The Sequence of Amino Acid Residues in Bovine Pancreatic Ribonuclease: Revisions and Confirmations*

DEREK G. SMYTH, WILLIAM H. STEIN, AND STANFORD MOORE

From the Rockefeller Institute, New York 21, New York

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Recent studies (1-3) have led to a revision of the published sequence of residues 11 to 18 in bovine pancreatic ribonuclease (4). In the experiments from this laboratory (1), the improved conditions for the Edman degradation (Konigsberg and Hill (5)) provided an understanding of some of the limitations inherent in the procedure employed by Hirs, Moore, and Stein (4). It was noted that difficulties can be encountered particularly in the determination of sequences of peptides containing glutamine, asparagine, serine, and threonine residues. These observations have stimulated a new examination of the amino acid sequence of the whole molecule to determine whether such complications had affected any of the conclusions drawn in regard to other parts of the chain.

Experience with the stepwise degradation has resulted in the following observations: (a) When the cyclization in the Edman degradation is performed in glacial acetic acid-anhydrous HCl, a newly liberated NH$_2$-terminal glutamine residue cyclizes to a pyrrolidone carboxylic acid residue. Since such a peptide has no α-NH$_2$ group, the degradation cannot proceed further. (b) When the process is followed by amino acid analysis of the residual peptide (the subtractive method), an incorrect conclusion can be drawn if any artifactual decomposition of amino acids occurs during hydrolysis, as has been observed for serine and threonine (1) and tyrosine (4). (c) These difficulties are avoided by the modified conditions of Konigsberg and Hill (5), in which the cyclization is performed at 25° in anhydrous trifluoroacetic acid and the residual peptide is purified by passage through a short column of Dowex 50-X2. (d) An internal asparagine or aspartic acid residue may form an imide in glacial acetic acid-HCl. (e) An internal asparagine or threonine residue is nearly quantitatively acetylated in acetic acid-HCl. When such an N-acetylated residue becomes NH$_2$-terminal, an O → N acyl shift may occur after removal of the acid. To the extent that it does, subsequent degradation is blocked. Acylation does not occur in trifluoroacetic acid at 25°.

With these facts in mind, it became essential to reexamine all the peptides on which the proposed structural formula for ribonuclease had been based (Hirs et al. (4)). Mainly through the use of the Edman degradation under the milder conditions, the following revisions of the structure have been documented: The sequence from residues 101 to 103 becomes GluNH$_2$-Ala-AspNH$_2$; the sequence from 87 to 89 becomes Thr-Gly-Ser; following asparagine at 94, the alanine and half-cystine at 95 and 96 are interchanged. These results, together with the previously reported revised sequence for residues 11 to 18, are incorporated in Fig. 1. Extensive new data in the present communication confirm the sequence deduced for the rest of the 124 amino acid residues in the ribonuclease molecule.

EXPERIMENTAL PROCEDURE

The materials and experimental procedures were as previously reported (1), with the following exceptions and additions.

Oxidized ribonuclease was prepared from chromatographically purified ribonuclease A (Worthington Biochemical Corporation), which contained over 90% ribonuclease A. NH$_2$-terminal analysis of the oxidized protein by the cyanate method (7) gave 0.87 residue of lysine per molecule.

Glycyl-L-serine was obtained from Cyclo Chemical Corporation; L-seryl-L-leucine and L-glutamine were obtained from Nutritional Biochemicals Corporation. The purity of the samples was checked chromatographically and by elementary analysis.

Analyses for neutral and acidic amino acids were performed with a 55-cm column (7) on the amino acid analyzer (8) at pH 3.25 and pH 4.25; analyses for the basic amino acids were made with an 8-cm column. The use of these shorter columns for the analysis of hydrolysates of simple peptides permits greater sensitivity and rapidity. Solutions for hydrolysis were deaerated before the tubes were sealed (9, 10). The results are not corrected for decomposition of serine and threonine during the hydrolysis. All hydrolysates were performed in 6 M HCl at 110° for 16 hours.

Isolation of Peptides—Chromatography on Dowex 50-X2 provided the initial fractionation for the isolation of the following peptides used in this study: O-Ttryp 2, 4, 5, 6, 8, 9, 10, 11, 14, 15 (cf. Hirs (11)). O-Tryp 7-9 and O-Trypt-16 were isolated by the alternative procedure described below, with the aid of gel filtration on Sephadex as the first step.

Oxidized ribonuclease, 300 mg (12), was digested with 3 mg of trypsin for 24 hours at 25°. The resulting peptides were separated on a column (150 × 0.9 cm) of Dowex 50-X2 at 35°. The column was developed first with a gradient from pH 3.1 to pH 4.3 (500-ml mixing chamber) as described previously (1, 11). At 575 ml, 1 M sodium acetate at pH 5.1 was added to the upper reservoir. The effluent was collected in 5-ml fractions, and 100-ml portions were analyzed with ninhydrin after alkaline hydrolysis (9, 13). Under these conditions, all peptides of the trypsin series (11), identified subsequently by amino acid analysis,
was warmed to dissolve the peptide for desalting. The salt-free
an equal volume of glacial acetic acid was added,
His procedure has been applied in this present work to a 6-hour
tryptic hydrolysate of 30 mg of oxidized ribonuclease to separate
exchange chromatography second, as described by Eaker (14).
resolved under these conditions. 0-Tryp 15 and 0-Tryp 16,
which overlap at 1800 ml on Dowex 50, were separated and
50% acetic acid as eluant. The two peptides were adequately
and resubmitted to gel filtration on the same size column with
eluant. The high tyrosine content of the heptapeptide, 0-Tqp
(seven residues) and 0-Tryp 11 (four residues) was concentrated
dilute acetic acid is used. The desalted mixture of 0-Tryp 10
14, causes it to be the most retarded of the group when the
organic nature of the solvent also minimizes the retardation of
peptides containing aromatic residues. The pooled effluent
fractions from the Dowex 50-X2 column were concentrated as
as much as 5-fold and the solution,1 up to 6 ml in volume, was
added to the Sephadex column. The effluent was collected in
2-ml fractions and 20-µl aliquots were taken for alkaline hydroly-
sis and ninhydrin analysis. The acetic acid was removed by
rotary evaporation from the fractions that contained the salt-
free peptide. The peptide was dissolved in water, and the
solution stored at -20° in a polyethylene bottle. Lyophiliza-
tion was not employed in these studies.

The peptides O-Tryp 10, 11, and 14 emerge together at 1400 ml
from the Dowex 50-X2 column. O-Tryp 14 was desalted and
separated from the other two peptides by gel filtration on a
column (150 X 0.9 cm.) of Sephadex G-25 (120 to 200 mesh) developed at 25° at approximately 5 ml per hour with 50% acetic acid as eluant. In this solvent, the column
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The large peptides emerge at 40 to 60 ml when the column
(150 X 0.9 cm,) of Sephadex G-25 is developed with 0.2 M acetic
acid. The four were then chromatographed at 50° on a column
(20 X 0.9 cm,) of Dowex 1-X2, which was eluted with pyridinium
formate buffers at pH 5.25, 0.1 M for 130 ml, and 0.4 M buffer
(64.4 ml of pyridine and 15.2 ml of 98% formic acid diluted to
1 liter) from 130 ml to 270 ml.

The first of four peaks from the Dowex 1 column contains a
peptide analogous to the 0-Tryp 4 of Hirs (11), but with the
NH₂-terminal glutamine residue (1 intact.2 The second peak
contains the pyrrolidone carboxylyl form of O-Tryp 4, together
with O-Tryp 7-9 (residues 38 to 61) which is present because
the arginylcysteicyl bond between residues 39 and 40 is very
slowly cleaved by trypsin (cf. Hirs et al. (13)). The mixture of
O-Tryp 7-9 and the pyrrolidone carboxylyl form of O-Tryp 4
was resolved on a column (5 X 0.4 cm,) of Dowex 50-X2 at room
temperature. The latter peptide was eluted in the first of three
4-ml portions of 0.2 M pyridinium formate at pH 5.25. O-Tryp
7-9 appeared in the first of three 4-ml portions of 0.2 M pyridinium
formate at pH 5.1. The yields of the peptides isolated from the
Dowex 1-X2 column were: O-Tryp 2 (in the fourth peak), 45%;
the two forms of O-Tryp 4, 52% and 32%; O-Tryp 7-9, 90%;
and O-Tryp 18 (in the third peak), 90%.

The conversion of the glutamine form of O-Tryp 4 to the pyr-
rolidone carboxylyl form, measured by chromatography on
Dowex 1, is 70% complete after 16 hours at pH 5.28 and 50°.
NH₂-terminal analysis, by the cyanate method (7), of the pep-
1 From the previous studies (1), it is known that O-Tryp 4,
which initially contains an NH₂-terminal glutamine residue, is
present in the hydrolysate also as the pyrrolidone carboxylyl
peptide. The latter peptide gives the peak labeled O-Tryp 4 in
the experiments of Hirs (11). In the present separations on col-
umns of Dowex 50-X2, the peptides following the O-Tryp 4 peak
were observed to be contaminated (up to approximately the posi-
tion of O-Tryp 8) by small amounts of a peptide with the same
amino acid composition as O-Tryp 4; this trailing results from the
gradual cyclization of the glutaminyl residue of the more retarded
form of the peptide during chromatography at pH 3. This sensi-
tive peptide is much more stable under the rapid conditions of
chromatography on Dowex 1 at pH 5.28.

Fig. 1. The sequence of amino acid residues in bovine pancreatic ribonuclease A, based on the experiments of Hirs et al. (4), Spackman et al. (6), and Smyth et al. (7), and this communication.)
tide from the first peak gave an uncorrected yield of 67% of one residue of glutamic acid (or glutamine); some cyclization of the terminal glutamine residue probably occurred before reaction of the —NH₂ group with cyanate. No NH₂-terminal residue was found when the pyrrolidone carboxylyl peptide, present in the second peak, was submitted to the same procedure.

Chymotryptic Hydrolysis of O-Tryp 2, 4, 9, and 16—The peptides (2 to 4 μmoles) were hydrolyzed as described by Hirs (11). The products of the hydrolysis of O-Tryp 2 were separated on a column (150 × 0.9 cm) of Dowex 50-X2 developed with sodium buffers; the results were similar to those in Fig. 1E of Hirs (11); the peptides were desalted on Sephadex. When a 15-cm column was used for a more rapid separation of (O-Tryp 2)Chr 5, an additional peptide, GluNH₂-Ser-Tyr, was isolated in 30% yield. This result confirms the conclusion that glutamine is NH₂-terminal in the parent peptide, and that it subsequently cyclizes to a pyrrolidone carboxylyl residue to form (O-Tryp 2)Chr 2 (cf. 15).

The products of the cleavage of O-Tryp 4 and 9 gave chromatographic patterns corresponding to those in Figs. 16 and 1H of Hirs (11). Hydrolysates of O-Tryp 16 were separated on a short column (5 × 0.4 cm) of Dowex 50-X2 developed with three 4-ml portions each of the following pyridinium formate buffers: 0.2 M, pH 3.2; 0.2 M, pH 5.1; 1 M, pH 5.1. The result resembling that obtained in Fig. 1F of Hirs (11). The yields were: (O-Tryp 16)Chr 1, 95%; (O-Tryp 16)Chr 4, 70%; and (O-Tryp 16)Chr 5, 84%.

O-Tryp 7–9 was hydrolyzed with chymotrypsin, and the products were separated on a column (150 × 0.9 cm) of Dowex 50-X2 operated at 35° with a gradient (500-ml mixer) from 0.16 M, pH 5.1. Hydrolysates of O-Tryp 16 were separated on a long column (150 X 0.9 cm) of Dowex 50-X2 developed with sodium citrate at pH 3.1 as eluant. The purified peptide was, therefore, usually carried through three stages without purification after each stage; the resulting peptide mixture was then submitted to precise chromatography on a long ion exchange column, as noted in the summaries of “Results.” Even when the peptides do not contain cysteic acid, the introduction of a chromatographic purification on a long ion exchange column proved advantageous after the completion of three or more stages of the degradation. Such a step is particularly efficacious after the removal of an acidic residue.

RESULTS

Edman Degradation—The results that confirm previously determined sequences are summarized in a table presented later in the text. The data that have led to revisions are summarized below. The numbers in parentheses next to each peptide are the residue numbers, beginning at the amino end of the chain. The molar ratios given after each step of the Edman degradation refer to the analyses of the residual peptide. The amino acid lost at each step is given in bold face type. The yields obtained after each step are calculated from the amino acid analyses on the basis of the amount of the peptide submitted to the step in question.

O-Tryp 2—AspNH₂-Gly-GluNH₂-Thr-AspNH₂-CysSO₂H-Tyr-GluNH₂-Ser-Tyr-Ser-Thr-MeSO₂-Ser-Ileu-Thr-Asp-CysSO₂H-Arg (67–85)

Composition: Asp, 2.96; Gly, 1.00; Glu, 2.00; Thr, 2.73; CysSO₂H, 2.00; Tyr, 1.75; Ser, 2.76; MeSO₂, 1.13; Ileu, 0.98; Arg, not determined, but known to be present from previous analyses of O-Tryp 2 (11). On paper electrophoresis at pH 5, the peptide carried a negative charge.

Edman Degradation—Arginine was not determined during these degradations.

First step (91%): Asp, 2.18; Gly, 0.98; Glu, 1.98; Thr, 2.68; CysSO₂H, 2.01; Tyr, 1.88; Ser, 2.77; MeSO₂, 1.08; Ileu, 0.92.

Second step (85%): Asp, 2.14; Gly, 0.41; Glu, 2.00; Thr, 2.43; CysSO₂H, 2.00; Tyr, 1.74; Ser, 2.76; MeSO₂, 1.09; Ileu, 0.96.

Third step (97%): Asp, 2.08; Gly, 0.38; Glu, 1.63; Thr, 2.60; CysSO₂H, 1.92; Tyr, 1.75; Ser, 2.86; MeSO₂, 1.02; Ileu, 0.86.

At this stage, the peptide mixture was chromatographed on a column (150 × 0.9 cm) of Dowex 50-X2 operated at 35° with 0.2 M sodium citrate at pH 3.1 as eluant. The purified peptide (yield, 24%) had the expected one residue of glutamic acid per molecule. Further degradation proceeded as follows:

Fourth step (98%): Asp, 2.04; Gly, 0.13; Glu, 1.09; Thr, 1.95; CysSO₂H, 2.00; Tyr, 1.82; Ser, 2.77; MeSO₂, 1.00; Ileu, 0.99.

Fifth step (73%): Asp, 1.19; Gly, < 0.04; Glu, 1.24; Thr, 1.64; CysSO₂H, 1.79; Tyr, 1.88; Ser, 2.78; MeSO₂, 1.00; Ileu, 1.05.

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Sixth step (100%): Asp, 1.30; Gly, < 0.04; Glu, 1.24; Thr, 1.73; CySOH, 1.23; Tyr, 1.56; Ser, 2.70; MeSO, 1.00; Ileu, 0.91.

The following results with the chymotryptic fragments confirm the sequence just given. The failure to obtain a more complete loss of glycine at the second step was duplicated in a second degradation of the peptide, and was encountered in the following degradation of (O-Tryp 2)Chy 3.

(O-Tryp 2)Chy 3 — AspNH₂-Glu-GluNH₂-Thr-AspNH₂-CySOH-Tyr (67-73)

Composition: Asp, 1.93; Gly, 1.18; Glu, 1.17; Thr, 0.92; CySOH, 1.00; Tyr, 0.96. On electrophoresis at pH 5, the peptide carried a negative charge.

Edman Degradation—

First step (98%): Asp, 1.09; Gly, 0.96; Glu, 1.00; Thr, 0.89; CySOH, 1.06; Tyr, 0.85.

Second step (100%): Asp, 1.06; Gly, 0.46; Glu, 0.99; Thr, 0.91; CySOH, 1.00; Tyr, 0.88.

Third step (90%): Asp, 0.98; Gly, 0.39; Glu, 0.44; Thr, 0.87; CySOH, 1.00; Tyr, 0.86.

At this stage, chromatography of the reaction mixture on a column (50 × 0.9 cm) of Dowex 50-XS at 35° with 0.2 M pyridinium formate at pH 3.2 as eluant provided a pure peptide (yield, 45%) of the following composition: Asp, 1.00; Thr, 0.83; CySOH, 1.01; Tyr, 0.88; Gly and Glu < 0.02.

Further degradation proceeded as follows:

Fourth step (96%): Asp, 0.92; Thr, 0.13; CySOH, 1.08; Tyr, 0.92.

Fifth step (91%): Asp, 0.40; Thr, 0.10; CySOH, 1.00; Tyr, 1.00.

Hydrolysis of (O-Tryp 2)Chy 3 by papain, followed by chromatography of the mixture on a column (50 × 0.9 cm) of Dowex 50-XS (200 to 400 mesh) developed at 50° with 0.2 M pyridinium formate at pH 3.2, provided two peptides. One, (O-Tryp 2)Chy 3Pap 1, obtained in 31% yield, moved rapidly on the column, was ninhydrin-negative in the absence of alkaline hydrolysis, and had the following composition: Glu, 1.14; Thr, 0.89; Asp, 1.08; CySOH, 1.00; Tyr, 0.78. Edman degradation caused no significant change in composition. These properties are consistent with the formation of a pyrrolidone carboxyllysine peptide, derived by cyclization of a glutaminyl peptide, after papain cleavage of the glycylglutaminyl bond in the parent peptide. The second papain cleavage product, (O-Tryp 2)Chy 3Pap 2, formed in 46% yield, had the composition: Asp, 0.99; Gly, 1.04; Glu, 1.00; Thr, 0.05. After a single step of the Edman degradation, the aspartic acid value had fallen to 0.20, proving that this peptide had NH₂-terminal aspartic acid (or asparagine) and was derived from the NH₂ end of (O-Tryp 2)Chy 3.

The improved conditions for the Edman degradation do not cause imide formation from an internal asparagine residues. When asparagine is initially NH₂-terminal, rearrangement may occur during preparation of the peptide. Failure to obtain a

When (O-Tryp 4)Chy 3, a peptide possessing an internal asparagine residue, was dissolved in anhydrous trifluoroacetic acid for 8 hours at 25°, subsequent electrophoresis at pH 3.2, both before and after exposure of the peptide to pH 9, showed no change in mobility. The opening of an imide to give a β linkage would result in a detectable change in the acidity of a peptide.

Further evidence is given by the ability of the Edman degradation to provide an estimation of the molar ratios at the NH₂-terminal asparagine residue; perhaps imide formation occurred when the peptide was warmed in acid solution during desalting. Support for this possibility was furnished when the peptide mixture was chromatographed after three steps of the degradation. Two peptides were obtained. The one moving faster was desalted on Sephadex and found to contain the same amino acids as (O-Tryp 2)Chy 3, except that only one residue of aspartic acid was present. Measurement of the absorption spectrum of the peptide at 220 to 330 nm with a recording spectrophotometer suggested that the phenylthiohydantoin moiety was still present. Aspartic acid would be formed in only poor yield on hydrolysis of the phenylthiohydantoin in 6 M HCl for 22 hours, thus accounting for the apparent deficit of one residue of this amino acid. Further evidence for the formation of a β-aspartyl isomer of (O-Tryp 2)Chy 3 was provided by the isolation from the chymotryptic digest of O-Tryp 2 of a very acidic peptide, (O-Tryp 2)Chy 1 (also isolated by Hirs (11), Fig. 1E), which had the same amino acid composition as (O-Tryp 2)Chy 3. On Edman degradation of this peptide, one residue of aspartic acid was apparently lost on the first stage, but on a second degradation, no further change in composition occurred. This result would be expected if a β-linkage were present (1).

The data obtained by chemical and enzymatic degradation of (O-Tryp 2)Chy 3 document thoroughly the revised sequence given here. In the earlier work, the Edman degradation had doubtless halted after the second step because ring closure occurred at the new NH₂-terminal glutamine residue. Analysis of the residual peptide after the third step showed an artificial loss of threonine, but no loss of glutamic acid. This situation is analogous to the one encountered in the reinvestigation of the sequence in positions 11 to 18 (1). The sequence from residues 69 to 71, formerly reported as Thr-AspNH₂-GluNH₂, is thus GluNH₂-Thr-AspNH₂.

O-Tryp 6 — Glu-Thr-Gly-Ser-Ser-Lys (86-91)

Composition: Glu, 0.89; Thr, 0.89; Gly, 1.11; Ser, 1.69; lysine was not determined but its presence is confirmed by analyses of one of the papain cleavage products. The peptide was neutral on electrophoresis at pH 5.

Edman Degradation—Lysine not determined.

First step (94%): Glu, 0.12; Thr, 0.86; Gly, 1.00; Ser, 1.72.

Second step (99%): Glu, 0.06; Thr, 0.22; Gly, 1.00; Ser, 1.77.

Third step (95%): Glu, 0.05; Thr, 0.15; Gly, 0.36; Ser, 1.77.

Fourth step (90%): Glu, 0.05; Thr, 0.08; Gly, 0.23; Ser, 0.96.

Serine served as a basis for calculation at the third step, on the assumption that it was unchanged; similarly, glutamic acid served as a basis for an estimation of the molar ratios at the fourth step.

The peptide was hydrolyzed with papain, and the hydrolysate was chromatographed on a column (50 × 0.9 cm) of Dowex 50-XS at 30° with 0.2 M pyridinium formate at pH 4 and 2 M pyridinium formate at pH 8.8 as eluants. The first peptide to emerge, (O-Tryp 6)Pap 1, was obtained in 93% yield and had the

Further evidence is given by the ability of the Edman degradation to provide an estimation of the molar ratios at the NH₂-terminal asparagine residues.

Vol. 238, No. 1

Bovine Pancreatic Ribonuclease
following composition. Glu, 1.00; Thr, 0.98; Gly, 0.98. It represents the NH₂-terminal half of O-Tryp 6; the absence of a serine residue is in agreement with the sequence just given. Further confirmation was provided by the isolation in 89% yield of a second cleavage product, (O-Tryp 6)Pap 2, which had the composition: Ser, 1.80; Lys, 1.00.

The data obtained by both chemical and enzymatic degradation document the revision of the sequence from residues 87 to 89, formerly reported as Ser-Thr-Gly, to Thr-Gly-Ser. The earlier conditions for the Edman degradation probably had resulted in an O→N acyl shift by an acetyl group introduced on the threonine residue during the first cyclization. Further effective degradation was thus blocked, as discussed below. The former data obtained with leukine aminopeptidase were misleading because of the carboxypeptidase action of the impure enzyme (cf. Smyth et al. (1)).

O-Tryp 14 — Tyr–Pro–AspNH₂–CySO₃H–Ala–Tyr–Lys (92–98)

Composition: Tyr, 1.75; Pro, 1.00; Asp, 1.00; CySO₃H, 1.04; Ala, 0.97; lysine was not determined, but its presence is confirmed by the analyses of one of the papain cleavage products. On electrophoresis at pH 5, the peptide was found to be neutral.

Edman Degradation — Lysine not determined.

First step (94%): Tyr, 0.82; Pro, 0.98; Asp, 1.00; CySO₃H, 1.03; Ala, 1.00.

Second step (95%): Tyr, 0.90; Pro, 0.37; Asp, 0.98; CySO₃H, 1.03; Ala, 1.00.

Third step (96%): Tyr, 0.90; Pro, 0.10; Asp, 0.33; CySO₃H, 1.94; Ala, 1.00.

Fourth step (70%): Tyr, 0.91; Pro, < 0.04; Asp, 0.24; CySO₃H, 0.42; Ala, 1.00.

At this stage, the reaction mixture was chromatographed at 25°C on a column (5 × 0.4 cm) of Dowex 50-X2 with 0.2 M pyridinium formate at pH 5.1 as eluant. A peptide of the following composition was obtained in 68% yield: Ala, 1.00; Tyr, 0.91. Lysine was not determined, since presumably it was present as the ε-phenylthiocarbamyl derivative. After a single step of the Edman degradation, the molar ratio of alanine to tyrosine had fallen to 0.23:1.00.

The stepwise degradation in this instance shows the successful application of the modified method to peptides containing tyrosine. The progressive losses of nonterminal tyrosine encountered under the earlier conditions (4) are avoided.

The data establish the sequence CySO₃H–Ala (95–96) instead of the previously assigned Ala–CySO₃H. The Edman degradation at 100°C had probably been stopped by the formation of a β linkage at the asparagine residue at position 94. The revised sequence is in agreement with the earlier data obtained with carboxypeptidase (4).

The revised sequence was further checked by cleavage of O-Tryp 14 with papain. The fragments (Tyr, Pro, AspNH₂, CySO₃H) and (Ala, Tyr, Lys) were isolated in 30% yield. The compositions confirm the conclusion that a cysteic acid residue comes before the alanine residue.

O-Tryp 8 — Thr–Thr–GluNH₂–Ala–AspNH₂–Lys (98–104)

Composition: Thr, 1.75; Glu, 1.05; Ala, 1.07; Asp, 1.00; lysine was not determined, but its presence was confirmed by analysis of one of the papain cleavage products. On electrophoresis at pH 5, the peptide had a positive charge.

Edman Degradation — Lysine not determined.

First step (90%): Thr, 0.96; Glu, 0.99; Ala, 1.01; Asp, 1.00.

Second step (86%): Thr, 0.27; Glu, 0.99; Ala, 1.11; Asp, 1.00.

Third step (76%): Thr, 0.14; Glu, 0.47; Ala, 1.09; Asp, 1.00.

Fourth step (83%): Thr, 0.09; Glu, 0.22; Ala, 0.49; Asp, 1.00.

O-Tryp 8 was hydrolyzed with papain and the mixture chromatographed on a column (50 × 0.9 cm) of Dowex 30-X8 developed at 35°C with 0.2 M pyridinium formate, pH 4.0, and 2 M pyridinium formate, pH 5.8. Two peptides were obtained. The first, isolated in 74% yield, had the composition Thr, 2.14; Glu, 1.00. The second, obtained in 75% yield, had the composition Ala, 0.95; Asp, 1.00; Lys, 1.01. Unhydrolyzed O-Tryp 8 was isolated in 20% yield.

The sequence for O-Tryp 8 given in the preceding paragraph is well supported. In the previous work, the formation of N-acytylthreonine (see the following) probably halted degradation almost completely after the first step; further degradation of the peptide was completely halted after the second step by the cyclization of the newly formed NH₂-terminal glutamine residue to a pyrrolidine carboxylyl residue.

Confirmatory Results on Other Peptides — The results presented above in detail furnish the basis for the present revisions in the published structure of ribonuclease. From this knowledge, it was clear that the conclusions presented earlier to support some of the other sequences in the chain required reexamination. For this reason, the relevant peptides were isolated and subjected to Edman degradation by the modified procedure now in use. In each case, the published sequence has been confirmed. The experimental data are therefore not presented in detail but are summarized in Table 15.7.

Confirmatory results have also been obtained by new cleavages of a number of the peptides by papain to ascertain that the grouping of the residues is in full accord with the formula. For example, digestion of peptide (O-Tryp 16)Chy 4 (11 residues) with papain followed by chromatography on a Dowex 50-X2 column (150 × 0.9 cm) at 35°C with a gradient from 0.2 M pyridinium formate at pH 3.25 to 2 M pyridinium formate at pH 5.1 provided three fragments: ((O-Tryp 16)Chy 4)Pap 1 (residues 110 to 112; yield, 75%; CySO₃H, 1.04; Glu, 1.00; Gly, 0.98); ((O-Tryp 16)Chy 4)Pap 2 (residues 113 to 115; yield, 72%; Asp, 1.00; Pro, 0.92; Tyr, 0.93); and ((O-Tryp 16)Chy 4)Pap 3 (residues 105 to 109; yield, 48%; 72-hour hydrolysate; His, 0.99; Ileu, 1.96; Val, 1.06; Ala, 0.93). In addition, peptide (O-Tryp 16)Chy 4 (yield, 25%) was recovered unchanged. Edman degradations were performed on two of these peptides (Table 1). The results from papain cleavage confirm fully the sequence assigned to the first 11 residues in the 20-residue COOH-terminal section of the molecule.

Peptide (O-Tryp 9)Chy 4 was hydrolyzed with papain and on chromatography as in the preceding paragraph yielded two fragments: ((O-Tryp 9)Chy 4)Pap 1 (residues 56 to 58; yield, 66%; Ala, 0.92; Val, 1.03; CySO₃H, 1.06); and ((O-Tryp 9)Chy
Results of Edman degradations that confirm sequences reported previously

Revised sections of the sequence are covered in Smyth et al. (1) and in the text of this communication. The degradations were performed as described in detail in the text. All the peptides of the trypsin and trypsin-chymotrypsin series gave amino acid analyses in full agreement with those of Hirs et al. (4).

<table>
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<tr>
<th>Name</th>
<th>Position of residues</th>
<th>First step</th>
<th>Fraction of expected residue lost [%]</th>
<th>Yield</th>
<th>Second step</th>
<th>Fraction of expected residue lost [%]</th>
<th>Yield</th>
<th>Third step</th>
<th>Fraction of expected residue lost [%]</th>
<th>Yield</th>
<th>Fourth step</th>
<th>Fraction of expected residue lost [%]</th>
<th>Yield</th>
<th>Fifth step</th>
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</table>

* Progressive losses of threonine and phenylalanine observed previously are avoided.
† Analysis for the last residue in the peptide was performed without hydrolysis.

---

4) Pap 2 (residues 59 to 61; yield, 63%; Ser, 0.94; Glu, 1.00; Lys, 1.03). On electrophoresis at pH 5, the first fragment carried a negative, and the second a positive charge. The stepwise degradations (Table I) confirm the earlier results.

**Allocation of Amide Groups**—Each peptide isolated in the present study was submitted to paper electrophoresis (cf. Smyth et al. (1) for conditions) as a check on purity. The results thus obtained (indicating an acidic, neutral, or basic peptide) provided information as to whether or not the carboxyl group of an aspartic acid or a glutamic acid residue was amidated. The results in every instance were in agreement with the assignments of asparagine and glutamine residues by Hirs et al. (4), except for residues 11 to 18 (1). When several potentially acidic residues are present in the same peptide, the electrophoretic data are not definitive, and the decision rests on the analysis for asparagine and glutamine after hydrolysis by leucine aminopeptidase (4).

**Model Experiments with Serine Peptides and with Glutamine**—To understand more fully why misleading results were observed when the Edman procedure employed by Hirs et al. was applied to certain serine-, threonine-, or glutamine-containing peptides, several model experiments were performed with simpler substances.

In the first experiments, glycyl-serine and n-glutamine were subjected to the cyclization procedure involving the use of hot glacial acetic acid-HCl. The peptide (1.5 mg), glutamine (1.5 mg), and alanine (1.1 mg) as an internal standard, were dissolved in 10 ml of glacial acetic acid, and HCl gas (dried by passage through concentrated H₂SO₄) was bubbled through the solution at 100°. At hourly intervals, 0.5-ml portions were withdrawn and analyzed at pH 3.25 and 50° on the 55-cm column of the amino acid analyzer. After 1 hour, glutamine could not be detected, and glycylserine had undergone a 90% conversion to a more rapidly eluted, ninhydrin-positive product considered to be glycyl-O-acetyl-L-serine. When the experiment was repeated at 50°, glutamine was found to have a half-life of approximately 1 hour and glycylserine a half-life of approximately 1.3 hours. In a parallel experiment, the same substances were maintained in trifluoroacetic acid at 25° for 4 hours. Both glutamine and glycylserine appeared to be completely stable under these conditions.

Proof that O-acetylation of serine peptides can occur in hot glacial acetic acid-HCl was provided by the isolation of crystalline glycyl-O-acetylserine after this treatment. Glycyl-L-serine (100 mg) was subjected to the conditions cited in the preceding paragraph at 100°. Chloride was precipitated with silver acetate and, after centrifugation, the supernatant solution was concentrated to dryness under reduced pressure. The product, crystallized from water-acetone, gave an analysis corresponding to a monoacetyl glycylserine.

C₉H₁₇O₃N₂ (193.2)

Calculated: C 41.2, H 5.9
Found: C 40.9, H 5.9

The preparation of O-acetylseryine and O-acetylthreonine, by dissolving the amino acids in glacial acetic acid in the presence
of hydrogen chloride, has recently been reported. The derivatives were isolated in excellent yield and were fully characterized (17).

To ascertain whether the presence of O-acetylserine (or threonine) residues in a peptide could account for the artificial losses of serine and threonine in the experiments of Hirs et al. (4), a mixture of glycy1-L-serine (1.5 mg) and alanine (1.1 mg, internal standard) was submitted to the cyclization conditions used previously (glacial acetic acid-HCl, 100°, 1 hour). The residue, after removal of solvent by rotary evaporation, was maintained at pH 8 and 25° for 16 hours in a pH-Stat. A portion of the product was hydrolyzed with acid and submitted to amino acid analysis. The results were compared with those obtained when glycy1serine was hydrolyzed without any prior treatment. Similar experiments were performed with (O-Tryp 4)Chy 3, which contains three internal serine residues, and with (O-Tryp 2)Chy 4, which contains an internal threonine residue. No significant losses of serine or threonine were observed in any of these experiments. The fact that losses of serine and threonine do occur when the earlier conditions for the degradation are used has been confirmed (1). It appears that these losses were associated with the fact that peptides were hydrolyzed after each stage without prior purification. In the improved procedure, the subtractive analysis is carried out with a purified peptide; in this case, the recoveries of serine, threonine, and tyrosine after hydrolysis are normal.

Since O-acetylation of an internal serine or threonine residue in a peptide occurs during cyclization in glacial acetic acid-HCl, it is to be expected that when this residue becomes NH₂-terminal, an O → N acetyl migration would take place on neutralization of the solution. To test this possibility, L-seryl-L-leucine (2 mg) was subjected to the "cyclization" conditions described previously. The product, after removal of solvent by rotary evaporation, was dissolved in 5 ml of water and the solution was adjusted to pH 6.5 by the cautious addition of 0.1 M NaOH. The solution was kept in a pH-Stat at 25° and, at 30 minute intervals, the alkali uptake was noted and 0.5-ml portions were removed for ninhydrin analysis. The experiment was repeated at pH 5.5 and pH 7.0. At pH 6.5, the concentration of --NH₂ groups had decreased by 50% in approximately 1 hour and the rate of alkali uptake corresponded to this rate of disappearance. At pH 5.5, the rate of disappearance of --NH₂ groups was much slower; at pH 7, the rate was similar to that at pH 6.5. These data are consistent with the assumption that a transfer of acetyl groups from the serine hydroxyl to the serine amino groups can take place under these conditions.

Detailed kinetic studies on the rates of the O → N acyl shift in O-acetylserine and O-acetylthreonine have been reported (17). The data show that transacylation proceeds rapidly at pH values near neutrality. Similar findings were reported by Elliott (18) in a careful study on O → N transformations in silk fibroin. With O-acetyl peptides, the α-NH₂ groups of which possess pK values lower than those of the corresponding amino acids, the O → N shift would be expected to occur readily at a lower pH value.

Therefore, the most likely stage where the O → N acetyl shift might take place in the earlier procedure for the Edman degradation is during the 16-hour extraction of the aqueous peptide solution with ether (4). Under more alkaline conditions, saponification of the acetyl group may be a competitive reaction.

GENERAL CONCLUSIONS

The revisions in the sequence (Fig. 1) do not affect the conclusions as to the pairing of the half-cystine residues reported by Spackman et al. (8). The confirmatory results in Table I, together with the fuller understanding of the experimental methods, leave us no reason to doubt the correctness of the formul as given in Fig. 1. Nevertheless, it should be emphasized, as it was initially (4), that a formula derived by degradative experiments should be regarded as a working hypothesis until it is confirmed by other means, preferably by synthesis.

SUMMARY

The determination of the sequence of amino acid residues in performic acid-oxidized ribonuclease has been repeated for all sections of the molecule for which earlier methods might have given doubtful results. The use of improved techniques for the isolation of the peptides and their stepwise degradation has grown from the experience provided by the recent revision of the sequence of residues 11 to 18; some of the problems associated with the earlier conditions for the Edman degradation on peptides containing glutamine, asparagine, serine, and threonine residues made it desirable to determine whether such complications had affected the conclusions drawn concerning the sequence of any other parts of the chain. The previously proposed sequence, with the 11 to 18 revision, has been confirmed with four exceptions. The revised sections are the following: residues 69 to 71, --Glu-NH₂-Thr-AspNH₂-- 87 to 89, --Thr-Gly-Ser-; 94 to 96, --AspNH₂-Cys-Ala-- 101 to 103, --GluNH₂-Ala-AspNH₂- Experience with the Edman procedure used earlier has shown three principal sources of difficulty. NH₂-terminal glutamine residues cyclize readily to residues of pyrroldione carboxylic acid; a peptide of asparagine or aspartic acid may rearrange from an α- to a β-peptide; peptides containing serine or threonine are acetylated in HCl-acetic acid, and subsequently undergo an O → N acyl migration. These difficulties are not present under the conditions recommended by Konigsberg and Hill for the cyclization step and the purification of the resulting peptides.

Acknowledgment—The experiments reported in this study have been carried out with the skillful assistance of Miss Anne Potts. We are indebted to Mr. S. Theodore Bella for the elementary microanalyses.

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Derek G. Smyth, William H. Stein and Stanford Moore


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