THE PURIFICATION AND PROPERTIES OF RIBONUCLEASES

by

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ABSTRACT

The purification of ribonucleases T_1 and T_2 from Takadiastase has been carried out to provide enzymes of high purity and known specificity for structural studies on s-RNA. These purifications involved acid treatment, acetone fractionation, gel filtration on Sephadex and ion-exchange chromatography on substituted celluloses. Satisfactorily pure RNase T_1 has been obtained and its specificity has been confirmed. Further purification of RNase T_2 might be desireable but some studies using chemically synthesized substrates have been carried out on the most highly purified fraction yet obtained.

Partial purification of an RNase present in a different diastase preparation involved heat, acid, acetone fractionation and anion exchange chromatography. Information is not yet available on its specificity because of the small quantities isolated.

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ABBREVIATIONS

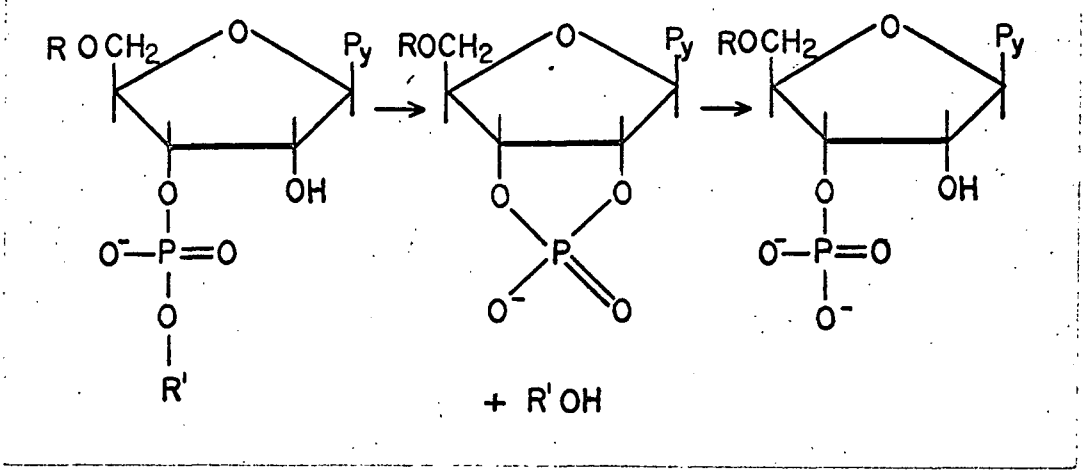
AMP	Adenosine monophosphate (3' or 2')
CMP	Cytidine monophosphate (3' or 2')
GMP	Guanosine monophosphate (3' or 2')
UMP	Uridine monophosphate (3' or 2')
CM-	Carboxymethyl-
DEAE-	Diethylaminoethyl
SE-	Sulfoethyl
TEAE-	Triethylaminoethyl
DCC	Dicyclohexylcarbodiimide
Poly A	Poly adenylic acid
Poly C	Poly cytidylic acid
Poly G	Poly guanylic acid
Poly I	Poly inosinic acid
Poly U	Poly uridylic acid
RNase	Ribonuclease
UV	Ultra violet
His 12	Histidine in the 12th position in pancreatic RNase
His 119	Histidine in the 119th position in pancreatic RNase
Lys 41	Lysine in the 41st position in pancreatic RNase

INTRODUCTION

Structural studies on ribonucleic acids, in particular on the soluble or transfer ribonucleic acids, require several highly purified and well characterized ribonucleases of known enzymatic specificity. This thesis describes the purification and some of the properties of two such ribonucleases, the T_1 and T_2 from Takadiastase.

Pancreatic Ribonuclease

The first discovered and most thoroughly studied ribonuclease (RNase) is that of bovine pancreas which was crystallized by Moses Kunitz in 1940 (1). Over 20 years later it became the first enzyme in which the primary sequence of amino acids was established. The sequence of reactions involved in the hydrolysis of RNA by this enzyme, and its specificity, were elucidated by Schmidt *et al.* (2), Markham and Smith (3), Brown and Todd (4), and Volkin and Cohn (5). It was shown that pancreatic RNase catalyzed two separate reactions, transesterification and hydrolysis (3,4). Both reactions were shown to be specific for phosphodiester linkages involving pyrimidine nucleoside 3'-phosphates (2,5). As shown below, transesterification produces a 2',3'-cyclic phosphate which is then opened in a hydrolytic step to produce the 3'-phosphate (4).



R = H or Nucleotide

R' = Alkyl group or Nucleoside

Complete hydrolysis of RNA by RNase produces, as a result of its pyrimidine specificity, uridine 3'-phosphate, cytidine 3'-phosphate, and a series of oligonucleotides composed of purine nucleotide residues terminated by a pyrimidine nucleoside 3'-phosphate. Reports by Hakim (6) that pancreatic RNase can hydrolyze the 2',3'-cyclic phosphates of adenosine and guanosine disagree with the results of Brown and Todd (4) and results obtained in this laboratory. Beers (7) has claimed than pancreatic RNase can hydrolyze an adenylic acid polymer (poly A) but no one has as yet confirmed this so it would probably be unwise to accept it at the present time.

Ribonucleases T_1 and T_2

Recently, considerable interest has centered around the ribonucleases of Takadiastase, an extract of Aspergillus oryzae. The presence of RNases in Takadiastase was first reported by Kuninaka (8) in 1954. In 1957 Egami and co-workers

showed the presence of at least two RNase activities and named them T_1 (major component) and T_2 (minor component). The T_1 enzyme was partially purified at that time and a few of its properties shown (9). The preliminary results suggested that RNase T_1 preferentially hydrolyzed those phosphodiester bonds distal to the 3'-phosphate of guanylic acid residues in RNA. They also demonstrated the intermediate formation of the 2',3'-cyclic phosphate of guanosine, and its subsequent hydrolysis to guanosine 3'-phosphate. Takahashi (10, 11) and Rushizky and Sober (12) have purified the RNase T_1 quite highly and the latter have confirmed its absolute specificity.

Partial purification of RNase T_2 was reported by Naoi-Tada et al. (13) who observed preferential hydrolysis of RNA at phosphodiester bonds distal to the 3'-phosphates of adenylic acid. The enzyme was able to hydrolyze adenosine 2', 3'-cyclic phosphate to the 3'-phosphate but the cyclic intermediates were not found in the RNA digests. The suggestion was made that RNase T_2 might have an absolute specificity for adenylic residues with contamination by another RNase. As yet no studies have been published on the relative rates of hydrolysis by this enzyme of the different linkages in RNA. Recent work by Rushizky and Sober has indicated that RNase T_2 is a completely non-specific enzyme which merely cleaves those bonds involving adenylic acid residues at a higher rate than others (14).

B. subtilis Ribonuclease

Another ribonuclease for which a distinct specificity was claimed is the extracellular RNase of Bacillus subtilis. Nishimura was the first to study this enzyme and he concluded that its specificity was complementary to that of pancreatic RNase. That is, it seemed to hydrolyze only phosphate ester bonds distal to the 3'-phosphates of purine nucleotides (15). Reinvestigation of this enzyme by Rushizky et al. (16), and by Whitfeld and Witzel (17) indicated a very complex series of preferences, with only a very few linkages completely resistant to hydrolysis.

Other Ribonucleases

Although the four ribonucleases described above are the most highly purified and well characterized many others have been reported. Ribonucleases are so widely distributed that probably all living cells produce one or more RNases. Many different plants have RNases, nearly all capable of hydrolyzing RNA to 3'-nucleotides or the nucleoside 2',3'-cyclic phosphates (18).

An unusual plant nuclease is that from Mung Bean sprouts which was studied by Sung and Laskowski (19). This enzyme hydrolyzes both RNA and DNA with the production of nucleoside 5'-phosphates. It shows no base specificity but seems to preferentially hydrolyze those bonds distal to the 5'-phosphates of adenosine and uridine (or thymidine in DNA). A somewhat similar RNase has been isolated from liver nuclei.

It likewise produces 5'-phosphates (20) but appears to be specific for RNA. Cunningham et al. (21) have described a nuclease from Staphylococcus pyogenes (Micrococcal nuclease) which degrades RNA and DNA to the 3'-phosphates of mono- and dinucleotides. This enzyme would probably not catalyze the transesterification reaction typical of the four RNases described previously. A great many other RNases have been reported other than these few, but generally have not been extensively studied.

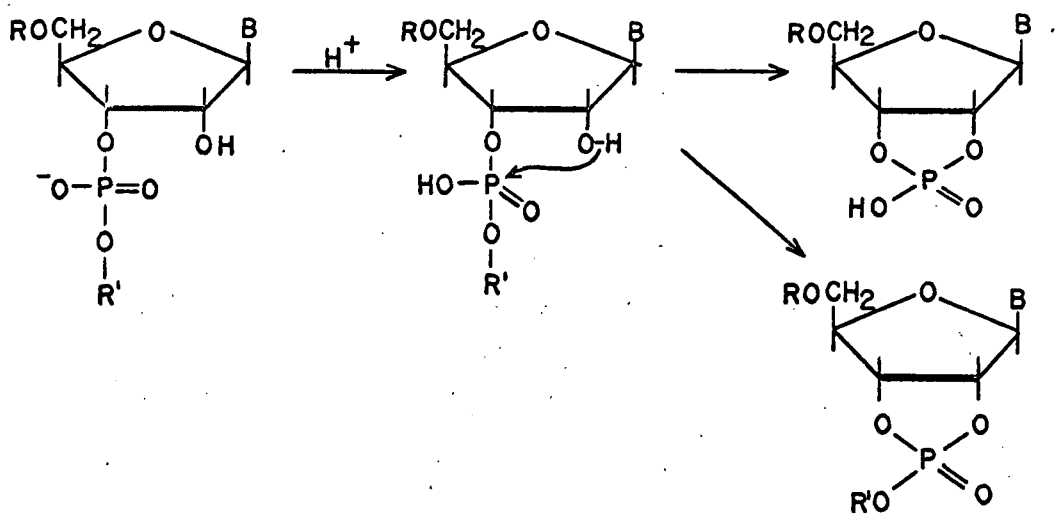
An interesting feature of most ribonucleases is their stability. In general they can be subjected to strong acids and high temperatures with very little loss of activity. These two properties have frequently been exploited in the schemes devised for the purification of RNases. These stability properties can be atributed to their fairly simple structures, at least in those cases where the structures are known. Pancreatic RNase, RNase T_1, the B. subtilis RNase, and Micrococcal nuclease have molecular weights under 14,000, and RNase T_2 is thought to have a molecular weight no greater than 30,000. A further demonstration of their remarkable stability is the report of Rushizky et al. (22) who found that pancreatic RNase, B. subtilis RNase, Micrococcal nuclease, and RNase T_1 could all be extracted from aqueous solution into phenol with full retention of activity on removal of the phenol. When one considers the ability of phenol to denature proteins this stability is remarkable.

Mechanisms of Hydrolysis

The mechanisms by which RNases hydrolyze RNA have not yet been very extensively studied, except in the case of pancreatic RNase. Even in this case the literature is meager when compared to that on the proteolytic enzymes. Mechanisms have been proposed for the chemical degradation of RNA and the simple phosphodiesters of nucleosides. It has been shown that in those cases, as well as in the enzymatic hydrolysis, nucleoside 2',3'-cyclic phosphates are intermediates.

Acid Catalysis

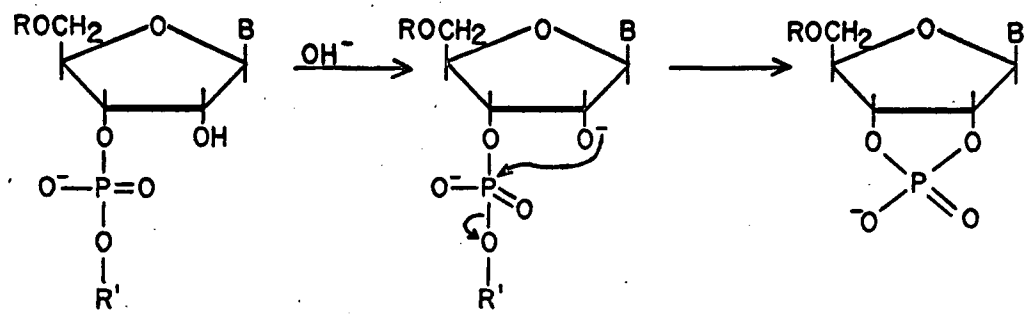
Protonation of the primary phosphate dissociation of these phosphodiesters causes a large increase in the electrophilicity of the phosphorus atom. This makes it more susceptible to nucleophilic attack by the 2'-oxygen atom and leads to the formation of either a 2',3'-cyclic diester by elimination of R', or a 2',3'-cyclic triester if OH^- is eliminated.



Attack by a water molecule would produce in the first case a mixture of the nucleoside 2'- and 3'-phosphates. In the second case attack by water would yield a mixture of the 2',3'-cyclic diester and the 2'- and 3'-phosphodiesters. These three products would be eventually hydrolyzed to 2'- and 3'-phosphates. The cyclic triester shown above has not been demonstrated as an intermediate in this reaction but its existance is necessary to explain the formation of the 2'-phosphodiesters. Szer and Shugar claim to have synthesized compounds of this structure (23) but information on its stability is not available. Brown et al. (24) synthesized dimethyl and dibenzyl esters of uridine 3'-phosphate and found them to be unstable at all pHs, decomposing to uridine 2'- and 3'- phosphomonoesters. This extreme instability is almost certainly due to the presence of the vicinal hydroxyl group.

Basic Catalysis

In alkali the 2'-hydroxyl group becomes ionized and the negatively charged oxygen carries out a nucleophilic attack on the phosphorus. Due to the primary phosphate dissociation only -OR' can act as a leaving group so only 2',3'-cyclic phosphate diesters can be formed as products of this transesterification.



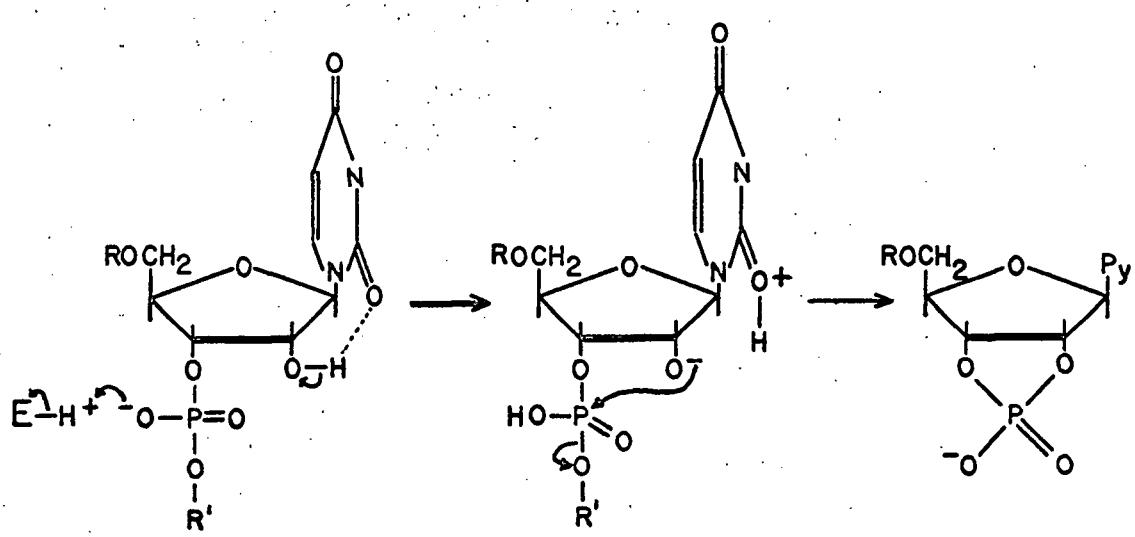
Two lines of evidence tend to eliminate the possibility of an intermediate cyclic triester as postulated for acid hydrolysis. First, no 2'-phosphodiesters have been isolated from alkaline digests although they have been found in acid hydrolysates. Second, alkaline hydrolysis in $H_2{}^{18}O$ of yeast RNA gave mononucleotides with only one atom of ^{18}O per atom of phosphorus. Formation of an intermediate cyclic triester would be expected to allow incorporation of two atoms of ^{18}O. Hydrolysis of the cyclic diester is carried out by direct attack by OH^- producing a mixture of the 2'- and 3'-phosphomonoesters.

Hydrolysis of RNA catalyzed by certain divalent cations has also been reported (25,26). The mechanism in this case appears to be similar to that catalyzed by acid.

Enzyme Mechanisms

Mechanisms for the enzymatic hydrolysis of RNA by pancreatic RNase have been proposed by Witzel (27) and Findlay et al. (28). Witzel suggests that the pyrimidine base is

not involved in binding to the enzyme but is specifically involved in catalysis by hydrogen bonding to the 2'-hydroxyl group. He proposes that this increases the nucleophilicity of the 2'-oxygen in the same way that alkali does and thus causes transesterification to occur forming the 2',3'-cyclic phosphate. In addition he suggests that the electrophilicity of the phosphorus is increased by protonation by the enzyme. The actual sequence of reactions he proposes is 1) Protonation of the phosphate by the enzyme 2) Nucleophilic attack of the phosphorus by the 2'-oxygen. In this second step the pyrimidine base is assumed to transfer the proton from the 2'-oxygen to the R'-oxygen causing the loss of that group.

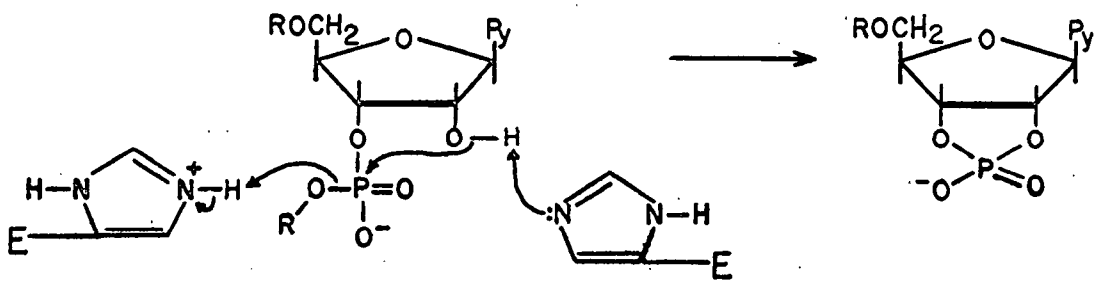


E = ENZYME

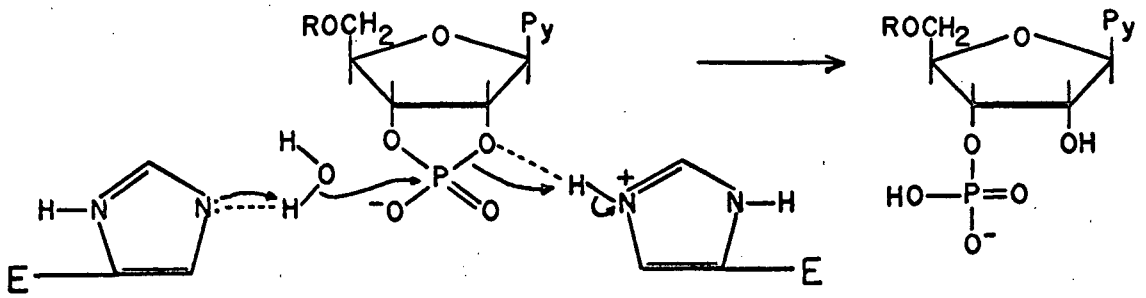
In the opening of the cyclic diester Witzel suggests that the enzyme again protonates the phosphate and that the pyrimidine activates a water molecule by hydrogen bonding to

it. The activated water molecule is then assumed to carry out a nucleophilic attack on the phosphorus while the pyrimidine base transfers one of the protons from the water molecule to the 2'-oxygen.

The mechanism proposed by Findlay et al. contrasts rather sharply with Witzel's in that they consider the pyrimidine to be involved only in the binding of substrate to enzyme, and not at all in catalysis. In their hypothesis two imidazole groups are involved, one protonated and the other not. In their first step the unprotonated histidine is H-bonded to the 2'-hydroxyl to increase the nucleophilicity of the oxygen while the protonated histidine transfers its proton to the oxygen of the leaving group.



In the second step they consider that the unprotonated imidazole H-bonds to a water molecule to activate it and that the protonated imidazole transfers a proton to the 2'-oxygen to weaken the P-O bond. In this way the cyclic phosphate is specifically opened to the 3'-phosphate.

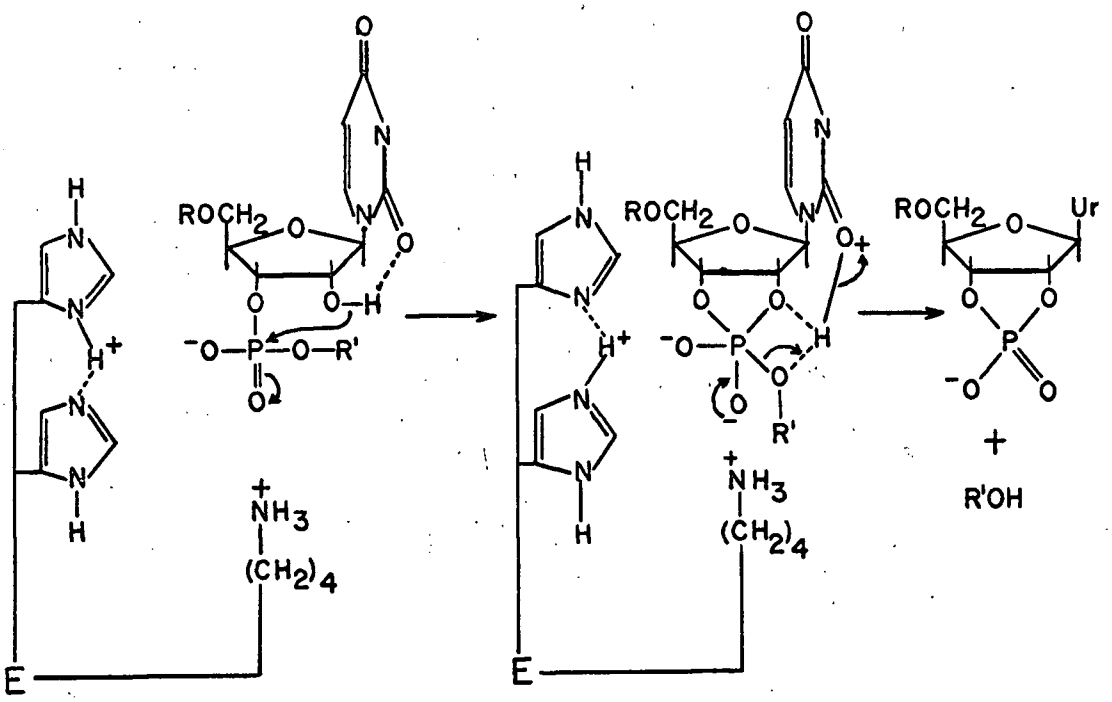


If one considers the pyrimidine to perform the function of the unionized imidazole one has a mechanism similar but not identical to Witzel's. This is due to Witzel's proposal that the phosphate is protonated by the enzyme and thus becomes very susceptible to nucleophilic attack. In the mechanism proposed by Findlay *et al.* the phosphate is always ionized. Witzel bases his mechanism on several good arguments. First, the Km is not particularly dependant on the nature of the pyrimidine base while the ES $\xrightarrow{k_3}$ E + P step is. He assumes Km to be a measure of the enzyme's ability to bind a given substrate while k_3 is assumed to be the rate of transesterification or hydrolysis. Further, his studies with dinucleoside phosphates indicate a very considerable effect of the 5'-linked nucleoside on k_3 but very little effect on Km. He interprets this as being due to π-interaction between the pyrimidine base and the second base. The most effective base for this assumed π-interaction is adenine, then guanine, cytosine and finally uracil. This π-interaction is assumed to

increase the ability of the 2-oxygen substituent on the pyrimidine to polarize the 2'-OH bond and thus cause a further increase in the nucleophilicity of the 2'-oxygen. The methods that Witzel has used were generally kinetic analysis of reactions using a wide variety of substrates. Findlay et al. based their mechanism on studies of one reaction--that of hydrolysis of cytidine 2',3'-phosphate. These included studies on pH dependence of the kinetic parameters for this reaction, the effects of inert organic solvents and alcohols, an attempt to determine the charge types of the groups at the active center, and the effects and interactions of inhibitors with the enzyme (29, 30, 31, 32). Their conclusions are that the active site contains two histidine residues, one protonated and the other not. The studies of Crestfield et al. (33, 34) have shown that alkylation of His-119 completely inactivates pancreatic RNase, while alkylation of His-12 does not completely inactivate it. It would seem then that Findlay et al. are probably correct in their finding of the involvement of two histidine residues at the active site. Witzel has a different interpretation of how the two histidines are involved (35). According to his hypothesis the pyrimidine base catalyzes both reactions when the phosphate is protonated. He finds that imidazolium ion does not catalyze the reaction and therefore the electrophilicity of the imidazolium proton must be increased. This he feels is brought about by having a hydrogen bond between an imidazole and an imidazolium ion. Several

groups, in particular Crestfield et al. (33), have found that carboxymethylation of RNase has a pH optimum at 5.5. This is about the same pH at which Witzel found the Km for several RNase substrates to be lowest. Carboxymethylation of N-1 of His-119 occurs in 92% yield, the other 8% of carboxymethylation occurring at N-3 of His-12. From this it has been postulated that they are sufficiently close together that when one imidazole is protonated it attracts an iodoacetate ion and orients it in such a way that the other imidazole is alkylated. Crestfield et al. suggest from this that the two histidine residues are about 5A apart (34). This conclusion is reached in part by the observation that if one residue is alkylated it protects the other from alkylation. Witzel now suggests that the enzyme-substrate complex in the ribonuclease reaction is an enzyme bound pentacovalent phosphotriester. Since Lys-41 has been implicated in the active site of the enzyme by several groups Witzel proposes that its function is to stabilize one of the negative charges on the phosphotriester. Further he proposes that no diester monoanion can act as an inhibitor of RNase unless it can form the dianionic intermediate. All dianions should therefore be inhibitors of RNase, and in a number of cases this has been shown.

The following diagram indicates Witzel's revised hypothesis.



All other ribonucleases which produce 3'-phosphates have been shown to follow the same sequence of reactions, that is, transesterification followed by hydrolysis of the intermediate 2',3'-cyclic phosphate. Some RNases open the 2', 3'-cyclic phosphates of some nucleotides very slowly and in some cases not at all.

Whitfeld and Witzel have published a note on the mechanism of RNase T_1 hydrolysis of RNA and find some differences between it and pancreatic RNase (36). For substrates they used a number of dinucleoside phosphates and found the effectiveness of the 5'-linked nucleosides in promoting transesterification decreased in the order cytidine, adenosine, guanosine, uracil. The relative effectiveness of the 3'-linked nucleosides decreased in the order guanosine, inosine, xanthosine, glyoxal guanosine.

Further they found that the rates of transesterification of the dinucleoside phosphates was between 125 and 400 times the rate of hydrolysis of guanosine 2',3'-cyclic phosphate. The cyclic phosphates of inosine, xanthosine, and glyoxal guanosine were not hydrolyzed. The conclusions they reached were that the T_1 enzyme differed markedly from pancreatic RNase in its kinetic behavior. Whereas pancreatic RNase is very strongly influenced by the nature of the 5'-linked nucleoside, T_1 is only influenced to a minor extent. They interpret this as meaning that the guanine base cannot play the same role in catalysis during reaction with T_1 as the pyrimidine bases play during reaction with pancreatic RNase. Instead they suggest that the guanine base is involved in binding to the enzyme and that the base must be protonated at N-1 or the oxygen on position 6.

McCully and Cantoni (37) have shown that RNase T_1 is unable to hydrolyze those phosphodiester bonds distal to 1-methyl-guanosine and 6-hydroxy-2-dimethylaminopurine nucleoside. This is in accord with the proposed requirement for protonation of the base at N-1. However, their methods would not have shown the formation of a 2',3'-cyclic phosphate of either of these nucleosides. Since the glyoxal derivative of guanosine is a substrate for the transesterification reaction but not the hydrolysis step the possibility remains that the methylated guanosines 3'-phosphodiesters may also be susceptible to this transesterification.

Whitfeld and Witzel have also studied B. subtilis RNase using in this case homopolymers of IMP, AMP, CMP and UMP (38). In addition they used several smaller substrates. They found that the enzyme catalysed transesterification of purine polynucleotides faster than pyrimidine and polynucleotides with 6-hydroxy substituents faster than those with 6-amino substituents. The rates were Poly I > poly A > poly U > poly C and they calculated that poly G would have been broken down even faster than poly I. They postulate that the distance between the 6-substituent and the catalytic center for transesterification is critical. Further they conclude that the 6-substituent must act as a proton donor. They found that addition of a 3'-phosphate to a dinucleoside phosphate increases the rate of transesterification and that a further increase results from addition of another nucleoside to this phosphate. Another point is that guanosine 2',3'-cyclic phosphate is the only cyclic phosphate that can be hydrolysed of the four arising from RNA.

As yet no kinetic studies have been published dealing with RNase T_2 and no mechanisms have been suggested. The most recent information on this enzyme in regard to its substrate preferences is found in the paper of Rushizky and Sober (14). They showed that AMP (3') is produced at a faster rate than the other mononucleotides. This in itself is unusual in that all seem to be liberated at rates unlike those shown by any other enzyme. There have been no determinations

of relative rates of hydrolysis of substrates of known structure and no inhibitors of this enzyme have been reported.

The purpose of the present investigations was to purify RNases T_1 and T_2 from Takadiastase and to determine specificities using synthetic substrates. Further, in the event of non-specific nuclease activity it was intended that relative rates of hydrolysis and transesterification be determined. An investigation of the effects of various inhibitors was also considered necessary to fully characterize the activities. The use of nucleoside 2',3'-cyclic phosphates and nucleoside 3'-phosphate propyl esters made possible the study of both the transesterification step and the hydrolytic step, without the complications introduced by the presence of a second base as found in studies using dinucleoside phosphates and dinucleotides.

MATERIALS AND METHODS

Takadiastase powder (Sanzyme R) was purchased from the Sankyo Co. Ltd., Tokyo, Japan. Another A. oryzae diastase preparation was purchased from Mann Laboratories, New York.

Fresh pressed yeast (Flieschmann) was purchased from Standard Brands Inc., Richmond, B. C.

The mixed 2'- and 3'-phosphates of adenosine, uridine and cytidine were purchased from Schwartz Bioresearch, Inc., Orangeburg, New York.

Guanosine 2'(3')-phosphate (mixed isomers) was purchased from C. F. Boehringer, Mannheim, Germany.

Adsorbents

DEAE-, CM- and SE-cellulose were purchased from Brown Company, Berlin, N. H. TEAE-cellulose was obtained from Calbiochem, Los Angeles.

Before use the adsorbents were suspended in tap water and fine particles removed by repeated decantation. Columns were packed in 2M ammonium carbonate under 2-4 lb. air pressure. All columns were washed with 2M ammonium carbonate until the optical density (260 mμ) of the effluent was as low as that of the influent solution. The columns were then washed with at least 3 column volumes of distilled water to remove ammonium carbonate. Columns were converted to other ion forms (if necessary) by washing with the appropriate solution. Prior to loading, all columns were equilibrated with the starting solution, which was also used to wash in the ap-

plied solution. All elutions were carried out using linear gradients of salts, and, in some cases pH.

Sephadex

Sephadex G-25 and G-75 were purchased from Pharmacia A. B., Uppsala, Sweden.

Columns of Sephadex were prepared from gel particles which had been allowed to swell for at least 48 hours. The fine particles were removed by repeated decantation and the coarse particles rinsed several times with the solution to be used. Columns were packed slowly under gravity to ensure even packing. When completed a piece of filter paper was put on top of the gel to prevent disturbances to the top. The columns were loaded in the usual manner for gel filtration on Sephadex.

Ribonuclease Assays

A. The standard assay procedure used involved the addition of 0.1 ml of properly diluted enzyme (up to 7 units/ml) to 0.4 ml of 0.2M ammonium acetate buffer (pH 4.5 for T_2, pH 7.5 for T_1). To this was added 0.5 ml of RNA solution (140 $O.D._{260}$/ml) and the mixture was incubated for 15 min at 37°C. At this time 0.25 ml of cold 25% perchloric acid plus 0.75% uranyl acetate was added. The precipitate was centrifuged out at 0°C and 0.5 ml of the supernatant was diluted to 5 ml. The $O.D._{260}$ of this solution was then determined using the proper blanks. A unit of enzyme activity was defined as that quantity of enzyme which would produce an increase of one O.D. unit. (An O.D.

unit is that amount of material per ml which, in a 1 cm light path gives a spectrophotometric reading of one.) This assay gave a linear response up to an $O.D._{260}$ of about 0.7.

All UV absorption measurements were carried out on either a Cary 11 Recording Spectrophotometer or a Zeiss PMQ II spectrophotometer. The Cary 11 was used for all spectra.

B. Assays using nucleoside 2',3'-cyclic phosphates and nucleoside 3'-phosphate propyl esters (or mixed 2'- and 3'-isomers) were carried out in 1 ml tubes with greased ground glass stoppers at 37°C. At various times aliquots were removed and spotted on paper (Whatman 1 or 40). The chromatograms were developed in a solvent system containing isopropanol, ammonium hydroxide, and water in a ratio 7:1:2. After development, the papers were dried and UV absorbing areas cut out. These were eluted with distilled water and the optical densities determined against the appropriate blanks from clear areas of the same R_f on the chromatograms.

TABLE 1

R_f Values of Substrates and Products in the Isopropyl Alcohol: Ammonium Hydroxide:Water (7:1:2) System (40)

Compound	R_f
Adenosine 2'(3')-phosphate	.08
Adenosine 2',3'-cyclic phosphate	.42
Adenosine 2'(3')-phosphate propyl ester	.56
Cytidine 2'(3')-phosphate	.07
Cytidine 2',3'-cyclic phosphate	.31
Cytidine 2'(3')-phosphate propyl ester	.54
Guanosine 2'(3')-phosphate	.05
Guanosine 2',3'-cyclic phosphate	.28
Guanosine 2'(3')-phosphate propyl ester	.47
Uridine 2'(3')-phosphate	.06
Uridine 2',3'-cyclic phosphate	.30
Uridine 2'(3')-phosphate propyl ester	.45

These R_f values tend to increase with the age of the solvent system and are accurate only for the freshly prepared system.

C. A third assay was used to follow rates of hydrolysis of the pyrimidine nucleoside 2',3'-cyclic phosphates. Hydrolysis of uridine and cytidine 2',3'-cyclic phosphates was followed directly by measuring the increase in $O.D._{275}$ and $O.D._{285}$ respectively produced on formation of the corresponding 3'-phosphates (39). For measurement of RNase T_2 activity incubations were carried out in the cell compartment of the spectrophotometer. The temperature (37°C) was maintained by passing oil from a thermostat-controlled circulating pump through the jacketed cell compartment. The cells were warmed to 37° in a water bath before the enzyme was added. Complete digestion of uridine 2',3'-cyclic phosphate produced a 29.6% increase in $O.D._{275}$ and cytidine 2',3'-cyclic phosphate a 33% increase in $O.D._{285}$.

EXPERIMENTAL AND RESULTS

Synthetic Substrates

A. Cyclic Phosphates

Preparation of Nucleoside 2',3'-cyclic phosphates was by the method of Smith, Moffat and Khorana (41) or by the modified procedure of Smith and Khorana (42). The first of these involved dissolving the nucleotide (mixed 2'- and 3'-isomers) in 2M ammonium hydroxide and formamide. To this was added a five-fold excess of dicyclohexylcarbodiimide (DCC) in t-butyl alcohol. The resulting solution was then refluxed for 2 1/2 hours, cooled, and ammonium hydroxide and t-butyl alcohol were removed by evaporation under reduced pressure. This was followed by addition of water, filtration, extraction with ether, and finally precipitation of the product from acetone solution as the barium salt by the addition of barium iodide. The quantitative yields claimed have not been achieved, but yields of 80% or better have been obtained. The modification referred to used an aqueous solution of triethylamine to dissolve the nucleotides followed by addition of a five-fold excess of DCC in methanol. The resulting clear solution was allowed to stand at room temperature for 8 hours (or more) and worked up in the same manner as before.

In order to avoid divalent cations in the preparations, a number of preparations have been purified directly, after extraction with ether, by chromatography on columns of DEAE-cellulose. The adsorbed nucleotides, cyclic and open,

were well separated by elution with a linear gradient of ammonium carbonate. Repeated evaporation to dryness under reduced pressure was sufficient to remove all of the salt.

B. Nucleoside Phosphate Propyl Esters

Nucleoside 2'- and 3'-phosphate propyl esters were prepared by the method of Tener and Khorana (40). As starting material nucleoside 2',3'-cyclic phosphates were used. They were subjected to acid catalyzed transesterification in an anhydrous solution of dioxane and n-propanol. The reaction was stopped by evacuating to remove the hydrochloric acid. Purification was then carried out chromatographically on paper in the isopropanol-ammonium hydroxide-water (7:1:2) system. Separation of the 2'- and 3'-isomers of the adenosine and guanosine phosphate propyl esters was carried out by chromatography on DEAE-and TEAE-celluloses, respectively.

Synthesis of the pure 3'-isomers of uridine and cytidine propyl phosphates was achieved enzymatically using pancreatic RNase. Incubation at room temperature of the enzyme and 2',3'-cyclic phosphate in the presence of 80% n-propanol produced the desired compounds (30). RNase was removed by forcing the solution very rapidly through a column of SE-cellulose which adsorbs RNase but not the nucleotides. Chromatography of the RNase-free solution on DEAE-cellulose gave the 3'-phosphate propyl esters.

In all of these syntheses identification of the UV absorbing material eluted from the ion-exchange columns was

by paper chromatography. Further, in synthesis of mixed 2'- and 3'-propyl esters the isomer eluted from anion exchange columns at lower ionic strength was the 2'-ester. This was proved by comparing their susceptibility to enzymatic hydrolysis.

RNA Preparation

High molecular weight yeast RNA was prepared by the method of Crestfield, Smith and Allan (43). This procedure involved the breaking of the yeast cell wall by extraction with hot sodium dodecyl sulfate. The solution was cooled rapidly to 0°C and cell debris was centrifuged off at 0°C. The RNA was precipitated from the supernatant by addition to two volumes of cold 95% ethanol. The precipitate was washed twice with 67% ethanol and left overnight in 80% ethanol. The precipitate was then collected by centrifugation, dissolved in distilled water and centrifuged at 25,000 RPM for one hour. The supernatant was made 1M in sodium chloride and the resulting precipitate collected. The yield reported by Crestfield et al. (43) was about 0.9 g per 100 g of fresh pressed yeast. However, in our hands the yields varied from .2 to .65 g per 100 g of pressed yeast, depending on its age. Much lower yields were obtained when active dry yeast was used instead of fresh pressed yeast.

Modifications of this procedure were introduced to reduce contamination and to same time. These included extraction of the aqueous RNA solution with phenol, followed by

extraction of the aqueous phase with ether. In this step all RNases and most other protein was eliminated. High speed centifugation was replaced by filtration through Celite to clarify the final solution.

RNA was stored frozen in small tubes at a concentration of 140 $O.D._{260}$ units per ml.

Pancreatic RNase

Preliminary experiments were carried out with pancreatic RNase to determine the feasibility of using nucleoside 2'(3')-phosphate propyl esters and nucleoside 2',3'-cyclic phosphates as substrates for the determination of the specificity of the RNases and of the rates of hydrolysis of the susceptible bonds.

All of these early experiments were carried out at 37° with incubation mixtures composed of equal parts of nucleoside 2'(3')-phosphate propyl ester solution (200 O.D./ml, 36% of which was the 2'-isomer) and buffered RNase solutions of varying concentration.

Kinetic studies on pancreatic RNase hydrolysis of these synthetic substrates are complicated by two factors. First is the presence of the 2'-isomer of the propyl phosphates. These esters are known to be strong competitive inhibitors of this enzyme (32). Second is the production by the reaction of nucleoside 3'-phosphates which are also potent competitive inhibitors. As a result it was extremely difficult to obtain data which followed zero or first order kinetics.

Since this work was carried out prior to the preparation of pure 3'-isomers of the nucleoside phosphate propyl esters no quantitative information was obtained on the course of the reaction with these substrates.

Incubation of pancreatic RNase A and B fractions with all four propyl esters showed that the propyl esters of guanylic and adenylic acids were completely resistant to both the A and B fractions under conditions in which very rapid hydrolysis of cytidylic and uridylic acid propyl esters occurred.

Mann Diastase

An A. oryzae diastase extract from Mann Laboratories, New York, was studied to determine the presence or absence of RNases (T_1 and T_2).

Incubation of an aqueous extract of this powder with the nucleoside 2'(3')-phosphate propyl esters led to the formation of nucleosides, thus indicating the probable presence of both an RNase activity and a phosphatase activity.

Heating the aqueous extract at pH 1.5 for two minutes at 80°C, followed by cooling and neutralization completely eliminated the phosphatase activity.

Incubation of a sample of this acid and heat treated solution with each of the four mixed 2'- and 3'-nucleoside phosphate propyl ester solutions showed the production of nucleoside 3'-phosphates in all cases.

Activity against the propyl ester of adenylic acid

was the greatest, with that against the esters of uridylic and cytidylic acids much less. Hydrolysis of the propyl ester of guanylic acid was not detectable until 24 hours had elapsed. Under the conditions used the time required for 25% hydrolysis of the susceptible 3'-substrates was two hours for adenosine 3'-phosphate propyl ester, ten hours for cytidine 3'-phosphate propyl ester and seventeen hours for uridine 3'-phosphate propyl ester.

When assayed using RNA as substrate practically no activity was observed. However, when 0.01M EDTA was added to the buffer solution activity was observed.

Purification of the enzyme was attempted by DEAE-cellulose column chromatography using a 2.2 cm column packed to a height of 22 cm. To this was applied 12 ml of the diastase extract (about 2 g of protein) which had been acid and heat treated as before. Elution was with 0.001M phosphate buffer (pH 7) and a 2 l linear gradient of sodium chloride to 0.5M. Eighty-five percent of the RNase activity applied was recovered in a 56 ml fraction which emerged from the column after 1100 ml of eluant had passed through. This fraction contained 36 $O.D._{280}$ units of protein.

Activity of this solution against the propyl esters was followed using a buffer which included EDTA (0.01M). In this case the rates of hydrolysis of all four propyl esters were of the same order of magnitude. Either the inclusion of EDTA or the column chromatography seemed to inactivate or re-

move some inhibitor present in the enzyme.

Acetone Fractionation

An attempt to fractionate the RNase activity of a crude, heat and acid treated extract with ammonium sulfate was unsuccessful, but acetone fractionation proved useful. It was found that the addition of increasing amounts of acetone to crude acid and heat treated enzyme solutions produced a precipitate. The precipitate which formed between one and three volumes of acetone at room temperature contained greater than 85% of the RNase activity.

Chromatographic Fractionation

DEAE-cellulose column chromatography (column size = 3.7 x 37 cm) was carried out on a solution which had been subjected to heat and acid treatment followed by acetone fractionation. A total of 280 units were loaded on and a peak of activity was recovered which contained about 190 units. This peak contained about 16.8 $O.D._{280}$ units of protein from approximately 5 g of material in the acetone fractionated extract.

Samples of this preparation were incubated with each of the four nucleoside phosphate propyl esters and each of the four nucleoside 2',3'-cyclic phosphates. No hydrolysis of any of these was observed, even after 48 hours.

One explanation for this anomalous result is that too low a concentration of enzyme was used. On the other hand it might represent the isolation of an RNase devoid of simple diesterase activity. Therefore, further studies on this frac-

tion are needed before a final conclusion can be reached.

Ribonuclease T_1 Purification

Two procedures have been reported for the preparation of RNase T_1 from Takadiastase, that of Takahashi (10,11) and that of Rushizky and Sober (12). In the present work the first preparation of the T_1 enzyme followed a modified procedure, derived from that of Takahashi.

A. Method of Takahashi (Modified)

1) Acid and Heat Treatment

80 g of Takadiastase powder was extracted with 150 ml of water for an hour and then centrifuged for an hour at 2000 RPM. The supernatant was removed and the precipitate again extracted with 150 ml of water as before. The process was repeated a third time. The combined aqueous extract was acidified slowly with hydrochloric acid to pH 1.5. It was then heated to 80°C for 2 minutes in 200 ml lots. The heated solutions were cooled as rapidly as possible and centrifuged at 2000 RPM and the precipitate discarded. The supernatant was brought to pH 5.8 with ammonium hydroxide and centrifuged again at 2000 RPM.

2) Acetone Precipitation

The supernatant was added to three volumes of acetone at 0°C and the precipitate which formed was collected by centrifugation at 2000 RPM. The supernatant acetone solution was discarded. The precipitate was extracted very thoroughly with 100 ml of 0.005M phosphate buffer (pH 7) and centrifuged at

9000 RPM for 30 minutes.

3) DEAE-cellulose chromatography - Column 1

The 9000 RPM supernatant was loaded on a DEAE-cellulose column (4 x 45 cm) which had previously been equilibrated with .005M phosphate (pH 7.0). Elution was carried out with a linear gradient from 0 to 0.5M sodium chloride, and with both chambers of the mixing apparatus 0.005M in pH 7.0 phosphate buffer. About 23,500 $O.D._{280}$ units were eluted from this column of which 1385 were in the region of maximum RNase activity. The total activity of this peak was about 520,000 units.

4) Phenol Extraction

RNase was extracted from the above solution with phenol by the method of Rushizky et al. (22). The enzyme was forced out of the phenol by the addition of ten volumes of diethyl ether which had been freed of peroxides by the addition of water and calcium hydride followed by filtration (44). The resulting solution was extracted three times with 0.1M ammonium bicarbonate (pH 7.8), using in each case a volume of the bicarbonate solution equal to the original volume of phenol. The combined aqueous phase was extracted three times with ether to remove phenol and freed of ether by partial evacuation.

5) DEAE-Cellulose Chromatography - Column 2

The resulting RNase solution was loaded directly onto another DEAE-cellulose column (2.2 x 105 cm) and eluted with

a linear gradient from 0.05M sodium phosphate (pH 7.0) to 0.15 M sodium phosphate plus .2M sodium chloride. Of the 300 $O.D._{280}$ eluted from this second column about 60 O.D. units were in the peak of maximum activity. This contained about 255,000 units of RNase which was phenol extracted as before and forced out of phenol as described previously.

6) DEAE-Cellulose Chromatography - Column 3

The aqueous solution recovered was loaded onto a third DEAE cellulose column (1 x 105 cm). This was eluted with a gradient of 1 l of 0.05M sodium phosphate (adjusted to pH 7.0) to 1 l of 0.1M sodium phosphate (pH 7.0) plus 0.2M sodium chloride. A sharp peak of activity was eluted which coincided with a peak of ultraviolet absorbing material (Fig. 1). This peak contained 220,000 of the 255,000 units of RNase activity applied and a total of 26.5 $O.D._{280}$. Using the extinction value of Rushizky and Sober (12) for RNase T_1 this corresponds to 15.5 mg of protein.

B. Method of Rushizky and Sober (Modified)

1) Acid Extraction

A preparation of RNase T_1 was carried out using modifications of the procedure described by Rushizky and Sober (12). 200 g of Takadiastase was mixed with 1 l of distilled water and stirred at room temperature for 2 1/2 hours. Concentrated sulfuric acid was added to bring the pH of a 1 in 100 dilution to 2.6 and the solution was allowed to stand in the cold room for 48 hours. The pH was raised to 5.3 with 51

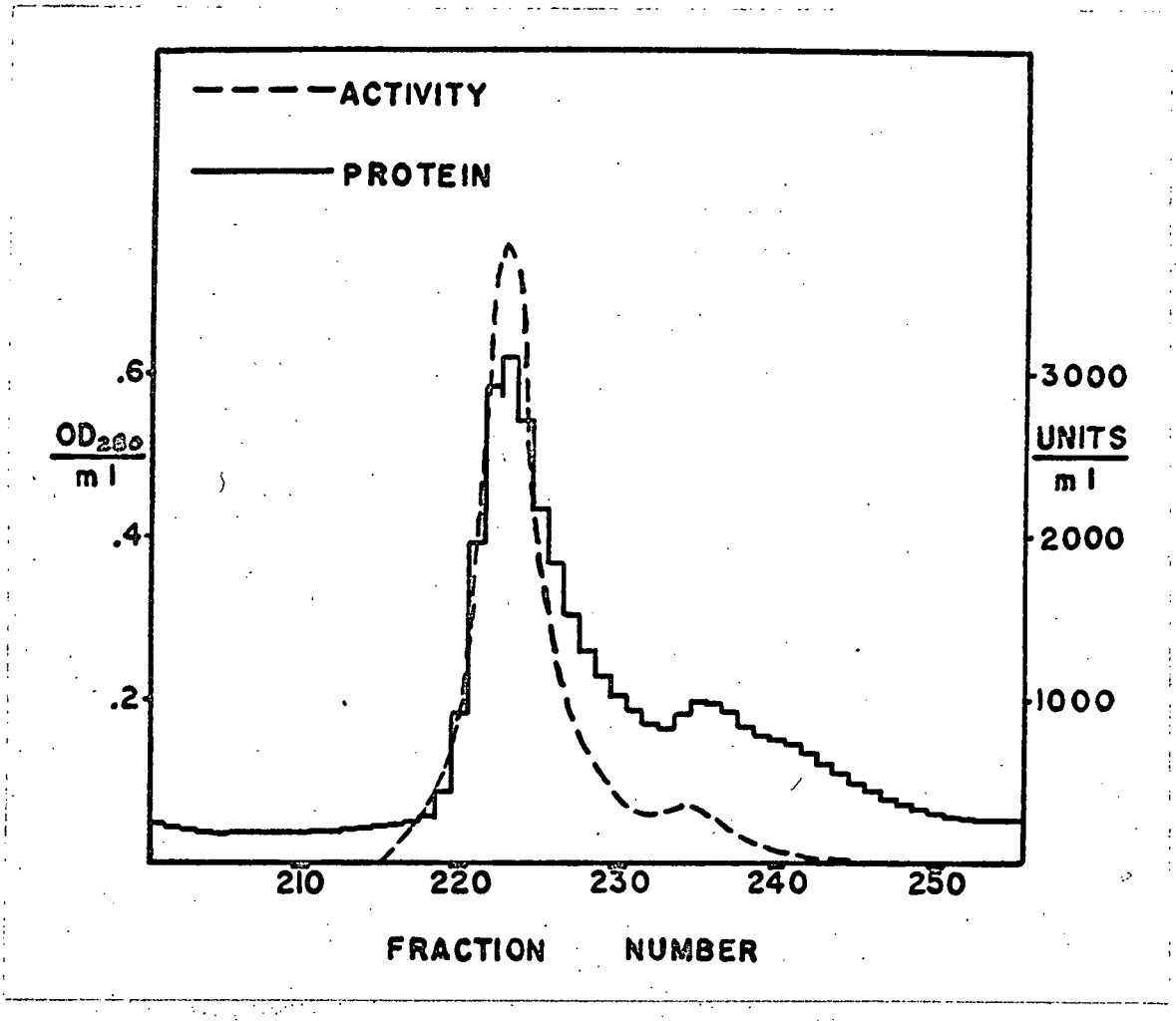


Figure 1. Third DEAE cellulose column chromatography of RNase T_1.

ml of concentrated NH_4OH. A clear dark solution was obtained by centrifugation at 8000 x g for one hour. This solution was exhaustively dialyzed against several changes of distilled water.

2) Acetone Precipitation

Ammonium acetate (pH 5.3) was added to bring the solution to 0.1M and it was added to 2.5 volumes of -15° acetone. The resulting precipitate was dissolved in distilled water and clarified by centrifuging for 45 min at 10,000 x g.

3) Gel Filtration

The volume of the 10,000 x g supernatant was reduced to 110 ml on a rotary evaporator at $30^{\circ}C$ and loaded on a column of Sephadex G-75 (7.5 x 42 cm) for gel filtration chromatography. The T_1 and T_2 activities were separated to a certain extent on this column. The T_1 fraction was further purified by DEAE-cellulose column chromatography, extraction into phenol, further DEAE-cellulose chromatography, gel filtration on Sephadex G-27, and a third chromatography on DEAE-cellulose.

TABLE II

RNase T_1 Purification

Procedure	$O.D._{280}$	Volume	Units	Specific Activity
Acid Extraction	103,820	1160	2,500,000	24
Acetone Precipitation	14,080	110	1,727,000	123
Sephadex G-75	8,232	980	1,234,800	150
DEAE Column I	1,567	1045	1,200,000	760
Phenol Extraction	656	650	540,000	820
DEAE Column II	148	290	485,750	3280
DEAE Column III	84	240	380,000	4530

Using the extinction value of Rushizky and Sober of

1.7/mg for RNase T_1, the quantity recovered is about 50 mg. Since 200 g of Takadiastase contain about 2.5×10^6 units of RNase activity, this may then be considered a 630 fold purification with 15.8% recovery.

Ribonuclease T_2 Purification

This preparation was carried out by modifications to the procedure described by Rushizky and Sober (14).

A. First Preparation

1) Acid Extraction

80 g of Takadiastase powder were mixed with 400 ml of distilled water and stirred at room temperature for two hours. The mixture was then cooled to 5° and 36N sulfuric acid added to lower the pH to 0.6. After 48 hr the pH was raised to 5.3 with ammonium hydroxide and the whole solution was centrifuged for one hour at 1000 x g.

2) Acetone Precipitation

The dark supernatant was added to 2.5 volumes of -16° acetone. The precipitate was collected and dissolved in 0.05M phosphate buffer (pH 7.0). This was centrifuged at 8000 x g and the precipitate discarded.

3) Sephadex G-25 Gel Filtration

The 8000 x g supernatant was concentrated and passed through a 600 ml bed volume column of Sephadex G-25. The Sephadex had been previously equilibrated with 0.05M phosphate (pH 7.0) and was used instead of the dialysis step recommended by Rushizky and Sober (14).

4) DEAE-Cellulose Chromatography - Column 1

The enzyme recovered from the Sephadex column was loaded directly onto a DEAE-cellulose (phosphate) column (3.0 x 80 cm). This column was eluted using a total of 4 l of eluant in a linear gradient from 0.05M phosphate (pH 7.0) to 0.15M phosphate plus 0.2M sodium chloride (pH 7.0). The T_2 activity was eluted before the T_1 but was not completely separated from it. The volume of the T_2 fraction was reduced to about 70 ml by using a rotary evaporator at low temperature ($30^{\circ}C$).

5) Sephadex G-75 Gel Filtration

A column of Sephadex G-75 (4 x 40 cm) was preequilibrated with 0.05M phosphate (pH 7.0) and loaded with 75 ml of concentrated RNase T_2. The $O.D._{280}$ of the effluent showed a small peak of absorption followed by a much larger peak. RNase T_2 activity was associated only with the earlier peak (Fig. 2).

6) DEAE-cellulose Chromatography - Column 2

The activity eluted off the Sephadex column was loaded directly on the second DEAE-cellulose column (3 x 80 cm). Elution was carried out exactly as with the first DEAE column. In this case the activity was eluted in a split peak, both portions of which had identical pH optima for activity. The whole region of RNase activity was again reduced in volume and subjected to dialysis against distilled water.

7) Attempted CM-cellulose Column Chromatography

The dialyzed T_2 solution was loaded onto a CM-cellu-

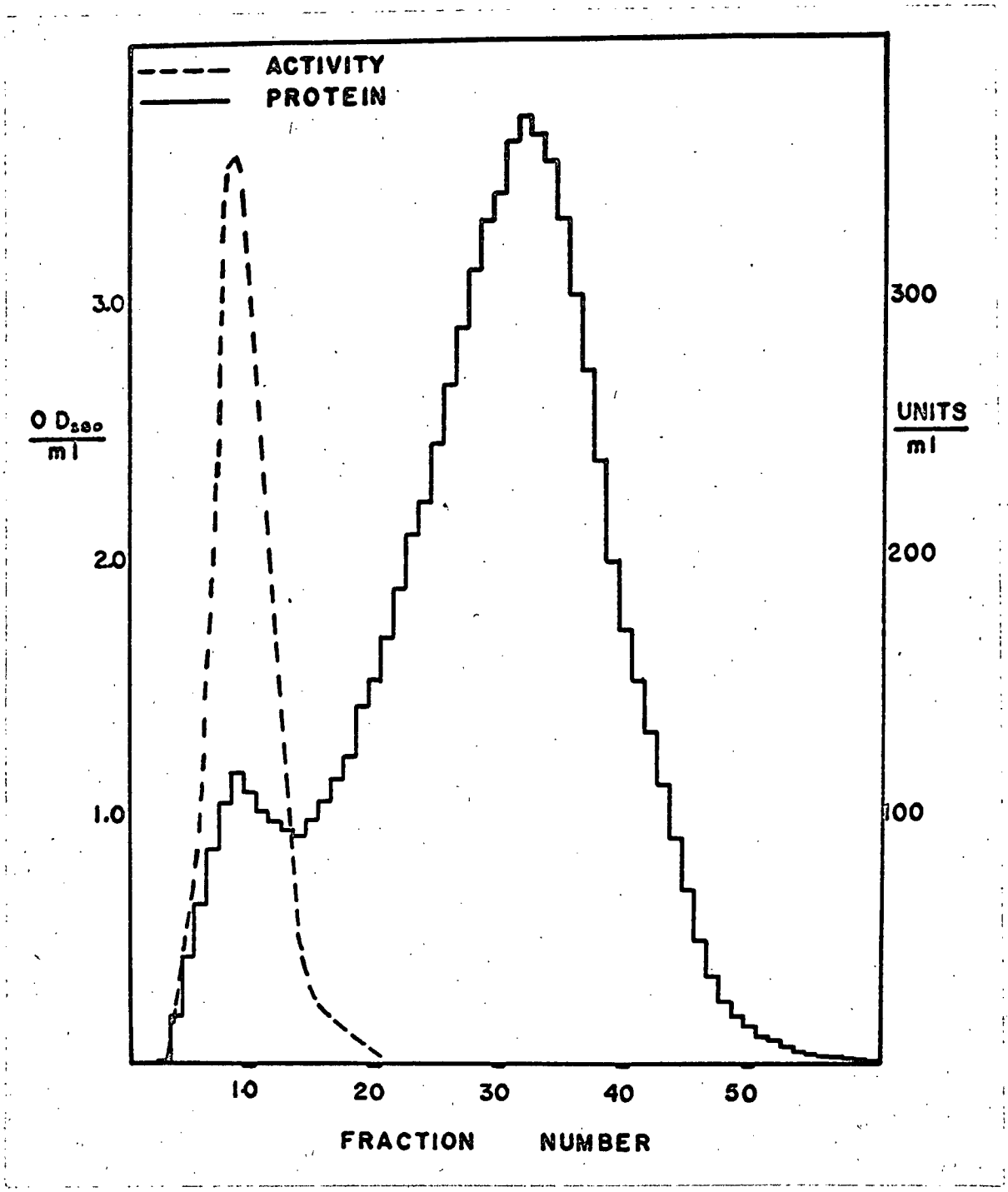


Figure 2. Sephadex G-75 chromatography of RNase T_2.

lose column (2.2 x 90 cm) which had previously equilibrated with 0.005M ammonium acetate (pH 4.35). It was washed in with one litre of the above solution and gradient elution began to a limit of 0.125M $(NH_4)_2CO_3$ (pH 7.6). At this point the activity was lost and it is now felt that it was probably not adsorbed and washed through the column at the front.

B. Second Preparation

1) DEAE-Cellulose Column Chromatography

A second preparation of RNase T_2 was carried out in conjunction with the second RNase T_1 preparation described previously. The RNase T_2 fraction separated from T_1 by gel filtration on Sephadex G-75 was chromatographed on DEAE-cellulose as described previously. The split peak of activity was again observed.

2) CM-Cellulose Column Chromatography - Column 1

After concentration and dialysis the enzyme was loaded onto a CM-cellulose column (3 x 90 cm) and washed in as described previously. In this case the front was collected and was found to contain nearly all of the protein applied to the column. A small peak of $O.D._{280}$ was eluted much later and found to have a high specific activity. However, the majority ($\geq$90%) of the activity was eluted with the front.

3) CM-Cellulose Column Chromatography - Column 2

This material which was not adsorbed was taken to a small volume on the rotary evaporator and dialyzed exhaustively against running tap water, then against distilled water and

finally against the starting buffer (ammonium acetate - .005 M, pH 4.35). The pH of the enzyme solution was then checked (4.35) and it was diluted with two volumes of the starting buffer. It was then loaded on the same, re-equilibrated CM-cellulose column. This time a greater proportion of the material was adsorbed, but again, the majority of the RNase activity passed through the column at the front (Fig. 3).

4) SE-Cellulose Column Chromatography - Column 1

Since this material was not adsorbed it was thought that SE-cellulose might adsorb the enzyme more firmly. Therefore a small column (2 x 20 cm) was packed with SE-cellulose and equilibrated with 0.1M acetic acid, adjusted to pH 3.5 with ammonium hydroxide. The enzyme solution was concentrated, dialyzed and made 0.1M in acetic acid, then adjusted to pH 3.5 with ammonium hydroxide. It was then loaded on the SE-cellulose column and washed thoroughly with the equilibrating solution. A linear gradient (total vol 1 l) was applied from the equilibrating solution to 0.6M ammonium carbonate (pH 7.8). The main peak of $O.D._{280}$ and RNase activity was eluted very early in the gradient. However, the material which washed through before the gradient was started comprised about 40% of the total $O.D._{280}$ applied but less than 5% of the activity applied.

5) SE-Cellulose Column Chromatography - Column 2

The most highly purified fractions eluted from the two CM-cellulose columns and those from the SE-cellulose column

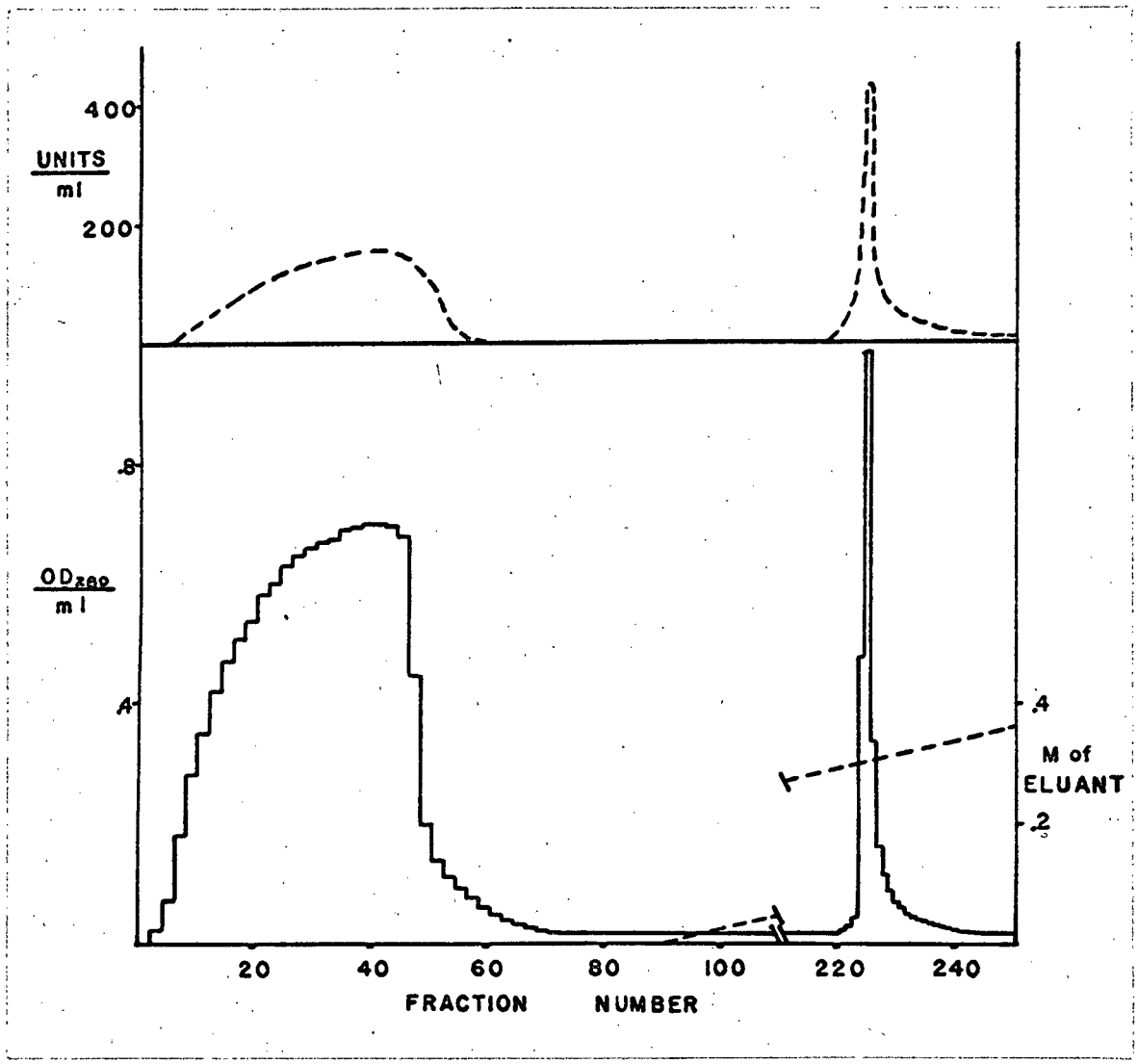


Figure 3. CM-cellulose column chromatography of RNase T_2.

were combined, concentrated and dialyzed. They were then applied to a large SE-cellulose column (3.3 x 90 cm) and washed in with 0.05M ammonium acetate (pH 3.5) and eluted with a 4 litre linear pH and salt gradient to .15M ammonium acetate, pH 4.5. The fractions of maximum specific activity were pooled and concentrated (Fig. 4). It was this fraction which was used for the synthetic substrate studies.

Determinations of the extent of purification and of the overall yield of this enzyme cannot be accurately made because of the far greater quantity of RNase T_1 present in the early stages. The most purified sample of this enzyme prepared to date had a specific activity of 415 units per $O.D._{280}$ unit.

Characterization of RNase T_1

A sample of high molecular weight yeast RNA was chromatographed on Sephadex G-200 and that portion which was eluted at the $O.D._{260}$ front was reserved for digestion with RNase T_1. 18.5 ml of this RNA (containing 1650 $O.D._{260}$ units) were incubated with 0.5 mg of RNase T_1 at room temperature and the pH was maintained above 7.3 by the dropwise addition of 0.1N NaOH. After 9 hr at room temperature *E. coli* alkaline phosphatase was added to the solution and incubation was continued at 37°C for 13 hours. At this time half of the material was removed and the other half left to incubate for a further 24 hours. After each portion was removed from the incubator, potassium hydroxide was added to 0.3M and the solutions then

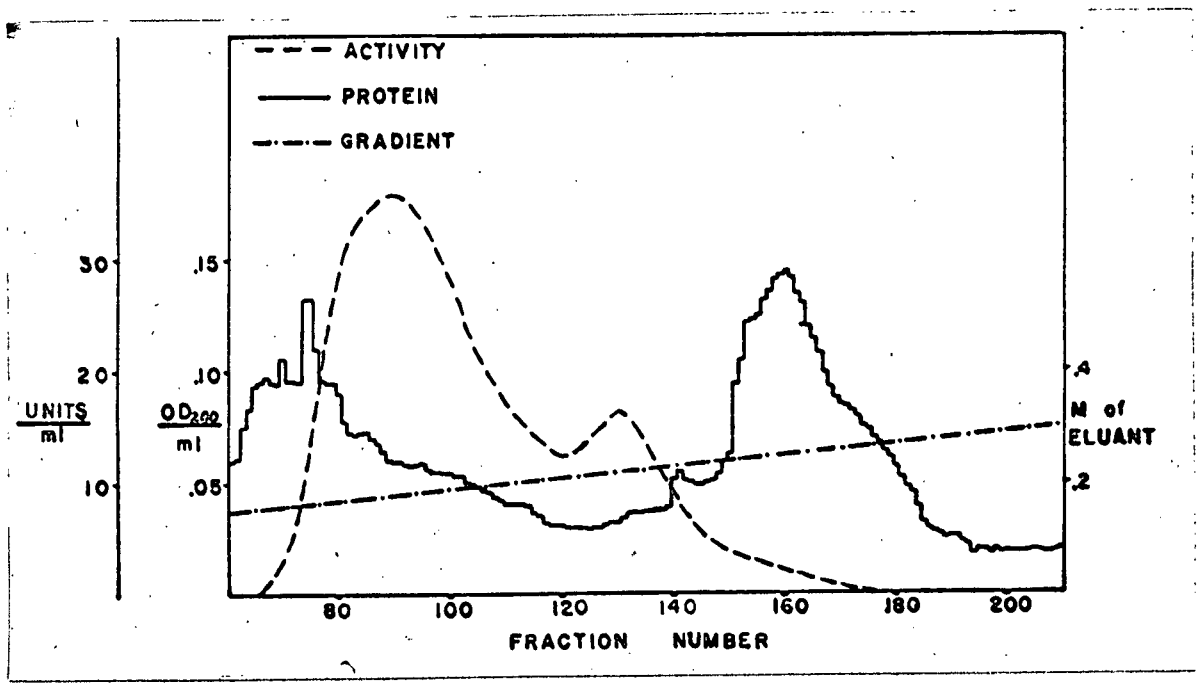


Figure 4. SE-cellulose column chromatography of RNase T_2.

incubated for 24 hours at 37°C. Each was neutralized with perchloric acid and cooled to 0°C. Potassium perchlorate was centrifuged off and samples were submitted to high voltage electrophoresis on Whatman 31 paper. (0.1M formate buffer, pH 3.1, 83 volts/cm). Standard samples of the four nucleosides and the four nucleotides were also applied as markers.

The electrophoretograms showed the presence of AMP, CMP, and UMP as well as guanosine in the two digestion mixtures. These spots were cut out and eluted, as were the regions where GMP and the 3 other nucleosides were expected to run. Spectra of all 8 spots in both experiments showed no GMP, no adenosine, cytidine or uridine. In contrast, the spots corresponding to guanosine, AMP, UMP and CMP contained from 7.5 to 9 O.D. units each. On this basis it can safely be assumed that greater than 99% of the hydrolysis catalyzed by the RNase T_1 preparation occured distal to guanylic acid residues, and that greater than 99% of all linkages distal to guanylic acid were hydrolyzed.

Characterization of RNase T_2

Measurements were made of the rates of hydrolysis of adenosine 3'-phosphate propyl ester, uridine 3'-phosphate propyl ester and the two purine nucleoside cyclic phosphates by the previously described chromatographic method. The spectrophotometric method was used to follow the hydrolysis of the pyrimidine nucleoside cyclic phosphates.

In the latter case the reactions followed pseudo

first order kinetics but in no case did the chromatographic data give an integral order.

It was shown that RNase T_2 would hydrolyze all four nucleoside 2',3'-cyclic phosphates but comparative rates and Km values have not yet been determined. Hydrolysis of uridine 2',3'-cyclic phosphate is faster than cytidine 2',3'-cyclic phosphate and cyclic adenylic acid was hydrolyzed faster than cyclic guanylic. Adenosine 3'-phosphate propyl ester was transesterified to the cyclic phosphate and then hydrolyzed faster than uridine-3'-phosphate propyl ester.

Preliminary studies of divalent cation inhibition have shown 0.001M cupric sulfate to be a very potent inhibitor of RNase T_2, at least for the hydrolysis of uridine 2',3'-cyclic phosphate. The same concentration of magnesium chloride brought about less than 15% inhibition of the same reaction.

DISCUSSION

Substrate Syntheses

A. Nucleoside 2',3'-cyclic Phosphates

The method used in the earlier work (41) gave satisfactory yields of all four cyclic phosphates but was inconvenient in that formamide was used as the solvent for the reaction and could not be removed except by chromatography on DEAE-cellulose, or else by precipitating the cyclic phosphates from acetone with barium iodide. The modified procedure of Smith and Khorana (42) did not use formamide and was therefore more convenient, but was of little value for cyclization of guanylic acid since this compound was too insoluble in methanol, the solvent used, to react with the DCC.

B. Nucleoside 2'(3')-phosphate Propyl Esters

Synthesis of the mixed isomers by the hydrochloric acid catalyzed transesterification method of Tener and Khorana (40) was in all cases successful and gave yields of greater than 60%. Separation of the isomers of adenosine 2'(3')-phosphate propyl esters was accomplished readily on DEAE-cellulose, but the separation of the guanosine derivatives was more difficult. The chromatographic methods used were incapable of separating the isomeric propyl esters of cytidylic and uridylic acids. However, the pure 3'-phosphate propyl esters of the pyrimidine nucleosides could be synthesized enzymatically, but due to the absolute requirement for some water in the incubation mixture only relatively low yields of ester were ob-

tained.

RNA Preparation

The method of Crestfield et al. (43) is adequate for preparing RNA suitable for an analytical substrate. The introduction of a phenol extraction step ensures the removal of all ribonucleases which may contaminate and degrade the preparation (22). In addition it was found that combining this phenol extraction step with filtration through a Celite bed makes ultracentrifugation unnecessary.

Pancreatic RNase

The preliminary studies carried out on pancreatic RNase using nucleoside 2'(3')-phosphate propyl esters showed the need for pure 3'-isomers. In no case was it possible to obtain zero or first order kinetics for the disappearance of the susceptible isomer. This was due to competitive inhibition by the 2'-isomer which was present as about 36% of the total ester concentration, and by the product, nucleoside 3'-phosphate. The kinetics of the transesterification reaction are difficult to follow because of the presence of the competing hydrolytic reaction. Further, this competing hydrolytic reaction makes determination of nucleoside 2',3'-cyclic phosphate not meaningful for kinetic studies so all that can be measured is the overall two step reaction. The inability of both the A and B fractions of pancreatic RNase to catalyze hydrolysis of the propyl phosphates of adenosine and guanosine disagress with the reported non-specificity of these fractions

(6) and is in agreement with the generally accepted pyrimidine specificity of pancreatic RNase.

Mann Diastase

The nature of the enzyme or enzymes present in the A. oryzae diastase preparation from Mann Laboratories has not yet been established. The possibility that RNase T_2 or a very similar enzyme is present is strongly indicated by the observation that a chromatographically purified preparation was able to break down all four nucleoside 2'(3')-phosphate propyl esters. Compared with Japanese Takadiastase this diastase preparation has a very low level of RNase activity. In contrast to the lack of specificity of the partially purified enzyme it was observed that a crude extract hydrolysed adenosine 2'(3')-phosphate propyl esters more rapidly than the corresponding pyrimidine derivatives while not causing detectable breakdown of the guanosine esters. This suggested that an inhibitor may have been present which was removed either by the addition of EDTA or by the DEAE-cellulose chromatography. Such an inhibitor might be very useful for structural studies of s-RNA if it could be shown to completely inhibit hydrolysis distal to one or more different nucleotide residues. Future work on the purification of this enzyme and on the nature of its inhibitors is proposed.

RNase T_1 Purification

RNase T_1 is a very stable enzyme and can be quite safely subjected to heat (80°C), acid (below pH 1), phenol,

and concentration on a rotary evaporator at 30°C with little detectable loss of activity. In its purification use was made of these stability properties.

In the first preparation of RNase T_1, using a procedure derived from that of Takahashi (10), a yield of 15.5 mg of RNase T_1 was obtained from 80 g of Takadiastase. This compares quite favorably with his reported yield of about 100 mg from a kilogram of Takadiastase (10). The two preparations had approximately the same specific activity. In a more recent publication Takahashi reported an increase in his average yield of RNase T_1 to about 150 mg per kilo (11). Modifications of Takahashi's procedure included the substitution of acetone fractionation for ammonium sulfate fractionation, a step in which he lost between 20 and 25% of his total RNase activity. The use of phenol to extract RNase T_1 from aqueous solution was reported by Rushizky *et al.* (22) to be an effective method for concentrating and desalting this, and other enzymes. In the present work phenol extraction was used instead of dialysis to avoid the losses of activity encountered in dialysis as a result of the small size of the protein. Since no large scale method has yet been described for the removal of RNase T_1 from the phenol the use of ether to force the enzyme into a slightly basic solution was investigated. In general this was fairly effective and gave a good recovery of activity when the diethyl ether used was previously treated with calcium hydride to destroy peroxides (44). The third DEAE-cellulose column chromat-

ography was not carried out by Takahashi but in this work doubled the specific activity of the RNase T_1 preparation. Also the sizes of columns and the volumes of elution gradients used by Takahashi were much smaller than those reported here.

The second RNase T_1 preparation was carried out by a procedure modified from that of Rushizky and Sober (12). This preparation resulted in the recovery of about 50 mg of RNase T_1 with a lower specific activity than the first. This yield is about 75% of that reported by Rushizky and Sober but due to different RNase assay procedures the specific activities cannot be compared. A definite improvement over Takahashi's procedure is the low temperature acid extraction used by the former workers. Elimination of the heat step and the use of even lower pH produces an increase in the specific activity of the preparation without undue loss of total activity. Gel filtration on Sephadex G-75 prior to DEAE-cellulose chromatography was introduced to eliminate low molecular weight materials and to separate RNase T_2 from T_1. This now seems to be impractical at this stage in the purification because of the bulk of the solution applied and its viscosity.

Phenol extraction after the first DEAE-cellulose column chromatography was less successful than in the previous experiment, and consequently was the point of major loss of activity during this preparation.

The homogeneity of the most purified preparation of RNase T_1 is indicated by the narrow band of activity eluted

from DEAE-cellulose which coincided with a sharp peak of protein (Fig. 1).

Future preparations of this enzyme would probably be best carried out by using column chromatography on Sephadex G-25 to remove salts between DEAE-cellulose chromatography steps.

RNase T_2 Preparation

The purification of RNase T_2 has not progressed to the state where the preparation can be considered as consisting primarily of a single molecular species. The presence of an overwhelming amount of RNase T_1 in the starting material makes estimation of the purification factor impossible. The use of a different assay procedure by Rushizky and Sober (14) makes comparison of specific activities difficult.

The phenol extraction technique, used successfully for desalting of RNase T_1 preparations could not be applied to the T_2 purification because it caused complete loss of activity. However, gel filtration of RNase T_2 preparations concentrated from ion exchange column effluents proved very satisfactory, both for desalting and for removal of lower molecular weight material (when Sephadex G-75 was used).

As mentioned previously the elution pattern of RNase T_2 obtained on DEAE-cellulose chromatography showed the enzyme to be eluted as a double peak of activity. This has been observed previously but as yet no explanations have been advanced (10,14). Similar observations have been made with pancreatic RNase when chromatographed on CM-cellulose or XE-64

ion-exchange resin. This has recently been shown to be due to several forms of the enzyme, all but one of which contain several glycosyl residues (45).

CM-cellulose chromatography of RNase T_2, using essentially the same conditions as those reported by Rushizky and Sober (14) was unsuccessful in every attempt made. In each case most or all of the activity passed through the column without being adsorbed. In two cases small quantities were adsorbed and eluted in a much more highly purified form but the quantities adsorbed were too small to make the procedure practical. The reason for its non-adsorption may have been ionic bonding to an acidic material present in the mixture. To circumvent this possibility a lower pH was tried. The pKa of protein carboxyl groups is sufficiently high that at pH 3.5 their ionization should be depressed and binding of acidic proteins to RNase T_2 should be reduced. Since this pH would also depress the ionization of CM-cellulose the more strongly acidic ion-exchanger, SE-cellulose, was used. Adsorption of nearly all of the RNase T_2 activity applied was accompanied by elimination of a significant quantity of inactive contaminating material.

Further work on the purification of this enzyme will be carried out using starch-gel electrophoresis (46). Complete separation of the two isomeric forms of RNase T_2 will also be attempted using DEAE-cellulose column chromatography.

Characterization of RNase T_1

The method used for this determination of specificity of RNase T_1 involved complete digestion of high molecular weight yeast RNA with RNase T_1, followed by E. coli phosphomonoesterase treatment and then complete hydrolysis with alkali. The finding of guanosine as the only nucleoside present and no guanylic acid indicates complete digestion by the enzyme and confirms the reported absolute specificity for those linkages distal to guanylic acid residues (12). A similar method was used by McCully and Cantoni (38) for determination of susceptible bonds in s-RNA which contains 1-methyl guanylic acid and N,N-dimethyl-2-amino-6-hydroxy purine nucleotide as minor constituents. They found that both of these were present in the complete digest as the nucleotides. From this they concluded that RNase T_1 would not attack linkages involving these methylated bases. This experiment was not conclusive though because if the transesterification step had occured to produce a 2',3'-cyclic phosphate which was resistant to hydrolysis then the same results would be found. The possibility that this may have been the case follows from experiments of Whitfeld and Witzel who showed transesterification of dinucleoside phosphates involving glyoxal guanosine but no hydrolysis of the resulting nucleoside 2',3'-cyclic phosphate (36). The non-critical nature of McCully and Cantoni's experiments makes necessary a re-examination of this problem. More meaningful information would be obtained if the phosphomonoesterase di-

gestion were carried out in the presence of an enzyme such as that described by Drummond et al. (47) which hydrolyzes all nucleoside 2',3'-cyclic phosphates forming the 2'-phosphates. In this case resistance of a 2',3'-cyclic ended oligonucleotide to phosphomonoesterase would be overcome and after alkaline hydrolysis an additional quantity of nucleoside would be present. This experiment will be attempted soon because a definite answer to this problem will be required before the T_1 enzyme can be used for structural studies of s-RNA.

Accurate quantitative measurements of the rate of hydrolysis of guanosine 2',3'-cyclic phosphate must also be made since it has been reported that this hydrolytic step is much slower than the transesterification step. Titration measurements of this hydrolysis may be not only a simpler but also a more accurate method for following this reaction than the chromatographic method.

Characterization of RNase T_2

The earlier reported specificity of RNase T_2 for bonds distal to adenylic acid residues (13) has been more recently shown to be incorrect (14). It is now thought that RNase T_2 digestion of RNA brings about complete hydrolysis to the 3'-mononucleotide level. The present studies with the most highly purified preparation of RNase T_2 confirmed this lack of base specificity by demonstrating that all four nucleoside 2',3'-cyclic phosphates are hydrolyzed to the 3'-phosphates. In addition it has been demonstrated that adenosine 3'-phosphate propyl ester and uridine 3'-phosphate propyl ester are broken

down, via the 2',3'-cyclic phosphates, to 3'-mononucleotides. This observation is contrary to that of Naoi-Tada et al. (13) who were unable to demonstrate the formation of cyclic phosphate intermediates in T_2 catalyzed hydrolysis of poly-adenylic acid. In preliminary studies of rates of hydrolysis of nucleoside 2',3'-cyclic phosphates the adenosine derivative was found to be opened at least twice as fast as the guanosine derivative. In digestions of adenosine 3'-phosphate propyl ester the cyclic intermediate was found, but it did not accumulate to the same extent ad the corresponding cyclic phosphate in the digestions of uridine 3'-phosphate propyl ester.

As discussed earlier, there are a number of limitations to procedures for following these reactions. Inhibition by the 2'-ester was eliminated from these studies by using the pure 3'-propyl esters which were obtained either by purification on chromatographic columns or by enzymatic synthesis. At present there is no means of accurately measuring the rate of the transesterification step independant of the hydrolytic step. Electrophoretic separation of dinucleoside phosphate digestions, as reported by Whitfeld and Witzel (36) for RNase T_1, could give some information on the first step reaction, but only if the rate of this reaction were much greater than the rate of the second step. Even in this case the only valid reaction rate measurements would be those made very early in the reaction, before the hydrolysis of the cyclic phosphates formed becomes an important competing reaction. If the hydro-

lytic reaction was occurring simultaneously with the transesterification reaction the result would be a decreased quantity of enzyme available for catalysis of transesterification. The reason for this decrease would be the existence of two different types of enzyme-substrate-complex.

In the hydrolytic step preliminary experiments indicate that reliable quantitative data may be obtained by the direct spectrophotometric method, at least for the measurement of hydrolysis of the pyrimidine nucleoside 2',3'-cyclic phosphates. The spectral differences between the 3'-phosphates and 2',3'-cyclic phosphates of purine nucleosides are too small to be accurately measured. In the case of the pyrimidine 3'-nucleotides the spectral difference is probably due to hydrogen bonding between the 2'-hydroxyl and the 2-oxygen substituent on the pyrimidine ring. This binding cannot occur in the cyclic phosphates. Similar spectral differences should also occur between the pyrimidine nucleosides and their 2'- or 2',3'-cyclic phosphates.

Unfortunately, titration cannot be used to follow the hydrolytic reaction because the pH optimum for RNase T_2 is 4.5 and at this pH the liberated phosphate groups are undissociated (pKa 6-7).

Finally, a number of divalent cations should be tested for their ability to inhibit the transesterification and hydrolysis of esters of all four 3'-nucleotides. If a differential inhibition could be demonstrated then possibly the T_2 en-

zyme would be of value in s-RNA structural studies. The preliminary observations on the effect of EDTA on the crude enzyme from the Mann diastase offer some hope for the success of these studies.

As a result of time limitations more extensive studies were not carried out but the preliminary results reported here are to be extended.

SUMMARY

1. Nucleoside 2',3'-cyclic phosphates and nucleoside 3'-phosphate propyl esters have been synthesized for use as substrates in studies on ribonucleases.
2. Ribonuclease T_1 has been highly purified from Takadiastase and studies on its specificity have been carried out.
3. Ribonuclease T_2 has been extensively purified and preliminary studies have been carried out on its relavive activities on different synthetic substrates.
4. A ribonuclease has been partially purified from an A. oryzae diastase extract and some of its properties are reported.

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