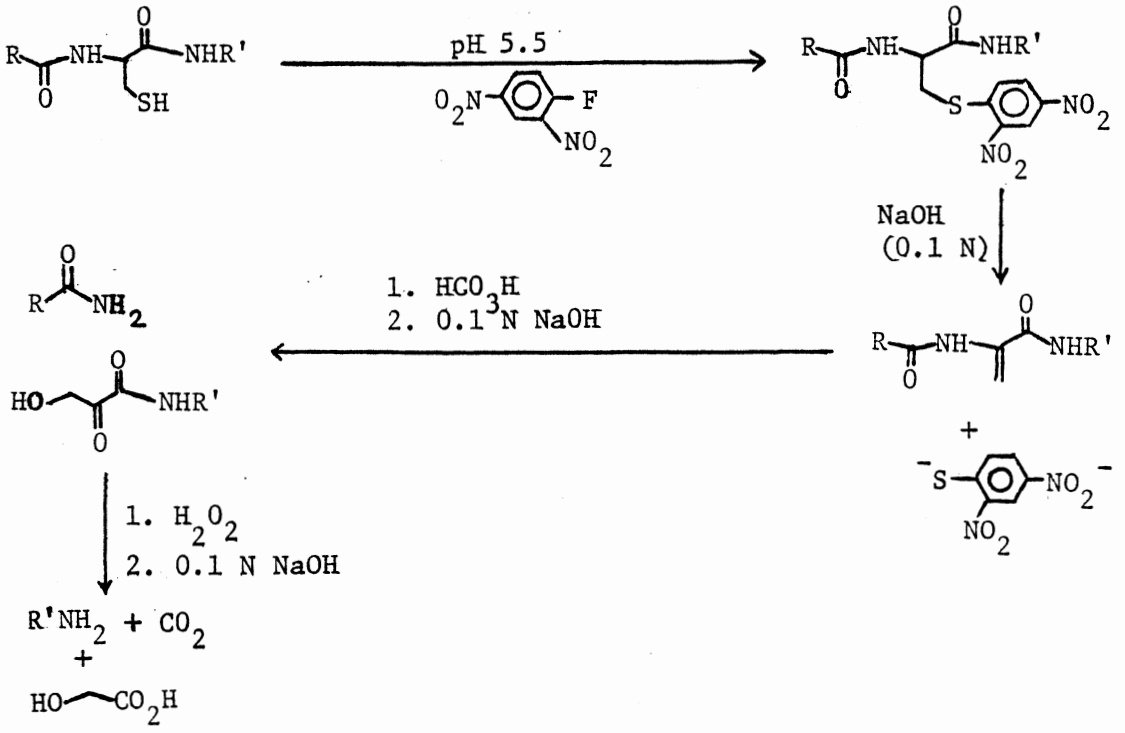
2,4-Dinitrofluorobenzene is widely used for H_2N-terminal labelling and also for cleavage of cysteine units in a peptide. The disadvantage of the reaction is that serine also combines with 2,4-dinitrofluoro-

TABLE VI

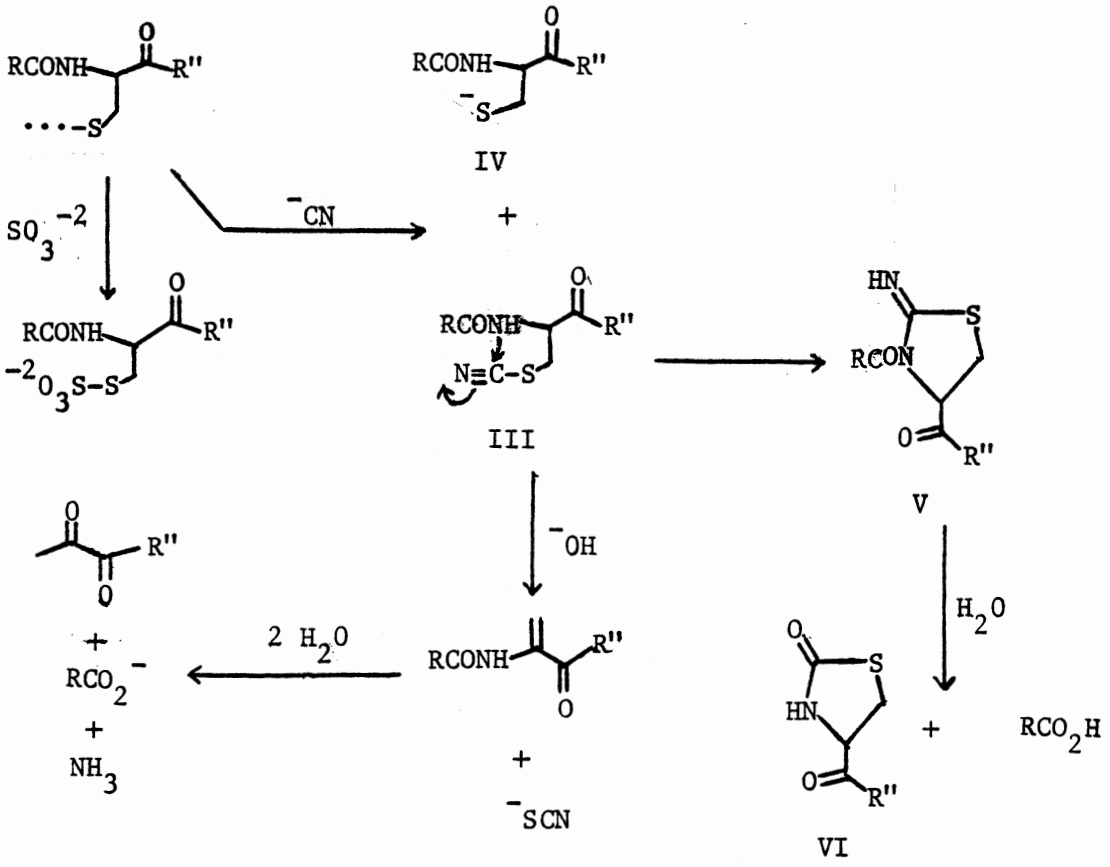
EFFECT OF VARIOUS S-ACYL GROUPS ON THE CLEAVAGE OF REDUCED GLUTATHIONE,  , by RC(O)Cl

Thiol Derivatives RC(O)	Percent Glutamic Acid Formed (Specific Fission)	Percent Glycine Formed (Nonspecific Fission)
$4\text{-}NO_2C_6H_4OC(O)$	30	2
$C_6H_5SC(O)$	47	3
$n\text{-}C_4H_9SC(O)$	10	3
$(CH_3)_2NC(O)$	7	5
$C_6H_5C(O)$	1	3

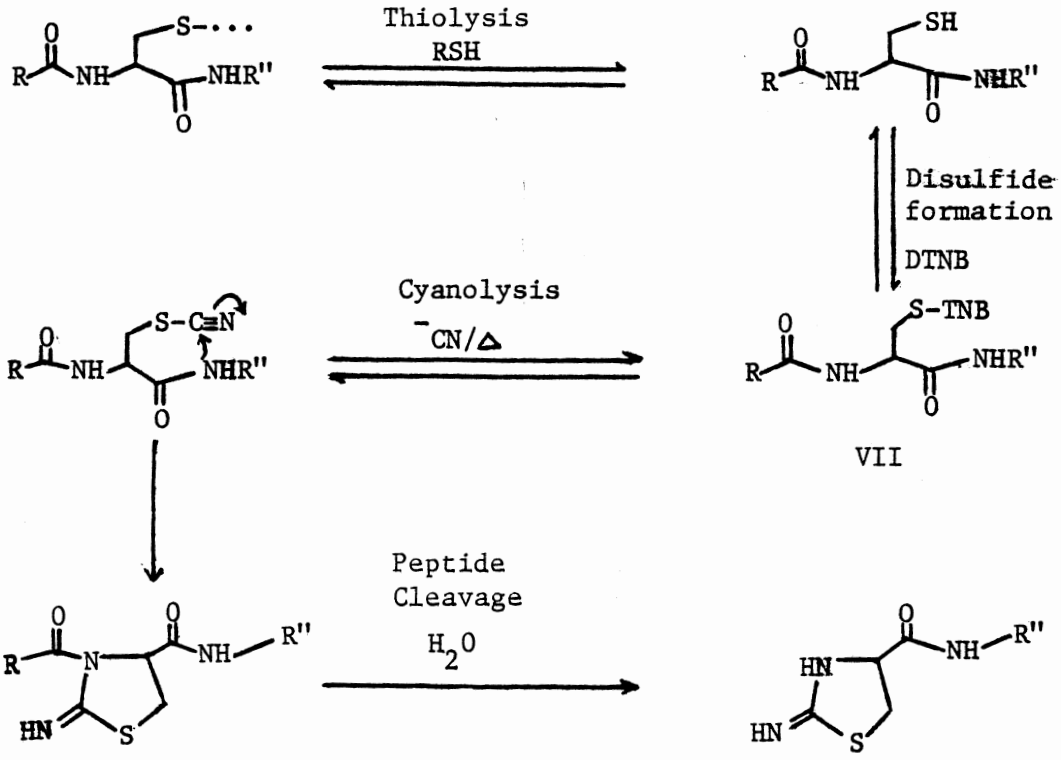
benzene and a similar cleavage ensues (44). Selective cleavage of cysteine residues has been studied with several polypeptides and proteins. The kinetics of the β-elimination reaction (step 2) can be measured by spectrophotometry at 408 nm, the absorption maximum of the thiodinitrophenolate anion (45-48) see below.



The S-S linkage can also be cleaved by CN^-. For example, treatment of glutathione with cyanide ion at pH < 8 results in formation of the thiocyano derivative III and the thiol anion IV. Then the thiocyano derivative III cyclizes to form the 2-iminothiazolidine derivative V which spontaneously fragments to glutamic acid and an iminothiazolidine VI (49-51).



Activated thiols can also react with CN^- (39-41). By treating a thiol with 5,5'dithiobis(2-nitrobenzoic acid), (DTNB), a reactive intermediate VII can form. Cyanide ion reacts rapidly with VII to give the thiazole with concomitant cleavage of the peptide link (39-41). One possible mechanism is outlined:


Thiolysis
RSH
Disulfide formation
DTNB
Cyanolysis
$^-CN/\Delta$
Peptide Cleavage
H_2O

VII

CHAPTER III

EXPERIMENTAL METHODS

Chemicals

Reduced glutathione was obtained from Sigma Chemical Company and stored in a freezer ($-20^{o}C$). Trichloroacetamide was obtained from ICN Pharmaceutical Inc. (K and K) and p-anisidine was obtained from MC & B Company and redistilled under vacuum. Pyridine was obtained from Eastman Company. Finally, other miscellaneous reagents such as KOH, NaOH, $O{=}PCl_3$, ethanol, acetic acid, formic acid, propionic acid, trifluoroacetic acid, and $K_3Fe(CN)_6$ were supplied by Mallinckrodt, Fisher Scientific, Pierce, Sigma, Eastman and Aldrich Chemical Company.

Laboratory

The project was divided into two major parts: 1) the organic syntheses portion with Dr. Berlin (Chemistry Department), and 2) the biochemical reactions and amino acids analysis with Dr. Liao (Biochemistry Department).

Synthesis of 2-Cyano-6-Methoxybenzothiazole

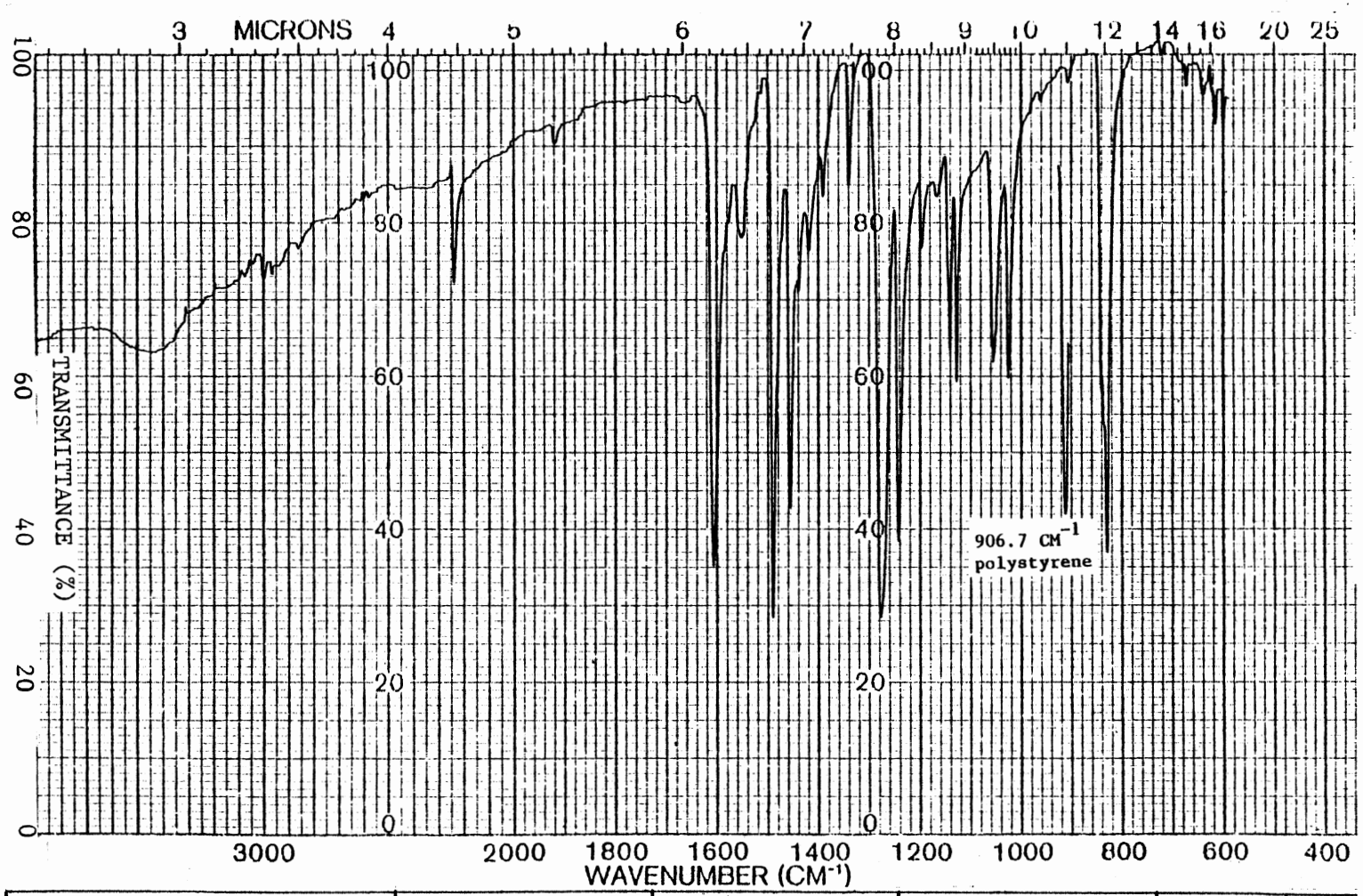
Exactly 48.72 g of KOH was dissolved by 500 ml of 95% ethanol in a three-neck one-liter flask equipped with two addition funnels and a gas inlet tube. The solution was stirred (magnetic), and H_2S was bubbled

into the basic ethanol solution for two hours. Then 47.1 g (0.29 mole) of trichloroacetamide in 335 ml of 95% ethanol was added dropwise through the addition funnel to the stirred solution. At the same time, 39.3 g (0.32 mole) of p-anisidine in 175 ml of ethanol was also added dropwise into the flask through the second funnel. The solution initially was deep red-orange and turned bright yellow after one half of the reactants were mixed at room temperature. After 2.5 hr, a solid had precipitated and was filtered. The crude product 4-methoxythiooxanilamide was purified by fractional crystallization (95% ethanol). The yield for the step was about 0.032 mole. After all the 4-methoxythiooxanilamide was dissolved in a 10% sodium hydroxide solution (94 g NaOH/936 ml H_2O) with vigorous stirring, potassium ferricyanide (105 g, 0.32 mole) was added dropwise at room temperature over a 2-hr period. A white precipitate formed and was filtered and then recrystallized (methanol). The yield of 2-carbamoyl-6-methoxybenzothiazole was 1.5 g ($\sim$ 22%), with a melting point of 255-257°C [Lit. (52-53) 255-257°C]. In a hood, 2-carbamoyl-6-methoxybenzothiazole (1.5 g, 0.008 mole) was dissolved in excess phosphorus oxychloride (10 ml, 0.11 mole), and the solution was boiled for 2 hr at 110°C with constant stirring. The solution turned red-pink during this period. Excess phosphorus oxychloride was removed under aspirator pressure. Moreover, vigorous magnetic stirring of the solution and with mild warming with a heat gun at the top part of the reaction vessel were necessary in order to prevent bumping of the solution. After a majority of the phosphorus oxychloride had distilled, ice was added to decompose the remaining phosphorus oxychloride and crude 2-cyano-6-methoxybenzothiazole precipitated. Sublimation of the crude product

at 0.2 mm/60°C gave a light yellow compound (0.8 g, 4.2×10^{-3} mole) with melting point 126-128°C. Recrystallization (hexane) of this compound gave silver-colored needles of 2-cyano-6-methoxybenzothiazole, mp. 128-129°C [lit. (52) mp. 126-128°C]. The IR and NMR spectra of the product are shown in Figures 2 and 3, respectively. A brief synthetic scheme and a possible mechanism are outlined in Figure 4 (52).

General Methods for Reacting Reduced Glutathione with 2-Cyano-6-Methoxybenzothiazole

A buffer solution was composed of 150 ml of pyridine, 100 ml of H_2O, 29 ml of N-ethylmorpholine and enough glacial acetic acid to give a solution of pH = 8.0 (some earlier experiments were conducted without pyridine). Due to the ease of oxidation of the reduced glutathione, freshly reduced glutathione solution (0.01 M) was made with the buffer immediately before each experiment. The solutions of 2-cyano-6-methoxybenzothiazole used were approximately 0.05 M (some experiments were conducted with 0.1 M and some with 0.01 M solutions) in ice-chilled acetone; the solutions were stored in freezer. Acetone was used as the solvent because the compound was not very soluble in water. The buffer solution (pH = 8.0) was not used, although 2-cyano-6-methoxybenzothiazole was quite soluble in the buffer, because the compound might react with pyridine over a long period of storage. For all of our later experiments, the internal standard valine was employed in the reactions. Its concentration was 0.001 M in a solution consisting of pyridine, N-ethylmorpholine, H_2O and acetic acid (pH 8.0 buffer as mentioned above).



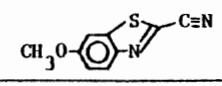
SAMPLE 	SOLVENT CONC. 2 mg/150 KBr CELL PATH REFERENCE PERIN-ELMER	SCAN Normal SLIT Normal OPERATOR Jeffrey Yeung DATE July 22, 80 PART No. 5102 1000	SINGLE B. T.D. SPEED ORD. EXP. T. CONST.	REMARKS M.P. 126-128°C

Figure 2. IR Spectra of 2-Cyano-6-Methoxybenzothiazole

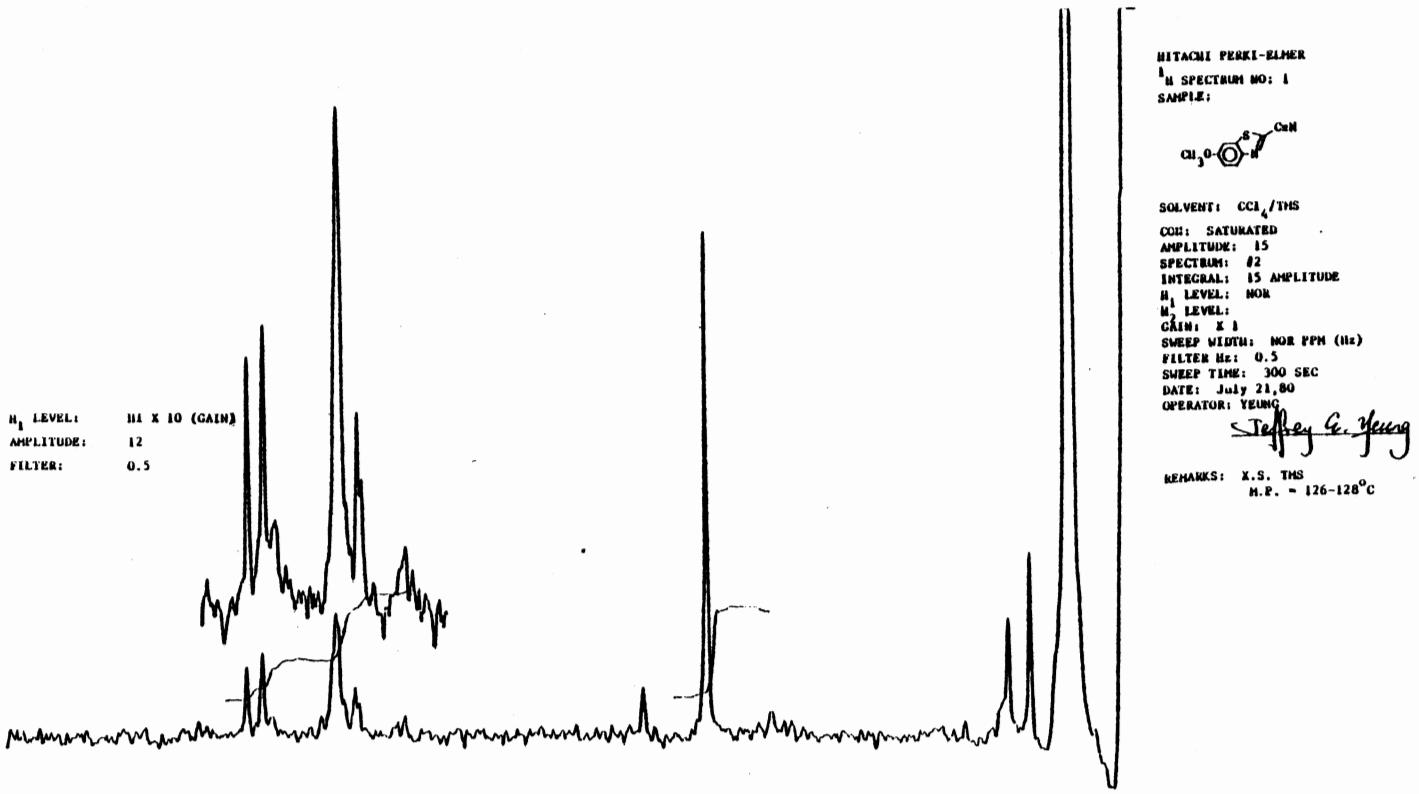


Figure 3. NMR Spectra of 2-Cyano-6-Methoxybenzothiazole

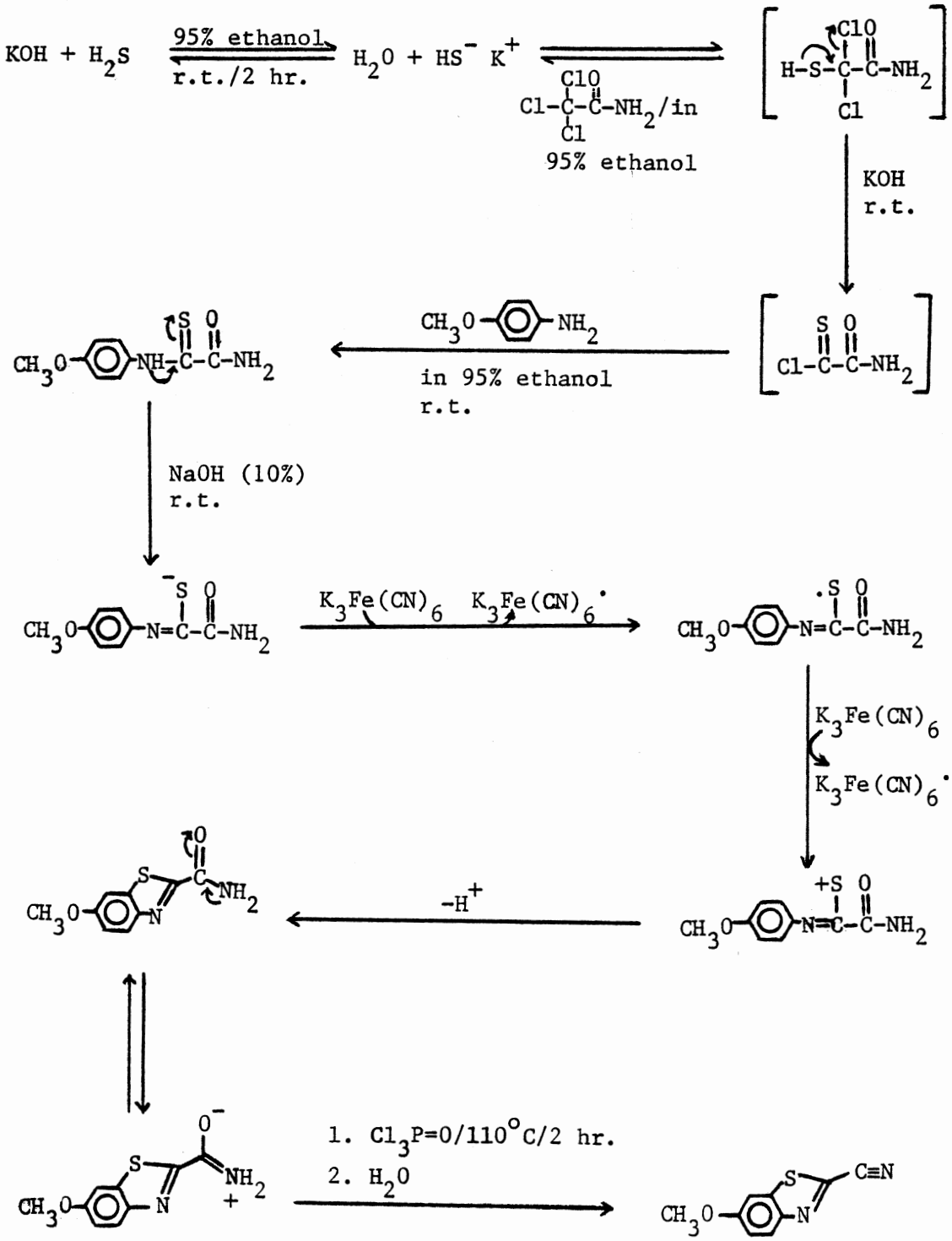


Figure 4. Synthetic Scheme for 2-Cyano-6-Methoxybenzothiazole

The general procedure for the treatment of glutathione with 2-cyano-6-methoxybenzothiazole consisted of the following steps. At least 1 equivalent of 2-cyano-6-methoxybenzothiazole (1 equivalent of 2-cyano-6-methoxybenzothiazole:1 equivalent of reduced glutathione) in acetone was added to a 3 ml test tube. The experiments were performed with evaporation of all acetone before the addition of the reduced glutathione. An explanation for this step is given in the Discussion Section of this thesis. To the 2-cyano-6-methoxybenzothiazole was added 10 µl of reduced glutathione solution and then 100 µl of valine (pH 8.0), the internal standard (the earlier experiments were done without valine as the internal standard in the pH 8.0 buffer). The test tube was purged with nitrogen and was then quickly covered with parafilm before being placed in a shaking incubator for 4 hr at 37°C. After the incubation, the parafilm was punctured and the tube was placed in a dessicator under a high vacuum (2 mm) at room temperature for 2 hr. Then the parafilm was removed and 200 µl of an acid was added (the most common acids used were trifluoroacetic, formic, acetic or propanoic). All of these acids have relatively low boiling points (see Table VII) for easy removal. Once again the tube was purged (N_2) with great caution and quickly covered with parafilm. At this point, it was noted that if the solution turned pale yellow, a high percentage of cleavage of glutathione was later observed. A bright yellow color indicated a medium percentage ($\sim$ 34%) of cleavage. In contrast, if the solution was crystal clear, almost no cleavage had occurred. The tube was returned to the incubator (37°C) for 4 hr of additional shaking. Again, the parafilm was punctured, and the acid was removed by evaporation under a high vacuum (2 mm). Finally, 100 µl of H_2O was added and

TABLE VII

SOME ORGANIC ACIDS AND THEIR PHYSICAL PROPERTIES

Carboxylic Acid	Structure	pK_a (H_2O/25°C)	MW	BP	Density (g/ml)	Solubility
trifluoroacetic	CF_3CO_2H	0.2	114.02	72.4	1.54	S:H_2O, alcohol, ether, acetic
Trichloroacetic	Cl_3CCO_2H	0.6	163.39	197.6	1.62	VS:H_2O; S:alcohol, ether
Dichloroacetic	Cl_2CHCO_2H	1.3	128.94	194.0	1.56	∞:H_2O, alcohol, ether
Malonic acid	$HO_2CCH_2CO_2H$	1.9 (pK_1)	104.06	140 (d)	1.62	VS:H_2O; S:alcohol, ether
Fluroracetic	CFH_2CO_2H	2.58	78.04	165.0	1.37	S:H_2O, alcohol
Chloroacetic	$ClCH_2CO_2H$	2.8	94.50	187.8	1.40	VS:H_2O, S:alcohol, ether
Formic acid	HCO_2H	3.7	46.03	100.7	1.22	∞:H_2O, alcohol, ether acetic
Acetic	CH_3CO_2H	4.8	60.05	117.9	1.05	∞:H_2O, ether, acetone, benzene
Propanoic	$CH_3CH_2CO_2H$	5.0	78.08	141.0	0.99	∞:H_2O, alcohol

*CRC Handbook of Chemistry and Physics, 60th ed., CRC Press, 1979-1980.

∞ = infinity solubility; VS = very solubility; S = soluble.

the solution was ready for the amino acid analyzer and high voltage paper electrophoresis.

Procedure for Sample Preparation for the Amino Acid Analyzer

After removal of the acid under vacuum, 100 µl of H_2O was added to the test tube. The sample was prepared for the amino acid analyzer by taking 20 µl of the above solution and then diluting it with 100 µl of Beckman Amino Acid Analysis buffer [sodium citrate (0.2 N buffer) and 0.5% thiodiglycol, pH 2.2]. Finally, the sample was loaded into a fully automated Amino Acid Analyzer constructed by Dr. Liao (56). After 4.5 hr of Amino Acid Analyzer operation time, the relative concentrations of each amino acid were printed out by the Autolab (System AA) via integrations of individual peaks on the chromatogram. The signals monitored were the absorbances at 440 nm and 570 nm. The retention time for all the experimental species were listed in Table VIII.

Preparation of High Voltage Paper Electrophoresis

All the work in this section was performed with disposable gloves. There was also used Whatman chromatography paper (56 cm x 47 cm) (see Figure 5). Before applying any sample to the paper, it was necessary to soak this chromatography paper with formic acid:acetic acid:H_2O, (25:100:875 ml) in a pH 1.9 buffer for 10 sec. Before the high voltage electrophoresis experiment was initiated, 5 µl of solution of 10 mg methyl green in 100 ml of H_2O (methyl green is a fast moving dye in the high voltage chamber) was applied to the paper to facilitate

TABLE VIII

RETENTION TIME FROM THE AMINO ACID ANALYZER

Compound	Retention Time (min)
S-Carboxymethylcysteine	30
Oxidized Glutathione	29
Reduced Glutathione	30
Glutamic Acid	45
Half Cystine	50
Glycine	62
Valine	84

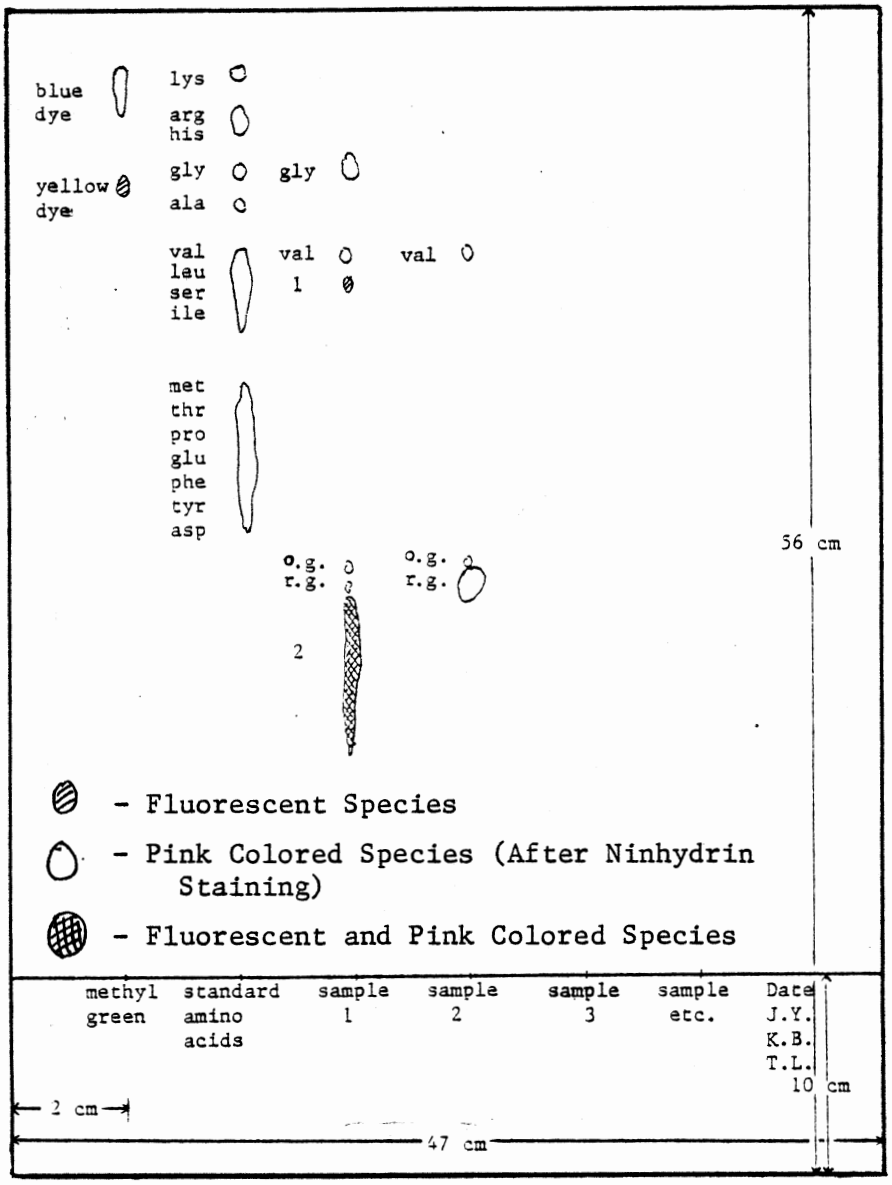
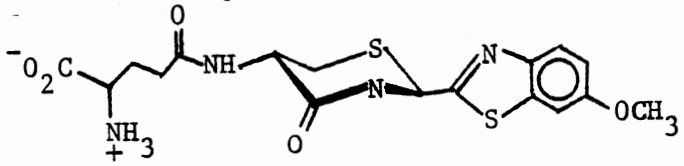


Figure 5. Distribution of Amino Acids, Methyl Green and the Representative Experimental Results

Sample 1: Reduced Glutathione, 2-Cyano-6-Methoxybenzothiazole and Valine (Internal Standard). 1 is an unknown fluorescent species; 2 is believed to be



Sample 2: Control; Reduced Glutathione and Valine

Sample 3: 2-Cyano-6-Methoxybenzothiazole (Washed off by the Chamber Organic Solvent)

detection of the migrating amino acids. Moreover, 10 µl of a standard solution of amino acids (2.5 µmole of each following amino acids:lysine, arginine, histidine, glycine, alanine, valine, leucine, serine, isoleucine, methionine, threonine, proline, glutamic acid, phenoalanine, tyrosine and aspartic acid per ml of H_2O) was applied to each paper in each electrophoresis experiment. The standard solution was purchased from Sondell Scientific Instruments, Inc. After each run, the exact order (55) of each amino acid which appeared on the paper chromatogram was noted and shown in Figure 5. Instead of using a 20 µl solution, as in the preparation of the amino acid analyzer sample, a 10 µl of solution was applied directly to the pre-soaked paper. The paper was then submerged into a cooled (16°C; pH = 1.9 buffer, the same buffer used as in soaking the paper), high voltage electrophoresis chamber. The experiment was initiated by setting the power level at 3000 volts for about 1 hr. The power was stopped when the methyl green dye had migrated to within about three to four inches from the end of the paper chromatogram. This chromatogram was dried in the hood overnight and then was checked for fluorescent spots under a UV and/or IR lamp. Finally, the last step of the experiment involved immersing the paper chromatogram into a staining solution which consisted of cadmium acetate (2 g), acetic acid (40 ml), ninhydrin (1 g) and acetone (200 ml). Each amino acid was detectable on the paper which had been treated with ninhydrin followed by heating in an oven (110°C) for 3 min immediately after the immersion.

Method for Protein Hydrolysis (6 N HCl)

The acid hydrolysis was done under high vacuum (2 mm) to prevent

the oxidation of reactive amino acids during the incubation period ($110^{o}C$/24 hr). The protein sample was dried in a pyrex test tube (3 ml) before it was treated with 200 µl of 6 N HCl. The air was evacuated from the test tube by a vacuum pump while at the same time a point near the open end was heated with a gas-oxygen combination torch. With slow rotation in the flame and constant pulling on both ends of the tube, it was possible to seal the tube. This tube was placed in an oven ($110^{o}C$) for 24 hr. The sealed tube, after the contents had undergone complete hydrolysis, was opened cautiously by applying a hot glass rod to a scratch mark on the pyrex tube. After the sealed tube was opened, H_2O and HCl were removed under vacuum (2 mm) at room temperature.

KF Calculation for Glutathione

It was possible to identify each amino acid, S-carboxymethyl-cysteine $H_3^{+}NCH(CO_2^{-})CH_2SCH_2CO_2^{-}$ and oxidized and reduced glutathione from the information provided by the amino acid analyzer according to the appropriate retention times. The retention times for all detectable species are listed in Table VIII. Using standard solutions, it was possible to determine constant KF which by definition is equal to the peak area (unit area)/amino acid concentration in nmole per ml. Each amino acid had an individual KF value.

The equivalent of glutathione could be calculated from the integrated peaks provided by the amino acid analyzer. Concurrently, the same sample of glutathione was acid-hydrolyzed and the resulting product was analyzed for the equivalents of glycine or glutamic acid residues. Reduced glutathione (100 µl, 0.01 M) and 100 µl of valine

(0.001 M) were added to a test tube. This solution was evaporated completely (2 mm) at room temperature to give a solid. Then 100 µl of H_2O was added to the solid, and 20 µl of the resultant solution was taken for the amino acid analysis. The remaining solution was again evaporated. The residue was hydrolyzed with 6 N HCl as described in Methods for Protein Hydrolysis (6 N HCl). To the final residue was added 80 µl of H_2O. Once again, 20 µl of this solution was taken for amino acid analysis. The analysis data for the samples examined, before and after acid hydrolysis, are in Table IX. With this data, it was possible to determine the KF for glutathione. The calculation for this KF value was done as follows:

Case 1:

$$\frac{144.24 \text{ nmole valine}}{140.96 \text{ nmole valine}} \times 88.06 \text{ nmole of glycine and glutamic acid}$$

$$\text{average} = \frac{8.54 \text{ unit area}}{KF}$$

$$KF = 0.0948 \text{ unit area/nmole}$$

Case 2:

$$\frac{148.08 \text{ nmole valine}}{126.00 \text{ nmole valine}} \times 78.94 \text{ nmole of glycine and glutamic acid}$$

$$\text{average} = \frac{8.57 \text{ unit area}}{KF}$$

$$KF = 0.0924 \text{ unit area/nmole}$$

AVERAGE KF = 0.0936 unit area/nmole

Determination of the Percentage of Reduced Glutathione by Sodium Iodoacetate

Due to the moisture and the long period of storage, some reduced

TABLE IX

BEFORE AND AFTER ACID HYDROLYSIS OF GLUTATHIONE WITH VALINE AS THE INTERNAL STANDARD

	Glycine nmole/ml	Glutamic nmole/ml	Glutathione unit area	1/2 Cystine nmole/ml	Valine nmole/ml
Before Acid Hydrolysis					
Case 1	0	0	8.54	0	144.24
Case 2	0	0	8.57	0	148.08
After Acid Hydrolysis					
Case 1	84.94	91.18	0	37.64	140.96
Case 2	78.51	79.36	0	26.35	126.00

glutathione might have been oxidized. The method for calculating the percentage of reduced glutathione was as follows. The cysteine unit in the reduced glutathione rapidly reacted with iodoacetate and formed S-carboxymethylcysteine which could be detected by the amino acid analyzer. The retention time for S-carboxymethylcysteine and KF were almost identical to these of aspartic acid (56). The retention times for the experimental species were listed in Table VIII. For all practical purposes, the author used the KF of aspartic acid which is 0.1298 for the S-carboxymethylcysteine in order to calculate its concentration.

The experimental approach was to use various amounts of excess iodoacetate to react with freshly prepared reduced glutathione. All of the following solutions were made in the pH 8.0 buffer: 10 µl of 0.01 M freshly-prepared, reduced glutathione, and 100 µl of 0.001 M valine were mixed in each of the six 3 ml pyrex test tubes. To the six labelled test tubes were added 0, 10, 30, 50, 100, and 200 µl of 0.1 M sodium iodoacetate, respectively. The mixture was purged thoroughly with N_2 and then allowed to stand at room temperature for 2.5 hr. The tubes were transferred to an incubator (37°C) for 4 hr. It was assumed that all cysteine had reacted with iodoacetate. Thus, tubes 1-6 were dried in vacuum (2 mm), and then acid hydrolysis (6 N HCl) was performed on the contents immediately. After the hydrolysis, glycine, glutamic acid and S-carboxymethylcysteine were analyzed in the amino acid analyzer. Partial results of the analysis (only the S-carboxymethylcysteine was shown) outlined in Figure 6. From this experiment, the conversion of reduced glutathione could be estimated as 92%.

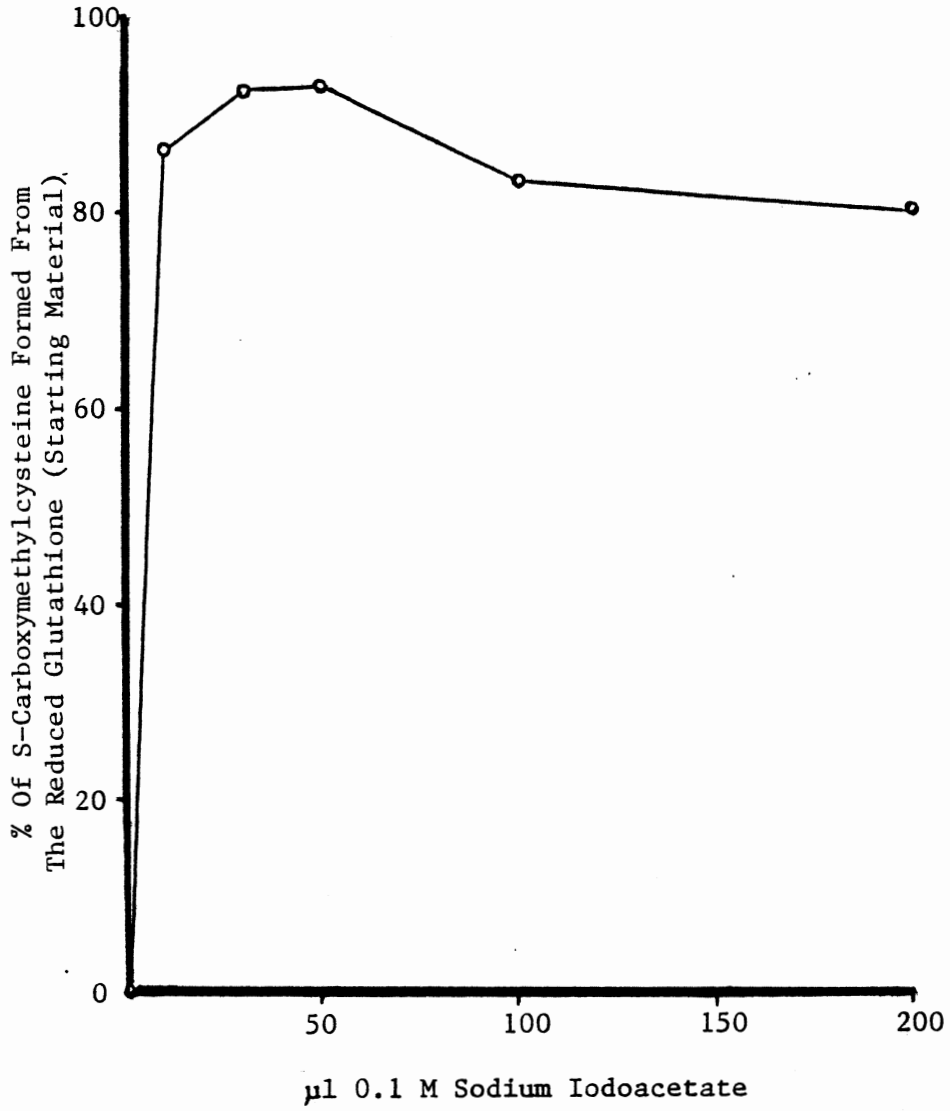


Figure 6. The Results from the Analysis for the Percentage of Reduced Glutathione by Sodium Iodoacetate

Sample Calculation for Reduced Glutathione Cleavage Percentage

Perhaps this could be the most crucial calculation within the entire thesis. For example, the data from the amino acid analyzer showed that unreacted glutathione had 3.75 unit area. Thus the amount of unreacted glutathione = 3.75/0.0936 = 40.06 nmole. With the same data from the analysis, the equivalents of glycine were also determined. For instance, if there was 65 nmole of glycine in the same sample with 40.06 nmole of unreacted glutathione (calculated from above), then the % of cleavage would obviously be, (65.00/65+40.06) X 100 = 62%.

However, from the sodium iodoacetate experiment (Figure 6), there was only 8% unreduced glutathione detected. Thus, the true reduced glutathione cleavage percentage would be (0.62/0.92) X 100 = 67.39%.

CHAPTER IV

RESULTS

In our efforts to cleave cysteine on the carbonyl side of the peptide bond with 2-cyano-6-methoxybenzothiazole, many solvents and reaction conditions were examined. The most successful solvent system employed 150 ml pyridine:100 ml H_2O:29 ml N-ethylmorpholine and enough acetic acid to obtain a pH 8.0 for the reduced glutathione/2-cyano-6-methoxybenzothiazole coupling step. Using the most successful solvent, as indicated in Table X, acetic acid provided 61.8% net cleavage. However, in the experiments the acetic acid/pyridine combination did not greatly affect the acid cleavage much. Among the less successful solvent combinations used before the acid cleavage to initiate coupling of 2-cyano-6-methoxybenzothiazole and reduced glutathione was ethanol/N-ethylmorpholine/H_2O/acetic acid (pH 8.0 buffer). The amount of cleavage only about 2-4%. Another combination of acetone/N-ethylmorpholine/H_2O/acetic acid (pH 8.0 buffer) was used before the acid cleavage step, but the yield was improved only to about 10-30% overall.

In the experimental results reported in Table XI, it was the author's mistake by not using the same amount of 2-cyano-6-methoxybenzothiazole as the experiments in Table X for the 2-cyano-6-methoxybenzothiazole-reduced glutathione coupling reaction in the pH 6.8 buffer. Otherwise one could definitively conclude the fact that pH 8.0 coupling was more efficient than the one in pH 6.8.

TABLE X

ACID CLEAVAGE STEP BY THE ACETIC ACID/PYRIDINE COMBINATION[1]

Tube[2]	Acetic Acid/Pyridine (v/v)	Glutathione	Glutamic nmole/ml	Glycine	% Cleavage[3]	Corrected %[4] Cleavage[2]
1	100/0	56.20	0	74.17	56.9	61.8
2	99/1	255.77	0	541.69	67.9	73.8
3	95/5	69.12	0	77.98	53.0	57.6
4	90/10	169.98	0	386.00	69.4	75.5
5	75/25	66.67	0	91.25	57.8	62.8

[1]To each tube was placed 20 µl of 0.05 M 2-cyano-6-methoxybenzothiazole, 10 µl of 0.01 M reduced glutathione and 100 µl of pH 8.0 buffer.

[2]The controls for tubes 1-5 (without 2-cyano-6-methoxybenzothiazole) displayed no cleavage at all.

[3]Calculations were based upon the amount of glycine which was cleaved divided by (the original equivalent of glutathione, which is the nmole of glutathione determined after the cleavage process + nmole glycine). The amount of glycine and glutathione after the cleavage reaction is listed in the Table.

[4]Only 92% glutathione was in reduced form in the starting material; therefore the true reduced glutathione's cleavage % was (% cleavage/92.0 x 100 or = corrected % cleavage.

TABLE XI

2-CYANO-6-METHOXYBENZOTHIAZOLE AND REDUCED GLUTATHIONE UNDERGOING NEUTRAL pH COUPLING[1]

Tube[2]	Acetic Acid/Pyridine (v/v)	Glutathione	Glutamic nmole/ml	Glycine	% Cleavage	Corrected % Cleavage
1	100/0	57.5	0	62.6	52.3	56.9
2	99/1	98.08	0	55.17	36.0	39.1
3	95/5	63.57	0	62.97	49.8	54.1
4	90/10	93.59	0	47.36	33.6	36.5
5	75/25	73.61	0	51.96	41.4	45.0

[1]To each tube, 5 µl of 0.05 M 2-cyano-6-methoxybenzothiazole, 10 µl of 0.01 M reduced glutathione and 100 µl of pH 6.8 buffer.

[2]The controls for tubes 1-5 (without 2-cyano-6-methoxybenzothiazole) displaced no cleavage at all.

The results reported in Table XII clearly indicated that the efficiency of acid cleavage decreased in the order: acetic > propanoic > formic > trifluoroacetic acid. Moreover, two hours of incubation in the acid was insufficient for the acid cleavage step.

From the Mechanism II in the Discussion, H_2O is also involved in the role of acidic catalytic cleavage process. Therefore the experiments in Table XIII employed the combination acid/pyridine/H_2O for the acid cleavage step. However, both pyridine and H_2O provided no substantial improvement over the results obtained from using pure acids.

The results of experiments reported in Table XIV were interesting. However, another cleavage occurred to give 7-10 nmole of glutamic acid ($\sim$ 5% equivalent of glutathione) which is extremely high. However, the improvement of the specific cleavage was obvious; in vacuum, the thiols were not being oxidized by the oxygen. Thus, the maximum amount of thiol was available to react with the 2-cyano-6-methoxybenzothiazole. Moreover, the 32°C incubation (instead of 37°C) might have helped the coupling step because the reaction entropy would favor the association (2-cyano-6-methoxybenzothiazole-glutathione coupling) at a lower temperature.

All of the high voltage paper chromatographic data for glutathione cleavage are quite reproducible. A representation of the typical experimental result is outlined in Figure 5 (page 32).

TABLE XII

TIME STUDY FOR ACID CLEAVAGE STEP WITH TRIFLUOROACETIC, FORMIC, ACETIC AND PROPANOIC ACID

Tube	Period for Acid Incubation (hr)	Glutathione	Glutamic nmole/ml	Glycine	Acid	% Cleavage	Corrected % Cleavage
1	6	21.26	0	0	TRIF.	0	0
2	0	88.14	0	5.08	TRIF.	5.4	5.9
3	2	94.20	0	11.31	TRIF.	10.7	11.7
4	6	79.91	0	28.93	TRIF.	26.6	28.9
5	6	152.03	0	0	FORM.	0	0
6	0	113.14	0	4.79	FORM.	4.1	4.41
7	2	71.90	0	30.08	FORM.	29.5	32.1
8	6	66.24	0	37.46	FORM.	36.1	39.3
9	6	150.32	0	0	ACET.	0	0
10	0	100.21	0	16.40	ACET.	14.1	15.3
11	2	62.39	0	78.79	ACET.	55.8	60.7
12	6	67.41	0	76.36	ACET.	53.1	76.4
13	6	114.74	8.08	0	PROP.	0	0

TABLE XII (Continued)

Tube	Period for Acid Incubation (hr)	Glutathione	Glutamic nmole/ml	Glycine	Acid	% Cleavage	Corrected % Cleavage
14	0	83.54	0	8.66	PROP.	9.4	10.21
15	2	50.43	5.29	55.73	PROP.	52.5	57.1
16	6	51.71	7.12	62.30	PROP.	54.6	59.4

All tubes contained 10 µl of 0.01 M reduced glutathione and 100 µl pH 8.0 buffer, and all contained 5 µl of 0.005 M 2-cyano-6-methoxybenzothiazole except for tube 1, 5, 9 and 13.

TABLE XIII

ACID CLEAVAGE WITH THE COMBINATION OF ORGANIC ACID/PYRIDINE/H_2O (VALINE AS AN INTERNAL STANDARD)

Tube	Acid/Pyridine/H_2O (V/V/V)	Glutathione	Glutamic nmole/ml	Glycine	Acid	% Cleavage	Corrected % Cleavage
1	100/0/0	37.39	0	53.84	ACET.	59.01	64.15
2	95/5/5	34.29	0	54.80	ACET.	61.51	66.86
3	90/10/0	39.74	0	55.15	ACET.	58.12	63.17
4	80/20/0	42.32	0	52.07	ACET.	55.17	59.97
5	90/5/5	35.26	0	50.30	ACET.	58.79	63.90
6	90/5/5	89.20	0	0	ACET.	0	0
7	80/10/10	30.55	0	41.92	ACET.	57.84	62.87
8	80/10/10	91.23	0	0	ACET.	0	0
9	60/20/20	39.96	0	56.96	ACET.	52.41	56.96
10	60/20/20	75.96	0	0	ACET.	0	0
11	95/0/5	37.18	0	47.00	ACET.	55.83	60.69
12	95/0/5	N.A.	N.A.	0	ACET.	(F.P.E.) 0	$\sim$ 0
13	90/0/10	34.08	0	47.53	ACET.	58.24	63.30

TABLE XIII (Continued)

Tube	Acid/Pyridine/H_2O (V/V/V)	Glutathione	Glutamic nmole/ml	Glycine	Acid	% Cleavage	Corrected % Cleavage
14	90/0/10	91.56	0	0	ACET.	0	0
15	80/0/20	32.69	0	34.46	ACET.	51.32	55.78
16	80/0/20	96.15	0	0	ACET.	0	0
17	100/0/0	30.88	0	35.69	PROP.	53.62	58.28
18	95/5/0	33.33	1.99	37.23	PROP.	52.76	57.35
19	90/10/0	31.94	0	36.3	PROP.	53.19	57.82
20	80/20/0	36.00	1.76	34.92	PROP.	49.24	53.35
21	90/5/5	33.54	0	34.30	PROP.	50.55	54.95
22	90/5/5	N.A.	N.A.	0	PROP.	(F.P.E.) 0	∿ 0
23	80/10/10	34.51	0	34.61	PROP.	50.07	54.43
24	80/10/10	N.A.	N.A.	N.A.	PROP.	(F.P.E.) 0	0
25	60/20/20	44.12	0	29.46	PROP.	40.04	43.51
26	60/20/20	N.A.	N.A.	N.A.	PROP.	(F.P.E.) 0	0
27	95/0/5	32.91	0	38.15	PROP.	53.69	58.36

TABLE XIII (Continued)

Tube	Acid/Pyridine/H_2O (V/V/V)	Glutathione	Glutamic nmole/ml	Glycine	Acid	% Cleavage	Corrected % Cleavage
28	95/0/5	N.A.	N.A.	N.A.	PROP.	(F.P.E.) 0	0
29	90/0/10	32.26	0	31.30	PROP.	49.24	53.52
30	90/0/10	N.A.	N.A.	N.A.	PROP.	(F.P.E.) 0	0
31	80/0/20	29.06	0	23.3	PROP.	44.50	48.37
32	80/0/20	N.A.	N.A.	N.A.	PROP.	(F.P.E.) 0	0

The controls were tubes 6, 8, 10, 12, 14, 16, 22, 24, 26, 28 and 30, which did not contain the 20 µl of 0.05 M 2-cyano-6-methoxybenzothiazole. All tubes contained 10 µl of 0.01 M reduced glutathione, 100 µl of 0.001 M L-Valine pH 8.0 buffer.

N.A. = not available

(F.P.E.) = from high voltage paper electrophoresis chromatogram.

TABLE XIV

COUPLING UNDER VACUUM OF 2-CYANO-6-METHOXYBENZOTHIAZOLE WITH REDUCED GLUTATHIONE FOR 3 HOURS AT 32°C

Tube	Acid/Pyridine (V/V)	Glutathione	Glutamic nmole/ml	Glycine	Acid	% Cleavage	Corrected % Cleavage
1	100/0	39.96	10.94	111.92	ACET.	73.69	80.1
2	95/5	49.34	0	84.31	ACET.	63.08	69.56
3	90/10	39.74	0	75.40	ACET.	65.48	71.18
4	50/50	51.49	7.94	64.37	ACET.	59.22	64.37
5	100/0	65.05	7.93	32.98	PROP.	33.64	36.61
6	95/5	46.79	6.79	33.76	PROP.	41.92	45.56
7	75/25	35.26	9.03	35.84	PROP.	50.41	54.79

To each tube, 10 µl of 0.01 M reduced glutathione, 20 µl of 0.05 M 2-cyano-6-methoxybenzothiazole and 200 µl of valine pH 8.0 buffer were added.

CHAPTER V

DISCUSSION

Mechanism

From the data which was presented in Chapter III, there appears to be two reasonable mechanisms to explain the observed cleavage of reduced glutathione. Of course, other mechanisms could be operational but the two given ones seem to fit the conditions used and the results obtained. Figures 7 and 8 contain the pathways we suggest for the cleavage of reduced glutathione with 2-cyano-6-methoxybenzothiazole.

Mechanism I and II

In mechanism I, formation of the crucial cleavage intermediate VIII depends upon the electronically rich cyano nitrogen to undergo nucleophilic attack on the peptide backbone. The more electron rich the nitrogen, the more favorable the nucleophilic attack would be. On the other hand, in mechanism II, the crucial cleavage intermediate IX depended upon the more electron deficient carbon in the cyano group to participate in the cyclization. The more electron deficient this carbon, the more favorable the cyclization should be.

The cyclization step in mechanism I or in mechanism II could be the rate limiting step for the entire cleavage process. Step 1 in both mechanisms as shown in Figures 7 and 8 is believed to be controlled by

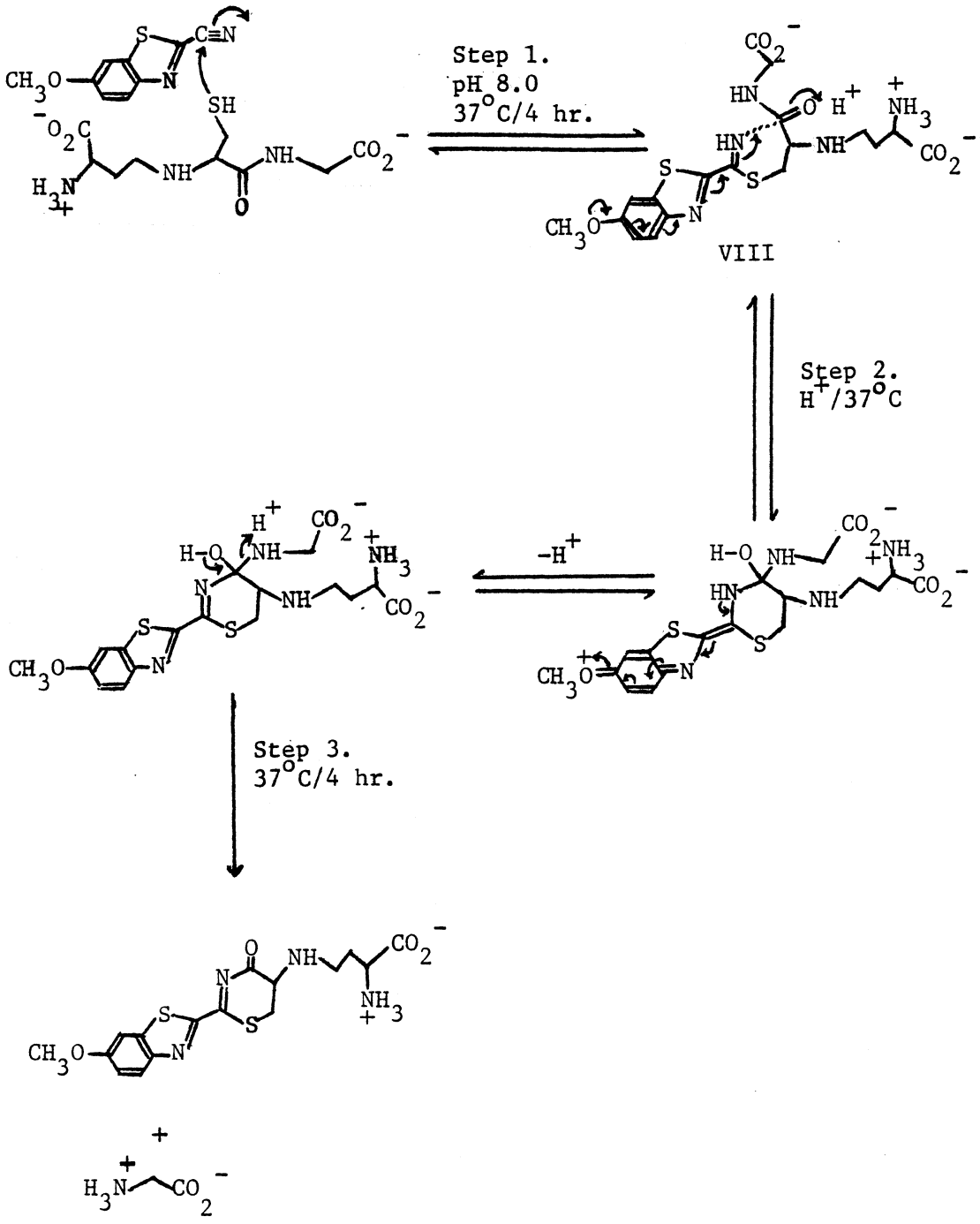


Figure 7. Proposed Mechanism I for Glutathione Cleavage by 2-Cyano-6-Methoxybenzothiazole

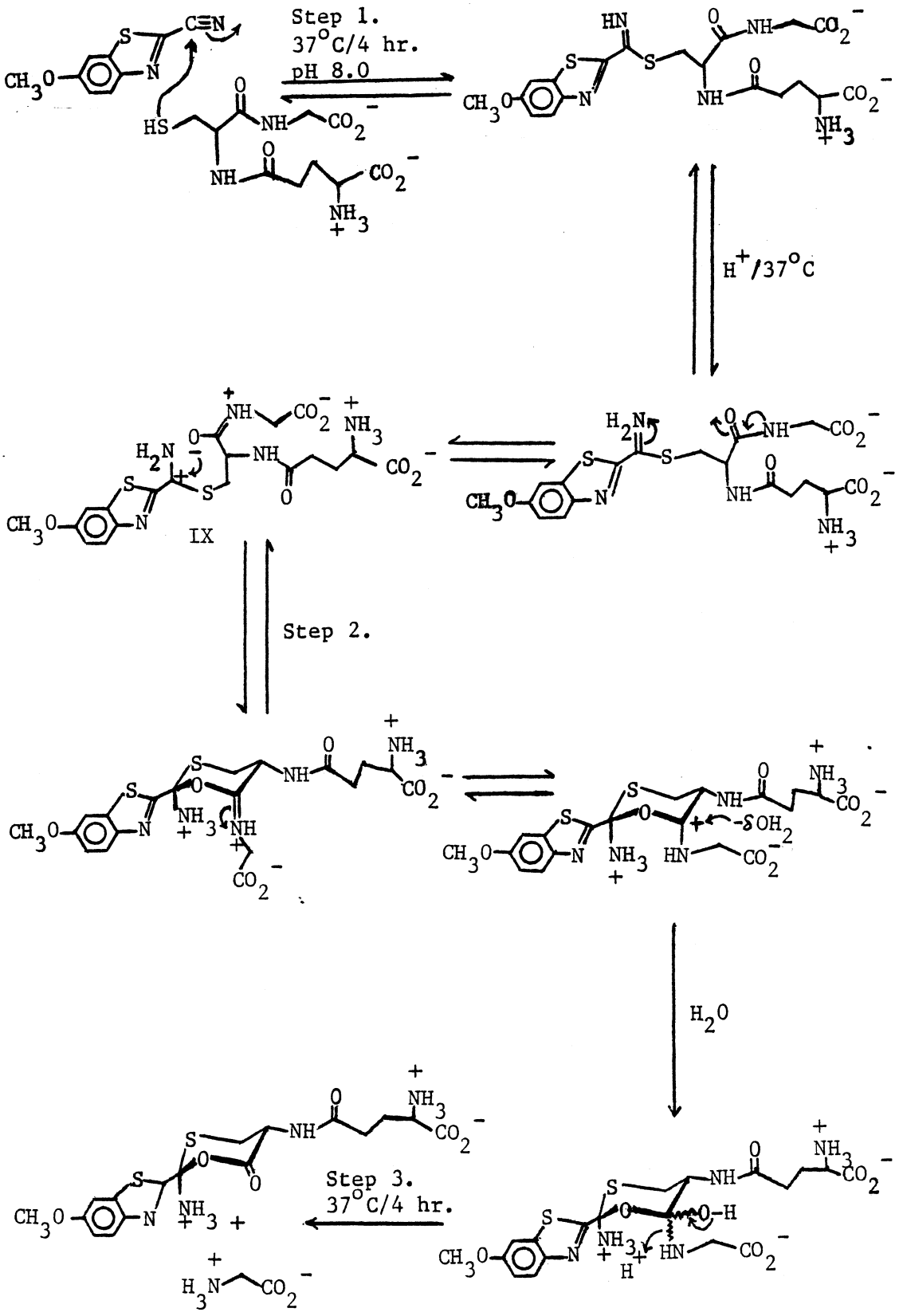


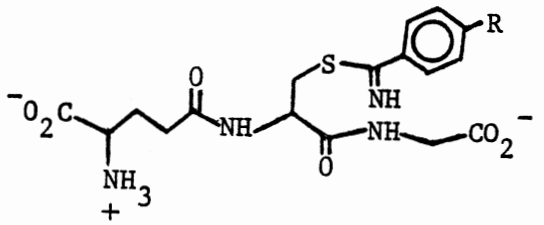
Figure 8. Proposed Mechanism II for Glutathione Cleavage by 2-Cyano-6-Methoxybenzothiazole

an equilibrium process. In essence, an excess of the 2-cyano-6-methoxybenzothiazole would react with the majority of the reduced glutathione and strongly favor the product of step 1 reaction. Similarly, step 3 also favored its product which was the result of cleavage, because in both mechanisms I and II the peptide carbonyl carbon (after the cyclic closure) became a highly unstable asymmetric center. Moreover, since step 3 was done under mild heating (37°C), the entropy of the reaction could strongly favor cleavage. In fact, the author believes step 3 is the fastest step for both mechanisms.

Since mechanisms I and II had exactly opposite characteristics for the limiting step (step 2), being electronically rich and poor, respectively, the author felt that he could distinguish which mechanism was operating for the cleavage by setting individual experiments with an electron deficient cyano derivative, 4-cyanophenol (HO-⟨⟩-CN) and an electronic poor cyano derivative, p-nitrobenzonitrile (O_2N-⟨⟩-CN). Both reagents were purchased from Aldrich Chemical, Inc. If mechanism I was operational, 4-cyanophenol would provide a higher concentration of glycine than p-nitrobenzonitrile. On the other hand, if mechanism II was operational, 4-cyanophenol would provide a lower concentration of glycine than p-nitrobenzonitrile. If the cyclic arrangement occurred at all with both reagents, the difference for peptide cleavage by 4-cyanophenol and p-nitrobenzonitrile would be expected to be overwhelming.

Unfortunately, by using trifluoroacetic, formic or acetic acid, no cleavage was detected from either reagent, even though both p-nitrobenzonitrile and 4-cyanophenol, from high voltage paper chromatography, had shown covalent attachments to the reduced glutathione. The covalent

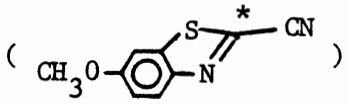
attachments were concluded from the fact that the R_f(s) of reacted glutathione shift extensively lower than the free reduced glutathione on the high voltage paper chromatogram. Presumably species such as



where R = NO_2 for p-nitrobenzonitrile, and R = OH for 4-cyanophenol, were formed. After experiencing the failure of this part of the experiment, verification of the proper mechanism was still inconclusive. Thus, additional evidence for a mechanism has yet to be obtained.

One More Possible Mechanism

We predicted thiol anion of cysteine undergoes nucleophilic attack on the cyano carbon of 2-cyano-6-methoxybenzothiazole. If the thiol anion attacked the α carbon* to the cyano group



it could possibly provide us with a reasonable cleavage mechanism as well. The third mechanistic speculation is formulated in Figure 9. The third mechanistic proposal is the most unlikely model for the following reasons: 1) Nucleophilic attack on the peptide carbonyl by a secondary amine is extremely difficult and 2) Steric effects are too great to favor the product of step 2 in Figure 9. Therefore, by eliminating

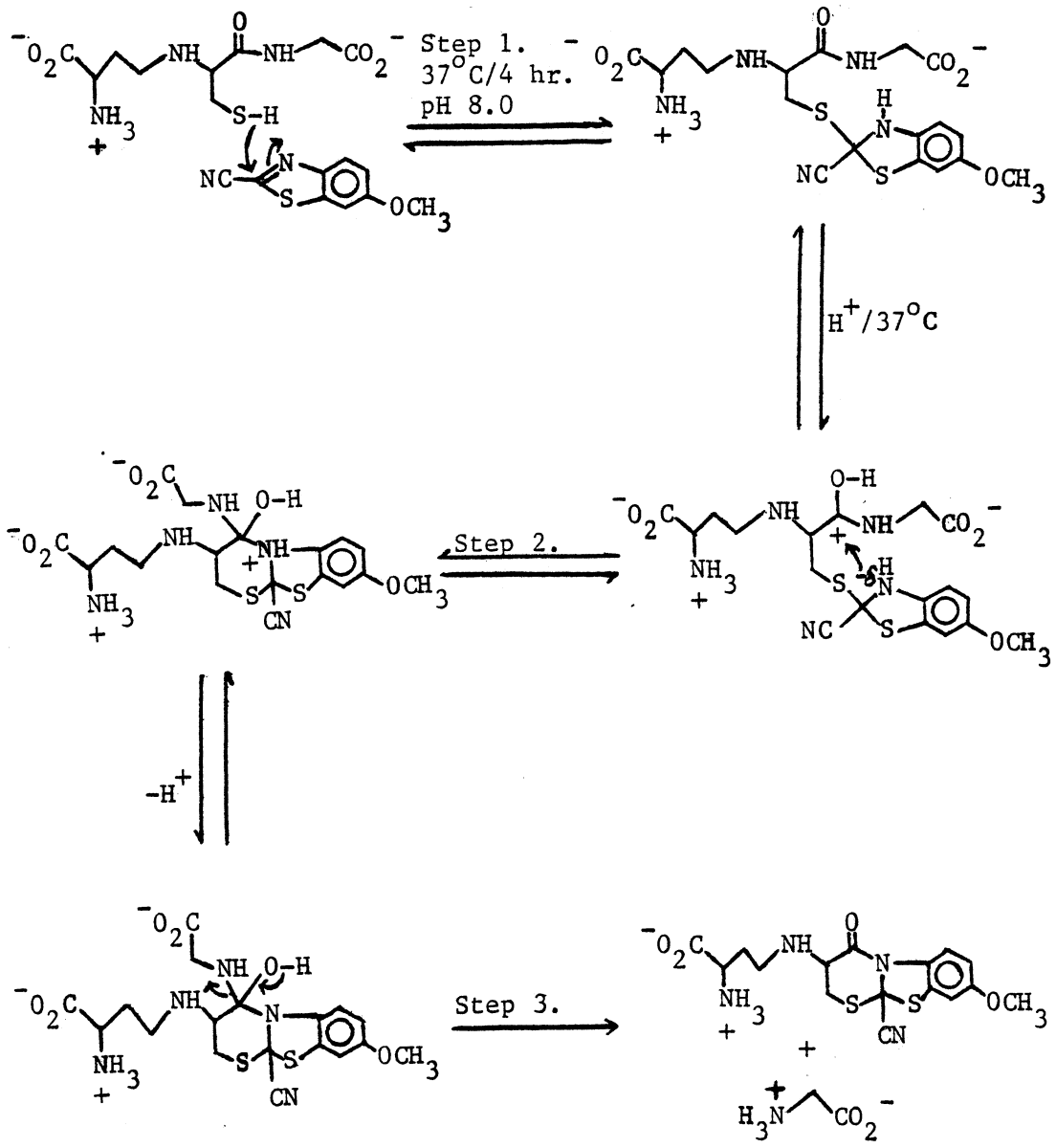
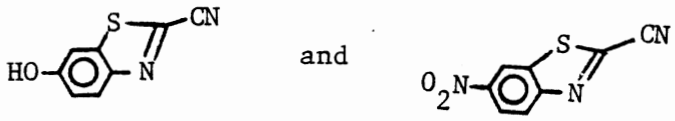


Figure 9. Proposed Mechanism III for Glutathione Cleavage by 2-Cyano-6-Methoxybenzothiazole

the mechanism III, mechanisms I and II are still the most possible ones.

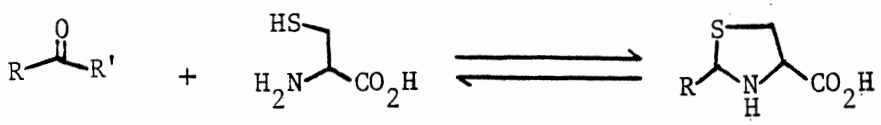
After the failure of simple mechanistic verification methods, as mentioned earlier, verifying the proper mechanism is not an easy matter. Isolating the final cleaved product and determining its structure by NMR and mass spectrometry would be the most reasonable approach to the problem. A simpler alternative, replacing 4-cyanophenol and p-nitrobenzonitrile with the relatives



shown might give evidence that one mechanism is more defensible than another. However, it may be difficult to rule out one mechanism completely based on any one simple test.

Reactions of Amino Thiols with Aldehydes and Ketones

The thiol group appears to be quite active toward carbonyls. Frequently the reaction is an equilibrium process which is pH dependent (57-63). A representative reaction of this kind follows:

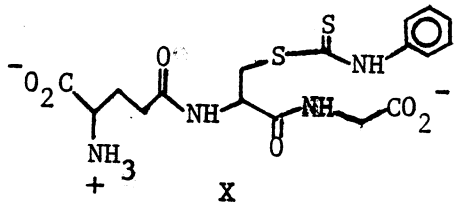


Thus, in the experimental procedure (Chapter III) in order to obtain a maximum concentration of glutathione in the reduced form, the carbonyl-containing acetone is evacuated before adding reduced glutathione.

Reactions Between Edman Reagent and

Reduced Glutathione

Since thiol is a good nucleophile, the thiol group of reduced glutathione may react with the Edman Reagent much easier than in the usual reaction between the H_2N-terminus and the Edman Reagent. In the pH 8.0 buffer, the S-Edman reagent linkage, X presumably is formed.



X

The conclusion is drawn from the fact that the R_f of the reacted glutathione (with Edman Reagent) shifted extensively lower than the free reduced glutathione on the high voltage paper chromatogram. However, treating X with trifluoroacetic acid at 37°C does not initiate the type of cleavage as with 2-cyano-6-methoxybenzothiazole. Actually, a reasonable cleavage mechanism can be summarized in Figure 10. However, the cleavage attempt is not successful so far.

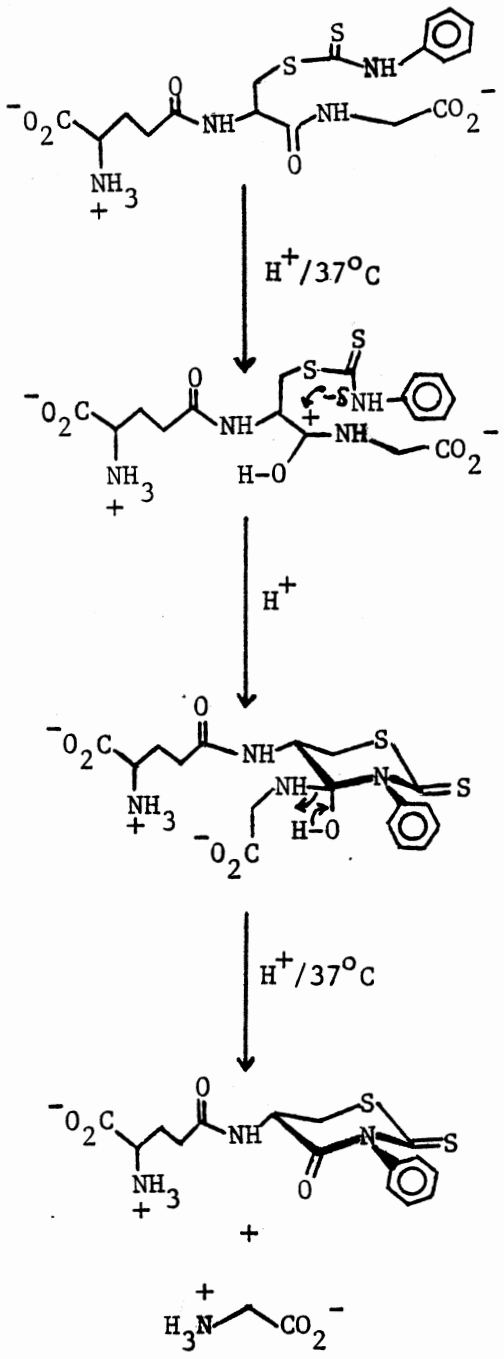


Figure 10. Proposed Mechanism for Glutathione Cleavage by the Edman Reagent

CHAPTER VI

SUMMARY

The object of this study was to explore the use of 2-cyano-6-methoxybenzothiazole as a peptide bond cleavage agent specific for the carbonyl side of cysteine residues in a peptide or protein. In this study, reduced glutathione was used as a simple model system to evaluate the efficiency and specificity of the cleavage under a variety of experimental conditions.

Results from the amino acid analysis of glycine which is one of the cleavage products showed that under the best conditions, 76% of cleavage of the reduced glutathione were observed. However, the sample of the reduced glutathione used in this study contained only 92% of the reduced form, based on the alkylation study with iodoacetate. Therefore, the maximum cleavage after correction can be as high as 80%. Cleavage of the other peptide bond in glutathione will result in the release of glutamic acid. Since very little glutamic acid could be detected, it was concluded that cleavage was limited only to release glycine. Thus the present study showed the reagent, 2-cyano-6-methoxybenzothiazole, is specific for cysteinyl peptide bond cleavage and provides a high yield. The cleavage process was relatively clean and left a new free H_2N-terminal. Thus the Edman reagent can then be used to sequence amino terminal. Thus the Edman reagent can then be used to sequence amino acid residues after the cysteine. Therefore, the 2-cyano-6-methoxyben-

zothiazole appears to have considerable potential as a useful reagent in protein chemistry and may have a broad scope in other utilities as well.

Several cleavage mechanistic speculations were mentioned. However, not enough evidence uncovered to substantiate any one of the three possible mechanisms yet.

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