CONVERSION OF CYSTEINE TO SERINE IN REDUCED GLUTATHIONE*

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Recently Gross and Witkop (1962) have demonstrated cleavage at the carboxyl group of methionine in RNase with cyanogen bromide and conversion of the methionine to a C-terminal homoserine lactone. Stimulated by this work, we thought that this method might be applicable also for cysteine containing peptides. Methylation of cysteinyl residues would result in formation of S-methyl-cysteine. Reaction of cyanogen bromide with this derivative should result in a sulfonium intermediate. Methylthiocyanate might possibly be eliminated via formation of a four-membered serine lactone. However, this involves the formation of a strained four-membered ring. Elimination should be favored by the attack of the carbonyl group on the amino side of S-methyl-cysteine. In this case there would be a five-membered cyclic intermediate, the oxazoline ring. This is also the intermediate postulated for the N to O acyl shift of serine residues in peptides. In acid, it would open to form a seryl ester linkage. In base the oxazoline ring would open to leave a peptide with serine in place of the

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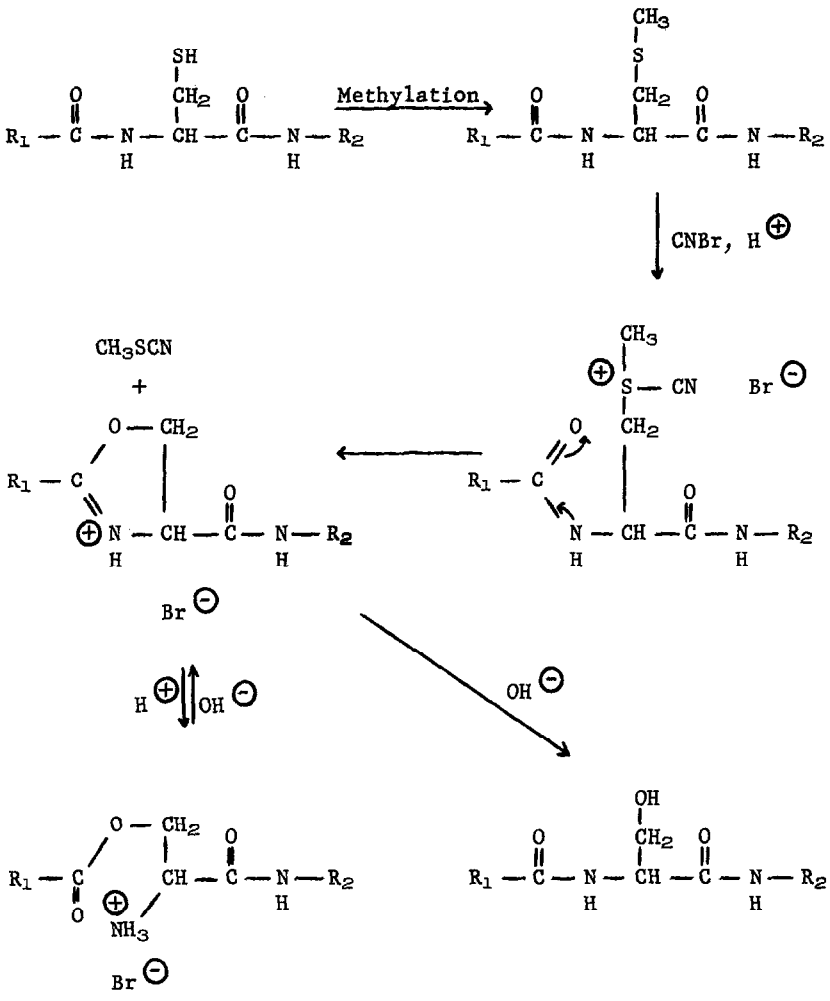
original cysteine. The pathway is summarized below:



We have tested this hypothesis using reduced glutathione as a substrate model.

Materials and Methods: One-half ml of concentrated NH_4OH was added to 10 ml of distilled water and bubbled with nitrogen for 10 minutes to remove oxygen. 200 mgm (0.65 mmole) of reduced glutathione (CalBiochem.) were added under nitrogen to this solution. To this was added 0.2 ml (0.456 g or 3.2 mmoles) of methyl iodide. The reaction was stirred vigorously with precautions taken to

exclude air. At half-hour intervals, samples were taken and tested by nitroprusside reaction. When a sample gave a negative test, the reaction was stopped by immediate lyophilization. In initial experiments the addition of cyanogen bromide after acidification of the S-methyl-glutathione resulted in the development of an orange color, which proved to be due to iodine formed from the HI bound by the peptide during methylation. Thus, it appears that cyanogen bromide oxidizes iodide. Since any iodine formed during the reaction might convert the sulfide to the sulfoxide and since the latter could not form a sulfonium salt with CNBr, it became necessary to remove the iodide before reaction with CNBr. The ion retardation resin AG 11A8 (Bio-Rad Laboratories) was used. A column 16 X 1.8 cm (25 g of resin) was prepared and washed with acid, base, salt solution, and distilled water successively as recommended by Bio-Rad Laboratories (1963). The last liter of water wash was bubbled with nitrogen to exclude oxygen. 180 mgm of S-methyl-glutathione were added to the column. About one-half was eluted with distilled water as a breakthrough peak and the rest was eluted with 0.17 N acetic acid. Both peaks were iodide free. This probably does not represent separation of two peptide species, but rather that the peptide binding capacity of the column had been exceeded. With a larger column and less peptide applied, ninhydrin positive material was eluted as one peak only after addition of 0.17 N acetic acid.

The reaction with CNBr was prepared as follows: 10 mgm (0.03 mmole) of iodide free S-methyl-glutathione were dissolved in 3 ml of nitrogen bubbled 0.1 N HCl. To this were added 106 mgm (1 mmole) of cyanogen bromide. The mixture was stirred at room temperature. After 24 hours the material was lyophilized. Increases in temperature to 45° C and time to 48 hours gave essentially the

same results by amino acid analysis. Reactions in 50%, 90%, and 98% formic acid gave results similar to that in 0.1 N HCl.

Acid hydrolyses of the peptide at different steps were performed and analyzed by a Technicon autoanalyzer.

Results: The results of the acid hydrolyses are shown in Fig. 1 and Table 1. The chromatograph of hydrolyzed reduced glutathione shows the expected amino acids plus a small peak of unknown nature appearing after glutamic acid with a 440 to 570 mμ absorption ratio of greater than one. The pattern of hydrolyzed S-methyl-glutathione shows almost complete loss of cysteine with the appearance of a large new peak after glutamic acid. This is S-methyl-cysteine. It appears in a position very close to where a proline standard appeared. Methylation of a known amount of cysteine with methyl iodide was used to determine a chromatographic

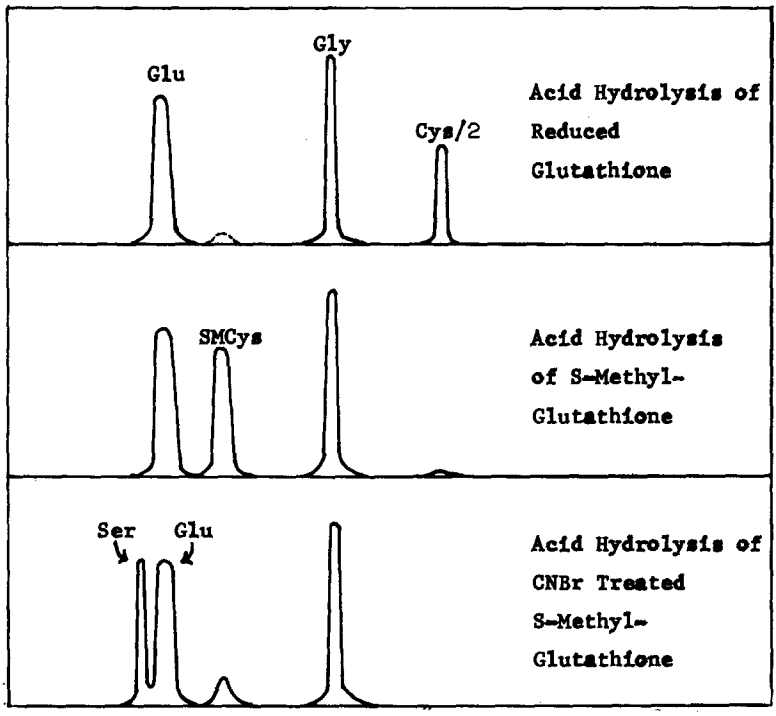


Figure 1. Chromatograms at different stages of acid hydrolyses of the peptide and products of reaction as indicated. Continuous line: 570 mμ absorption. Dashed line: 440 mμ absorption. SMCys = S-methyl-cysteine

integration constant of 15.4 for S-methyl-cysteine. Also shown is a chromatograph of the hydrolyzed product of CNBr treated S-methyl-glutathione. Here a new peak appears in the position of serine and there is a great diminution in the S-methyl-cysteine peak.

Table I gives the quantitative values of these chromatographs in molar ratios with respect to glycine. As is seen, the proportion of glutamic acid to glycine is fairly constant. About 50% of the cysteine is converted to serine.

Table I

Results of Amino Acids Obtained from Acid Hydrolyses

Substrate	Ser	Glu	S-M-Cys	Gly	Cys/2
Reduced Glutathione		0.92		1.00	0.38
S-Methyl-Glutathione		0.92	1.01	1.00	
CNBr treated S-Methyl-Glutathione	0.51	0.90	0.14	1.00	

The above data is not indicative of the mechanism by which serine is formed. If, however, serine were formed via the lactone, free glycine should appear quantitatively after the CNBr reaction. If serine were formed via the oxazoline ring, there would appear a neutral triamino acid compound, γ-glutamyl-O-serylglycine. This would have two α amino groups and two α carboxyl groups. In base this would rearrange to form the tripeptide, γ-glutamylserylglycine, having one α amino group, two α carboxyl groups, and one primary alcohol group. Such an acidic peptide should appear very early in a standard amino acid chromatograph. This, of course, is without the consideration of molecular size. Accordingly, chromatographs of CNBr treated S-methyl-glutathione were performed before and after treatment with 0.5 M Tris buffer pH 8. Figure 2 gives the

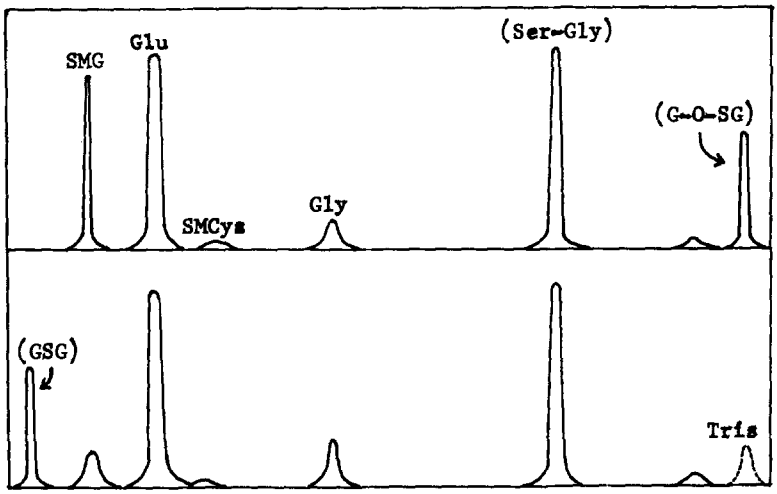


Figure 2. Chromatograms of CNBr treated S-methyl-glutathione. Upper pattern: products untreated and kept in 0.1 N HCl. Lower pattern: products placed in 0.5 N Tris buffer, pH 7.98, for 18 hours before chromatography. Continuous line: 570 mμ absorption. Dashed line: 440 mμ absorption. Compounds in parentheses only tentatively identified without confirmation with standards. SMG = S-methyl-glutathione; SMCys = S-methyl-cysteine; G-O-SG = γ-glutamyl-O-serylglycine; GSG = γ-glutamylserylglycine.

chromatographic patterns. A small amount of free glycine appears. This may be due to a portion of the reaction going via the lactone mechanism. However, it might be due to nonspecific acid hydrolysis during the CNBr reaction. Hofmann (1964) noted nonspecific peptide cleavage during the reaction of trypsinogen with CNBr in 0.1 N HCl. A large amount of glutamic acid is seen. This is probably due to hydrolysis of the γ-glutamyl-O-serylglycine ester under the mild acid conditions of the reaction. Accordingly, one large peak has been tentatively designated as serylglycine. A third peak shows a marked change in chromatographic position after Tris buffer treatment, in the direction expected of the serine containing tripeptide. It has been so designated tentatively though not characterized chemically as yet. A peak appears with a 440 to 570 mμ absorption ratio of greater than one. This is Tris buffer.

Discussion: In this simple model system, it appears that application of the Gross and Witkop techniques to a cysteine residue within a peptide allows for conversion to serine with partial cleavage. However, this model is atypical since a γ-glutamyl-cysteine bond was studied. We plan to extend these studies to peptides with α-carboxyl cysteine bonds. Studies are also in progress in an attempt to increase the yield of serine conversion.

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