The Thiolation of Ribonuclease

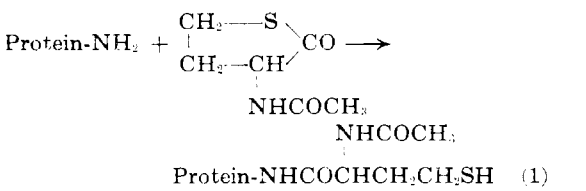
FREDERICK H. WHITE, JR., AND ARMANDO SANDOVAL

From the Laboratory of Cellular Physiology and Metabolism, National Heart Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Maryland

Received June 4, 1962

The thiolating agent, *N*-acetyl homocysteine thiolactone, reacts with the primary amino groups of proteins to introduce the *N*-acetyl homocysteinyl residue. The appearance of sulfhydryl groups during the thiolation of ribonuclease, as well as of *S*-carboxymethyl-homocysteinyl and *S*-carboxymethylcysteinyl residues after reaction of the thiolated protein with iodoacetate, indicated close to three *N*-acetyl homocysteinyl residues added per average molecule of protein after prolonged reaction. Chromatographic examination on CM-cellulose, however, showed the presence of a slow-forming component containing five *N*-acetyl homocysteinyl residues, while other components were less extensively thiolated. In general, this reaction had the effect of lowering the enzymatic activity toward ribonucleic acid and cyclic phosphate, when studied as a function of *p*H, although certain of the chromatographic components exhibited specific activities toward ribonucleic acid that were higher than the native activity, when assayed at low *p*H values. Disulfide interchange proceeded as a secondary reaction during thiolation and was followed kinetically by analysis of the thiolated carboxymethylated protein for its content of *S*-carboxymethylcysteine. When approximately one disulfide bond per average protein molecule was interchanged, there was little effect on the enzymatic activity. With three disulfide bonds interchanged, activity was still observed toward both substrates, although it was appreciably diminished. Therefore, it appears that not all of the native disulfide bonds are essential for the expression of enzymatic activity.

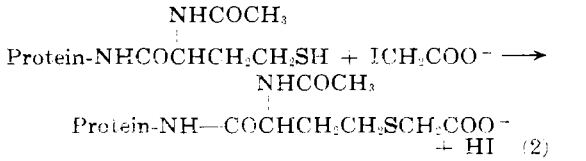
The reaction of primary amino groups in RNase[1] with compounds containing thiolester or thiolactone bonds, to introduce SH groups into the protein molecule (thiolation), proceeds as shown in (1).



Protein-NH_2 + CH_2—S, CH_2—CH, CO → NHCOCH$_3$, NHCOCH$_3$

Protein-NHCOCHCH_2CH_2SH (1)

N-acetyl homocysteine thiolactone, shown above, was used as the thiolating agent throughout the present work. The product of (1) reacts with iodoacetate as shown in (2). This carboxymethylation step has no apparent effect on the enzymatic activity of thiolated RNase. Oxidation of SH groups may therefore be prevented by this reaction and the thiolated protein rendered stable for further study.



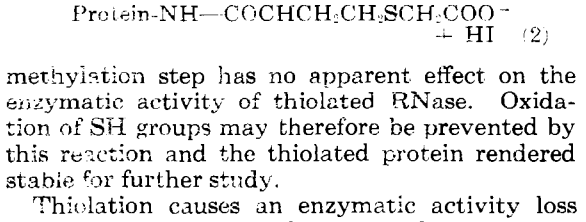
Protein-NHCOCHCH_2CH_2SH + ICH_2COO^- → NHCOCH$_3$

Protein-NH—COCHCH_2CH_2SCH_2COO^- + HI (2)

Thiolation causes an enzymatic activity loss that is more pronounced when performed in the presence of urea (White, 1960). In the absence of urea there is first an increase in activity toward RNA to 140% of the specific activity of native RNase, followed by a relatively slow decrease. Concomitantly, there is only a loss of activity toward uridine-2′,3′-cyclic phosphate (White, 1961a). Because of these activity changes, the thiolation of RNase affords an approach for studying the relationships between enzymatic activity and molecular structure.

Under the present conditions of reaction, an apparent maximum of 3 moles of *N*-acetyl homocysteinyl residue was introduced per mole of protein, although a slow-forming component was detected chromatographically that contained five such groups. Other chromatographically separated components of thiolated carboxymethyl RNase were less extensively thiolated.

Disulfide interchange proceeds as a secondary reaction during thiolation of RNase as shown in (3). The possibility exists that disulfide interchange may be intermolecular as well as intramolecular. There is no reason to believe that the reaction stops at the point shown in (3). Instead, the SH group of the product could react



Protein-NHCOCHCH_2CH_2SH ⇌ S—S

NHCOCH$_3$

Protein-NHCOCH

SH S-S-CH_2CH_2 (3)

[1] The abbreviations used are: AHT, *N*-acetyl homocysteine thiolactone; DNP, dinitrophenyl; Tris, tris(hydroxymethyl)aminomethane.

with another disulfide bond in either the same or a different molecule and the interchange could be continued until a "scrambling" of all disulfide bonds occurs. As shown in the present work, disulfide interchange may also be initiated by the free *N*-acetyl homocysteine that results from the slow breakdown of *N*-acetyl homocysteine thiolactone during thiolation.

Reaction of the product of (3) with iodoacetate would proceed in a manner analogous to that shown for (2), but with *S*-carboxymethylcysteine formed instead of *S*-carboxymethylhomocysteine. Disulfide interchange was followed by analyzing hydrolysates of thiolated carboxymethyl RNase for their content of *S*-carboxymethylcysteine. This reaction was observed to involve approximately one disulfide bond per average thiolated RNase molecule with little or no effect on the enzymatic activity. A product with three interchanged disulfide bonds exhibited appreciably diminished activities toward RNA and cyclic phosphate.

Methods

Thiolation and Carboxymethylation of RNase.—Beef pancreatic RNase (Lot #R31B-204, from the Sigma Chemical Company) was dissolved to 10 mg/ml in 0.1 M ammonium bicarbonate, to which enough 5% aqueous trimethylamine had been added to raise the *p*H to 8.0. An amount of *N*-acetyl homocysteine thiolactone equal to the weight of RNase to be thiolated was added to the RNase solution. This procedure was generally employed for 50 mg of RNase, but occasionally 100 or 200 mg were thiolated at one time. Nitrogen was passed through the solution in a test tube for 15 minutes. The tube was tightly stoppered and allowed to stand at room temperature for the desired time of reaction. Thiolation was ended by precipitation of the protein with 10 volumes of acetone–1 N HCl (40:1) at −5°. After washing with this solvent and ether, as described earlier (Sela *et al.* 1959), the protein was redissolved in water through which nitrogen had been passed for at least 15 minutes at room temperature. The solution could then be lyophilized and the weight of the dried product determined. The thiolation of RNase resulted in yields of from 70 to 90%. The thiolated RNase was carboxymethylated by reaction with iodoacetate exactly as described earlier for reduced RNase (White, 1961b).

A study of the variation of thiolation with *p*H involved use of 0.2 M boric acid–sodium borate buffers of *p*H 7, 8, 9, and 10, other conditions being as described above. However, at *p*H 10, the capacity of this buffer system was not sufficient to maintain a constant *p*H during thiolation. Therefore, aqueous 25% trimethylamine was added by a *p*H-stat (Coleman autotitrator) for the initial 6 hours of reaction. The reaction mixture was contained in an open beaker, and nitrogen was passed continuously through it. After this time there was no further change in *p*H, and the reaction mixture was placed in a test tube, flushed with nitrogen, stoppered, and kept at room temperature for the remaining time of reaction.

When RNase was thiolated in the presence of iodoacetate, the amount of iodoacetic acid used was equal in weight to the protein. The acid was first dissolved in 0.1 M ammonium bicarbonate, and the *p*H was adjusted to 8.0 with aqueous trimethylamine. RNase was then dissolved in the same kind of buffer to produce a protein concentration of approximately 20 mg ml. The iodoacetate was then added with enough additional buffer to lower the protein concentration to 10 mg ml. The *N*-acetyl homocysteine thiolactone was added to start the reaction, and the mixture was treated as outlined above in the absence of iodoacetate.

For kinetic experiments involving assays for enzymatic activity during thiolation, aliquots were removed from the reaction mixture, which was then gassed with nitrogen as before and the tube stoppered. A few thiolations were performed in the presence of 8 M urea for 24 hours at *p*H 8, other conditions being the same as above, with 0.1 M ammonium bicarbonate as the buffer Under the same conditions, except in the absence of *N*-acetyl homocysteine thiolactone, thiolated RNase before and after carboxymethylation was incubated to determine the effects of urea on disulfide interchange and enzymatic activity.

Determination of the Extent of Thiolation.—Thiolation was routinely followed by spectrophotometric titration with mercuribenzoate to determine the SH content, as described earlier for reduced RNase (Sela *et al.*, 1959). After carboxymethylation, amino acid analysis was employed for estimation of *S*-carboxymethylhomocysteine and *S*-carboxymethylcysteine to determine the extent of thiolation. Thiolated carboxymethyl RNase was first hydrolyzed in a vacuum in 6 N HCl (2.5 to 5 mg of protein per ml) for 17 to 20 hours at 110°. The hydrolysate was dried in a vacuum over phosphorous pentoxide and sodium hydroxide pellets and then analyzed on a Spinco model MS amino acid analyzer (Moore *et al.*, 1958) for *S*-carboxymethylhomocysteine and *S*-carboxymethylcysteine. The sum of these amino acids, expressed as moles of residue per mole of RNase, was taken as equivalent to the number of protein amino groups thiolated.

Analyses for *S*-carboxymethylhomocysteine and *S*-carboxymethylcysteine were also carried out by dinitrophenylation of the protein hydrolysate and paper chromatographic separation of the DNP-amino acids. For this procedure, the alkylating agent was iodoacetic acid-1-C^{14}, with a specific activity of 0.2 mc μmole. The dinitrophenylation was performed by the method of Levy (1954) with the modification that the reaction mixture, after ether extraction and acidification, was extracted three times with toluene to remove the bulk of the unwanted DNP-

amino acids. The remaining water solution was extracted exhaustively with ether, and the combined ether extracts were evaporated to dryness over a warm water bath in a current of air. After redissolving in a few drops of acetone, they were applied to Whatman No. 3 MM paper in a circular area of about 1 cm^2, containing between 50 and 200 μg of sample. As many as ten samples could be chromatographed on the same paper, with dimensions of 20 × 57 cm, descending for 24 hours in a closed cylindrical glass tank, 30.5 cm in diameter and 61 cm in height, in the organic phase of secondary butanol and 0.8 N NH_4OH (1:1). Under these conditions, the spot containing DNP-*S*-carboxymethylhomocysteine separated completely from that containing DNP-aspartic acid, -glutamic acid, and -*S*-carboxymethylcysteine.[2] These spots had R_F values of 0.28 and 0.32, respectively.[3] The paper was dried in the dark at room temperature. The DNP-aspartic-glutamic and DNP-*S*-carboxymethylhomocysteine spots were excised and placed in separate tubes. Five ml of water was added to each DNP-aspartic-glutamic spot and 0.5 ml. to each DNP-*S*-carboxymethylhomocysteine spot. The tubes were allowed to stand in a water bath at 65° for 10 minutes, and after they had cooled to room temperature, the optical density of each solution was determined in a Beckman model DU spectrophotometer at 360 mμ. Each solution was acidified with one drop of 1 N HCl and extracted five times with ether; the ether extracts were dried and then redissolved in 2 ml of water. Each of the water solutions was mixed with 10 ml of phosphor solution (Bray, 1960), and the resulting samples were counted in a Packard Tricarb liquid scintillation counting system, model #314 EX.

Calculation of Moles of S-Carboxymethylhomocysteine and S-Carboxymethylcysteine per Mole of RNase from Data Obtained by Spectrophotometry and Scintillation Counting.—The content of *S*-carboxymethylcysteine is given by[4]:

$$x = \frac{ACRa}{M_a - CR_a} \times 1.16 \qquad (4)$$

where x = moles of *S*-carboxymethylcysteine per mole of RNase; A = moles of aspartic and glutamic acids per mole of RNase (= 27 [Hirs *et al.*, 1956]); C = micromoles of iodoacetic acid-1-C^{14} in blank[5]/counts per minute of iodoacetic acid-1-C^{14} in blank = 5.60×10^{-6}; R_a = corrected[6] counts per minute of DNP-aspartic-glutamic eluate; M_a = μmoles of DNP amino acid in DNP-aspartic-glutamic eluate = $(D_a/\epsilon_a) \times 1000 \times$ volume of DNP-aspartic-glutamic eluate in ml; D_a = optical density at 360 mμ of DNP-aspartic-glutamic eluate; and ϵ_a = molar extinction coefficient of DNP-aspartic-glutamic eluate.[7]

The content of *S*-carboxymethylhomocysteine was calculated from the counting data as follows[4,8]

$$y = \frac{CR_s(A + x)}{M_a} \times 1.16 \times 1.05 \qquad (5)$$

where y = moles of *S*-carboxymethylhomocysteine per mole of RNase; R_s = corrected[6] counts per minute of DNP-*S*-carboxymethylhomocysteine eluate. As a check on the content of *S*-carboxymethylhomocysteine, this value was also calculated from the spectrophotometric data as follows[8]:

$$y = \frac{M_s(A + x)}{M_a} \times 1.05 \qquad (6)$$

where M_s = μmoles of DNP-*S*-carboxymethylhomocysteine in eluate = $(D_s/\epsilon_s) \times 1000 \times$

[2] This spot is hereafter referred to as the DNP-aspartic-glutamic spot, whether or not DNP-*S*-carboxymethylcysteine was present in any given sample.

[3] The spots were identified by chromatography of the synthetic amino acids. *S*-Carboxymethylhomocysteine was synthesized by the method of Armstrong (1951) and *S*-carboxymethylcysteine according to Harris *et al.* (1944). The amino acids were then reacted with fluorodinitrobenzene (Sanger, 1945) to obtain the DNP derivatives.

[4] By extraction of solutions containing known quantities of DNP-aspartic acid, -glutamic acid, -*S*-carboxymethylhomocysteine, or -*S*-carboxymethylcysteine with ether after acidification, followed by spectrophotometric determination of the quantities recovered, an average extraction factor of 1.16 was found. This factor was applied to all determinations of *S*-carboxymethylcysteine and *S*-carboxymethylhomocysteine content that were made by scintillation counting of ether extracts.

[5] Prepared by dissolving 2.0 ml of aqueous iodoacetic acid-1-C^{14} (0.2 mc/μmole, 0.5 to 3.0 μg/ml) in 10 ml of phosphor solution (Bray, 1960). These concentrations resulted in a range of cpm expected for the sample solutions. By this procedure, 1 μg of the iodoacetic acid exhibited 959.7 cpm.

[6] Corrections were made for the color quenching effect of DNP amino acids by the method of Baille (1960).

[7] The molar extinction coefficients for DNP-aspartic acid and DNP-*S*-carboxymethylhomocysteine were found to be 1.81×10^4 and 1.75×10^4 respectively. DNP-glutamic acid and DNP-*S*-carboxymethylcysteine possess the same coefficient of 1.48×10^4 (Sela *et al.*, 1959). The values for DNP-aspartic acid and DNP-glutamic acid were averaged to obtain 1.65×10^4 for the DNP-aspartic-glutamic eluate.

[8] A chromatographic correction factor for *S*-carboxymethylhomocysteine was determined by paper chromatography, as described in the text, of a mixture containing 15 moles of aspartic acid and 12 moles of glutamic acid per 1 to 5 moles of *S*-carboxymethylhomocysteine. Spectrophotometric determinations of the concentrations of the eluates indicated an average loss of 5.0% for *S*-carboxymethylhomocysteine relative to the quantities of aspartic acid and glutamic acid recovered. Therefore, a correction factor of 1.05 was applied to all determinations of *S*-carboxymethylhomocysteine content based on spectrophotometric reading or scintillation counting of eluates from paper chromatography. Similar chromatographic comparison between *S*-carboxymethylhomocysteine and *S*-carboxymethylcysteine indicated no such loss of the latter.

volume of eluate in ml; D_s = optical density at 360 mμ of DNP-*S*-carboxymethylhomocysteine eluate; and ϵ_s = molar extinction coefficient of DNP-*S*-carboxymethylhomocysteine.[7]

These equations are based on the assumption that the concentration of DNP amino acid is directly proportional to its optical density at 360 mμ. They take into account the effect of the variable content of DNP-*S*-carboxymethylcysteine on the optical density of the DNP-aspartic-glutamic eluate.

Assays for the Determination of RNase Activity.—RNase was assayed according to Anfinsen *et al.* (1954) with RNA prepared from yeast by the method of Crestfield *et al.* (1956) and with uridine-2′,3′-cyclic phosphate as substrates, as described previously (White, 1961b), with the exception that the 0.2 M sodium acetate buffer was replaced by buffers that were combinations of sodium cacodylate, Tris, and acetic acid, for studies on the variation of enzymatic activity with *p*H. For the preparation of these buffers, a series of buffer solutions varying in *p*H from 4.5 to 8.1 was prepared by mixing 0.2 M sodium cacodylate with 0.2 M acetic acid in the appropriate amounts. Another series of buffer solutions of corresponding *p*H values was prepared by mixing 0.2 M Tris with 0.2 M acetic acid. Equal volumes of buffer solutions of the same *p*H from these two series were combined to obtain a series of mixed buffers for use in the *p*H study. Both substrates were used within a few hours after dissolving in the buffer of the desired *p*H. Components of thiolated carboxymethyl RNase, after separation of carboxymethyl-cellulose columns, were prepared for *p*H optimum studies by pooling the fractions comprising each of the peaks, dialyzing for 17 hours at 5° against water, and lyophilizing. Each sample was dissolved in buffer solution to the appropriate concentration for assay, determined by weight.

Results

Effects of pH on Thiolation of RNase.—A preliminary experiment was performed to determine the effects of *p*H on the extent of thiolation. At *p*H 7 no reaction was evident. At *p*H 8, 2.1 SH groups were found per mole of RNase in 7 hours. In the same time interval there appeared 3.1 SH groups per mole at *p*H 9 and 6.2 SH groups at *p*H 10. The RNase thiolated at *p*H 8 showed an activity toward RNA that was 130% of the specific activity of native RNase. Samples reacted at *p*H 9 and 10 had activities of 117% and 60% respectively. A *p*H of 8 was chosen for a more detailed study of the thiolation of RNase, the results of which follow.

Loss of AHT During Thiolation.—It has been reported that AHT undergoes conversion to *N*-acetyl homocysteine at alkaline *p*H values (Benesch and Benesch, 1956; Abadi and Wilcox, 1960). To determine the extent to which this reaction proceeds under the present conditions of thiolation at *p*H 8, the disappearance of thiolactone bond was followed by the decrease in optical density at 238 mμ in a control thiolation mixture (containing no RNase) by the procedure of Benesch and Benesch (1956). It was estimated that the half-life of AHT under these conditions is 59 hours. Since AHT is present in excess at the start of thiolation (86 moles of AHT per mole of RNase) it did not appear likely that the slow loss of reagent would contribute appreciably to the reaction kinetics of thiolation.

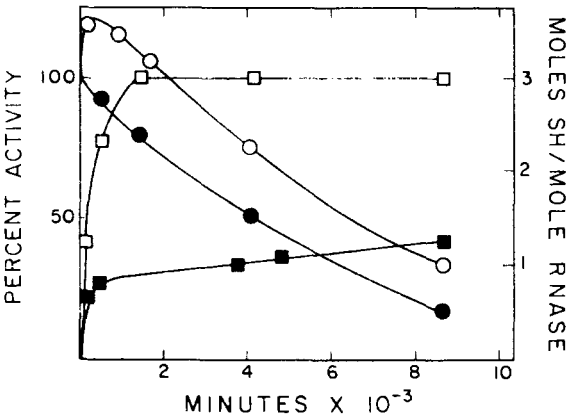


FIG. 1.—Variation of SH content, SH in interchange, and enzymatic activities of RNase with time of thiolation. ○, activity with RNA as the substrate; ●, activity with uridine-2′,3′-cyclic phosphate as the substrate; □, total moles of SH per mole of RNase, determined by titration with mercuribenzoate; ■, moles of SH in interchange, per mole of RNase, determined by analysis of the dinitrophenylated hydrolysate of thiolated carboxymethyl RNase for *S*-carboxymethylcysteine as described in the text.

Activity Changes During Thiolation.—The activities of RNase toward RNA and uridine-2′,3′-cyclic phosphate were followed as shown in Figure 1. There was a rise to 125% of the specific activity of native RNase at 600 minutes, followed by a decrease to 29% at 8690 minutes. Maxima as high as 175% have occasionally been observed at the earlier interval, but there is at present no apparent explanation for this variability. The cyclic phosphatase activity decreased throughout the reaction, and it therefore appears that a disparity in the specificities of RNase is produced by thiolation. A control, prepared as for the thiolation of RNase but containing no AHT, was assayed at intervals throughout the reaction period, without significant change in the activities toward RNA and uridine cyclic phosphate. Complete inactivation has never been observed toward either substrate. After 14 days under these conditions, the activities toward RNA and uridine cyclic phosphate were 8% and 2%, respectively. Figure 1 indicates that nearly 3 moles of SH are introduced per mole of RNase in 1560 minutes of thiolation, but the SH content does not appear to increase beyond this time.

Chromatography of Thiolated Carboxymethyl RNase.—The carboxymethyl derivative of thio-

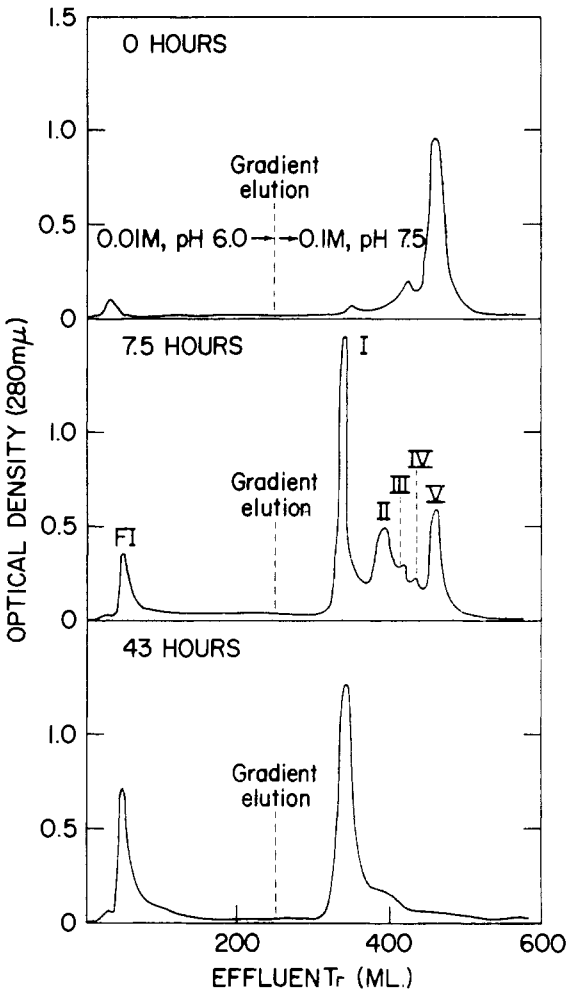


FIG. 2.—Separation of components of thiolated carboxymethyl RNase on carboxymethyl-cellulose at 0, 7.5, and 43 hours of thiolation. Chromatography was performed as described earlier (White, 1961b) with sodium phosphate buffers on columns that were 2.5 cm in diameter and 18 to 20 cm in height, each containing 75 mg of protein sample. Carboxymethyl-cellulose was prepared by the method of Peterson and Sober (1956), and by their method of titration contained 0.87 μmoles of ionizing groups per gram.

lated RNase, rather than the form containing free SH groups, was used for chromatography on carboxymethyl-cellulose because of the possibility of oxidation of the SH groups on the column. Carboxymethylation did not appear to change the specific activity of thiolated RNase toward RNA or the cyclic phosphate. The results of chromatography, shown in Figure 2, indicate the appearance of six components after 7.5 hours of thiolation; after 43 hours of thiolation, peaks II through V disappear almost entirely, leaving peaks FI and I. Peak FI appears as a slow-forming and minor component, particularly up to 7.5 hours, but continues to increase in size between 7.5 and 43 hours, while the other peaks diminish. This peak is the most extensively thiolated, with 4.99 moles of *S*-carboxymethylhomocysteine and *S*-carboxymethylcysteine per mole of protein (Table I) and possibly contains the final product or products of thiolation. Peak V contains no *S*-carboxymethylhomocysteine or *S*-carboxymethylcysteine and appears to be residual native RNase A.

The variations of activities of the thiolated, carboxymethylated components with *p*H is shown in Figure 3 with RNA as the substrate. Peaks FI, I, and II possess more than the native activity at low *p*H values. However, throughout most of the *p*H range their activities are less than the native. Peaks III and IV show less activity throughout the entire *p*H range. With cyclic phosphate as the substrate (Fig. 4) all peaks exhibit less than native activity at all *p*H values.

Inclusion of iodoacetate during thiolation for 7.5 hours resulted in activities of 66% and 40% for RNA and cyclic phosphate respectively. A parallel experiment, conducted in the absence of iodoacetate, produced 120% and 95% respectively. However, reaction of the product of the latter experiment with iodoacetate for the usual 15 minutes (White, 1961b) had no apparent effect on either of these activities. Although there is ample opportunity for reaction of iodoacetate with groups other than sulfhydryl (Gundlach *et al.*, 1959) when it is included for the 7.5-hour period, chromatography of the product on carboxymethyl-cellulose produced a pattern that was not significantly different from that in Figure 2. Therefore, nonspecific reactions between RNase and iodoacetate did not contribute noticeably to the chromatographic heterogeneity.

Disulfide Interchange.—It is well known that alkaline *p*H values and the presence of SH groups favor disulfide interchange in proteins, and this reaction was therefore studied to determine the extent to which it occurs during thiolation, as well as its influence on chromatographic heterogeneity and enzymatic activity. There are two sources of SH groups during thiolation, and these are free *N*-acetyl homocysteine (resulting from the breakdown of AHT during thiolation) and *N*-acetyl homocysteinyl residues (resulting from thiolation of RNase). An incubation of 50 mg of RNase with 50 mg of *N*-acetyl homocysteine in 0.1 M NH_4HCO_3 resulted in the introduction, after 24 hours, of 1.1 SH groups per mole of RNase. This value was not exceeded in an additional 24 hours of incubation. Therefore disulfide interchange of native disulfide bonds in RNase with free *N*-acetyl homocysteine may have proceeded to a maximum of approximately one bond per mole of protein. The extent to which this reaction will influence the kinetics of thiolation (Fig. 1) is difficult to ascertain, since a continuously increasing concentration of *N*-acetyl homocysteine must be taken into account. No distinction is made in the present results between the disulfide interchange that was initiated by SH

TABLE I

S-CARBOXYMETHYLCYSTEINE (SCMC) AND S-CARBOXYMETHYLHOMOCYSTEINE (SCHC) CONTENTS OF PEAKS FROM CARBOXYMETHYL-CELLULOSE CHROMATOGRAPHY OF THIOLATED CARBOXYMETHYL RNASE

Component	SCMC[b]	SCHC			SCMC plus av. SCHC[e]
		Counts[c]	Spec.[d]	Av.	
Peak FI,[a] 7.5 hour thiolated	2.91	1.71	2.45	2.08	4.99
Peak I, 7.5 hour thiolated	0.16	1.57	1.75	1.66	1.82
Peak II, 7.5 hour thiolated	0.05	0.66	0.77	0.72	0.77
Peak III, 7.5 hour thiolated	0.89	0.87	0.98	0.93	1.82
Peak IV, 7.5 hour thiolated	0.45	0.63	0.29	0.46	0.91
Peak V, 7.5 hour thiolated	0	0	0	0	0

[a] Peak numbers refer to components of thiolated carboxymethyl RNase from the experiment illustrated in Figure 2. [b] Moles of SCMC per mole of RNase, determined by scintillation counting of eluate from chromatography of DNP derivatives as described in the text. This value is taken as a measure of the extent of disulfide interchange. [c] Moles of SCHC per mole of RNase, determined by scintillation counting of eluate from chromatography of DNP derivatives as described in the text. [d] Moles of SCHC per mole of RNase, determined spectrophotometrically from eluates containing DNP-SCHC after paper chromatography as described in the text. [e] The sum of moles of SCMC and average moles of SCHC per mole of RNase, taken as a measure of the number of amino groups thiolated.

TABLE II

DETERMINATION OF MOLES OF S-CARBOXYMETHYLHOMOCYSTEINE (SCHC) AND S-CARBOXYMETHYLCYSTEINE (SCMC) PER MOLE OF RNASE BY SCINTILLATION COUNTING

	Minutes					
	370		2865		8690	
	SCMC	SCHC	SCMC	SCHC	SCMC	SCHC
cpm[a]	1253	3665	1350	3438	1470	2329
μmoles[b]	0.494	0.0307	0.348	0.0271	0.267	0.0162
Corrected cpm[c] $\times 10^{-3}$	2.51	3.90	2.11	3.56	2.06	2.44
Moles per mole of RNase[d]	0.92	1.50	1.21	1.97	1.42	1.77

[a] Counts per minute per eluted spot, determined by scintillation counting. [b] μmoles of DNP amino acid per eluted spot, determined spectrophotometrically as described in the text. The values for SCMC were determined from the DNP-aspartic-glutamic eluate and therefore include the optical density contributions of DNP-aspartic acid and -glutamic acid. [c] Counts per minute per eluted spot, corrected for color quenching (Baille, 1960). [d] Calculated from the corrected counts per minute as described in the text.

groups of free *N*-acetyl homocysteine and that by SH groups of *N*-acetyl homocysteinyl residues.

Most of the data presented for *S*-carboxymethylhomocysteine and *S* - carboxymethylcysteine content of thiolated carboxymethyl RNase were obtained by spectrophotometric reading and scintillation counting of eluates from paper chromatograms of the DNP derivatives because of the relative ease and rapidity of these techniques. A typical paper chromatogram is given in Figure 5, showing separation of DNP–S-carboxymethylhomocysteine from DNP - aspartic - glutamic. The faster moving components are unidentified. Table II shows data obtained in this way for three time intervals during thiolation. The validity of the results was checked by the amino acid analyzer.[9] From the data presented in Table III, it was concluded that the paper chromatographic method was adequate for the present study.

The kinetic experiment in Figure 1 shows that disulfide interchange proceeded rapidly in the initial stages of reaction to involve nearly one SH group per mole of RNase, but thereafter never exceeded this amount by more than 0.35. There is no obvious correlation between the kinetics of this reaction and the accompanying activity losses, and therefore the observed activity losses would appear not to be directly attributable to disulfide interchange as measured in this work.

Table I indicates a variable disulfide interchange, as measured by the moles of *S*-carboxymethylcysteine per mole of RNase, for the components of thiolated carboxymethyl RNase, separated by carboxymethyl-cellulose. Peaks I and II have undergone little interchange, whereas peaks FI and III and IV have interchanged to the extents of 2.91, 0.89, and 0.45 moles of native disulfide bond per mole of protein respectively. It is of interest that peak FI, despite its extensive thiolation and interchange, still possesses some enzymatic activity. At *p*H 5, the activity toward RNA is nearly the same as native (Fig. 3). At

[9] The mixed disulfide between cysteine and homocysteine was detected between the peaks for isoleucine and leucine (Frimpter, 1961), whenever disulfide interchange occurred. No attempt was made to measure its concentration by integration of the peak because it was never completely separated from isoleucine.

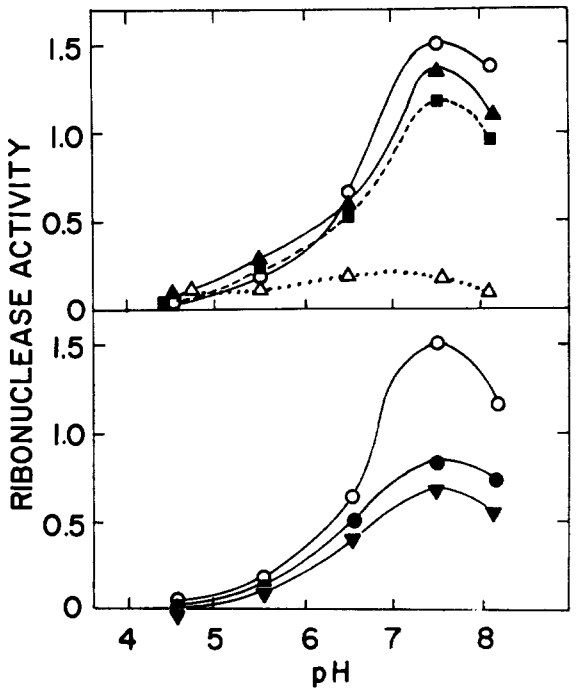


FIG. 3.—Enzymatic activities of components of 7.5 hour-thiolated carboxymethyl RNase as functions of *p*H, with RNA as the substrate. ○, native RNase; △, peak FI; ▲, peak I; ■, peak II; ▼, peak III; ●, peak IV. RNase activities are expressed as the differences between the optical density readings at 260 mμ of the assay solutions and their corresponding blanks (without enzyme) according to the procedure of Anfinsen *et al.* (1954). At *p*H values below 6.5, 1 to 2 μg of enzyme was included for 2.5 ml of reaction mixture. At *p*H 6.5 and above, 0.2 to 0.4 μg of enzyme was used, and the final optical density differences were multiplied by 5 to render all points comparable with regard to enzyme concentration.

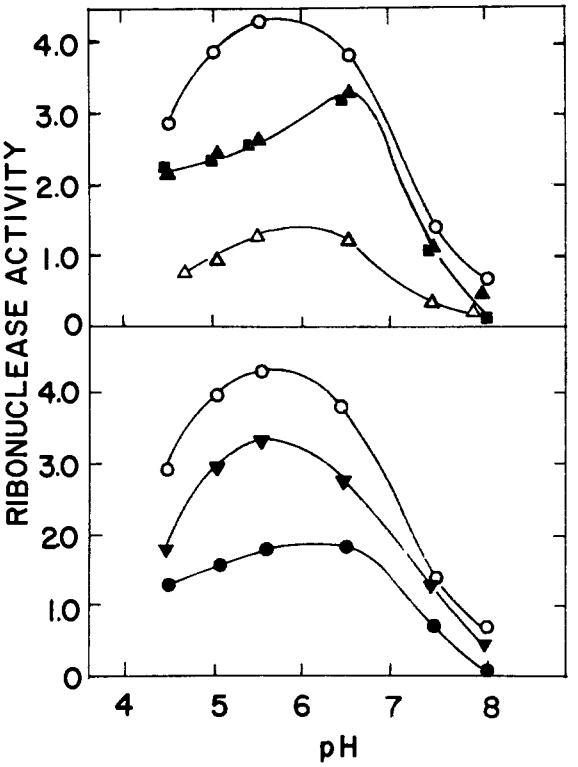


FIG. 4.—Enzymatic activities of components of 7.5 hour-thiolated carboxymethyl RNase as functions of *p*H, with uridine-2′,3′-cyclic phosphate as the substrate. RNase activities are expressed as 100 × optical density increase per 2 minutes. ○, native RNase; △, peak FI; ▲, peak I; ■, peak II; ▼, peak III; ●, peak IV.

higher *p*H values, however, the activity of this peak fails to increase significantly, and at *p*H 7.5, for example, it possesses only 13.3% of the native specific activity. Its activity toward uridine cyclic phosphate is less than native throughout the entire *p*H range (Fig. 4).

As has been reported by several other workers (Halwer, 1954; Hospelhorn and Jensen, 1954; Loewy and Edsall, 1954), disulfide interchange is inhibited by SH-binding reagents such as iodoacetate. When this reagent is included during the thiolation of RNase, no detectable interchange occurs, as shown by the absence of *S*-carboxymethylcysteine in the hydrolysate. However, inclusion of this reagent as mentioned above (Chromatography of Thiolated Carboxymethyl RNase) does not alter the chromatographic pattern. Therefore, it can be stated that disulfide interchange does not influence the chromatographic heterogeneity of thiolated RNase as it is manifested in the present work.

Thiolation, as presently described, but in 8 M urea for 24 hours, caused complete inactivation toward RNA and cyclic phosphate. In contrast, if RNase is thiolated for 24 hours in the absence of urea, most of the activity toward these substrates is retained. Incubation of the product of the latter experiment at room temperature in 8 M urea in the absence of thiolating agent caused complete inactivation within 24 hours. However, activity was retained when the product of thiolation in the absence of urea was incubated with iodoacetate for 15 minutes and then treated with urea under the above conditions. It appears likely that urea destroys the activity of thiolated RNase by enhancing disulfide interchange and that iodoacetate has this protective action by conversion of homocysteine to *S*-carboxymethylhomocysteine; there are then no SH groups remaining through which disulfide interchange might be perpetuated. That urea increases the rate of disulfide interchange was shown by carboxymethylation, acid hydrolysis, dinitrophenylation, and amino acid analysis of the above thiolated, urea-incubated RNase. The result was a complete conversion to *S*-carboxymethylcysteine, with no trace of *S*-carboxymethylhomocysteine, indicating that disulfide interchange had proceeded to completion.

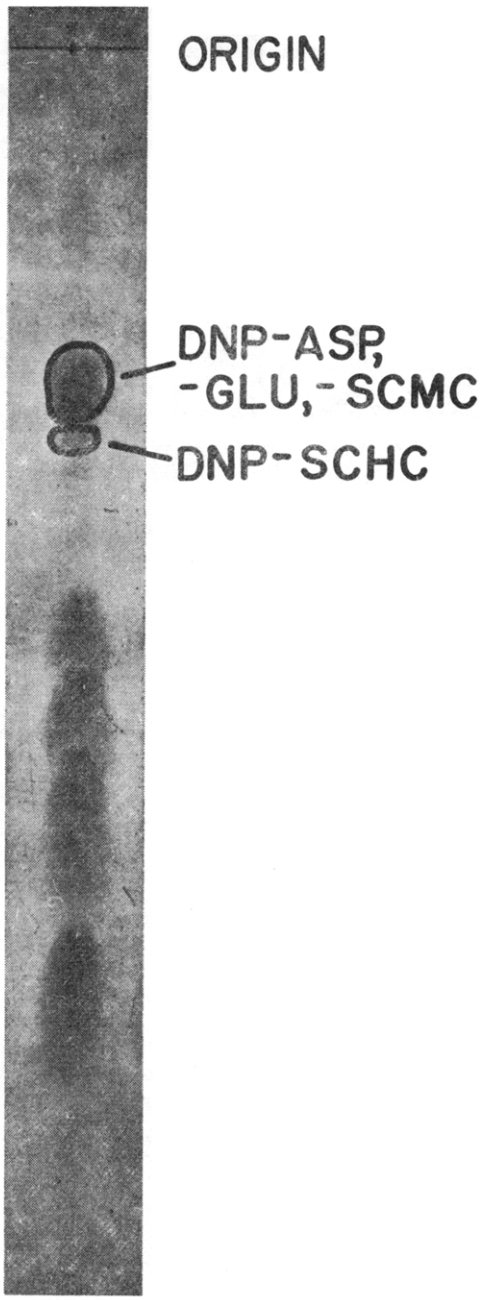


FIG. 5.—Paper chromatogram of dinitrophenylated hydrolysate of peak I (after treatment as described in the text), from 7.5 hour-thiolated RNase (see Fig. 2), showing the spot containing DNP–aspartic acid, –glutamic acid, and –*S*-carboxymethylcysteine (DNP-aspartic-glutamic spot), and the spot containing DNP–*S*-carboxymethylhomocysteine.

DISCUSSION

During the thiolation of RNase, the enzymatic activity toward RNA first increases, but eventually declines. The cyclic phosphatase activity decreases steadily from the start of the reaction. This disparity in specificities is confirmed by the studies of activity *vs.* *p*H for the chromatographically separated components of thiolated carboxymethyl RNase. Several other workers have reported differences in the specificities of RNase toward its substrates, after various alterations of the RNase molecule (van Vunakis *et al.*, 1960; Kalman *et al.*, 1955; Nelson and Hummel, 1961). Their results, as well as the present disparities, could be explained by a preferential attack on one of two active centers (Rogers and Kalnitsky, 1957) or on one of two binding sites.

At least three other effects must be considered in finding explanations for the observed activity changes during thiolation: (1) the gain of SH groups; (2) the loss of primary amino groups; (3) secondary reactions, *e.g.*, disulfide interchange between the SH groups of *N*-acetylhomocysteinyl residues and native disulfide bonds. The initial increase in activity toward RNA may possibly result from loss of amino groups, in view of the work of van Vunakis *et al.* (1960), who observed similar increases toward RNA after partial deamination of RNase. The gain of SH groups may be ruled out as a factor in alteration of either of the specificities of RNase, since their carboxymethylation does not alter the activity of thiolated RNase under the present conditions of reaction. There is no obvious contribution of disulfide interchange to the observed activity changes. However, there is the possibility of a continued disulfide interchange, perpetuated by the SH groups of cysteinyl residues, which could eventually destroy enzymatic activity by a "scrambling" of the native disulfide bonds.

The chromatographic heterogeneity of thiolated carboxymethyl RNase is not caused by disulfide interchange or reactions of iodoacetate with groups other than sulfhydryl, since inclusion of iodoacetate during thiolation prevents disulfide interchange and enhances nonspecific carboxymethylation without altering the chromatographic pattern. Positional isomerism concerning the points of attachment of the *N*-acetyl homocysteinyl residues, as suggested by Abadi and Wilcox (1960) for thiolated α-chymotrypsinogen, as well as the extent of thiolation, may contribute to the number of components appearing on chromatography. Until further evidence of homogeneity is obtained, it cannot be assumed that these components are homogeneous with respect to the extent either of thiolation or of disulfide interchange. Therefore all values presently reported for content of SH, *S*-carboxymethylcysteine, or *S*-carboxymethylhomocysteine are for the average protein molecule.

Disulfide interchange to the extent of one disulfide bond per average protein molecule did not adversely affect the enzymatic activity, while peak FI contained close to three interchanged disulfide bonds and remained partially active toward RNA and cyclic phosphate. Therefore it may be that not all of the native disulfide bonds are essential for enzymatic activity. It is felt that more specific conclusions with regard to the

TABLE III

COMPARISON OF DATA OBTAINED BY COUNTING, SPECTROPHOTOMETRY, AND MERCURIBENZOATE TITRATION

Minutes of Thiolation	SCMC Determined by: Counts[a]	Column[b]	Av.	SCHC Determined by: Counts[a]	Column[b]	Spect.[c]	Av.	Sum[d] of avs.	SH[e]
370	0.92	0.71	0.82	1.50	1.52	1.82	1.61	2.43	2.30
2865	1.21	0.84	1.03	1.97	1.93	2.31	2.07	3.10	2.98
8690	1.42	1.25	1.34	1.77	1.61	1.81	1.73	3.07	3.05

[a] Moles of SCMC or SCHC per mole of RNase, taken from Table II. [b] Moles of SCMC or SCHC per mole of RNase, determined with the amino acid analyzer (Moore *et al.*, 1958; Spackman *et al.*, 1958). [c] Moles of SCHC per mole of RNase, calculated from micromoles of DNP amino acid per eluted spot (Table II) as described in the text. [d] The sum of average moles of SCMC per mole of RNase and average moles of SCHC per mole of RNase, taken as a measure of the extent of thiolation. [e] Obtained by spectrophotometric titration with mercuribenzoate.

extent of interchange and the corresponding effects on activity must await further proof of homogeneity. Furthermore, it is not yet established whether disulfide interchange occurs between specific disulfide bonds and homocysteinyl residues which are attached to specific lysyl residues, whether some of these reacting groups are specific and others are not, or whether none is specific.

ACKNOWLEDGMENT

We wish to thank Mrs. Juanita Cooke for operation of the automatic amino acid analyzer.

REFERENCES

Abadi, D. M., and Wilcox, P. E. (1960), *J. Biol. Chem. 235*, 396.
Anfinsen, C. B., Redfield, R. R., Choate, W. L., Page, J., and Carrol, W. L., (1954), *J. Biol. Chem. 207*, 201.
Armstrong, M. D. (1951), *J. Org. Chem. 16*, 749.
Baille, L. A. (1960), *Int. J. Appl. Radiation and Isotopes 8*, 1.
Benesch, R., and Benesch, R. (1956), *J. Am. Chem. Soc. 78*, 1597.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Crestfield, A. M., Smith, K. C., and Allen, F. W. (1956), *J. Biol. Chem. 216*, 185.
Frimpter, G. W. (1961), *J. Biol. Chem. 236*, PC51.
Gundlach, H. G., Stein, W. H., and Moore, S. (1959), *J. Biol. Chem. 234*, 1754.
Halwer, M. (1954), *J. Am. Chem. Soc. 76*, 183.
Harris, S. A., Easton, N. R., Heyl, D., Wilson, A. N., and Folkers, K. (1944), *J. Am. Chem. Soc. 66*, 1757.
Hirs, C. H. W., Stein, W. H., and Moore, S. (1956), *J. Biol. Chem. 219*, 623.
Hospelhorn, V. D., and Jensen, E. V. (1954), *J. Am. Chem. Soc. 76*, 2830.
Kalman, S. M., Linderstrøm-Lang, K., Ottesen, M., and Richards, F. M. (1955), *Biochim. Biophys. Acta 16*, 297.
Levy, A. L. (1954), *Nature 174*, 126.
Loewy, A. G., and Edsall, J. T. (1954), *J. Biol. Chem. 211*, 829.
Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.
Nelson, C. A., and Hummel, J. P. (1961), *J. Biol. Chem. 236*, 3173.
Peterson, E. A., and Sober, H. A. (1956), *J. Am. Chem. Soc. 78*, 751.
Rogers, W. I., and Kalnitsky, G. (1957), *Biochim. Biophys. Acta 23*, 525.
Sanger, F. (1945), *Biochem. J. 39*, 507.
Sela, M., White, F. H., Jr., and Anfinsen, C. B. (1959), *Biochem. Biophys. Acta 31*, 417.
Spackman, D., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
van Vunakis, H., Leikhim, E., Delaney, R., Levine, L., and Brown, R. K. (1960), *J. Biol. Chem. 235*, 3430.
White, F. H., Jr. (1960), *J. Biol. Chem. 239*, 383.
White, F. H., Jr. (1961a), *Fed. Proc. 20*, 221.
White, F. H., Jr. (1961b), *J. Biol. Chem. 236*, 1353.