THE ACTIVITY OF RIBONUCLEASE AFTER DIGESTION WITH CARBOXYPEPTIDASE*

by

GEORGE KALNITSKY AND WILLIAM I. ROGERS

Department of Biochemistry, College of Medicine, State University of Iowa, Iowa City, Iowa (U.S.A.)

INTRODUCTION

During the past 30 years, a great deal of progress has been made in the isolation and identification of enzymes, coenzymes and intermediates of a large number of biochemical reactions. Despite the fact that the mechanism of action of coenzymes is fairly well understood, we are still largely in the dark concerning the mechanism of action of enzymes. Numerous attempts have been made to simplify the problem and to gain further insight into it by working with enzyme models, *i.e.*, non-enzymically catalyzed reactions similar to those which occur biologically. Many workers have demonstrated the necessity of specific chemical groups, such as sulfhydryl, phenolic and primary amino, for the activity of various enzymes[1-4]. In addition, certain chemical groups have been found to be non-essential for the activity of some enzymes[1-3]. This has of course contributed to the idea that the whole, intact protein may not be necessary for enzyme activity, but that an active site of the enzyme may represent a small fraction of the active molecule. An analogous situation is found in chemical studies of protein hormones and antibodies where protein fragments have been isolated possessing the biological activity of the original molecule[2,5].

Since the classical work of SUMNER, and of NORTHROP AND KUNITZ in demonstrating the protein nature of enzymes, no genuine example of the persistence of enzymic activity after hydrolysis has been described until recently. Although final proof is lacking, the isolation of 3 different chymotrypsins, by KUNITZ[6] in 1938 may possibly have been the first example of this kind with reference to enzymes. More recently several preliminary communications have presented evidence that the intact enzyme molecule is not essential for enzyme activity[7-12]. Enzymically active dialyzable fragments are formed during the autodigestion of pepsin[7]. Chymotrypsin[8], lysozyme[9] and ribonuclease[10] when digested with carboxypeptidase and divested of at least their C-terminal end groups, maintain full enzymic activity. Partial digestion of ribonuclease by subtilisin also yields a fully active enzyme[11]. Denaturation of ribonuclease in 8 *M* urea does not inactivate the enzyme[12].

Crystalline ribonuclease (RNase) was selected as a suitable starting material in this work, since it is quite stable, has a relatively simple structure, requires no

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coenzyme or metal ion and can be readily assayed[13]. In addition, RNase is obtainable in electrophoretically homogeneous form and is relatively inexpensive. Among the variety of tools available for degrading RNase, carboxypeptidase (CPase) digestion was selected as the method of choice, since this enzyme is now used generally for determining the C-terminal end groups of proteins[9, 14, 15] and is available in crystalline form.

The present communication presents evidence which indicates that a considerable part of the crystalline enzyme ribonuclease is not essential for catalytic activity. A qualitative and quantitative analysis is presented of the amino acids which are split off, and it is calculated that approximately 15% of the residues may be lost before any inactivation occurs.

METHODS

Crystalline RNase was purchased from Armour and Company. Paper electrophoresis of this enzyme at four different pH's (pH 4.6, 5.9, 6.8 and 8.5; ionic strength, 0.12) showed only one protein spot. However, when the RNase was chromatographed on Amberlite IRC-50(XE-64)* according to HIRS, MOORE AND STEIN, two peaks were obtained, with approximately 90% of the activity in one peak[16]. Enzyme activity paralleled the protein determinations which were carried out according to the method of LOWRY *et al.*[17].

The RNase assay was essentially that described by ANFINSEN *et al.*[18]. Four ml of 0.2*M* acetate buffer, pH 5.0 (containing the RNase) was incubated with 1.0 ml of 1.0% sodium ribonucleate, for 25 minutes, at 30° C. The reaction was stopped by the addition of 1.0 ml of 0.75% uranyl acetate in 25% $HClO_4$. After stirring and centrifuging the precipitate, a 0.10 ml aliquot of the supernatant was added to 3.0 ml of distilled water, and the optical density was measured at 260 mμ using a Beckman DU Spectrophotometer. A reagent blank and at least four standards were run with every assay. The activity of the RNase was proportional to the logarithm of its concentration up to 4 or 5 μg of the enzyme. The sodium ribonucleate used was purchased from Nutritional Biochemicals Corp. Schwarz ribonucleic acid (RNA) neutralized to pH 5.0 gave essentially similar results, as did Schwarz RNA purified by acid ethanol precipitation[19] or by repeated neutral alcohol precipitation (with 0.05*M* NaCl)[20, 21]. A purified RNA preparation[22] obtained from Dr. C. LAMANNA (preparation 15A) gave almost identical results in this assay system.

Carboxypeptidase recrystallized 5 times was purchased from Pentex Biochemicals. The enzyme was generally washed several times and suspended in LiCl before use[23]. The CPase was not treated with di*iso*propylfluorophosphate (DFP) since our objective was to determine the extent to which RNase could be degraded without inactivation.

Digests were carried out by adding CPase to RNase in LiCl solutions, adjusting the pH to 7.6–8.1 and incubating at 25° C for varying periods of time. Aliquots were then taken and diluted for RNase assay. The amino acids liberated from the RNase molecule were separated by descending paper chromatography and quantitatively determined by direct photometry of the ninhydrin spots, according to the method of ROLAND AND GROSS[24]. Six standards of each amino acid were run with every experiment. No amino acids could be detected upon chromatographing either CPase or RNase, incubated under the experimental conditions described above. In addition certain amino acids were identified by specific color reactions. Histidine was identified by treatment with a freshly diazotized *p*-bromo aniline solution followed by spraying with half-saturated Na_2CO_3 to develop the characteristic red color[25]. The color reaction for proline involved spraying with an isatin solution, yielding a blue color[26]. Cystine was determined by treatment with Na_2SO_3 and phosphotungstic acid[27]. Bacteriological examination of several digests showed that very few and in some cases no bacteria, were present before, during or at the end of the digestion period.

The separation of digested from undigested RNase molecules by paper chromatography was carried out as follows: aliquots of controls or of digests were placed on 1 inch wide Whatman No. 1 filter paper strips and subjected to descending chromatography in secondary-butanol:water (3:1) for 15 hours at 3° C. Areas which contained the original spot and successive 0.5 cm strips were then cut out and each strip was eluted in 4.0 ml of 0.2*M* acetate buffer (pH 5.0) for 3.5 hours at room temperature (approximately 23° C). Aliquots of each eluate were then tested for RNase activity and for protein concentration.

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RESULTS

The first amino acid which appeared in any quantity as a result of the digestion of RNase by CPase was valine in accord with other experiments of this type[10,18]. That valine is indeed the C-terminal residue of RNase has been substantiated by hydrazinolysis[28,29]. Thus, in Table I, experiment 1, 8 of the 17 amino acids in the RNase molecule[30] were detected as a result of short-term digests with no decrease in the total activity. Assuming that one mole of the C-terminal end group valine had come from each mole of RNase, it appeared that approximately 7% of the RNase molecules present had been digested. In experiment 2, with a slightly longer period of digestion serine and threonine also appear. It is evident that more than one residue each of alanine and threonine are released. Again, there is no inactivation of the RNase with this progressive hydrolysis of the molecule.

TABLE I

PRODUCTS OF THE DIGESTION OF RIBONUCLEASE WITH CARBOXYPEPTIDASE

Experiment number	*1*	*2*	*3*	*4*	*5*	*6*
Amino acids liberated			$100 \times \frac{\mu Moles\ Amino\ acid}{\mu Moles\ RNase}$			
Valine (9)*	7	8	17	40	60	100
Leucine (2)	5	5	13	27	57	60
Phenylalanine (3)	+	5	+	20	47	60
Alanine (12)	+	23	>47	75	200	394
Tyrosine (6)	3	8	20	40	40	127
Methionine (4)	3	0	19	21	53	53
Isoleucine (3)	3	6	17	25	80	127
Lysine (10)	6	6	—	29	40	67
Serine (15)	0	8	26	44	+	80
Threonine (10)	0	14	>35	89	93	153
Aspartic acid (16)	0	0	24	28	20	107
Glutamic acid (12)	0	0	11	12	+	+
Glycine (3)	0	0	15	25	+	100
Arginine (4)	0	0	—	0	40	80
Histidine (4)	0	0	—	0	40	93
Cystine (4)	0	0	0	0	0	0
Proline (5)	0	0	0	0	0	0

+: present but not measurable; —: not analyzed; 0: not detectable.

* Figures in parenthesis indicate the number of residues of each amino acid in the RNase molecule, according to HIRS, STEIN AND MOORE[30].

Experiment 1: 42 mg (3 μM) of RNase and 3.3 mg (0.1 μM) of 5×-washed CPase were adjusted to pH 8.1 in 5% LiCl (final concentration) in a total volume of 1.0 ml. The time of digestion was 0.5 hour.

Experiment 2: 35 mg (2.5 μM) of RNase and 2.5 mg (0.08 μM) of CPase were adjusted to pH 7.6 in 1.5% LiCl (final concentration), in a total volume of 0.50 ml. The time of digestion was 1.5 hours.

Experiment 3: 126 mg (9 μM) of RNase in $5 \cdot 10^{-3} M$ phosphate buffer, pH 7.7, and 10 mg (0.3 μM) of CPase were adjusted to pH 7.8 in 5% LiCl (final concentration) in a total volume of 0.95 ml. The time of digestion was 5.5 hours.

Experiment 4: 84 mg (6 μM) of RNase and 3.3 mg (0.1 μM) of 3×-washed CPase were adjusted to pH 7.9 in 2.5% LiCl (final concentration) in a total volume of 1.0 ml. The time of digestion was 10 hours.

Experiment 5 and 6: 21 mg (1.5 μM) of RNase and 3.3 mg (0.1 μM) of 5×-washed CPase were adjusted to pH 8.1 in 5% LiCl (final concentration) in a total volume of 1.0 ml. The times of digestion were 8.5 and 18.5 hours respectively.

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In experiment 3 (Table I) the molar ratio of RNase to CPase was maintained at 30 to 1 as in the previous experiments but the time of digestion was lengthened to 5.5 hours with the result that several additional amino acids appeared. In experiment 4 (Table I) the relative amount of CPase was decreased but the time of digestion was increased to 10 hours. The values for valine and other amino acids suggested that about 40% of the RNase molecules were attacked, and that digestion had proceeded to quite an appreciable extent in these molecules. Relatively large amounts of valine, alanine, tyrosine, serine and threonine were liberated. Of the seventeen amino acids in the RNase molecule thirteen amino acids were liberated as products of the digestion of RNase and no decrease in RNase activity was observed. No arginine, histidine, cystine or proline were found in this experiment.

In experiment 5, with a smaller ratio of RNase to CPase, appreciable amounts of histidine and arginine appeared for the first time as digestion products. At this stage 15 of the 17 amino acid constituents of the RNase molecule had been liberated. It is noteworthy that no significant decrease in enzymic activity occurred (94% of the original RNase activity was retained).

By increasing the digestion time (experiment 6) further digestion took place, so that the value for the C-terminal end group was no longer used as an estimate of the percent digestion (see further, Table III). Four of the 12 alanine residues, more than 1 of the 3 isoleucine residues, and 1 of the 3 residues of glycine have been liberated with little or no significant loss in RNase activity. The other amino acids were liberated in smaller proportions. Again 94% of the original RNase activity was still present after this extensive degradation.

Some information as to the rates of liberation of various amino acids was obtained in experiment 3 and is presented in Fig. 1. It appears that at least seven amino acids, valine, methionine, glutamic acid, glycine, isoleucine, leucine and tyrosine are liberated at approximately the same rate (lower curve). Each point on this curve represents an average of about 4 determinations for each amino acid. After 4.5 hours of digestion approximately 10% of the RNase molecules had lost at least each of these seven amino acids; and after 8.5 hours of digestion approximately 19% of the RNase molecules had been divested of each of these seven amino acids (in addition to the other amino acids indicated). Aspartic acid, serine, alanine and threonine are liberated much more rapidly, as would be expected, since they have the highest number of residues per mole of RNase, 16, 15, 12 and 10, respectively[30].

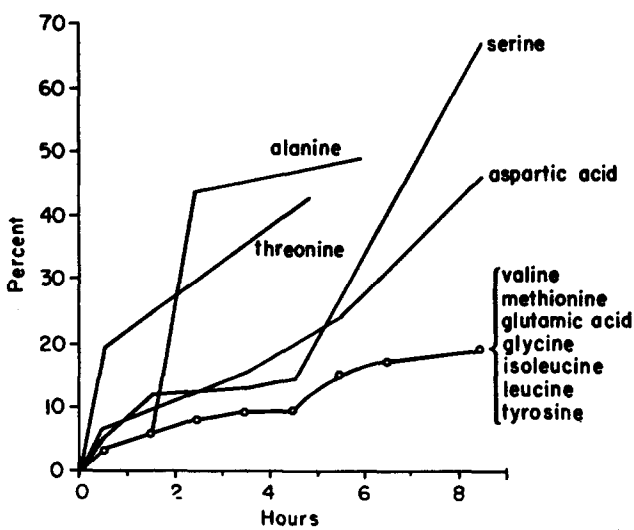

Fig. 1. The rates of liberation of a number of amino acids from the RNase molecule by the action of CPase.

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However, the values for glutamic acid, which by this criterion should also appear in large amounts, are notably low in all digests.

In this same experiment (Table I, No. 3) aliquots for RNase assay were taken at various intervals during the digestion. The results are presented in Table II. There was no decrease from the original RNase activity at any time during the digestion period. It was interesting that after 4.5 and 5.5 hours of digestion, the potency of the RNase appeared not to diminish, but on the contrary, to increase. This increased activity has been observed in 7 other experiments. In general an increase of 20% or more coincided with the digestion of 5–17% of the RNase molecules as measured by the liberation of valine. CPase did not affect the enzymic activity of undigested RNase.

TABLE II

ASSAY OF DIGESTED RNASE

Digestion time hours	*Activity remaining. %*
1.5	103
2.5	111
3.5	103
4.5	129
5.5	121
6.5	105
8.5	109

Since histidine is associated with the activity of RNase[31] it was of interest to determine the amounts of this amino acid liberated up to and including slight inactivation of the enzyme (Fig. 2). It is apparent that the first mole of histidine liberated from the RNase molecule by the action of the CPase preparation is not essential, whereas the second molecule of histidine liberated is essential to the activity of the enzyme. Similar patterns were also obtained with arginine, lysine, isoleucine and valine.

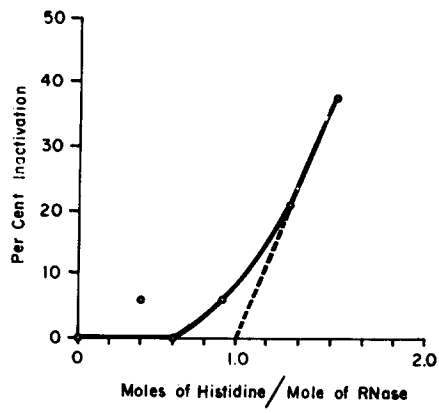

Fig. 2. Correlation of moles of histidine liberated per mole of RNase with inactivation of the enzyme.

Attempts were made to separate the digested from undigested molecules using paper electrophoresis. In our hands, this did not appear to be a sensitive enough tool, to accomplish the separation. In addition, the RNase was largely inactivated during the process.

In further attempts to achieve this separation (of digested from native RNase) the method of HIRS, MOORE AND STEIN was employed. The elution pattern obtained with 700 μg of undigested RNase from Amberlite IRC-50 (XE-64) was quite typical[16]. After only slight digestion (1.5 hours; experiment 2, Table I) an essentially identical elution pattern was obtained with an equivalent amount of digested RNase (Fig. 3). The slight displacement is not significant, but there is a difference in the height of the peaks. Protein determinations in general followed RNase activity. However, fraction number 33, at the height of the main peak of the digested sample, showed a specific activity of 1.28 (μg RNase per μg of protein in the sample).

In taking aliquots for the determination of amino acids by the 2-BuOH–3% NH_3 (3:1) solvent system, it had previously been noticed after chromatographing and staining with ninhydrin, that controls containing RNase, or RNase plus CPase at zero time (no digestion occurring) remained at the origin, whereas after digestion had taken place the protein streaked. This suggested a possible method for separating digested from native RNase molecules. Areas corresponding to the origin, 2–3 cm

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and 4–5 cm from the origin were cut out of both control and experimental chromatograms. Each strip was eluted in 4.0 ml of 0.2 *M* acetate buffer (pH 5.0) for 3.5 hours at room temperature. Aliquots of this eluate were then assayed for RNase activity. Very appreciable amounts of RNase were found in the streaked area below the origin on the experimental strips, none in the corresponding areas of the control strips. This indicated at least a partial separation of active digested RNase from undigested RNase molecules. However, the RNase which had migrated, and that which had remained at the origin could not be differentiated by chromatography on Amberlite IRC-(XE-64). Their elution patterns were essentially identical.

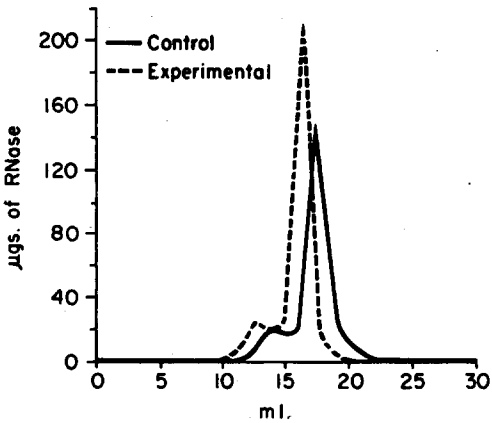

Fig. 3. Elution patterns of RNase from Amberlite IRC-50(XE-64). Experimental, a 10 µl aliquot of digest described in experiment 2, Table I, containing 700 µg of RNase, was placed on the column, with 112% recovery. The control contained 700 µg of native RNase (101% recovery).

In addition, it is of interest to point out that active RNase withstood treatment with CPase for 5.5 hours, followed by 51 hours of chromatography in secondary-butanol: 3% NH_3 (3:1) at room temperature, and development of the ninhydrin color by dipping in a ninhydrin-acetone solution. Also of interest is the fact that quantitative recoveries of native RNase could be obtained within one or two hours after the development of the blue ninhydrin color. Later, there appeared to be a gradual deepening of the ninhydrin color accompanied by a progressive loss of enzymic activity with approximately 67% of the RNase activity remaining one day after staining with ninhydrin.

In an attempt to obtain quantitative recoveries, the chromatography was repeated in secondary-butanol: 3% NH_3 (3:1), at 3° C, and the staining procedure was omitted. The circles corresponding to the origin and successive 1 cm strips were cut out and eluted as before and analyzed for RNase activity. From control strips which contained 1300 µg RNase, 1224 µg (94%) were recovered at the origin; none was found below the original spot of application. On experimental strips, containing a similar amount of digested RNase, only 44% of the original RNase put on the strip was recovered. Therefore it appeared that the native RNase could withstand this treatment whereas the digested RNase could not.

To afford more quantitative recoveries of the digested RNase, the solvent system was changed to secondary-butanol: H_2O (3:1) and the time of chromatography was shortened to 15–16 hours. The results of several typical experiments described in Table I are presented in Table III. Under these conditions it was found that native RNase (alone or with CPase at zero time) did not migrate and could be quantitatively recovered at the origin; none was detected below this point in over a dozen experiments. The % digestion was therefore calculated as follows:

$$\% \text{ D} = 100 \times \frac{(\text{Total RNase} - \text{RNase remaining at origin})}{\text{Total RNase}}$$

Approximately 24% of the RNase had migrated from the origin in experiment 2,

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Table III; 95% of the enzyme activity and 96% of the protein were recovered. CPase is not eluted from the paper probably because of its insolubility at pH 5.0, and is therefore not determined by this protein method.

TABLE III

SEPARATION OF DIGESTED FROM UNDIGESTED RNASE

Distance travelled from origin (cm)	*Micrograms of RNase*						
	Control	*Exp. 2*	*Exp. 4*	*Exp. 5*	*Exp. 6*	*Exp. 7*	*Exp. 8*
Origin	1255	977	750	111	109	355	430
0.5	0	136	112	12	10	55	79
1.0	0	104	91	8	11	50	88
1.5	0	89	71	11	12	44	91
2.0	0	0	66	10	7	45	127
2.5	0	0	75	6	4	50	162
3.0	0	0	54	2	0	0	88
3.5	0	0	0	0	0	0	174
4.0			0	0	0	0	134
4.5			0	0	0	0	96
5.0			0	1	1	0	38
5.5							0

Experiments 2 through 6 are those described in Table I. Experiments 7 and 8 contained 84 mg (6 μM) of RNase and 3.28 mg (0.1 μM) of 5×-washed CPase in a total volume of 1.0 ml. The pH was adjusted to 8.1. The times of digestion were 0.5 and 34 hours for experiments 7 and 8 respectively. In experiment 8 there were 1.10 moles of valine liberated per mole of RNase.

The μgs of RNase applied to each strip were: control, 1372; experiment 2, 1372; experiment 4, 1680; experiments 5 and 6, 210; experiment 7, 823; experiment 8, 2520.

An aliquot of experiment 4 was also analyzed in this solvent system. It was found that 39% of the total RNase and 36% of the protein put on the strip migrated during paper chromatography. These figures correlate very well with the 40% digestion obtained on the basis of the C-terminal valine analysis in experiment 4, Table I. Also, in this experiment, 90% of the protein but only 73% of the RNase activity was recovered after chromatography.

In experiment 5, it appeared that approximately 60% of the RNase molecules had been attacked (according to the value of valine) with no significant loss in activity. Paper chromatography of an aliquot of the digest showed that 52% of the RNase remained at the origin indicating that about 48% of the RNase had been digested (total recovery of RNase was 78%). Virtually the same results on chromatography were obtained for experiment 6. The apparent discrepancy between 50% of the RNase having migrated in this system and the value of 100% for valine in Table I can be accounted for by the fact that probably more than one residue of valine is liberated per mole of RNase.

In experiments 7 and 8 respectively, 57 and 83% of the RNase molecules were digested with no decrease in total activity. That the protein becomes less stable with progressive digestion is suggested by the fact that only 73 and 60% of the enzymic activity was recovered after chromatography.

DISCUSSION

It is readily apparent that all 126 amino acid residues of the protein are not required for full enzymic activity of RNase. Indeed, after digestion of 5–17% of the terminal

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valine residues (for example, Table I, experiment 3) an increase in activity of about 20% was noticed. The precise nature of the mechanism of "stimulation" is not known.

According to the work of WEIL AND SEIBLES[31], histidine is important to the activity of the enzyme. Our data indicate that the first histidine molecule which came off was not essential for the full activity of RNase (Fig. 2). However, the second molecule was required for enzymic activity. Before the first moles of histidine and arginine were hydrolysed from the protein, 13 other amino acids were liberated (Cf. Table I, experiments 4 and 5) with no decrease in enzymic activity. An additional mole each of alanine and threonine were also released (Table I, experiment 2). From this it may be calculated that about 15% of the RNase molecule was not essential for enzymic activity. That the entire molecule is needed for the stability of RNase is suggested by the loss in enzymic activity of digested protein during chromatography.

Paper chromatography employing the solvent system 2-butanol: H_2O (3:1) has proved useful in separating the digested from the undigested molecules of RNase. Undigested and slightly digested RNase are quite stable under these conditions and can be recovered quantitatively. The results obtained with this method, when compared with the amino acid values resulting from the degradation of RNase are thus useful in determining the approximate range of per cent digestion. With very slight digestion (less than 10%) the migration values appear to be somewhat higher than would be expected from the amino acid data. This might possibly be due to artificially low amino acid values caused by the occurrence of transpeptidation reactions. Possible combination with CPase seems unlikely since on a single strip CPase and undigested RNase do not migrate in this system.

It is known that some crystalline CPase preparations contain trace amounts of trypsin and chymotrypsin. STEINBERG[32] has shown than another DFP-sensitive proteolytic contaminant, which is neither trypsin nor chymotrypsin, was present in crystalline CPase preparations. Recently GRANT AND ROBBINS[33] have isolated still another DFP-sensitive proteolytic enzyme from hog pancreas, which they call pankrin. It is probable that the CPase used in our experiments contains proteolytic impurities. This is borne out by comparing the order of liberation of the amino acids reported here with the known residues[29] of the carboxyl end of RNase. Since the foreign protease does not affect the activity of RNase until over 15% of the residues have been hydrolysed, it will be of interest to determine the nature of its attack in order to gain a better understanding of the relationship between the active center(s) of RNase and the rest of the molecule.

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SUMMARY

On incubating ribonuclease (RNase) with a crystalline carboxypeptidase preparation, a number of amino acids were liberated. Of the 17 amino acids in the RNase molecule, all except cystine and proline have thus far been quantitatively determined as products of this hydrolytic reaction with no loss in the original activity of the RNase. During the early phases of the digestion, the activity of the RNase was increased.

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A separation of the digested from the undigested RNase molecules has been achieved by chromatography of the digests in secondary-butyl alcohol:water (3:1) at 3° C for 15 hours. The native RNase molecules did not migrate under these conditions and were quite stable, whereas the digested RNase molecules migrated and were less stable. The data obtained with this procedure correlates reasonably well with the amino acid determinations as a measure of the per cent of the RNase molecules digested.

The extent of digestion thus far achieved approximates 80% of the RNase molecules present, with no loss in enzymic activity. It is calculated that approximately 15% of the amino acid residues which constitute RNase are not essential for the enzymic activity, but appear to be necessary for the stability of the molecule.

REFERENCES

1 H. S. OLCOTT AND H. FRAENKEL-CONRAT, *Chem. Revs.*, 41 (1947) 151.
2 R. R. PORTER in *The Proteins*, Edited by H. NEURATH AND K. BAILEY, Vol. I, part B, (1953) p. 988.
3 J. B. SUMNER AND G. F. SOMERS in *Chemistry and Methods of Enzymes*, Academic Press, Inc., N.Y. (1953) p. 9.
4 E. S. G. BARRON, *Advances in Enzymology*, II (1951) 201.
5 C. H. LI, in *The Proteins*, Edited by H. NEURATH AND K. BAILEY, Vol. II, part A (1953) 595.
6 M. KUNITZ, *J. Gen. Physiol.*, 22 (1938) 207.
7 G. E. PERLMAN, *Nature*, 173 (1954) 406.
8 J. A. GLADNER AND H. NEURATH, *J. Biol. Chem.*, 206 (1954) 911.
9 J. I. HARRIS, C. H. LI, P. G. CONDLIFFE AND N. G. PON, *J. Biol. Chem.*, 209 (1954) 133.
10 G. KALNITSKY AND E. E. ANDERSON, *Biochim. Biophys. Acta*, 16 (1955) 302.
11 S. M. KALMAN, K. LINDERSTRØM-LANG, M. OTTESEN AND F. M. RICHARDS, *Biochim. Biophys. Acta*, 16 (1955) 297.
12 C. B. ANFINSEN, W. F. HARRINGTON, A. HVIDT, K. LINDERSTRØM-LANG, M. OTTESEN AND J. SCHELLMAN, *Biochim. Biophys. Acta*, 17 (1955) 141.
13 M. LASKOWSKI in *The Enzymes*, Edited by J. B. SUMNER AND K. MYRBACK, Academic Press, N.Y., Vol. I, part II (1951) p. 957.
14 J. LENS, *Biochim. Biophys. Acta*, 3 (1949) 367.
15 J. I. HARRIS, *J. Am. Chem. Soc.*, 74 (1952) 2944.
16 C. H. W. HIRS, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 200 (1953) 493.
17 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
18 C. B. ANFINSEN, R. R. REDFIELD, W. L. CHOATE, J. PAGE AND W. R. CARROLL, *J. Biol. Chem.*, 207 (1954) 201.
19 E. VISCHER AND E. CHARGAFF, *J. Biol. Chem.*, 176 (1948) 715.
20 B. MAGASANIK AND E. CHARGAFF, *Biochim. Biophys. Acta*, 7 (1951) 396.
21 B. MAGASANIK in *The Nucleic Acids*, Edited by E. CHARGAFF AND J. N. DAVIDSON, Academic Press, New York, Vol. I (1955) p. 391.
22 M. F. MALLETTE AND C. LAMANNA, *Arch. Biochem. Biophys.*, 47 (1953) 174.
23 J. A. GLADNER AND H. NEURATH, *J. Biol. Chem.*, 205 (1953) 345.
24 J. F. ROLAND AND A. M. GROSS, *Anal. Chem.*, 26 (1954) 502.
25 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, New York (1955) p. 95.
26 *Ibid.*, p. 97.
27 R. J. BLOCK, *Anal. Chem.*, 22 (1950) 1327.
28 H. FRAENKEL-CONRAT, personal communication.
29 C. B. ANFINSEN, personal communication.
30 C. H. W. HIRS, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 211 (1954) 941.
31 L. WEIL AND T. S. SEIBLES, *Arch. Biochem. Biophys.*, 54 (1955) 368.
32 D. STEINBERG, *Compt. Rend. Lab. Carlsberg, Ser. Chim.*, 29 (1954) 159.
33 N. H. GRANT AND K. C. ROBBINS, *J. Am. Chem. Soc.*, 77 (1955) 2027.

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