Relations of Reactivity to Structure in Pancreatic Ribonuclease. II. Positions of Residues Alkylated in Certain Conditions by Bromoacetate*

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ABSTRACT: The specific alkylation of a methionine residue in ribonuclease A, which occurs with bromoacetate in water at pH 3 to 7, is shown to be at position 30. Several methods were used to confirm this location, including the demonstration of the predicted labeled peptide in a gel filtration after cyanogen bromide cleavage, and determination of the products released from labeled peptides when an intramolecular peptide bond cleavage was produced by attack of the *S*-carboxymethyl group. The quantitative results in the various peptide analyses show also that not more than 10% of the alkylation occurs at methionine-29, and none is detectable at methionines-13 and -79. The selective carboxymethylation at residue 30, and its various characteristics, can be reconciled with the presently available three-dimensional structure of ribonuclease A. Changes in this reactivity are taken to be an index of local conformational changes occurring in the protein, as in a transition that is observed with pH change near pH 6. The rate constants for carboxymethylation at methionine-30 in native ribonuclease, and for all four methionines at pH 2, are those expected for partly obstructed thioether groups. Peptide separations also have shown that, after reaction with bromoacetate at pH 5.8 (35°), the site of the dicarboxymethylation of imidazole that occurs is the same as that of the N-1-carboxymethylation, namely histidine-119. When carboxymethylation proceeds at a greatly reduced rate in the presence of pyrophosphate ions, there is no direction of the reagent to sites other than those alkylated in the non-complexed protein.

The positions of the groups alkylated in the polypeptide chain of ribonuclease A, in reactions with halogenoacetate in the pH range 2–7, have previously been defined by peptide analysis in certain specific cases. These cases are (i) after reaction with bromoacetate at pH 7 (at 37°) in the presence of phosphate ions, when almost all of the CM groups introduced were shown to be at histidine-119 (Stein and Barnard, 1959); (ii) after reaction with iodoacetate at pH 5.5 at 25°, when about eight-ninths of the CM groups were determined to be at histidine-119 and one-ninth at histidine-12 (Crestfield *et al.*, 1963b); (iii) after reaction of ribonuclease A dimer with iodoacetate at pH 5.5 (25°), when histidines-119 and -12 were alkylated as above and (in the alkylated dimer molecules) a minor reaction also occurred at methionine-29 or methionine-30 (Fruchter and Crestfield, 1965), with apparently about equal frequency at each of those two methionines. In addition, recent reports have described a slower reaction with iodoacetamide at pH 5.5 that occurs at histidine-12, and also at an unidentified methionine (Fruchter and Crestfield, 1967), and a reaction with methyl iodide which occurs apparently at methionine-29 only (Link and Stark, 1968).

In the analysis of the bromoacetate reaction throughout the pH zone 2–7 (Goren and Barnard, 1970), we have separated three protein products each of which has a constant chromatographic behavior and analysis no matter what the composition of the reaction mixture was (in that pH range), although their respective amounts vary greatly with the reaction conditions. We here report peptide analyses that show that histidines-119 and -12 are, as expected, the sites of carboxymethylation in these conditions, and in the presence or absence of competitive inhibitors. The analyses are extended to show that the 119 position is also the site of the formation of di-CM-histidine, and that the position of the specific methionine reaction (Goren and Barnard, 1970) which occurs in native, monomeric ribonuclease A and in 119-His-CM-ribonuclease[1] is, in the case of bromoacetate, residue 30.

Experimental Procedures

Materials. Ribonuclease A, subtilisin (subtilopeptidase A), and all materials not otherwise specified were as used by Goren and Barnard (1970) or by Markus *et al.* (1968). Iodoacetic acid (Eastman) was recrystallized from ligroin–diethyl ether and stored in the dark at −30°. Sephadex G-25 (beads, coarse mesh) and G-50 (beads, coarse mesh), DEAE-Sephadex A-25 (fine mesh) from Pharmacia, Bio-Gel P-6 and P-2 (100-200 mesh), Aminex Q-15S resin from BioRad, and

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[1] Abbreviations used are as in part I of this work (Goren and Barnard, 1970) except for: RCM-, reduced and carboxymethylated; T-, tryptic; and ribonuclease peptide abbreviations as used by Hirs *et al.* (1956) (but using the prefix RCM where appropriate), by Richards and Vithayathil (1959), and by Gross and Witkop (1962).

IRC-50 (CG-50, Type III) from Mallinckrodt were each prepared as recommended by the manufacturer. Dithiothreitol was from P-L Biochemicals. Trypsin (Mann, Type TL, "chymotrypsin-free") had (by activity on benzoyl-L-tyrosine ethyl ester) less than 4 parts of chymotrypsin per 10,000 parts of trypsin.

Methods. Analyses of the various [^{14}C]CM-amino acid derivatives were by the flow-cell methods described by Goren *et al.* (1968). The reaction of ribonuclease A with bromoacetate was as described by Goren and Barnard (1970), as were all other methods not otherwise specified. Chromatography of the protein derivatives, however, employed CM-cellulose CM-52 (Whatman), the microgranular form replacing CM-1. Improved resolution is obtainable with CM-52, but the elution media used with CM-1 are then inapplicable. Satisfactory conditions were obtained with a linear gradient from 0.04 M Tris-HCl (at pH 7.80) to 0.10 M Tris-HCl–0.10 M NaCl (at pH 8.40), on a column 0.9 × 25 cm. This method is used routinely now, other details being as for the CM-1 method.

For peptide analyses, a column (0.9 × 18 cm) of Aminex Q-15S resin (spherical beads) was attached to the Beckman amino acid analyzer, and the usual analyzer buffers (pH 3.28, 4.25 and 5.28) were applied following the method used with another resin by Crestfield *et al.* (1963a). The flow-cell was attached to the exit line from the column (Goren *et al.*, 1968) prior to the entry of the ninhydrin stream. The analyzer was equipped with high-sensitivity cuvets (Beckman) to give sufficient sensitivity for ninhydrin measurement of the small amounts of peptides used. It was subsequently found that a pH-gradient elution system gives better resolution of the tryptic peptides derived from RCM-ribonuclease than does the stepwise application of the three standard buffers; the gradient is linear, from pH 3.25 to 4.5, in 0.2 M citrate buffer.

For the phenyl isothiocyanate reaction, the method of Sjoquist and Sjoquist (1963) was followed, up to the stage of extraction of the PTH. The PTH-containing fraction was taken up in ethyl acetate–methyl ethyl ketone (1:1) and the residual peptide fraction in water, and each was used for radioactivity measurement and analysis.

Heating of Met-CM-ribonuclease. Two types of specimen were used: (1) ribonuclease reacted with 0.025 M [^{14}C]bromoacetate at pH near 6.1 for 10–17 hr (35°) to give samples containing 0.7–0.9 [^{14}C]CM-methionine residues per molecule (as well as maximal alkylation at histidines-119 and -12); and (2) ribonuclease alkylated at pH 2.8 with 2 mM pyrophosphate present (Goren and Barnard, 1970) with essentially only methionine alkylated. Each was deionized as previously. Aliquots (containing 3000 cpm each) of specimen 2 were heated in 5 mM acetic acid (pH 4) solution (deaerated and sealed *in vacuo*) in a boiling-water bath for periods from 1 to 10 hr. Each sample was then diluted with pH 2.2 buffer and analyzed for amino acids on the 60-cm column at 35° and pH 3.28 (Goren *et al.*, 1968). Two front-running radioactive peaks were measurable and were identified (Goren *et al.* 1968) as containing, respectively, glycollic acid and *S*-methylthioglycollic acid. The latter peak increased in size faster and was the major product.

Another sample of specimen 2 was heated in deaerated water adjusted to pH 7.5, sealed, at 110° ± 1°, 14 hr. The solution, combined with a slight precipitate which formed and which was dissolved in 25 mM acetic acid, was analyzed for labeled products as above, and for N-terminal groups by the cyanate method of Stark and Smyth (1963), modified as noted elsewhere (Markus *et al.*, 1968); the final product in the preceding heating experiment, and that of ribonuclease A similarly heated, were also analyzed thus.

From specimen 1, a sample was oxidized by performic acid (Hirs, 1956), freed of reagents by freeze-drying from large volumes of water, and heated at 100° in 1 ml of water in a sealed, deaerated tube for 2 hr. It was then passed through a column (1 × 105 cm) of Sephadex G-25 in 0.2 N acetic acid. A protein peak carrying 70% of the original radioactivity emerged at the void volume, followed by four or five unresolved peaks of peptide material, and a final peak containing labeled *S*-methylthioglycollic and glycollic acids carrying about 20% of the original radioactivity.

Samples of type 1 were used in similar experiments, in acetic acid at pH 3 for 1 hr at 110°, and at pH 4.5 for 16 hr at 90°. Gel filtration on Sephadex G-25, as above, was applied to the latter sample (which had not been oxidized), when a similar labeled protein peak emerged, as well as peptide material in several succeeding peaks; the radioactivity in the latter peaks was, in sum, only 25% of that in the first, major peak.

Subtilisin Treatment. Modifications of the methods of Gordillo *et al.* (1962) and Doscher and Hirs (1967) were used. The protein (5–20 mg/ml) was treated with subtilisin (0.09 mg/ml) at 0°, pH 8.0 (0.1 M Tris-HCl), 90 min. When active enzyme was used the production of RNase-S was sometimes followed by its trypsin-sensitive ribonuclease activity, as described elsewhere (Markus *et al.*, 1968), when about 60% of the theoretically possible trypsin-sensitive activity was produced up to the termination point. After termination by the addition of 1.0 ml of 0.05 N HCl, the subtilisin digest was immediately placed on a column (0.9 × 100 cm) of Sephadex G-25 and eluted with 0.05 N HCl.

Pure ribonuclease A was treated thus, yielding a preparation of S-peptide which was refiltered in the same conditions, when the material in the single peak revealed by A_{220} measurements showed, on amino acid analysis, the composition (Gordillo *et al.*, 1962) of S-peptide. In a second digestion, ribonuclease A was alkylated with 0.025 M [^{14}C]bromoacetate for 8 hr at 35°, in 0.025 M sodium acetate–0.005 M sodium pyrophosphate buffer, pH 5.8. After termination of the reaction, the mixture was chromatographed on CM-52 cellulose (Figure 1). The material in peak II (Met-CM-ribonucleases) was pooled, concentrated, deionized by passage through a G-25 column (0.9 × 100 cm) in 0.05 N HCl, and rotary evaporated to dryness from excess water. For a third digestion, the material in peak III (Figure 1) was deionized similarly.

A still purer preparation of S-peptide was made from a ^{14}C-labeled Met-CM-ribonuclease, starting with the slightly different procedure of Glick *et al.* (1967). The protein (containing 90% ribonuclease A) was in peak II, separated in CM-cellulose chromatography (sample 7, Table I, Goren and Barnard, 1970), after an alkylation at pH 3.3. After maximal formation, by subtilisin digestion, of ribonuclease-S (as measured by the trypsin-sensitivity assay), the sample was extracted with trichloroacetic acid (5%, 1 hr at 4°) and the extract filtered on a Sephadex G-50 column (0.9 × 50 cm) in 50% acetic acid, revealing a single peptide peak at 20–34 ml of elution volume. After rotary evaporation from large volumes of water, this product was refiltered as before on

TABLE 1: Alkylated Residues in Products of Reaction in Presence of 5 mM Pyrophosphate, at pH 5.8 for 5 hr.[a]

Peak[b]	1-CM-His	Di-CM-His	Di-CM-His	CM-Met	CM-Lys		Enzymic Act.
					α	ϵ and di-CM	
I	61	16	1	20	0	1	0
II	3	0	3	**94**	0	0	100
III	0.8	0.3	82	20	0.6	0	0
IA	33	5.4	3.5	17	38	3	0

[a] Within each peak, the percentage of its radioactivity in each of the CM residues present is shown. The enzymic activity on C2'3'p is also shown (last column), relative to that of ribonuclease A (fractions of a percentage point were not determined). [b] The peaks of Figure 1; peak IA is the minor labeled peak centered at fraction 51, and contained 2.5 CM groups per protein molecule.

G-25, when only a pure S-peptide peak was obtained, centered at 38 ml; this was unlabeled. The corresponding S-protein was purified from the redissolved trichloroacetic acid precipitated fraction by a filtration on a G-75 column (0.9 × 50 cm) in 50% acetic acid (separated at 15–24-ml elution volume, and characterized by its reactivation with authentic S-peptide, prepared as above). This S-protein fraction was labeled and contained only [^{14}C]CM-methionine products when a hydrolysate was analyzed by the flow-cell method.

Tryptic Peptides. The ribonuclease derivative was reduced and carboxymethylated with iodoacetate by a modification of the method of Crestfield *et al.* (1963a). The medium was as described there, but was at pH 8.4 (using Mann Ultra-Pure Tris), and reduction was in a deoxygenated solution for 2.5 hr at 30°. In the later experiments, the mercaptoethanol (200-fold excess over the protein) was replaced by dithiothreitol (100-fold excess), at 25°. After the treatment (under N_2) with iodoacetate, the product was immediately gel filtered on a Bio-Gel P-2 column (1.5 × 40 cm) in water.

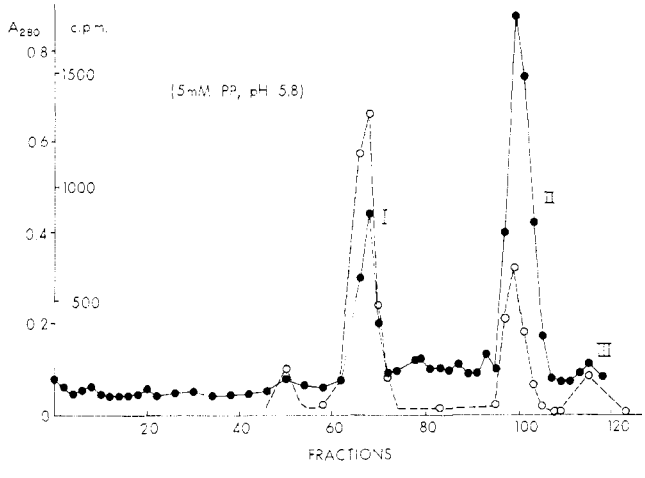

FIGURE 1: Products of alkylation of ribonuclease A at pH 5.8 in the presence of 5 mM pyrophosphate, 5 hr. Chromatography is on CM-52 CM-cellulose in a pH–salt gradient (see Methods). The 2-ml fractions were analyzed for protein (A_{280}, ●) and radioactivity (cpm in 25-μl aliquot, ○). Similar results, with a correspondingly higher labeling of peak II, were obtained after a parallel reaction for 8 hr.

The column and the alkylation reaction chamber were wrapped in aluminum foil to exclude light. The 1-ml fractions were analyzed for protein (A_{278}) and radioactivity. The reagent-free protein located thus was evaporated, redissolved in Tris-HCl buffer (pH 7.0), and treated with 0.003% trypsin at room temperature. Excess pH 2.2 buffer was finally added.

This treatment was applied (using an 8-hr tryptic digestion) to the protein isolated from peak I of a CM-cellulose chromatogram (see Figure 2 of Goren and Barnard, 1970) of the products of alkylation at pH 5.8, 35°, 40 min, and also to about 10 times that quantity of ribonuclease A. The combined digest was chromatographed (in an experiment of Mr. M. H. Gold) on a column (0.9 × 15 cm) of IRC-50, using a linear gradient from water to glacial acetic acid. ^{14}C content was counted in aliquots of the 1.2-ml fractions. Cuts (about 8 ml) were taken, for analysis, of the centers of the two radioactive peaks.

Met-CM-ribonuclease (peak II, Figure 1) was also reduced, carboxymethylated, and gel filtered (on Sephadex G-25), and its tryptic digest (20 hr) analyzed as above on the Aminex Q-15S column. The Aminex column was then equilibrated in 0.2 M citrate buffer (pH 3.28) without thiodiglycol or detergent. This column and eluent were used to chromatograph the bulk of the trypsin-treated RCM-(Met-ribonuclease); the ^{14}C-containing peak was collected from the flow-cell outlet line just after its radioactivity had been detected. This radioactive peptide was evaporated to dryness, redissolved in 1.0 ml of water, and applied to a column (0.9 × 12 cm) of IRC-50 (H^+) (Crestfield *et al.*, 1963b) with elution by, in sequence, 5 ml of water, 10 ml of 5% (v/v) acetic acid, 20 ml of 20% acetic acid, and 25 ml of 50% acetic acid. The four effluents were evaporated and each was redissolved in 1.0 ml of water. The sodium citrate was in the water and the 5% acetic acid fractions, and gave a white precipitate as these were concentrated. The 20% acetic acid fraction contained at least 80% of the original radioactivity.

Ribonuclease A samples that had been alkylated either at pH 5.6 or at pH 6.0 (at 35°, for 45 min with 0.025 M [^{14}C]-bromoacetate) and chromatographed on CM-cellulose were also used. The material in peak II in each case (see Figure 2 of Goren and Barnard, 1970) was combined (in about equal amounts) and deionized. The protein was then oxidized with performic acid (Hirs, 1956) at −7°, and the product was isolated by gel filtration on a G-25 column (0.9 × 100 cm)

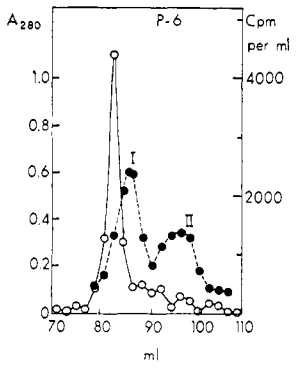

FIGURE 2: Separation on Bio-Gel P6 of the peptides from the oxidized C protein derived from Met-[^{14}C]CM-ribonuclease (●--●, A_{280}; ○—○, cpm per ml).

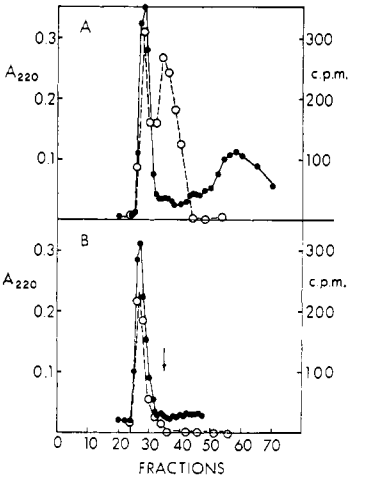

FIGURE 3: Separation on Sephadex G-25 (see Methods) of *S*-protein and *S*-peptide fractions of subtilisin digests (●—●, A_{220}; ○--○, cpm in 25-μl aliquot; 1-ml fractions collected). (A) The products of cleavage of the His-N3-CM-ribonuclease (peak III, Figure 1); (B) the products of cleavage of Met-CM-ribonuclease plus ribonuclease A (peak II, Figure 1). The arrow indicates the position where the peak of S-peptide obtained from a similar filtration (not shown) of pure ribonuclease A, after a parallel subtilisin digestion, falls when refiltered through this column. The material in the corresponding fractions here (34–38) was collected, hydrolyzed, and analyzed, and showed that S-peptide is again located here. The wide peak around tube 55 (in A) contained no peptides (as shown by hydrolysis and analysis), but is due to the buffer components.

eluted with 0.05 N HCl; this *O*-(Met-CM-ribonuclease) retained all of the radioactivity. It was treated with trypsin (20 hr) as described above, and the digest was gel filtered similarly on G-25 in 0.05 N HCl. The radioactive peak material was pooled, dried, redissolved in 1.0 ml of water, and adjusted to pH 4.0 with 2 N NaOH. It was sealed under vacuum, held at 100° for 2 hr, and immediately gel filtered on the G-25 column as before.

Cyanogen Bromide Treatment. Deionized Met-CM-ribonuclease (9 mg) from peak II (Figure 1), dissolved in 1 ml of 0.05 N HCl, was deoxygenated, treated with about 12 mg of CNBr, and stirred 24 hr at room temperature. After freeze-drying, gel filtration on a G-25 column (0.9 × 125 cm) in 0.05 N HCl separated a C-protein peak (detected by A_{278}) containing all the radioactivity, while none was in the later C-peptide peak (detected by A_{220}). The C-protein was oxidized by performic acid as described above. The reaction mixture was immediately applied to a column (1.0 × 150 cm) of Bio-Gel P-6, eluted in water. The material in the single radioactive peak (Figure 2) was pooled, dried, dissolved in water at pH 5.0, deaerated, and maintained in a sealed, evacuated tube at 100° for 2 hr. Analyses were then made on the 60-cm and 15-cm analyzer columns.

Results

Products of Alkylation of Ligand-bound Ribonuclease. It was desired, firstly, to confirm that the species produced by alkylation of ribonuclease combined with specific ligands are identical with those produced from the free enzyme. While this would be so if the reaction occurs exclusively with the unliganded protein, the latter fraction can become minute at quite low concentrations of the more tightly bound ligands, while the complexes then formed must be distinctly different in structure to the free protein, as judged from their large differences from the latter in behavior in urea denaturation (Nelson *et al.*, 1962; Barnard, 1964), in proteolytic cleavage at numerous points in the chain (Markus *et al.*, 1968), and in various optical properties (Cathou and Hammes, 1965; Deavin *et al.*, 1966; Markus *et al.*, 1968). The more tightly bound ligands strongly retard the formation by bromoacetate of *N*-1-CM-histidine, *N*-3-CM-histidine and CM-methionine residues (Goren and Barnard, 1970), but it is conceivable that some direction of the reagent to different sites occurs in the complex. We have, therefore, analyzed the complex with one of those ligands, pyrophosphate ion, after reaction.

Ribonuclease A was treated with [^{14}C]bromoacetate at pH 5.8, 35°, in 5 mM sodium pyrophosphate, for 5 and 8 hr. The products were separated by CM-cellulose chromatography (Figure 1); three main protein peaks corresponded to those obtained (Goren and Barnard, 1970) after alkylation without inhibitor, as did their complements of CM residues (Table I), with CM-methionine content raised due to the extended reaction period.

The protein in a smaller, front peak (IA, Figure 1) was inactive and multiply substituted (Table I). After the 8-hr alkylation and with a less steep initial salt gradient in the chromatography, a rather smaller second peak (IB) was also clearly resolved there, closer to peak I. This protein, too, was inactive and contained 1.6 CM groups per molecule, in about the same distribution as in peak IA, except that the 3-CM-histidine content was raised to 25% of the CM residues in the peak. Essentially all of the alkylated lysine, therefore, is in these fast-running peaks. ε-Carboxymethylation of lysine is largely suppressed by the pyrophosphate, as observed previously (Goren and Barnard, 1970). The results indicate that carboxymethylation of lysine-1 slowly occurs in the His-CM-ribonucleases, together with (to explain peak IB) the very slow *N*-3-carboxymethylation at histidine-105 or -48.

Identification of Sites of Histidine Alkylation in Pyrophosphate-Retarded Reaction. His-*N*-3-CM-ribonuclease (peak III, Figure 1), obtained from alkylation of the pyrophosphate complex, was cleaved with subtilisin, yielding on gel filtrations (Figure 3A) S-peptide (residues 1–20 or 1–21: Doscher and

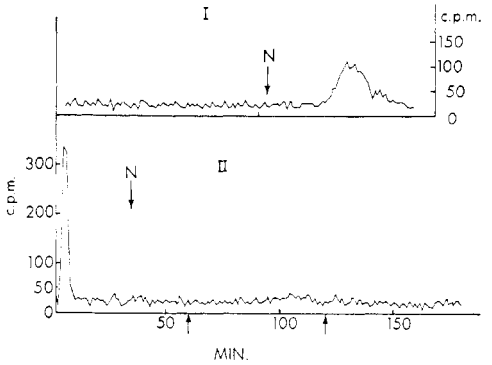

FIGURE 4: Analysis of the labeled tryptic peptides from RCM-ribonuclease derivatives, separated on an Aminex Q-15S resin column. I, di-CM-histidine-enriched fraction; N marks the position of the only significant peak in the parallel ninhydrin chromatogram of this run. II, CM-methionine-containing peptide; N marks the first significant peak in the parallel ninhydrin chromatogram, containing the peptides RCM-T2, RCM-T4, and RCM-T9. In each case, the two buffer changes were made where shown by the lower arrows.

Hirs, 1967, identified here by its elution position and its composition) carrying one [^{14}C]CM group. The prior labeled peak present (Figure 3A) is due to incomplete cleavage by the subtilisin treatment used (Markus *et al.*, 1968). This reaction went only to 60% yield in the case of ribonuclease A, and with the inactive CM-ribonuclease about 60% of the total ^{14}C ran in the S-peptide fraction (Figure 3A). Hence, that S-peptide is (on average) fully labeled at one residue. Since essentially only 3-CM-histidine was labeled in this protein (Table I), histidine-12 (the only histidine residue in the S-peptide) is again the site of the minor histidine carboxymethylation, occurring at nitrogen-3 there as in the non-inhibited case.

This 12-His-*N*-3-CM-ribonuclease ran in the CM-cellulose chromatography (Figure 1) identically with that product from a normal carboxymethylation of ribonuclease at pH 5.6 (Goren and Barnard, 1970). The *N*-1-CM-histidine-containing protein from the pyrophosphate-retarded reaction coincided in CM-cellulose chromatography with 119-His-*N*-1-CM-ribonuclease separated (Goren and Barnard, 1970) after the normal alkylation, and it seems reasonable to conclude that those two products, also, are identical.

Sites of N-1- and Dicarboxymethylation at pH 6. Ribonuclease A (ligand free) was [^{14}C]carboxymethylated at pH 6.0, and peak I (Goren and Barnard, 1970) was separated as the major product, containing all the *N*-1-CM- and di-CM-histidine residues. It was reduced and *S*-carboxymethylated (using dithiothreitol and iodoacetate), and (with added carrier RCM-ribonuclease A, in tenfold excess) was digested by trypsin. The digest was chromatographed on IRC-50 (H^+) with elution by a linear gradient from 0 to 100% acetic acid (Crestfield *et al.*, 1963b). The radioactivity was present solely in twin peaks, partly overlapping, with maxima at 55% and 68% acetic acid in the effluent. The major component, in the second peak, was hydrolyzed and analyzed, and the radioactivity was found in *N*-1-CM-histidine (89%) and di-CM-histidine (11%); the amino acid analysis was that of peptide RCM-T16 (residues 105–124), accounting quantitatively (without corrections) for all the residues therein within a maximum range of ±0.2 residue. This agrees with the known monoalkylation at histidine-119 by bromoacetate (Stein and Barnard, 1959) and with the finding of Crestfield *et al.* (1963b) that the *N*-1-CM-histidine-containing form of RCM-T16 is eluted by about 60% acetic acid in such a chromatography. Since the nonalkylated RCM-T16 peptide was in large excess here, it served as a marker to indicate that the IRC-50–acetic acid chromatography proceeded as expected. The other histidine-containing tryptic peptides from RCM-ribonuclease are eluted much earlier in this chromatography (Crestfield *et al.*, 1963a,b).

The di-CM-histidine-containing fraction was, therefore, examined further. The material in the first labeled peak in the IRC-50 chromatography described above was collected and analyzed. It contained 85% of the di-CM-histidine of the total sample, having 50% of its radioactivity in that derivative and 50% in 1-CM-histidine. Amino acid analysis showed a composition that corresponded to 90% of RCM-T9 (residues 40–61) and about 10% of RCM-T16 (these being the carrier nonalkylated peptides, since the labeled material was too low in amount for such analysis). It was concluded, therefore, that a di-CM-histidine-containing form of RCM-T16 was present and this migrated rather faster on the IRC-50 column, coinciding with peptide RCM-T9 and also overlapping with some of the monoalkylated form of RCM-T16. This was confirmed by analyzing a sample of the same fraction in the Aminex peptide-separation system (Figure 4, I). The single radioactive peak was eluted in a region corresponding to the elution position of peptide RCM-T16 carboxymethylated at histidine-119 (Crestfield *et al.*, 1963b). There was only one really appreciable ninhydrin-positive peak (marked N in Figure 4, I) and this was devoid of label and emerged where authentic RCM-T9 chromatographs; it was, therefore, attributed to the latter. It was concluded, hence, that none of the label is on histidine-48. It is, in any case, known that the histidine-containing peptides other than RCM-T16 move in very different positions (Crestfield *et al.*, 1963a,b) from that found here for the di-CM-histidine-containing peptide in the chromatographic system of Figure 4. It is clear that the labeled peak separated on the Aminex column (Figure 4, I) is the peptide RCM-T16 alkylated at histidine, the mono- and di-CM-histidine forms of this peptide not being separated in such chromatographic systems.

To confirm that the substitution is indeed on histidine-119 (and not on histidine-105, which is present and is N-terminal in peptide RCM-T16), a sample of the above-described di-CM-histidine-enriched fraction obtained in the IRC-50 chromatography was taken through one stage of Edman degradation. The labeled di-CM- and *N*-1-CM-histidine together yielded 6040 cpm in this sample before that treatment, and of this only 580 cpm was removed from the peptide in the degradation. Analyses showed that the removal of the N-terminal had proceeded satisfactorily and that di-CM and *N*-1-CM-histidine again accounted for all of the radioactivity in the residual peptides. Hence, histidine-105 was not the site of any significant labeling.

Attempted Cleavage of the Protein Chain at CM-methionine. A method potentially available for location of the alkylated methionine is the cleavage of the polypeptide chain at the adjacent peptide bond, when heated, due to the intramolecular attack of the carbonyl of the methionine residue when the

TABLE II: Stability of Met-CM-ribonuclease.

Alkylation Conditions[a]	CM-Met per Molecule	Incubation Conditions[b]	CM Groups per Molecule	
			Initial	Final
pH 6.2, 180 min	0.25	pH 7.8, 5 mM Tris-HCl	1.16	1.15
	0.25	25 mM acetic acid, 5°, 6 days	1.15	1.15
pH 2.0, 100 min	0.94	pH 7.8, 5 mM Tris-HCl 48 hr, 25°	0.94	0.99

[a] Reaction was with 0.025 M [^{14}C]bromoacetate, 35°. [b] The deionized CM-ribonuclease was exposed to the conditions shown, and then again deionized on the DEAE-Sephadex column.

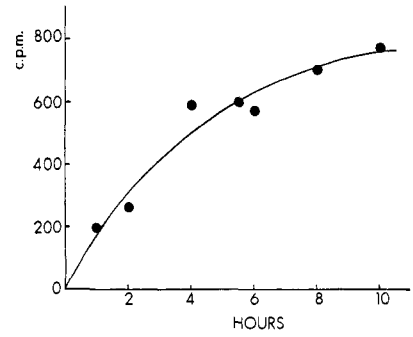

FIGURE 5: Cleavage of Met-[^{14}C]CM-ribonuclease at 100°. At the intervals shown, samples were removed and analyzed by chromatography; the total radioactivity released (shown as cpm) was in *S*-methyl-[^{14}C]thioglycollic acid and to a minor extent in [^{14}C]-glycollic acid. The theoretical yield would release 3000 cpm.

latter is in the sulfonium form (Lawson *et al.*, 1962). The chain cleavage next to alkylated methionine has been employed successfully in sequence studies on chymotrypsin by Tang and Hartley (1967) and in locating the methionine alkylated in chymotrypsin by Lawson and Schramm (1965). Those reactions were obtained in peptides containing CM-methionine, by heating for about 1 hr at 100°, when C-terminal homoserine lactone was produced. This must mean that an equivalent amount of *S*-methylthioglycollic acid is liberated.

Met-[^{14}C]CM-ribonuclease samples were heated at 100° in sealed tubes at pH 4, and the extent of cleavage was measured by the concurrent release of *S*-methyl-[^{14}C]thioglycollic acid (Figure 5). The reaction was slow, and leveled off at about 30% cleavage. The same limiting yield was obtained in other reactions at pH values from 3 to 7.5, and also when the performate-oxidized derivative of the Met-CM-ribonuclease was heated in water. This slow cleavage is complicated by a slight but interfering hydrolysis elsewhere (probably at aspartic acid residues; Schultz *et al.*, 1962), since when the products from the heating of either the oxidized *or* the unoxidized protein were gel filtered, a number of peptides were seen to have been formed. N-Terminal analyses by the cyanate method (Stark and Smyth, 1963), applied in two cases to the unoxidized samples at the end of the heating period, showed (in addition to the normal 1.0 residue of N-terminal lysine) 0.2–0.4 residue per protein molecule of N-terminal aspartic acid, methionine and serine, and others at very minor levels. Native ribonuclease A similarly heated showed, however, most of these same new end groups. This method was, therefore, discarded.

Stability of Met-CM-ribonuclease. The possibility was considered that in the course of many hours of exposure to buffered or acetic acid media in the course of purification, a part of the CM group complement on methionine might be lost, either through a chain cleavage reaction or by simple fission as occurs in CM-methionine at higher temperature (Gundlach *et al.*, 1959). However, with ribonucleases having either the specific or a more general methionine alkylation, no loss of label occurred (Table II) on extended exposure to media used for handling the protein products.

Location of the Alkylated Methionine. Material from peak II (as in Figure 1), containing Met-[^{14}C]CM-ribonuclease and ribonuclease A (obtained from an 8-hr alkylation at pH 5.8 in pyrophosphate medium) was cleaved by subtilisin in the conditions used previously, which give 60% yield of ribonuclease-S without significant side reactions. The S-peptide produced was separated by gel filtration (Figure 3B) and was seen to be unlabeled. A repeat of this experiment using twice the amount of material gave the same result. Hence, the methionine in S-peptide, residue 13, is not a site of carboxymethylation in these conditions.

The comparable material in a peak II fraction (Goren and Barnard, 1970), obtained by chromatography after alkylation (without inhibitor) at pH 3.3, was also cleaved by subtilisin. The S-peptide was separated (Richards and Vithayathil, 1969) and further purified by two gel filtrations; the amino acid composition was then as reported (Gordillo *et al.*, 1962), including 0.9 methionine residue per molecule. It, again, contained no significant counts above background. The corresponding S-protein fraction was purified in G-75 filtrations, and contained all of the radioactivity (6200 cpm), as [^{14}C]CM-methionine, of the digested parent protein. This level of radioactivity was such that, if 2% of the CM groups present were in the S-peptide, this would have been measured. In view of these results on the products from pH 3.3 and pH 5.8 alkylations, it was concluded that methionine-13 is totally unavailable in this pH range.

Further material from peak II (Figure 1) containing Met-[^{14}C]CM-ribonuclease was reduced, *S*-carboxymethylated, and digested with trypsin. The peptides were analyzed on the Aminex resin. In this system, the tryptic peptides of RCM-ribonuclease are separated in a series of fractions, as shown by Crestfield *et al.* (1963b) using a similar resin; the first ninhydrin-positive peak in this chromatogram contains a mixture of the peptides RCM-T-2, RCM-T4, and RCM-T9. In the present case, only one radioactive peak was observed on the chromatogram (Figure 4, II), emerging several minutes before the first ninhydrin-positive peak. The specific radioactivity of the preparation was so high that a single, labeled peptide could be insufficient in amount to be (without hydrolysis) ninhydrin positive.

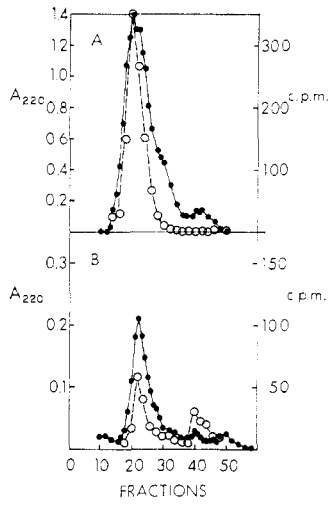

FIGURE 6: Filtration on Sephadex G-25 (see Methods) of the tryptic peptides of oxidized Met-[^{14}C]CM-ribonuclease. (A) First filtration; (B) refiltration of the labeled peak in A, after treatment at pH 4.0, 100°, 2 hr. The second ^{14}C peak was subsequently analyzed for amino acids and radioactive products with and without hydrolysis (105°) in 6 N HCl, 12 hr. (●—●) A_{220}; (○--○) cpm in 25-μl aliquots.

This [^{14}C]CM-peptide was collected, purified on an IRC-50–acetic acid column, and hydrolyzed. Amino acid analysis (Table III) indicated that this peptide was alkylated RCM-T4 (residues 11–31). The advance in the chromatogram (Figure 4, II) of the labeled peptide relative to RCM-T4 itself is consistent with the entry of one CM group. Since residue 13 has been ruled out (above) as the alkylated methionine, the observations indicate that in this alkylation either methionine-29 or methionine-30 reacts.

The Met-CM-ribonuclease fraction (peak II) was also isolated after alkylations at pH 5.6 and pH 6.0, and combined and used to prepare oxidized tryptic peptides. On Sephadex G-25, this digest gave one radioactive peak, while peptide material was both in this peak and in subsequent, overlapping peaks (Figure 6A). In the tryptic digest of O-ribonuclease, there are four large peptides (O-T2, O-T4, O-T9, and O-T16) which emerge in this initial single peak on G-25 (Smyth *et al.*, 1963). All four methionines are in peptides O-T2 (residues 67–85) and O-T4 (residues 11–31), so that the behavior seen in Figure 6A is as expected. The labeled peak was, therefore, collected, and the material therein was heated at 100° for 2 hr. On refiltration (Figure 6B) a new labeled peak, in the low-molecular-weight region, was seen to have been produced. This peak was found, by hydrolysis and analysis, to contain lysine as the sole ninhydrin-positive constituent: 0.5 μmole of lysine was found per μmole of the equivalent amount of parent Met-[^{14}C]CM-ribonuclease used, and an identical amount of lysine (only) was also found in an aliquot of that peak analyzed with no hydrolysis. The radioactivity in the peak was in *S*-methyl-[^{14}C]thioglycollic acid, and this radioactivity corresponded to 0.35 μCi/μCi of the total radioactivity present after heating (Figure 6B). These results show that methionine-30 is carboxymethylated, since this is the only methionine in ribonuclease which is followed by a lysine residue. The latter is expected to be released as free lysine when the peptide is heated to give the chain cleavage referred to above. This cleavage appears to occur here to the extent of 50%, a reasonable yield for this case. The expected ^{14}C-containing moiety is found, to a somewhat lesser extent, attributable to some volatility.

For confirmation, a Met-CM-ribonuclease fraction (peak

TABLE III: Amino Acid Analysis of Peptides.

Amino Acid	Radioactive Peptide[a]	Alkylated RCM-T4[b]	Alkylated RCM-T2[b]	Peak II (Figure 2)[c]
S-CM-cysteine	*d*	1	2	0 (0)
Aspartic acid	4.0[e]	3	3	3.0 (3)
Threonine	0.8	1	3	0.9 (1)
Serine	8.0[e]	6	3	3.7 (6)
Homoserine[f]		0.3	0.3	0.9 (1)
Glutamic acid	2.0	2	2	2.8 (1)
Proline	0	0	0	0 (0)
Glycine	0	0	1	1.0 (0)
Alanine	1.2	2	0	2.0 (2)
Valine	0	0	0	0 (0)
Methionine	0.5[g]	2.3	1.3	0[j] (0)
Isoleucine	0.2	0	1	0 (0)
Leucine	0.2	0	0	0 (0)
Tyrosine	1.0	1	2	0.2 (1)
Phenylalanine	0	0	0	0 (0)
Lysine	1.0[h]	1	0	(0)
Histidine	1.0	1	0	(0)
Arginine	0	0	1	(0)
CM-Methionine	1.0[i]	0	0	0 (0)

[a] Hydrolysate (20 hr) of peptide in the labeled peak (Figure 4, II) obtained from the chromatography of the tryptic digest of RCM (CM-Met-ribonuclease). The ^{14}C flow-cell analysis of this hydrolysate showed only the products derived from [^{14}C]CM-methionine (see text). All values are uncorrected, and are expressed as residues per mole of peptide. [b] Theoretical content of peptide RCM-T4 (residues 11–31) alkylated at one methionine or RCM-T2 (residues 67–85). [c] Hydrolysate (20 hr) of the unlabeled peptide in peak II of Figure 2. Numbers in parentheses represent the theoretical content for residues 14–29. [d] Not measured; appeared to be mainly oxidized (see note *g*). [e] Amino acid analyzer under the conditions used did not separate methionine sulfoxide or sulfone from aspartic acid, nor homoserine from serine. [f] Includes homoserine lactone. [g] Low yield expected, since this peptide had to be isolated on a column devoid of antoxidant. Methionine oxidation products are presumed to account for the increase in the peak at the aspartic acid position here. [h] All values are relative to this, taken as one residue per mole. [i] Calculated on basis of radioactivity (prior to hydrolysis). CM-methionine breakdown products were present in hydrolysate, but were not used for calculation. [j] A small amount of methionine sulfone (~0.2 residue, measured in a single analysis) was found, attributed to the nonalkylated ribonuclease A initially also present, part of this cleaving at Met-30 but not Met-29, and then being oxidized at Met-29.

II, Figure 1) was treated with cyanogen bromide (which will cleave at nonalkylated methionines only) and the C-protein and C-peptide peaks were separated on a G-25 gel column. The profile obtained by Gross and Witkop (1962) was quantitatively reproduced, and the label was found to be totally in the C-protein. This is further evidence that methionine-13 is devoid of CM groups, since the parent protein carried 0.5 CM-methionine groups per molecule and the yield of C-peptide would be only half of the normal if residue 13 were alkylated, whereas a normal yield (80%) was found here. The C-protein was oxidized (Hirs, 1956) and the peptides were separated on Bio-Gel P-6 (which excludes compounds above molecular weight about 6000). The radioactivity was essentially all in the largest peptide fraction present (Figure 2). This also excludes methionine-29 as the alkylation site, since if it were, the label would be in the smallest peptide present, residues 14–30. Peak II in Figure 2 contained the peptide of residues 14–29 (Table III).

The single radioactive peak (Figure 2) material was heated at 100°, 2 hr, and analyzed (Table IV). Only homoserine lactone was found, as well as the expected products from the [^{14}C]CM moiety. In view of the partial volatility of the latter products (Goren *et al.*, 1968), the results are in good agreement with expectation for chain cleavage adjacent to a terminal CM-methionyl group. This can only come from position 30 as N-terminal, and with methionine-29 not alkylated, so that the 29–30 bond was cleaved by CNBr. The yield of homoserine lactone and the fact that the released labeled group was not in excess over it confirmed the absence of alkylation at methionine-79.

TABLE IV: Analysis of Products after Cleavage at 100° of the Radioactive Peptide.[a]

Product	Cpm	Moles per Mole of Peptide[b]
S-Methylthioglycollic acid	3820	0.71
Glycollic acid	668	
Total radioactive products[c]	4477	
Homoserine lactone	0	1.1
Homoserine	0	0.0

[a] The peptide was isolated (see Figure 2) from the oxidized C-protein prepared from Met-[^{14}C]CM-ribonuclease. The analysis was made without acid hydrolysis. [b] Calculated on the basis of the total radioactivity in the peptide before treatment and the known specific activity of the [^{14}C]CM group. [c] An aliquot equal to that analyzed was used to measure the total radioactivity in the sample, in the flow-cell system in the same conditions.

Discussion

When ribonuclease A is complexed to certain dianions its carboxymethylation at histidine is much retarded (Goren and Barnard, 1970); we show here that the sites alkylated (119 and 12) and their relative rates of reaction are unaltered. Reaction with the small amount of free enzyme in equilibrium with the complex would reasonably account for the alkylation observed, although the measurements do not rule out an additional very slow alkylation of the complex itself. Effective direction of the reagent, in the complex, in a manner different to that in the free enzyme is, however, excluded.

More unexpected is the finding that in ribonuclease A the carboxymethylation at histidine-119 is followed by the entry of a second CM group into the same imidazole ring. This reaction is slow relative to the first alkylation there, but faster than a nonspecific histidine reaction. The evidence obtained excludes the significant occurrence of this reaction at the other histidines.

The reaction at methionine is shown to occur at residue 30. Alkylation at methionine-13 is excluded by several pieces of evidence, of which that on the unlabeled S-peptide is the most quantitative, setting an upper limit of about 2% for alkylation at that site. (In a preliminary report (Glick *et al.*, 1967) a rather higher limit was set, but the products separated there show, on reexamination by the present methods, the results cited here, the earlier S-peptide preparation having been impure.) Similarly, no products derived from a 79-Met-CM-ribonuclease were detectable here by any of several methods. While it was rather straightforward to establish that alkylation had not occurred at positions other than 29 or 30, the allocation between the latter two gave some difficulty. The monoalkylated methionylmethionyl sequence complicated the peptide analyses, such that Edman degradation of the entire tryptic peptide involved could not give quantitative results and was not employed here.

We rely upon two independent lines of evidence to distinguish alkylation at position 30 from possible alternative or concurrent alkylation at 29. Firstly, when the only labeled tryptic peptide, which contains these two methionines, was subjected to intramolecular attack by its CM group, no products were detectable except those due to fission of bond 30–31. Secondly, after cyanogen bromide cleavage and oxidation of the whole protein, the radioactivity was almost entirely in the largest peptide, as expected if bond 30–31 was *not* cleaved, due to a CM group on the thioether at 30. Not more than 5% (Figure 2) was in the small peptide corresponding to cleavage at 30 and not at 29. It might conceivably be, however, that the label in the large peptide is, in part, due to an incomplete CNBr cleavage at bond 30–31 in a hypothetical 29-Met-CM-ribonuclease: Link and Stark (1968) obtained a yield of only 40–52% from CNBr cleavage there in a methylated analog. CNBr cleavage appears, however, to be of the usual efficiency in the present derivative, with a CM group at position 30; since gel filtration was employed and recovery appeared to be essentially quantitative, the pattern of separated peptides (Figure 2) gives information on this and is consistent with the expected cleavages. Further evidence to exclude the above-noted complication is provided by the results in Table IV. If methionine-29 were appreciably carboxymethylated, either bond 30–31 would be cleaved by CNBr, in which case the 5% of the carbon-14 in the corresponding small peptide represents the limit of alkylation there; or the cleavage is inefficient at bond 30–31, in which case the yield of the terminal residue (Table IV) would be reduced by the corresponding amount, an effect which is seen to be negligible. Taking into account the probably experimental error range, an upper limit of 10% for an alternative alkylation at methionine-29 is deduced, this being equivalent

TABLE V: Second-Order Rate Constants (k) for Halogenoacetate Alkylations of Sites in Ribonuclease.

Reagent	Site	Reaction conditions			$k \times 10^4$ M^{-1} sec^{-1}
		Urea, M	pH	Temp, °C	
Bromoacetate	His-119		5.6	35	307.8
	His-12		5.6	35	34.2
	Met-30		6.0–7.0	35	19
	Methionines	8	4.8	35	63[a,b]
	Methionines		2.0	35	20[a,c]
Iodoacetate	Methionines	8	5.8	40	75[a,b,d]
	Methionine		5.5	25	1.5[e]
Bromoacetate	(His-119 + His-12)		5.5	25	205[f]
Iodoacetate	(His 119 + His 12)		5.5	25	58.4[f]

[a] Value for reaction at one methionine, assuming that all four proceed at the same rate in the unfolded protein. [b] The equivalent value of k for the unfolded protein in water, *i.e.*, correcting for the solvent effect alone, on the basis of model reactions in 8 M urea and in water (Stark and Stein, 1964), would be about 20% lower. [c] Mean value calculated from the results at pH 2.0 and 2.1 (Table VIII of Goren and Barnard, 1970). [d] Stark and Stein (1964). [e] Fruchter and Crestfield (1965). Ionic strength 0.18. [f] Heinrikson *et al.* (1965). Ionic strength 0.11.

to an efficiency of 50% for CNBr cleavage at the 30–31 bond in that derivative.

The peptide bond cleavage by a CM-sulfonium group at 100° is seen in the course of these observations to be strongly determined by the macromolecular environment. Both in intact, and in oxidized, ribonuclease, it proceeded slowly and to a maximum of 30% (Figure 5). Steric interference with effective positioning for the intramolecular attack is presumably the reason for this. When the oxidized peptide of residues 11–31 was used, the reaction proceeded more rapidly, to give 50% yield in 2 hr. Similarly, when the peptide deduced to have the CM-methionine group as N-terminal was heated, the reaction was quantitative in 2 hr. The greater ease of this reaction at a CM-methionyl residue when N-terminal in a peptide has been shown by Tang and Hartley (1967) and by Lawson *et al.* (1962).

The conclusion reached, that alkylation occurs to the extent of 90% or more at methionine-30 and 10% or less at methionine-29, contrasts with the converse finding of Link and Stark (1968) for the reaction of methyl iodide with methionine in ribonuclease. The difference may be due to the nonpolar character of methyl iodide compared to bromoacetate ion, and perhaps to protein structural changes induced at the interface with the separate methyl iodide phase used. Exclusion of bromoacetate from 29 or 30 cannot be deduced conclusively from models of the structure at present available. In the structure of ribonuclease A phosphate (Kartha *et al.*, 1967, and G. Kartha, personal communication) the sulfur atoms of residues 29 and 30 are both in a hydrophobic region, although 29 is more exposed toward the medium on one side. These two side chains point in different directions. We deduce from the model that access to the sulfur at position 30 from the solvent is likely to be possible from the opposite side, through the active center cleft. This access is possible but restricted, especially by the lysine-41 side chain as seen in the crystal structure, but if this chain is flexible in solution, the reagent would apparently be able to attack the sulfur at 30 from that side.

The entry of the methyl iodide molecule into a superficial but hydrophobic zone in the one case, and the entry of the bromoacetate ion through a polar channel in the other, would permit all the observations made to be reconciled with the structure. The lack of inactivation is expected, since both 29 and 30 are far enough removed from the active center region at histidine-119 and -12. The inhibition by a complexed nucleotide of the bromoacetate reaction (Goren and Barnard, 1970) but not of methylation (Link and Stark, 1968) is ascribed to steric blockade of 30 in the complex. The simple dianions also bind in the active center cleft, of course, but the additional contact of the base can be seen on a model to give a greater blockade; a tightening of the structure in the nucleotide complex can be inferred from the decrease in dissociation constant compared to that for phosphate dianion (see Figure 9 of Goren and Barnard, 1970) and from other evidence (Markus *et al.*, 1968), and is seen directly in the crystallographic structure to occur in the active center region of the complex (G. Kartha, personal communication).

The reactivity evidence points, in any event, to a partial accessibility, in free ribonuclease A in solution, to several reagents in the 29–30 region. The pH dependence of the methionine carboxymethylation (Goren and Barnard, 1970) shows an upswing at pH 5.5–6 (which, consistent with the interpretation put forward above, is absent (Link and Stark, 1968) in the methyl iodide reaction). Residue 30 is the site on both sides of this transition, since samples from both pH zones were pooled for the peptide analysis. This increase in reactivity must be an index of a structural change in the protein occurring in this pH range. A conformational equilibrium of ribonuclease with a similar pH profile was deduced by French and Hammes (1965) from temperature jump studies and (involving histidine-48, which is near methionine-30) by Roberts *et al.* (1969) from histidine titrations by nuclear magnetic resonance spectroscopy.

The rate constants for the various bromoacetate reactions studied, obtained from experiments conducted as described by Goren and Barnard (1970), and those for iodoacetate

reactions measured by others, are collected in Table V. The reaction in water at pH 2 at each of the four methionines occurs at about the same rate as that of the single residue at pH 6. The intrinsic reaction rate with the protonated reagent in acid would be expected, from model studies (Stark and Stein, 1964), to be slightly lower than the rate near neutrality, but this does not account for all of the decrease found here on moving from urea to aqueous acid media. Hence, all four methionines in the acid-unfolded protein react as though partly obstructed, presumably due to residual hydrophobic structures which disappear in 8 M urea. A comparable, partly obstructed situation appears to exist for methionine-30 in the native protein.

At pH 3.1 (35°), the reaction of the other methionines becomes quite discernible, increasing with further acidity (see Figure 5 and Table VIII of Goren and Barnard, 1970). Enzymic activity is lost in this reaction, this being attributed to the inability of the protein thus modified to re-form the native structure in the neutral assay medium. The thermal transition temperature of ribonuclease is 34° at pH 2.1 and 41° at pH 2.7, from the spectroscopic data of Hermans and Scheraga (1961) in 0.15 M KCl (and at the low ionic strengths used in all the alkylation studies, the decrease in monoanion concentration would reduce each of these values by 3°, as estimated from the data of Ginsburg and Carroll (1965) for pH 2.1). Neumann *et al.* (1962) have reported a reaction with iodoacetate (0.016 M) at pH 2.7, 40°, for 3 hr, when the major product (as separated on IRC-50 phosphate) was inactive and contained 3.5 CM-methionine residues per ribonuclease molecule. This rapid polysubstitution is quite similar to our time course at pH 2.1 (Table VIII of Goren and Barnard, 1970). The inactivating reactions at pH 2.1, 35° (Table V), or at pH 2.7, 40°, are, therefore, in the zone of unfolding detected spectrally. The increase in reaction at 35° in the pH range 3.6–2.8 (Figures 5 and 7, and Table VIII, of Goren and Barnard (1970)) is in advance of the major unfolding, and, from its lack of association with inactivation, probably involves only one methionine in addition to residue 30. It is plausible to suppose that this is residue 29, but this requires confirmation by peptide analysis of material reacted in this zone. A local loosening prior to the major unfolding appears, in any case, to be detected by the methionine reactivity.

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