On the Alkylation of Amino Acid Residues at the Active Site of Ribonuclease*

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SUMMARY

Ribonuclease A has been subjected to reaction at pH 8.5 with a series of homologous α-bromo acids and with iodoacetamide. At this pH, carboxyalkylation occurs predominantly at the 3 amino acid residues which have been implicated in the catalytic function of the enzyme: histidine-12, histidine-119, and lysine-41. The histidine reactions proceed at rates which vary from 3 to 25% of those observed at pH 5.5. The extent of reaction at the three positions is dependent upon the structure of the alkylating agent. In the reaction with bromoacetate, the derivative alkylated at lysine-41 accounts for the major proportion of the reaction products, while with n-α-bromo-n-butyrate, histidine-12 is the predominant site of alkylation. In general, the reactions at the histidine residues display the same stereospecificity toward a reagent at pH 8.5 as observed at pH 5.5; reagents of the D configuration favor reaction at histidine-12, while their L antipodes alkylate the 119-position preferentially. No selectivity for the optical isomers of a given reagent is observed for the alkylation of lysine-41. Alkylation of the enzyme with iodoacetamide at pH 8.5 yields a complex mixture of products of which, in the initial stages of the reaction, the derivatives mono- and dicarboxamidomethylated at lysine-41 are prominent.

The three products substituted at lysine-41 that have been characterized, namely the carboxymethyl, the carboxamidomethyl, and the dicarboxamidomethyl derivatives, all are essentially inactive.

The active site histidine residues in the carboxymethyllysine-41 and carboxamidomethyllysine-41 derivatives react with bromoacetate at pH 5.5 at rates, respectively, 0.8 and 0.25 that of native ribonuclease. The effect of the D-substituent is in each case to depress the alkylation of histidine-12 in relation to that at histidine-119. These findings indicate that although the chemical modification of lysine-41 inactivates ribonuclease, it does not substantially inhibit the alkylation of the histidine residues which have been implicated as part of the active site of the enzyme. From the information currently available, a tentative working model has been proposed for the spatial relationships of the chemically reactive nitrogen atoms of histidine-12, histidine-119, and lysine-41 in ribonuclease.

To date, evidence has been presented which suggests that 3 of the 124 residues in bovine pancreatic ribonuclease are present at the active site of the enzyme. These are the histidine residues at positions 12 and 119 and the lysine residue at position 41. The importance of the histidine residues to the catalytic activity of the enzyme seems to be well established (see Reference 1 for a review and references) and data have been provided which indicate that nitrogen 3 of histidine-12 and nitrogen 1 of histidine-119 are about 5 Å apart (2).

The first experiments implicating lysine-41 were provided by Hirs, Halmann, and Kycia (3), who showed that when ribonuclease is alkylated at pH 8.0 with 1-fluoro-2,4-dinitrobenzene, an inactive monosubstituted enzyme derivative may be isolated in which the ε-amino group of lysine-41 has been substituted. Protection against alkylation and inactivation was afforded by 3'-cytidylate and phosphate ion, which also inhibit the carboxyalkylation of the active site histidines (4, 2). On the basis of his study of the dinitrophenylation reaction, Hirs (5) suggested that lysine-41 is part of an anion-binding site in or near the active site of ribonuclease, and that substitution of a dinitrophenyl radical on the ε-amino group of lysine-41 may cause inactivation owing to a change in the native conformation of this region of the molecule. Further evidence in support of lysine-41 as a component of the active site has been provided by Cooke, Anfinsen, and Sela (6), who correlated the loss of activity of ribonuclease during alkylation with the extent of substitution at lysine-41. Reaction at lysine-41 was also inhibited by phosphate ion. In a more recent investigation, Carty and Hirs (7) have permitted the enzyme to react at pH 8 with 4-sulfonoxy-2-nitrofluorobenzene and have isolated a monosubstituted derivative bearing a sulfonoxy nitrophenyl group on lysine-41 which is less than 0.04% as active as native ribonuclease.† Because of the bulk of the inactivating substituents thus far introduced at lysine-41, and in view of evidence that conformational changes may accompany dinitrophenylation at this position (5), it is still uncertain as to whether the ε-amino group plays a role either in the binding or cleavage of substrate.

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Nevertheless, the findings taken as a whole suggest that the active site of ribonuclease may be composed of a cationic cluster (8) involving histidine-12, histidine-119, and lysine-41.

If this is the case, and if lysine-41 were properly positioned relative to the 2 histidines, it might furnish the positively charged site to which anionic alkylating agents are bound during the alkylation of the histidine residues at pH 5.5. This possibility has been considered (2), but recently it has seemed more likely that it is the protonated form of one of the imidazoles which furnishes the electrostatic attraction for the reagent, which then reacts with the unprotonated form of the other imidazole (9, 2). A choice between these two possibilities might be provided by studying the effects of substituents on lysine-41 upon the alkylation of histidine residues 12 and 119 at pH 5.5. This study would also be of interest because thus far all procedures which inactivate the enzyme, such as solution in 8 M urea or rupture of the disulfide bonds, also abolish the high reactivity of the histidines at the active site (9).

Accordingly, the present communication describes the reactions of ribonuclease at pH 8.5 with a number of $\alpha$-bromo acids differing in chain length and optical configuration and with iodoacetamide. During the course of this work several new, inactive derivatives of ribonuclease have been prepared which contain carboxymethyl or carboxamidomethyl substituents on the $\epsilon$-amino group of lysine-41. These derivatives have, in turn, been used to study the effects of 41-substituents relative to both the rate and the course of the alkylation of histidine residues 12 and 119 at pH 5.5. The data presented herein are consistent with the hypothesis that the chemical integrity of lysine-41 is essential for the enzymatic function of ribonuclease but not for the alkylation of histidine residues 12 and 119. Moreover, these studies provide information with regard to the spatial arrangement of the reactive nitrogen atoms of histidine-12 and histidine-119 relative to the $\epsilon$-amino group of lysine-41.

**EXPERIMENTAL PROCEDURE**

**Materials**—Two commercial preparations of ribonuclease A, both purchased from Worthington, were used. One, a lyophilized, phosphate-free preparation (RAF-6045), had been shown previously to consist of 88% ribonuclease A and 12% aggregates and minor protein components (10). This source of ribonuclease A was found to be satisfactory for the preparation of derivatives carboxyalkylated at histidine residues 12 and 119, since the contaminating protein did not interfere with the formation or the final purification of the desired products (11). However, the carboxymethyl- and carboxamidomethyllysine-41 derivatives of ribonuclease which are described in the present communication are not conveniently separable from the minor protein components present in the RAF-6045 preparation. Therefore, before this material was used for the preparation of derivatives substituted at lysine 41, the contaminating protein was removed by chromatography on sulfoethyl SephacDEX C-25 as described earlier (11, 12).

Worthington has currently available a preparation of ribonuclease A (RASE 6501-02) which has been purified by this same sulfoethyl Sephadex procedure. This highly purified enzyme, which is obtained as a 1% solution in 0.1 M phosphate at pH 6.47, was found to be free of aggregates and minor components, and could be used directly after transfer of the enzyme to the desired solvent by gel filtration.

Nonlabeled bromoacetic acid and iodoacetamide were purchased from Eastman and K and K Laboratories, respectively. Iodoacetamide-1-14C with a specific radioactivity of 1.25 mC per mmole was obtained from Tracerlab, and bromoacetic acid-2-14C with a specific activity of 4.8 mC per mmole was purchased from Nuclear-Chicago. The radioactive iodoacetamide used in the present studies was prepared by recrystallizing from water a mixture containing the labeled compound and a 50-fold excess by weight of nonlabeled material. The specific radioactivity of the resulting iodoacetamide-1-14C, determined by bottle counting as described in the following section, was 49,000 cpm per $\mu$ mole. All preparations of iodoacetamide were recrystallized from water prior to use. A stock solution of radioactive bromoacetic acid was prepared by dissolving 1.39 mg of the labeled compound and 12.5 mg of nonlabeled material in 1.0 ml of 0.1 M acetate buffer at pH 5.5. The specific radioactivity of the bromoacetic acid-2-14C solution was 400,000 cpm per $\mu$ mole. Portions of the stock solution were adjusted to pH 5.5 just prior to use.

The d and L isomers of $\alpha$-bromopropionate and $\alpha$-bromo-n-butyrate were synthesized from the optically active amino acids as described earlier (11).

**Carboxypeptidase B** treated with diisopropyl fluorophosphate (COB-DFP, Lot 27) was purchased from Worthington.

**Radioactivity Counting**—Radioactivity measurements were performed with the liquid scintillation system described earlier (13) modified as follows. For bottle counting, 20- to 100-$\mu$l samples were added directly to a vial (2.5 × 5 cm) containing 16 ml of the scintillation liquid. The scintillation liquid was prepared by dissolving 12 g of 2,5-diphenyloxazole and 480 mg of $p$-bis-$2'(5'$-phenyloxazoyl)benzene in 800 ml of a 6:1:1 mixture of spectral grade dioxane, anisole, and 1,2-dimethoxyethane. The counting efficiency for $^{14}$C was 80%. When monitoring the radioactivity of the effluent from ion exchange columns, a 1-mL flow cell was used and the counts were printed at 1-min intervals.

**Amino Acid Analyses**—The instrumentation employed has been described in detail by Fruchter and Crestfield (14). When it was necessary to resolve $\epsilon$-carboxymethyllysine from methionine, the neutral and acidic amino acids were analyzed on a 150-cm column operated at an accelerated flow rate of 55 ml per hour (15) with the recording equipment originally described by Spackman, Stein, and Moore (16).

**Chromatography on Amberlite IRC-50**—Three chromatographic systems in which IRC-50 was employed were used. These will be referred to as the analytical and preparative IRC-50:NaCl systems and the IRC-50:phosphate system. Amberlite IRC-50 (CG-50, 400 to 600 mesh, Mallinckrodt) equilibrated in 0.2 M phosphate buffer, pH 6.47, containing 0.1 M sodium chloride was fractionated as described earlier (11) into several portions differing in their rates of sedimentation in this medium. Each of the three chromatographic columns described in the following paragraphs contained a different fraction of resin.

Alkylation mixtures were monitored as described before (11) by chromatography of diluted aliquots on the analytical IRC-50:NaCl system devised by Crestfield (17). The column (0.9 × 6 cm) contained a fraction of the IRC-50 resin which sedimented from the phosphate buffer at a rate of 0.07 to 0.11 cm per min. This procedure has been used extensively in determining both the rate and the course of the carboxyalkylation of histidine residues 12 and 119 in ribonuclease (2, 11) and has been similarly applied to the analysis of reaction mixtures in which lysine residues are modified. The isolation of milligram quantities of the carboxyalkylhistidine ribonucleases has been achieved by chromatography of reaction mixtures on columns of IRC-50.
(18-20) or sulfoethyl Sephadex (11) eluted with phosphate buffer. Crestfield, Stein and Moore (2) first established that the elution position of ribonuclease A relative to those of its histidine-substituted derivatives is different when elution is accomplished with NaCl than with phosphate buffer, owing to the greater phosphate binding capacity of the native enzyme. Although e-carboxamidomethyllysine-41 ribonuclease is adequately resolved from ribonuclease A and other products by chromatography on sulfoethyl Sephadex eluted with 0.1 mM phosphate buffer at pH 6.47, this system does not separate the unmodified enzyme from its e-carboxamidomethyllysine-41 derivative. When chromatographed on the analytical IRC-50:NaCl system, however, ribonuclease A and e-carboxamidomethyllysine-41 ribonuclease are completely resolved.

It therefore seemed desirable to use a preparative IRC-50:NaCl column to obtain milligram quantities of e-carboxamidomethyllysine-41 ribonuclease. A column (2 × 25 cm) was prepared from the IRC-50 fraction which sedimented in the phosphate buffer at a rate of 0.04 to 0.07 cm per min. The preparative column was washed thoroughly with 0.266 molal NaCl and was inserted in place of the analytical column in the recording spectrophotometric system described previously (17). The sample addition device was not used. When the system was operated at a flow rate of 60 ml per hour, the pressure was less than 20 psi. Under these conditions, the column afforded excellent resolution of the products formed from the reaction of ribonuclease and iodoacetamide at pH 8.5.

The e-carboxamidomethyllysine-41 derivative isolated by chromatography on the preparative IRC-50:NaCl column was found to contain 25 to 30% of a product monocarboxamidomethylated at lysine-1. These two products were not resolved when chromatographed in the IRC-50:NaCl system or on a column of sulfoethyl Sephadex eluted with 0.1 mM phosphate buffer at pH 6.47. Separation of the two derivatives was achieved by chromatography on the IRC-50:phosphate system of Hirs, Moore, and Stein (18) as modified for rapid flow by Crestfield, Stein, and Moore (20). The sedimentation rate in 0.2 M phosphate buffer of the IRC-50 fraction used in this column was 0.11 to 0.25 cm per min. After preparation, the column (0.9 × 30 cm) was washed thoroughly with 0.2 M phosphate buffer at pH 6.44 to remove all of the phenol present in the equilibrating buffer. The column was fitted with a sample addition device (17) and the effluent stream was continuously monitored by means of the equipment described previously (17), except that the light path of the microflow cell was 20 mm rather than 14 mm. Buffer was pumped through the system at a rate of 18 ml per hour by a Milton Roy Minipump. After use, the column was flushed with the phosphate buffer containing 0.1% phenol and stored in this condition.

Preparation of e-Carboxamidomethyllysine-41 Ribonuclease—A 1 to 2% solution of highly purified ribonuclease A in 0.05 M Tris buffer at pH 8.5 was alkylated at room temperature in the dark with a 2-fold molar excess of bromoacetate. It was found preferable to use low concentrations of reagent and allow longer periods of time for reaction in order to minimize undesirable side reactions. The extent of alkylation was determined at various times by analyzing diluted portions of the reaction mixture on the IRC-50:NaCl system described earlier (17, 11). In the reaction of 1.1 mM ribonuclease A with 2 mM bromoacetate at pH 8.5, a 70% conversion of the enzyme to a variety of carboxymethylated products was obtained in 48 hours. The yield of e-carboxamidomethyllysine-41 ribonuclease was routinely of the order of 30%.

To isolate the derivative, the reaction mixture was adjusted to pH 5.5, and a sample containing 50 to 75 mg of protein was added directly to a column (0.9 × 55 cm) of sulfoethyl Sephadex C-25 (cf. Reference 21). The column was eluted with 0.1 M phosphate buffer, pH 6.47, containing 0.05% phenol. Aliquots of the effluent fractions were submitted to alkaline hydrolysis and ninhydrin analysis (20). Fractions containing the e-carboxamidomethyllysine-41 derivative were pooled and stored at 4°C until further use. The derivative may be somewhat further purified by chromatography on the same column of sulfoethyl Sephadex eluted with 0.1 M phosphate buffer at pH 6.25.

Preparation of e-Carboxamidomethyllysine-41 Ribonuclease and e-Dicarboxamidomethyllysine-41 Ribonuclease—To 3 ml of a 1 to 2% solution of highly purified ribonuclease A in 0.95 M Tris buffer at pH 8.5 were added 12 mg of solid recrystallized iodoacetamide or iodoacetamide-1-13C. After 6 hours of reaction in the dark at room temperature, the protein components of the reaction mixture were transferred to solution in 0.0266 molal NaCl by gel filtration on a column (2 × 34 cm) of Sephadex G-25 (bead form). Fractions containing protein were pooled, and the resulting solution was concentrated 8- to 10-fold by rotary evaporation. The concentrate was added to the preparative IRC-50:NaCl column described in an earlier section, and 0.266 molal NaCl was pumped through the column at a rate of 60 ml per hour. Since the absorption of the effluent at 210 mμ was continuously recorded (17), the desired derivatives could be collected as they emerged from the flow cell in the spectrophotometric unit. The solutions of the e-carboxamidomethyllysine-41 derivatives thus obtained varied in protein concentration between 100 and 500 μg per ml. The protein was concentrated in each case by ultrafiltration (20, 19) and finally obtained in any desired solvent by gel filtration. From a typical 6-hour reaction mixture containing initially 1.1 mM ribonuclease A and 22 mM iodoacetamide, the final yields of the mono- and dicarboxamidomethyllysine-41 derivatives were 7% and 3%, respectively, of the initial protein.

The e-mono carboxamidomethyllysine-41 derivative isolated by this procedure contains 25 to 30% of the derivative monosubstituted at lysine-1. This contaminant may be largely removed from the 41 derivative by chromatography on the IRC-50:phosphate system described above. Further purification of the e-dicarboxamidomethyllysine-41 derivative was achieved by chromatography on a column (0.9 × 55 cm) of sulfoethyl Sephadex C-25 eluted with 0.1 M phosphate buffer, pH 6.47, containing 0.05% phenol.

Identification of Lysine-41 as Site of Alkylation—Proof that lysine-41 had undergone chemical modification was based upon the analysis of a peptide containing this amino acid residue which had been isolated from the carboxymethyl and carboxamidomethyl ribonuclease derivatives of interest. The derivatives selected were those which were predominant during the early stages of reaction and which were found on amino acid analysis to be missing a single residue of lysine. Each lyophilized protein derivative was reduced, carboxymethylated, and hydrolyzed with trypsin by the procedures of Crestfield, Moore, and Stein (22), except that the reaction was scaled down 5-fold. A pure peptide, containing residues 40 to 61, was obtained in approximately 90% over-all yield by chromatography of the 8-hour tryptic digest on a column of IRC-50 (H+ form) with a linear gradient of acetic acid concentration (see Fig. 8 in Reference 11).
RESULTS

Preparation and Proof of Structure of ε-Carboxymethyllysine-41 Ribonuclease—In Fig. 1 are presented chromatograms obtained during the reaction of ribonuclease and bromoacetate at pH 8.5. Portions of the reaction mixture were chromatographed at various times on the analytical IRC-50: NaCl system described earlier (17, 11). Three major peaks are clearly evident throughout the course of the alkylation. On the basis of their chromatographic behavior and relative quantity, it seemed likely that two of the three products (designated as 1-CMHis-119 and 3-CMHis-12) were the carboxymethylhistidine derivatives of ribonuclease described by Crestfield, Stein, and Moore (19). These are formed exclusively when the alkylation is carried out at pH 5.5. As the time of reaction is extended to 48 hours, additional derivatives appear on the leading and trailing edges of the peak designated as ε-CMLys-41.

In order to prepare quantities of the various carboxymethylated products sufficient for further analysis, 3 ml of the 48-hour reaction mixture containing 50 to 75 mg of protein were adjusted to pH 5.5 and the solution was immediately chromatographed on a column of sulfoethyl Sephadex eluted with 0.1 M phosphate buffer at pH 6.47. A typical chromatogram is shown in Fig. 2.

The abbreviations used for the derivatives of ribonuclease are: 1-CMHis-119 RNase, 1-carboxymethylhistidine-119 ribonuclease; 3-CMHis-12 RNase, 3-carboxymethylhistidine-12 ribonuclease; ε-CMLys-41 RNase, ε-carboxyamidomethyllysine-41 ribonuclease; ε-diCMLys-41 RNase, ε,N-carboxyamidomethyllysine-41 ribonuclease; ε-CAMLys-41 RNase, ε-carboxamidomethyllysine-41 ribonuclease; CAMLys-1 RNase, carboxamidomethyllysine-1 ribonuclease.

This peptide, RCM-Trp-9, contains, in addition to lysine-41, lysine-61 at the carboxyl terminus. In each case, amino acid analysis revealed that 1 of the 2 lysines had been alkylated. In order to rule out the unlikely possibility of reaction at lysine-61, this residue was removed by treating 0.5 ml of a solution containing 0.275 μmole of the peptide in 0.025 M Tris buffer at pH 7.5 with 3 μg of carboxypeptidase B at room temperature for 4 hours.

In order to prepare quantities of the various carboxymethylated products sufficient for further analysis, 3 ml of the 48-hour reaction mixture containing 50 to 75 mg of protein were adjusted to pH 5.5 and the solution was immediately chromatographed on a column of sulfoethyl Sephadex eluted with 0.1 M phosphate buffer at pH 6.47. A typical chromatogram is shown in Fig. 2.

The designations used for the tryptic peptides are the same as those of Hirs et al. (23, 24) except that the prefix RCM is employed to indicate that the peptides have been derived from reduced, carboxymethylated ribonuclease or its derivatives.
On the basis of their chromatographic behavior (11) and amino acid analysis (19), the two most retarded products were shown to be, in the order of elution, 1-CMHis-L19-RNase and 3-CMHis-L12-RNase. Amino acid analysis of the derivative corresponding to the peak designated as e-CMLys-41-RNase (Fig. 2) indicated a loss of 1 residue of lysine and the presence of 1 residue of e-carboxamidomethyllysine. Gundlach, Stein, and Moore (25) demonstrated that, when valine is eluted from the 150-cm column of the amino acid analyzer in buffer at pH 4.25, e-carboxamidomethyllysine appears on the chromatogram just ahead of methionine. It has been found that if valine is eluted at pH 3.25, all other conditions being the same, e-carboxamidomethyllysine emerges between methionine and isoleucine. When analyses are carried out on the 60-cm column with the accelerated procedure of Spackman (15), e-carboxamidomethyllysine is eluted in the position of methionine. This poses no difficulties in the amino acid analysis of the peptide containing lysine-41, however, since it contains no methionine.

Proof that the major product of the reaction of ribonuclease and bromoacetate at pH 5.5 was carboxamidomethylated at the e-amino position of lysine-41 was furnished by the isolation and analysis of the peptide RCM-Tryp-9 from a tryptic digest of the reduced carboxamidomethylated protein derivative. The amino acid analysis given in Table I reveals a loss of 1 of the 2 lysine residues present in the unmodified peptide and the presence of 1 residue of e-carboxamidomethyllysine. Although RCM-Tryp-9 contains lysine-61 at the carboxyl terminus in addition to lysine-41, alkylation presumably had not occurred at lysine-61, since, if it had, the tryptic cleavage at this position which is required for the liberation of RCM-Tryp-9 would not have taken place. Reaction at lysine-61 was ruled out completely by treating the 22-residue peptide RCM-Tryp-9 with carboxypeptidase B. This yielded 1 eq of lysine. The hydrolysate resulting from the carboxypeptidase B treatment was subjected to gel filtration on a column (0.9 x 50 cm) of Sephadex G-25 eluted with 50% acetic acid. Analysis of the 20-residue peptide thus obtained revealed no lysine, a loss of 1 residue of glutamic acid, and the presence of 1 residue of carboxamidomethyllysine. Apparently, both lysine-61 and glutamine-60 were removed during hydrolysis with carboxypeptidase B. These results, presented in Table I, establish the structure of the derivative corresponding to the peak designated as e-CMLys-41-RNase in Figs. 1 and 2. The two derivatives emerging ahead of e-CMLys-41-RNase (Fig. 2) have not been characterized further.

Preparation and Proof of Structure of e-Carboxazidomethyllysine-41 Ribonuclease and e-Dicarboxazidomethyllysine-41 Ribonuclease—In contrast to the alkylation with bromoacetate, the reaction of ribonuclease A and iodoacetamide is quite complex. A wide variety of products are formed, as may be seen in Fig. 3, which shows the course of the iodoacetamide reaction as determined by chromatographic analysis of portions of the reaction mixture on the analytical IRC-50:NaCl system. After 3 hours, the major product is a derivative (labeled e-CMLys-41) eluted just ahead of ribonuclease. The concentration of this material reaches a maximum after about 6 hours of reaction, at which time a second product, designated as e-dCMLys-41, is also present in high concentrations. As the time of alkylation is extended to 25 and 50 hours, the e-CMLys-41-RNase is gradually converted, together with the unreacted enzyme, to a variety of front-running components, eluted near the position of e-dCMLys-41-RNase.

Initial attempts to isolate e-CMLys-41-RNase by chromatography on sulfonated Sephadex as described above for the corresponding carboxymethyl derivative were unsuccessful. Under these conditions, the monocarboxazidomethyl derivative is not resolved from ribonuclease A. Separations were therefore carried out on a preparative column of IRC-50 eluted with 0.266 molal NaCl. As may be seen in Fig. 4, the higher resolution afforded by this larger column permitted the isolation of a number of carboxazidomethyl derivatives in addition to those observed on the analytical system.

In order to facilitate subsequent chemical characterization of the products, the enzyme was alkylated with iodoacetamide-1-14C

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>No. of residues in RCM-Tryp-9 (residues 40 to 61)</th>
<th>No. of residues in RCM-Tryp-9 treated with carboxypeptidase B (residues 40 to 59)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>2</td>
<td>2.0a</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3</td>
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<tr>
<td>Alanine</td>
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<td>2.00b</td>
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<tr>
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<tr>
<td>Leucine</td>
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<td>Serine</td>
<td>2</td>
<td>1.86</td>
</tr>
<tr>
<td>Threonine</td>
<td>1</td>
<td>0.97</td>
</tr>
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</table>

* The glutamic acid content is low by 1 residue, hence there is a loss not only of carboxyl-terminal lysine-61, but also of the next residue, glutamine-60.

* The molar ratios of the amino acids present in RCM-Tryp-9 peptides were calculated assuming 2.00 residues of aspartic acid. The corresponding values for the peptide treated with carboxypeptidase B were calculated assuming 2.00 residues of aspartic acid.

* Not corrected for decomposition during acid hydrolysis (22 hours at 110°C).

* The glycine color constant was employed in calculating the concentration of the lysine derivatives.
Alkylation of Active Site of Ribonuclease

Reagent
RNase A

\[ \text{Effluent ml} \]

**FIG. 3.** Chromatograms obtained at various times during the reaction of iodoacetamide and ribonuclease A at pH 8.5. The reaction conditions and method of analysis are described in the legend to Fig. 1, except that the initial concentration of reagent was 22 mM.

and the carboxamidomethylated derivatives were fractionated by chromatography on the preparative IRC-50:NaCl column. On the basis of their specific radioactivities, it was concluded that the peaks labeled a and ε-diCAMLys-41- in Fig. 4 represent disubstituted products, and that the derivatives corresponding to Peaks b, c, and ε-CAMLys-41- are monosubstituted.

Amino acid analysis of the two major products shown in Fig. 4 revealed a loss of a single residue of lysine. The more retarded of the two, ε-CAMLys-41-RNase, gave 1 residue of ε-carboxymethyllysine, and the faster moving derivative, ε-diCAMLys-41-RNase, yielded 1 residue of ε-dicarboxymethyllysine. The product designated as ε-CAMLys-41- in Fig. 4 proved to be chromatographically homogeneous both on the IRC-50:NaCl system and on sulfoethyl Sephadex eluted with 0.1 M phosphate buffer at pH 6.47. However, several lines of evidence, to be discussed below, suggested that this monosubstituted 41-derivative was only 75 to 80% pure. Therefore, a chromatographic system was sought which would separate ε-CAMLys-41-RNase from its contaminants. Partial resolution of two monosubstituted derivatives was achieved by chromatography of the mixture on the IRC-50:phosphate system described in "Experimental Procedure." As may be seen in Fig. 5, the contaminant, designated as CAMLys-1-RNase, amounts to about 30% of the total protein. Its structure was ascertained by analysis of the NH₂-terminal tridecapeptide before and after removal of carboxymethyllysine-1 by a single stage of the Edman degradation procedure, with the modification of Konigsberg and Hill (26). The tridecapeptide was obtained by cyanogen bromide cleavage of 1 to 2 mg of the derivative by the procedure of Gross and Witkop (27). Analysis of the tridecapeptide before the Edman degradation gave 1 residue of lysine and 1 residue of material eluted in the position of ε-carboxymethyllysine. Analysis of the dodecapeptide after removal of NH₂-terminal lysine-1 gave 1 residue of lysine and no carboxymethyllysine, thus indicating that the contaminating protein had been substituted at lysine-1. Model reactions between lysine and bromoacetate at pH 5.5 yield a major product which is chromatographically similar to ε-carboxymethyllysine. It is not possible at present, therefore, to tell whether the lysine-1 derivative is substituted at the ε or at the α position.

Because of the scarcity of material, peptide analysis of ε-CAMLys-41-RNase was carried out on a 14C-labeled preparation contaminated with about 25% of CAMLys-1-RNase. For this reason, the tryptic peptide containing lysine-41, RCM-Tryp-9, contained only about 78% of the radioactivity of the original sample. As may be seen in Table I, amino acid analysis of...
In Table II are presented the enzymatic position of the c-carboxymethyllysine peak. The faster moving derivative (Fig. 4) is e-dicarboxamidomethyllysine-41 ribonuclease, and the more retarded product is e-carboxamidomethyllysine-41-derivative. Although the 3% activity is rather constant across the e-CAMLys-41-RNase peak, it should be considered a maximal value. It could easily result from a slight degree of contamination.

The activity of e-diCAMLy-41-RNase was less than 0.2% that of native ribonuclease. This derivative was assayed after purification on sulfoethyl Sephadex as described previously.

### Table II

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity (^a)</th>
</tr>
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<tbody>
<tr>
<td>RNase A</td>
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<tr>
<td>e-CMLys-41-RNase (^b)</td>
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<td>e-CMLys-41-RNase (^c)</td>
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<tr>
<td>e-CAMLys-41-RNase (^d)</td>
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<tr>
<td>e-diCAMLy-41-RNase (^e)</td>
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<tr>
<td>CAMLy-1-RNase (^f)</td>
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<tr>
<td>Peak a (^g)</td>
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<tr>
<td>Peak b (^h)</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) The activities are based upon turnover numbers, which, since saturating concentrations of substrate were not used, are characteristic only for the assays conducted under the stated conditions. The turnover number is micromoles of 3'-cytidylate formed per second per 

\(^b\) The derivative was isolated from the preparative reaction mixture containing RNase and bromoacetate at pH 8.5 by chromatography on a column (0.9 X 55 cm) of sulfoethyl Sephadex eluted with 0.1 M phosphate buffer at pH 6.47 (cf. Fig. 2).

\(^c\) The derivative was isolated after rechromatography of the material described above on a column (0.9 X 55 cm) of sulfoethyl Sephadex eluted with 0.1 M phosphate buffer at pH 6.25 (cf. "Experimental Procedure").

\(^d\) The activity given is the minimal value obtained after the removal of CAMLy-1-RNase (cf. Fig. 5).

\(^e\) The derivative was isolated by chromatography on the preparative IRC-50:NaCl system and was further purified on a column (0.9 X 55 cm) of sulfoethyl Sephadex eluted with 0.1 M phosphate buffer at pH 6.47 to assay.

\(^f\) Activities were determined for the derivative isolated as described in Fig. 5.

\(^g\) Peaks a, b, and c (cf. Fig. 4) were collected during chromatography on the preparative IRC-50:NaCl column of reaction mixtures containing RNase A and iodoacetamide.
In order to determine the effect of the size and the optical configuration of the reagent upon the rate and the course of the alkylation at pH 8.5, the carboxamidomethyl derivatives represented by Peaks a, b, and c in Fig. 4 were fully active.

**Reaction of Ribonuclease A with d and L Antipodes of α-Bromopropionate and α-Bromo-n-butyrate at pH 8.5**—In order to determine the effect of the size and the optical configuration of the reagent upon the rate and the course of the alkylation at pH 8.5, ribonuclease was allowed to react with some of the reagents which had been used previously in a similar study of the reactions at pH 5.5 (11). The elution curves shown in Fig. 6 were obtained by chromatography on the analytical IRC-50:NaCl system of portions of reaction mixtures containing ribonuclease and the reagent at pH 5.5 (11). The elution curves shown in Fig. 1, the Pl-derivative is in each case eluted first, and the over-all rate constant is equal to the sum of the individual constants. This treatment is therefore analogous to that applied previously (11) in characterizing the reactions at pH 5.5. It must be stressed, however, that although these assumptions are valid for the alkylation at pH 5.5, where the products are formed simultaneously and independently, they may not be at pH 8.5, where secondary alkylations of the products are known to occur. Therefore, the values for the individual rate constants which appear in Table III represent minimal approximations of the actual rates of production of the monocarboxyalkyllysine-41, histidine-119, and histidine-12 ribonuclease derivatives.

The over-all reactions at pH 8.5 proceed at rates which vary from 20 to 100% of those at pH 5.5. The 25-fold decrease in the over-all rate of reaction as one proceeds from bromoacetate to the C4 reagents is somewhat less than the 100-fold difference observed at pH 5.5 (11). This decrease is just about equally reflected by the decrease in the individual rate constants for the reactions at lysine-41 and histidine-119. The 3-fold difference between the reactivity of bromoacetate and n-α-bromopropionate at histidine-12 is comparable to the 5-fold decrease observed at pH 5.5.

**Table III**

Rates of reaction of ribonuclease A with several α-bromo acids at pH 8.5 and 25°

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Reaction with ribonuclease</th>
<th>Lysine-41</th>
<th>Histidine-119</th>
<th>Histidine-12</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromoacetate</td>
<td></td>
<td>260</td>
<td>110</td>
<td>18</td>
<td>411</td>
</tr>
<tr>
<td>α-Bromopropionate</td>
<td></td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>L-α-Bromopropionate</td>
<td></td>
<td>5.5</td>
<td>1.8</td>
<td>0.8</td>
<td>10</td>
</tr>
<tr>
<td>D-α-Dibromo-n-butyrate</td>
<td></td>
<td>1.5</td>
<td>0.6</td>
<td>8.7</td>
<td>15</td>
</tr>
<tr>
<td>L-α-Bromo-n-butyrate</td>
<td></td>
<td>1.5</td>
<td>0.4</td>
<td>0.6</td>
<td>3.3</td>
</tr>
<tr>
<td>DL-α-Bromovalerate</td>
<td></td>
<td>1.6</td>
<td>0.2</td>
<td>0.08</td>
<td>2.1</td>
</tr>
</tbody>
</table>

In Table III are presented approximate values of the individual second order rate constants for the alkylation of lysine-41, histidine-119, and histidine-12 at pH 8.5 by bromoacetate, the D and L isomers of the C4, α-bromo acids, and the racemic C4 acid, α-bromovalerate. These constants were derived from the over all second order rate constant calculated in each case on the basis of the disappearance of ribonuclease A. Since the reactions were monitored chromatographically on the IRC-50:NaCl system, both the disappearance of native enzyme and the formation of products were recorded during the course of the alkylation. It must be stressed, however, that these assumptions are valid for the alkylation at pH 5.5, where the products are formed simultaneously and independently, they may not be at pH 8.5, where secondary alkylations of the products are known to occur.
The stereospecificity of the histidine reactions noted earlier (11) at pH 5.5 is maintained at pH 8.5. At pH 5.5, the $C_3$ and $C_4$ reagents of the $D$ configuration were found to react preferentially at histidine-12, while their $L$ antipodes reacted primarily at histidine-119. As indicated by the chromatograms in Fig. 6 and the constants in Table III, this is also generally true for the histidine reactions at pH 8.5. The only exception is that $L$-$\alpha$-bromo-$\alpha$-butyrate reacts somewhat more extensively at histidine-12 than at histidine-119. This finding may reflect a conformational change in going from pH 5.5 to pH 8.5 which results in a hindrance of the reactions at histidine-119 with the $C_4$ reagents of either the $D$ or the $L$ configuration. There is no apparent stereospecificity in the alkylation of lysine-41, however. The $D$ and $L$ antipodes of a given reagent react at this position at similar rates.

In general, alkylation at histidine-12 is diminished less as the pH is raised than is that at histidine-119. The ratios of the $D$-$\alpha$-bromopropionate in 0.05M Tris buffer at pH 8.5, with increasing amounts of NaCl added to give the desired ionic strength. All reaction mixtures contained 0.05 M Tris buffer at pH 8.5, with increasing amounts of NaCl added to give the desired ionic strength. Reactions were allowed to proceed in the dark at 25°C.

Although the rates of reaction of bromoacetate and $\alpha$-bromopropionate at lysine-41 differ in proportion to the reactivities of the two reagents (11), the rate of reaction at this position with $\alpha$-bromo-$\alpha$-butyrate is about three times slower than one would expect on the basis of its reactions with the histidine residues. This finding suggests that certain steric requirements must be satisfied in order to alkylate lysine-41, and that these inhibitory effects become prominent as the chain length of the alkylating agent is extended from 3 to 4 carbon atoms. The $C_3$ reagent, $\alpha$-bromovalerate, reacts primarily at lysine-41 and at a rate comparable to that of the $C_4$ reagent. In agreement with the results at pH 5.5, the $C_3$ reagent reacts more at histidine-119 than at the 12-position; both histidine reactions are much less prominent than the one at lysine-41.

**Effect of Ionic Strength and Cu$^{++}$ on Alkylation of Ribonuclease A by $\alpha$-Bromopropionate at pH 8.5**—In order to study the effects of variations in the reaction conditions upon the course of the alkylation, it is desirable to use a reagent which reacts at nearly equal rates at the three positions of interest: histidine-119, histidine-12, and lysine-41. This permits easier detection of slight variations in the ratios of the products which result from changes in the composition of the reaction mixture. The reagent which best satisfies this condition is $\alpha$-bromopropionate. During the early stages of the reaction of ribonuclease with $\alpha$-bromopropionate in 0.05M Tris buffer at pH 8.5, the derivatives substituted at lysine-41, histidine-119, and histidine-12 account for 40%, 25%, and 34%, respectively, of the total reaction products.

In Fig. 7 is shown the effect of ionic strength on the individual second order rate constants for the reaction of $\alpha$-bromopropionate at lysine-41 (A—A), histidine-12 (W—W), and histidine-119 (O—O) at pH 8.5. The initial concentrations of ribonuclease and reagent were 0.6 mM and 13.1 mM, respectively. Owing to the occurrence of secondary alkylations, the values presented for the reactions at pH 8.5 are subject to the same qualifications mentioned earlier with regard to the constants in Table III.

The reactions carried out at pH 5.5 are inhibited by the presence of 0.01 M Cu$^{++}$ to the extent of 76%, 60%, and 55% for bromoacetate, $\alpha$-bromopropionate, and $\alpha$-bromo-$\alpha$-butyrate, respectively. The inhibitory effect is in each case greatest on the reaction at histidine-119. Thus, although the rate of reaction of each reagent at histidine-12 is diminished less than 2-fold by the presence of 0.01 M Cu$^{++}$, the alkylation of histidine-119 is reduced approximately 5-fold in each case. At pH 8.5, however, the over-all rate of reactions conducted in 0.01 M Cu$^{++}$ is not inhibited but is actually accelerated to a slight degree. The increased rate thus observed occurs to nearly the same extent at the three major sites of reaction, nitrogen 1 of histidine-119, nitrogen 3 of histidine-12, and the $\epsilon$-amino group of lysine-41.

Since, in the reaction at pH 8.5, the cupric ion exists in the form of coordination complexes with ammonia and Tris (cf. Table IV), its binding potential for the enzyme is undoubtedly lower than is the case at pH 5.5.

**Alkylation at pH 5.5 of Derivatives of Ribonuclease Substituted at Lysine-41**—In Fig. 8 is shown the time course of the reaction of ribonuclease A, $\epsilon$-CM-Lys-41-RNase, and $\epsilon$-CAM-Lys-41-RNase with bromoacetate at pH 5.5. The latter derivative was freed of CAM-Lys-1-RNase as described earlier (Fig. 5). Similar rates of reaction are observed for the native enzyme and the derivative carboxamidomethylated at lysine-41. However,
TABLE IV

<table>
<thead>
<tr>
<th>Reagent</th>
<th>pH 5.5</th>
<th>pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>In 0.01 M Cu++</td>
</tr>
<tr>
<td><em>Lysine-41</em> Histidine-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Relative activity</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>% Reaction</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Reaction rate, $k_2 (10^{-4}$ sec$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromoacetate</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>n-α-Bromopropionate</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>n-α-Bromo-n-butyrate</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Figure 8.** Rates of reaction of ribonuclease (C—A), e-CAMLys-41-RNase (●—●), and e-CMLys-41-RNase (■—■) with bromoacetate in 0.1 M acetate buffer at pH 5.5. Reactions were carried out in the dark at 25°. The initial concentrations of protein (b) and bromoacetate (a) were 0.15 mM and 35 mM, respectively, and x is the number of moles of product formed per liter in the given time interval.

if the derivative contains a negatively charged carboxymethyl group at lysine-41, the rate of reaction is diminished 4 to 5-fold.

In order to determine whether or not substituents at lysine-41 alter the 12:119-product ratio, e-CAMLys-41-RNase, e-CMLys-41-RNase, and ribonuclease A were carboxymethylated at pH 5.5 with bromoacetate-214C. The total protein from each reaction mixture was then subjected to cleavage with cyanogen bromide as described by Gross and Witkop (27). The levels of radioactivity of the NH$_2$-terminal tridecapeptide and the C-protein fragment thus obtained are presented in Table V. On amino acid analysis, all of the radioactivity in the tridecapeptide was accounted for as 3-carboxymethylhistidine. Since, in each case, the cleavage at methionine-13 occurred to the extent of 95 to 100%, the radioactivity in the tridecapeptide and in the 110-residue C-protein fragment reflects the degree of reaction at histidine-12 and histidine-119, respectively. In earlier studies (11), the course of the alkylation of ribonuclease A was followed chromatographically, and it was found that the reaction proceeded to the extent of 91% at histidine-119 and 9% at histidine-12. As may be seen in Table V, very similar values are obtained when the radioisotope procedure is used. The results given in Table V indicate that the reaction at histidine-12 is diminished relative to that at histidine-119 by substituents on the ε-amino group of lysine-41. With each 41-derivative, the product alkylated at histidine-12 was reduced from 9% to 5% of the total reaction product.

Since bromoacetate reacts predominantly at histidine-119, it is not an ideal choice upon which to base conclusions regarding the inhibitory effects of 41-substituents on the reaction at histidine-12. A better reagent in this regard is n-α-bromo-n-butyrate, which reacts with ribonuclease preferentially at the 12-position (11). Moreover, with this reagent, alkylation of histidine-12 may be followed by amino acid analysis, since the product of the reaction, 3-(1-carboxy-n-propyl)histidine, is clearly resolved from the remaining amino acids on both the 50- and the 150-cm columns of the amino acid analyzer (11). Because of the lower reactivity of e-CMLys-41-RNase at pH 5.5 (Fig. 8), e-CAMLys-41-RNase was chosen as a more suitable derivative for reaction with the C$_1$ reagent. A reaction mixture was prepared containing 0.034 mM e-CAMLys-41-RNase and 0.1 M n-α-bromo-n-butyrate in 0.1 M acetate buffer at pH 5.5. After 10 hours of reaction, chromatography of an aliquot on the analytical IRC-50:NaCl system indicated that 75% of the starting protein had been converted to faster moving products. Amino acid analysis of the total protein present in a 2.0-m1 portion of the 100-hour reaction mixture revealed the presence of 0.30 residue of 3-(1-carboxy-n-propyl)histidine. Since the reaction had proceeded 75% to completion, and since in the alkylation of native ribonuclease A under these conditions the 3-(1-carboxy-n-propyl)-histidine-12 derivative accounts for 76.4% of the total reaction
TABLE V

| Effect of substituents at lysine-41 on carboxymethylation of histidine-12 and histidine-119 in ribonuclease |

Ribonuclease A and derivatives substituted on the ε-amino group of lysine-41 were allowed to react with bromoacetate-2-^{14}C (400,000 cpm per mole) in 0.1 M acetate buffer at pH 5.5. After 50 to 75% of the starting protein had been converted to products, the entire reaction mixture was in each case applied to a column (2 X 30 cm) of Sephadex G-25 eluted with 5% acetic acid. The protein fractions were pooled, and the solution was lyophilized with the precautions described earlier (21). The lyophilized protein was then subjected to cyanogen bromide cleavage and the amino-terminal tridecapeptide was separated from C-protein as described by Groess and Witkop (27). The yield of tridecapeptide was 95 to 100%.

<table>
<thead>
<tr>
<th>Protein alkylated</th>
<th>Radioactivity</th>
<th>Reaction at histidine-12</th>
<th>Reaction at histidine-119</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH-terminal</td>
<td>C-Protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tridecapeptide</td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>ε-CMLys-41-RNase</td>
<td>18,760</td>
<td>286,080</td>
<td>6</td>
</tr>
<tr>
<td>ε-CM Lys-41-RNase</td>
<td>2,250</td>
<td>50,530</td>
<td>4</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>29,185</td>
<td>210,140</td>
<td>0</td>
</tr>
</tbody>
</table>

*a Determined by bottle counting of effluent fractions with the liquid scintillation system described in "Experimental Procedure."

**The reaction mixture contained initially 0.25 mM ε-CMLys-41-RNase and 4 mM bromoacetate-2-^{14}C in a total volume of 3.0 ml. The time of reaction was 48 hours.

The reaction mixture contained initially 0.20 mM ε-CMLys-41-RNase and 1.5 mM bromoacetate-2-^{14}C in a total volume of 0.6 ml. The time of reaction was 43 hours.

The reaction mixture contained initially 0.82 mM ribonuclease A and 2.0 mM bromoacetate-2-^{14}C in a total volume of 1.25 ml. The time of reaction was 63 hours.

A variety of α-bromo acids of varying chain length and optical configuration. The extent of substitution at any given position depends largely upon the structure of the α-bromo acid employed. For example, in the alkylation at pH 8.5 with the α isomers of α-bromopropionate and α-bromo-n-butyrate, the monosubstituted histidine derivatives account for 45% and 73%, respectively, of the total products formed. Therefore, monocarboxylalkylhistidine ribonucleases are formed during the alkylation with α-bromo acids at pH 8.5, and, depending upon the reagent employed, may represent the major proportion of the reaction product. These observations may be compared to those of Hirs, Halman, and Kycia (28), who found that in the reaction with 1-fluoro-2,4-dinitrobenzene at pH 8.0, dinitrophenylation of at least 1 lysine residue occurred before reaction of tyrosine and histidine residues could be detected.

The reactions of histidine-12 and histidine-119 at pH 8.5 proceed at rates which vary, depending upon the reagent used, from ½ to 2% of those observed at pH 5.5. In general, the 12-substituted products are favored with respect to the 119-derivatives as the pH is raised from 5.5 to 8.5. Although this finding might suggest a conformational change in this pH range, such an alteration could not be very great since the specific activity of the histidine reactions is nearly the same at the two pH values (Fig. 6 and Table III). It should also be stressed that at both pH 5.5 and pH 8.5, alkylation at histidine-119 takes place on nitrogen 1, while reaction at histidine-12 occurs on nitrogen 3.

It is noteworthy that lysine-41 is modified preferentially by a variety of reagents. In the reactions with 1-fluoro-2,4-dinitrobenzene (28) and 4-sulfonoxy-2-nitrofluorobenzene (7) at pH 8.0, and with iodoacetamide and bromoacetate at pH 8.5, the derivatives present in highest concentration during the initial stages of the alkylation of ribonuclease are modified at lysine 41. Hirs et al. (28) postulated that the enhanced reactivity of lysine-41 might be due either to a selective absorption of the aromatic side chain of the reagent to a hydrophobic region near to the ε-amino group, or to an abnormally low pK of lysine-41, which might be increased by its existence as a positively charged center in the enzyme. Since bromoacetate and iodoacetamide lack hydrophobic side chains which might participate in anchimeric facilitation of the alkylation reactions, it would appear that the unusually high reactivity at lysine-41 is due primarily to the low pK value of its ε-amino group relative to those of the other 9 lysine residues in the molecule.

The high reactivity of lysine-41 may also be a result of its presence in a relatively accessible region of the molecule. This accessibility is manifested by the lack of stereospecificity in the alkylation of lysine-41 by the optical antipodes of the C1 and C2 α-bromo acids, and by the ease of dissubstitution by iodoacetamide. The alkylation of ribonuclease by iodoacetamide gives a host of products which may be resolved by various chromatographic procedures. One of the first of the carboxamidomethyl derivatives to be formed is ε-CM Lys-41-RNase. One would expect that the introduction of the first carboxamidomethyl group would lower still further the pK of the ε-amino group relative to those of the other 9 lysine residues in the molecule.

From their experiments on the pH dependence of the inactivation of ribonuclease by iodoacetate, Gundlach, Stein, and Moore (25) concluded that alkylation at pH 5.5 and pH 8.5 was confined to histidine and lysine residues, respectively. Indeed, it has since been shown that at pH 5.5, reaction of the enzyme with iodoacetate (2) and with a series of α-bromo acids ranging in chain length from 2 to 3 carbon atoms (11) occurs exclusively either on imidazole nitrogen 1 of histidine-119 or on nitrogen 3 of histidine-12. The results of the present investigation show, however, that in the reaction with bromoacetate at pH 8.5, about 70% of the total product results from the carboxymethylation of lysine residues, primarily lysine-41, and that the remaining 30% arises by virtue of the same imidazole modifications encountered at pH 5.5. These three positions in ribonuclease, nitrogen 1 of histidine-119, nitrogen 3 of histidine-12, and the ε-amino group of lysine-41, are the most reactive at pH 8.5 toward the introduction of the first carboxamidomethyl group.
Thus, the results of this and earlier investigations all suggest that, in the absence of divalent anions (cf. Reference 28), lysine-41 is a particularly reactive nucleophile in ribonuclease at pH 8.5, and that its chemical integrity is essential for the activity of the enzyme. If this is true, one would expect that reaction of the enzyme at pH 8 to 8.5 with any alkylating agent specific for the unprotonated form of the amino group would lead to inactivation after the modification of only 1 to 2 lysine residues. At least in one known instance, the reaction with O-methylisourea, this is not the case. Klee and Richards (29) have shown that ribonuclease is not significantly inactivated by this reagent until essentially all 10 of the lysine residues have been guanidinated. Therefore, either the 41-position is the last to be guanidinated by this positively charged reagent, or else the ribonuclease derivative bearing a guanido group at lysine-41 is almost fully active.

One of the major objectives of the present investigation was to determine the effect of substituents on the ε-amino group of lysine-41 upon the rate and course of the carboxyalkylation of histidine residues 12 and 119. The ε-dinitrophenyllysine-41 derivative of Hirs et al. (3) was not an ideal choice for this study because of its limited solubility and because of the possibility that the bulky dinitrophenyl substituent had affected a conformational change in the active center (3). These considerations prompted attempts to isolate derivatives of ribonuclease substituted at lysine-41 with groups of low molecular weight, thus minimizing the possibility of conformational alterations. The carboxymethyl- and carboxamidomethyllysine-41 derivatives described in this paper are especially well suited for studying the reactivities of histidine-12 and histidine-119. They are inactive and quite soluble, and their 41-substituents are small. Although structurally similar, the groups at lysine-41 differ considerably in their effects on the charge at the active site and on the pK of the ε-amino group. The rates for carboxymethylation at pH 5.5 of histidine residues 12 and 119 in ε-CAMLys-41-RNase and ε-CMLys-41-RNase were found to be about 0.8 and 0.25 that of ribonuclease, respectively, under similar conditions (Fig. 8). Thus, although the chemical modification of lysine-41 results in the inactivation of ribonuclease, the reactivity of the histidine residues is, at least in the case of the carboxamidomethyl derivative, almost as great as in the native enzyme. The more pronounced reduction in the rate of reaction of the carboxymethyllysine-41 derivative might well be expected, owing to charge repulsion of the bromoacetate anion by the negatively charged substituent. It was also shown that the effect of the 41-substituent on the relative rates of carboxymethylation of histidine residues 12 and 119 is the same whether the adduct on the ε-amino group is carboxymethyl or carboxamidomethyl. In both cases there was a 50% reduction in the yield of the 12-derivative from what would be expected in the absence of the 41-substituent.

It would seem, therefore, that although substituents on the ε-amino group of lysine-41 do not prevent alklyation of the active site histidines, they do inhibit the reaction preferentially at histidine-12 in comparison to that at histidine-119. Since this inhibitory effect is the same for 41-substituents of widely differing charge, it is tempting to speculate that the ε-amino group of lysine-41 is relatively near to nitrogen 3 of histidine-12, and that the greater inhibition of the reaction at the 12-position may be due to steric hindrance. Although it seems unlikely that the carboxymethyl and carboxamidomethyl groups at lysine-41 would give rise to conformational alterations which would affect the ratio of the histidine products in a similar fashion, this possibility cannot at present be eliminated.

The mechanism whereby substituents at lysine-41 inactivate ribonuclease and the question as to what role lysine-41 may play in the catalytic process are still undecided. However, the results of these and of other chemical modification studies have supplied information from which certain aspects of the active center may be deduced. The studies of Spark et al. (9) and Crestfield et al. (2) suggested that the unusually high reactivity of the active site histidines might be due to an electrostatic facilitation whereby the protonated form of one of the imidazole rings binds the anionic reagent, which then alkylates the unprotonated form of the other. Furthermore, Crestfield et al. (2) were led to conclude that the reactive nitrogens of histidine residues 12 and 119 are about 5 Å apart. As an alternative (2, 5, 8), it has been considered that the ε-amino group of lysine-41 might provide this positive orienting charge. This latter possibility now seems less likely since a negatively charged carboxymethyl group at the 41-position does not drastically reduce the over-all rate of alkylation of histidine-12 and histidine-119 at pH 5.5. These considerations support the hypothesis (2) that the reactive nitrogens of histidine-12 and histidine-119 are solely responsible for the facilitation of the alkylation at pH 5.5 and are, therefore, approximately 5 Å apart. Because of the preferential inhibition of the reactions at histidine-12 by the 41-substituents, it is tentatively proposed that the ε-amino group of lysine-41 is of the order 7 to 10 Å from nitrogen 3 of histidine-12 and somewhat further removed from nitrogen 1 of histidine-119. Support for this model derives from the observation that, although at pH 5.5 only one of the two imidazole rings may be alkylated in a given enzyme molecule (2), a disubstituted derivative in which lysine-41 and one or the other of the two histidines are modified is readily obtained under these same conditions.

Since the alkylation of the histidine residues at the active site is assumed to consist of two phases, electrostatic binding of the reagent and alkylation of the imidazole ring, it might be argued that the 41-substituents hinder reaction at histidine-12 by blocking the ionic interaction at histidine-119. This interpretation, therefore, suggests that the ε-amino group of lysine-41 is closer to histidine-119 than to histidine-12. Since, however, both neutral and charged 41-substituents have the same effect on the alkylation of the histidines, it seems unlikely that the hindrance is electrostatic in nature and, therefore, the model proposed above seems the more plausible.

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