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# Modification of the Methionine Residues in Ribonuclease*

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To shed further light on the relationship between the enzymic activity and the chemical structure of ribonuclease the methionine residues in the enzyme have been modified by reaction with iodoacetic acid to form the carboxymethylsulfonium derivatives or with hydrogen peroxide to form the sulfoxides. These reactions do not occur at neutrality but take place when the protein is unfolded by urea or by exposure to pH 2–3. Both reactions at pH 2–3 are inhibited markedly by sulfate ions. Chromatographic separation of products with varying degrees of substitution was performed on columns of Amberlite IRC-50. Alkylated derivatives containing more than one carboxymethyl group were inactive. An active monosulfoxide derivative was isolated, but products in which more than one methionine residue had been oxidized were inactive. Inactivation probably results from inability of the altered protein to refold into the native conformation. In addition, methods are described for the determination of the acid-labile carboxymethylsulfonium and methionine sulfoxide residues in a protein. The methods depend upon the facts that sulfonium derivatives are stable to performic acid oxidation and the sulfoxides are stable to alkylation with iodoacetate. Hence, oxidation of alkylated proteins yields the acid-stable methionine sulfone only from unsubstituted methionine residues, whereas exhaustive alkylation followed by oxidation yields sulfone only from methionine sulfoxide residues.

Numerous chemical modifications of ribonuclease have been studied in the past in efforts to learn the nature of the amino acid residues required for the catalytic activity of the enzyme. Relatively few attempts have been made, however, to modify selectively the methionine residues in the molecule, largely owing to a scarcity of suitable reagents. Recently, Gundlach *et al.* (1959a,b) observed that iodoacetic acid reacted readily with methionine to form the carboxymethylsulfonium salt. Although the reaction with the free amino acid was relatively insensitive to pH, alkylation of the methionine residues in ribonuclease, with loss of enzymic activity, was found to occur easily only under acidic conditions (pH 2–3). Apparently, at neutral pH the methionine residues are buried in the interior of the ribonuclease molecule and are unavailable to iodoacetate. Indeed, Stark *et al.* (1961) observed that sulfonium salt formation would occur at neutral pH only if the organized structure of the molecule were first disrupted either by reduction of the disulfide bonds or by exposure to a denaturing agent such as 8 M urea or 4 M guanidinium chloride. From the work of Vithayathil and Richards (1960), the formation of a monosubstituted active derivative might be envisioned. They found that alkylation of the methionine residue in the S-peptide obtained by cleavage with subtilisin decreased greatly the tendency of the S-peptide and the S-protein to combine, but did not alter the enzymic activity of the combination.

Loss of activity following chemical modification of a group in a protein may depend not only upon the group modified but also upon the modification introduced. For this reason, we have explored the possibility, suggested by the work of Toennies and Callan (1939), of using hydrogen peroxide as a selective reagent for the conversion of methionine residues to residues of the sulfoxide. Instances of this conversion are known. Photooxidation of phosphoglucomutase and chymotrypsin in the presence of methylene blue has been found by Ray and Koshland (1960) to transform a methionine residue to the sulfoxide, but alteration of a histidine residue occurred simultaneously. Oxidation of methionine to the sulfoxide by atmospheric oxygen or hydrogen peroxide has recently been shown by Dedman *et al.* (1961) to be responsible for the loss of activity of ACTH earlier observed by Dixon (1956) and by Dedman *et al.*

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(1957). The hormone could be reactivated by reduction with sulfhydryl compounds. Purified parathyroid hormone, which, like ACTH, lacks cysteine or cystine, has been found by Rasmussen (1959) to be reversibly inactivated by oxidation and reactivated by reduction. Ledoux (1954) reported that ribonuclease could be inactivated by air or by hydrogen peroxide and ascribed his results to oxidation of sulfhydryl groups. Since ribonuclease is now known to be devoid of sulfhydryl groups, there must be a different chemical basis for these observations.

Because methionine sulfoxide has been found by Ray and Koshland (1960) to revert to methionine on acid hydrolysis, the usual hydrolytic methods of preparing proteins for amino acid analysis cannot be used prior to the estimation of the sulfoxide content of proteins. Ray and Koshland recommend alkaline hydrolysis prior to amino acid analysis. An alternative method of determining methionine sulfoxide is presented in this communication; the sulfoxide-containing protein is treated with iodoacetate to alkylate the unaltered methionine residues, and the resulting derivative is oxidized with performic acid to convert the sulfoxide to the sulfone. The sulfone is fully stable to acid hydrolysis, and its amount in the hydrolysate provides a measure of the initial sulfoxide content of the protein.

## Experimental

*Ribonuclease Preparations.*—Several different preparations of ribonuclease were used in these studies, including Armour (Lot 381059), Sigma (Lot R 69-52, which was found by chromatography, amino acid analysis, and activity to be similar to the Armour preparation), and ribonuclease A purified from Armour ribonuclease by chromatography on Amberlite IRC-50 and desalted by dialysis or by a modification of the procedure of Dixon (1959).

*Assay of Ribonuclease.*—The activity of ribonuclease was determined with 2′,3′-cyclic cytidylic acid as substrate by a modification of the titrimetric method of Davis and Allen (1955) essentially as described by Gundlach *et al.* (1959a) except that 0.35 M NaCl was used instead of 0.25 M $Na_2SO_4$.

*Reaction of Ribonuclease with Iodoacetate.*—To a solution of ribonuclease (6 mg per ml) was added an equal volume of a solution of 6 mg per ml of iodoacetic acid (The Matheson Co., Inc.). The pH was immediately adjusted to the desired value with N HCl. The mixture was incubated at 40° in a glass-stoppered tube and 10-μl portions were removed periodically for assay of ribonuclease activity. To terminate the reaction, the mixture was poured onto a 0.9 × 5 cm bed of Amberlite IRC-50 (XE-64) which had been equilibrated with 5% (*ca.* 0.9 N) acetic acid. The resin bed was washed with 50 ml of 5% acetic acid, and the adsorbed protein was eluted with 15 ml of 50% acetic acid (1:1 glacial acetic acid–water) as described by Dixon (1959). The 50% acetic acid was removed in a rotary evaporator at 40°. When the reaction mixture was to be chromatographed, it was forced into the column rapidly by air pressure to terminate the reaction, and analyzed by the procedure of Hirs *et al.* (1953). Phenol (0.1%) was incorporated into the eluent as a preservative. Prior to amino acid analysis, samples isolated from columns (*cf.* Fig. 1) were desalted by gel filtration through a column of Sephadex G-25 (Pharmacia Laboratories Inc., Rochester, Minn.) equilibrated with 5% acetic acid. All of the samples (except those from peaks I and III, Fig. 1) were also passed through IRC-50 according to the procedure of Dixon described above.

*Determination of Methioninecarboxymethylsulfonium Residues.*—Since the methioninesulfonium salt is unstable to acid hydrolysis, an indirect method has been devised for its determination. The same approach has been used independently by Vithayathil and Richards (1960), based upon the observation of Gundlach *et al.* (1959b) that the

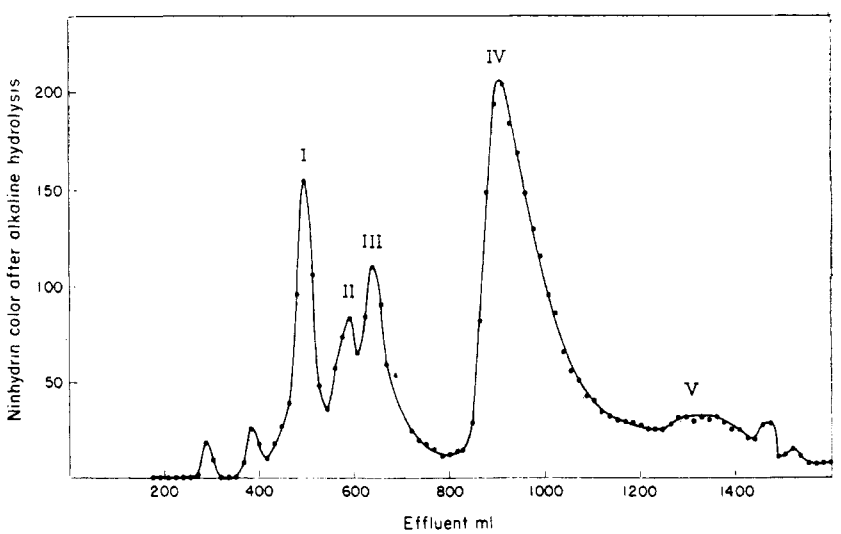


FIG. 1.—Chromatographic separation of the products of the reaction of ribonuclease A with iodoacetic acid. The column of IRC-50 (4.0 × 30 cm) was employed at pH 6.45 under the conditions described by Hirs *et al.* (1953). The total load on the column was 100 mg of protein. Alkylation was carried out at pH 2.7 and 40° for 3 hours.

sulfonium salt is not oxidized by performic acid. The protein sample (about 10 mg in 0.25 ml of 99% formic acid) is oxidized at 0° with performic acid (0.5 ml) by the procedure of Hirs (1956) in order to convert methionine residues to residues of the sulfone. The amount of sulfonium salt is, therefore, equal to the difference between the methionine content of the untreated protein and the methionine sulfone content of the hydrolysate obtained from the carboxymethylated, oxidized derivative.

*Reaction of Ribonuclease with Hydrogen Peroxide.*—Hydrogen peroxide (Merck and Co.) was added to a solution of ribonuclease maintained at 30° and previously adjusted to the desired pH and ionic environment. The final concentration of enzyme was 10 mg per ml; the concentration of oxidant was varied from 0.1 to 1.0 M. To determine ribonuclease activity, 50-μl portions were pipetted into 0.4 ml of 0.1 M acetate buffer at pH 7 which contained 100 μg of catalase (Worthington Biochemical Corp., Freehold, N. J.) per ml. After 5 to 10 minutes, 50-μl portions of this solution were assayed. To terminate the oxidation, the main body of the solution was rapidly brought to pH 6.5-7.0 with 6 N NaOH, and several 5-μl portions of a 1% catalase solution were added at 2-minute intervals until evolution of gas ceased. The total amount of catalase never exceeded 1.5% of the ribonuclease.

Chromatography of the reaction products was carried out according to the method of Hirs *et al.* (1953).

*Determination of Methionine Sulfoxide.*—Alkaline hydrolyses were performed in screw-cap Teflon vials (Microchemical Specialties Co., Berkeley, Calif.) at 110° for 16 hours. A silicone rubber washer was used in the cap to insure a vapor-tight fit. About 5 mg of protein was dissolved in 0.7 ml of water and 0.3 ml of 50% NaOH was added. After hydrolysis, the samples were cooled and acidified (below pH 2.2) with 0.95 ml of 6 N HCl and 0.5 ml of 1.1 M citric acid containing, per 100 ml, 5 ml of thiodiglycol (Pierce Chemical Co., Rockford, Ill.). The solution was diluted to 5 ml with water prior to amino acid analysis.

The indirect determination depends upon the resistance of the sulfoxide residues to alkylation. The protein is exhaustively alkylated by treatment with iodoacetic acid at pH 2 (adjustment made with HCl) overnight, as described above. The residue after removal of the 50% acetic acid was submitted to oxidation by performic acid, as already described, and the hydrolysate analyzed for amino acids. The methionine sulfone content of an acid hydrolysate is equal to the methionine sulfoxide originally present in the molecule.

*Amino Acid Analyses.*—Analyses were performed with columns of IR-120 (Moore *et al.*, 1958) and the automatic recording equipment of Spackman *et al.* (1958). Samples for analysis were hydrolyzed in 6 N HCl at 110° for 22 hours in evacuated, sealed tubes. Oxidized glutathione was determined chromatographically as described by Spackman *et al.* (1960).

The integration constants used for homoserine, the lactone, and carboxymethylhomocysteine were 23.3, 18.9, and 25.4, respectively, determined on an amino acid analyzer for which the factors for the neighboring amino acids, serine, alanine, and histidine, are 27.2, 27.2, and 29.8.

The color yields for homoserine and homoserine lactone·HBr using the manual ninhydrin method (Moore and Stein, 1954) were 0.98 and 0.82, respectively, relative to leucine. Because of the interconversion of homoserine and the lactone (Armstrong, 1949) during chromatography, the recorder constants for these two compounds compensate for small losses on the column. In addition, chromatography of the lactone results in the appearance of another derivative in about 10% yield in the position of glutamic acid. The approximate constant reported was obtained by chromatographing a sample of homoserine lactone·HBr on the 15-cm column of the amino acid analyzer and subtracting from the weight of the initial sample the amount of homoserine observed to be present in a peak from the 150-cm column.

The homoserine (Mann Research Laboratories, New York, N.Y.) was recrystallized from 70% ethyl alcohol. To prepare homoserine lactone·HBr, homoserine was heated under reflux for one hour with 48% hydrobromic acid and the product was isolated as described by Armstrong (1948).

*Anal.* Calcd. for $C_4H_8O_2NBr$: C, 26.4; H, 4.46. Found: C, 26.65, H, 4.55.

## Results

*Determination of Methioninecarboxymethylsulfonium Residues.*—In order to follow the alkylation of methionine residues in ribonuclease by iodoacetate, a method of determining the carboxymethylsulfonium salt is needed. Gundlach *et al.* (1959b) showed that the sulfonium salt decomposed on acid hydrolysis to yield homoserine (and its lactone), S-carboxymethylhomocysteine, and methionine. They also observed that at 100° at pH 6.5 the sulfonium salt of free methionine was desulfurized completely and yielded homoserine (and its lactone) as the only product. Unsuccessful attempts were made to achieve a similarly specific cleavage as the basis of a method for the determination of the sulfonium salt content of iodoacetate-treated ribonuclease. With the alkylated protein, however, methionine, homoserine (and its lactone), and S-carboxymethylhomocysteine were all formed, even when the carboxymethylated protein had first been heated at 100° for an hour at pH 10 in borate buffer, in 8 M urea (pH 8.5 at end of heating period), or in 1.5% Duponol at pH 6.9. Alkaline hydrolysis in 6 N NaOH for 17 hours at 110° also failed to yield homoserine quantitatively.

The indirect method required for the determination of the extent of sulfonium salt formation is outlined in the Experimental section. The effectiveness of the method was demonstrated by analysis of ribonuclease that had been exhaustively alkylated with iodoacetate at pH 2.8; oxidation yielded only 0.17 residue of methionine sulfone, indicating a 96% conversion of the methionine residues to the sulfonium salt.

*Reaction of Ribonuclease with Iodoacetate at pH 2.*—Chromatography of the reaction mixture ob-

tained on 90% inactivation of ribonuclease A by iodoacetate at pH 2 gave the pattern of peaks shown in Figure 1. The material in peak I had the same specific activity as ribonuclease A. The material in the other peaks was inactive. All of these peaks appear relatively early in the reaction. The sum of peaks II and III remains fairly constant, whereas peak I decreases and peak IV, the major product, increases as the reaction progresses.

Analyses of the fractions included in peaks I-V of the chromatogram reproduced in Figure 1 showed that these fractions contained 0.6, 2.3, 1.2, 3.4, and 3.0 residues of sulfonium salt respectively (*cf.* Table I). In addition, Fraction III contained approximately 0.5 residue of carboxymethylhistidine with a corresponding loss in histidine content. The minor components (Fractions II, III, and V) gave amino acid analyses which, besides the changes in the methionine residues, were atypical for ribonuclease A. Apparently contaminants, possibly fragments produced in a side-reaction, were present.

The major product (Fraction IV) at pH 2.7 contains nearly all of its methionine in the alkylated form, a result in agreement with the observations of Stark *et al.* (1961) on the alkylation of ribonuclease at neutral pH in urea solutions.

*Effect of Sulfate Ions.*—It has been found that the rate of inactivation of ribonuclease by iodoacetate at pH 2-3 is extraordinarily sensitive to the presence of sulfate ion. As may be seen in Figure 2, as little as $4 \times 10^{-4}$ M sulfate causes a fivefold decrease in the extent of inactivation of ribonuclease A in 2 hours. This corresponds to a molar ratio of sulfate to ribonuclease of only 1.9.

The sulfate effect was first observed as a puzzling variation in the inactivation rate with different samples of the enzyme. Ribonuclease A, which had been prepared by chromatography on IRC-50 (phosphate buffer as eluent [Hirs *et al.*, 1953]) and desalted by dialysis, was inactivated readily by iodoacetate at pH 2-3, as Gundlach *et al.* (1959a) had reported. Armour and Sigma preparations, on the other hand, were scarcely inactivated at all (10-15%) under the same conditions. The Armour sample (Lot 381059) has been found (Hirs *et al.*, 1956) to contain 0.8% sulfate sulfur, which, from the data given in Figure 2, is sufficient to account for its low reactivity. This preparation of ribonuclease has been shown by Taborsky (1959) to contain a small quantity of nucleotide-like material which also may be inhibitory. The Sigma sample, though it has not been analyzed, probably contains inorganic sulfate, since its inactivation rate is enormously increased (to 80% in 80 minutes) by passage through Dowex-1 in the acetate form, a procedure which would replace all anions by acetate.

The inhibitory effect of sulfate upon the iodoacetate reaction probably is a function of the dianionic character of the sulfate ion. Several observations support this contention. In the first place, the extent of inactivation of sulfate-containing preparations of Armour ribonuclease increases about fourfold as the reaction medium is acidified from pH 2.9 to pH 2.2. At the lower pH there is an appreciable conversion to the monovalent bisulfate ion. A similar pH sensitivity is not noted with sulfate-free ribonuclease A. Phosphate, with which ribonuclease A undoubtedly is slightly contaminated, is a monovalent anion below pH 3 and would not be so inhibitory. In the second place, as can be seen in Figure 3, the extent of inactivation of the sulfate-containing Armour preparation can be more than doubled by the addition of a small amount of chloride ion, although the reactivity toward iodoacetate still is far less than that of ribonuclease A. Chloride also partially reverses the inhibitory effect of sulfate upon the alkylation of ribonuclease A. Chloride ion alone, however, somewhat inhibits the alkylation of ribonuclease A.

In contrast to iodoacetic acid, iodoacetamide reacts very slowly with ribonuclease at pH 2-3.

*Reaction of Ribonuclease with Hydrogen Peroxide.*—The rate of inactivation of ribonuclease (Armour) by hydrogen peroxide increases markedly with decreasing pH. At pH 1, even in the presence of a small amount of sulfate, inactivation by 0.3 M $H_2O_2$ is complete in 30 minutes. At pH 2.1 inactivation in 30 minutes is 10% at 0.1 M $H_2O_2$, 30% at 0.3 M $H_2O_2$, and 65% at 1 M $H_2O_2$. At pH 4.7,

TABLE I

DETERMINATION OF THE METHIONINECARBOXYMETHYLSULFONIUM CONTENT OF SAMPLES OF ALKYLATED RIBONUCLEASE

Samples isolated from the chromatogram shown in Figure 1 were used. Complete amino acid analyses were carried out in each case. Conversion of cystine to cysteic acid and some loss of tyrosine occurred on performic acid oxidation. The molar ratios given are calculated by assuming that the average of the μmoles of glutamic acid and alanine found is equal to 12.0 residues, in accordance with the composition of ribonuclease A.

| | Amino Acid Residues per Molecule | | | | | |
|---|---|---|---|---|---|---|
| Ribonuclease Sample | Methionine | Methionine Sulfone | Homoserine and Lactone | S-Carboxymethyl Homocysteine | Total | Sulfonium Salt (Calcd.) |
| Fraction I | 3.46 | 0 | 0.22 | 0.23 | 3.9 | 0.6[a] |
| Fraction II | 2.06 | 0 | 0.69 | 1.09 | 3.8 | 2.5[a] |
| Fraction II after performic acid oxidation | 0.67 | 1.67 | 1.00 | 0.86 | 4.2 | 2.3[b] |
| Fraction III | 3.12 | 0 | 0.30 | 0.49 | 3.9 | 1.1[a] |
| Fraction III after performic acid oxidation | 0.43 | 2.76 | Trace | Trace | >3.2 | 1.2[b] |
| Fraction IV | 1.36 | 0 | 0.90 | 1.63 | 3.9 | 3.6[a] |
| Fraction IV after performic acid oxidation | 0.99 | 0.62 | 0.95 | 1.76 | 4.3 | 3.4[b] |
| Fraction V after performic acid oxidation | 0.77 | 1.00 | 1.10 | 1.23 | 4.1 | 3.0[b] |

[a] Estimated from the quantity of decomposition products by the procedure of Stark *et al.* (1961). [b] Calculated on the basis of 4.0 residues of methionine per molecule.

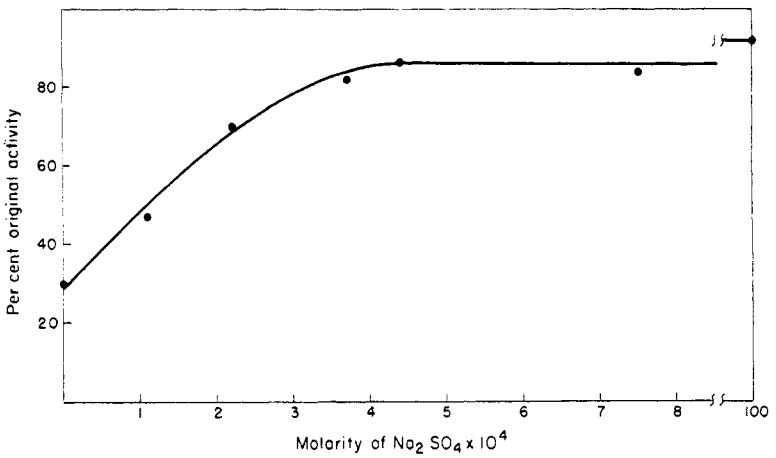


FIG. 2.—The effect of sulfate on the inactivation of ribonuclease A by iodoacetic acid at 40° and pH 2.7.

on the other hand, even 1 M $H_2O_2$ causes only 10% inactivation in 30 minutes, and at pH 6.6 no inactivation is brought about in 30 minutes by 0.3 M $H_2O_2$ in 0.01 M phosphate buffer.

The rate of inactivation by peroxide at pH 2, like the alkylation by iodoacetate, is slower in the presence of the divalent sulfate ion than in the presence of the monovalent perchlorate ion. A molar ratio of sulfate to ribonuclease A of 2.7 causes a threefold decrease in the extent of inactivation by 0.1 M $H_2O_2$ in 30 minutes. Chloride could not be studied, since in acidic peroxide it is of course oxidized to chlorine, which can cause side-reactions, notably with tyrosine and cystine residues.

When a sample of ribonuclease (Sigma) 50% inactivated by $H_2O_2$ at pH 2 was chromatographed on a column of Amberlite IRC-50, the result shown in Figure 4 was obtained. The largest peak, labeled I, had a specific activity which was similar to that of ribonuclease A and was in the position normally occupied by the unmodified enzyme. A small, poorly resolved peak (II) and a larger zone (peak III) contained no activity. Rechromatography of the material in peak I gave a single sharp peak inseparable upon mixed chromatography from ribonuclease A.

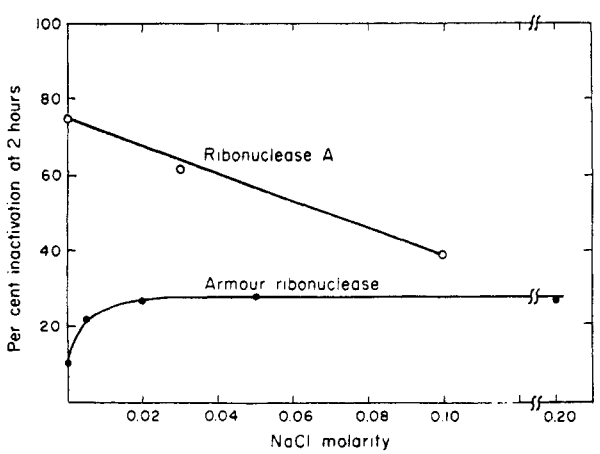


FIG. 3.—The effect of chloride upon the inactivation of ribonuclease by iodoacetic acid at 40° and pH 2.8.

Amino acid analyses of the material isolated from peaks I, II, and III (Fig. 4) and desalted by dialysis yielded the results given in Table II. The methionine sulfoxide content of the three samples was determined both after alkaline hydrolysis and by the indirect method. Ribonuclease A, when similarly analyzed, contains only a trace of methionine sulfoxide. The two methods gave roughly concordant values (comparing methionine sulfoxide in the alkaline hydrolysate and methionine sulfone from the alkylated-oxidized samples) for the peak I and peak II materials, which had only one and two methionine sulfoxide residues per molecule respectively, but the indirect procedure yielded appreciably higher results with the material from peak III, which had nearly four methionine sulfoxide residues. Ray and Koshland (1960) have reported that pure methionine sulfoxide is quantitatively recovered after alkaline hydrolysis, but we have experienced losses of from 10 to 25%. Alkaline hydrolysis also causes a complete disappearance of cystine, appreciable losses of aspartic acid, and severe losses of serine and threonine; a considerable portion of the last two compounds appears in the hydrolysate as glycine and alanine. Arginine is converted to ornithine. Tyrosine, on the other hand, appears to be more stable to alkaline than to acid hydrolysis. Losses of tyrosine on acid hydrolysis are particularly large when the protein contains methionine sulfoxide or, as has been noted earlier (Sanger and Tuppy, 1951), after performic acid oxidation. Much of the tyrosine that has disappeared may be accounted for as chlorotyrosine. The presence of methionine sulfoxide residues in the protein also causes the disappearance of some cystine, only part of which is recovered as cysteic acid. Both of these effects may be noted by comparing the half-cystine and tyrosine values, given in Table II, for the material obtained from peaks I, II, and III.

A demonstration that losses of cystine and tyrosine on acid hydrolysis are proportional to the quantity of methionine sulfoxide present is provided by the data in Figure 5, which were obtained by hydrolyzing samples of ribonuclease A in the presence of increasing amounts of methionine sulfoxide. During hydrolysis the sulfoxide is reduced, with the

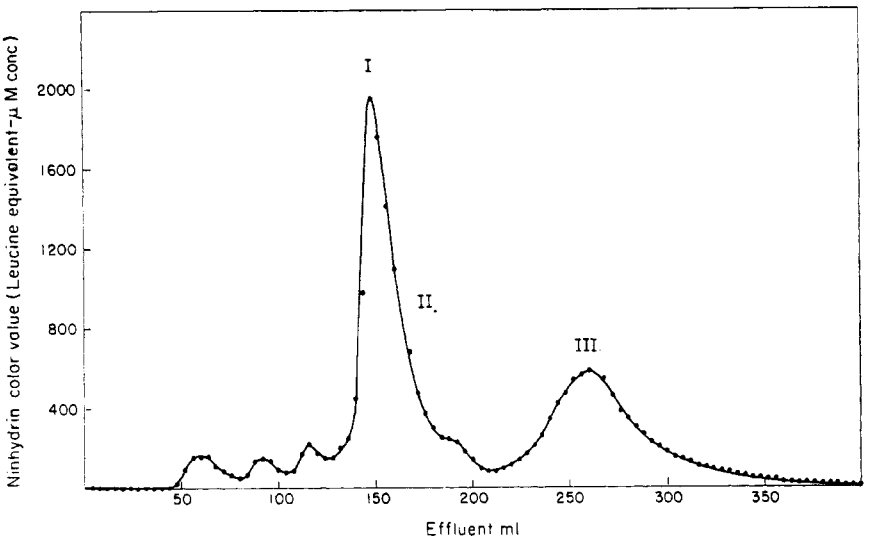


FIG. 4.—Chromatographic separation of the products of the reaction of ribonuclease (Sigma) with $H_2O_2$. The column of IRC-50 (2.0 × 60 cm) was employed at pH 6.47 under the conditions described by Hirs *et al.* (1953). The ribonuclease (200 mg) was treated for 50 minutes at 30° with 0.1 M $H_2O_2$ at pH 2.1 (pH adjusted by addition of $HClO_4$).

probable formation of chlorine from the HCl, which in turn oxidizes part of the cystine and chlorinates some of the tyrosine. Material with the chromatographic behavior of chlorotyrosine (Hirs, 1956) is found in such hydrolysates.

The cystine and cysteic acid values obtained on acid hydrolysis of the material from peak I and peak III are about what might be expected from the methionine sulfoxide content when allowance is made for the decomposition of cystine (up to 20%) that is often observed to occur on acid hydrolysis of unoxidized ribonuclease (Hirs *et al.*, 1954). The tyrosine figures, however, are lower than would be expected from the data in Figure 5.

Inactivation of ribonuclease by hydrogen peroxide at pH 6.5 yielded a markedly heterogeneous reaction mixture, most of the components of which moved more rapidly than ribonuclease A on columns of IRC-50.

*Effect of Hydrogen Peroxide upon Methionine, Cystine, Oxidized Glutathione, and Tryptophan.*—As a background for the use of hydrogen peroxide to modify proteins, the effect of this reagent upon several simple compounds was studied briefly with the aid of the amino acid analyzer. Under the conditions (pH 2) used for the oxidation of ribonuclease, methionine was converted quantitatively to the sulfoxide. Although cystine residues in ribonuclease appear to be unaffected, free cystine was decomposed completely, only 20% being accounted for as cysteic acid. Oxidized glutathione, on the other hand, appeared to be unaffected by hydrogen peroxide. Tryptophan also exhibited no detectable change in chromatographic behavior under the oxidizing conditions used and was recovered quantitatively.

*Methionine Sulfoxide Content of Several Proteins.*—If methionine sulfoxide residues were present in proteins, they would not have been detected by the usual analytical procedures, almost all of which require preliminary acid hydrolysis. Because free methionine is so easily converted to the sulfoxide, it seemed possible that protein preparations might contain appreciable amounts of this oxidation prod-

TABLE II

AMINO ACID ANALYSES OF SAMPLES OF RIBONUCLEASE ISOLATED CHROMATOGRAPHICALLY AFTER REACTION WITH HYDROGEN PEROXIDE

The samples were isolated from the chromatogram shown in Figure 4 and desalted by dialysis. Complete amino acid analyses were done in each case. Except for the values reported, no differences in consumption were observed between the materials from Peaks I, II, and III. None of the figures is corrected for losses during hydrolysis.

| Sample (*cf.* Fig. 4) | Method of Hydrolysis | Number of Amino Acid Residues per Molecule | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Me | MeSO | $MeSO_2$ | Half-Cys | $CySO_3H$ | Tyr | Cl-Tyr |
| Peak I | Acid | 3.6 | <0.1 | <0.1 | 6.5 | 0.1 | 5.0 | 0.3 |
| Peak I | Alkali | 2.1 | 0.9 | <0.1 | <0.1 | <0.1 | 5.4 | |
| Peak I (fully alkylated, and performic acid oxid.) | Acid | 0.7[a] | <0.1 | 0.8 | <0.1 | 7.6 | <0.1 | |
| Peak II | Acid | 3.7 | <0.1 | <0.1 | Lost | 0.3 | 4.4 | 0.3 |
| Peak II | Alkali | 1.5 | 1.8 | <0.1 | <0.1 | <0.1 | 5.3 | |
| Peak II (fully alkylated, and performic acid oxid.) | Acid | 0.4[a] | <0.1 | 1.9 | <0.1 | 8.0 | 2.4 | 2.5 |
| Peak III | Acid | 3.5 | <0.1 | <0.1 | 5.6 | 0.4 | 3.8 | Lost |
| Peak III | Alkali | <0.1 | 2.7 | <0.1 | <0.1 | <0.1 | 5.3 | |
| Peak III (fully alkylated, and performic acid oxid.) | Acid | 0.1[a] | <0.1 | 3.6 | <0.1 | 9.8 | 1.3 | 4.4 |

[a] The quantities of methionine obtained after alkylation and performic acid oxidation are derived exclusively from S-carboxymethylsulfonium salt residues and have not been corrected for losses owing to formation of homoserine and its lactone and S-carboxymethylhomocysteine during acid hydrolysis.

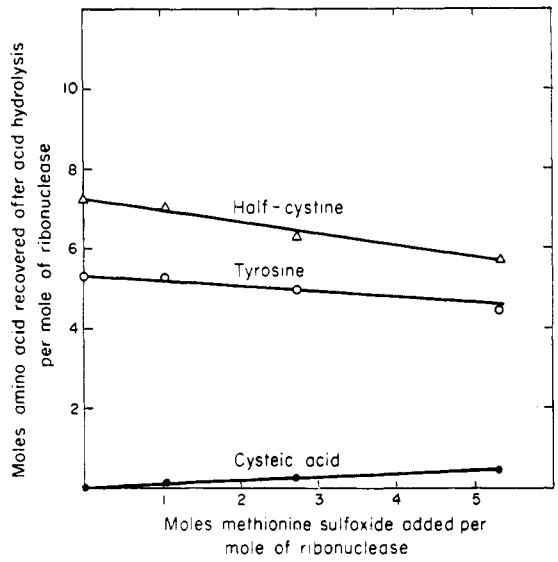


FIG. 5.—Effect of the presence of added methionine sulfoxide during acid hydrolysis of ribonuclease upon the subsequent amino acid analysis.

uct. However, examination of alkaline hydrolysates of the following preparations failed to reveal more than a trace of methionine sulfoxide: lysozyme (Armour and Co., Chicago, Ill.), trypsinogen, pepsin, catalase, and chymotrypsinogen (Worthington Biochemical Corp.), bovine serum albumin (Pentex Inc., Kankakee, Ill.), β-lactoglobulin (preparation analyzed earlier [Stein and Moore, 1949]), and a proteinase isolated from a strain of group A *Streptococcus* (Elliott, 1950).

## DISCUSSION

The relative unavailability of the methionine residues in ribonuclease to anionic iodoacetate or neutral peroxide, except under conditions known to cause unfolding of the molecule, indicates that in the native, active enzyme methionine residues probably are not present in a portion of the protein which is available to the much larger substrate molecules, cyclic cytidylic acid or RNA.

If unfolding of the molecule is necessary to render the methionine residues reactive, the striking reduction in the reactivity of these residues toward both an anionic and a neutral reagent which is observed in the presence of sulfate ion is most logically interpreted as an inhibition of the unfolding process by the divalent anion. Probably the sulfate ion is bound to two positively charged groups in the enzyme and acts as a bridge or tie to prevent unfolding of the molecule and unmasking of the methionine residues. Ribonuclease is known to have a marked affinity for polyvalent anions (Crestfield and Allen, 1954), and the ability of such anions to prevent the unfolding action of urea has already been noted by Sela *et al.* (1957). The ability of monovalent chloride ions partially to counteract the effect of sulfate may be explained by a competition for the cationic sites in the molecule.

If substitution at the methionine residues is possible only after unfolding of the peptide chain, the inhibitory effects upon enzymic activity of such substitution may probably be ascribed to an inability of the modified chain to refold into the conformation required for activity. It might be expected that the extent of this inhibitory effect would depend somewhat upon which of the four methionine residues is modified as well as upon the nature of the modification introduced.

The results in Figure 1 and Table II show that the principal product of alkylation is an inactive derivative containing three to four modified methionine residues. Thus, as observed by Stark *et al.* (1961), once the alkylation starts it tends to go to completion. But there is a measurable amount (0.6 residue per molecule) of the sulfonium salt of methionine in the active Fraction I. Therefore, the possibility remains that there is formed a small amount of a monocarboxymethyl derivative that is active and that is eluted at the ribonuclease A position from IRC-50 columns.

Oxidation with hydrogen peroxide has yielded evidence for the presence of at least one active derivative which contains one methionine sulfoxide residue per molecule. The homogeneity of the active material in peak I, Figure 4, has not been demonstrated unequivocally; the amino acid analyses suggest, however, that oxidation of methionine sulfur is the sole chemical modification that has been brought about.

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# Spectrometric Evaluation of the Approximate pK of the Carboxyl Group in 2,4-Dinitrophenyl-Amino Acids

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The absorption at 360 mμ shown by 2,4-dinitrophenyl-amino acids in aqueous solution is very sensitive to changes in hydrogen ion concentration in the pH range 1 to 5. Twenty-one 2,4-dinitrophenyl derivatives have been examined for changes in absorbancy at 360 mμ at various hydrogen ion concentrations, and the approximate pK of the carboxyl group in many of these compounds has been evaluated from a curve relating absorbancy to pH. The effect of ionization of the carboxyl on the contribution to absorbancy at 360 mμ by the chromophore is highly dependent on the distance of the carbon carrying the chromophore system from the carboxyl group. When this distance exceeds three carbons, carboxyl ionization has little effect on absorbancy. The observed changes in spectra would be consistent with resonance stabilization of the anion.

The determination of 2,4-dinitrophenyl(DNP)-amino acids is generally done by the measurement of their absorbancy in solution in glacial acetic acid or aqueous sodium bicarbonate at 340 and 360 mμ respectively (Fraenkel-Conrat *et al.*, 1954), or by measurement of the absorbancy in the visible region of the spectrum after reduction of the compounds with sodium borohydride in aqueous sodium bicarbonate (Ramachandran, 1961). Molar absorbancy values of DNP-amino acids in acetic acid or acid solutions are somewhat lower than the values obtained for solutions in aqueous bicarbonate, and the peak found at 360 mμ in alkaline solutions is shifted to a lower wave length of 340–350 mμ in acid solutions. We present in this communication data on the dependence of the absorbancy of the compounds on the hydrogen ion concentration of the medium, pointing to the possibility of evaluation of the approximate pK values of the carboxyl groups from a curve relating absorbancy to pH. Potentiometric determination of the pK of the carboxyls is difficult owing to the very low solubility of most DNP-amino acids in water; in such cases spectrometric methods are useful if the spectrum changes with changes in hydrogen ion concentration of the solution (Gillam and Stern, 1954).

## EXPERIMENTAL AND RESULTS

*2,4-Dinitrophenyl Derivatives.*—DNP derivatives of glycine, DL-valine, L-phenylalanine, L-isoleucine, L-tryptophan, L-alanine, L-arginine, L-aspartic acid, L-asparagine, DL-glutamic acid, L-proline, β-alanine, DL-isoserine, L-lysine (ε-DNP), DL-ornithine (δ-DNP), aminoethanol, α-, β-, and γ-aminobutyric acids, and α- and β-aminoisobutyric acids were prepared with 1-fluoro-2,4-dinitrobenzene (Sanger, 1945) and had physical constants in agreement with those recorded in the literature (Fraenkel-Conrat *et al.*, 1954; Rao and Sober, 1954; Green and Kay, 1952). DNP-β-aminobutyric acid, DNP-DL-α-aminobutyric acid, DNP-β-aminoisobutyric acid, and DNP-DL-isoserine had melting points of 166–8°, 190°, 154°, and 145–8° respectively.

*Spectrum of DNP-Arginine.*—Figure 1 shows the spectrum of DNP-arginine in aqueous solution at pH 5.8 and 2.0. At the lower pH the peak at 360 mμ found in solutions at pH 5.8 is shifted to the lower wave length of 350 mμ, and the absorbancy is lower. The trough at 300 mμ is likewise shifted to the lower wave length of 297 mμ at the more acid pH. Gradual changes in pH from 5 to 2 resulted in a family of curves which also shifted gradually from A to B, and the common isobestic point was found close to 348 mμ.

*Spectrometric Determinations of pK.*—The DNP-amino acids were dissolved in a series of buffers (to give molar concentrations in the range $2 \times 10^{-5}$ to $10^{-4}$) whose pH decreased from the alkaline side down in steps to acid pH values where the change in spectrum ceased—namely, until the lowest absorbancy at 360 mμ had been reached. Buffer solutions of known pH were made by using hydrochloric acid, sodium acetate, acetic acid, sodium bicarbonate, and sodium carbonate. Buffering constituents were usually present in a concentration of 0.2

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