Total Synthesis of Bovine Pancreatic Ribonuclease A. Part 6.[1] Synthesis of RNase A with Full Enzymic Activity †

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Protected bovine pancreatic RNase A was synthesized by azide condensation of the three fragments Z(OMe)-(RNase 21—124)-OBzl, Z(OMe)-(RNase 9—20)-$NHNH_2$, and Z-(RNase 1—8)-$NHNH_2$. After removal of the protecting groups by methanesulphonic acid, establishment of the disulphide bridges by aerial oxidation, and purification by affinity chromatography and ion-exchange chromatography on CM-cellulose, a synthetic protein was obtained which was indistinguishable by chemical and enzymatic criteria from natural RNase A. Comparable results were also obtained when HF was used as a deprotecting reagent. The unambiguous synthesis of a protein having full enzymic activity has thus been accomplished for the first time.

In the previous parts of this series,[1] we have described the synthesis of the protected S-protein Z(OMe)-(RNase 21—124)-OBzl and the protected S-peptide Z-(RNase 1—20)-$NHNH_2$, which cover the entire amino-acid sequence of bovine RNase A. We now report the synthesis of the protected RNase A, the removal of all protecting groups, and the subsequent establishment of the disulphide bridges. The synthetic protein obtained is indistinguishable from natural bovine RNase A by several chemical criteria, as well as by enzymic assay using specific substrates.

As an initial approach to the synthesis of the protected RNase, the azide condensation [2] of Z-(RNase 1—20)-$NHNH_2$ and a TFA-treated sample of Z(OMe)-(RNase 21—124)-OBzl was performed. In spite of earlier expectations, we encountered great difficulty in driving the reaction to completion. The reaction mixture remained ninhydrin positive, even after two additions of 6 equivalents of the acyl component at 72-h intervals. Acid hydrolysis of the isolated product revealed the incorporation of the S-peptide portion to be only *ca.* 22%. We therefore returned to our original strategy, *i.e.* building up the peptide backbone from small fragments, rather than condensation of the icosapeptide with the S-protein in one step.

As described previously,[1e] we synthesized the protected S-peptide from three fragments, Z-(RNase 1—8)-$NHNH_2$ (C), Z(OMe)-(RNase 9—13)-NHNH-Troc (B), and Z(OMe)-(RNase 14—20)-NHNH-Troc (A). We next examined the most efficient method of utilizing these.

Of the possible routes, it did not seem advantageous to condense fragment (A) with the S-protein unit, since this fragment still carries the base-sensitive Asp(OBu^t)-Ser sequence.[3,4] The following route was therefore chosen: condensation of the two sub-units (A) and (B) to form Z(OMe)-(RNase 9—20)-NHNH-Troc and, after removal of the Troc group, condensation with TFA-treated S-protein to obtain Z(OMe)-(RNase 9—124)-OBzl and condensation of (C) to obtain Z-(RNase 1—124)-OBzl, as illustrated in the Scheme.

Condensation of Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-$NHNH_2$, derived from (B), with the TFA-treated sample of Z(OMe)-Asp(OBu^t)-Ser-Ser-Thr-Ser-Ala-Ala-NHNH-Troc (A) proceeded smoothly, as reported,[1e] to give the protected dodecapeptide Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-NHNH-Troc, from which the Troc group was removed by Zn.[5] The resulting dodecapeptide hydrazide, Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met-(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-$NHNH_2$ (29) (positions 9—20), was selected as one fragment for the synthesis of RNase. As mentioned previously, the β-carboxy-protecting group of Asp was removed during the TFA treatment to suppress α ⟶ β rearrangement in the subsequent reactions.

Chain elongation of the S-protein unit was next carried out according to the Scheme. The Z(OMe) group was removed from Z(OMe)-(RNase 21—124)-OBzl by treatment with TFA [6] and the N^{α}-deprotected peptide was dissolved in DMSO–HMPA; as reported in the preceding paper, no single solvent dissolved the N^{α}-deprotected peptide. After neutralization with Et_3N, this amino-component was condensed with 10 equivalents of the acyl component derived from Z(OMe)-(RNase 9—20)-$NHNH_2$. After 72 h, an additional 10 equivalents of the acyl component were added. After an additional 48 h, the reaction mixture became almost ninhydrin negative, when tested by a t.l.c. scanner. In order to drive the coupling reaction to completion, two portions each of 5 equivalents of the azide were further added at intervals of 24 h. After 7 days, the product was precipitated from DMSO by addition of MeOH and purified by gel-filtration on Sephacryl S-200 [1c] with DMSO–H_2O (95 : 5 v/v) as eluant (Figure 1a). The unchanged acyl component used in excess was easily separated from the desired product. Examination of the product by t.l.c. was still unsuccessful, since the spot stayed at the origin with all solvent systems examined. However, no contamination by the acyl component was observed by t.l.c. Amino-acid ratios in the acid hydrolysate revealed nearly 100% incorporation of the acyl component, when the recovery of Phe or Leu (2 residues each) was taken as a standard for the calculation. The final condensation was that of this product, Z(OMe)-(9—124)-OBzl, with Z-(RNase 1—8)-

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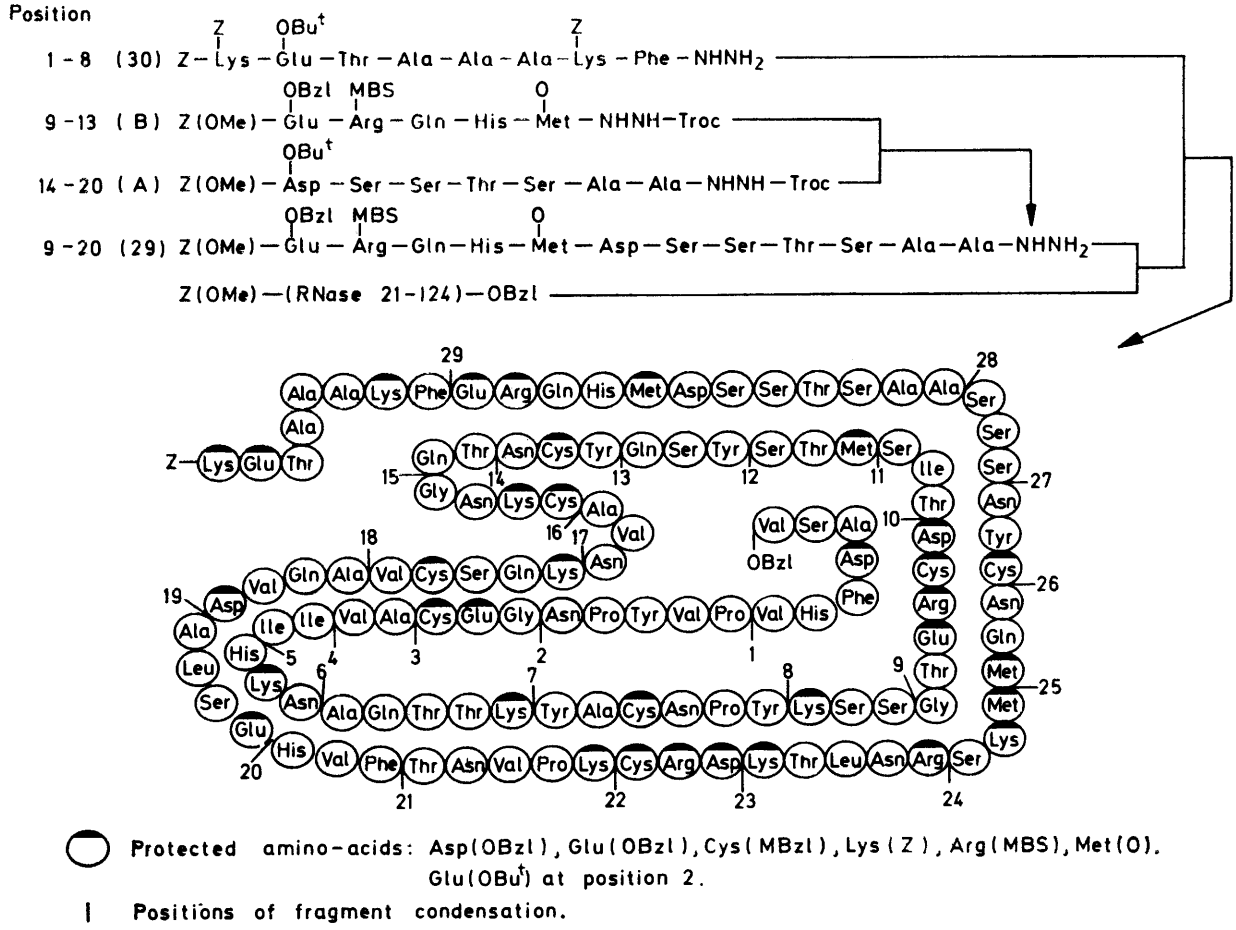
$NHNH_2$ (30) (C). The reaction was performed essentially as described above, using a total of 30 equivalents of the acyl component. The product was purified by gel-filtration on Sephacryl S-200 with DMSO–H_2O (95 : 5 v/v) as eluant (Figure 1b), when any unchanged acyl component was cleanly separated from the desired protected RNase.

In this final condensation, one Phe residue was incorporated into the chain. The amino-acid composition of the protected RNase could also be calculated by taking either the recovery of Leu or Phe as a standard. Two moles of Leu were incorporated into the peptide chain at positions 51 and 35 and the ratios of Leu(2) and Phe(2) were in excellent agreement with each other,



SCHEME Synthetic route to the protected tetracosahectapeptide ester, Z-(RNase 1—124)-OBzl (protected RNase)

Amino-acid ratios of synthetic RNase and intermediates

	Protected peptides			Air	Affinity-purified		CM-	Natural
	9—124	1—124	Reduced	oxidized	Peak 1	Peak 2	purified	RNase
Asp	15.36 (15)	15.34 (15)	14.84	16.17	13.75	15.54	15.06	14.99 (15)
Thr	8.52 (9)	9.39 (10)	9.00	8.99	8.84	9.89	9.90	9.97 (10)
Ser	12.17 (15)	11.93 (15)	11.66	10.88	12.63	14.61	13.95	14.37 (15)
Glu	11.67 (11)	12.64 (12)	12.08	12.27	12.05	12.59	12.48	12.44 (12)
Pro	3.78 (4)	4.11 (4)	4.10	3.52	4.24	4.43	4.35	4.43 (4)
Gly	3.09 (3)	3.09 (3)	3.02	3.17	3.27	3.73	3.31	3.40 (3)
Ala	8.89 (9)	12.11 (12)	11.84	11.65	10.81	11.90	12.59	12.16 (12)
Cys	3.11 (4)	2.82 (4)	3.50	2.27	2.20	3.25	3.65	3.72 (4)
Val	8.71 (9)	8.71 (9)	8.53	8.71	8.00	8.81	9.25	8.88 (9)
Met	3.33 (4)	3.31 (4)	3.68	1.75	3.00	3.08	3.65	3.85 (4)
Ile	1.94 (3)	1.99 (3)	2.13	1.96	1.84	2.08	2.35	2.31 (3)
Leu	2.00 (2)	2.00 (2)	2.00	2.00	2.00	2.00	2.00	2.00 (2)
Tyr	6.32 (6)	6.19 (6)	6.06	2.49	2.98	5.65	5.82	5.66 (6)
Phe	2.03 (2)	3.04 (3)	2.91	2.37	1.87	2.59	3.03	3.09 (3)
Lys	8.37 (8)	10.57 (10)	10.17	11.97	8.88	10.85	10.67	10.82 (10)
His	3.20 (4)	3.36 (4)	3.24	3.11	2.80	3.29	3.93	4.03 (4)
Arg	3.92 (4)	3.87 (4)	3.68	4.10	3.45	4.13	4.00	4.20 (4)
Recovery (%)	88	85	95	70	73	77	79	80

prior to the final condensation. Their ratios, Leu(2) : Phe(3), were now found to be 1.97 : 3.00. The coupling efficiency in the final step was thus judged to be quantitative (Table).

Starting with 1.6 g of the protected S-protein, we were able to obtain 0.98 g of the protected RNase having a high degree of homogeneity. Of the 30 successive fragment condensations performed in the present synthesis, the most laborious step was the condensation of the relatively large fragment (29) with the N^{α}-deprotected S-protein. Each of the 30 fragments and their 97 intermediates were fully characterized by elemental analysis. Each fragment condensation was performed at least three times so as to obtain sufficient material. All products were examined by amino-acid analysis. It must be emphasized that our synthesis of the protected RNase is supported by a considerable accumulation of chemical evidence, as reported in this series of six papers.

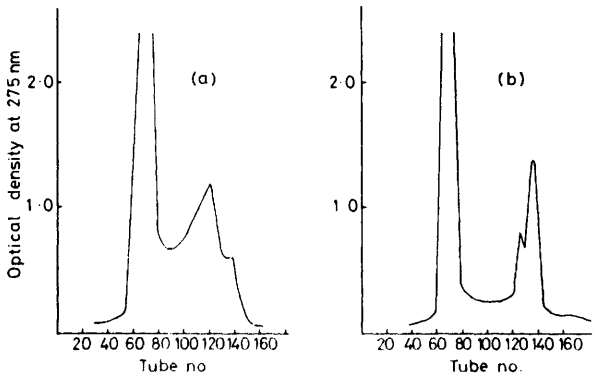


FIGURE 1 Purification of (a) Z(OMe)-(RNase 9—124)-OBzl and (b) Z-(RNase 1—124)-OBzl by gel-filtration on Sephacryl S-200

As stated in Part 1,[1a] use of methanesulphonic acid (MSA) [7] was our original plan for the deprotection step of the synthesis. Prior to the deprotection, the first problem with which we were concerned was whether cysteine could be recovered from Cys(MBzl) using this acid. In the presence of various cation scavengers, Z(OMe)-Cys(MBzl)-OH was treated with MSA, as well as with HF [8] for comparison, and the treated samples were subjected to amino-acid analysis. When the sample was treated with MSA in the presence of anisole or *m*-cresol, a nearly quantitative recovery of cysteine was obtained; however in the presence of thioanisole or other sulphur compounds the samples gave several unidentified peaks and recovery of cysteine remained less than 75%. Thus it became apparent that cation scavengers play an important role in the satisfactory recovery of cysteine from Cys(MBzl) in the MSA deprotecting procedure. In our experiments, the deprotection using HF–anisole gave some side products, but a satisfactory result was obtained when the HF–*m*-cresol system was employed. Considering also the recent investigations of the scavenger system reported by Lundt *et al.*[9] (see later), we decided to try two methods of deprotection, *i.e.* MSA and HF with *m*-cresol as a cation scavenger.

It has been reported that when acid hydrolyses of protected peptides containing Cys(MBzl) are performed in the presence of phenol,[10] an unidentified peak with a retention time of 28 min occasionally appears on the short column of the amino-acid analyser. Under these conditions our synthetic protected RNase was also found to give this same peak. Through model experiments, this compound was identified as *p*-hydroxyphenylcysteine,[11] derived from the sulphoxide of Cys(MBzl) in the 6N-HCl–phenol system. It was also obtained when the sulphoxide of Z(OMe)-Cys(MBzl)-OH was exposed to MSA, as well as to HF, in the presence of phenol. When this treatment was performed in the presence of anisole, *p*-methoxyphenylcysteine was obtained in both acidolytic deprotections. Z(OMe)-Cys(MBzl)-OH, containing no sulphoxide group, gave no such side-product, even in the presence of phenol or anisole.

These results suggested that the Cys(MBzl) residues of our synthetic protected RNase were partially oxidized to the corresponding sulphoxide during the synthesis, and that satisfactory recovery of cysteine could never be expected unless reduction preceded deprotection. The use of thiophenol as a reducing agent overcame this difficulty. However, under these conditions, Met(O) residues [12] were also reduced to Met, thus removing their protection from attack by carbonium cations resulting from exposure of the Z, Bzl, or MBzl group to MSA. Fortunately, the *S*-alkylated compounds, if formed, can be removed by incubation with thiols.[13] We believe that, in addition to the reduction of the sulphoxide of Cys(MBzl) prior to deprotection, the selection of *m*-cresol instead of anisole as a scavenger is one of the elements which guides our synthesis of RNase to its successful completion.

It is known that in the MSA–anisole system the methyl group of anisole is transformed into Met to form the *S*-methyl-Met sulphonium salt;[14] however, the MSA–cresol system has no property as an alkyl donor. Furthermore, *m*-cresol is more efficient than anisole in not only suppressing the side-reaction of Cys(MBzl) mentioned above, but also another side-reaction involving modification of the Tyr residue [15] during the MSA and HF deprotection, *i.e.* partial *O*-sulphonation of Tyr by Arg(MBS) and partial intermolecular alkylation at the 3-position by attack of carbonyl cations derived from the protecting groups. Intramolecular alkylation by Tyr(Bzl) is also a well-known important side-reaction;[8,16] however, since unprotected Tyr was used in the present synthesis, this was of no concern.

As we have pointed out, *m*-cresol suppresses most side-reactions which may occur during the MSA deprotection. In addition, in deprotection with acid, one has to take into account the N ⟶ O shift at the Ser and Thr residues. This side-reaction was reported to occur during HF deprotection [17,18] and also during MSA deprotection by Fujino *et al.*[19] Treatment with mild base is known to be

effective in reversing this rearrangement. Another possible side-reaction is the α→β rearrangement of Asp(OBzl) or Asp(OBu^t).[3] We removed the protecting group from Asp at position 14 adjacent to Ser, since this residue is known to cause side-reactions of this type. Although Suzuki *et al.*[20] found little tendency for these side-reactions to occur during MSA deprotection, their complete prevention seems impossible.

The protected RNase was treated with thiophenol to reduce the partially formed sulphoxide of Cys(MBzl), using Z(OMe)-Cys(MBzl)(O)-OH and Z(OMe)-Met(O)-OH as the markers. The Met(O) residues were also reduced under these conditions. We first describe the MSA deprotection.

The reduced peptide under nitrogen was exposed to MSA in the presence of *m*-cresol at 25 °C for 60 min. The amount of the scavenger was estimated as 20 equivalents per protecting group (33 protecting groups). Thus a total of 660 equivalents were employed. In order to remove the large number of protecting groups [*i.e.* 8 × Cys(MBzl), 4 × Glu(OBzl), 1 × Glu(OBu^t), 4 × Asp(OBzl), 4 × Arg(MBS), 10 × Lys(Z), and the N and C-terminal protecting groups Z and Bzl], this treatment was repeated. Drastic conditions seemed necessary for the complete removal of so many protecting groups to avoid obtaining a complicated mixture of partially deprotected peptides. The deprotected peptide was then reduced with 2-mercaptoethanol and dithiothreitol in 0.2M-Tris-HCl buffer containing 4M-guanidine-HCl at pH 8.6. This pH was that selected by Anfinsen and Haber [21] for reductive cleavage of the disulphides of natural bovine RNase. These basic conditions seemed suitable to us for the prevention of any possible N ⟶ O shifts at the Ser and Thr residues. This treatment was also judged effective for reduction of any remaining Met(O) residues, if present, and for removal of the *S*-alkyl groups from the Met residues, if such products were formed during the deprotection. Any aggregated disulphide complex, if formed, could then be reduced during this treatment.

The reduced product was next isolated by gel-filtration on Sephadex G-25 with 0.1N-AcOH as eluant. The reduced product emerged from the column as a single component and the eluates of the desired fractions were then diluted in 0.05M-Tris-HCl buffer at pH 8.2 to a concentration of 0.02 mg ml^{-1}. The dilute solution was kept at 25 °C for 2 days to establish the 4 intramolecular disulphide bridges,[22] the progress of the oxidation being monitored by Ellman's reagent.[23] The entire solution was concentrated by lyophilization and the oxidized product was then dialysed against distilled water for desalting. Some water-insoluble material, presumably highly aggregated products or materials still bearing protecting groups, was removed at this stage. The oxidized product, isolated by gel-filtration on Sephadex G-75 with 0.05M-NH_4HCO_3 as eluant [Figure 2(a)], had 9—12% of the activity of natural RNase on yeast RNA.[24] Starting from the protected RNase, the yield of crude, but active, product was 54%. Gutte and Merrifield [25] attempted to purify the deprotected peptide through the corresponding *S*-sulphonate. Our preliminary test indicated that the oxidative sulphitolysis [26] necessary to prepare the *S*-sulphonate converted Met to Met(O) in 10% yield. Thus, we felt that this procedure would only be effective in purifying Cys-peptides which contain no Met, such as insulin,[27] and would not be applicable to our present synthesis.

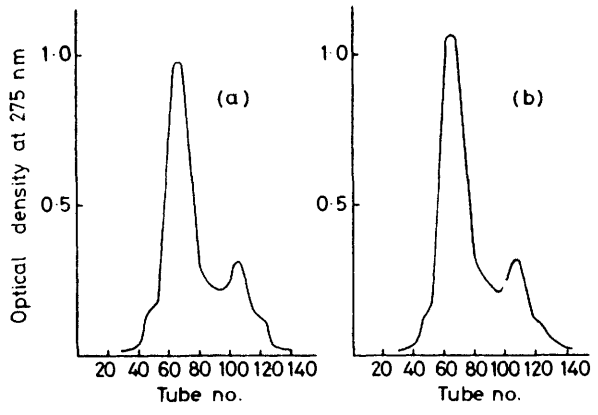


FIGURE 2 Purification of the air-oxidized product by gel-filtration on Sephadex G-75: (a) MSA-deprotected product, (b) HF-deprotected product

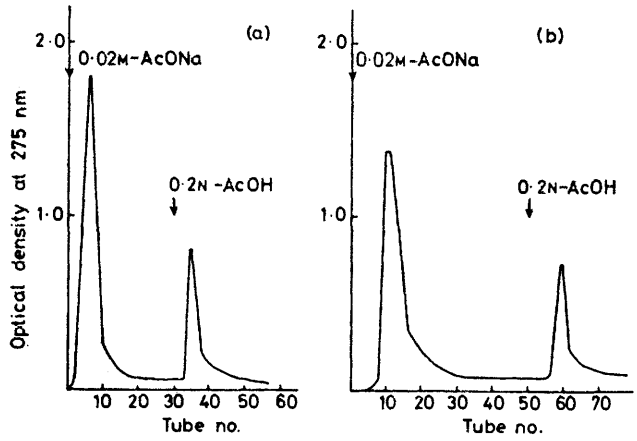


FIGURE 3 Purification of the G-75-purified product by affinity chromatography on Sepharose-(4B)-5′-(4-aminophenylphosphoryl)-uridine-2′(3′)-phosphate: (a) MSA-deprotected product (b) HF-deprotected product

We next decided to purify the crude active product by affinity chromatography on Sepharose-(4B)-5′-(4-aminophenylphosphoryl)uridine-2′(3′)-phosphate.[28] This procedure worked well [Figure 3(a)]. The material isolated from the fractions which passed through the column without retention was inactive. The product eluted with 0.2N-AcOH exhibited an activity of 74—82% against yeast RNA, when examined at three different concentrations. The yield of this purification step was 14%. Thus, to reach this point, approximately 92% of material was lost during the conversion of the inactive protected peptide into the enzymatically active product. Various side reactions involved in the deprotection and

the misleading disulphide bridge formation may be the main reasons responsible for lowering the yield.

Examination of the active component by disc electrophoresis at pH 4.3 revealed the presence of a single band, which was matched with that of natural RNase, and an adjacent faint band. When the amino-acid compositions in the acid hydrolysate of active and inactive compounds were compared, no significant differences were observed, except for recoveries of Tyr and Cys (Table). Recoveries of these two amino-acids in the active component were much closer to those of natural RNase, compared to those of the inactive component. The activity we obtained at this stage was nearly equivalent to that reported by Gutte and Merrifield [25] who reported that the supernatant of the ammonium sulphate fractionation of the trypsin-resistant material exhibited an activity of 78%. This solution, which was estimated to contain 0.41 mg of a protein, was claimed to be the most active component which they obtained. However, this fraction was not chemically characterized.

In order to remove the trace contaminant revealed by disc-electrophoresis, we submitted the affinity-purified product to ion-exchange chromatography on CM-cellulose.[22a] The chromatographic pattern eluted by a gradient with pH 7.5 (0.1M-sodium phosphate buffer) revealed the presence of a major peak with a small shoulder to the fore [Figure 4(a)]. The small front fractions were discarded. The main fractions were desalted by gel-filtration on Sephadex G-25 and the product was examined by disc electrophoresis and amino-acid analysis. The purified product behaved as a single component in the field of disc electrophoresis at pH 4.3 and its mobility was identical with that of natural RNase. The amino-acid compositions of the synthetic protein was finally in excellent agreement with that of RNase, including those of Tyr and Cys residues (Table). The synthetic protein, with a high degree of homogeneity, exhibited essentially the same activity as that of natural RNase, when yeast RNA was used as a substrate and the activity measured at three different concentrations. The values obtained were in the range 104—107%.

Next, the physicochemical properties and biological activities of our synthetic protein were examined. We first confirmed that the elution pattern of the synthetic protein from the CM-cellulose column was comparable to that of natural RNase. In addition to the mobilities of both compounds on disc electrophoresis, we confirmed that the specific rotation, u.v. absorption, and Michaelis constant of our synthetic protein were coincident with those of natural RNase.[22a,29]

In order to examine further the specific enzymatic activity of the synthetic protein, the activity against 2',3'-cyclic cytidine phosphate [30] was examined. Results comparable to that of natural RNase were obtained in this assay. The synthetic protein had no ability to digest calf-thymus DNA.[31] We were thus able to ascertain the specific activity of our synthetic enzyme by three different assay systems.

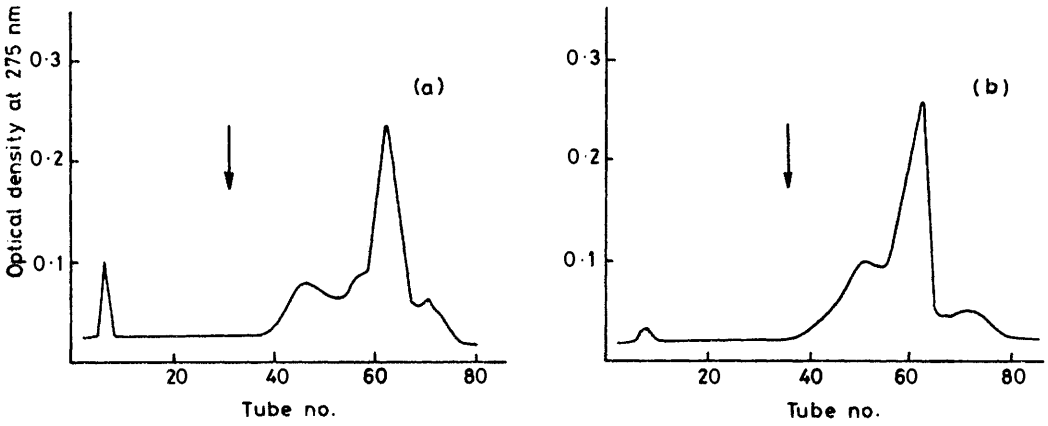


FIGURE 4 Purification of the affinity-purified product by ion-exchange chromatography on CM-cellulose: (a) MSA-deprotected product, (b) HF-deprotected product. Arrow indicates start of gradient elution with 0.1M-phosphate buffer (pH 7.5)

In order to confirm the results obtained from the MSA deprotection, HF deprotection was conducted in the presence of *m*-cresol and the product was treated in the manner mentioned above. The crude air-oxidized product isolated by gel-filtration on Sephadex G-75 [Figure 2(b)] possessed a slightly higher activity (17%) than that of the MSA-deprotected product, although the amount of water-insoluble material was somewhat greater than in the former case. The affinity-purified product [Figure 3(b)] was as active as that of the MSA-deprotected product (82%). After purification on CM-cellulose [Figure 4(b)], we were able to obtain the fully active component in nearly the same yield.

From the experimental data cited above, it can be ascertained that our synthetic protein has a high degree of homogeneity and possesses physicochemical properties and specific enzymatic activity comparable to those of natural RNase. We conclude that the unambiguous synthesis of a protein with full activity has thus been accomplished for the first time.

EXPERIMENTAL

General experimental procedures are essentially the same as described in Part 1[1a] of this series. The azide condensation was performed according to Honzl and Rudinger.[2] A large excess of the azide component was employed by subdividing into 2 to 4 portions in a certain interval. For measurement of a small amount of Et_3N and *N*-methylmorpholine, Pipetman P200 (Gilson Co) was used. For dialysis, cellulose tubing [VT351 (Lot No. V9H-1894)] was purchased from Nakarai Chemical Co. For column chromatography a Dainihon Seiki model DNS-DFC-100 fraction collector was used. U.v. absorption was measured by a Hitachi model 200-20 spectrophotometer. A Hitachi wavelength-tunable effluent monitor (034-0029) was used to determine the enzymic activity against 2′,3′-cyclic cytidine phosphate. For affinity chromatography, Sepharose-(4B)-5′-(4-aminophenylphosphoryl)-uridine-2′(3′)-phosphate, was prepared according to Wilchek and Gorecki.[28] Natural bovine pancreatic RNase (Type 1-A, Lot No. 47C-0422), 2′,3′-cyclic cytidine monophosphoric acid sodium salt (Lot No. 76C-7510), bovine pancreatic DNase I (Lot No. 127C-0347) and yeast RNA (Type XI, Lot No. 124C-8150) were purchased from Sigma Chemical Co. Calf-thymus DNA (Lot No. 38N687P) was purchased from Worthington Biochemical Co.

Condensation of Z-(RNase 1—20)*-NHNH$_2$ and H-(RNase* 21—124)*-OBzl.*—Z(OMe)-(RNase 21—124)-OBzl (213 mg, 14.2 μmol) was treated with TFA–anisole (1.8 ml; 5 : 1 v/v) in an ice-bath for 90 min, then dry ether was added. The resulting powder was washed with ether, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMSO–HMPA (1 : 1 v/v; 1 ml) containing 10% Et_3N in DMF (79 μl, 56.8 μmol). To this ice-chilled solution were added the azide [from Z-(RNase 1—20)-NHNH$_2$ (253 mg, 86.9 μmol)] in DMF–DMSO–HMPA (1 : 1 : 1 v/v; 1.5 ml) and 10% Et_3N in DMF (0.12 ml, 86.9 μmol) and the mixture was stirred at 4 °C for 72 h. Additional azide (prepared from the same amount of the hydrazide) in DMF–DMSO–HMPA (1.5 ml) and 10% Et_3N in DMF (0.12 ml) were added and stirring was continued for a further 72 h. Although the solution still remained positive to ninhydrin, H_2O (60 ml) was added. The resulting powder was collected by filtration, washed with 3% AcOH, 5% $NaHCO_3$, and H_2O, and precipitated from DMSO with MeOH. The product was dissolved in 5% H_2O–DMSO and the solution was applied to a column of Sephacryl S-200 (3 × 142 cm), which was eluted with the same solvent. Individual fractions (each 5.1 ml) were collected and the absorption at 275 nm was determined. Two peaks were detected; peak 1 (154 mg, tube nos. 55—75, a mixture of the protected RNase and the unchanged amino-component) and peak 2 (299 mg, tube nos. 116—131, the rearrangement product of the azide). Amino-acid ratios in acid hydrolysate of the peak 1 substance: Asp 14.46, Thr 8.15, Ser 10.74, Glu 10.19, Pro 3.74, Gly 3.00, Ala 8.17, Val 8.64, Met + Met(O) 2.66, Ile 1.96, Leu 2.00, Tyr 6.08, Phe 2.22, Lys 8.91, His 2.96, Arg 3.27 (recovery of Leu 85%). Incorporation of new amino-acids was *ca.* 22%.

Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-NHNH$_2$, Z(OMe)-(RNase 9—20)*-NHNH$_2$* [29].—Z(OMe)-(RNase 9—20)-NHNH-Troc (4.77 g, 2.45 mmol) dissolved in a mixture of DMF–HMPA (1 : 4 v/v; 50 ml) and AcOH (10 ml) was treated with Zn (1.6 g, 10 g atom equiv.) at room temperature for 5 h, during which time the starting material disappeared (t.l.c.). Some material which precipitated during the reaction was dissolved by slight warming to 40 °C and the solution was filtered. The filtrate was concentrated and H_2O (200 ml) was added. The resulting oily precipitate was treated with a saturated solution of EDTA. Storage in a refrigerator afforded a powder, which was precipitated from DMSO with MeOH to afford the *protected dodecapeptide hydrazide* (3.37 g, 78%), m.p. 246 °C (decomp.) $[\alpha]_D^{25}$ −17.2° (*c*, 0.9 in DMSO), R_{F_3} 0.62. Amino-acid ratios in acid hydrolysate: Glu 2.08, Arg 1.10, His 1.12, Met + Met(O), 0.74, Asp 1.03, Ser 2.74, Thr 0.97, Ala 2.00 (average recovery 91%) (Found: C, 48.3; H, 6.0; N, 14.7. $C_{73}H_{104}N_{20}O_{28}S_2 \cdot 3H_2O$ requires C, 47.96; H, 6.07; N, 15.33%).

Z(OMe)-(RNase 9—124)*-OBzl, [Z(OMe)-Glu(OBzl)-Arg-(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-*(21—124)*-OBzl].*—Z(OMe)-(RNase 21—124)-OBzl (829 mg, 55.1 μmol) was treated with TFA–anisole (7 ml; 6 : 1 v/v) and the N$^{\alpha}$-deprotected peptide, isolated as above, was dissolved in DMSO–HMPA (1 : 1 v/v; 6 ml) containing Et_3N (30 μl, 220 μmol). To this ice-chilled solution were added the azide [from Z(OMe)-(RNase 9—20)-NHNH$_2$ (977 mg, 10 mol equiv.) in DMSO–DMF (1 : 1 v/v, 4 ml) and Et_3N (76 μl, 551 μmol) and the mixture was stirred at 4 °C for 72 h. Three additions of the azide (10, 5, and 5 mol equiv.) were carried out at 48 and 24 h intervals. After the final addition of the azide, the mixture was stirred for a further 24 h, while the ninhydrin intensity on the origin of the t.l.c. plate disappeared, when examined by a Shimadzu dual-wavelength t.l.c. scanner. The mixture was then stirred at room temperature for 6 h. Addition of H_2O (60 ml) afforded a powder, which was collected by filtration, washed with 3% AcOH, and precipitated from DMSO with MeOH to give the crude product (2.41 g). The crude product (500 mg each), dissolved in 5% H_2O–DMSO (5 ml), was applied to a column of Sephacryl S-200 (5 × 126 cm), which was eluted with the same solvent at a flow rate of 72 ml h^{-1}. Individual fractions (each 15 ml) were collected and the absorption at 275 nm was determined. Fractions of the front peak [Figure 1(a), tube nos. 56—80] were combined, the solvent was evaporated off, and the residue was precipitated from DMSO with MeOH to give a *powder* (620 mg, 68% total yield after 5 purifications), m.p. 242 °C (decomp.) $[\alpha]_D^{25}$ −26.8° (*c*, 0.7 in DMSO), R_{F_3} 0 (Found: C, 51.9; H, 5.85; N, 12.4. $C_{765}H_{1047}N_{161}O_{225}S_{16} \cdot 64H_2O$ requires C, 51.72; H, 6.67; N, 12.70%).

Z-(RNase 1—124)*-OBzl, [Z-Lys(Z)-Glu(OBu^t)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-*(9—124)*-OBzl, Protected RNase.*—Z-(OMe)-(RNase 9—124)-OBzl (616 mg, 37.1 μmol) was treated with TFA–anisole (7 ml; 6 : 1 v/v) and the N$^{\alpha}$-deprotected peptide, isolated as above, was dissolved in DMSO–HMPA (1 : 1 v/v, 5 ml) containing Et_3N (31 μl, 223 μmol). To this ice-chilled solution were added the azide [from Z-(RNase 1—8)-NHNH$_2$ (658 mg, 10 mol equiv.)] in DMSO–DMF (1 : 1 v/v, 2 ml) and *N*-methylmorpholine (41 μl, 371 μmol), and the mixture was stirred at 4 °C for 72 h. Three additions of the azide (10, 5, and 5 mol equiv.) were carried out at 48 and 24 h intervals. After the final addition of the azide, stirring was continued for an additional 24 h. The solution was then stirred at room temperature for 6 h and H_2O (100 ml) was added. The resulting powder was collected by filtration, washed with 3% AcOH, and precipitated twice from DMSO with MeOH to give the crude product. The product was purified by column chromatography on Sephacryl S-200 (5 × 132 cm) with 5% H_2O–DMSO as eluant. Individual fractions

(each 15 ml) were collected and the absorption at 275 nm was determined. Fractions corresponding to the front peak [Figure 1(b), tube nos. 60—85] were collected and the solvent was removed by evaporation. Treatment of the residue with MeOH afforded a *powder* (505 mg, 77%), m.p. 233 °C (decomp.), $[\alpha]_D^{25}$ −25.1° (*c*, 0.5 in DMSO), R_{F_s} 0 (Found: C, 53.15; H, 6.45; N, 12.8. $C_{823}H_{1127}N_{171}O_{239}S_{16}\cdot 42H_2O$ requires C, 53.40; H, 6.59; N, 12.94%).

Reduction of the Sulphoxides of Cys(MBzl) and Met Residues of the Protected RNase.—Thiophenol (3.72 ml, 1 280 mol equiv.) was added to a solution of the protected RNase (502 mg, 28.3 μmol) in HMPA–NMP (2 : 1 v/v; 10 ml) and the solution was incubated under N_2 at 90° for 25 h, while standard samples of Z(OMe)-Cys-(MBzl)(O)-OH and Z-(OMe)-Met(O)-OH were completely reduced under identical conditions. It was confirmed that Z(OMe)-Asp(OBzl)-OH and Z(OMe)-Glu(OBzl)-OH remained intact under these conditions. The solvent was evaporated off and the residue was treated with MeOH. The resulting powder was precipitated from HMPA with MeOH to give the reduced protected RNase (452 mg, 90%), m.p. 239 °C (decomp.), $[\alpha]_D^{25}$ −24.4° (*c*, 0.7 in DMSO). Recovery of cystine in acid hydrolysates of the protected RNase, before and after reduction, were 2.82 and 3.50 (theoretical value 4).

Deprotection of the Protected RNase by MSA.—Under N_2, the reduced RNase, Z-(RNase 1—124)-OBzl (100 mg, 5.63 μmol) was treated with freshly distilled MSA (3.9 ml) in the presence of *m*-cresol (0.39 ml, 660 mol equiv.) in an ice-bath for 10 min and at 25 °C for 60 min. Dry ether was added and the resulting powder was retreated with MSA under conditions identical with those above. The deprotected peptide, precipitated by ether, was then dissolved in an 0.2M-Tris–HCl buffer (3 ml) containing 4M-guanidine HCl and the pH of the solution was adjusted to 8.6 with 5% methylamine in H_2O. After addition of 2-mercaptoethanol (0.35 ml, 800 mol equiv.) and dithiothreitol (174 mg, 200 mol equiv.), the solution was incubated, under N_2, at 50 °C for 24 h, while a marker, *S*-methylmethionine, was completely reduced to methionine. 2-Mercaptoethanol (0.18 ml, 400 mol equiv.) was further added and the solution was incubated for an additional 40 h. The pH of the solution was then adjusted to 3 with 3N-HCl and the solution was applied to a column of Sephadex G-25 (1.6 × 90 cm), which was eluted with 0.1N-AcOH. Individual fractions (each 4 ml) were collected and absorptions at 275 nm were determined. Fractions (tube nos. 23—40) containing the reduced substance were then subjected to air oxidation.

Air Oxidation of the Deprotected Peptide.—The fractions (tube nos. 23—40) obtained from gel-filtration on Sephadex G-25 were combined and the solution was diluted with H_2O (3 l) and the entire volume was brought up to 4 l with 0.2M-Tris–HCl buffer at pH 8.2. The protein concentration of this solution was 0.02 mg ml^{-1}. The solution was kept standing at room temperature for 2 days, during which time the Ellman test dropped from 0.097 to the constant value, 0.001. After adjusting the pH to 3.0 with 2M-HCl, the entire solution was lyophilized. The residue was dialysed with cellulose tubing against distilled H_2O (1 l) three times for desalting and the solution was lyophilized to give a hygroscopic powder (169 mg). The crude oxidized substance was dissolved in 0.05M-NH_4HCO_3, pH 8.4 (2 ml), and some insoluble material (*ca.* 6 mg) was removed by centrifugation. The supernatant solution was applied to a column of Sephadex G-75 (2.8 × 95 cm), which was eluted with the same buffer. Individual fractions (each 5.5 ml) were collected and their absorptions at 275 nm were determined. The compound emerged from the column as a single component with slight tailing. The fractions corresponding to the main peak [Figure 2(a), tube nos. 57—80] were combined and the solvent and NH_4HCO_3 were removed by repeated lyophilization to give a fluffy powder (42.5 mg, 54%). The activity against yeast RNA was 12%.

In the preliminary run, starting from the protected RNase (56 mg), the yield of the crude oxidized product was 28.2 mg and the activity was 9%.

Affinity-chromatographic Purification of the Oxidized Product.—The oxidized product (40.1 mg) was dissolved in 0.02M-AcONa (pH 5.2) (1 ml) and the solution was applied to a column of Sepharose-(4B)-5′-(4-aminophenylphosphoryl)-uridine-2′(3′)-phosphate (0.8 × 12.3 cm, Vb 6.2 ml). The first eluant used was 0.02M-AcONa, pH 5.2, with which one peak was detected at 275 nm, when individual fractions (each 3 ml) were collected. The fractions corresponding to this peak [Figure 3(a), tube nos. 3—15] were collected and the solution was lyophilized. The residue was desalted by gel-filtration on Sephadex G-25 with 0.1 N AcOH as eluant. Lyophilization gave a fluffy powder (22.4 mg, inactive). After elution of peak 1, the column was eluted with 0.2N-AcOH. The 2nd peak [Figure 3(a), tube nos. 33—40] was detected. Lyophilization of the solvent of peak 2 followed by desalting on Sephadex G-25 afforded a white fluffy powder (5.5 mg, 14%). The yield of this affinity-purified product from the protected RNase was 7.4%. The activity against yeast RNA was 82% (85% in 2 μg per 2 ml, 78% in 4 μg per 2 ml and 84% in 8 μg per 2 ml).

Purification of the Affinity-purified Product by Ion-exchange Chromatography on CM-cellulose.—The affinity-purified sample (4.61 mg) was dissolved in a small amount of 0.01M-sodium phosphate pH 6.0 buffer and the solution was applied to a column of CM-cellulose (1 × 11.8 cm), which was eluted with the same buffer (64 ml) and then by gradient elution with pH 7.5 0.1M-phosphate buffer (250 ml) through a mixing flask containing the above 0.01M-phosphate buffer (120 ml). Individual fractions (2.0 ml each) were collected and the absorption at 275 nm was determined. Fractions corresponding to the main peak [Figure 4(a), tube nos. 59—65], present in the gradient eluates, were combined and the solvent was removed by lyophilization. The residue was desalted by gel-filtration on Sephadex G-25 with 0.1N-AcOH as eluant. After lyophilization, the product was obtained as a white fluffy powder (yield 2.82 mg, 61%). Amino-acid ratios in the 6N-HCl (48 h) hydrolysate are given in the Table (Found: C, 41.55; H, 3.75; N, 13.7%); $[\alpha]_D^{23}$ −70.8° (*c*, 0.7 in 0.1M-KCl) (lit.,[22a] −73.3° in 0.1M-KCl).

Comparison of Synthetic RNase and Natural RNase.—(a) *Chromatographic comparison on CM-cellulose.* Natural RNase (6.1 mg) was applied to a column of CM-cellulose (1 × 11.8 cm), which was eluted with pH 6.0, 0.01M-sodium phosphate buffer and subsequently by gradient elution with pH 7.5 0.1M-phosphate buffer as mentioned in the purification of synthetic RNase. The maximum of the peak coincided with that of synthetic RNase (tube no. 62).

(b) *Disc electrophoretic mobility.* Disc electrophoresis was performed on 15% polyacrylamide gel at pH 4.3 at 2 mA per tube for 2 h, using Methyl Green as a marker. The sample was stained with Coomassie Brilliant Blue R (Sigma). The affinity-purified sample (30 μg) exhibited a main band with a faint impurity, 0.46 and 0.41 × Methyl Green, respectively, from the origin towards the cathode. The

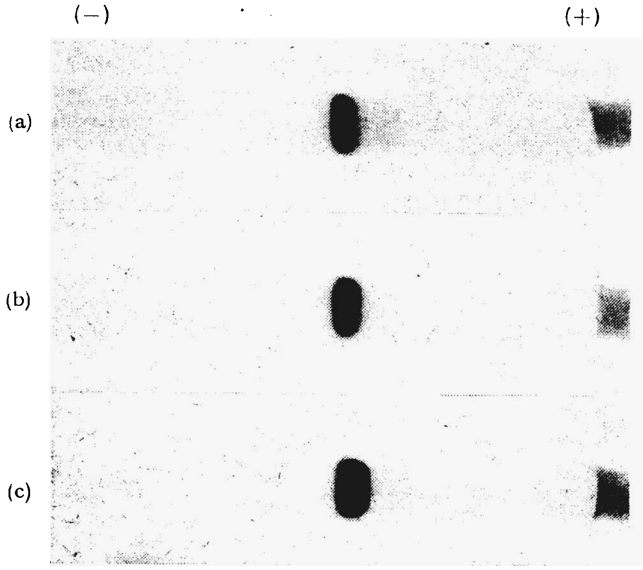


FIGURE 5 Disc electrophoresis of synthetic RNase A at pH 4.3: (a) affinity-purified synthetic RNase (30 μg), (b) CMC-purified synthetic RNase (20 μg), (c) synthetic RNase + natural RNase (each 20 μg)

CMC-purified sample (20 μg) behaved as a single component with the mobility of 0.46 × Methyl Green from the origin towards the cathode. The identical mobility was confirmed by disc electrophoresis of a mixing sample of synthetic RNase and natural RNase (20 μg each) in the same tube (Figure 5).

(c) *Ultraviolet spectrum.* The u.v. absorption spectrum of synthetic RNase (1.771 mg/ml) was compared with that of natural RNase (1.826 mg ml^{-1}) in 0.1M-KCl solution and the data are shown in Figure 6. The spectra were superimposable. The maximum molar extinction at 277.5 nm was 9 700, when the literature value,[22a] $\varepsilon_{max.}$ 9 800, was taken as the basis for the calculation.

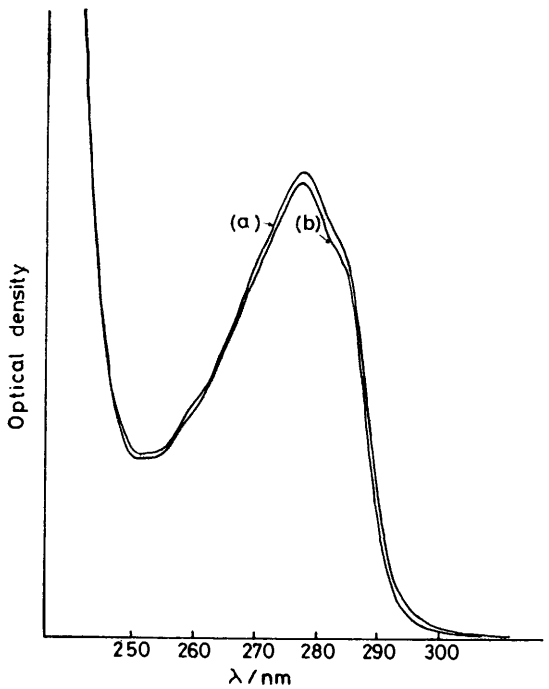


FIGURE 6 U.v. spectra of (a) natural and (b) synthetic RNase A

(d) *Michaelis constant.* According to the Kunitz method,[24] the value was determined by measuring the initial velocities of RNA hydrolysis spectrophotometrically at 300 nm. Samples of synthetic RNase and natural RNase (each 5 μg in 2 ml H_2O) were mixed with 2 ml of yeast RNA solutions (4.00, 2.00, 1.00, 0.50, 0.40, 0.33, 0.25 mg per ml) in pH 5.0 0.1M-sodium acetate buffer (total volume 4 ml). The absorption at 300 nm was measured for 10 min (Figure 7). The Michaelis constants were then calculated from Lineweaver–Burk plots. The K_M values were 1.22 and 1.20 mg ml^{-1} for synthetic and natural RNase respectively (lit.,[29] 1.25 mg ml^{-1}).

Evaluation of Enzymic Activity of Synthetic RNase.—(a) *Yeast RNA as substrate.* The Kunitz method [24] was applied to determine the activity of synthetic RNase at each purification step. Solutions containing yeast RNA (2 mg) in pH 5.0 0.1M-sodium acetate buffer (2 ml) were mixed with a solution of synthetic RNase (each 2 μg, 4 μg, and 8 μg) in H_2O (each 2 ml). The reactions were followed spectrophotometrically at 300 nm for 10 min. A solution of natural RNase (2 μg, 4 μg, and 8 μg each) in H_2O (2 ml) was allowed to react with yeast RNA as a standard. The specific activities of the synthetic RNase samples were calculated from the initial slope of the curves. An average activity of the CMC-purified sample (Figure 8) was 105% (107% in 2 μg per 2 ml, 104% in 4 μg per 2 ml and 104% in 8 μg per 2 ml).

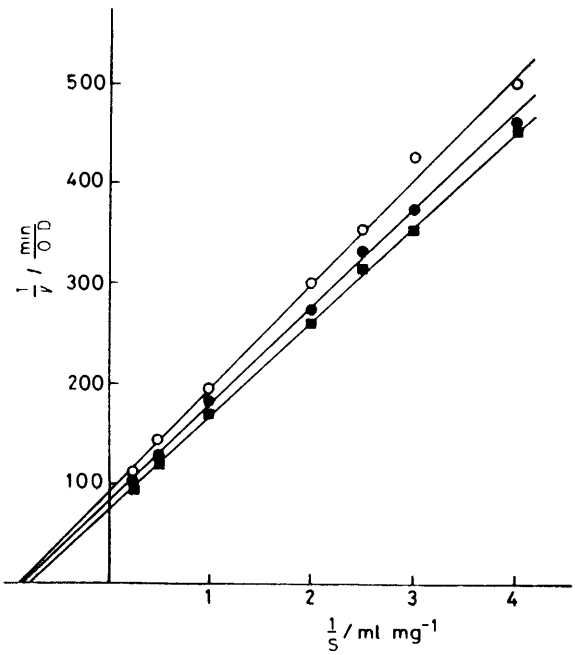


FIGURE 7 Calculation of Michaelis constant (K_M) for synthetic RNase A: ●, MSA-deprotected synthetic RNase, K_M 1.22 mg ml^{-1}; ■, HF-deprotected synthetic RNase, K_M 1.26 mg ml^{-1}; ○, natural RNase, K_M 1.20 mg ml^{-1} (lit.,[29] 1.25 mg ml^{-1})

(b) *2′,3′-Cyclic cytidine phosphate as substrate.* The Fruchter and Crestfield method [30] was applied to determine the specific activity of synthetic RNase purified by ion-exchange chromatography on CM-cellulose. A solution of synthetic RNase (20 μg) in H_2O (20 μl) was mixed with a

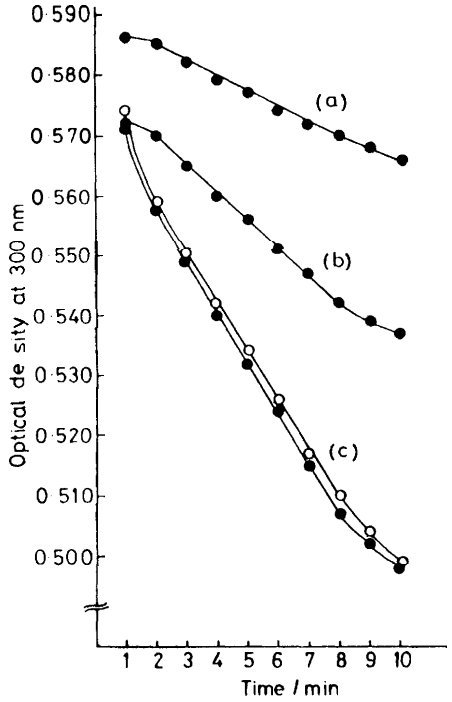


FIGURE 8 Enzymic activity of synthetic (●) compared with natural (○) RNase A with yeast RNA as substrate: (a) 2 μg per 2 ml, activity 107%; (b) 4 μg per 2 ml, activity 104%; (c) 8 μg per 2 ml, activity 104%

solution of 2′,3′-cyclic cytidine phosphate (1.4 mg) in 2 ml of pH 7.48 Tris-NaCl buffer (0.012M in Tris, 0.025M in NaCl, and 0.01M in HCl). After stirring for 10 min at 0 °C, 0.2M-mercuric chloride (50 μl) was added to stop the reaction. In order to obtain a better separation of 3′-cytidine phosphate from undigested substrate, the reported procedure was modified as follows. The reaction mixture was applied to a column of Amberlite CG-400 (0.9 × 6.7 cm) equilibrated with pH 7.48 Tris-NaCl buffer (0.012M in Tris, 0.025M in NaCl, and 0.010M in HCl). The column was then eluted by a linear concentration gradient with pH 7.48 Tris-NaCl buffer (0.060M in Tris, 0.125M in NaCl, and 0.050M in HCl) through a mixing flask (200 ml) containing the former buffer at a flow rate of 130 ml h^{-1}. The optical

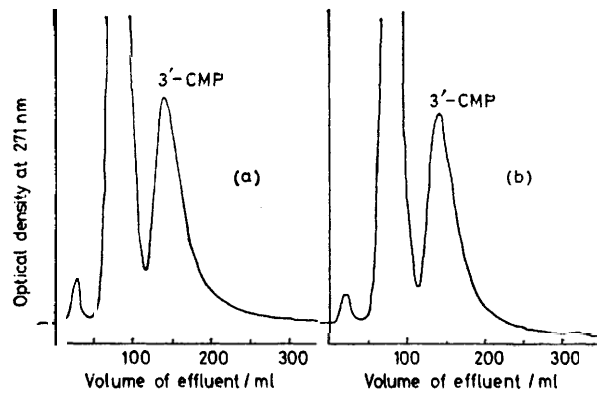


FIGURE 9 Enzymic activity of synthetic RNase A towards 2′,3′-cyclic cytidine phosphate as substrate [Amberlite CG-400 (0.9 × 6.7 cm) column, gradient elution with Tris-HCl-NaCl buffer (ph 7.48)]: (a) synthetic RNaseA (20 μg), activity 104%; (b) natural RNase A (20 μg).

density of the effluents at 271 nm was recorded by a Hitachi wavelength-tunable effluent monitor with a 10 mm light-path flow-cell. After elution of undigested substrate, the area of peak 2 (3′-cytidine phosphate) was compared with that of natural RNase (20 μg) as a standard (Figure 9). Three parallel experiments were performed and an average activity of 104% was found in synthetic RNase.

(c) *DNA as substrate.* Referring to the Kunitz method [31] for the DNase assay, the behaviour of synthetic RNase against calf-thymus DNA was examined. A solution of DNA (0.12 mg) in pH 5.0 sodium acetate buffer (3 ml) containing 0.005M-$MgSO_4$ was mixed with a solution of synthetic RNase (40 μg and 100 μg each) and natural DNase I (10 μg and 20 μg each) in H_2O (1 ml) at 25 °C. The change of the optical densities was followed spectrophotometrically at 260 nm for 10 min (Figure 10). The absorption of the

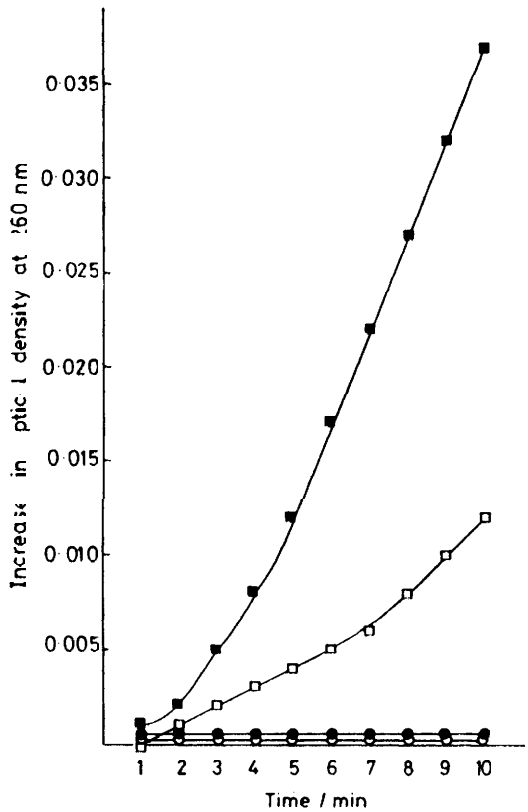


FIGURE 10 DNase activity with synthetic RNase A, using calf-thymus DNA as substrate (Kunitz method): ■, natural DNase I, 20 μg; □, natural DNase I, 10 μg; ●, synthetic RNase A, 100 μg; ○, synthetic RNase A, 40 μg

solution containing synthetic RNase showed no increase up to 2 h.

Deprotection of the Protected RNase by HF.—The protected RNase reduced by thiophenol (100 mg) was treated with HF (*ca.* 4 ml) in the presence of *m*-cresol (0.39 ml, 660 mol equiv.) in an ice-bath for 120 min. The excess of HF was removed by evaporation and dry ether was added. The resulting powder was dissolved in 0.2M-Tris-HCl buffer (3 ml) containing 4M-guanidine-HCl. The pH of the solution was adjusted to 8.6 with 5% methylamine. The solution was incubated under N_2 in the presence of 2-mercaptoethanol (0.35 ml, 800 mol equiv.) and dithiothreitol (174 mg, 200 mol equiv.) at 50 °C for 24 h and, after

further addition of 2-mercaptoethanol (0.18 ml, 400 mol equiv.), at room temperature for 40 h, as described for deprotection by MSA. After adjustment of the pH to 3 with 3N-HCl, the solution was applied to a column of Sephadex G-25 (1.6 × 68 cm) with 0.1N-AcOH as eluant for desalting. Individual fractions (each 3 ml) were collected.

Air Oxidation of the HF-Deprotected Product.—Fractions corresponding to the front peak (tube nos. 18—34) were combined and the solution was diluted with H_2O (3 l). The protein concentration was brought to 0.02 mg ml^{-1} with pH 8.2, 0.2M-Tris-HCl buffer (1 l). After stirring for 15 min, the solution was kept standing at room temperature for 2 days, while the Ellman colour intensity dropped from 0.118 to a constant value of 0.004. The solution was adjusted to pH 3 with 2N-HCl and lyophilized. The residue was dialysed three times against distilled H_2O (1 l) using cellulose tubing (VT 351). The content of the tube was lyophilized to give a hygroscopic powder (172 mg). The crude product was dissolved in a small amount of 0.05M-NH_4HCO_3 and insoluble material was removed by centrifugation (8 mg). The supernatant was applied to a column of Sephadex G-75 (2.8 × 95 cm, V_b 585 ml) with 0.05M-NH_4HCO_3 as eluant as described for deprotection by MSA. Fractions corresponding to the main peak [Figure 2(b), tube nos. 57—80] were combined and the solvent was removed by lyophilization to give a fluffy powder (47.1 mg, 60%). The activity against yeast RNA was 17%.

Purification of the Air-oxidized Product by Affinity Chromatography.—The crude air-oxidized product (44.12 mg) was dissolved in a small amount of 0.02M-AcONa and the solution was applied to an affinity column (2.2 × 8.6 cm), and was eluted with the same buffer (150 ml) and then with 0.2N-AcOH (100 ml) as described for deprotection by MSA. Peak 1 [Figure 3(b), tube nos. 9—40] in 0.02M-AcONa eluates gave, after desalting, an inactive material (26.13 mg). Peak 2 (tube nos. 57—74) in 0.2N-AcOH eluates gave an active material (4.79 mg, yield 11%). The activity against yeast RNA was 82% (79% in 2 μg per 2 ml, 82% in 4 μg per ml, and 84% in 8 μg per 2 ml).

Purification of the Affinity-purified Product by Ion-exchange Chromatography on CM-Cellulose.—The affinity-purified sample (3.99 mg) was applied to a column of CM-cellulose (1 × 11.8 cm), which was eluted with 0.01M-sodium phosphate buffer (pH 6.0) (64 ml) and then by gradient elution with 0.1M-phosphate buffer (pH 7.5) through a mixing flask containing 0.01M-buffer (120 ml). Fractions corresponding to the main peak [Figure 4(b), tube nos. 59—65) were combined and the solvent was removed by lyophilization. The residue was desalted by gel-filtration on Sephadex G-25 with 0.1N-AcOH as eluant. After lyophilization, a white fluffy powder was obtained (3.02 mg, 76%; total yield 5%). The activity against yeast RNA was 106% (108% in 2 μg per 2 ml, 107% in 4 μg per 2 ml, and 103% in 8 μg per 2 ml). Michaelis constant, K_M 1.26 mg ml^{-1}. Amino-acid ratios in 6N-HCl (48 h) hydrolysate (numbers in parentheses are theoretical values): Asp 14.74(15), Thr 9.65(10), Ser 13.84(15), Glu 12.49(12), Pro 4.45(4), Gly 3.31(3), Ala 12.47(12), Cys 3.70(4), Val 8.67(9), Met 3.71(4), Ile 2.38(3), Leu 2.00(2), Tyr 5.96(6), Phe 3.07(3), Lys 10.21(10), His 3.59(4), Arg 3.70(4) (recovery of standard Leu 79%).

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