Relations of Reactivity to Structure in Pancreatic Ribonuclease. I. An Analysis of the Various Reactions with Bromoacetate in the pH Range 2–7*

H. Joseph Goren† and Eric A. Barnard

ABSTRACT: The carboxymethylation of ribonuclease A received a more detailed scrutiny. The pH dependences of the individual reactions at histidine-119, histidine-12, and lysine have been established, and related to their apparent pK_a values. The protein products after reaction at various pH values between 2 and 7 were separated and related to alkylations at these sites. Improved preparation methods are specified.

Facilitated formation of the *N,N*-1,3-dicarboxymethyl derivative of histidine-119 occurs in a second phase of the reaction with bromoacetate. Slow but significant alkylations also occur at histidine-48 and histidine-105. A concurrent carboxymethylation at a single methionine thioether group has been investigated in ribonuclease A monomer. A number of characteristics of this methionine reaction were determined, including its lack of effect upon enzymic activity and its lack of increase with temperature. It is not inhibited by phosphate or sulfate, but it is by the firmer ligands pyrophosphate and cytidine 2′- and 3′-phosphates. All ligands binding at the active center, however, inhibit the histidine-119 and histidine-12 alkylations, equally at each site. The pH dependences of these effects are considered in relation to the structure of the complexes. Ribonuclease when unfolded in acid can be carboxymethylated at all four methionines, and dianionic ligands do not reverse the unfolding to protect. An incipient unfolding in acid is revealed by the increase in availability to bromoacetate of methionine in ribonuclease near pH 3 (at 35°).

The specific, inactivating reaction of bromoacetate with histidine in pancreatic ribonuclease has been known for over a decade (Stein and Barnard, 1958; Barnard and Stein, 1959a,b). It was shown that the main product is an inactive ribonuclease carrying one CM group located on the imidazole ring of histidine residue 119. An investigation of the similar reaction with iodoacetate at pH 5.5 (Gundlach *et al.*, 1959b; Crestfield *et al.*, 1963a,b) confirmed the position of the major attack at histidine-119, and showed in addition a minor extent of reaction (about one-ninth) at histidine-12.

The ribonuclease–halogenoacetate reaction is of much interest since its highly specific characteristics, documented in the references cited, are such as to place it in a valuable class of enzyme modifications, namely those which utilize an interaction of the reagent with the active center of the enzyme to label covalently and specifically a group therein. This deduction of active center character for the two histidines, 12 and 119, has recently been vindicated in the three-dimensional structure established for the protein (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967), which shows these two imidazoles to be close to each other in the region where substrate-like molecules bind.

The course of the halogenoacetate–ribonuclease reaction has not been reported previously in detail with regard to all sites of substitution. This reaction has proven to be complex. We have consistently found that the extent of alkylation of ribonuclease by bromoacetate at pH 4 to 6 is greater than the extent of the inactivation, indicating some attack at one or more sites other than histidine. We have reported briefly (Glick *et al.*, 1967; Barnard and Goren, 1967) that this can be specified as an attack at methionine, which at pH 4–6 occurs without removing enzyme activity.

Reaction at methionine residues has previously been observed with more drastic treatment, as at pH 2.8 and 40° (Gundlach *et al.*, 1959b; Neumann *et al.*, 1962) or in 8 M urea (Stark and Stein, 1964), and was ascribed to the unfolding of ribonuclease; it also occurs slowly in ribonuclease dimer (Fruchter and Crestfield, 1965b). However, the use of ^{14}C-labeled reagent and a scintillator flow-cell method of analysis (Goren *et al.*, 1968) has enabled us to recognize a significant attack at a methionine site occurring in a higher pH range, where the unfolding seen in acid (Hermans and Scheraga, 1961) is undetectable physically. The identity of this site, and the detailed characteristics of the various alkylations in ribonuclease in the pH zone 2–7, involving a number of features hitherto unreported, are considered in this and the succeeding paper (Goren and Barnard, 1970), with structural interpretations.

Materials and Methods

Ribonuclease. Bovine pancreatic ribonuclease A (Type

* From the Molecular Enzymology Unit, Departments of Biochemistry and Biochemical Pharmacology, State University of New York, Buffalo, New York 14214. *Received September 25, 1969.* Supported by a grant (GM-11603) from the National Institutes of Health, U. S. Public Health Service. Summaries of parts of this work have been presented (3rd Meeting of the Federation of European Biochemical Societies, Warsaw, April 1966; Barnard and Goren, 1967; Glick *et al.*, 1967).

† Predoctoral trainee, National Institutes of Health (Grant FR-5454), and recipient of a predoctoral fellowship of the Graduate School, State University of New York at Buffalo, to which a dissertation including a part of this work was submitted in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel.

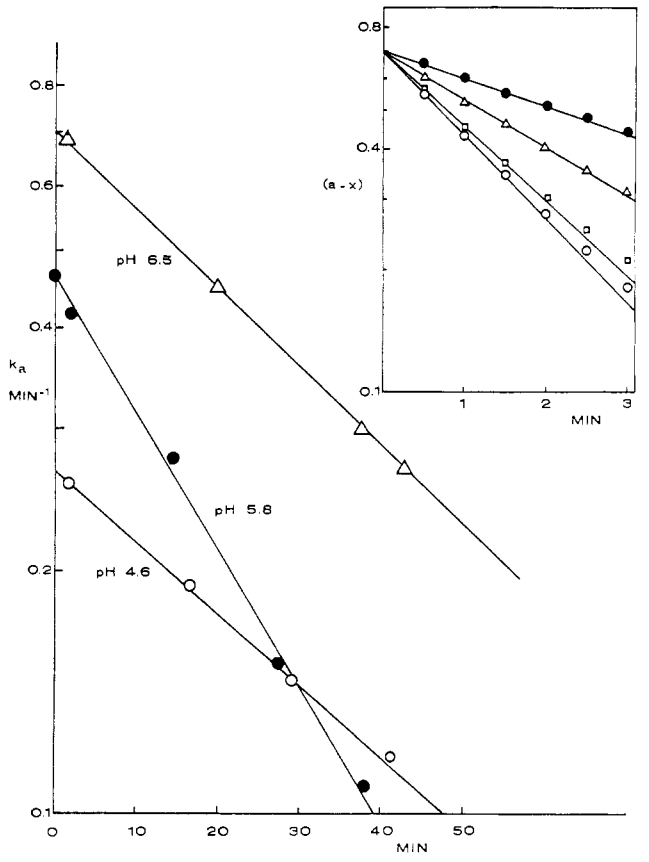

FIGURE 1: The method used for following the inactivations by bromoacetate. The insert shows a set of semilogarithmic plots of assays (on C2′3′p) of samples removed after four successive times from a reaction mixture: a is the initial absorbance value and x the value at the time (on the abscissa) in the assay reaction. The first-order plot deviates slightly from linearity (due to product inhibition) but its initial slope, k_a, is proportional to the activity in the range used (Barnard, 1964). The values of k_a are plotted semilogarithmically for the various reaction periods, showing a pseudo-first-order inactivation. Three such plots are shown, illustrating a part of the variation of the inactivation with pH.

IIA, Sigma Chemical Co.) was purified by a method that avoids exposure of the purified enzyme to phosphate. This involved preliminary dialysis (18 hr) in extracted (Goren *et al.*, 1968) Visking tubing, of the Type IIA material against water; this material was chromatographed on CM-cellulose with details as described below for CM-ribonuclease chromatography. Fraction D (Taborsky, 1959) was separated,[1] dialyzed against water overnight, and finally deionized through a column (about 8 × 0.9 cm) of MB-3 mixed-bed resin, with immediate neutralization by dilute HNO_3 of the emergent isoionic protein. To show that the mixed-bed resin treatment has no deleterious effect, similar samples were prepared in which the latter stage was replaced by passage through a column of DEAE-Sephadex A-50 (Pharmacia Inc) of adequate size, equilibrated with 0.05 M KNO_3 (Fisher Certified grade), with final dialysis of the protein before use; these gave identical results in alkylations, and were not generally used. The pure enzyme was never freeze-dried before reaction, to avoid possible aggregation (Fruchter and Crestfield, 1965a,b), but was stored for short periods in solution at 4° in water. The ribonuclease A was homogeneous in rechromatography of a sample, and homogeneous and monomeric by sedimentation equilibrium analysis (by Dr. M. Derechin) in the Spinco Model E ultracentrifuge.

Bromoacetate and 1-[^{14}C]bromoacetate were the white recrystallized samples described by Goren *et al.* (1968). Iodoacetamide (Eastman) was recrystallized from ethylene dichloride–ethyl ether, dried *in vacuo* in the dark, and stored at −20°. 1-[^{14}C]Iodoacetamide was obtained in crystalline form without free halogen, from Volk Radiochemical Labs, at 1.84 mCi/mmole. C2′3′p was,[2] for routine assays, purchased from Schwartz BioResearch Inc. as the barium salt (99% pure), and used as such. For exact enzymic activity determinations, a synthesized pure sample[3] of C2′3′p (K^+ salt) was employed. A3′p was from Boehringer Co., while preparations[3] of pure A2′3′p and C2′p were as noted by Barnard (1964), the paper chromatographic evidence for homogeneity being as given there. RNA (yeast) was from Worthington, prepared by the method of Crestfield *et al.* (1955). Urea was purified as described by Barnard (1964). Other reagents were as used by Goren *et al.* (1968). CM-cellulose was Whatman CM-1. After removal of fines, it was equilibrated at room temperature in succession with 0.1 N HCl–1 M NaCl, deionized water, 1 M $NaHCO_3$, 0.1 N NaOH, and water, extracted with 95% (v/v) ethanol, and dried. SE-Sephadex (C-25, Pharmacia, fine mesh) was cycled similarly. Glass-distilled, deionized water (conductivity 0.5–1 × 10^{-6} ohm^{-1}) was used throughout.

Analyses. Radioactivity measurement (by liquid scintillation counting), protein hydrolysis, and amino acid analysis were performed as previously (Goren *et al.*, 1968); so also was spectrophotometric protein concentration determination, except in the cases where the CM-protein was denatured, as after reaction below pH 2.8, where the method of Lowry *et al.* (1951), as modified by Layne (1957), was used for that purpose. Analysis of the ^{14}C-containing products in hydrolysates of carboxymethylated ribonuclease was by the flow-cell procedures that have been described in detail (Goren *et al.*, 1968).

Enzymic activity was measured by the ultraviolet spectroscopic assay method (Barnard, 1964) on C2′3′p as substrate, at pH 7.0 (I = 0.2, 25°), reading at 287 mμ using a Cary Model 15 spectrophotometer. The first-order initial rate constant, k_a, was determined from the curves (see Figure 1) at an initial substrate concentration of 0.4 mM. For activity on RNA as substrate, the spectroscopic method of Shapira

[1] We shall refer to the major ribonuclease fraction obtained in the CM-cellulose system as ribonuclease A, to avoid a multiple terminology, although it was designated ribonuclease D by Taborsky (1959) and Shapira and Parker (1960), who showed that it was identical with the great bulk of ribonuclease A as separated on IRC-50/phosphate (Hirs *et al.*, 1953). Their evidence, and that of Fruchter and Crestfield (1965a) using another separation, shows that the usual form of ribonuclease A may contain other minor components; these are removed in the system we use.

[2] Abbreviations are listed in *Biochemistry 5*, 1445 (1966), except for: 119-His-CM-ribonuclease, the protein with a CM group on the imidazole of histidine-119, etc.; SE-, sulfoethyl-.

[3] These samples of C2′3′p, C2′p, and A2′3′p were kindly supplied by Dr. H. Witzel (Chemisches Institut, Marburg, Germany), the characterization of the C2′3′p being as noted by Gassen and Witzel (1967).

(1962a,b) was employed, but using continuous recording on the spectrophotometer.

Reaction of Ribonuclease with Bromoacetate. For analytical studies, the reaction was carried out in a thermostat at 35.0° (or other stated temperature) ± 0.1°. Preincubated solutions of ribonuclease and sodium 1-[^{14}C]bromoacetate, containing sodium acetate and adjusted to the pH value required, were mixed to give a medium that was (unless stated otherwise) 0.025 M in bromoacetate and 0.025 M in acetate. Ribonuclease final concentration was usually at 3 mg/ml, and was always within the range 0.5–10 mg/ml. In the pH range investigated, the buffers specified were shown to be sufficient to take up the small proton output from reagent decomposition and reaction without a change of more than 0.02 pH unit, except for those reactions over several hours at pH 6.5 or above, where occasional NaOH addition was made to keep the pH constant. To follow the loss in enzymic activity, aliquots containing about 60 μg of ribonuclease could be taken directly (to initiate a reading within about 20 sec of withdrawal) into a preequilibrated (25.0°) assay cuvet.

To follow the incorporation of [^{14}C]CM groups, the reaction was usually arrested in each sample by the addition of 0.5 M sodium sulfate (to 0.02 M final concentration) and of 10% (v/v) 2-mercaptoethanol (5 μl to a 0.5-ml sample). Bromoacetate and its products were then removed quantitatively on a small column of DEAE-Sephadex A-25 (Goren *et al.*, 1968). It was later noted that if the application to this column is rapid enough, arrest by the addition of mercaptoethanol and sulfate is not necessary.

Column Chromatography of Carboxymethylated Ribonucleases. At termination of the [^{14}C]bromoacetate reaction, the mixture was at once run onto a column (0.9 × 21 cm) of CM-cellulose, equilibrated with 5 mM Tris chloride buffer at pH 7.8. A modification of the methods of Taborsky (1959) and Shapira (1962b) was employed. Both salt and pH gradients were applied here, using in reservoir 1 (stirred, and connected to the column head) 400 ml of 5 mM Tris chloride at pH 7.80, and in reservoir 2, 400 ml of 5 mM Tris chloride in 0.15 M NaCl solution at pH 8.00. Operation was at room temperature, flowing at about 6 ml/hr, and collecting 1–2-ml fractions.[4] The protein in the individual peaks was dialyzed, or concentrated and deionized by passage through a column (0.9 × 25 cm) of Bio-Gel P2 (BioRad Laboratories) in water, or on an MB-3 column as for ribonuclease A.

Preparation of Specific Histidine-Alkylated Ribonucleases. The reaction with bromoacetate is conducted at pH about 5.6 at 35°, for a short period only. The histidine reaction is then so fast that virtually no methionine is alkylated in peak I (see sample 1, Table I). Ribonuclease (deionized) in sodium acetate (0.025–0.04 M, pH 5.6) is used in any concentration up to about 20 mg/ml. Bromoacetic acid (0.5 M) is prepared in the acetate medium, with adjustment to pH 5.6 by cautious addition of NaOH solution (50%, then 1 N) before making up to volume. To initiate reaction, 50 μl of this solution is taken per milliliter of the reaction mixture, at 35°. When about 60–70% of the enzymic activity has been lost (about 20 min, at 35°), sodium sulfate (20 mM final concentration) and mercaptoethanol (to about 0.04 M) are added; 1 M Tris solution is then added to about 5 mM final concentration, with pH adjustment to 7.8. The mixture is chromatographed on CM-cellulose as described above or (preferably) as described by Goren and Barnard (1970) (where CM-52–40 mM Tris is used). The chromatogram is as in Figure 2A; peak I (119-His-1-CM-ribonuclease) is pooled (discarding the front one-quarter of the peak), and absence of detectable enzyme activity in this pool is checked. Deionization can be by concentration to 2 ml and passage through a column (0.9 × 30 cm) of Bio-Gel P-2 in water or any required medium, when the protein emerges quantitatively at the breakthrough. It can be stored at 4° in solution for several weeks, or indefinitely if sterilized by passage through a Millipore filter, or it can be frozen or freeze-dried from water (with very slight aggregation). 12-His-3-CM-ribonuclease (peak III) can be similarly prepared simultaneously, in small amount.

[4] More recently, we have found still better results using the microgranular CM-52 cellulose (Whatman) in place of CM-1, in modified conditions (see Goren and Barnard, 1970).

TABLE I: Residues Alkylated in Proteins Separated in CM-Cellulose Chromatography.[a]

Sample[b]	Peak Number	^{14}C Content (% of Total ^{14}C) N-1-CM-His	Di-CM-His[c]	N-3-CM-His	CM-Met[c]
1	I	85	15	0	0
	III	0	0	100	0
2	I	81	18	0	0
	II	0	0	0	100
3	I	77	20	0	3
5	I	74	17	0	9
	II	0	0	0	100

[a] Peaks were numbered as designated in Figure 2. [b] Samples were as in Table II. [c] For discussion of these products, see later text. Peaks I and II also contained about 1% CM-lysine in each.

Results

Reaction with Bromoacetate at pH 5.6. Near this pH value, where the inactivation is fastest, the reaction is conveniently performed using 0.025 M bromoacetate, with buffering by 0.025 M sodium acetate. The inactivation (Figure 1) then has a half-time of about 16 min at 35°. It obeys pseudo-first-order kinetics for most of its course.

The products from such a reaction can be separated by chromatography on CM-cellulose using a salt and pH gradient (Figure 2A). Here, reaction is not taken to completion, so that an unchanged peak of ribonuclease A is present. Two other distinct peaks are always obtained. The major product, 119-His-CM-ribonuclease (Stein and Barnard, 1959; Crestfield *et al.*, 1963a), separates here as a faster running peak (I), which is enzymically inactive and is labeled to the extent of about one CM group per molecule. A small peak (III) emerges after ribonuclease A, with only a trace of enzymic activity and carrying one CM group per molecule. This peak was assigned (see below) to 12-His-CM-ribonuclease.

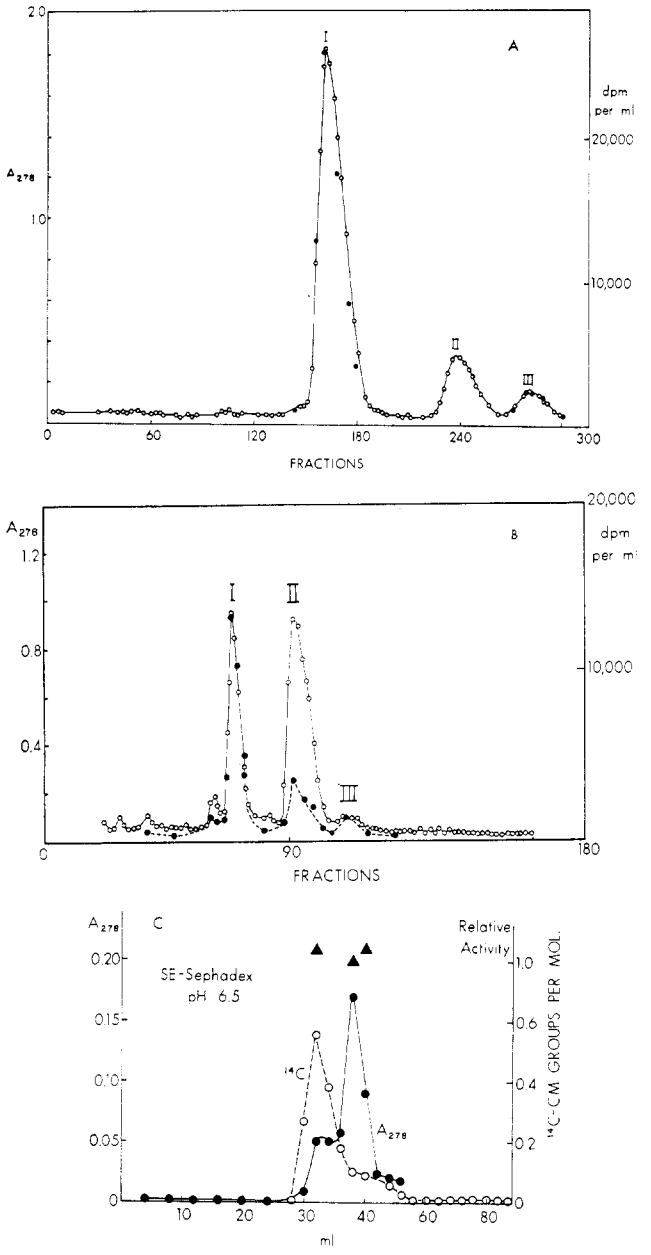

FIGURE 2: Chromatographic separation of ribonuclease derivatives. (A) After reaction at pH 5.6 (30 min, 35°, 0.04 M [^{14}C]bromoacetate); chromatography on CM-cellulose. Protein is measured by A_{278} (○), and ^{14}C as disintegrations per minute per milliliter of effluent (●) on a scale normalized so that it coincides with the protein scale at 1.0 [^{14}C]CM group per ribonuclease molecule. (B) As for A, but after reaction at pH 4.2 (sample 2 of Table II). (C) Chromatography on SE-Sephadex of material in peak II separated as in B. The column was 0.9 × 62 cm, equilibrated in and eluted with 0.1 M sodium phosphate, pH 6.5 (Fruchter and Crestfield, 1965a). Protein content (●), [^{14}C]CM groups per molecule (○), and the enzymic activity (on C2′3′p) relative to that of ribonuclease A (▲) are plotted.

The ratios of these peaks varied considerably with the conditions of the alkylation (Table II).

Amino acid analyses of the proteins separated in the three main peaks of Figure 2A showed no significant difference in any amino acid except for histidine, which was (in the labeled peaks only) low by about one residue. Possible small changes in methionine could not be determined by conventional amino acid analysis but were examined, here and after other reaction conditions, by the isotopic method as described below.

TABLE II: Analysis of CM-Cellulose Chromatograms.

Sample	Reaction Conditions[a]	Proportion of Peaks[b] Peak I	Peak II	Peak III
1	pH 5.5, 37 min, 35°, 0.033 M acetate	73	21	6
2	pH 4.2, 44 min, 35°, 0.023 M acetate	36	58	6
3	pH 3.8, 32 min, 35°, 0.024 M acetate	21	79	0
4	pH 3.75, 40 min, 30°, 0.050 M acetate	10	90	0
5	pH 4.1, 37 min, 37°, 0.023 M acetate	30	65	5
6	pH 4.2, 100 min, 35°, 0.026 M acetate	63	26	11
7	pH 3.3, 90 min, 35°, 0.022 M acetate	14	84	2

[a] All contained 0.025 M bromoacetate, except samples 1 (0.040 M) and 7 (0.018 M). [b] Peaks were numbered as in Figure 2 (peak II containing native ribonuclease A), and expressed as a percentage of the total protein in the chromatogram.

Analysis of Sites of Substitution in CM-Ribonucleases. Each of the protein peaks obtained in the chromatographies was analyzed (Table I) after hydrolysis, by a sensitive scintillation flow-cell chromatographic method (Goren *et al.*, 1968). The major labeled peak (I, Figure 2A) showed 1-CM-histidine as almost the sole labeled product after reaction at pH 5.6, (Figure 3), and the same was true (with the exception of some di-CM-histidine, discussed below) at other pH values in the range 3–6 (Tables I and III). The minor labeled peak (III in Figure 2A) gave rise to only 3-CM-histidine. The usefulness of the 1-CM-histidine content as a measure of the derivative I, and of the 3-CM-histidine content as a measure of the derivative III formed, was confirmed in each case where both types of analysis were made, as shown typically in Table III.

Peak I is 119-His-CM-ribonuclease (see above) and peak III is 12-His-CM-ribonuclease. Those products were chromatographed by Crestfield *et al.* (1963b) on an IRC-50 phosphate column and identified. We have confirmed our assignation of the peaks in the CM-cellulose system by first separating these derivatives in the system of Crestfield *et al.*, after bromoacetate reaction at pH 5.6 and at pH 4.0; on rechromatography of each derivative on CM-cellulose, the expected peak, as in Figure 2, was obtained. However, the present CM-cellulose chromatographic method was used in all further studies, since the resolution of ribonuclease A and 119-His-CM-ribonuclease was not complete in the IRC-

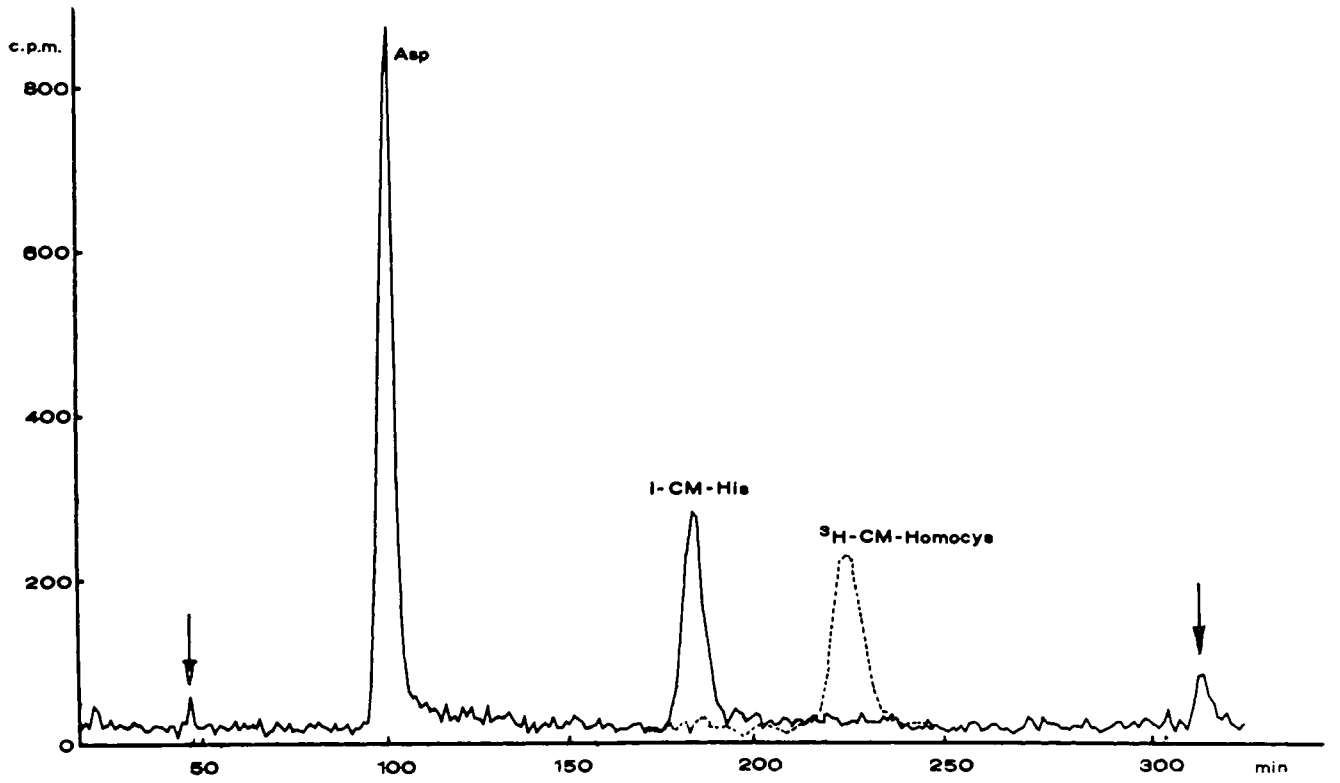

FIGURE 3: Analysis (on the 60-cm analyzer column) using the flow-cell scintillation counting system of the hydrolysate of peak I of Figure 2A. ^{14}C (——) and ^{3}H (---) cpm are plotted per minute of effluent flow. The ^{3}H is from added [^{3}H]CM-homocysteine added as marker to confirm the absence of this CM-methionine hydrolysis product in the ^{14}C-labeled derivatives. Asp represents [^{14}C]aspartic acid added as internal standard. The arrows mark the positions of the two peaks that arise from the di-CM-histidine (Goren *et al.*, 1968).

TABLE III: Agreement of Analysis of Labeled Residues with CM-cellulose Chromatographic Analysis.[a]

Peak	CM Groups per Mole of Protein	Contribution[b] of Individual Peaks to Total						
		Activity[c]	CM groups	1-CM-His[d]	Di-CM-His[d,e]	CM-His	CM-Met[e]	CM-Lys[e]
I	1.02	0	0.83	0.61 (0.55, 0.68)	0.15 (0.15, 0.11)	0.01	0.05	0.01
II	0.13	0.11	0.01	0	0	0	0.2	0
III	0.9–1.0[f]	0.01	0.08	0	0	0.08	0	0
Sum		0.12	0.92	0.61	0.15	0.09	0.07	0.01
Overall[g]		0.11[h]	0.90	0.55	0.14	0.10	0.09	0.02

[a] Reaction was with 0.025 M [^{14}C]bromoacetate in 0.035 M acetate, pH 6.02, at 35° for 45 min. [b] Contribution of the component shown per molecule of the total protein in the chromatogram. [c] The enzymic activity (on RNA) contributed by the protein in the peaks, as a fraction of the original activity of the total ribonuclease in the sample. [d] Peak I was not homogeneous with respect to 1-CM-His, and the analysis of material from the center of the peak is shown (to which the slight deviation from the overall value for 1-CM-His is attributed). The first third and last third were also analyzed separately, giving the values shown, respectively, in the parentheses. [e] For discussion of these products, see text. [f] Range arises from the inaccuracy of measurement of the small amount of protein in this peak. [g] Analysis of a deionized aliquot of the mixture after reaction, without CM-cellulose chromatographic separation. [h] The overall activity (on C2′3′p) in the sample, as a fraction of the original activity therein.

50 phosphate system. Crestfield *et al.* (1963b) showed that an IRC-50–NaCl system did give complete resolution, but this was not used here since in that unbuffered system the 12-His-CM-ribonuclease peak and the retarded ribonuclease A peak (which was of special interest here; see below) were much spread out.

Dicarboxymethylation of Histidine. One of the minor products present in the hydrolysates of the CM-ribonuclease

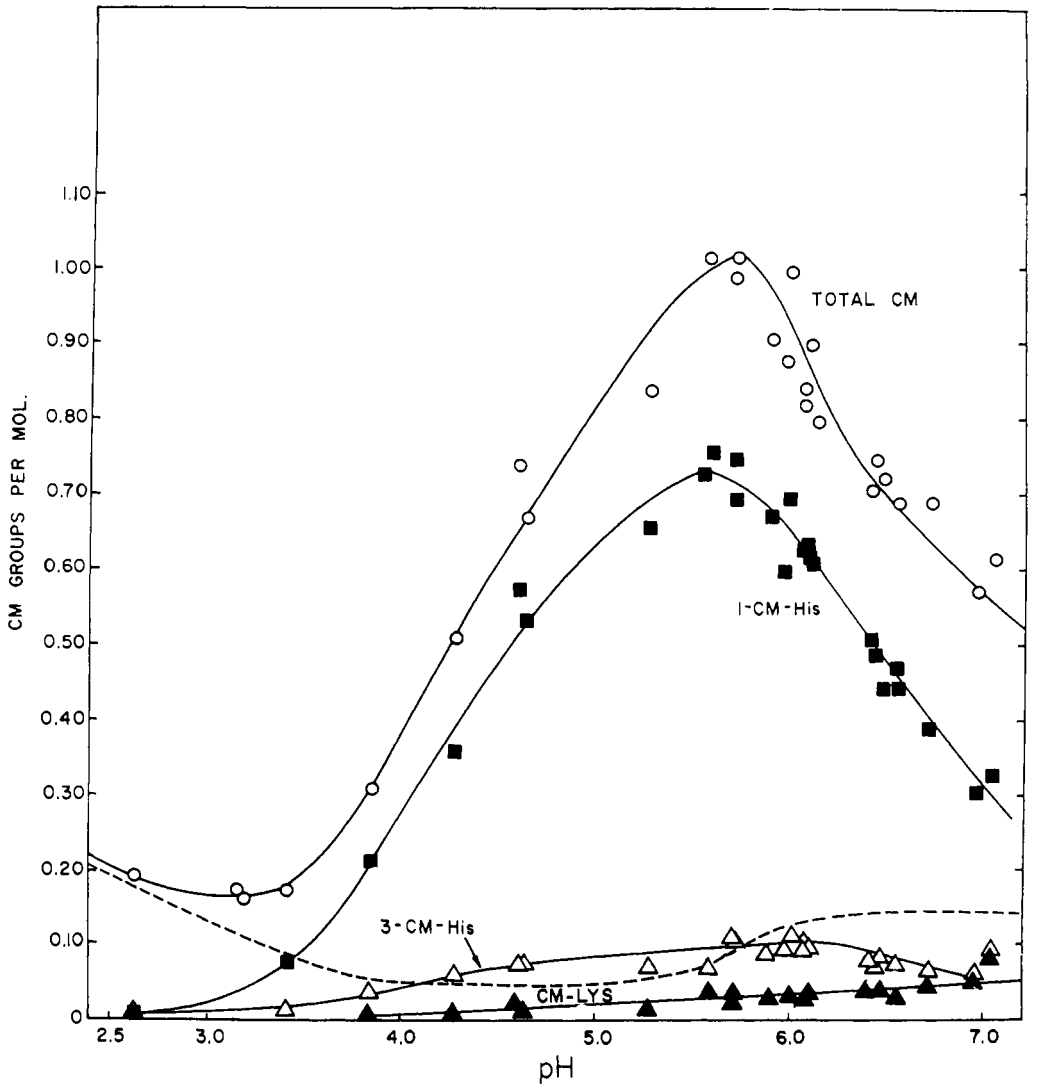

FIGURE 4: [^{14}C]CM groups per ribonuclease molecule introduced in reaction with 0.025 M [^{14}C]bromoacetate, 45 min, 35°, at various pH values. 1-CM-histidine and di-CM-histidine residues (combined) (■), 3-CM-histidine (△), and CM-lysine (sum of all forms, ▲) are plotted as residues per molecule (curves drawn by eye). The broken line represents CM-methionine (curve drawn by eye, from Figure 5).

preparations was *N,N*-1,3-di-CM-histidine. This compound[5] was prepared by Goren *et al.* (1968) from CM-poly-L-histidine; it was shown that di-CM-histidine gives rise to two characteristic peaks which can be found in the flow-cell analysis of some CM-ribonuclease hydrolysates (see Figure 3). The formation of a di-CM-histidine residue in the ribonuclease alkylation has not hitherto been reported.

Its extent of formation in some of the protein derivatives is noted in Tables I and III. It is noteworthy that it only occurred in peak I, containing 119-His-CM-ribonuclease. Analysis across this peak (Table III) showed that the di-CM-ribonuclease runs slightly faster than the mono-CM-ribonuclease. It is probable that with a change of chromatographic conditions the former, more acidic component could be separated from the latter, but this has not been investigated.

Evidence from peptide analysis is presented by Goren and Barnard (1970) that the position of disubstitution is again histidine-119. Therefore, the amount of di-CM-histidine in the hydrolysate of a CM-ribonuclease mixture was added to that of 1-CM-histidine in deducing the amount of 119-His-CM-ribonuclease.

The Carboxymethylations at Various pH Values. It has previously been shown (Goren *et al.*, 1968) that the overall reaction at pH 4–7 is approximately pseudo first order, up to about one CM group introduced per molecule. A detailed pH profile of the alkylation was obtained here by measuring the [^{14}C]CM incorporations after a standard treatment (0.025 M bromoacetate, 35.0°, 45 min) at many pH values in the range 2.5–7 (Figure 4). This treatment gives sufficient labeling for accurate analysis of the substitution sites at all pH values studied, but does not saturate any one site even at the optimum.

[5] That evidence has recently been extended (H. J. Goren and E. A. Barnard, in preparation) to give a chemical identification of this product from CM-ribonuclease as the expected *N,N*-1,3-di-CM-histidine structure.

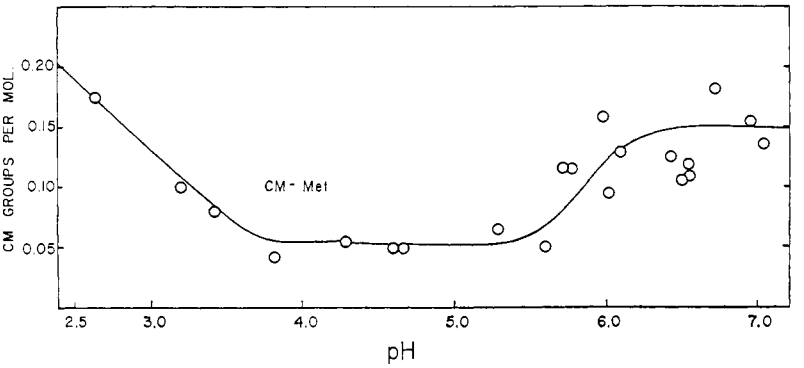

FIGURE 5: CM-methionine residues per ribonuclease molecule after reaction at various pH values (as in Figure 4).

The curves for the extent of alkylation at histidines-119 and -12, deduced from the analysis of the total mixture (Figure 4), lie in close agreement with the amounts of those derivatives as measured at several pH values by separation of the CM-ribonuclease species (Table II, samples 1–5, results adjusted to 45-min reaction). This is a confirmation that the assignment made of the N-1-CM- (plus di-CM-) and N-3-CM-histidine products to positions 119 and 12, respectively, holds throughout the pH range studied.

It is readily seen (Figure 4) that the total incorporation of CM groups always exceeds the sum of the substitutions at the two histidines. The deficit was found in the analyses to be largely accounted for by CM-methionine. This sulfonium salt breaks down in the protein hydrolysis (Gundlach *et al.*, 1959a) and its determination at low levels (<0.5 residue/protein molecule) is of low accuracy by established methods (see Goren *et al.*, 1968). All of the hydrolysis products of CM-methionine have been characterized in the isotopic analysis system, permitting such determination (Goren *et al.*, 1968). Despite the rather low levels, the pH profile of the methionine alkylation (Figure 5) was reproducibly obtained, and was confirmed by several determinations of larger contents after longer reaction times (see below). The increased reactivity from pH 5.6 to 6.4, and below pH 3.8, were distinct features of this methionine alkylation.

When the separated CM-ribonuclease peaks (Figure 2) were analyzed, peak II was invariably shown to contain CM-methionine as the sole labeled residue (Tables I and III). The methionine-labeled component in peak II was not separated from the larger amount of unchanged ribonuclease A in that peak, in the CM-cellulose chromatographic conditions used. However, in all such chromatographic analyses of the reaction mixtures, peak II contained this labeled component running slightly faster than the major ribonuclease A of that peak, as illustrated in Figure 2B. Peak II material always had a specific enzymic activity indistinguishable from that of ribonuclease A on C2′3′p and, when tested (Table III), on RNA. Peak II (separated after an alkylation at pH 6.0, 45 min) was also examined by electrophoresis in cellulose acetate (at 300 V, room temperature, using the methods noted by Lazarus *et al.*, 1966). It gave a single band migrating identically with pure ribonuclease A, in 0.1 M Tris-HCl, pH 8.0 or pH 10.4, and also in 0.1 M sodium phosphate at pH 6.5. However, when peak II was rechromatographed on SE-Sephadex (Figure 2C), the CM-methionine-containing protein was separated (although not completely) from the ribonuclease A present. Its enzymic activity is seen to be, again, identical with that of ribonuclease A.

Above about pH 5, lysine alkylation was a very minor additional reaction (Figure 4), accounting for the rest of the [^{14}C]CM incorporation. The rate constants for the histidine reactions were determined for a number of pH values, assuming pseudo-first-order behavior (Figure 6). It is seen

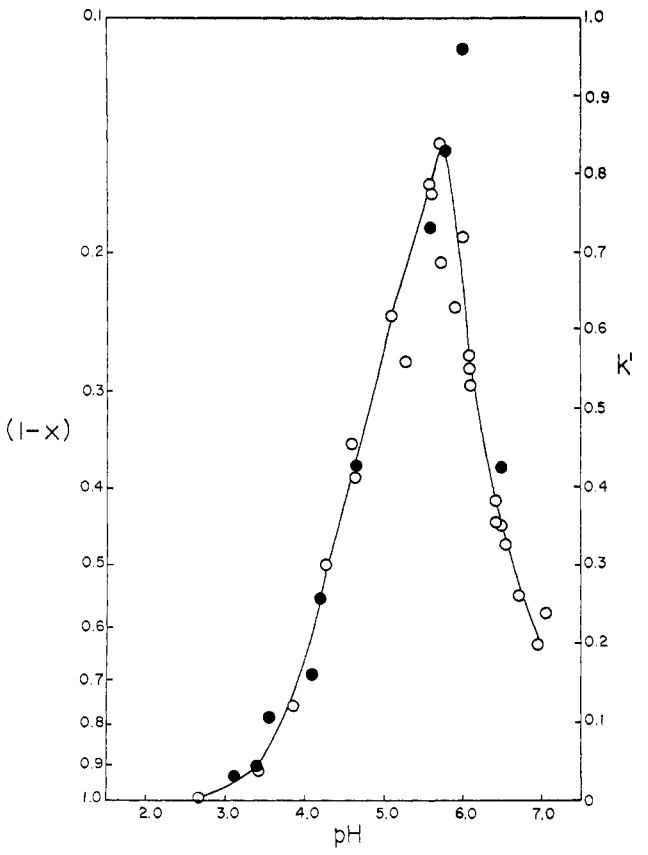

FIGURE 6: Inactivation and histidine-alkylation pH profile. The sum (x) of the histidine-119 and histidine-12 residues per molecule after a 45-min reaction was deduced from measurements of the hydrolysis products (see text); ($1 - x$) plotted logarithmically (○) is proportional, therefore, to the pseudo-first-order rate constant for the combined alkylations. Also plotted are k' values (●), proportional to the rate constant for the enzymic inactivation in the same reaction.

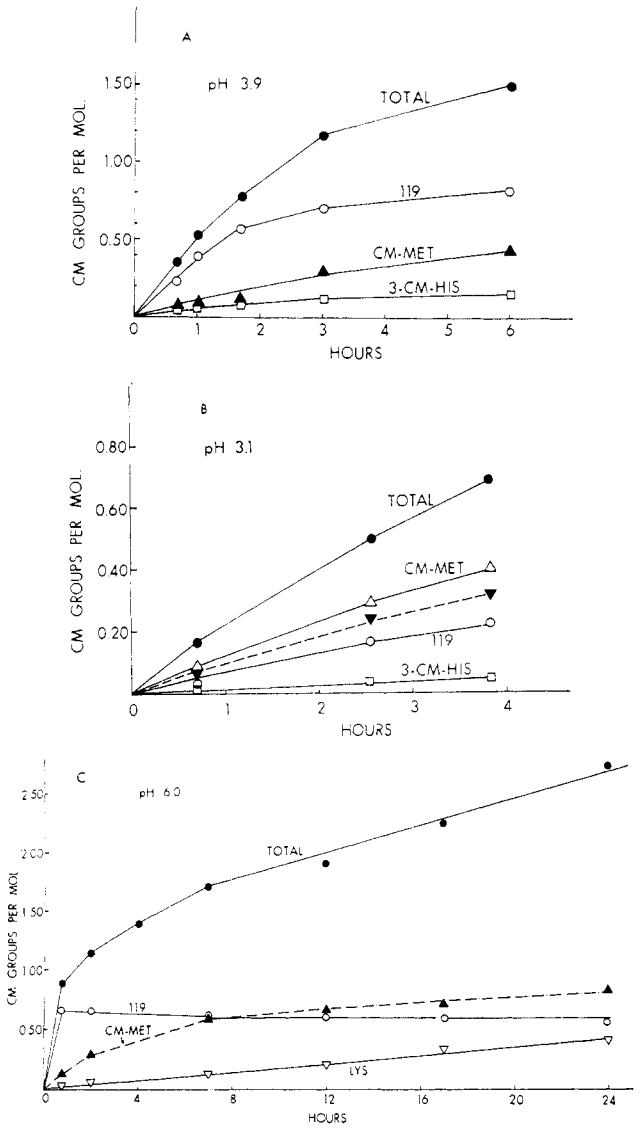

FIGURE 7: Alkylation products formed during reactions with 0.025 M [^{14}C]bromoacetate, 35°, at (A) pH 3.9, (B) 3.1, and (C) 6.0. 119 (○) represents the sum of 1-CM- and di-CM-histidine residues per molecule. The broken line in the pH 3.1 reaction plot represents the fractional inactivation of the enzyme.

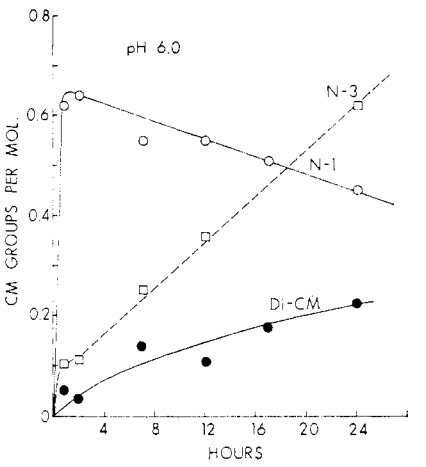

FIGURE 8: Alkylation products formed during reaction at pH 6.0 (0.025 M [^{14}C]bromoacetate, 35°). N-1-CM-His (○), di-CM-His (●), and N-3-CM-His (□) residues per molecule are plotted.

that the true pH dependence for the total histidine alkylation exhibits a sharp maximum at pH 5.7 (at 35°, I = 0.05). Also plotted in Figure 6 is the rate constant for enzymic inactivation determined at a number of pH values. This shows an identical pH dependence. Down to pH 3.1, the fractional inactivation corresponds within experimental error to the fraction of one CM group introduced into histidines-119 and -12, taken together. Only below pH 3.1, therefore, does methionine reaction lead to any inactivation.

Enzymic Activity of the His-CM-ribonucleases. 119-His-1-CM-ribonuclease, obtained from the center of peak I (Figure 2A), had no detectable activity in the standard assay on C2′3′p, even when about 20 times the normal enzyme concentration was used. At a similar relative concentration using the very sensitive assay of Shapira (1962a,b) on RNA the specific activity was measurable, being 0.2% of that of pure ribonuclease A. Since this was further reduced about tenfold in a rechromatography, the residual activity is believed due to contamination by traces of ribonuclease A not entirely separated in this chromatographic step.

A test was made of the possibility that after full unfolding of this inactive derivative, a refolding might give some partly active molecules in another conformation. Deionized 119-His-1-CM-ribonuclease was incubated at 29° in 8 M urea at pH 6.1 (0.05 M imidazole–0.1 M NaCl). In these conditions this protein is fully unfolded within 1 min (Barnard, 1964). After 30 min, the urea was removed by dialysis against four changes of water which fully refolds ribonuclease A (Barnard, 1964). The derivative was then tested as above on C2′3′p, where it was totally devoid of activity. Hence, no activity can be restored by unfolding and refolding, in contrast to the case of 12-His-3-CM-ribonuclease refolded from 50% acetic acid (Yang and Hummel, 1964). The material in peak III had a trace of activity (Table III).

Carboxymethylations at Longer Reaction Times. Analyses of the products were made at intervals through longer reaction periods (Figure 7). At pH 3.9, the alkylations at histidine-119 (N-1-CM- and di-CM-residues combined) and at histidine-12 (N-3-CM-residues) were levelling off at 6 hr, to a sum of about one alkylated residue per molecule. The total CM incorporation continues to rise, however; this is due to the methionine reaction. The same appears to be true at pH 3.1 (Figure 7). At these lower pH values, however, such lengthy reaction periods would be needed to reach one CM-methionine residue per molecule that we would incur the risk of complications due to other reactions, and this limit was not tested there. At pH 6, where the methionine reaction is about threefold faster, saturation at one, and not at four, methionine residues was, in fact, approached after a 24-hr reaction (Figure 7). The methionine alkylation was pseudo first order for a reaction at one group per protein molecule, at the three pH values used. It is concluded that only one residue is involved, and that any carboxymethylation at the others must be extremely slow.

The inactivation reaction, at a pH as low as 3.1, was not significantly greater than the sum of the 119 and 12 histidine

TABLE IV: Labeled Products after [^{14}C]Bromoacetate Reaction of 119-His-1-CM-Ribonuclease.

Treatment[a]	CM-His-119[b]	N-3-CM-His	CM-Lysine		CM-Met
			α	ϵ	
0.025 M [^{14}C] bromoacetate, pH 5.4, 35°, 5 hr	0.037	0.218	0.078	0.134	0.324

[a] The 119-His-1-CM-ribonuclease starting material was obtained as peak I (see Figure 2) in chromatography, after a reaction with 0.025 M sodium bromoacetate (unlabeled) in 0.025 M acetate at pH 5.8, 35°, 45 min. After the [^{14}C]bromoacetate retreatment and deionization, the CM residues introduced in that second treatment (only) were measured. [b] Taken as the sum of N-1-CM-His and di-CM-His residues (the latter being about one-third of that sum).

alkylations (Figure 7), despite the accumulation of this CM-methionine.

At pH 6.0, the CM incorporation is clearly biphasic (Figure 7C). In the rapid initial phase, histidines-119 and -12 are alkylated, removing (see Figure 6) all active ribonuclease. The second phase of CM incorporation all occurs, therefore, in species already alkylated once. It is seen (Figure 7C) that the methionine alkylation proceeds in the same way throughout, so that it occurs equally well on native and histidine-alkylated ribonuclease molecules.

The production of 3-CM-histidine residues at pH 6.0 after full inactivation of the enzyme proceeds steadily, but slowly, further (Figure 8). In this later phase, we do not take 3-CM-histidine as an index of histidine-12 substitution. The much more rapid initial phase corresponds to the period when ribonuclease A molecules are being substituted either to give 119-His-1-CM-ribonuclease or 12-His-3-CM-ribonuclease (see above). These results suggest that histidines-48 and/or -105 (probably both) can be alkylated at pH 6, at very slow rates. The mean of these rates is about 150 times slower than that of histidine-119.

When the formation (at pH 6.0) of 1-CM-histidine and of 1,3-di-CM-histidine is plotted separately (Figure 8), it is seen that an actual decline occurs in the 1-CM-histidine product at longer times, which is fully accounted for by the increase in 1,3-di-CM-histidine residues, to give (Figure 7C) a constant sum. This is taken to show that the second CM group enters only at a 1-CM-histidine residue, *i.e.*, at histidine-119. Evidence from peptide analysis to support this is provided by Goren and Barnard (1970). The second carboxymethylation at 119 is seen (Figure 8) to occur at a much slower rate than the first.

Alkylation of Lysine. The α-CM, ϵ-di-CM, and ϵ,ϵ-di-CM derivatives of lysine have been identified in the flow-cell system (Goren *et al.*, 1968). Lysine reaction becomes appreciable in ribonuclease at pH 6 and above (Figures 4 and 7). Both α- and ϵ-alkylated species are always formed. The α-CM-lysine must come from position 1; this lysine residue accepts a second CM group, at the ϵ position, as readily as the first, since the α,ϵ-di-CM-lysine concentration becomes appreciable even when the α-CM-lysine concentration is still quite low. Thus, when the total of CM-lysine residues per protein molecule was 0.41 (Figure 7), there were 0.20 α-CM-lysine, 0.15 ϵ-CM-lysine, and 0.11 α,ϵ-di-CM-lysine CM groups per molecule.

In the isolated protein derivatives separated after a brief reaction (Table I), both α- and ϵ-lysine products were found in peak I, and no CM-lysine in peak III. However, after longer reaction at pH 6 or above, two small, new protein peaks are separated in the CM-cellulose chromatograms ahead of peak I, and the lysine products are contained in these (Goren and Barnard, 1970).

Independence of the Methionine and Histidine Alkylations. The analyses of peak I (Tables I and III) show that (except at pH 5.5 at an early stage) reaction at histidine and simultaneously (to a small extent) at methionine residues has occurred in the protein therein.

After much longer reactions, the excess of CM-methionine in that protein (Goren and Barnard, 1970) brings the content of CM residues to well above 1 per protein molecule: some of the 119-His-CM-ribonuclease molecules must have accepted a CM group at a methionine residue. To confirm that this process occurs, and to estimate its rate, peak I was isolated (as in Figure 2) from a mixture after a reaction without isotope, in conditions which introduced very little CM-methionine into it; after deionization, the material in this peak was resubjected to the [^{14}C]bromoacetate reaction and analyzed (Table IV). The 119-His-CM-ribonuclease starting material accepted CM groups at methionine at about the same rate as when (Figures 5 and 7) native ribonuclease A is reacted. It is also seen that further alkylation at histidine continues slowly, almost entirely at the N-3 position. This confirms that the secondary alkylation at other histidine(s) noted above proceeds on the protein molecules already alkylated at histidine-119.

The 12-His-3-CM-ribonuclease peak analyses shown earlier (Tables I and III) did not reveal CM-methionine, but this does not exclude that the latter is formed in this species, since this peak, III, was very small there (Table II). After much longer reaction times, a content can be measured (Goren and Barnard, 1970) of CM-methionine in peak III about equal to the proportion in peak I. Hence, the methionine alkylation proceeds in 12-His-3-CM-ribonuclease as in the other species.

Preparation of His-CM-ribonucleases. It is now clear that a preparation of 119-His-1-CM-ribonuclease will in general not contain that species alone, unless special evidence is presented to the contrary. This is due to the concurrent reaction at a methionine in the same molecule, and the entry of a second CM group at histidine-119. It seems reasonable to suppose that preparations (Crestfield *et al.*, 1963a) made by methods using iodoacetate instead of bromoacetate will contain the same products. The di-CM-histidine may not be measured readily with ninhydrin detection in amino

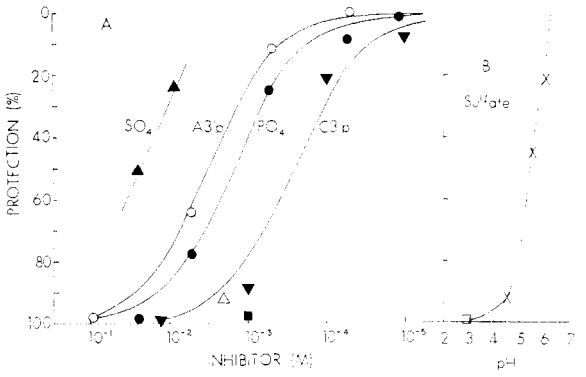

FIGURE 9: Inhibition by dianions of the inactivation reaction of bromoacetate at 35°. The protection is expressed as the percentage decrease in the pseudo-first-order rate constant for the inactivation reaction. (A) Reactions in the presence of the named ion at pH 6.0. The curves are theoretical dissociation curves drawn assuming dissociation constants (K_I) for the complexes: 2.3×10^{-2} M, sulfate (▲); 3×10^{-3} M, A3′p (○); 1.5×10^{-3} M, phosphate (●); 2.2×10^{-4} M, C3′p (▼). Dissociation constants have been reported of 0.5×10^{-2} M for sulfate (pH 5.6, 30°; Nelson *et al.*, 1962), and 2.1×10^{-3} M for phosphate and 1.1×10^{-4} M for C3′p (pH 6.0, 25°; Anderson *et al.*, 1968). For pyrophosphate (△) and C2′p (■), only single points are shown, since, while inhibition was also observed at lower concentrations, the amount of enzyme there was approaching the amount of inhibitor. (B) Increase in the protection by sulfate (10 mM, ×, or 1 mM, □) as the pH of reaction is decreased.

acid analysis since it is not large in amount and much of it is in a product that runs under leucine (Goren *et al.*, 1968). We have not yet separated the contaminant 119-His-di-CM-ribonuclease from the 119-His-1-CM-ribonuclease by chromatography, although as noted above, there are indications that this should be possible. Met-CM-ribonuclease can be separated from ribonuclease A by chromatography on SE-Sephadex–0.1 M phosphate (Figure 2C). It is necessary, for its separation from all other components, to chromatograph twice, firstly in the CM-cellulose system to isolate the mixture of ribonuclease A and Met-CM-ribonuclease (Figure 2b), and secondly to resolve this mixture, for example on SE-Sephadex. A convenient method for the preparation of the derivatives monoalkylated at histidine-119 or -12 is described above (see Methods). The 119-alkylated derivative prepared thus will have only about 5% of its CM-histidine residues disubstituted, and methionine alkylation will be less than 1%. An additional chromatography on SE-Sephadex (as above) can be applied if required, to obtain a complete purification of the 119-His-1-CM-ribonuclease species.

Effects of Anionic Ligands on the Alkylations. A number of dianions known to act as competitive inhibitors of ribonuclease activity were employed. Their capacities to suppress the alkylations at the identified sites in the protein were measured (Tables V and VI). In addition, the decrease in the bromoacetate inactivation reaction rate constant, produced at several dianion concentrations, was used to measure the affinity of the dianion for the active center in several cases (Figure 9); this latter method makes use of the complete correlation observed (Figure 6) between the inactivation and the sum of the alkylation reactions at histidines-119 and -12.

Those dianions which protected histidines-119 and -12 from bromoacetate (Table V; Figure 9) were those which, at the concentrations and pH values used, are significant competitive inhibitors (Nelson *et al.*, 1962; Ukita *et al.*, 1961) of the enzymic activity. The protections observed could be increased toward completion by sufficient increase in the concentration of ligand added (Table V). It appears, therefore, that these histidines in the enzymic–ligand complex are virtually inert to bromoacetate, so that the observed alkylation rate constant is a measure of the free enzyme only. This was confirmed by showing for several inhibitors (Figure 9) that the observed protections fit, within the experimental error, theoretical dissociation curves for the respective complexes. The dissociation constants used for these curves are in reasonable agreement with those determined for these complexes by other methods (see Figure 9). The dissociation constants for pyrophosphate and for C2′p were not estimated thus, since these ions are bound too firmly ($K \sim 10^{-5}$–10^{-6} M at our ionic strength, from the values of Hummel and Witzel (1966) and Deavin *et al.* (1966)); at the concentration needed for the present measurements the complexed inhibitor was not negligible in amount compared to the free inhibitor. The simple dissociation approximation is invalid then, and an exact treatment for that case (*cf.* Barnard, 1964) was not applied, due to the insufficient accuracy of the measurements

TABLE V: Inhibition of Alkylation at Residues in Ribonuclease.[a]

Compound	Concentration (M × 10^3)	pH	% Inhibition of Alkylation[b] at: His-119	His-12	Met
Phosphate	95	6.3	86	78	0
	10	5.6	32	36	0
	10	3.9	32	42	0
	5	5.8	21	21	0
Sulfate[c]	10	6.0	11	18	0
	10	5.5	39	45	0
	10	4.5	92	92	0
	5	4.5	80	79	0
	5	4.0	88	100	0
Acetate	35	5.6	0	0	0
β,β-Dimethylglutarate	25	6.2	0	0	0
Pyrophosphate[c]	5	7.0	67	38	28
	2	6.3	89	81	45
A2′3′p	3.6	5.9	0	10	0
C2′p	1	6.0	92[d]	84[d]	30[d]
C3′p	15	6.2	100[e]	100[e]	100[e]
	1	5.9	90	86	80

[a] All reactions were for 45 min (35°) except where noted, in 0.025 M sodium acetate (the latter being omitted when dimethylglutarate was used, or increased to 0.035 M in the acetate experiment). [b] The decrease in CM incorporation at the site specified, expressed as a percentage of the incorporation there in a precisely identical control reaction in the absence of inhibitor (see Figure 4 for the extent of the latter reactions). [c] For results at lower pH, see Table IX. [d] 2-hr reaction period. [e] No products formed after 1.5 hr.

TABLE VI: Effect of Inhibitors on Alkylation of Lysine in Ribonuclease at 35°.

Compound	Concentration (M × 10^3)	Reaction Period (Hr)	pH	CM-Lys Residues per Molecule (Unprotected)	% Inhibition of Alkylation[a] at α-NH_2	% Inhibition of Alkylation[a] at ϵ-NH_2
C2'p	1	2	6.0	0.062	0	74
C3'p	15	1.5	6.2	0.07	~0[b]	100
Pyrophosphate	5	8	5.8	0.150	10	70

[a] See note *b* to Table V. ϵ-NH_2 includes both ϵ-CM-Lys and α,ϵ-di-CM-Lys residues. [b] The incorporation of CM groups was not determined exactly here, since the high nucleotide level interfered with protein determination. The very low radioactivity in the product was found to be exclusively in α-CM-lysine residues, such that the protection there was small or nil.

at low free enzyme concentrations. The data of Figure 9A and Table V serve, however, to show that the order of effectiveness of these dianions (at pH 6) in active-center histidine protection is: C2'p > pyrophosphate, C3'p > phosphate > A3'p > sulfate ≫ A2'3p. Sulfate (but not phosphate) was much more effective at much lower pH values, and was examined across a pH range (Figure 9B; Table V).

β,β-Dimethylglutarate is an example of a dianion with a wider separation of the charges and which is not inhibitory of the enzymic activity (Gassen and Witzel, 1967). It gave no depression of the histidine alkylation at relatively high concentration (Table V). The nucleotide monoanion, A2'3'p, is an activator (Wieker and Witzel, 1967); it was not significantly inhibitory of the alkylations (Table V). The monoanion, acetate, had no effect on the reaction when increased in concentration to 35 mM.

Methionine alkylation was not affected (above pH 3.0) by any ion other than the nucleotides and pyrophosphate, which were significantly inhibitory of it. These same inhibitors also retarded lysine alkylation (Table VI). The lysine reaction in free ribonuclease is so slow that the protection could not be determined quantitatively except above about pH 6. Since the various [^{14}C]CM-lysines are easily separated and measured by the flow-cell chromatographic method (Goren *et al.*, 1968) and since they suffer no hydrolytic decomposition, small amounts of these could then be determined accurately (Table VI), giving, therefore, relative initial velocities. These showed that pyrophosphate and the nucleotides tested gave strong inhibition of the alkylation at the reactive ϵ-NH_2 group of ribonuclease, but none at the α-NH_2.

Evidence is reported by Goren and Barnard (1970) that when the ribonuclease–ligand complex is present the alkylations at the histidines and methionine indeed occur at the same positions in the protein chain as in native ribonuclease.

Changes in the Alkylations with Temperature. Two pH values, where the histidine alkylation rate differs by more than tenfold at 35°, were used in studies at 25–40° (Figure 10), using a 45-min reaction period. At pH 3.5, the overall CM-group incorporation increased by a factor of 1.6 for a 10° increase. The rate of increase in the 119-His-CM-histidine formation fell off, however, with temperature increase. At pH 6.0 this product is approaching at 45 min (at 35°) its optimal yield of about 0.7 residue per molecule of total protein, so that the change in rate is not determined in these conditions. This rate constant was measured kinetically at 35°, pH 5.6, in a separate experiment (Table V of Goren and Barnard, 1970); it is again about 1.6 times that at 25°. The formation of di-CM-histidine shows a fairly normal temperature coefficient. More extensive analysis to obtain data for the derivation of the activation parameters ΔH^* and ΔS^* was not pursued, since (when rates are calculated) the observations on histidine alkylation in the 25–40° range do not fit the Arrhenius equation. Methionine alkylation also showed abnormal behavior (Figure 10), the rate being almost constant from 25 to 40°.

Effects of Other Variables on the Reaction. It has been stated (Crestfield *et al.*, 1963a) that methionine alkylation in ribonuclease may be due to the effects of small amounts of

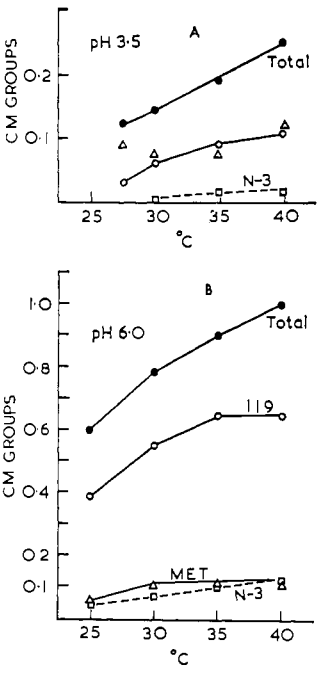

FIGURE 10: Alkylation products after reactions (0.025 M [^{14}C]bromoacetate, 45 min) at various temperatures at pH 3.5 and at pH 6.0. (●) Total reaction; (○) 119-His-CM-ribonuclease; (□) 3-CM-histidine; (△) CM-methionine; all as CM groups per molecule of protein. Di-CM-histidine values (included in 119-His-CM-ribonuclease), when measured separately at pH 6, fell on the same line as that shown (---) for N-3-CM-histidine.

TABLE VII: Comparison of Iodoacetamide and Bromoacetate Alkylations in Ribonuclease.[a]

Reagent	pH	% of 1 CM Group per Ribonuclease Molecule in Products	
		1-CM-His[b]	3-CM-His
Iodoacetamide	4.08	0.0	2.0
Bromoacetate	4.10	34.0	5.0
Iodoacetamide	5.45	0.0	1.5
Bromoacetate	5.45	72.0	7.5

[a] Reaction was with 0.025 M reagent in 0.025 M acetate at 35° for 45 min. CM-Methionine was also present in all cases (see Figure 5, and Fruchter and Crestfield, 1967). [b] Includes a small amount of di-CM-histidine in bromoacetate cases (see earlier results), but this was absent in the iodoacetamide cases.

copper in the medium. While precautions were taken here (see Methods) to exclude foreign metal ions from the reaction components, it was also shown (at pH 6.2) that the amounts of CM-methionine and CM-histidines formed were totally unaffected by the presence of 10^{-3} M EDTA.

Change of ribonuclease concentration, in the range 0.5–10 mg/ml, did not affect the carboxymethylations; their rates show apparent first-order kinetics, with respect to the protein concentration.

When the reagent was changed to an uncharged analog, iodoacetamide, histidine-119 alkylation was absent (Table VII), but slow reactions to form N-3-CM-histidine and CM-methionine still occurred.

In the hydrolysates of the [^{14}C]carbamylmethyl-ribonuclease so obtained, the same labeled products (only) were present as are obtained (Goren *et al.*, 1968) from [^{14}C]CM-methionine residues. Rates of a reaction of iodoacetamide at methionine(s) in ribonuclease have been given by Fruchter and Crestfield (1967).

Alkylation in the Region of Protein Unfolding. Below pH 3.4, methionine alkylation is faster than all other reactions (Figures 4, 5, and 7). Analysis (Table VIII) showed, firstly, that at pH 2.8 the alkylation of methionine produces partial inactivation. This inactivation is totally inhibited by low concentrations of pyrophosphate or sulfate (which remain largely dianionic at this pH), but much of the methionine carboxymethylation reaction still persists in the complexed protein. After alkylation in the presence of pyrophosphate (Table VIII) the protein products were chromatographed as in Figure 2, when about 90% of the protein in the chromatogram was found in peak II; this was fully active (on C2′3′p) and contained [^{14}C]CM-methionine as the sole labeled residue. The labeled component ran in the front half of peak II, which was at the position of ribonuclease A. It is concluded that this labeled active species is the same as that found (Figure 2) with identical chromatographic position and properties after the alkylations at higher pH values, and identified (Goren and Barnard, 1970) as 30-Met-CM-ribonuclease.

Secondly, at still lower pH values (Table III), methionine alkylation proceeded readily to 4 residues per molecule and (for the overall methionine reaction) was several times more rapid than at any pH value above 2.8 (*cf.* Figures 5 and 7); this is a reactivity counterpart to the known unfolding of ribonuclease in these acid media (Hermans and Scheraga, 1961; Ginsburg and Carroll, 1965). Differences between various methionines are apparent across this range (Table VIII), and at least one methionine can always be alkylated without enzymic inactivation. Pyrophosphate at low ratios is, in contrast to its effect at pH 2.8, not inhibitory when at pH 2.1 (although still about 80% dianionic in these conditions). This is true when, as in each case here, the dianion is added to the already unfolded protein: if the complex is formed first at neutral pH, marked hysteresis effects can occur on acidifying ribonuclease, accounting for the different effects of dianions observed by Ginsburg and Carroll (1965) and Neumann *et al.* (1962). The results of Table VIII show that the dianions can complex at pH 2.8 (at 35°) to prevent unfolding, but cannot do so at pH 2.1, nor can they (at low concentrations) reverse the unfolding in acid.

Discussion

Although the reaction of halogenoacetate with ribonuclease has been well studied in several laboratories, the evidence collected here offers significant new information on this evidently complex reaction, and on its relationship to the active center structure. Included in this information are the pH dependencies of the alkylations at histidines-119 and -12, the entry of a second CM group occurring only at N-3 of 1-CM-histidine-119, the characteristics of the carboxymethylation of one methionine in native ribonuclease A monomer, the effects of several variables upon the component reactions, and the availability of additional methionines as thermal unfolding commences.

It has been known for some time that nucleotides and other dianions inhibit the inactivation of the histidine alkylation (Barnard and Stein, 1959b; Ross *et al.*, 1962; Crestfield *et al.*, 1963b) and that, conversely, histidine-alkylated ribonuclease has lost the specific binding of these ligands (Barnard and Ramel, 1962; Hummel and Witzel, 1966). In the present study, the inhibitory effects have been compared for a series of these ligands, with respect to three groups in the protein. A distinction is drawn here between the nucleotides and pyrophosphate on the one hand and other dianions on the other hand, since only the former inhibit the reaction at the alkylatable methionine.

The capacity of the ions to protect the active center histidines from alkylation runs parallel to their effectiveness as competitive inhibitors of the enzymic activity (see Figure 9, and Deavin *et al.*, 1966). The deduction that this is due to a common binding site in the active center region is confirmed by the finding of a bound phosphate adjacent to histidines-119 and -12 in the structure (Kartha *et al.*, 1967). It is interesting that the nucleoside cyclic phosphate which we tried is ineffective, as are dianions in which the charges are well separated. This suggests that the essential feature of the interaction, in the active center, of the phosphate group of a substrate is that it involves an incipient dianionic form. This has been proposed also from kinetic evidence (Wieker and Witzel, 1967). The present evidence permits us to further specify this process, in

TABLE VIII: Alkylations of Ribonuclease at Low pH Values at 35.0°.

pH	Bromoacetate[a] (M)	Reaction (hr)	Inhibitor: Ion	Inhibitor: Concn (M)	Inhibitor: Moles/mole of protein	CM Groups per Molecule: CM-Met	CM Groups per Molecule: Total	Activity (%)[b]
3.1	0.025	6	None			0.52	0.90	60
2.8	0.025	6	None			1.07	1.19[c]	43
2.8	0.025	6	Sulfate	5×10^{-4}	16	0.79	0.8	95
2.8	0.025	8	Pyrophosphate	2×10^{-3}	2	0.53[d]	0.54	100
2.0	0.025	1.67	None			0.93	0.94	55
2.1	0.050	1.17	None					42
2.1	0.050	3.0	None			1.90	1.94	15
2.1	0.050	4.0	None			2.30	2.32	
2.1	0.050	16	None			3.86	3.98	0
2.1	0.050	1.17	Pyrophosphate	5×10^{-4}	3			50
2.1	0.050	3.0	Pyrophosphate	5×10^{-4}	3		1.9	20

[a] The total concentration of bromoacetic and acetic acids and their anions present was 0.050 M, with HCl added to the stated pH. The protein was at the stated pH prior to adding the inhibitor. [b] Where noted, activity was determined on RNA, and is expressed as a percentage of the original activity. The reacted protein to be assayed was first placed in pH 7.4 Tris-HCl–NaCl (I = 0.3) for at least 10 min at room temperature, to give opportunity for refolding. [c] Even at this low pH, histidine alkylation appears to occur predominantly at residue 119, since the product other than CM-methionine found in the hydrolysate was largely N-1-CM-histidine, at 5 times the level of N-3-CM-histidine present. [d] These protein products were subsequently separated by chromatography and shown to be one species to the extent of 90% (see text).

that for each inhibitor histidines-119 and -12 are equally affected in the dianionic interaction (Table V). The pH dependencies of these interactions also support this proposal. For sulfate (but not phosphate), the interaction becomes stronger as the pH decreases below 6 (Table V; Figure 9B).

Sulfate, a weak, competitive ribonuclease inhibitor and ligand at neutral pH (Nelson *et al.*, 1962; Barnard, 1964), is known to be strongly bound to the enzyme at pH values near 3 (Ginsburg and Carroll, 1965; Neumann *et al.*, 1962). The binding of phosphates, on the other hand, is maximal near pH 5.6 (Anderson *et al.*, 1968) and so is that of small substrates (Herries *et al.*, 1962; Witzel and Barnard, 1962; Wieker and Witzel, 1967). These differences, taken together with our findings, suggest that the acidic form of the enzyme, wherein histidines-119 and -12 are both protonated, binds the sulfate dianion strongly, and binds an effectively dianionic form of the phosphate ligands in a similar manner: it would not be possible to distinguish between an imidazolium binding to PO^- in the dianion and an imidazole binding to POH in the monoanion. The decline in the binding of phosphates (but not sulfate) below pH 5.6 can be understood as due to the phosphate secondary protonation (modified by the effect thereon of the complex formation). The monoanion cannot bind to the fully protonated active center, and the dianion cannot bind well when both imidazoles are un-ionized, giving a bell-shaped dependence. Consistent with this scheme is the proton uptake, or output, of ribonuclease when binding sulfate, or phosphate, respectively, observed in this pH range by Hummel and Witzel (1966). As those authors noted, however, the pH dependencies of the binding phenomena do not permit us to distinguish between an attachment across an imidazolium and an imidazole as one case, and a binding to an imidazolium and an ammonium group (lysine-41) as another case, if the latter binding is indirectly determined by an adjacent uncharged imidazole.

The alkylation results, in conjunction with the structural confirmations in the succeeding paper (Goren and Barnard, 1970), establish that the attacks at histidines-119 and -12 occur throughout the pH range 2.5–7, in a fairly constant ratio through that range; those reactions account totally for the inactivation by bromoacetate at 35° from pH 3.1 to 6.5. In the protein structure (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967) these two residues and the ϵ-NH_3^+ of lysine-41 are indeed together in a polar cluster in the substrate-binding region. The great enhancement of the bromoacetate reaction at these particular imidazole N atoms is clearly due to a favorable, substrate-like interaction of the reagent across two of the groups mentioned. However, pending further analysis, interaction across either of the two systems postulated (imidazole–imidazolium or imidazole–lysine) could account for the pH profile of histidine alkylations (Figures 4 and 6). In either case, a requirement for the existence of a conjugate imidazolium–imidazole pair explains the two limbs of the profile. In the second case, however, the uncharged imidazole must be a secondary determinant, for example as in some hydrogen-bonded system (Hummel and Witzel, 1966; Gassen and Witzel, 1967). If the pH dependence of the histidine-119 reaction is treated as due to the dissociation of two conjugate ionizing groups, of dissociation constants K_{119} and K_{12}, then the factor generating the pH dependence of the rate constant is $[1 + K_{12}/[H^+] + [H^+]/K_{119}]^{-1}$. This can be used to fit the experimental curve (for this reaction at N-1, in Figure 4, converted into a rate constant scale as in Figure 6), with a reasonably good fit given by values of

pK_{12} = 6.2 and pK_{119} = 4.8. Values of pK_{12} = 6.2 and pK_{119} = 5.8 (in 0.2 M acetate, 32°, *i.e.*, not too different from our medium, 0.05 M acetate–bromoacetate, 35°) have recently been deduced by Meadows *et al.* (1968) from nuclear magnetic resonance titration data. It is clear from inspection of that curve in Figure 4 that any such close pair of values as the latter cannot fit it. If both sets of data are equally valid, then the apparent pK_a values differ from the true, microscopic values because the system determining the observed behavior is more complex than an imidazolium and an imidazole group in isolation. Other equilibria must be involved. One possibility is the ionization of a carboxyl group, suggested from other evidence by Cathou and Hammes (1965) to be in the active center (where, indeed, such can be seen in that general region in the X-ray structure, notably aspartic acid-121). The interaction of a carboxylate would explain the low pH side of the alkylation profiles, and possibly that of phosphate binding discussed above.

The present results with [^{14}C]iodoacetamide complement a study by Fruchter and Crestfield (1967). The sensitive isotopic method, applied to the initial reaction period, shows that the alkylation rate at histidine-119 is less than 0.2% of that rate with bromoacetate, at pH 4–5.5. The amount of *N*-3-carbamylmethylhistidine found (Table VII) is consistent with the reaction at histidine-12 shown to occur by Fruchter and Crestfield (1967), at a rate that is slower than that with halogenoacetate, but which is still significant. A bell-shaped pH profile of that reaction was shown by those authors, and interpreted in terms of a requirement for a protonated group of pK_a = 6.2 ± 0.2 (at 24°) assigned to histidine-119, and, again, a carboxyl of pK_a = 3.5–4.3, proposed to form a dipolar combination with histidine-119. In view of the protonation of histidine-12 in this region (see above), the interpretation of the pH dependence of the iodoacetamide reaction is probably more complex than this. In fact (and especially when allowance is made for temperature and salt differences in the media), the pH profiles for the iodoacetamide and bromoacetate reactions are very similar, despite the fact that histidine-119 is exceptionally reactive in the latter and totally inert in the former. It seems likely that a similar system determines each of these reactions, although the amide presumably hydrogen-bonds and acquires a different orientation and reactivity to that of the charged reagent.

The initial reaction with bromoacetate occurs in a rapid phase, which ceases when one histidine residue per protein molecule is substituted, either at position 119 or at 12, but not at both. However, a much slower but distinct second phase of histidine alkylation is observable at pH 6 or above (Figure 8); this indicates that one or both of histidines-105 and -48 can slowly be carboxymethylated there. This reaction appears to occur with a great preference for the N-3 position; that position is favored in alkylation of free histidine, but the high selectivity here (as also at histidine-12) is presumably due to impeded access in the protein structure on the N-1 side of the imidazole group concerned. Residues 105 and 48 are each titratable, with pK_a values of 6.7 and 6.4 (Meadows *et al.*, 1968), and appear to be accessible from the exterior in the structure (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967), 105 more so than 48.

The carboxymethylation of one methionine is a feature of interest. This reaction in ribonuclease has been noted previously, but has not been well characterized. It must be distinguished clearly from the alkylation of methionine in unfolded ribonuclease, where it has been shown, in 8 M urea (Stark and Stein, 1964), or in acid (see Results above) that all four methionines react readily, producing inactivation. Crestfield *et al.* (1963b) found decomposition products of CM-methionine in an amino acid analysis of ribonuclease derivatives only after vigorous alkylation, and deduced that all the methionines at pH 5.5 are essentially inaccessible. Fruchter and Crestfield (1965a,b), in a study of the histidine alkylation of dimers of ribonuclease, reported also that both the dimers and the monomer contained CM-methionine, after a long reaction with iodoacetate at pH 5.5; a derivative of monomeric ribonuclease A alkylated only at methionine was not separated. The reaction in native ribonuclease was also noted by Glick and Barnard (1964) and Glick *et al.* (1967). Discussion of the methionine alkylations is made elsewhere (Goren and Barnard, 1970).

The dicarboxymethylation of a histidine in ribonuclease was also demonstrated here. The parallel decline of 1-CM-histidine (Figures 7 and 8) indicates that only residue 119 is involved, a deduction confirmed by peptide analysis (Goren and Barnard, 1970). This second alkylation of the ring is slower than any of the single imidazole substitutions (Figure 8), but still appears to be a facilitated reaction. It probably arises from bridging by the first N-1-CM group to a positive center in the enzyme, already suggested by the enhanced stability of that derivative to denaturation (Barnard, 1964; Yang and Hummel, 1964); such bridging could alter the position of the imidazole at 119 to permit some attack of bromoacetate at nitrogen-3.

In the temperature range 25–40° (Figure 10), the histidine reactions (even when corrected for the depletion of the protein) show a tendency to level off with temperature increase, suggesting that the structure that facilitates the rapid reaction is very susceptible to thermal mobility. The methionine reaction shows a very marked independence of temperature, perhaps for a similar reason.

A reaction at lysine-1 was observed, equally at the α- and ϵ-NH_2 groups. This residue is not protected by complexing at the active center (Table VI); the results are in line with its remoteness therefrom in the structure (Kartha *et al.*, 1967) and its accessible situation. The slow reaction seen, at pH 5–7, to give the ϵ-CM derivative most probably occurs at lysine-41 predominantly, since that is the fastest carboxymethylation occurring at pH 8.5 (Heinrikson, 1966). The increase in rate with pH suggests the start of the titration of a group with a p*K* well above 7. This reaction is well suppressed by the ligands (Table VI), in line with the situation of lysine-41 in the active center region. An electrostatic interaction of that group with the dianion would fit these and other data on lysine -41. Structural relations of the various bromoacetate reactivities are further considered in the succeeding paper (Goren and Barnard, 1970).

Acknowledgments

We wish to thank Mr. David Saunders for skillful assistance with the amino acid analyzer and Dr. D. M. Glick for some preliminary experiments and for performing the test of refolding of 119-His-CM-ribonuclease from urea.

References

Anderson, D. G., Hammes, G. G., and Walz, F. G., Jr. (1968), *Biochemistry 7*, 1637.

Barnard, E. A. (1964), *J. Mol. Biol. 10*, 235.

Barnard, E. A., and Goren, H. J. (1967), *Fed. Proc. 20*, 448.

Barnard, E. A., and Ramel, A. (1962), *Nature 195*, 243.

Barnard, E. A., and Stein, W. D. (1959a), *Biochem. J. 71*, 19P.

Barnard, E. A., and Stein, W. D. (1959b), *J. Mol. Biol. 1*, 339.

Cathou, R. E., and Hammes, G. G. (1965), *J. Amer. Chem. Soc. 87*, 4674.

Crestfield, A. M., Smith, K. C., and Allen, F. W. (1955), *J. Biol. Chem. 216*, 185.

Crestfield, A. M., Stein, W. H., and Moore, S. (1963a), *J. Biol. Chem. 238*, 2413.

Crestfield, A. M., Stein, W. H., and Moore, S. (1963b), *J. Biol. Chem. 238*, 2421.

Deavin, A., Mathias, A. P., and Rabin, B. R. (1966), *Nature 211*, 252.

Fruchter, R. G., and Crestfield, A. M. (1965a), *J. Biol. Chem. 240*, 3868.

Fruchter, R. G., and Crestfield, A. M. (1965b), *J. Biol. Chem. 240*, 3875.

Fruchter, R. G., and Crestfield, A. M. (1967), *J. Biol. Chem. 242*, 5807.

Gassen, H. G., and Witzel, H. (1967), *Eur. J. Biochem. 1*, 36.

Ginsburg, A., and Carroll, W. R. (1965), *Biochemistry 4*, 2159.

Glick, D. M., and Barnard, E. A. (1964), 148th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1964, Abstract No. C83.

Glick, D. M., Goren, J. H., and Barnard, E. A. (1967), *Biochem. J. 102*, 7c.

Goren, H. J., and Barnard, E. A. (1970), *Biochemistry 9*, 974.

Goren, H. J., Glick, D. M., and Barnard, E. A. (1968), *Arch. Biochem. Biophys. 126*, 607.

Gundlach, H. G., Moore, S., and Stein, W. H. (1959a), *J. Biol. Chem. 234*, 1761.

Gundlach, H. G., Stein, W. H., and Moore, S. (1959b), *J. Biol. Chem. 234*, 1754.

Heinrikson, R. L. (1966), *J. Biol. Chem. 241*, 1393.

Hermans, J., Jr., and Scheraga, H. A. (1961), *J. Amer. Chem. Soc. 83*, 3283.

Herries, D. G., Mathias, A. P., and Rabin, B. R. (1962), *Biochem. J. 85*, 127.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1953), *J. Biol. Chem. 200*, 493.

Hummel, J. P., and Witzel, H. (1966), *J. Biol. Chem. 241*, 1023.

Kartha, G., Bello, J., and Harker, D. (1967), *Nature 213*, 862.

Layne, E. (1957), *Methods Enzymol. 3*, 447.

Lazarus, N. R., Ramel, A. H., Rustum, Y., and Barnard, E. A. (1966), *Biochemistry 5*, 4003.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Meadows, D. H., Jardetzky, O., Epand, R. M., Ruterjans, H. H., and Scheraga, H. A. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 766.

Nelson, C. A., Hummel, J. P., Swenson, C. A., and Friedman, L. (1962), *J. Biol. Chem. 237*, 1575.

Neumann, N. P., Moore, S., and Stein, W. H. (1962), *Biochemistry 1*, 68.

Ross, C. A., Mathias, A. P., and Rabin, B. R. (1962), *Biochem. J. 85*, 145.

Shapira, R. (1962a), *Anal. Biochem. 3*, 308.

Shapira, R. (1962b), *Anal. Biochem. 4*, 322.

Shapira, R., and Parker, S. (1960), *Biochem. Biophys. Res. Commun. 3*, 200.

Stark, G. R., and Stein, W. H. (1964), *J. Biol. Chem. 239*, 3755.

Stein, W. D., and Barnard, E. A. (1958), Abstracts of the IVth International Congress of Biochemistry, Vienna, p 21.

Stein, W., and Barnard, E. A. (1959), *J. Mol. Biol. 1*, 350.

Taborsky, G. (1959), *J. Biol. Chem. 234*, 2915.

Ukita, T., Hagatsu, H., and Waku, K., (1961), *J. Biochem. (Tokyo) 50*, 550.

Wieker, H. J., and Witzel, H. (1967), *Eur. J. Biochem. 1*, 251.

Witzel, H., and Barnard, E. A. (1962), *Biochem. Biophys. Res. Commun. 7*, 289.

Wyckoff, H. W., Hardman, K. D., Allewell, N. M., Inagami, T., Johnson, L. N., and Richards, F. M. (1967), *J. Biol. Chem. 242*, 3984.

Yang, S. T., and Hummel, J. P. (1964), *J. Biol. Chem. 239*, 3775.