

PURIFICATION AND CHARACTERIZATION OF RIBOURIDYLYL
TERMINAL TRANSFERASE AND RIBOCYTIDYLYL TERMINAL
TRANSFERASE ACTIVITIES FROM RNA TUMOR
VIRUS-INFECTED AVIAN MYELOBLASTS

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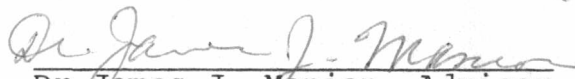
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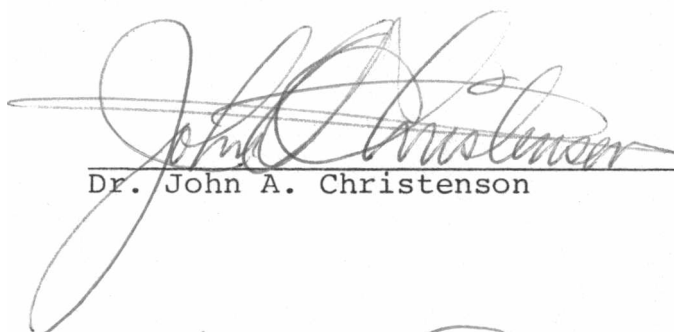
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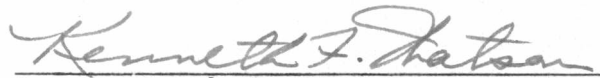


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This thesis for honors recognition has been approved
for the Department of Biology.


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ABSTRACT

Two enzymes, one which catalyzes the addition of one or more cytidine 5'-monophosphate residue from cytidine 5'-triphosphate to the 3'-terminus of a ribonucleic acid primer, referred to as ribocytidylyl terminal transferase, and one which catalyzes the addition of one or more uridine 5'-monophosphate residue from uridine 5'-triphosphate to the 3'-terminus of a ribonucleic acid primer, referred to as ribouridylyl terminal transferase, have been purified from RNA tumor virus-infected avian myeloblasts. The purification procedure involved cation and anion exchange chromatography. Both enzymes were stimulated by the divalent cations Mg^{++} and Mn^{++} with optimal concentrations of 0.0001 M Mn^{++} and between 0.001 M and 0.004 M Mg^{++} . The molecular weight of ribocytidylyl terminal transferase was approximately 60,000 and the molecular weight of ribouridylyl terminal transferase was approximately 56,000.

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INTRODUCTION

Ribonucleotidyl terminal transferases are enzymes that catalyze the addition of ribonucleotide residues (CMP, UMP, AMP, or GMP) to the 3'-hydroxyl group of preformed ribonucleic acid (RNA). The enzymes use ribonucleotide triphosphates (CTP, UTP, ATP, or GTP) as substrates. RNA ribonucleotidyl transferases have also been referred to as poly (A) polymerase, poly (G) polymerase, poly (C) polymerase, and poly (U) polymerase. Among these homopolynucleotide polymerases, only poly (A) polymerase has been extensively studied. The other three specific homopolynucleotide polymerases are less well documented in vitro and their role in vivo is poorly understood.

Our research was directed toward purifying and characterizing two of these relatively unresearched homopolynucleotide polymerases: ribouridylyl terminal transferase (poly (U) polymerase) and ribocytidylyl terminal transferase (poly (C) polymerase). The activities were derived from RNA tumor virus-infected avian myeloblasts (immature white blood cells).

The in vivo function of this enzyme class is largely unknown and deserves investigation in itself. However, our

work was directed toward determining in vitro characteristics and properties of these ribonucleotidyl terminal transferases leading to in vitro applications. A greater understanding of the in vitro capabilities of this enzyme is important for application in molecular biology, especially in recombinant DNA research. Many types of RNA do not contain a 3'-homopolymer sequence which is necessary for the enzyme reverse transcriptase to synthesize a complete DNA strand copy. An application of ribonucleotidyl terminal transferases would then be to synthesize a 3'-homopolymer tail on selected RNA strands. This would enable a DNA strand to be synthesized from this selected RNA strand, hence a gene could be synthesized.

LITERATURE REVIEW

There are riboadenylate terminal transferases specific for AMP that have been extensively studied (10, 14, 25, 26, 16, 20, 29, 23). Terminal ribouridylate transferases specific for UMP (2, 17, 21, 23), terminal ribocytidylate transferases specific for CMP (6, 9), and terminal riboguanylate transferase specific for GMP (3), have not been as extensively studied. These ribonucleotidyl terminal transferase activities differ from RNA polymerase activity which requires a template DNA to synthesize RNA by a complementary copying mechanism (5, 27). They also differ from polynucleotide phosphorylase, an enzyme that normally degrades RNA at its 3'-terminus, but in the presence of high concentrations of ribonucleoside diphosphates, adds ribonucleotides terminally (15). However, in studies with eukaryotic cells, polynucleotide phosphorylase presents few experimental difficulties since it appears in prokaryotes only.

Modern investigation of nucleic acid biosynthesis began in 1955 with Grunberg-Manago and Ochoa's discovery of an enzyme activity in Azobacter vinelandii extracts that polymerized nucleoside diphosphates into phosphodiester bonds characteristic of those found in natural RNAs (15). A short

time later, a DNA polymerase activity was reported by Kornberg and his co-workers using E. coli extracts (18).

Not only did these experiments prove the feasibility of studying polynucleotide biosynthesis with isolated enzyme systems, but they also supplied the basic experimental technique for investigations in this field. The technique entails measuring incorporation of radioactively labeled mononucleotides into polynucleotide. Early studies of RNA synthesis were hampered by a lack of knowledge of the different variety of RNA species that occurred. There was confusion in data interpretation which was only gradually resolved as the identification and characterization of natural RNA species was achieved.

The first detailed description of a ribonucleotidyl transferase appeared in 1960 (10). This was a study on riboadenylyl terminal transferase or poly (A) polymerase. At the time, and for some time afterwards, there was only a limited availability of radio labeled nucleotide substrates other than those of adenine. Hence, detection of poly (A) polymerase was favored. Evidence for polymerization of long sequences of AMP residues by partially purified extracts of calf thymus nuclei was obtained from a nearest-neighbor analysis of the reaction product synthesized from $\overline{\alpha - ^{32}\text{P}}$ ATP. The results showed that 99% of the incorporated ^{32}P was in the dinucleotide A-A. Another experiment was done to find the average length of the sequences by end group analysis of the product, which had been synthesized using ^{14}C labeled

ATP (11). The results indicated an average length of 50 to 100 nucleotides. Using extracts of Ehrlich ascites cells, it was shown that a different enzyme system was responsible for nonterminal incorporation of ATP into polynucleotides (cited in 14).

Since 1960, ribonucleotidyl terminal transferases have been described from a wide variety of organisms including bacteria (25, 26), yeast (16), plants (20, 2), and higher animals (10, 6, 29). By 1965, several laboratories had reported at least partial purification of poly (A) polymerase from calf thymus nuclei (10), and chick embryo allantoic membranes (30). The enzyme isolated from E. coli was reportedly crystallized (1). The common characteristics of the purified enzymes were a marked specificity for ATP as a substrate and a requirement for an RNA primer to which the poly (A) was added.

Also emerging from these early investigations was a general description of the reaction mechanism involving the sequential addition of adenylate residues to a primer rather than being generated by a template copying mechanism. Evidence supported a non-transcriptional process for the biosynthesis of these poly (A) sequences (10, 30) in that labeled nucleotides were found to be covalently attached to an RNA primer at the 3' terminus, whereas complementary RNA copies would be expected from a template copying mechanism.

Experimental data that sustained interest in continued research on poly (A) was also reported in these early studies.

Some results appeared to have biological importance. Infection of E. coli with T2, T4, or T5 phage caused a marked and immediate loss of poly (A) polymerase activity (24). This effect was not observed for other enzyme systems active in E. coli RNA metabolism. The decline seemed to be caused by synthesis of a protein inhibitor of poly (A) polymerase. These results suggested a role for these reactions in macromolecule metabolism. Another discovery that spurred interest was that poly (A) sequences were found in calf thymus nuclear extracts (11). This result was later confirmed when a poly (A) fraction lacking other nucleotides was isolated from calf thymus nuclear extracts using oligo(dT)-cellulose columns (12).

Early work also sometimes found other specific homopolynucleotide polymerases present in the same extracts with poly (A) polymerase activity (9, 28, 4 cited in 14). Some preliminary results showed separation of poly (U) polymerase from poly (A) polymerase found in extracts of rat liver cytoplasm (cited in 14). Also, a poly (C) polymerase was separated from poly (A) polymerase of calf thymus extracts (9). These other specific homopolynucleotide polymerases could not be linked to poly (U), poly (C), or poly (G) sequences in cells, so they have not been extensively studied.

In the late 1960s, most laboratory work was directed toward detection and characterization of poly (A) sequences in animal cells. Not as much work was directed toward polymerase activity (cited in 14).

Interest in polymerase reactions was greatly renewed in 1971 when poly (A) sequences were found to be covalently bound to heteronuclear RNA and messenger RNA molecules of animal cells (7, 19, 13). The poly (A) sequences were later identified at the 3'-terminus of the RNA (22). Evidence was also presented for post-transcriptional addition of the poly (A) sequences (8). Currently, laboratories are concentrating on increasing the purity of poly (A) polymerase to define the properties of the reaction more clearly (14). Its cellular function is unknown except for the possible polyadenylation of RNA in eukaryots (14).

In 1969, the presence and fractionation of an RNA-dependent RNA polymerase activity from RNA tumor virus-infected chick white blood cells was reported (32). In later studies to further purify this activity, it was apparent that the enzyme activity was present in several fractions during purification and each fraction was distinguished by the specific ribonucleoside triphosphate utilized (ATP, CTP, or UTP) (31). This led to the hypothesis that a mixture of ribonucleotidyl transferases was being observed. This was the first time that 3 polymerase activities have been observed in one cell type.

MATERIALS AND METHODS

All work was done at 0-4°C unless otherwise noted. All absorbances were taken in a Beckman DU-8 spectrophotometer at 280 nm and 260 nm.

Preparation of Carboxymethyl Sephadex Column

Ten grams of CM-Sephadex C-50 beads were equilibrated by washing twice with a 500 ml volume of 0.5 N HCl. The beads were then washed with double distilled water until the pH was approximately 4. The beads were then washed twice with 500 ml volumes of 0.5 N KOH followed by extensive washing with water. The beads were finally resuspended in 500 ml of double distilled water.

A Pharmacia column (1.5 x 30 cm) was plugged and filled to 2/3 volume with 0.01 M potassium phosphate buffer, pH 8, containing 5% v/v glycerol. Equilibrated beads were then added to the column and allowed to settle. After a base was established, the column was allowed to flow at a rate of approximately 1 ml per min as the rest of the beads settled. The final column measured 1.5 cm by 20 cm and was capped with 0.5 cm of CM-Sephadex G-50 beads. The column was equilibrated with 0.01 M potassium phosphate buffer, pH 8, containing 5% v/v glycerol. After equilibration, the pH input to the column and pH of the output from the column were approximately equal.

Preparation of Diethylaminoethyl Anion Exchange Columns

Twenty grams of Whatman DEAE-52 cellulose were suspended in 0.01 M potassium phosphate buffer, pH 7.6. The fibers were allowed to settle and the fine cellulose fibers were poured off. The fibers were then washed with 0.5 M potassium chloride buffer.

Columns were filled to 2/3 volume with 0.01 M potassium phosphate buffer, pH 7.4, containing 5% v/v glycerol. The beads were then added and allowed to settle to form a column that measured 0.9 cm by 10 cm. The column was then equilibrated with 0.05 M potassium phosphate buffer, pH 7.4, containing 5% v/v glycerol. After equilibration, the pH input to the column and pH output from the column were approximately equal.

Preparation of Radiolabeled Nucleoside Triphosphate Substrate

A 50-nM stock solution of unlabeled UTP containing $\gamma\text{-}^3\text{H}\text{UTP}$ (specific activity 52 Ci/mMole) was prepared to a final specific activity of 50 counts per min per pMole. This represented 1 uCi of $\gamma\text{-}^3\text{H}\text{UTP}$ per ul stock solution. The solution was diluted with 0.01 M Tris buffer, pH 8.2, to give a final concentration of 50 nMoles nucleoside triphosphate per 10 ul of triphosphate mix. When added to the enzyme reaction mixture which had a total volume of 50 ul, this gave a concentration of 10 nMoles nucleoside triphosphate per 50 ul assay.

The same procedure was followed for the preparation of the $\angle^{-3}\text{H}\overline{\text{CTP}}$ mix ($\angle^{-3}\text{H}\overline{\text{CTP}}$ has a specific activity of 24 Ci/mmole).

Cell Disruption

RNA tumor virus-infected avian myeloblasts which had been stored at -70°C were allowed to slowly thaw at room temperature. The cells were then suspended in 0.05 Tris buffer, pH 7.4, containing 0.005 M MgCl_2 , 0.0005 M EDTA, 0.1 M sucrose and 0.001 M DTT at a ratio of 1 ml buffer per gram of cells. The cells were then homogenized in a Dounce homogenizer with approximately 20 passes at 1,500 rpm. The homogenate was then transferred to glass Corex centrifuge tubes.

Centrifugation of Cell Homogenate

The homogenate was centrifuged at 1,500 rpm in a HB4 Sorvall swinging bucket rotor for 8 min. The supernate (S-0) was transferred to plastic centrifuge tubes. The pellet was discarded. The supernate (S-0) was centrifuged for 35 min at 11,000 rpm in a HB4 Sorvall rotor. The supernate (S-1) was removed and saved. The pellet was saved at -40°C for RNA extraction at a later date.

Protamine Sulfate Precipitation

The supernate (S-1) was made 5% v/v glycerol and its absorbance at 260 nm was recorded. Protamine sulfate was added to the supernate (S-1) at a level of 0.04 mg per A_{260}

unit. The addition was done slowly and the mixture was constantly stirred for 15 min. The mixture was then centrifuged for 15 min at 11,000 rpm in a HB4 Sorvall rotor. The supernate (S-2) was removed and the pellet was discarded.

Application to CM-Sephadex Column

The supernate (S-2) was made 5% v/v in glycerol and 0.001 M DTT. It was then applied to a CM-Sephadex cation exchange column which had been equilibrated as previously described. The column was then washed with 0.01 M potassium phosphate buffer, pH 8, containing 5% v/v glycerol and 0.001 M DTT. Fractions were collected in 6-ml aliquots until the A_{280} was less than 0.1 (using the wash buffer as a blank). The column was then eluted with 0.1 M potassium phosphate buffer, pH 8, containing 5% v/v glycerol and 0.001 M DTT. Fractions were collected in 2-ml samples until the A_{280} was less than 0.1. The column was then eluted with 0.3 M potassium phosphate buffer, pH 8, containing 5% v/v glycerol and 0.001 M DTT. Fractions were collected in 2-ml samples until the A_{280} was less than 0.1.

Standard Assay Procedure

Fractions taken from the column that had high absorbances were assayed. A 10- μ l sample of column fractions was added to the reaction mixture. If the activity had been concentrated then a 1- or 2- μ l sample was added to the reaction mixture. The enzyme fraction was the last component to be added to

the reaction mixture. The other components of the reaction mixture (listed in the order that they were added) were: 10 ul basal mixture (which contained 0.08 M Tris buffer, pH 8.2, 0.008 M $MgCl_2$ and 0.001 M DTT), 0.01 M Tris buffer, pH 8.2 (the amount added was enough to bring the final volume of the reaction mixture to 50 ul), 10 ul of labeled triphosphate (either 3H -CTP or 3H -UTP), and 10 ul of myeloblast RNA (which was at a concentration of 2 mg per ml). The reaction mixtures were incubated at 37°C in a water bath for a specified period of time. After incubation, the total volume of each reaction was spotted on a Whatman DE-81 anion exchange filter. The filters were then washed six times with 5% w/v Na_2HPO_4 and then twice with double distilled water. The filters were then dried under a heat lamp, placed in 4 ml of DE-81 scintillation fluid, and then placed in a Beckman LS-230 Liquid Scintillation System which measured the counts per min on the filters. A 1-ul aliquot of the labeled triphosphate mixture (3H -CTP or 3H -UTP) was spotted on a filter, dried, and placed directly in scintillation fluid for counting. The counts per min from this sample were used to determine the specific activity of the tritiated substrate solution.

Concentration of Fractions

Fractions from the separate elutions of the columns that showed activity were separately pooled and the volumes were recorded. Enough saturated ammonium sulfate was added

to the pooled fractions to reach 50% of saturation. The fractions were allowed to sit for 30 min in ice and were then centrifuged for 30 min at 12,000 rpm in a HB4 Sorvall rotor. The supernates were taken off and their total volume was recorded. The pellets were resuspended in small volumes of 0.05 M potassium phosphate buffer, pH 8, containing 10% v/v glycerol and 0.001 M DTT. More saturated ammonium sulfate was added to the supernates to reach 70% saturation. The mixture was left standing for 30 min and then centrifuged for 30 min at 12,000 rpm in a HB4 Sorvall rotor. The supernates were drained and stored at 0°C and each pellet was resuspended in a small volume of 0.05 M potassium phosphate buffer, pH 8, containing 10% v/v glycerol and 0.001 M DTT. The pellets were then assumed to have concentrated activity for assay reactions.

Application to DEAE Column

The 0-50% ammonium sulfate precipitate pellet suspension and the 50-70% ammonium sulfate precipitate pellet suspension from the 0.3 M potassium phosphate elution of the CM-Sephadex column were pooled and diluted 10-fold with 0.05 M potassium phosphate buffer, pH 8, containing 10% v/v glycerol and 0.001 M DTT. The 50-70% pellet suspension from the 0.1 M potassium phosphate elution of the CM-Sephadex column was diluted 10-fold with 0.05 M potassium phosphate buffer, pH 8, containing 10% v/v glycerol and 0.001 M DTT. The absorbances were then taken. Both activities were then

applied to DEAE columns which had been equilibrated by methods previously described. The columns were washed with 0.01 M potassium phosphate buffer, pH 7.4, containing 5% v/v glycerol and 0.001 M DTT. Fractions were collected in 2-ml samples until A_{280} was less than 0.1. The columns were then eluted with 0.1 M potassium phosphate buffer, pH 7.4, containing 5% v/v glycerol and 0.001 M DTT. Fractions were collected in 2-ml samples until A_{280} was less than 0.1. The columns were then eluted with 0.3 M potassium phosphate buffer, pH 7.4, containing 5% v/v glycerol and 0.001 M DTT. Fractions were collected in 2-ml samples until A_{280} was less than 0.1. Selected fractions were assayed for activity. The fractions with the highest activity from each column were pooled separately. The enzyme activities were then concentrated by ammonium sulfate precipitation methods previously described except only a 0-70% precipitation was done. The pellets (two of them) were suspended in small volumes of 0.05 M potassium phosphate buffer, pH 8, containing 10% v/v glycerol and 0.001 M DTT. The activities were assumed concentrated.

Velocity Gradient Centrifugation

There were two buffers involved in the gradient. The first was a light buffer consisting of 50 mM KPO_4 , pH 7.4, 10% glycerol, and 0.1 M KCl. Fifty ml of this buffer were prepared. The second buffer was a heavy buffer

composed of 50 mM KPO_4 , pH 7.4, 30% glycerol, and 0.1 M KCl. Fifty ml of the heavy buffer were prepared.

A gradient maker with a magnetic stir bar was used to produce the linear gradient. The stir bar was put on the heavy side (left tube) of the gradient maker. Beckman SW-41 centrifuge tubes were used to collect the formed gradient.

Of the light buffer, 5.9 ml was poured into the right tube of the gradient maker. The left tube of the gradient maker was filled with 5.9 ml of heavy buffer. DTT was added to only those gradients that contained enzyme. Two enzyme gradients with DTT were made.

Next, the molecular weight markers were prepared at 10 mg/ml. The first marker fraction consisted of 20 ul of ovalbumin, 20 ul myoglobin, and 160 ul of double distilled water. The second marker fraction consisted of 20 ul of bovine serum albumin, 20 ul of myoglobin, and 160 ul of double distilled water.

The enzyme was diluted 1:4 by volume with 50 mM KPO_4 buffer and 1 mM DTT to reduce its density prior to layering on the gradient.

All samples including protein markers were then layered onto the preformed gradients, placed in Beckman SW-41 rotor cups, and centrifuged at 40,000 rpm for 72 hrs.

The gradients were dripped by needle puncture and 0.4-ml fractions were collected. The presence of protein was determined by $A_{280/260}$.

Standard Assay Variations

Kinetics assays were similar to standard assays except the volume of each component was increased five or six times, resulting in enough volume in one reaction tube for five or six 50- μ l reaction assays. 50- μ l aliquots were withdrawn from the mixture at specific time intervals, spotted on Whatman DE-81 filters and assayed in the usual manner.

Enzyme activity titrations were run to find optimal enzyme and RNA concentrations by varying the volumes of each when added to the reaction mixture. The volumes of the other components were the same as usual. The final volume was brought to 50 μ l with 0.01 M Tris buffer, pH 8.2.

Competitive activity assays were run using 1 or 2 μ l of concentrated enzyme, 10 μ l of basal mixture, 10 μ l of labeled triphosphate mix (^3H -UTP or ^3H -CTP) and 10 μ l of myeloblast RNA. Unlabeled triphosphate was added in varying volumes. The reaction was brought to a 50- μ l volume with 0.01 M Tris buffer, pH 8.2.

Titrations with Mn^{++} and Mg^{++} concentrations were run in very small increments. Varying volumes of the salts of these ions were added to a standard reaction to determine optimal concentrations for basal mixtures.

RNA Extraction and Preparation

Phenol was thawed at room temperature and equilibrated first with 0.5 M Tris, pH 11 (equal volume) followed by equilibration with TNE buffer pH 8.8 (10 mM Tris, 100 mM

NaCl, 1 mM EDTA). The mixture was agitated and the phases allowed to separate. Each time the aqueous layer was then poured off and an equal volume of TNE was added two times. The pH of the aqueous phase was checked to be near pH 7.0, if not, the equilibration continued. The pellets that were saved from the enzyme preparation were thawed. Each pellet was from approximately 10 g of myeloblast cells. Ten ml of TNE were added to each pellet. One percent SDS was added to help dissolve the pellets. The two pellets were then poured together into a 125-ml flask and an equal amount of phenol was added. The mixture was shaken vigorously for 10 min. The mixture was divided into two 30-ml Corex centrifuge tubes. The mixture was centrifuged at 8,000 rpm for 5 min in HB4 Sorvall rotor at 10-20°C, to separate the phases. The top aqueous phase was removed and an equal volume of phenol was added and the mixture was shaken for 10 min. The above centrifugation technique was again employed--8,000 rpm for 10 min at 10-20°C in 30-ml Corex centrifuge tubes. The top aqueous layer was pipetted out and put in a sterilized bottle. One-tenth volume of 4 M NaCl and two volumes of ethyl alcohol were then added to the aqueous layer and stored at -20°C overnight to precipitate the RNA. The RNA was collected by repeated centrifugation in one tube. The time, speed, and temperature was 10,000 rpm for 15 min at 10°C on HB4 rotor. A 70% ethyl alcohol solution was prepared and used to wash the pellet to remove phenol. Following this the mixture was

agitated and centrifuged at 10,000 rpm for 10 min at 10°C on HB4 rotor. The pellet was drained, evacuated and resuspended in 5 ml of 50 mM Tris, pH 7.4, 5 mM MgCl₂, 2.5 uM CaCl₂, 100 ug/ml DNAase I. The solution was extracted with an equal volume of phenol. The aqueous phase was collected by centrifugation, and one-tenth volume of 4 M NaCl, and two volumes of ethyl alcohol was added, and put in a sterilized bottle.

The RNA-EtOH mixture was stored at -20°C. As RNA was needed, it was centrifuged out of solution using 10,000 rpm for 15 min at -5°C in a Sorvall HB4 swinging bucket rotor.

Myeloblast RNA was used in experiments at a concentration of 2 mg/ml. To obtain this, the following procedure was used: Myeloblast RNA was salt precipitated and resuspended in ethanol at 0.55 mg RNA/ml ethanol. Four ml were spun at 10,000 rpm in the HB4 rotor for 15 min at -5°C. The ethanol was removed to leave 2.2 mg of RNA. This RNA was suspended in 1.1 ml of 10 mM Tris, pH 7.4, to give a 2 mg/ml concentration.

RESULTS

Cation exchange chromatography of terminal ribonucleotidyl transferases utilizing CTP and UTP as substrates is described in Figs. 1 and 2.

The 0.01 M potassium phosphate buffer wash of the CM-Sephadex column did have good absorbance, but upon assay showed negligible enzyme activity. It is evident that our activity was bound to the CM-Sephadex column. This column resulted in some degree of purification, since an absorbance peak was registered by the 0.01 M potassium phosphate wash but little activity was found. The 0.1 M potassium phosphate elution had a good absorbance peak and a good ribocytidylyl terminal transferase activity. The 0.3 M potassium phosphate elution yielded a small absorbance peak but did have good peaks of both poly (C) and poly (U) polymerase activity. The enzyme activities in these cases were closely associated with absorbance peaks. Only fractions that had the highest activities were pooled for ammonium sulfate precipitation and concentration. This led to further purification by elimination of extraneous proteins.

Figs. 3, 4, 5, and 6 show the absorbances of and activities of fractions from the DEAE columns. Figs. 5 and 6 show the results of pooled and concentrated (0-70%

ammonium sulfate concentration) activity obtained from the 0.1 M elution of the CM-Sephadex column. Figs. 5 and 6 show the results of the pooled and concentrated (0-70% ammonium sulfate concentration) activity obtained from the 0.3 M elution of the CM-Sephadex column. Negligible absorbances and activities were found with the 0.1 M and 0.3 M buffer elutions of this DEAE column. The activities did not bind to the DEAE columns. The concentrated pool from the 0.1 M elution of the CM-Sephadex column and the pool from the 0.3 M elution of the CM-Sephadex column were washed from the column with 0.01 M potassium phosphate. However, the peak activities did elute ahead of the major protein peak. This eliminated more extraneous protein at ammonium sulfate concentration, since only peak activity fractions were pooled. A good poly (C) polymerase peak was observed in the concentrated pool from the 0.1 M elution of the CM-Sephadex column after it was applied to the DEAE column and assayed. A good poly (U) peak was observed in the concentrated pool from the 0.3 M elution of the CM-Sephadex column after it was applied to the DEAE column.

Using either a 0-50% ammonium sulfate precipitation and a 50-70% ammonium sulfate precipitation or a 0-40% ammonium sulfate precipitation and a 40-70% ammonium sulfate concentration with the active fractions from the DEAE column to which the 0.1 M elution of the CM-Sephadex column had been applied, it seemed that one could concentrate the enzyme activity in the high concentration pellet. This did

not work as well for the active fractions from the DEAE column to which the 0.3 M elution of the CM-Sephadex column had been applied. A 0-70% ammonium sulfate concentration was used to concentrate this activity.

Figs. 7, 8, and 9 represent ion concentration titrations run to find optimal concentration of divalent cation for the basal mixture used in the enzyme reaction.

Fig. 10 represents a kinetic assay run to find optimal incubation time for the enzymes.

Fig. 11 represents a competitive assay run to determine if other enzymes were present in our fractions, and to what extent they utilized RNA.

Fig. 12 shows the molecular weight determination of the poly (U) polymerase and poly (C) polymerase enzymes.

Figs. 13 and 14 represent trials run to determine optimal RNA and enzyme levels.

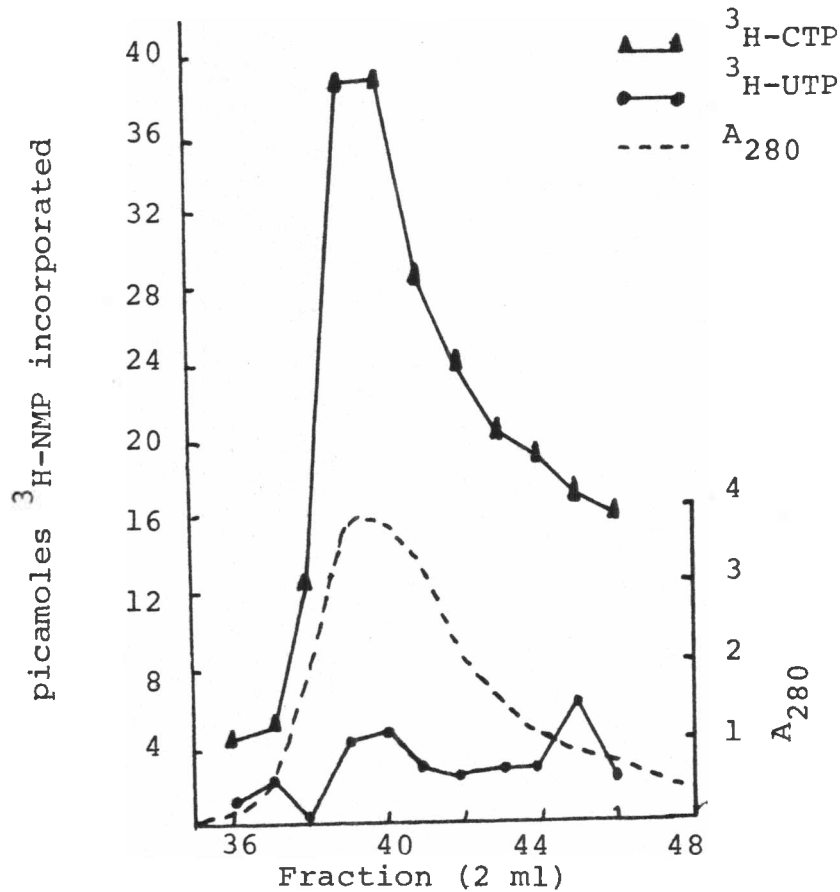


Fig. 1. Absorbances and enzyme activities of fractions collected from the 0.1 M potassium phosphate buffer elution of the CM-Sephadex column. The supernate (S-2) was applied to the column, washed with 0.01 M potassium phosphate buffer and then eluted with 0.1 M buffer. Enzyme activity was determined by the standard assay procedure.

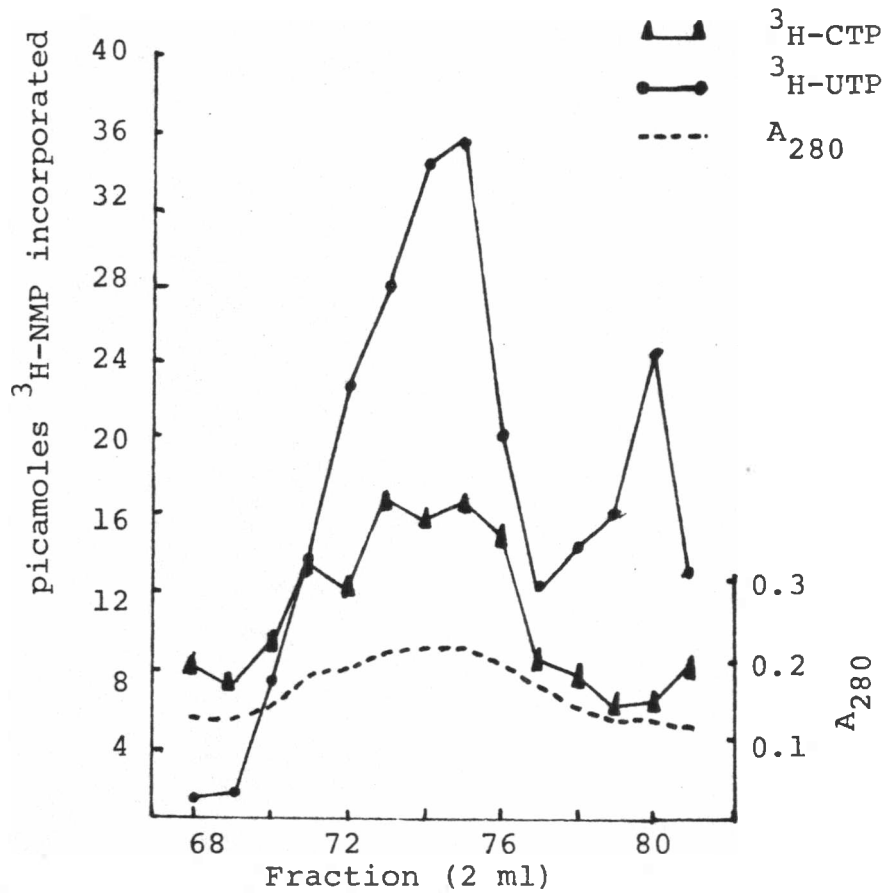
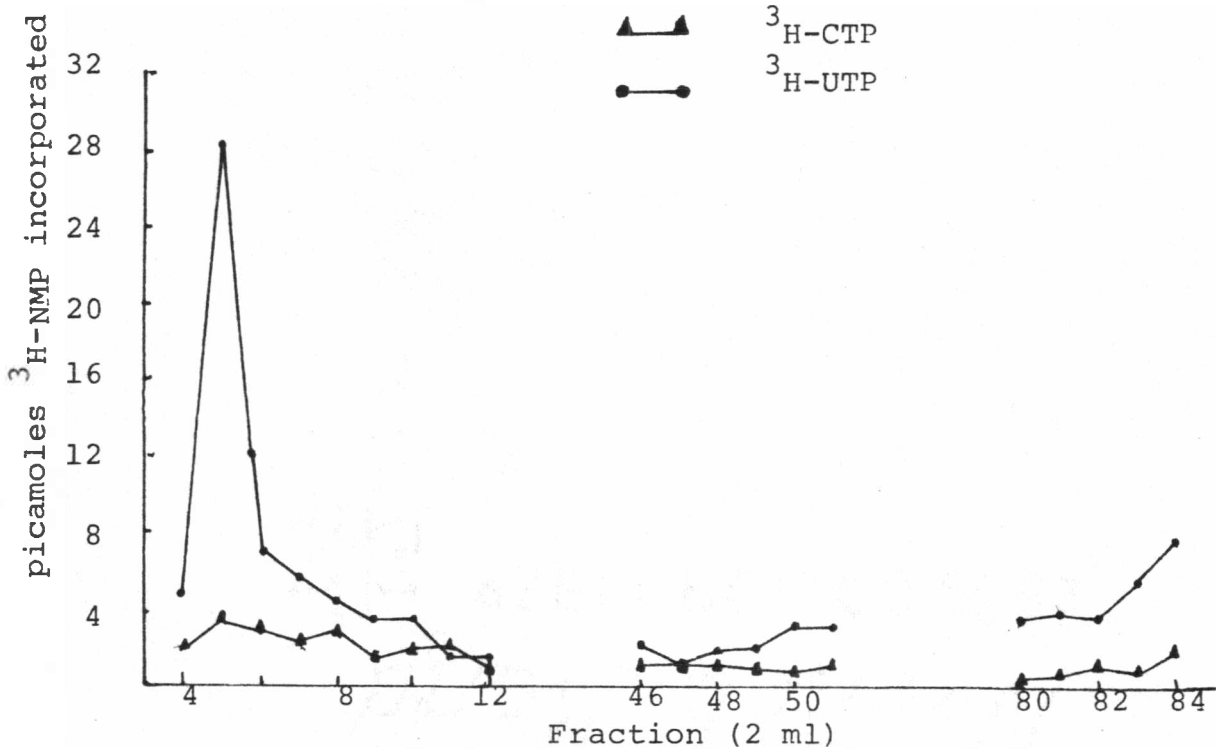
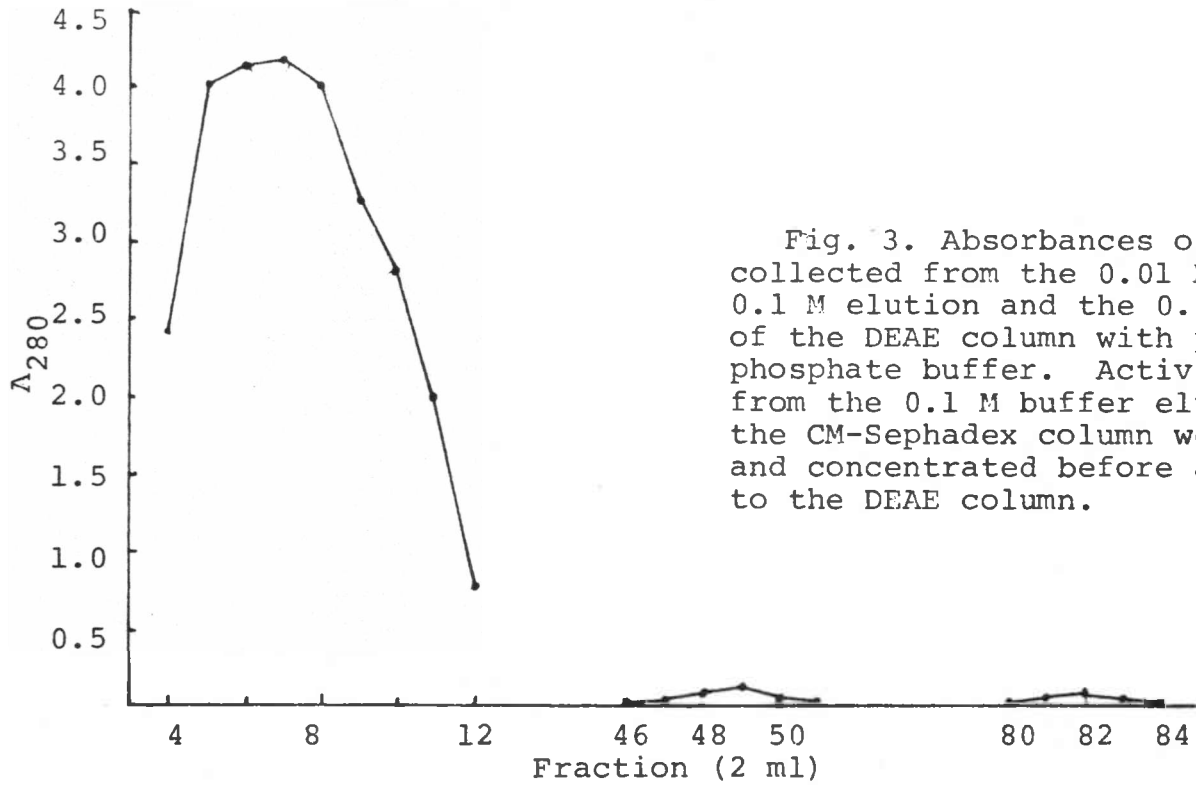


Fig. 2. Absorbances and enzyme activities of fractions collected from the 0.3 M potassium phosphate buffer elution of the CM-Sephadex column. The supernate (S-2) was applied to the column, washed with 0.01 M potassium phosphate buffer, and eluted with 0.1 M potassium phosphate buffer before the 0.3 M elution. Enzyme activity was determined by the standard assay procedure.



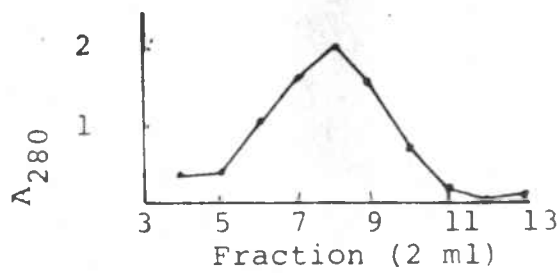


Fig. 5. Absorbances of fractions collected from the 0.01 M wash of the DEAE column with potassium phosphate buffer. Active fractions from the 0.3 M elution of the CM-Sephadex column were pooled and concentrated before application to the DEAE column. There were no recordable absorbances from the 0.1 M buffer elution and the 0.3 M elution of this same column.

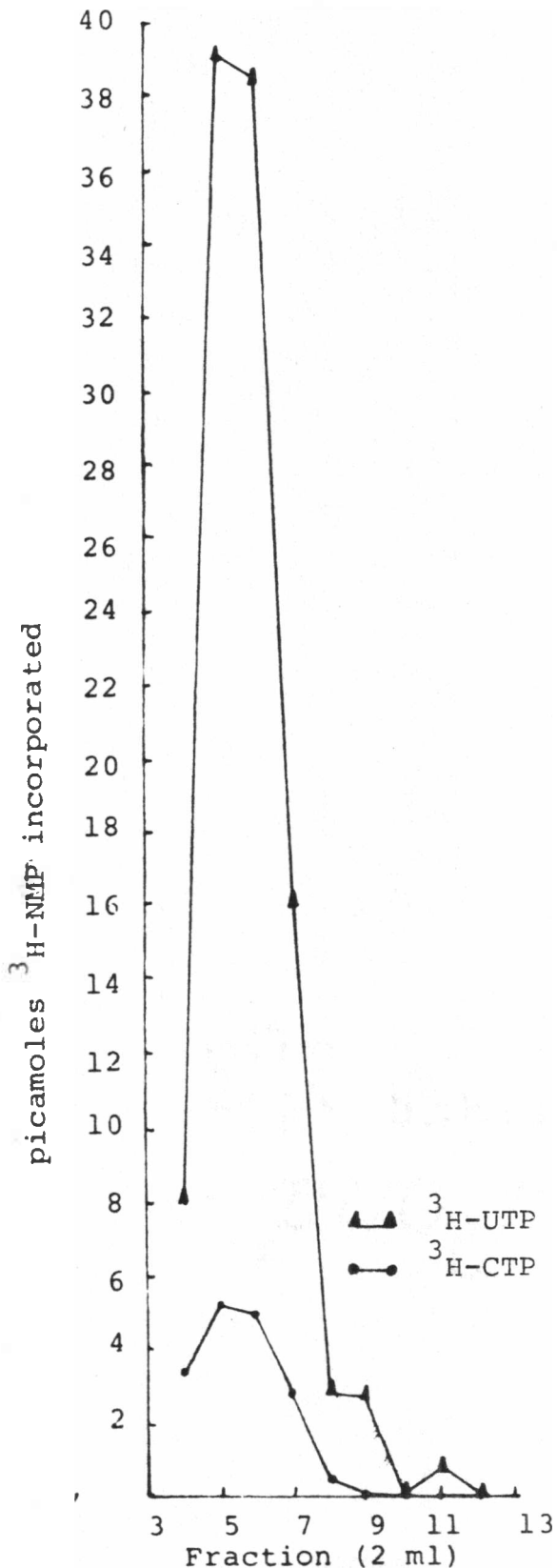


Fig. 6. Enzyme activities of fractions collected from the 0.01 M wash of the DEAE column with potassium phosphate buffer. Active fractions from the 0.3 M elution of CM-Sephadex column were pooled and concentrated before application to the DEAE column. Negligible activities were found in fractions collected from the 0.1 M elution and 0.3 M elution of the same column. Enzyme activity was determined by standard assay procedures.

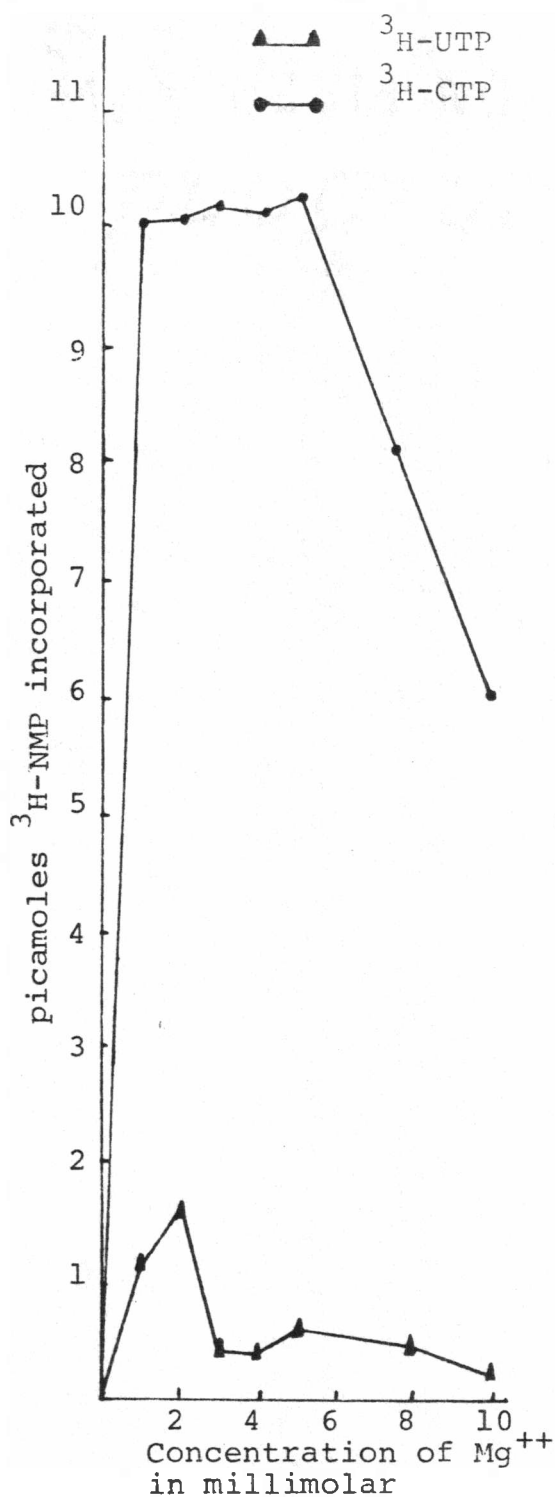


Fig. 7. Mg titration for enzyme activity of pooled and concentrated fractions from the 0.01 wash of the DEAE column to which pooled and concentrated fractions from the 0.1 M elution of the CM-Sephadex column had been applied. Fractions were pooled and this assay was run using enzyme concentrated in the 40-70% pellet from ammonium sulfate precipitation.

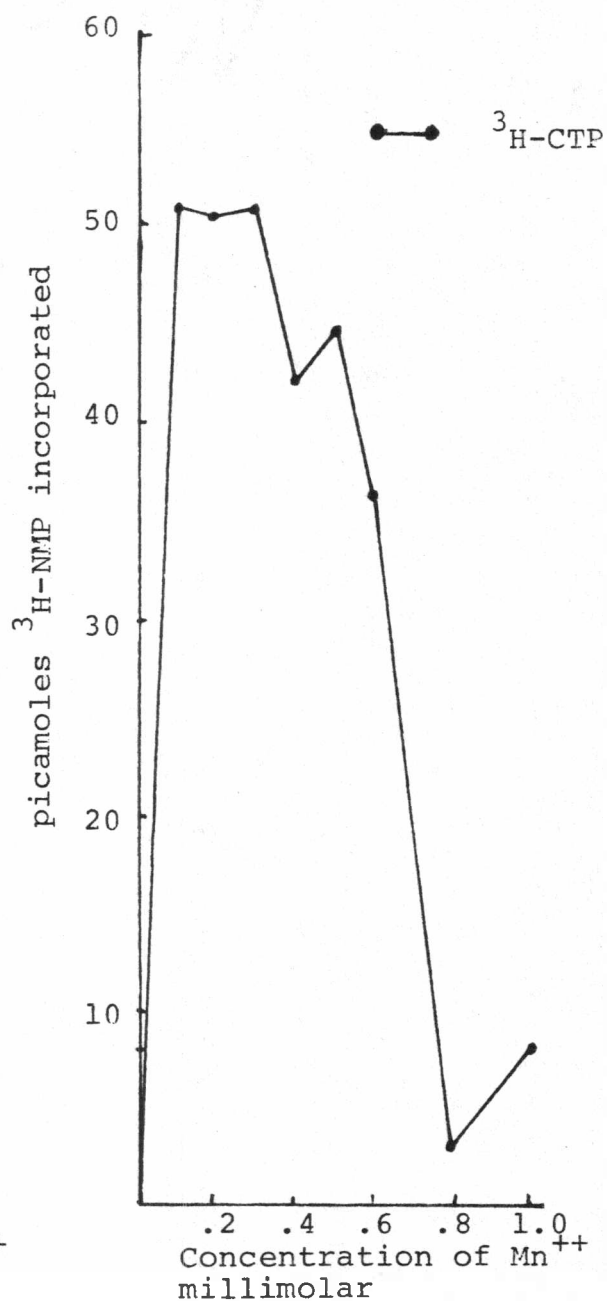


Fig. 8. Mn titration for enzyme activity of pooled and concentrated fractions from the 0.01 wash of the DEAE column to which pooled and concentrated fractions from the 0.1 M elution of the CM-Sephadex column had been applied. Fractions were pooled and this assay was run using enzyme concentrated in the 40-70% pellet from ammonium sulfate precipitation.

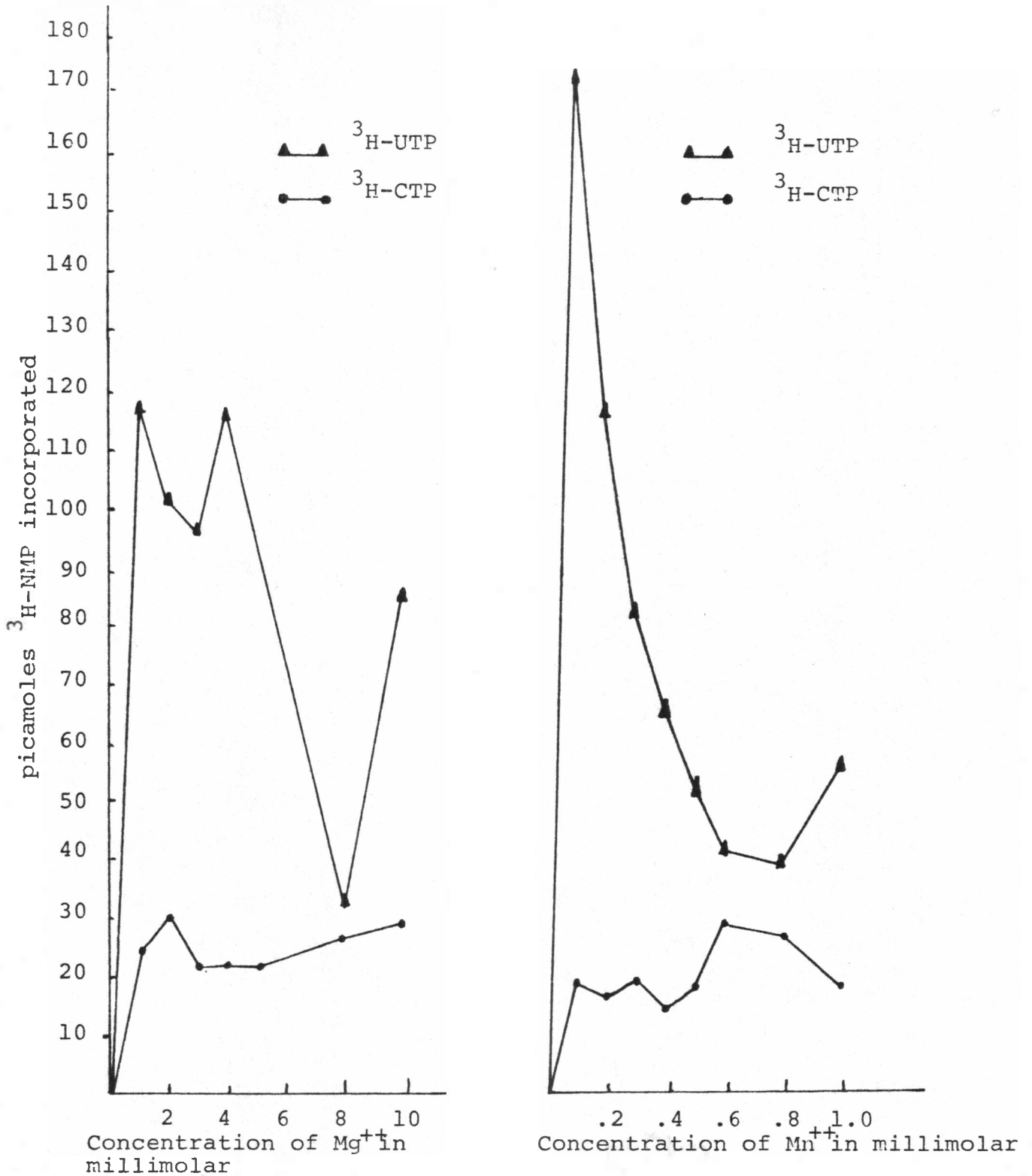


Fig. 9. Mg and Mn titrations for enzyme activity of pooled and concentrated fractions (0-70% ammonium sulfate concentrations) from the 0.01 M wash of the DEAE column to which pooled and concentrated fractions from the 0.3 M elution of the CM-Sephadex column had been applied.

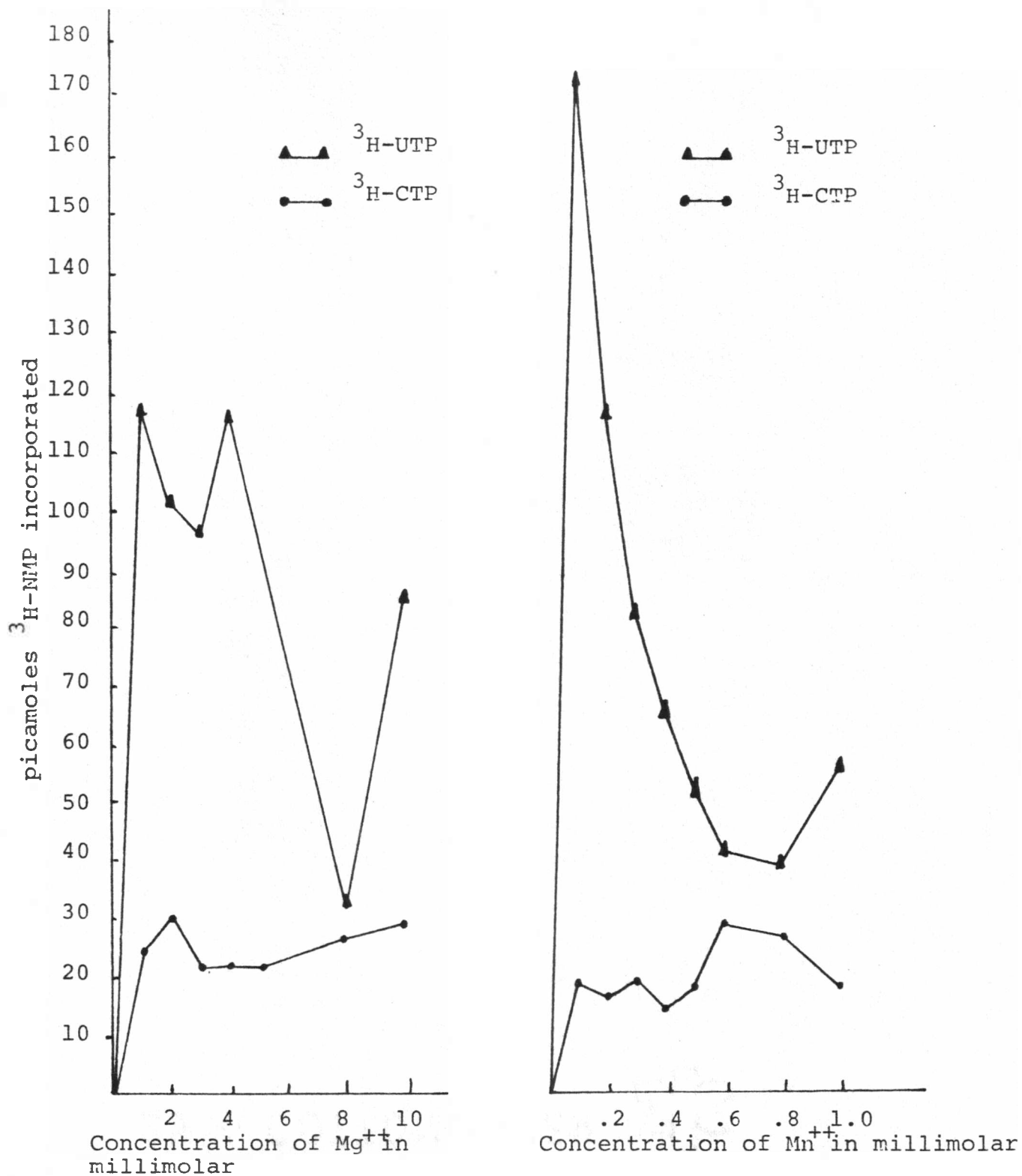


Fig. 9. Mg and Mn titrations for enzyme activity of pooled and concentrated fractions (0-70% ammonium sulfate concentrations) from the 0.01 M wash of the DEAE column to which pooled and concentrated fractions from the 0.3 M elution of the CM-Sephadex column had been applied.

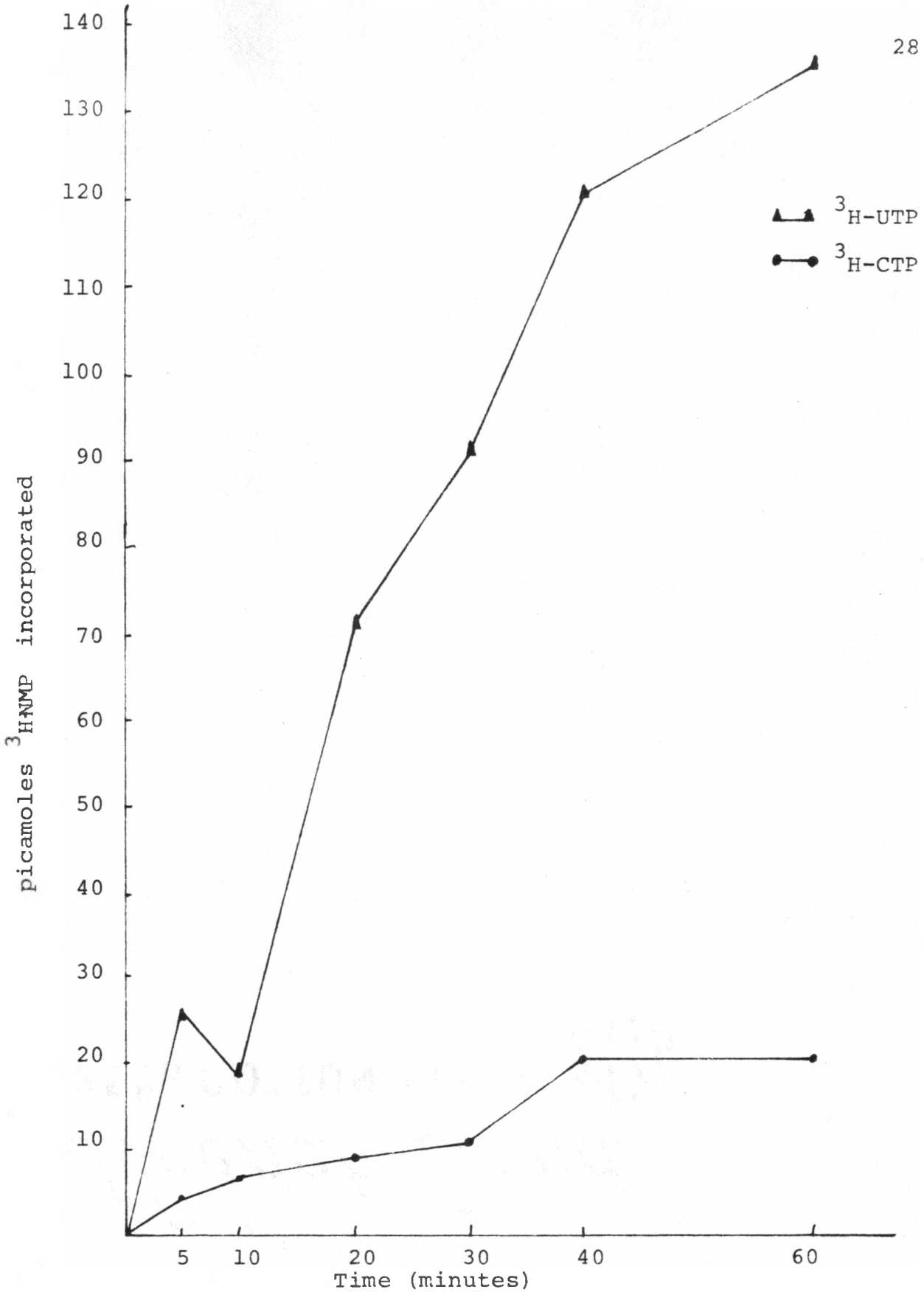


Fig. 10. Kinetics assay of enzyme activities at selected time intervals. The poly (U) activity is derived from the 0.3 M elution of the CM-Sephadex column and then 0.01 M wash of the DEAE column. The poly (C) activity is derived from the 0.1 M elution of the CM-Sephadex column and then 0.01 M wash of the DEAE column.

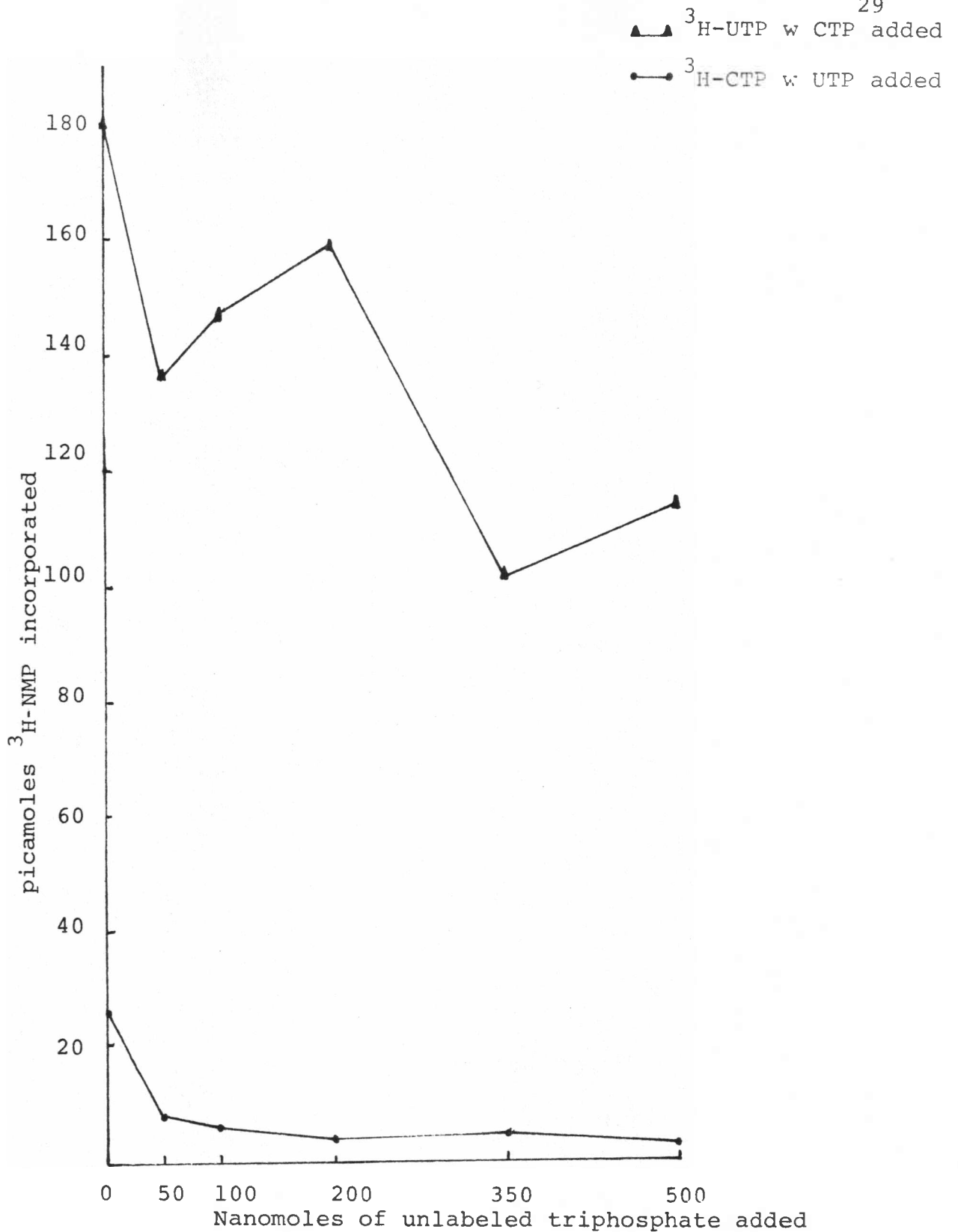
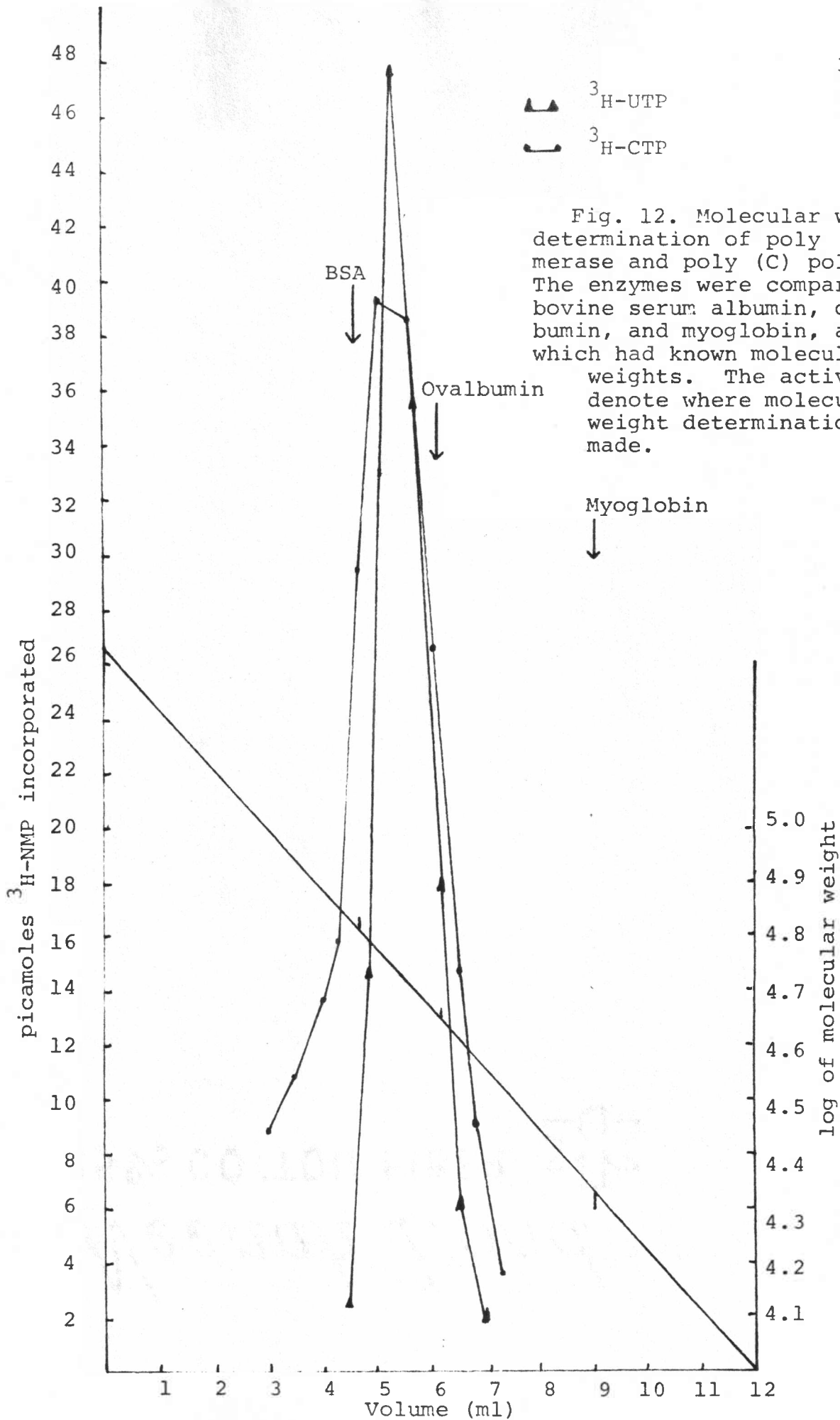


Fig. 11. Competitive assay run using enzyme activity derived from the 0.3 M buffer elution of the CM-Sephadex column and the 0.01 M buffer wash of the DEAE column. Unlabeled CTP was added to the assay specific for poly (U) polymerase activity and unlabeled UTP was added to the assay specific for poly (C) polymerase activity.



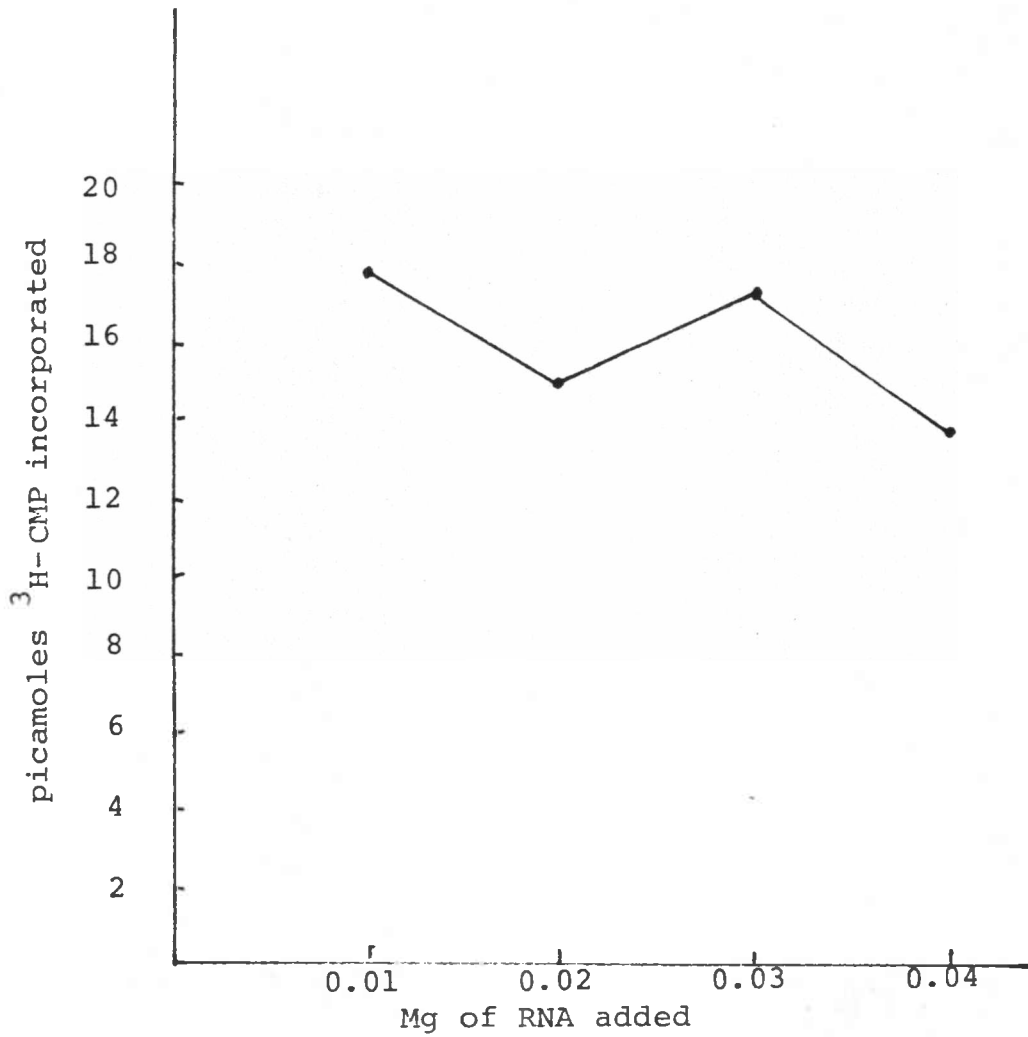


Fig. 13. RNA titration for maximum poly (C) polymerase activity using a fixed amount of enzyme in each assay (10 ul of unconcentrated enzyme derived from the 0.1 M elution of CM-Sephadex column).

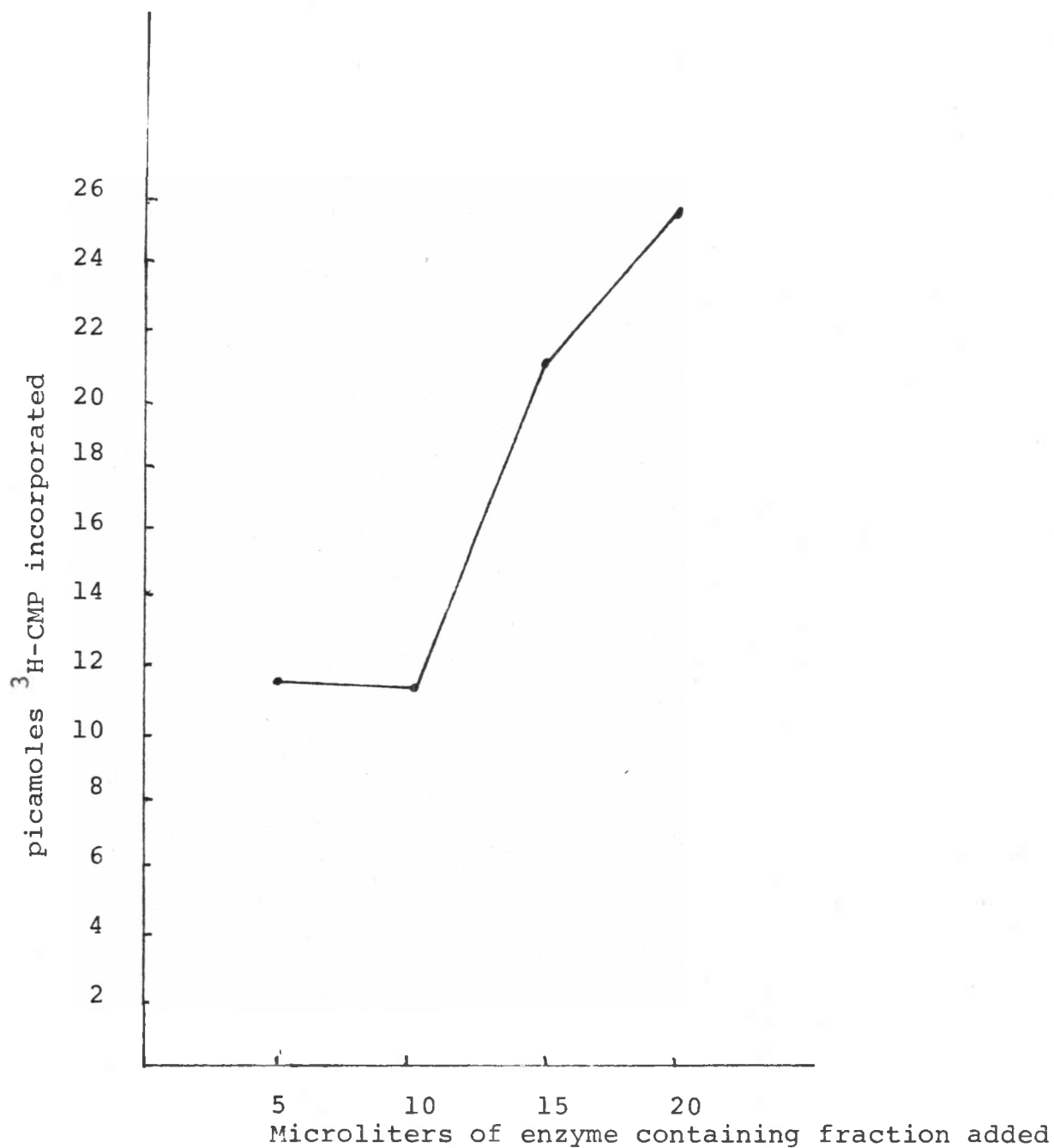


Fig. 14. Enzyme activity titration for maximum poly (C) polymerase activity using a fixed amount of RNA in each assay (10 μl RNA with a concentration of 2 mg/ml).

DISCUSSION

The activity peaks observed seemed to indicate that a separation of poly (U) polymerase and poly (C) polymerase had been achieved by washing the CM-Sephadex column with 0.1 M potassium phosphate buffer and 0.3 M potassium phosphate buffer.

These were the only purification procedures undertaken in our research. An SDS gel electrophoresis of the enzyme fractions at this stage indicated that there was still some heterogeneity in the concentrated activities after DEAE chromatography (31) (data not shown). The next logical step in the purification procedure would be to use affinity chromatography on poly (A) and poly (G) columns.

It appears that our choice of 0.005 M MgCl_2 in our basal mixture was a poor one. The results from titration studies with Mg^{++} and Mn^{++} ion indicate that we should have been using MnCl_2 in our basal mixture. A concentration of 0.0001 M MnCl_2 significantly increased the activity of both enzymes, the poly (C) polymerase derived from the 0.1 M buffer elution of the CM-Sephadex column and the poly (U) polymerase derived from the 0.3 M buffer elution of the CM-Sephadex column.

The kinetic studies indicate that long incubation times yield a greater activity for both enzymes. While we did

not run a trial past 60 min, it can be seen that the highest activity was achieved for both enzymes at 60 min. It would be interesting to run trials for longer periods of time to find the optimum length of incubation. Another interesting experiment would be running the reactions at different temperatures to find the optimum temperature for the enzyme. This would require more water baths than were at our disposal.

The results from the competitive assay show a decrease in enzyme activity (either poly (U) polymerase or poly (C) polymerase) when unlabeled RNA was added to the reaction. That is, the activity of poly (U) polymerase was decreased when CTP was added to the reaction and the activity of poly (C) polymerase was decreased with the addition of UTP. This points to the conclusion that these enzymes are capable of reacting with more than one substrate. When the enzyme was provided with two substrates, one labeled and the other not, one can see that the competitive nature of the enzyme would decrease incorporation of labeled substrate. The enzymes aren't specific for any one substrate but merely favor one over the other. This is feasible, since there are many structural similarities between CTP and UTP. A more comprehensive study is needed in this area to fully account for the results that we found.

The velocity sedimentation yielded very good results. Two good activity peaks were obtained for both enzymes. By running proteins of known molecular weight along with

our concentrated activities from the DEAE column, we were able to calculate an approximate molecular weight for both enzymes. The known proteins and their molecular weights were: bovine serum albumin, 66,500; ovalbumin, 45,000; and myoglobin, 16,900. Our results indicate a molecular weight of approximately 59,600 for ribocytidylyl terminal transferase and a molecular weight of approximately 55,600 for ribouridylyl terminal transferase. A molecular weight of 50,000 had been reported for ribouridylyl terminal transferase isolated from Vigna unguiculata leaves (33).

Enzyme and RNA concentration titrations were run to optimize conditions in our experiments only. Although they give a rough idea of ideal concentrations, the values for ideal enzyme concentrations would differ from preparation to preparation and would vary depending on how concentrated the enzymes might be. The values for ideal RNA concentrations would depend on the initial concentration of RNA (ours was 2 mg/ml). The amount of RNA added would depend directly on how much enzyme is present.

We did not monitor the presence of ribonuclease activity and one can speculate that it might be present at each stage of purification. It might even become more concentrated along with transferase activities. To fully characterize the transferase activities, nuclease activities will have to be removed. It could significantly lower the effectiveness of the transferase enzymes, and nuclease activity would have to be controlled in practical applications of the transferase enzymes.

Since this work is new, comparison to other work cannot be done in detail. It must await new data from subsequent studies on ribonucleotidyl terminal transferases.

APPENDIX

List of abbreviations used throughout this paper:

- RNA - ribonucleic acid
DNA - deoxyribonucleic acid
UTP - uridine 5'-triphosphate
CTP - cytidine 5'-triphosphate
ATP - adenosine 5'-triphosphate
GTP - guanosine 5'-triphosphate
UMP - uridine 5'-monophosphate
CMP - cytidine 5'-monophosphate
AMP - adenosine 5'-monophosphate
GMP - guanosine 5'-monophosphate
- MgCl₂ - magnesium chloride
EDTA²⁻ - ethylenediaminetetraacetic acid
DEAE-cellulose - diethylaminoethyl-cellulose
CM-Sephadex - carboxymethyl-Sephadex
DNAase - deoxyribonuclease
Mg - magnesium ion
Mn - manganese ion
- poly A - polyadenylic acid
poly C - polycytidylic acid
poly G - polyguanylic acid
poly U - polyuridylic acid
- Tris - tris (hydroxymethyl) aminomethane
N - normal
HCl - hydrochloric acid
KOH - potassium hydroxide
rpm - revolutions per minute
E. coli - Escherichia coli
- ³H-CTP - tritium labeled cytidine 5'-triphosphate
³H-UTP - tritium labeled uridine 5'-triphosphate
- Na₂HPO₄ - sodium phosphate
DTT - dithiothreitol
M - molar
MnCl₂ - manganese chloride
ml -² milliliter
ul - microliter
NaCl - sodium chloride

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