Alkylation of the Methionine Residues of Ribonuclease in 8 M Urea*

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The identity of some of the amino acid residues at the active site of ribonuclease is now known (see Crestfield, Stein, and Moore (1) for references and discussion) and the availability of other functional groups in the molecule has been studied extensively. For example, Anfinsen, Sela, and Cooke (2) have investigated the reduction and reoxidation of disulfide bonds in poly-alanyl ribonuclease and have found that the reduced protein can be reoxidized to a fully active conformation even after as many as 60 alanine residues have been added to the e-amino groups of lysine residues. Since 7 of the 10 e-NH₂ groups reacted, these workers concluded that 7 lysine residues are on the surface of the protein and do not participate in maintaining or reforming the active three-dimensional structure. White (3), on the other hand, has recently concluded that either lysine 37 or histidine 48, or both, although not part of the active site, are also on the surface of the enzyme and participate in side chain interreactions which aid in the regeneration of the native conformation. The present paper provides an example of a complementary approach. If a functional group which is buried in the interior of ribonuclease is modified by carrying out a chemical reaction under conditions known to cause reversible denaturation, the capacity of the modified, unfolded protein to refold to an active conformation can be studied after the denaturant is removed.

The side chain of methionine is one of the few groups likely to be found in the hydrophobic interior regions of native globular proteins (4), which can be modified readily and selectively. Substitution of the thioether sulfur by oxidation or alkylation leads to the introduction of a charge or dipole where none existed before, and, consequently, the stability of the native conformation may be decreased because the new polar group is solvated better in water than in a region of low polarity. Therefore, such a modification might render the native conformation of a protein unstable and hence inactivate the enzyme without direct involvement of the catalytic or binding sites.

Methionine and its derivatives are alkylated readily by iodoacetate (5) or iodoacetamide (6) to form sulfonium salts, at rates which have been reported to change little over the range pH 2 to 8. However, alkylation or oxidation of methionine residues in ribonuclease takes place much more rapidly at low pH than at neutral pH (7), but alkylation can be made to occur rapidly at neutral pH in the presence of 4 M guanidinium chloride or 8 M urea (8). Substitution leads to inactivation in all cases. To explain these findings, it was suggested that the methionine residues of native ribonuclease are not accessible to iodoacetate unless the native conformation of the protein is disrupted by low pH or denaturing solvents. Stark, Stein, and Moore (8) also noted that the 3 tyrosine residues out of 6 in ribonuclease which ionize at an abnormally high pH, titrate normally after an average of 3 methionine residues have reacted with iodoacetate. They took this to indicate that inactivation occurred because the three-dimensional structure of the protein had been grossly perturbed. An analogous explanation has been given by Vithayathil and Richards (9) to account for their observation that modification of the single methionine residue in the S-peptide fragment of ribonuclease markedly decreases the ability of the peptide to bind to S-protein, but does not affect very much the activity of the complex once it forms.

In the present study, potentiometric measurement of iodide liberation is employed to determine the rate at which methionine residues in ribonuclease are alkylated, and these results are compared with data obtained in a similar fashion with model compounds. It is found that all 4 residues are modified at the same or similar rates. Therefore, the rate at which the capacity to regenerate enzymic activity is lost can be correlated with the disappearance of unmodified ribonuclease and with the formation of alkylated derivatives.

EXPERIMENTAL PROCEDURE

Materials

Bovine pancreatic ribonuclease A was obtained from the Worthington Biochemical Corporation, Lot RAF 6045. The following composition was determined by Dr. A. M. Crestfield: 88% ribonuclease A, 5% aggregates not dissociated by heat, and 4% fast components resolved on sulfopropyl-Sephadex in 0.1 M phosphate (10). Methionine (Merck and Company, Inc.), methionine amide-HCl (Schwarz BioResearch, Inc.), and carbobenzyloxy-methioninamide (a sample synthesized many years ago by standard methods (11)) were analyzed for carbon, hydrogen, and nitrogen. The values found agreed with theory to within 0.2%, except for the carbon analysis of the methioninamide-HCl, which differed by 0.7%. N-Acetylmethionine (California Corporation for Biochemical Research) was recrystallized. Analysis for carbon and hydrogen gave values which were within 0.2% of theory. Iodoacetate acid and iodoacetamide (various commercial preparations) were recrystallized several times from water until colorless, then dried in a vacuum and stored at −20°C. Analyses by the potentiometric method (see below) showed no significant contamination with iodide. Urea was recrystallized according to Benesch, Lardy, and Benesch (12) and stored at 4°C.
Solutions were made immediately before use to minimize the formation of cyanate (13).

**Rates of Alkylation**—Reaction of any functional group with iodoacetic acid or iodoacetamide can be followed conveniently by determining the rate of appearance of iodide ion. In our early experiments, portions of the reaction mixtures were titrated with AgNO₃ in the presence of the fluorescent indicator erythrosin (14). This procedure, designated the titrimetric method, was suitable for studying the rates of alkylation of small molecules but not for studying the alkylation of ribonuclease, since sufficient sensitivity was obtained only at high concentrations of protein, and these interfered with the end point. Nevertheless, some results with ribonuclease were obtained. The potentiometric method of Watts, Rabin, and Crook (15) was found to be more suitable, but only after extensive modification, as described under "Potentiometric Method." Since protons are neither consumed nor liberated in the reactions of iodoacetate and iodoacetamide with thioueter sulfur, no buffer is needed to keep the pH constant. (If a buffer is used, carboxylic acids should be avoided since carboxylate anions react slowly with these alkylation agents.) The pH values of the reaction mixtures were measured with a Radiometer pH meter, model 4; temperature was kept at 40 ± 0.1°.

**Titrimetric Method**—Portions of the reaction mixtures were pipetted into a 10-fold or greater excess of 50% acetic acid. After a few drops of erythrosin had been added, iodide was titrated with 0.01 M AgNO₃ (Reagent Grade, Mallinckrodt). A stock solution of AgNO₃ was stored in the dark and titrated daily against freshly made solutions of KI (Reagent Grade, Mallinckrodt). When very small amounts of iodide were titrated, the end point could be seen only when a portion of standard KI solution was added to provide a more substantial precipitate of AgI upon which the indicator could be adsorbed. Since equal concentrations of the two reactants were used, second order rate constants were calculated from the slopes of linear plots of the reciprocal of concentration against time.

**Potentiometric Method**—The change in iodide concentration in reaction mixtures can be followed without removing samples for analysis by noting the change in potential of a silver-silver iodide electrode immersed in the solution. Our attempts to prepare useful silver electrodes by chemical plating of platinum foil, as suggested by Watts et al. (15), were without success. We therefore prepared them by electroplating, essentially as described by Jones and Hartmann (17). Pieces of No. 25 platinum wire were sealed into short sections of soft glass tubing so that about 0.5 cm of wire was left exposed at one end and a longer piece at the other. The tip of the short end was fused to avoid sharp edges. The surface area of each electrode is about 0.08 cm². Four electrodes were cleaned by anodization in concentrated HNO₃ to avoid some results with ribonuclease. The entire apparatus was carefully grounded.

In a typical experiment, the dependence of potential on the concentration of iodide was determined, both with solutions of KI in KNO₃ and in the complete system without iodoacetate or iodoacetamide. An excellent (±1%) linear relationship was obtained between potential and the logarithm of iodide concentration. The concentrations studied were between 10⁻⁴ and 10⁻² M in both instances. The line for the reaction system is displaced downward by about 3 mV, but the slopes of both lines correspond exactly, within experimental error, to the expression

\[-\Delta \text{mV/log [iodide]} = 62.2\] the value predicted by the Nernst equation for a 10-fold change in iodide concentration at 40°. At constant iodide concentration, the potential of an electrode was constant to within ±2 mV for several days. Tween 80 (0.02%, Nutritional Biochemicals Corporation) was included in the system, as recommended by Watts et al. (15), to prevent coating of the electrodes by protein.

To determine the rate of iodide liberation in the absence of thioueter sulfur, iodoacetate or iodoacetamide was dissolved in 8 M urea and immediately placed in the reaction vessel at 40°. In a typical experiment, the iodide concentration of a 4.5 × 10⁻² M solution of iodoacetic acid at pH 3.5 increased by 1.5 × 10⁻⁴ M per minute, only a small percentage of the rate of iodide liberation during the reaction with protein. All of the data gathered by the potentiometric method have been corrected appropriately. If the titrimetric method is used, this rate of iodide liberation is not detectable. After about 10 minutes, enough readings had been obtained for a good estimate of the velocity of the blank reaction; a portion of a solution in 8 M urea of the compound to be alkylated was then introduced. After a few minutes, readings of the potential against time were begun; such readings could be taken as often as every 30 seconds or as infrequently as every few hours, depending on the velocity of the reaction. The iodide

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1 In a test experiment, iodide was produced at about 10⁻² μmole per minute in a solution which contained 100 μmoles of iodoacetate in 5 ml of 0.2 M sodium acetate buffer, pH 5.7, at 40°. For a discussion of the nucleophilicity of carboxyl groups, see Dawson, Pycock, and Smith (16).

2 The details of this method were worked out in close cooperation with Mr. Thomas W. Schleich, who wishes to acknowledge several very helpful conversations with Dr. Duncan A. MacInnes.

3 This instrument was chosen because of its high input resistance and low grid current. Use of the Radiometer pH meter, model TTTI, as recommended by Watts, Rabin, and Crook (15), resulted in slow polarization of our electrodes because of the applied grid current.
The enzyme were carried out in 0.35 M nucleic acid as substrate, and that the assay and dilutions of enzyme were performed by the titrimetric procedure of Crestfield (Fig. 4 of Reference 18), which simplified evaluation of the rate constants. Under these conditions, a plot of logarithm of thioether sulfur concentration against time was linear (see Fig. 2). A second order rate constant was calculated from the slope (designated S) by the expression

$$k = -2.3S/(A - B)$$

in which $A$ and $B$ are the initial concentrations of iodine- and sulfur-containing compound, respectively.

Rates of Inactivation—Loss of ribonuclease activity was determined with reaction mixtures identical with those used in determining the rates of alkylation. (Both rates were also compared with the amino acid analyses of samples withdrawn from the reaction mixtures; see "Discussion" and Table II.) Portions of the solution containing enzyme and alkylating agent were diluted 5000- to 50,000-fold with 0.35 M NaCl to stop the reaction; dilutions were chosen so that the level of activity that was measured was always the same, within a factor of 2. Each dilution was assayed in duplicate. Activity was determined at 30°C by the titrimetric procedure of Crestfield (Fig. 4 of Reference 18), except that the barium salt of 2,3'-cyclic cytidylic acid (Schwarz BioResearch, Inc., Lot CNC 6303) was used in place of ribonuclease as substrate, and that the assay and dilutions of enzyme were carried out in 0.35 M NaCl rather than in 0.25 M Na$_2$SO$_4$. The concentrations of substrate and enzyme were 1.0 mg per ml and 0.1 to 0.2 $\mu$g per ml, respectively. Chromatography of the substrate on Amberlite XE-119, kindly performed by Dr. A. M. Crestfield, revealed only a single symmetrical peak; no cytidylic acid was present. During an assay, the pH of the unbuffered system drops rapidly from pH 7.5 to about pH 6.5, then more slowly to about pH 5.3 as the cyclic phosphate is hydrolyzed. After 24 hours, the extent of hydrolysis is measured by back-titrination of the sample with dilute NaOH and by comparison of the value obtained with a standard curve.

Amino Acid Analyses—The data summarized in Table I show, in general, that alkylations of thioether sulfur with iodoacetate are from 2 to 3 times faster than those with iodoacetamide and that the rates with both reagents decrease slowly with decreasing pH. Hellström (23), in experiments on the alkylation of several acidic thioethers with iodoacetic acid, has found a similar effect of pH and reports that it is restricted to the acid range. The rate of alkylation of methionine is faster in 8 M urea than in water (compare Experiment 3 with 6, and Experiment 4 with 7) and slowest in 20% ethanol (compare Experiment 4 with 9). Such an increase in rate with increasing concentration was obtained from potentials by the use of the equation referred to above. In all the experiments in which the potentiometric method was used, the concentration of alkylating agent was much greater than that of the substrate alkylated, which simplified evaluation of the rate constants. Under these conditions, a plot of logarithm of thioether sulfur concentration against time was linear (see Fig. 2). A second order rate constant was calculated from the slope (designated S) by the expression

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**Table I**

Rate constants for reaction of methionine, derivatives of methionine, and ribonuclease A with iodoacetate and iodoacetamide at 40°C

The liberation of iodide was followed either titrimetrically (T) or potentiometrically (P), as described in "Experimental Procedure."

<table>
<thead>
<tr>
<th>Experiment and substance alkylated</th>
<th>Alkylation</th>
<th>Solvent</th>
<th>Apparent pH</th>
<th>Method</th>
<th>$k$</th>
<th>$x^{-1} \text{ hr}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Methionine</td>
<td>Iodoacetate</td>
<td>8 M urea</td>
<td>3.5</td>
<td>P</td>
<td>8.9</td>
<td>7.6</td>
</tr>
<tr>
<td>2. Methionine</td>
<td>Iodoacetate</td>
<td>8 M urea</td>
<td>3.7</td>
<td>T</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>3. Methionine</td>
<td>Iodoacetate</td>
<td>8 M urea</td>
<td>5.8</td>
<td>T</td>
<td>12.1</td>
<td></td>
</tr>
<tr>
<td>4. Methionine</td>
<td>Iodoacetamide</td>
<td>8 M urea</td>
<td>4.0</td>
<td>P</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>5. Methionine</td>
<td>Iodoacetamide</td>
<td>8 M urea</td>
<td>7.0</td>
<td>T</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>6. Methionine</td>
<td>Iodoacetate</td>
<td>H$_2$O</td>
<td>6.2</td>
<td>T</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>7. Methionine</td>
<td>Iodoacetamide</td>
<td>H$_2$O</td>
<td>5.3</td>
<td>T</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>8. Methionine</td>
<td>Iodoacetate</td>
<td>20% ethanol</td>
<td>2.4</td>
<td>T</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>9. Methionine</td>
<td>Iodoacetate</td>
<td>20% ethanol</td>
<td>3.6</td>
<td>T</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>10. Carbobenzyloxymethioninamide.</td>
<td>Iodoacetate</td>
<td>20% ethanol</td>
<td>2.7</td>
<td>T</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>11. Carbobenzyloxymethioninamide.</td>
<td>Iodoacetamide</td>
<td>20% ethanol</td>
<td>4.6</td>
<td>T</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>12. N-Acetylhomethionine</td>
<td>Iodoacetate</td>
<td>20% ethanol</td>
<td>2.3</td>
<td>T</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>13. N-Acetylhomethionine</td>
<td>Iodoacetate</td>
<td>8 M urea</td>
<td>5.1</td>
<td>P</td>
<td>20.4</td>
<td></td>
</tr>
<tr>
<td>14. N-Acetylhomethionine</td>
<td>Iodoacetamide</td>
<td>8 M urea</td>
<td>3.5</td>
<td>P</td>
<td>11.8, 13.1</td>
<td>7.3</td>
</tr>
<tr>
<td>15. N-Acetylhomethionine</td>
<td>Iodoacetamide</td>
<td>8 M urea</td>
<td>4.6</td>
<td>P</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>16. N-Acetylhomethionine</td>
<td>Iodoacetamide</td>
<td>8 M urea</td>
<td>4.0</td>
<td>P</td>
<td>6.1, 6.0</td>
<td>5.1, 5.4</td>
</tr>
<tr>
<td>17. Methioninamide</td>
<td>Iodoacetate</td>
<td>8 M urea</td>
<td>3.5</td>
<td>P</td>
<td>1.6, 1.9</td>
<td>15.4, 15.6</td>
</tr>
<tr>
<td>18. Methioninamide</td>
<td>Iodoacetamide</td>
<td>8 M urea</td>
<td>3.5</td>
<td>P</td>
<td>22.9</td>
<td></td>
</tr>
<tr>
<td>19. Ribonuclease A</td>
<td>Iodoacetate</td>
<td>8 M urea</td>
<td>5.8</td>
<td>T</td>
<td>27.2</td>
<td></td>
</tr>
<tr>
<td>20. Ribonuclease A</td>
<td>Iodoacetamide</td>
<td>8 M urea</td>
<td>4.9</td>
<td>P</td>
<td>4.4, 4.3</td>
<td>4.4, 4.3</td>
</tr>
</tbody>
</table>
dielectric constant is to be expected for a reaction in which charged species are generated, since a higher dielectric constant favors their solvation. The thioether sulfur atom of methionine and its derivatives, insulated from the carboxyl and amino functions by two methylene groups, should not be highly susceptible to electronic effects. However, rates of alkylation are faster when the amino group of methionine is acylated, thus eliminating the electron-withdrawing positive charge, as in N-acetylmethionine and carbobenzyloxy-methioninamide. Methioninamide reacts more slowly than methionine (compare Experiment 1 with 17, and Experiment 4 with 18), probably because the positive charge on the amino group is partially neutralized in the dipolar ion. Since the rates of alkylation by iodoacetate of carbobenzyloxy-methioninamide and N-acetylmethionine are essentially the same (Experiments 10 and 12), charge-charge repulsion must play a very minor role under the experimental conditions used.

Alkylation of Ribonuclease—The methionine residues of ribonuclease are alkylated in 8 M urea at rates which are closely comparable to those for model compounds. The difference in rate between iodoacetate and iodoacetamide is somewhat greater for ribonuclease than for N-acetylmethionine (compare Experiment 14 with 19, and Experiment 15 with 22), but similar to that for methioninamide. Rates of alkylation of ribonuclease are shown in Fig. 1, A and B, along with rates of inactivation under the same conditions. Since iodoacetate and iodoacetamide were present in large excess, the alkylation can be shown as a pseudo-first order plot (Fig. 2). The data in Table II show that the extent of alkylation determined by the release of iodide corresponds very closely to that determined by amino acid analysis of the modified protein. It is emphasized that only methionine residues were found to have been modified.

**Properties of Carboxamidomethylmethioninesulfonium Iodide**—A small amount of this compound was prepared so that its mode of decomposition upon heating in acid and its behavior upon treatment with performic acid could be studied. Iodoacetamide was used in place of iodoacetic acid in the method of Gundlach, Moore, and Stein (5), but the product was not isolated. Carboxamidomethylmethioninesulfonium iodide chromatographed as a double peak which partially overlapped the position of ammonia on the 15-cm column of the amino acid analyzer at pH 5.25. In a single experiment, HCl was added to a portion of an aqueous solution of the salt to a final concentration of 6 M, and the mixture was heated at 110°C for 22 hours in an evacuated, sealed tube. Methionine represented 25% of the products, the remainder being accounted for by homoserine, homoserine lactone, and carboxymethylhomocysteine. No methionine sulfone was formed when the salt was treated with performic acid ac-
TABLE II

Determination of sulfonium salt content of methionalkyribonuclease by amino acid analysis and by release of iodide

Samples withdrawn from reactions being followed by potentiometric determination of iodide were diluted with 4 volumes of cold 50% acetic acid and immediately transferred to columns of Sephadex G-25 (1.9 X 35 cm) equilibrated with this solvent. After development of the columns, fractions which contained protein were pooled; hydrolysis was carried out as described in the text. It was assumed that 30% of the carboxymethylsulfonium salt and 25% of the carboxamidomethylsulfonium salt reverted to methionine during the hydrolysis (see the text). As noted previously (8), no evidence was obtained that any amino acid save methionine had been altered, and, except for methionine and derivatives of the sulfonium salts, the relative amounts of amino acids corresponded closely to those found in hydrolysates of ribonuclease A.

<table>
<thead>
<tr>
<th>Alkylating agent</th>
<th>Methionine as sulfonium salt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From iodide determination</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>30</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>38*</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>79</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>84</td>
</tr>
</tbody>
</table>

* The rate of formation of iodide was not followed in this case; the iodide to be expected was calculated from the known rate constant.

Reactivation of Ribonuclease—The lower curves in Fig. 1, A and B, show the rate at which the ability of refolded ribonuclease to catalyze the hydrolysis of cytidine 2',3'-cyclic phosphate at pH 7.5 to 5.3 is lost, under conditions of reaction exactly the same as those used in determining the rates of alkylation shown in the upper curves. The correspondence was checked by hydrolyzing two samples withdrawn from iodoacetate-containing reaction mixtures at 35 minutes, with the results shown in Table II (Line 2). Since the extent of reaction calculated from the rate constant corresponds to that found by amino acid analysis, the two curves in each figure can be compared directly.

DISCUSSION

Since ribonuclease is known to be extensively unfolded in 8 M urea (25-27), the 4 methionine residues might be expected to react at the same or similar rates; alkylation would then follow second order kinetics throughout the entire course of the reaction. The data of Fig. 2 do fall on a straight line, indicative of second order kinetics, if the concentration of methionine residues is taken simply as 4 times the concentration of ribonuclease, determined independently by amino acid analysis.

The most difficult case to distinguish from the one in which all 4 residues react at identical rates is the one in which a single residue reacts at a rate different from the other 3. For example, if a single residue were to react with iodoacetate at one-half or twice the velocity of the other, the point in Fig. 2 that corresponds to 84% reaction would deviate from the straight line by slightly more than 0.03 log unit. Such a deviation is about equal to the limit of experimental error at this point. If 2 residues were to react at twice the rate of the other 2, the deviation from linearity at 84% reaction would be about 0.05 log unit and should be detectable. The deviation would be even greater if 1 residue to react at twice the rate of the others and at 4 times the rate of the 4th.

Second order kinetics would also be followed if the first alkylation of each protein molecule were rate-limiting. In this case, if the protein were unfolded only partially, reaction at the first site might facilitate further unfolding and render other residues of methionine more accessible. However, for the rate constant to be equal to 15 M⁻¹ hour⁻¹ for the over-all reaction with iodoacetate at pH 3.5 (see Table I), the subsequent alkylations would have to occur with constants much larger than 15. By comparison with the rates of alkylation observed with model compounds, this seems to be an unlikely possibility. Furthermore, such a mechanism would require that the reaction mixture contain mainly unreacted ribonuclease and tetraalkyribonuclease, and only very small amounts of the intermediates. At half-reaction, about half of the initial amount of ribonuclease would remain unreacted and, since the tetrasubstituted protein is inactive, the activity would be reduced to half its original value. The experimental data shown in Fig. 1 clearly do not conform to such a possibility. The available evidence strongly suggests, therefore, that the methionine residues of the protein unfolded by 8 M urea react with approximately equal probability. Hence, the amount of each of the 15 possible ribonuclease derivatives modified at methionine residues can be calculated at each stage of the reaction.

The relationship between activity and degree of alkylation is shown in a somewhat different manner in Fig. 3. In Curve I, the fraction of total protein calculated to be present as unreacted ribonuclease is plotted against the amount of methionine that has not reacted; Curve II shows the amount of protein calculated to be monosubstituted. Both curves are constructed on the assumption that all 4 methionine residues have reacted at the same rate. The residual activity is given by the areas (alkylation by iodoacetate) and the open circles (alkylation by iodoacetamide). In addition, data from an earlier paper (8) are shown as filled squares. The two sets of data are very different, presumably because different conditions were used for the measurement of ribonuclease activity. For example, in the present work, the activity was measured over a 24-hour period and, during this time, the pH of the medium decreased gradually from 7.5 to 5.3. The previous measurements, the on the other hand, were performed within a few minutes and at a constant pH of 7.5. The discrepancy between the two sets of points may mean that the activities of the alkylated derivatives decrease more rapidly with decreasing pH than does the activity of unmodified ribonuclease. Such variations between the pH-activity profiles of native and polyalkyribonuclease have been observed by Wellner, Silman, and Sela (28). This question has not been evaluated now be-
A study has been made of the role of methionine residues in maintaining the native conformation of ribonuclease. Since the methionine sulfur atoms are buried in the native protein, alkylation has been carried out in 8 M urea, and the capacity of the modified, unfolded protein to refold to an active enzyme has been investigated after removal of the denaturant. The rates at which the methionine residues of ribonuclease react with iodoacetate and iodoacetamide in 8 M urea at 40° were determined by observing the amount of iodide released during the reaction, with the aid of the silver-silver iodide electrode. The rate constants are similar to those for the alkylation of methionine and several of its derivatives under the same conditions, showing that the methionine residues of ribonuclease have a “normal” reactivity in 8 M urea. The reactions with ribonuclease adhere closely to second order kinetics throughout their course, and the extent of alkylation found by kinetic measurement corresponds closely to that calculated from amino acid analyses of mixtures of the modified proteins. These observations have led to the conclusion that, in 8 M urea at 40°, all of the 4 methionine residues in ribonuclease react at rates which are identical or very similar. Therefore, the complete composition of any reaction mixture can be calculated if the average degree of alkylation is known. Since homogeneous partially alkylated products have not yet been obtained, it is not possible to relate with assurance the activity of ribonuclease derivatives to the extent of alkylation. It seems likely, however, that monosubstituted derivatives will prove to be at least partially active.

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