# PARTIALLY HYDROLYZED RIBONUCLEASE WITH ENZYMIC ACTIVITY*

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## INTRODUCTION

A direct attack on the role of the protein in enzyme-catalyzed reactions has been made in several laboratories during the past few years. That the "active center" of biologically active proteins may be comprised of several amino acid residues held together in the correct spatial configuration was suggested by evidence that the entire protein is not required for the activity of several enzymes and hormones.

In previous communications[1,2] some of the amino acid residues not required for the activity of RNase (ribonuclease) were determined. It was demonstrated that about 15% of the residues could be removed from the protein molecules without any loss of enzymic activity[2]. Results from other laboratories have indicated that enzymically active dialyzable fragments were formed during the autodigestion of pepsin[3] and that chymotrypsin[4] and lysozyme[5], when divested of at least their C-terminal end groups by digestion with a carboxypeptidase preparation, maintained their full enzymic activity. Partial digestion of RNase by subtilisin also yielded a fully active enzyme[6]. Mercuri-papain could lose up to 120 (of its 180) amino acid residues from the N-terminal end by the action of an amino peptidase preparation. After the removal of the mercury, there appeared to be no loss of activity[7]. Chymotrypsinogen, degraded by either chymotrypsin, carboxypeptidase or subtilisin, can be activated by trypsin[7a]. There was no loss of activity of the hypophysial growth hormone after 30% hydrolysis by chymotrypsin[8].

The purposes of this work are: (1) to show that partial inactivation of the enzyme corresponds to quantitative changes in the elution pattern of the enzyme protein from Amberlite IRC-50, and (2) to correlate the liberation of amino acids with the loss of enzymic activity to obtain a better estimate of the maximum size of the active moiety. From these data, and from information available as to the partial structural formula of oxidized RNase, it is estimated that the major portion of the RNase molecule is not essential for its catalytic activity.

## EXPERIMENTAL

### *Methods and materials*

The crystalline RNase, purchased from Armour and Company, has been described previously[2]. The RNase assay was essentially that of ANFINSEN *et al.*[9]. Four ml of 0.2 *M* acetate buffer, pH 5.0 (containing the RNase) was incubated with 1.0 ml of 1% sodium ribonucleate for 9 or 25 minutes

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at 30°C. The reaction was stopped by the addition of 1.0 ml of 0.75% uranyl acetate in 25% perchloric acid. After stirring and centrifuging the precipitate, a 0.10 ml aliquot of the supernatant was added to 3.0 ml of distilled water. The optical density of this solution was measured at 260 mμ using a Beckman DU spectrophotometer. A reagent blank and four standards were run with every assay. The activity was proportional to the optical density for the 9-minute incubation and proportional to a logarithmic function of the optical density for the 25-minute assay.

Carboxypeptidase (CPase) recrystallized 5 times was purchased from Pentex Incorporated. The enzyme was washed with water several times and suspended in 5% lithium chloride before use[10]. The CPase was not treated with di*iso*propylfluorophosphate. Digests were carried out by adding washed CPase to RNase in 5% lithium chloride solutions, adjusting the pH to 7.9–8.1, and incubating with gently stirring for various periods of time. In experiments 1 through 8 (except experiment 7), the final concentration of RNase was 21 mg per ml or 1.5 μmoles per ml. In experiment 7, the RNase concentration was 84 mg or 6 μmoles per ml. The molar ratios of RNase/CPase employed were as follows: Experiment 1, 7.8:1; experiments 2, 5 and 8, 3.6:1; experiments 3 and 4, 4.5:1; experiment 6, 15.6:1; experiment 7, 60:1. The molecular weight of RNase was taken as 14,000[11] and that of CPase as 34,400[12,18]. The time of digestion was as follows: experiment 1 (Table I), 43.5 hours; experiments 2, and 8 and experiment 5 (Table II), 20 hours; experiment 3, 12 hours; other experiments as indicated. Protein determinations were made by the method of LOWRY *et al.*[13]. Amino acids were determined quantitatively by paper chromatography[14] and by ion exchange[15]. In addition, histidine was identified by treatment with a freshly diazotized *p*-bromoaniline solution followed by spraying with half-saturated $Na_2CO_3$ to develop the characteristic red color[16]. RNase was chromatographed on Amberlite IRC-50 (XE-64)* according to the method of HIRS *et al.*[17].

To check the possibility of microbial contamination, aliquots of various digestion mixtures were streaked out on blood agar plates. After 48 hours of aerobic and anaerobic incubation, there was no evidence of contamination in any of these experiments.

### *Elution patterns from Amberlite IRC-50 (XE-64)*

The elution pattern of digested but fully active RNase was virtually the same from IRC-50 as the elution pattern of undigested RNase (Fig. 1). When digestion had proceeded to such an extent that partial inactivation of the RNase activity was achieved, however, there was a significant change in the elution pattern (Fig. 2). The relative position of the two enzyme peaks was found to be the same as in Fig. 1 and the enzyme content of the first peak to be eluted was still about 10% of the total activity recovered. The protein peaks, however, were markedly changed (Fig. 2). The first peak to be eluted in this experiment contained more protein than the second.

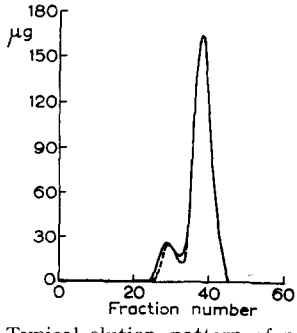


Fig. 1. Typical elution pattern of native and active, digested ribonuclease. Solid line, ribonuclease activity, 102% recovered; dashed line, protein, 93% recovered.

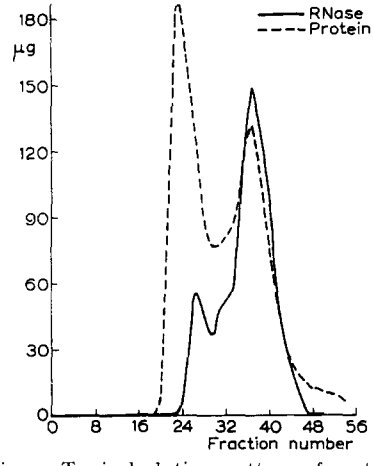


Fig. 2. Typical elution pattern of partially inactivated ribonuclease. Solid line represents the ribonuclease activity; dashed line represents protein. Conditions as in Table I, experiment 3.

* We are greatly indebted to Rohm and Haas Company for furnishing the Amberlite IRC-50 (XE-64).

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Data are presented in Table I to show the quantitative changes which have occurred in the elution patterns of partially inactivated RNase. The 1050 $\mu$g control is a representative example indicating the good recovery of undigested enzyme with about 10% of it eluted in the first peak and the remainder in the second. Digests 1, 2 and 3 were typical experiments illustrating the correlation between inactivation measured as decrease of RNase activity and shift of protein from the second peak to be eluted to the first. Since all of the RNase molecules exhibited the characteristic elution pattern of Fig. 1, whether or not they were digested, 10% of the total protein should have been eluted in the first peak, in the form of active RNase. The remaining protein in that peak should have been inactive RNase fragments. The per cent of the total which this inactive protein represented was considered the per cent protein shifted. If this protein shift were due to the different mobility of inactivated RNase, the protein shift should be equivalent to the per cent inactivation measured by enzyme assay. It may be seen in Table I that there was a good correlation between protein shift and per cent inactivation measured by the 9-minute assay.

TABLE I

CORRELATION OF % INACTIVATION WITH CHANGE IN ELUTION PATTERN

| | *Control* | *Expt. 1* | *Expt. 2* | *Expt. 3* |
|---|---|---|---|---|
| Protein applied to column, $\mu$g | 1050 | 1050 | 1050 | 2070 |
| Protein recovered: | 93% | 91% | 122% | 123% |
| First peak eluted, $\mu$g | 117 | 410 | 757 | 1232 |
| Second peak eluted, $\mu$g | 860 | 550 | 528 | 1305 |
| RNase recovered: | 102% | 50%* | 41%* | 66%** |
| First peak eluted, $\mu$g | 156 | 53 | 51 | 228 |
| Second peak eluted, $\mu$g | 912 | 474 | 436 | 1134 |
| % Inactivation: | | | | |
| 25-minute assay | | 58 | 63 | — |
| 9-minute assy | | 43 | 47 | 40 |
| Protein shift (calculated) | | 33 | 49 | 43 |

* 25-minute assay ** 9-minute assy

*Amino acid liberation*

Since the intact RNase molecule was found not to be essential for catalytic activity[1,2], experimental conditions were adjusted so that graded amounts of inactivation could be obtained with the liberation of significant quantities of amino acids. Fig. 3 shows the effect of increasing the time of digestion and relative concentration of CPase or RNase activity. At a molar ratio of 60:1 (RNase: CPase) no inactivation occurred after 34 hours of incubation although significant amounts of amino acids were liberated. No inactivation occurred before 8 hours of incubation at a ratio of 16:1. On longer digestion, however, activity was gradually lost. The 8-hour lag period was shortened by gradually increasing the relative concentration of CPase until inactivation was obtained during the first hour of incubation with 4 moles of RNase per mole of CPase in the digestion mixture (Digest 5, Fig. 3).

The possibility of interference from peptides in determining the amino acids

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TABLE II

CORRELATION OF DATA OBTAINED BY ION EXCHANGE AND PAPER CHROMATOGRAPHY

| | *Expt. 8* | *Expt. 5* |
|---|---|---|
| *Method:* | *Ion-exchange* | *Paper* |
| *Amino acid* | *Moles of amino acid/mole of RNase* | |
| Valine | 2.2 | 2.4 |
| Leucine | 0.6 | 1.0 |
| Phenylalanine | 0.5 | 0.7 |
| Alanine | 4.7* | 5.3 |
| Isoleucine | Ca. 2 | 2.0 |
| Histidine | 2.0 | 2.6 |
| Aspartic acid | 1.0** | 1.0 |
| Tyrosine | 0.7 | 2.4 |
| Methionine | 0.7 | 1.7 |
| Glutamic acid | 0.2** | glu and ser |
| Arginine | — | glu and asp§ |
| Lysine | 1.1 | lys and glu |
| Serine | 2.6 | ser*** |
| Glycine | * | *** |
| Threonine | 1.8 | *** |
| Factor to convert primary data to that reported: | 2 | 400 |

* Glycine and alanine were found in one peak.
** In addition, 1.0 mole of (glutamine and asparagine)/mole of RNase were found.
*** Determined on one strip, these "spots" consisted of one long spot.
§ No arginine was found.

liberated was eliminated in two ways. First, quantitative data were checked with an independent method (chromatography on Dowex-50). A digestion mixture which had been about 50% inactivated was chromatographed on an ion-exchange resin in addition to paper chromatography (Table II). The values for valine, leucine, phenylalanine, alanine, isoleucine, aspartic acid and histidine corresponded to the values obtained with paper chromatography. The other resin values were somewhat lower. In addition, ninhydrin-positive material was eluted between the prominent amino acid peaks. These lesser peaks have been noticed by other workers during the chromatography of acid hydrolysates of proteins[15,18,19] and attributed to peptides. Secondly, unstained sections of the paper chromatograms of digests which corresponded to ninhydrin-positive spots corresponding to known amino acids were eluted. The eluates were hydrolyzed and rechromatographed to determine the possible presence of peptides. All of the amino acid spots (Table II) were found to contain only one amino acid, with the following exceptions: equivalent amounts of lysine and glutamic acid were found in the "lysine" spot; only aspartic acid and glutamic acid were found in the "arginine" spot; and the "glycine" and "threonine" spots contained traces of other ninhydrin-staining substances. The demonstration of the presence of peptides in these experiments is further evidence for the occurrence of a proteolytic impurity in the CPase preparations[20,21].

Knowledge of the kinds and amounts of amino acids liberated during partial inactivation of RNase may provide information about those residues associated with

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TABLE III

AMINO ACIDS LIBERATED WITH PARTIAL LOSS OF ENZYMIC ACTIVITY

| *Amino acid* | *Moles of amino acid/mole of RNase* | | | | | |
|---|---|---|---|---|---|---|
| | *Experiment* 6 | 1 | 6 | 1 | 1 | 1 |
| Valine (9)* | 1.0 | 1.1 | 1.3 | 1.5 | 2.7 | 3.2 |
| Alanine (12) | 3.9 | 1.5 | 3.7 | 1.7 | 4.3 | 3.1 |
| Aspartic acid (15) | 0.8 | 0.5 | 1.3 | 0.7 | 1.1 | 0.9 |
| Phenylalanine (3) | 0.6 | 0.7 | 0.8 | 0.8 | 1.2 | 1.3 |
| Leucine (2) | 0.6 | 0.5 | 0.6 | 1.1 | 1.5 | 1.5 |
| Isoleucine (3) | 1.3 | 0.5 | 1.3 | 0.9 | 2.0 | 1.7 |
| Histidine (4) | 0.9 | 1.2 | 1.3 | 2.1 | 4.1 | 3.9 |
| % Inactivation | 6 | 14 | 21 | 29 | 30 | 33 |
| Hours of digestion | 18 | 12 | 32.5 | 18 | 36.5 | 30 |

* These figures indicate the number of residues of each amino acid in the RNase molecule[11,22].

the "active center" of the enzyme. The data in Table III include only those amino acids for which comparable values were obtained by the two different methods and which could be determined without the interference of peptides or corrections for the autolysis of the CPase preparation. (In control experiments, no amino acids were liberated from either RNase or CPase, incubated separately, under conditions reported previously[2] or under conditions described in Table III. However, with very high concentrations of CPase, such as in experiment 5, Table II, only traces of methionine, leucine, isoleucine and phenylalanine were detected from control strips containing CPase alone.) It was found that 3 of the 9 moles of valine, 3 to 4 of the 12 moles of alanine and approximately 1 of the 15 moles of aspartic acid were liberated from the ribonuclease molecule with 70% of the enzymic activity remaining. The other four amino acids in Table III, phenylalanine, leucine, isoleucine and histidine, are present in small numbers in the RNase molecule. A large proportion of each of these amino acids was liberated with only slight inactivation of the enzyme.

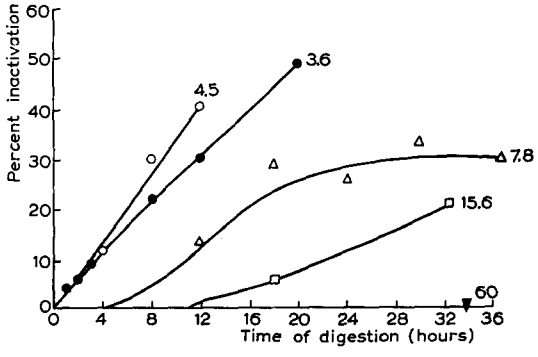


Fig. 3. Inactivation of RNase with CPase preparations. The moles of RNase: mole of CPase employed in each experiment was as follows: Expt. 4, 4.5:1; Expt. 5, 3.6:1; Expt. 1, 7.8:1; Expt. 6, 15.6:1; Expt. 7, 60:1.

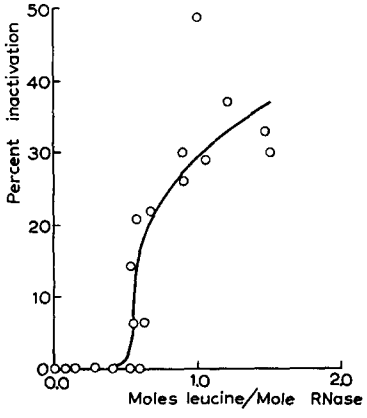


Fig. 4. Correlation of leucine liberation with loss of RNase activity.

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A correlation between the inactivation of RNase and the liberation of leucine, isoleucine and histidine is presented in the following figures.

Of the two leucine residues in the RNase molecule[11,22], at least 0.5 mole of leucine could be detected in the digestion mixtures before any enzymic activity was lost, and 1.5 moles of leucine were liberated with only 30% inactivation (Fig. 4). Therefore, it appears that the leucine residues are probably not directly related to the active "center" of the RNase molecule.

One of the three isoleucine residues in the RNase molecule could be liberated without any loss of enzymic activity (Fig. 5). Loss of a second isoleucine residue appeared to be proportional to the loss of enzymic activity. The line on the graph in Fig. 5 represents the curve which would be obtained experimentally if the liberation of the second and third isoleucine residues were proportional to the inactivation of the enzyme.

A correlation of the liberation of the four histidine residues in the RNase molecule with the loss of enzymic activity is presented in Fig. 6. The first mole of histidine could be liberated with no loss of enzymic activity, and release of the second mole was accompanied by a proportionate loss of enzymic activity. At this point, however, a departure from proportionality occurs and with about 60% of the enzymic activity remaining, 3 moles of histidine could be detected in the digestion mixtures. In three separate digestion mixtures with different molar ratios of RNase to CPase, 3.8–4 moles of histidine per mole of RNase could be liberated at various intervals. In these experiments, therefore, *all* of the RNase molecules had been divested of *all* of their histidine residues, with 55–70% of the original enzyme activity still intact.

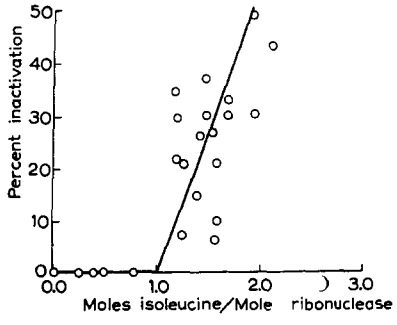


Fig. 5. Correlation of isoleucine liberation with loss of RNase activity.

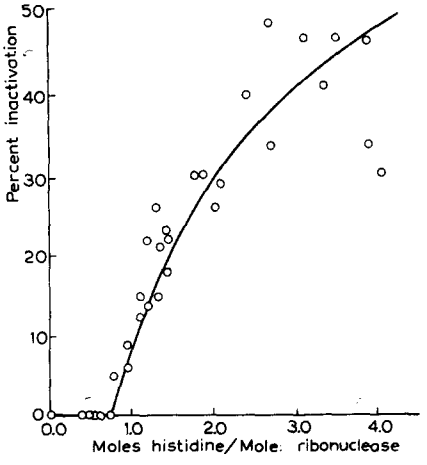


Fig. 6. Correlation of histidine liberation with loss of RNase activity.

## DISCUSSION

WEIL AND SEIBLES[23] have suggested that histidine may represent an important factor, which in some yet unknown fashion is related to RNase activity (measured as acid produced during the RNase-catalyzed hydrolysis of RNA). According to Fig. 6 histidine does not appear to be essential for RNase action (measured as optical density of small nucleotides produced by the action of RNase on RNA). An unequivocal solution of this apparent contradiction will come only with the isolation and charac-

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terization of the active center of RNase. However, it should be noted that the assay methods were different. The mechanism of action of RNase is not a simple hydrolysis but an intramolecular transphosphorylation followed by hydrolysis[24]. The rate of formation of titratable acid groups is much slower than the rate of formation of acid-soluble split products, *i.e.*, the hydrolysis is slower than the transphosphorylation[25,24]. In addition it has been shown that pyrimidine nucleotides inhibit the hydrolytic action of RNase[26]. If the active center of RNase had two active sites, a transphosphorylation and an hydrolytic site and if histidine were necessary for the hydrolytic site only, it might be possible to inactivate the hydrolytic site and liberate histidine with only slight inactivation of the transphosphorylation site. Indeed KALMAN *et al.*[6] have shown that by digesting RNase with subtilisin, the two activities, measured by independent methods, were not lost at the same rate. In addition, evidence has been presented for the participation of histidine in the hydrolytic activity of other enzymes such as chymotrypsin[27,28], lysozyme[29], cholinesterase[30], histidase[31], intestinal maltase and oligo-1,6-glucosidase[32].

An estimation of the amino acids which were liberated during partial inactivation of RNase (Table III) indicated that considerable amounts of the amino acids which are found in small numbers in the protein[11,22] could be liberated with more than half of the enzymic activity remaining. The excellent work of HIRS, MOORE AND STEIN in elucidating a partial structural formula of oxidized RNase[33,22,34], and their generous co-operation in making this information available, makes possible a more meaningful interpretation of the above data. Thus far, liberation of 1 of the 3 isoleucine residues, 2 of the 2 leucine residues and 3 of the 4 histidine residues from RNase does not significantly affect its activity under the conditions of these experiments. According to the information available[22,34], the 2 leucine residues (and 2 of the 4 histidine residues) are contained in the 79 amino acid residues from the N-terminal portion of RNase. Since the release of the leucine residues does not significantly affect the enzymic activity of the RNase, and since this portion of the molecule does not contain any isoleucine, one plausible interpretation of these data is that this large portion of the RNase molecule (64%) does not appear to be directly concerned with its catalytic activity. The remaining 36% of the RNase molecule, constituting the 45 amino acid residues from the C-terminal end, contains all 3 of the isoleucine and 2 of the 4 histidine residues[22,34] and should therefore contain the "active center" of RNase.

The 20 residue peptide which constitutes the C-terminal end of RNase contains 2 isoleucine residues which lie close together, plus 2 histidine residues, and no leucine residues[22,34]. Since the liberation of 1 or 2 isoleucine residues appears to be associated with inactivation of the enzyme as assayed in these experiments, then it is possible that the "active center" may be situated relatively close to the C-terminal end and contained in less than 20% of the original enzyme molecule. This would be in agreement with the finding that the loss of the C-terminal tetrapeptide causes inactivation of RNase[35]. However, unequivocal location of the "active center" must await its isolation and characterization and the correlation of the various methods of assay for RNase.

## SUMMARY

Crystalline ribonuclease (RNase) was digested with crystalline carboxypeptidase untreated with di*iso*propylfluorophosphate.

The elution patterns of the digested RNase from Amberlite IRC-50 (XE-64) were found to

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be constant unless inactivation occurred during the digestion period. The quantitative differences between the elution patterns of fully-active and partially-inactivated RNase (measured by protein determination) correlated with the per cent inactivation of the enzyme (measured by RNase assay).

With approximately 70% of the enzymic activity remaining, RNase may be divested of 3 of its 9 moles of valine, 3 to 4 of the 12 moles of alanine, at least 1 of the 15 moles of aspartic acid 1.3 of the 3 moles of phenylalanine, 1.5 of the 2 moles of leucine, 2 of the 3 moles of isoleucine and 2 to 4 of the 4 moles of histidine.

The liberation of both leucine residues in the RNase molecule is not clearly related to enzyme inactivation, whereas the liberation of 1 of the 4 histidine residues and 1 or 2 of the 3 isoleucine residues appears to be associated with inactivation of the enzyme. From these data, and from the partial structural formula of the RNase molecule made available by the work of HIRS, MOORE AND STEIN, it is estimated that the major portion of the RNase molecule is not essential for its catalytic activity, under the conditions of these experiments, and that the "active center" of this enzyme is located relatively closer to the C-terminal end of the molecule.

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