

**DNA - DEPENDENT RNA POLYMERASE FROM AN
EXTREMELY HALOPHILIC BACTERIUM**

**DNA - DEPENDENT RNA POLYMERASE FROM AN
EXTREMELY HALOPHILIC BACTERIUM**

by

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SCOPE AND CONTENTS:

This thesis describes the isolation and investigation of a DNA - dependent RNA polymerase from the extreme halophile Halobacterium cutirubrum.

The enzyme system was analyzed under conditions of very high ionic strengths which are characteristic of the internal salt concentrations of extreme halophiles and at much lower ionic strengths found in conventional bacterial systems. The enzyme was found to have activity in a wide range of salt concentrations when attached to its DNA template in the form of a DNA - Membrane - Protein complex. The enzyme, however, lost the ability to function at high ionic strengths when freed from this complex.

The properties of the isolated DNA - dependent RNA polymerase from the halophile were then compared to the properties of the same enzyme isolated from the non-halophilic bacterium, Escherichia coli. Both enzymes were found to have the same approximate molecular weights and to share the same substrate requirements. The enzymes differed,

however, in their response to inhibitors specific for RNA synthesis.

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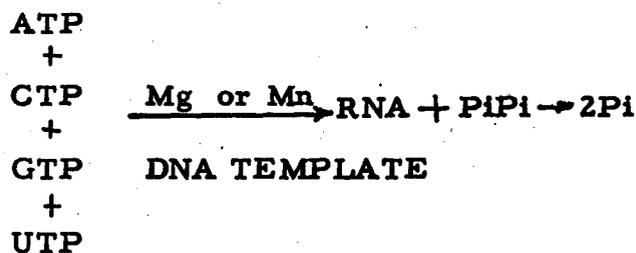
I. INTRODUCTION

(a) General

DNA-dependent RNA polymerase plays a very important role in the functioning of all living cells. It is the enzyme directly responsible for the production of cellular RNA, mRNA, tRNA and rRNA.

Under the direction of a DNA template, the enzyme catalyzes the sequential assembly of ribonucleotides into a complementary copy of the DNA sequence transcribed. The RNA copy then enters the cell's normal metabolic pathways.

Schematically the enzymic reaction can be illustrated as follows:

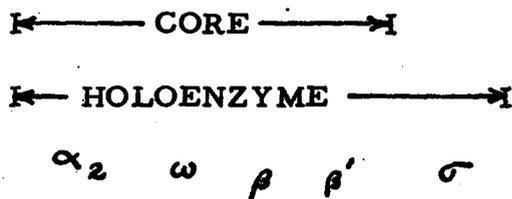


Because of the enzyme playing such a key role in the functioning of cells, it has been isolated for study from various bacterial cells. Some of the bacterial species studied are Escherichia coli (Chamberlain, 1962; Furth, 1962; Burges, 1969), Azotobacter vinelandii (Ochoa, 1961; Burma, 1961; Lee-Huang, 1969), Micrococcus lysodeikticus (Nakamoto, 1964), Bacillus stearothermophilus (Remond-O'Donnel, 1969) and Pseudomonas indigofera (Tani, 1968).

Study of this enzyme system has not, however, been confined to bacteria alone. The enzyme has also been isolated from yeast mitochondria (T'sai, 1967) and various animal tissues (Ballard, 1964; Furth, 1965; Ramuz, 1965). The major difficulty with these preparations and preparations of the enzyme from other non-bacterial sources, is the poor purification that has been to date accomplished. With the exception of viral preparations, most non-bacterial DNA-dependent RNA polymerase preparations have a specific activity in the range of a crude bacterial extract, and therefore a thorough study has not been possible.

To date the most intensively studied DNA-dependent RNA polymerase is that isolated from the common enteric bacterium E. coli. First isolated in 1962 by Chamberlain and Berg, the enzyme was finally prepared in an electrophoretically pure form in 1969 by Burgess.

The enzyme has a molecular weight of about 500,00 (Burgess, 1969). It is made up of five different sub-units as follows:



with molecular weights of 39,000; 9,000; 155,000; 165,000; and 100,000 respectively. How these sub-units interact and what role they play in the activity of the enzyme is presently under study.

The CORE is the real functional unit of the enzyme, and is the complex responsible for the synthesis of RNA. The HOLOENZYME is the total enzyme. Sigma, the sub-unit whose presence produces the complete enzyme from its functional unit, has been studied intensively and its role has been elucidated.

Although the core enzyme can under certain conditions synthesize RNA, sigma is the factor responsible for the specific initiation of RNA synthesis (Burgess, 1969; Darlix, 1969).

Sigma has also been implicated in the regulation of bacteriophage development (Bautz, 1969; Travers, 1969; Ekkehard, 1969; Travers, 1970a; Summers, 1970). The mode of action of sigma seems to be related to its ability to bind to promotor sites on the DNA template and initiate RNA synthesis in a specific place (Bautz, 1970). How sigma is able to select the specific promotor on which to bind is still under study.

In relation to the initiation of RNA synthesis, there is also a specific termination factor which has been isolated from E. coli. Unlike sigma, this factor is not an integral part of the RNA polymerase molecule. It acts independently and mediates the termination of RNA synthesis at a specific point on the DNA being transcribed (Roberts, 1970; Darlix, 1971). This factor has been given the name rho. It has a molecular weight of about 200,000 and is thought to act by binding to the RNA polymerase - DNA template

complex, causing this complex to come apart, halting RNA synthesis.

Other factors have been found which act in other regulatory capacities. Ψ_r factor acts by controlling the synthesis of ribosomal RNA (Travers, 1970b). M factor stimulates RNA synthesis prior to the effect of sigma and may be bound to ribosomes (Davison, 1969). As study is intensified, other factors are coming to light which help explain the control mechanisms of RNA synthesis.

With so much information available on the properties of DNA-dependent RNA polymerase from non-halophilic bacteria, it was felt that this enzyme would be a good candidate for study in halophilic bacteria. Non-halophilic enzymes rapidly lose activity as ionic strength is increased above 0.5 M. Most halophilic enzymes however, require very high ionic strengths for activity. By comparing the two enzymes, differences in the properties of E. coli and Halobacterium cutirubrum might be related to the special properties required for enzymes to function at very high ionic strengths.

(b) Halophiles

It seems appropriate at this point to consider why halophiles are such an interesting system for enzyme studies.

By definition, extreme halophiles are bacteria which grow optimally at a salt concentration greater than 25% (Larsen, 1962). There are only two genera which fit into this classification, Halobacterium and Halococcus. Of these two genera, Halobacterium has been the more commonly studied.

Halobacterium is a pseudomonad, pleomorphic, gram negative aerobic and pigmented with carotenoids which are thought to protect the bacterium from intense sunlight. There are five species which are differentiated by intensity of pigment, ability to produce nitrites from nitrates and the ability to produce gas from glucose fermentation (Kushner, 1968). The species are halobium, salinarium, cutirubrum, marismortui and trapicanum. Halobacterium cutirubrum requires 20 - 30% NaCl in its medium for growth (Larsen, 1962). The requirement for NaCl is quite specific and the NaCl cannot be replaced by other monovalent cations and still yield optimum growth (Brown, 1955; Boring, 1963; Stevenson, 1966).

Although the exterior salt concentration is about 4.0 M as NaCl, the interior of the cell selectively concentrates KCl while still maintaining a high intracellular NaCl concentration. The bacterium has an ion selectivity excluding sodium and concentrating potassium. Christian and Waltho (1962) found that in a growth medium containing 4.0 M as NaCl and 0.032 M as KCl, the interior of the

halophile, H. salinarium, contained salt concentrations equivalent to 4.6 M as KCl and 1.3 M as NaCl. Consequently, the macromolecules of the halophile must function in an environment super saturated in monovalent cations.

Normal non-halophilic proteins would denature under such extreme ionic conditions. Study of halophilic proteins indicate that modifications in halophilic enzyme structure may have taken place which may account for their resistance to high salt concentrations.

Studies on the membrane structure of halophiles indicate a large percentage of amino acids present in their proteins which are acidic in nature, ie. glutamic and aspartic acid (Brown, 1963).

Studies carried out on the ribosomal proteins of halophiles reinforce these findings (Bayley, 1966). Because of the large amount of acidic amino acids present in halophilic proteins, it is generally felt that large concentrations of negative ions are present in the enzyme structure of halophiles. The extremely high cation concentration of the environment of these proteins might therefore act to neutralize these charges and allow the protein to take up an active configuration. The exact role of these charge interactions is unknown and we can only speculate on how these interactions yield enzymes resistant to denaturation in high ionic strengths.

The structural differences between proteins of halophiles and

conventional bacteria are also reflected in the enzyme activity found as a function of salt concentration. Studies on malic, succinic and isocitric dehydrogenases from Micrococcus halodenitrificus and Pseudomonas salinaria species indicate that these enzymes are most active at concentrations of KCl ranging from 1.0 M to 3.0 M (Baxter, 1956). KCl seems to be the ion which yields the best activity in in vitro enzyme assays. This can readily be correlated with the high in vivo intracellular concentrations of KCl.

In some cases enzymes not only require KCl for optimum activity, but are completely inactivated in the absence of a sufficiently high concentration of KCl. Lactic dehydrogenase from H. salinarium is an example of such a labile enzyme. Baxter (1959) speculated that the removal of the cationic environment allowed the negative ions on the enzyme surface to repel each other, thereby unfolding the enzyme by mutual charge repulsion, and causing denaturation. Because of the unique adaption halophiles have made to an environment of such high ionic strength, it would be interesting to attempt to relate this adaption to physical changes in enzyme structure. This can be done by comparing the enzyme structure of halophiles to the enzyme structure of conventional bacteria. To this end, this project was designed.

The isolation of a single enzyme like DNA-dependent RNA polymerase would allow us to study in a comparative manner the differences in

the two enzymes. Any variation in properties might then be related to the mechanism of halophile adaption and evolution to high salt environments.

This thesis describes the beginning of such a comparative study.

II. MATERIALS AND METHODS

Materials:

Yeast extract and casamino acids were obtained from Difco Labs.; inorganic salts, TCA, sodium dodecyl sulphate and glycerol from Fisher Scientific Ltd. and Canlab Ltd.; bovine serum albumin and salmon sperm DNA from Calbiochem Ltd.; whole yeast RNA from Mann Research Labs. Ltd.; crystalline pancreatic RNase and catalase were obtained from Worthington Biochemicals Ltd.; Sephadex gels from Pharmacia Ltd.; unlabelled ribonucleoside triphosphates and monophosphates were obtained from P-L Biochemicals Ltd.; streptovaricin, rifampicin, actinomycin D and acriflavin were a gift from Dr. K. B. Freeman; Whatman filter paper was obtained from Mandel Scientific Ltd.; perchloric acid from Allied Chemicals Ltd.; Omni-fluor from Beckman Ltd.; hydrogen peroxide from BDH Ltd; E. coli RNA polymerase was a gift from Dr. H. P. Ghosh; gammaglobulin from Nutritional Biochemicals Ltd.; tris from Sigma Chemicals Ltd.; 2-mercaptoethanol was obtained from Eastman Chemicals Ltd.; and radioactivity labelled ribonucleotides were obtained from New England Nuclear Ltd.

Methods:**(i) Growth of Bacteria**

H. cutirubrum was grown in a complex medium which was essentially the medium described by Sehgal and Gibbons (1960) with the addition of ferrous sulphate. The preparation of the medium is outlined on Table I.

Cells were grown at 37 C under continuous aeration in a 13 liter New Brunswick Microferm Fermentor for 24 hours.

The routine volume of the culture was about 12 liters which had been inoculated with 600 ml of a 24 hour H. cutirubrum culture grown in a New Brunswick Incubator - Shaker. At 24 hours the cells are in mid-log phase of their growth cycle

(Lou, 1970).

Cells were harvested in either the Sharples continuous centrifuge or in the Sorval centrifuge. The cell paste was washed twice in NaCl wash, a solution containing 4.3 M NaCl, 0.03 M KCl and 0.08 M MgSO₄ (Bayley, 1964). All procedures were carried out at 4 C.

TABLE I

**COMPOSITION OF THE COMPLEX MEDIUM FOR THE
GROWTH OF HALOPHILIC BACTERIA**

Yeast Extract	10 grams
Casamino acids	7.5 grams
KCl	2.0 grams
Mg SO ₄ · 7H ₂ O	20 grams
Sodium Chloride	250 grams
Sodium Citrate	3.0 grams
Ferrous sulphate 7 H ₂ O (4.98% acidified with 1.0 ml N HCl/100 mls)	1.0 ml.
Distilled water	800 mls.

The mixture was adjusted to pH 7 - 8 with NaOH and autoclaved for 5 minutes. After cooling the precipitate was filtered, the volume of the medium brought to one liter and the final pH adjusted to pH 6.2 with HCl. The medium was autoclaved for 20 minutes to sterilize.

(ii) Preparation of the Enzyme

The washed cells were suspended in buffer D¹ (3.0 M KCl; 0.01 M Tris - HCl pH 7.66; 0.1 M MgAc; 0.008 mM 2 - mercaptoethanol) in a ratio of one g wet weight of cells to three ml of D¹. The cells are unstable in D¹ and many of them lyse when introduced into the buffer. Large clumps of cells and any unlysed cells were broken in a glass-teflon Potter-Elvehjem homogenizer kept at 0 - 4 C. The extract was centrifuged at 4 C and 27,000 r.p.m. for 15 minutes in a Sorval centrifuge. The pellet consisted primarily of unbroken cells and cell wall debris and was discarded. The supernatant was centrifuged at 129,000 x g for 180 minutes in a number 65 fixed-angle rotor in a Beckman ultracentrifuge. The result of this centrifugation was a three layered separation. The top layer and pellet were discarded. The center layer which contained a DNA-membrane-protein complex was retained. This complex was suspended in half the original volume of D¹ and dispersed with the teflon homogenizer. This DNA-membrane-protein solution was then treated with electrophoretically purified DNase in a ratio of 1 mgm DNase/30 mls of preparation. The DNase treated extract was centrifuged at 48,000 x g for 15 minutes in the Sorval and the supernatant was centrifuged at 229,000 x g for 90 minutes in the type 65 fixed-angle rotor in the Beckman ultracentrifuge. The supernatant of this centrifugation was dialyzed overnight against the low salt buffer

(0.01 M Tris-HCl, pH 7.66; 0.1 M MgAc; 0.008 mM 2 - mercaptoethanol).

The dialyzed preparation was then centrifuged at 48,000 x g for 15 minutes in the Sorval and the supernatant was layered on a G - 100 Sephadex column which had been equilibrated in the low salt buffer. The sephadex K 25/45 type column was used. The column was packed by the use of a Sephadex column extension which allowed all the slurry to be placed into the column with one application. The flow rate was about 20 mls./hour with a final bed height of 40 cms. The routine volume of the extract applied to the column was about 4.0 mls. The remaining extract was frozen and stored in liquid nitrogen in 4.0 ml. aliquots for easy use.

The G - 100 elution was monitored by reading the optical density of two ml. fractions in a Unicam SP-800 spectrophotometer at 280 m μ . Fractions were also assayed for enzymic activity. Active fractions were pooled and stored in liquid nitrogen.

(iii) Assay of the Enzyme

The development of the assay system for the DNA-dependent RNA polymerase will be described fully in Section III of this thesis. The assay system is described here for the readers' convenience.

The assay system was in a final volume of 125 μ l., and consisted of: 10 μ l. 2.5 M NH_4Cl - 0.375 M Tris-HCl, pH 8.05; 50 μ l. enzyme preparation suspended in low salt buffer (or where the assay was carried out at high ionic strength, the enzyme was suspended in D¹); 30 μ l. template DNA at a concentration of 1 mgm. DNA/ml. H_2O ; 10 μ l. of 0.004 M unlabelled nucleoside triphosphates each of GTP, CTP, ATP; 5 μ l. of ^{14}C UTP (New England Nuclear NEC 433, specific activity of 395 uc./ μ M.); and 20 μ l. H_2O . Dry KCl was added where required to bring the final KCl concentration to the desired level.

The resultant concentration of ions in the assay mixture was calculated to be as follows:

KCl	0 to 3.8 M
NH_4Cl	0.2 M
Tris-HCl, pH 8.05	0.035 M
MgAc	0.04 M
NTP's (each)	0.032 mM
^{14}C UTP	10 pM
Template (where added)	0.24 mgm./ml.

No allowance was made for volume change due to the addition of dry KCl in the calculation of these final concentrations.

The incubation mixture was set up in an ice bath and then incubated for 30 minutes at 37 C. After incubation, the reaction was stopped by rapid chilling and the labelled ribonucleoside triphosphate (UTP in the routine assay) was diluted out with 100 fold the amount of unlabelled ribonucleoside triphosphate. The reaction mixture was precipitated with ice cold 10% trichloroacetic acid (TCA), washed twice in fresh ice cold TCA with centrifuging, and the final washed precipitate was collected on 0.45 μ pore size Millipore filters. The filters were dried for thirty minutes at 60C and counted in the Beckman Liquid Scintillation Counter using Beckman Omni-fluor as the scintillator.

(iv) Glycerol Gradients

Glycerol gradients were prepared using a Buchler Gradient Former with equal amounts of 5% and 20% glycerol in each chamber. Gradients were made to conform to the ionic conditions of the low salt buffer. Gradients were run in a Beckman type SW 27 swinging bucket rotor using 17 ml. capacity buckets at 24,000 r.p.m. for 24 hours. Thirty-two fractions were collected by piercing the bottom of the centrifuge tube. The fractions were assayed according to the assay procedures required for the detection of the protein under study. When gradients were run on proteins in buffers other than the normal low salt buffer, the gradient ionic strength was adjusted accordingly.

(v) Assay of Catalase and Gamma Globulin

The enzyme catalase was used as a marker in the glycerol gradients to determine the approximate molecular weight of the DNA-dependent RNA polymerase. Fractions from the glycerol gradients were assayed for the presence of enzyme by a modification of previously described methods (Beers, 1952).

The enzyme substrate was prepared by adding 0.3 ml. 30% hydrogen peroxide to 100 mls. 0.05 M Na_2HPO_4 , pH 7.0. To this substrate was added 10 μl . of the gradient fraction and the disappearance of optical density at 240 $\text{m}\mu$ was noted as a function of time, using distilled water as a blank. The change in optical density after one minute was recorded.

Gamma globulin was another marker used to help establish the molecular weight of the halophilic enzyme. The protein was located in the gradient by presence of optical density at 280 $\text{m}\mu$.

(vi) Determination of Protein

Protein concentration was determined by the method of Lowry (1951) with bovine serum albumin as the standard.

(vii) Preparation of H. cutirubrum DNA

DNA from the halophile H. cutirubrum was prepared by phenol extraction using the method of Kirby (1957) as modified by Lou (1970).

(viii) Assay of E. coli RNA polymerase

RNA polymerase from E. coli was assayed by the method of Chamberlain and Berg (1962), with post-incubation treatment of the product carried out as in the halophile system.

(ix) Nearest Neighbour Analysis

Nearest neighbour analysis was carried out by the method of Lane (1963). To carry out the analysis of the product of the DNA-dependent RNA polymerase from the halophile, the incubation mixture was scaled up 20 times using ATP with p³² in the alpha position as the label (specific activity 2.68 c/mM). The incubation time was increased to one hour instead of thirty minutes to provide still more product for analysis. After incubation, to the reaction mixture was added 0.2 mgm. whole yeast RNA, 125 μ l. sodium dodecyl sulphate (10% solution in H₂O), 2.5 mls. water saturated phenol and 2.5 mls. chloroform containing 1% iso-amyl alcohol. The mixture was agitated and then heated at 60 C for two minutes. The mixture was then centrifuged in a laboratory clinical centrifuge for ten minutes at room temperature. The upper aqueous layer was collected.

The phenol-chloroform extraction was repeated twice more and to the final aqueous layer was added an equal volume of 10% TCA. The precipitate was centrifuged out at 10,000 r.p.m. in the Sorval centrifuge. To the pelleted precipitate was added 2 mls. 95% ethanol. The precipitate was air dried and then suspended in 0.3 ml. of 0.3 M KOH. The extracted nucleic acids were hydrolyzed at 37 C overnight.

The pH of the extract was then adjusted to about 3.5 with perchloric

acid and the extract was allowed to stand on ice for four hours.

The salt crystals were then centrifuged out in the Sorval at 10,000 r.p.m. for 10 minutes.

The supernatant was spotted with appropriate markers on number 1 Whatman filter paper which had been previously saturated with $(\text{NH}_4)_2\text{SO}_4$. The paper was run by descending chromatography in an 8:2 water:ethanol solvent system overnight.

Locations of the spots were determined by display of color under ultraviolet light. One-half inch strips were cut from the reaction product runway, dried for thirty minutes at 60 C and counted on the Beckman Scintillation Counter using Beckman Omnifluor as the scintillator.

(x) Lipid Analysis

The method of lipid analysis was carried out by the procedure provided by Dr. F. Bell (personal communication).

A G - 100 Sephadex preparation of enzyme was freeze dried overnight. The powder was extracted by homogenization with an all glass Potter-Elvehjem tissue homogenizer into chloroform-methanol in a ratio of 2:1 . The precipitate was centrifuged down at 2,500 r.p.m. in a laboratory clinical centrifuge. The lipid containing supernatant was air-dried and the lipid powder was re-dissolved in 0.2 ml. fresh chloroform and spotted with known markers on thin layer plates coated with silica gel G which had been activated by heating at 100 C. The plates were run by ascending chromatography using hexane-diethyl ether-acetic acid in a ratio of 70:30:1 as the developing solvent. After chromatography, the plates were sprayed with Rhodamine G (0.25% in ethanol) and the bands visualized by ultraviolet absorption.

Quantitation was carried out by comparative chromatography with known amounts of lipid.

III. ISOLATION AND ASSAY OF ENZYME

(a) Isolation

The isolation procedure was outlined in the Methods section of this thesis. A schematic representation of this procedure is presented on Figure 1.

The enzyme extract was assayed for enzymic activity at various stages of the preparation. The results of these assays are presented in Table II. It should be noted that no values are quoted for the specific activities of the preparation stages. Such values can only be calculated when assay conditions are standardized for all activity determinations. In the preparation of this enzyme extract, the enzyme was assayed both with endogenous DNA template before DNase treatment, and with exogenous DNA template after DNase treatment. Because in one case the enzyme used endogenous template, while in the other exogenous template was added, the reaction systems are not equivalent and comparison of enzyme activity cannot be made.

All assays were carried out at low ionic strength, so that crude extract, the 229,000 x g DNA-membrane-protein complex and the 229,000 x g supernatant were dialyzed against low salt buffer before being assayed.

FIGURE 1

THE PREPARATION OF THE ENZYME

Washed cells suspended in Buffer D' (3ml./gm. wet weight)

Cell suspension teflon homogenized

Extract centrifuged at 27,000 x g for 15 min.

Supernatant centrifuged at 129,000 x g for 180 min.

DNA-membrane-protein "Complex" collected

Complex treated with 1 mg./30 ml. DNase

Extract centrifuged at 48,000 x g for 15 min.

Supernatant centrifuged at 229,000 x g for 90 min.

Extract dialyzed against Low Salt Buffer overnight

Extract centrifuged at 48,000 x g for 15 min.

Supernatant layered on a G - 100 Sephadex column

Void Volume collected and pooled

DNA - free Enzyme

D'

3.0 M KCl
0.01 M Tris-HCl pH 7.66
0.1 M MgAc
0.008 mM 2 - mercaptoethanol

Low salt Buffer

0.01 M TrisOHCl pH 7.66
0.01 M MgAc
0.008 mM 2 - mercaptoethanol

TABLE II

INCORPORATION OF ^{14}C UTP INTO A COLD TCA
INSOLUBLE PRODUCT

C.P.M. (pM) / mgm. protein

- corrected for controls ¹

Crude extract ²	19,600 (22.9)
229 K x g Complex ²	21,400 (25.2)
229 K x g Supernatant ³	120 (0.1)
G - 100 Eluant ³	12,300 (14.1)

The assay was carried out as described in Materials and Methods, without the addition of dry KCl. The results are representative of more than three independent preparations.

- 1) Control was carried out by omitting the unlabelled ribonucleoside triphosphates (cf. Table VIII).
- 2) Endogenous DNA was used as template.
- 3) Salmon sperm DNA was used as template.

Note that all the enzymic activity was associated with the DNA-membrane-protein complex, and was only released when this complex was treated with DNase. The DNase was then removed by Sephadex chromatography from the preparation to allow the addition of exogenous template.

(b) Elution of DNase on a Sephadex G - 100 Column

When a protein preparation is passed through a G - 100 Sephadex column, the column fractionates protein molecules of a molecular weight ranging from 5,000 to 100,000 (Sephadex-Gel Filtration in Theory and Practice). DNase has a molecular weight of about 65,000 and should therefore be retarded upon passage through the column.

It is very important that the DNase used in the preparation of the extract be removed before any assay is carried out with the addition of fresh exogenous DNA. The added template DNA is sensitive to any DNase present and will be hydrolyzed. Once hydrolyzed, the DNA can no longer act as a template for the enzymic reaction. This effect is illustrated in Table III using the isolated enzyme. There was a ten fold decrease in activity in the presence of DNase.

To test the fractionation characteristics of DNase on G - 100 Sephadex, a standard column was run using Sephadex Blue - Dextran (M. W. 2,000,000) and DNase. The results of chromatography on such a column are illustrated in Figure 2. As can be seen from the figure, the DNase was well fractionated away from the void volume. Since isolated halophilic DNA-dependent RNA polymerase elutes in the void volume (cf. Figure 14), chromatography on G - 100 Sephadex provides an effective means of separating the DNA-dependent RNA polymerase from the DNase.

TABLE III

THE EFFECT OF DNase ON THE ENZYME SYSTEM
C.P.M. (pM) incorporated as ^{14}C UMP / mgm. protein

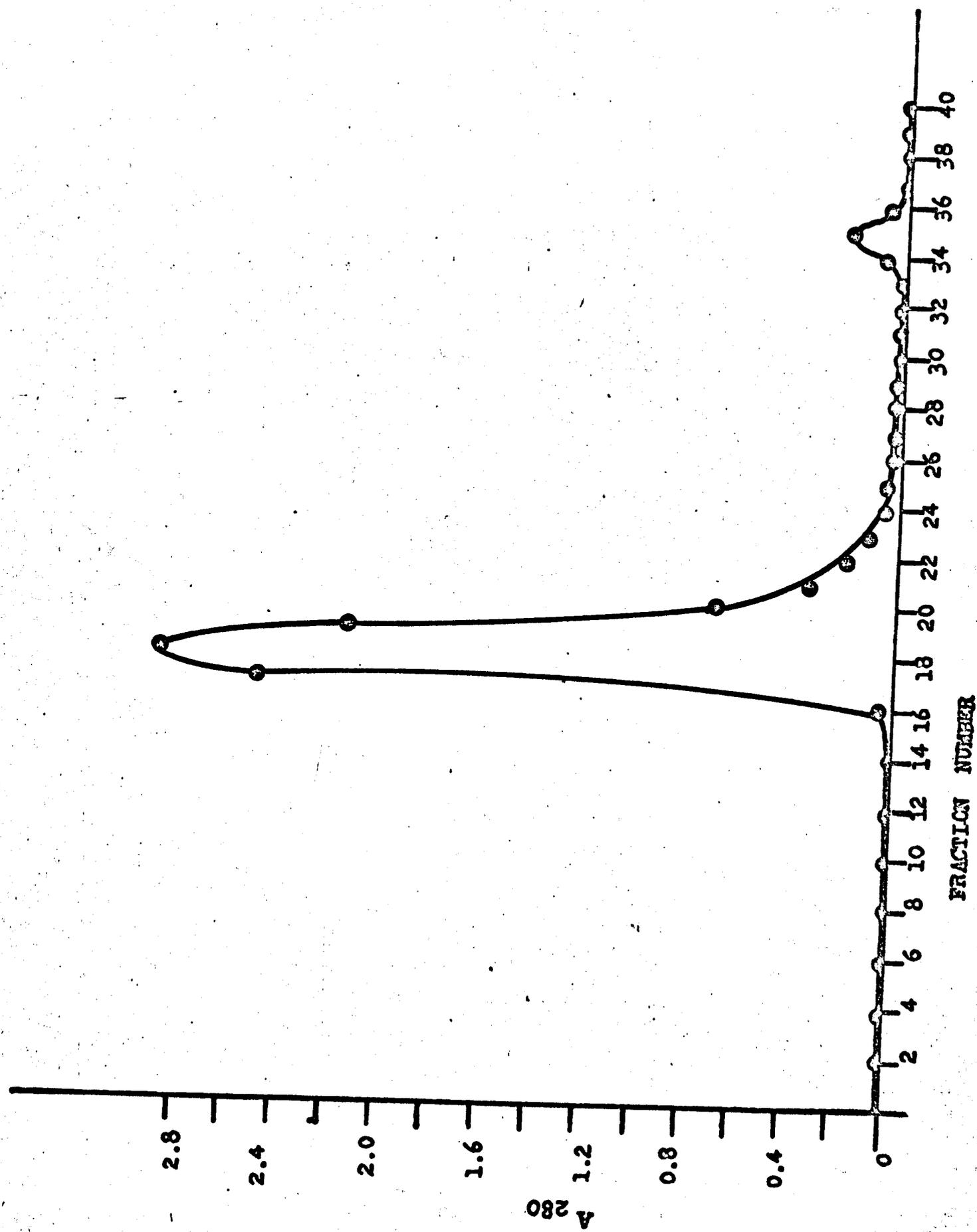
No DNase Addition	<u>20,400</u> (24.2)
No DNase Addition, cold NTP's	<u>1,800</u> (2.2)
DNase Addition	<u>2,200</u> (2.5)
DNase Addition, cold NTP's	<u>1,400</u> (1.8)

The assay was carried out as described in Materials and Methods without the addition of dry KCl. In the cases where DNase was present, 1 $\mu\text{gm.}$ of DNase was added at the beginning of the incubation.

FIGURE 2

ELUTION PROFILE OF A MIXTURE OF BLUE - DEXTRAN AND DNase PASSED THROUGH A G - 100 SEPHADEX COLUMN

A G - 100 Sephadex column was packed in low salt buffer as described in Materials and Methods. The column was layered with 2.0 mls. 0.2% Blue - Dextran and 0.5 mgms. DNase. Two ml. fractions were collected and optical density of the fractions was read at 280 m μ . The first peak contained Blue - Dextran and was identified as the void volume, while the second peak was DNase.



280
A

FRACTICN NUMBER

(c) Enzyme Saturation Study

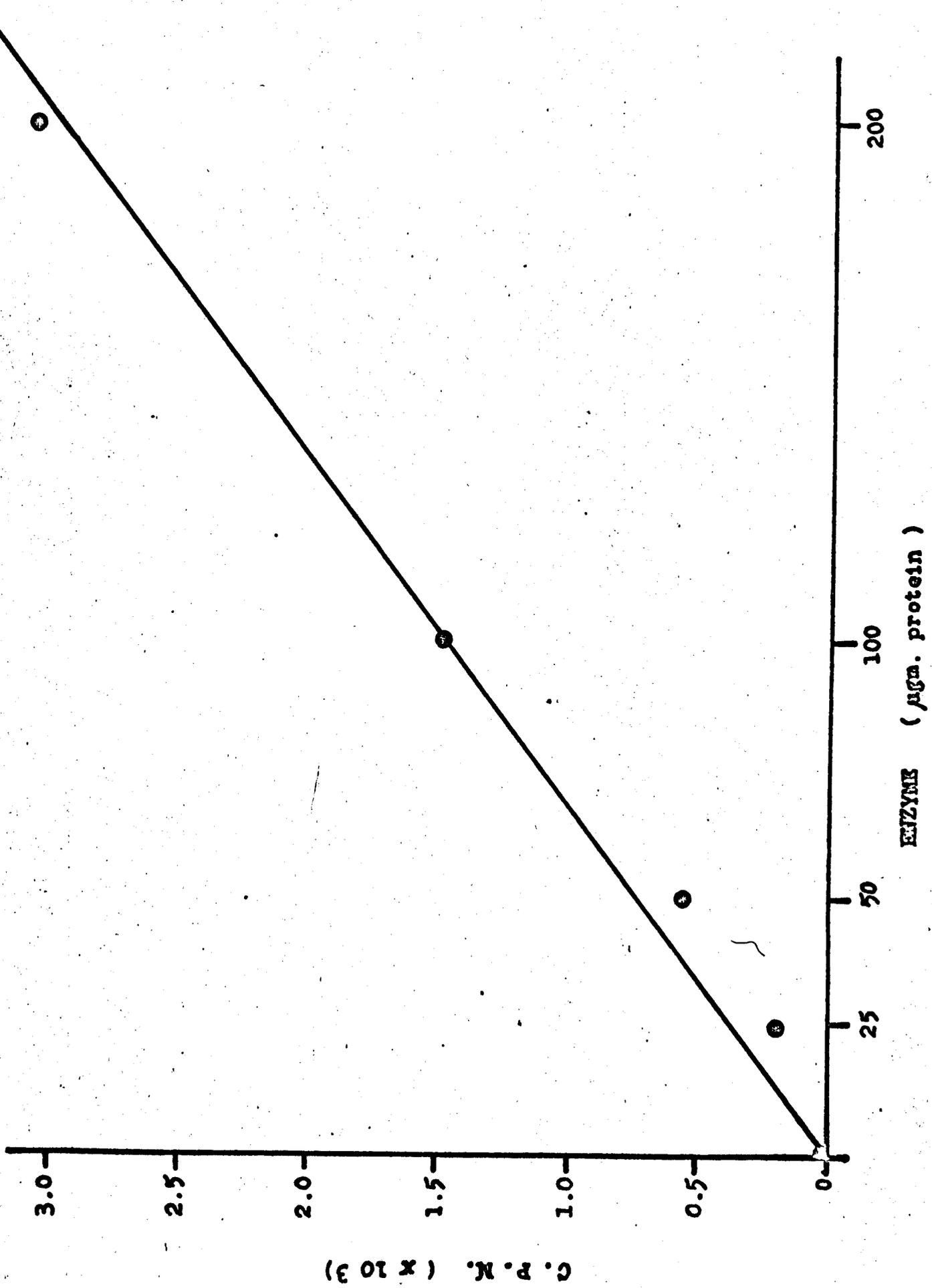
In the standard assay of a G - 100 eluant, the protein content of the reaction mixture was about 0.1 mgm. / 125 μ l. In studying the effect of altered conditions on the behaviour of the enzyme system, it is very important to be working within the linear range of the enzyme. In this way, the enzyme system will be sensitive to any changes in environment.

Figure 3 shows a plot of enzyme activity as a function of protein concentration. Note that the enzyme activity was linear in the protein concentration used.

FIGURE 3.

ENZYME ACTIVITY PLOTTED AGAINST PROTEIN CONCENTRATION

The assay was carried out as described in Materials and Methods without the addition of dry KCl. Protein content of the incubation mixture was varied as indicated.



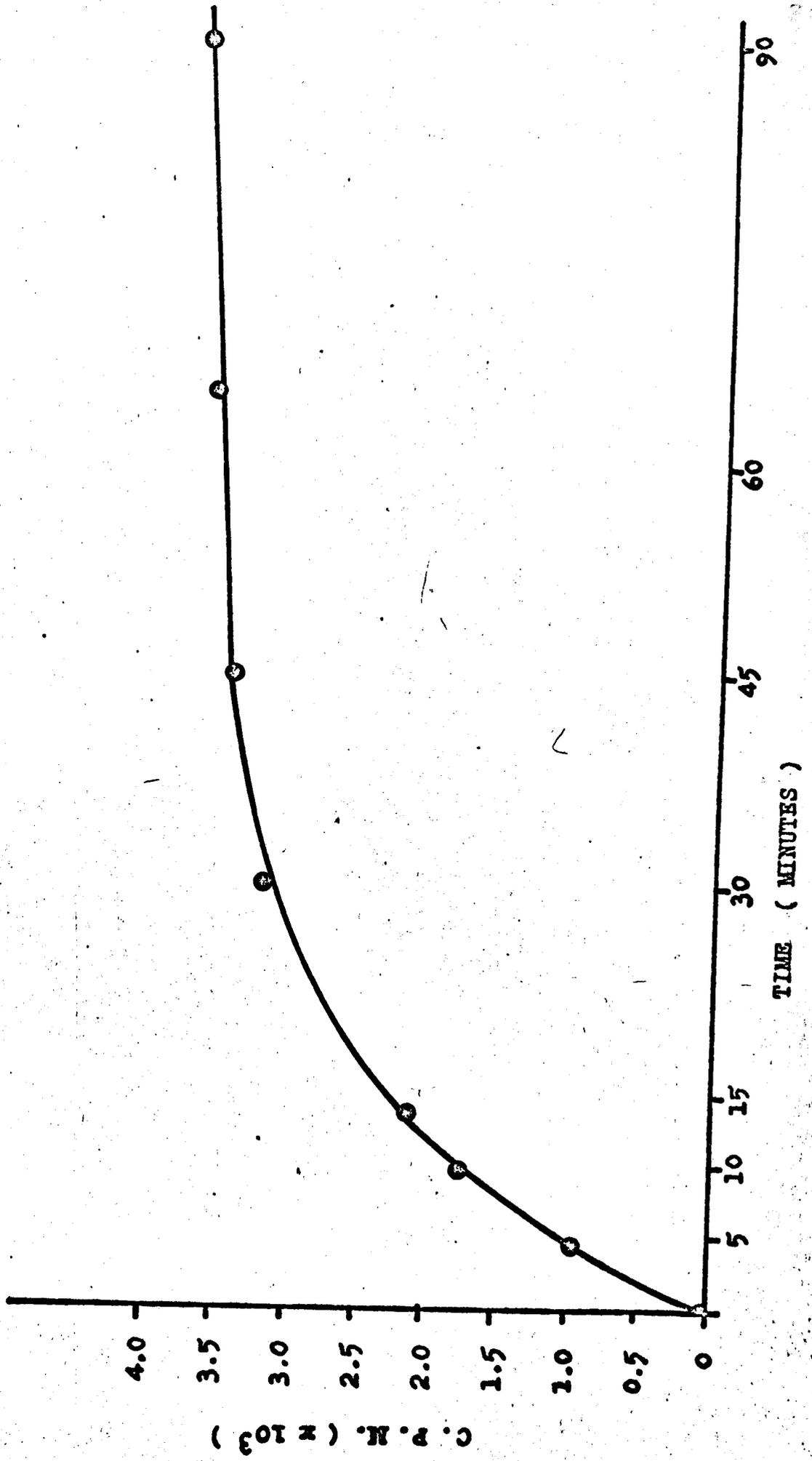
(d) Time Course of the Reaction

An important consideration in studying the enzyme system was incubation time. To find the optimum incubation time, incorporation of radioactive label into a TCA insoluble product was measured as a function of time. The results of such an experiment are illustrated in Figure 4. The enzyme activity reaches a plateau after about forty-five minutes. Thirty minutes was therefore selected as the routine incubation time.

FIGURE 4.

TIME COURSE OF THE REACTION

The assay was carried out as described in Materials and Methods without the addition of dry KCl. At indicated times the incubation mixture was placed on ice, diluted with cold UTP and precipitated with ice cold TCA.



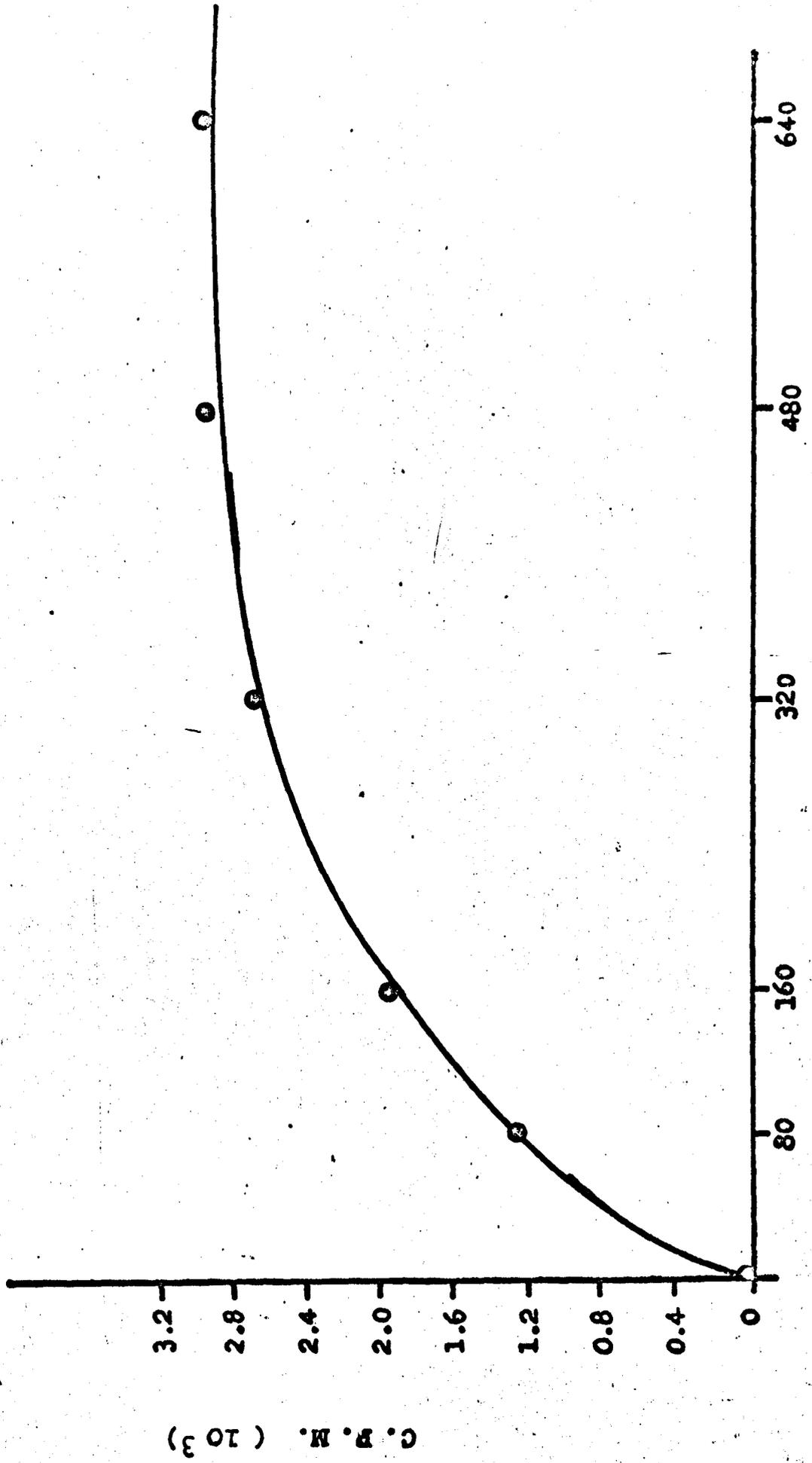
(e) Substrate Requirements

Ribonucleoside triphosphates are the substrate of the enzymic reaction. It was desirable to find the saturating concentration of substrate in order to be quite certain that the substrate would not be limiting when the enzyme was assayed. An experiment was carried out in which enzyme activity was plotted as a function of substrate concentration. The results of such an experiment are outlined in Figure 5. In routine assays, a concentration of 0.32 mM of each unlabelled ribonucleoside triphosphate was used.

FIGURE 5

**ENZYME ACTIVITY PLOTTED AGAINST THE CONCENTRATION
OF SUBSTRATE**

The assay was carried out as described in Materials and Methods without the addition of dry KCl. The concentration of each cold unlabelled ribonucleoside triphosphate was varied together as indicated. The radioactively labelled UTP was held constant in all cases.



ДИ ЕАСН НТР

(f) Template Requirements

In the standard assay system, salmon sperm DNA was used as template for the enzymic reaction. To determine the amount of DNA required to saturate the enzyme system, the DNA content of the assay mixture was varied and the enzyme activity determined. The results of such an experiment are illustrated in Figure 6. The enzyme activity plateaus at an addition of 20 - 30 μ gms. of DNA. In the standard assay, 30 μ gms. of template DNA were used. This amount corresponds to a final DNA concentration of 0.24 mgms./ml.

In studying the template requirements of the enzyme, experiments were also carried out on the source and state of the DNA. In Table IV there are represented results of studies carried out using a variety of templates, including various DNA's, denatured DNA and RNA. The activity of the enzyme is dependent on the type of template DNA used and the state of the DNA, native or denatured, with denatured DNA yielding 30% more enzyme activity. RNA and synthetic polyribonucleotides will not act as a template.

FIGURE 6

**ENZYME ACTIVITY PLOTTED AGAINST AMOUNT OF
TEMPLATE DNA PRESENT**

The assay was carried out as described in Materials and Methods without the addition of dry KCl. Template DNA was varied as indicated.

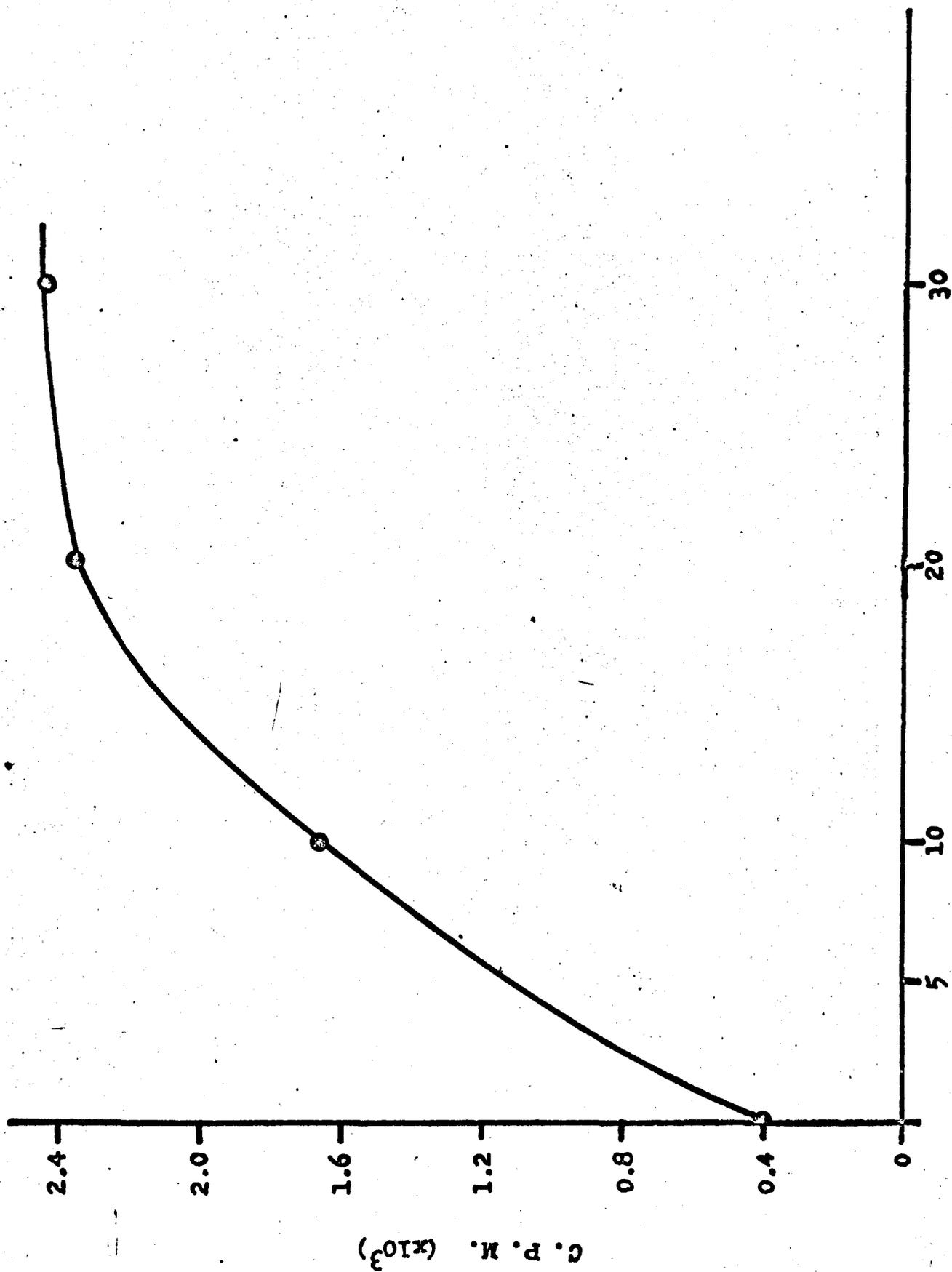


TABLE IV

THE EFFECT OF TEMPLATE ON ENZYME ACTIVITY

<u>TEMPLATE</u>	<u>% INCORPORATION</u>
Salmon Sperm DNA	100 ¹
<u>E. coli</u> DNA	10
Calf Thymus DNA	67
<u>H. cutirubrum</u> DNA	90
Heat Denatured Salmon Sperm DNA	130
Poly C	6
Whole Yeast RNA	6
- DNA	6

1) This represents 31,200 C.P.M. (36.2 pM) incorporated/mgm. protein

The assay was carried out as described in Materials and Methods. All templates were at the standard concentration of 0.24 mgm./ml. Salmon sperm DNA was denatured by heating at 100 C. for 10 minutes and then rapid cooling on ice.

(g) The Effect of Divalent Cations

All DNA-dependent RNA polymerases studied to date require a divalent cation for activity (Fuchs, 1967; So, 1967; Steck, 1968; Carrol, 1970). The response of the enzyme to magnesium was tested. The results of such a test are illustrated in Figure 7 where the enzyme activity is plotted as a function of magnesium concentration. The activity is poor at low magnesium concentrations and has an optimum activity at 40 mM. Complete removal of magnesium from the enzyme system causes the irreversible loss of enzymic activity. Manganese was tested for its ability to replace magnesium in the reaction mixture. A G - 100 eluant was dialyzed against the low salt buffer in which the magnesium was replaced by manganese (0.1 M $MnCl_2$; 0.01 M Tris - CH1 pH 7.66; 0.008 mM 2 - mercaptoethanol). The results of this experiment are outlined in Table V. The enzyme in the presence of manganese is only 50% as active as in the presence of magnesium.

FIGURE 7

ENZYME ACTIVITY AS A FUNCTION OF MAGNESIUM CONCENTRATION

The assay was carried out as described in Materials and Methods without the addition of dry KCl. The concentration of magnesium was varied as indicated. The value of 10 mM is the resultant magnesium concentration that results when the enzyme in low salt buffer is added to the 125 μ l. incubation mixture.

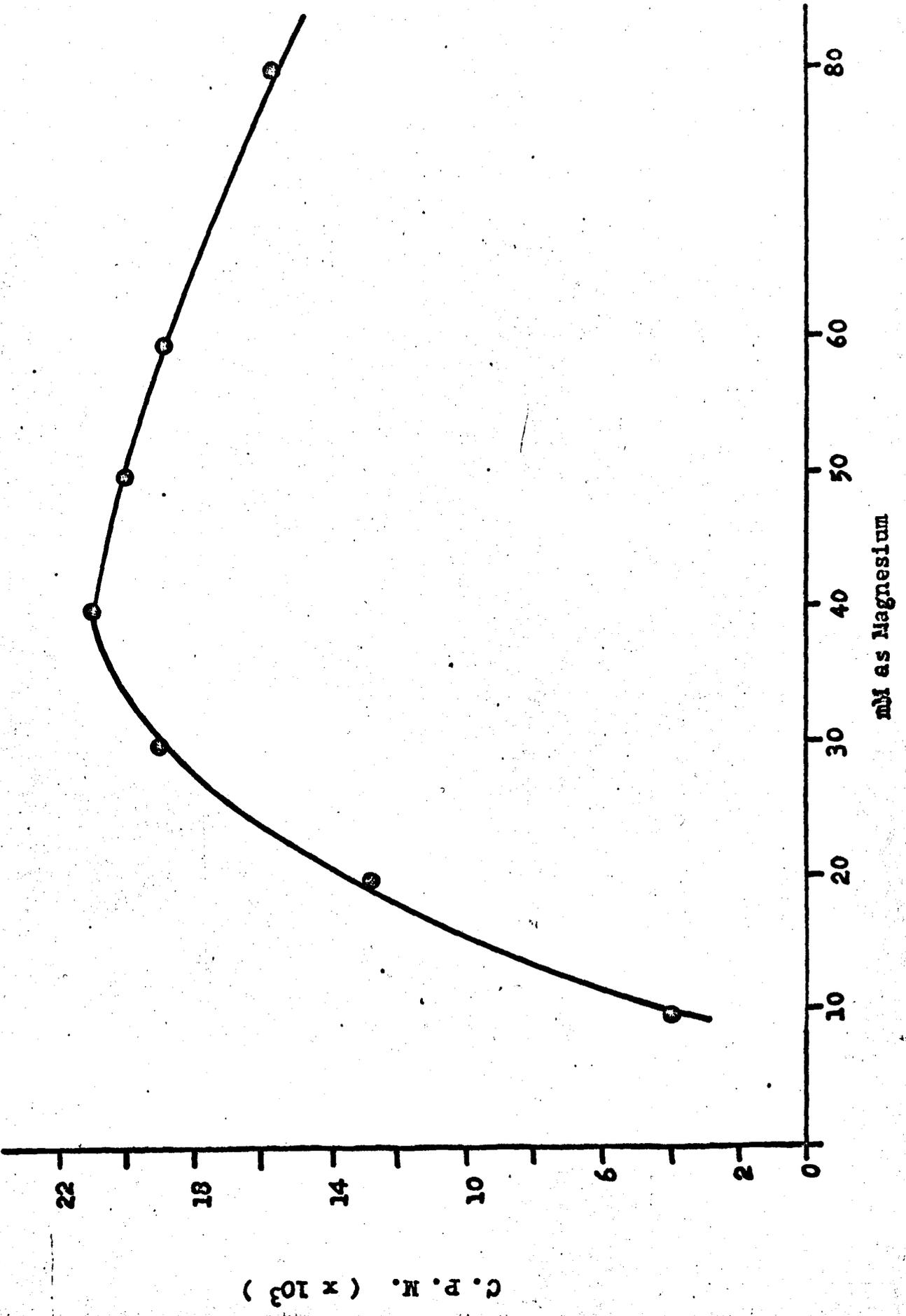


TABLE V

THE EFFECT OF DIVALENT CATIONS ON THE INCORPORATION OF C¹⁴ UMP INTO AN ACID INSOLUBLE PRODUCT

C.P.M. (pM) / mgm. protein

- corrected for controls

Manganese	6,100 (7.2)
Magnesium	12,300 (15.5)

The assay was carried out as described in Materials and

Methods without the addition of KCl. Magnesium was replaced by manganese by dialysis.

Controls were carried out by omitting the unlabelled ribonucleoside triphosphates (cf. Table VIII).

(h) The Effect of Monovalent Cations

To date all the DNA-dependent RNA polymerases studied require a monovalent cation for activity (Fuchs, 1967; So, 1967).

The reaction mixture has 0.2 M as NH_4Cl (cf. Methods).

At this concentration of cation, the enzyme has optimum activity

This is illustrated in Figure 8, where the enzyme activity is plotted as a function of NH_4Cl concentration.

Enzyme activity was also plotted as a function of KCl concentration. The replacement of NH_4Cl by KCl did not alter the results of the enzyme assay substantially. The enzyme maintained maximum activity at 0.1 - 0.2 M as KCl . This is illustrated in Figure 9. Because monovalent cations are so important in the environment of the halophile, the role of monovalent cation will be further discussed in Section IV.

The final standard enzyme assay system based on the preceding results is presented in Table VI.

FIGURE 8

**ENZYME ACTIVITY AS A FUNCTION OF AMMONIUM CHLORIDE
CONCENTRATION**

The assay was carried out as described in Materials and Methods without the addition of dry KCl. Dry ammonium chloride was added to bring the final incubation mixture to the indicated concentrations.

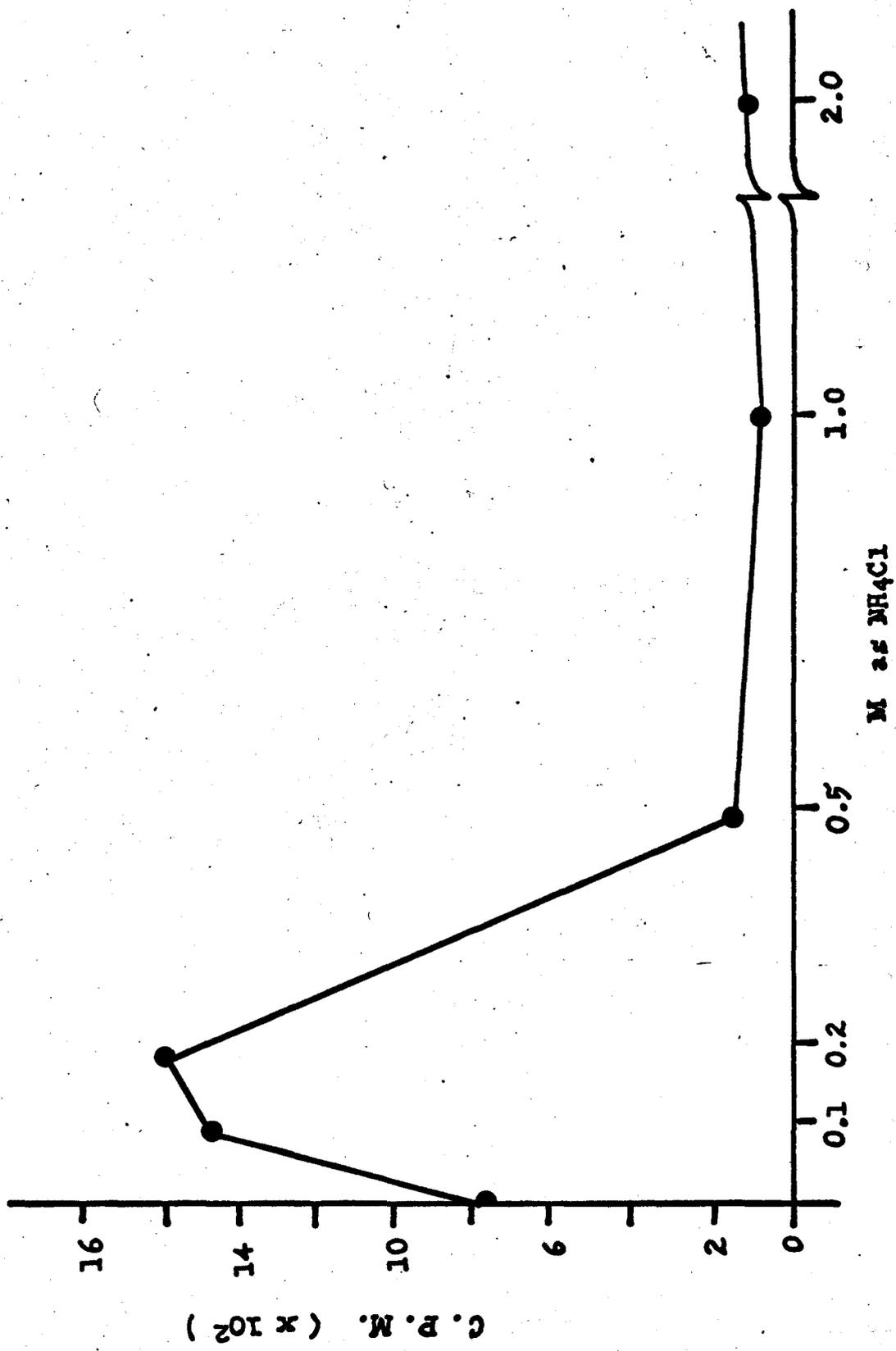


FIGURE 9

**ENZYME ACTIVITY AS A FUNCTION OF
KCl CONCENTRATION**

**The assay was carried out as described in Materials and
Methods with the addition of dry KCl to yield the concentrations
indicated.**

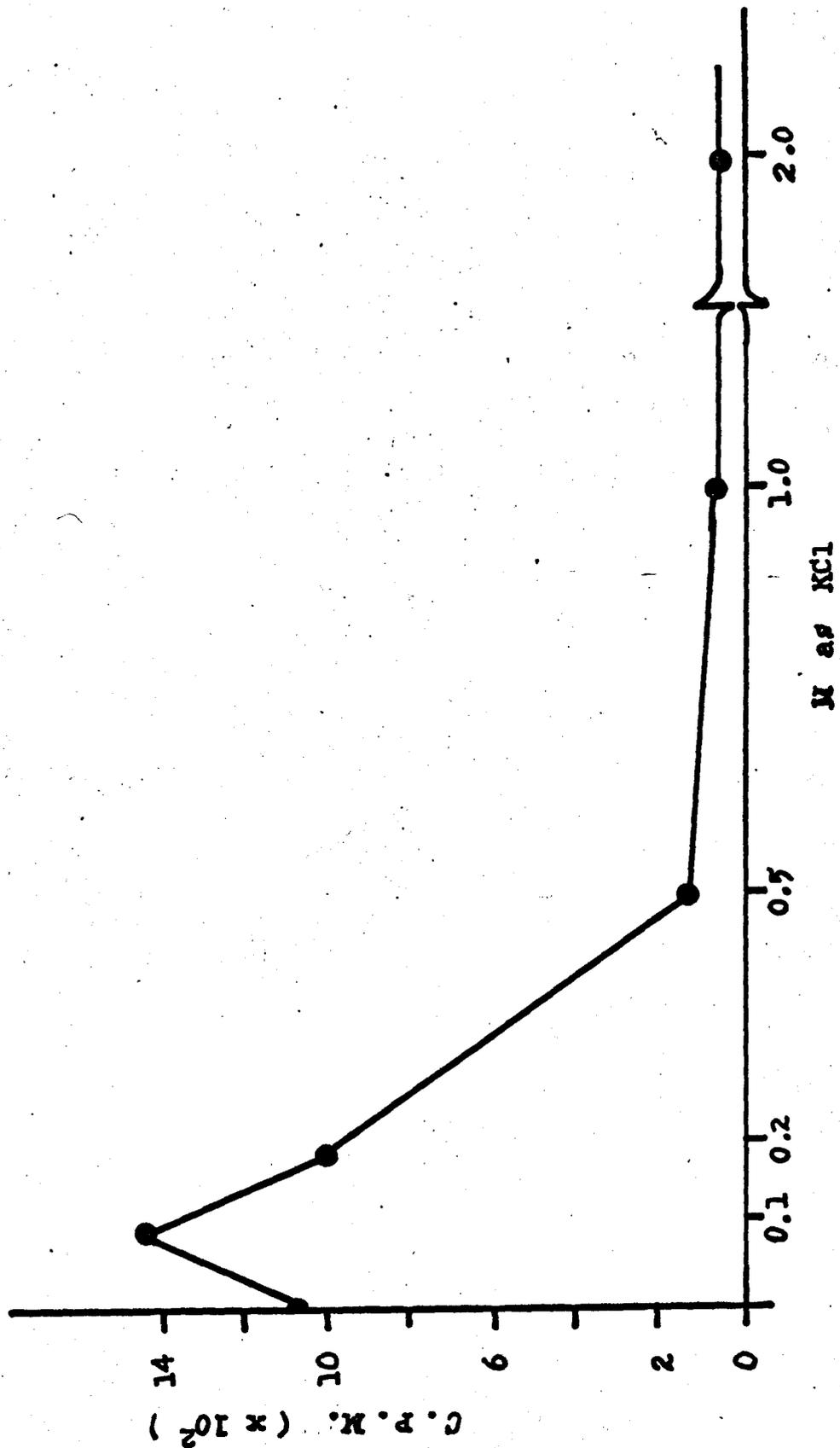


TABLE VI

THE ENZYME ASSAY SYSTEM SUMMARIZED

Final Salt Concentrations

KCl	from 0 to 3.8 M
NH ₄ Cl	0.2 M
Tris - HCl pH 8.0	0.035 M
MgAc	0.04 M

Substrate, Enzyme and Template

NTP's (each)	0.32 mM
C ¹⁴ UTP	10 pM
Protein (routine)	0.8 Mgm./ml.
DNA ¹	0.24 mgm./ml.

The reaction mixture was incubated for 30 minutes at 37 C.

The reaction was stopped by chilling on ice and rapidly diluting with 100 X unlabelled UTP. The product was precip-

itated with ice cold TCA and filtered on 0.45 μ Millipore filters.

The filters were dried and read by liquid scintillation (cf. Methods).

1. Omitted when "Complex" assayed

IV. THE ROLE OF MONOVALENT CATIONS

Of primary interest in the study of enzymes isolated from extreme halophiles is their relationship to high intracellular ionic strengths. Experiments were carried out to determine the ability of the isolated DNA-dependent RNA polymerase to function at high ionic strengths. In Section III, data was presented which showed the ability of the enzyme to function in high ionic strength by re-introduction of KCl and NH_4Cl into the assay system. (cf. Figures 8 and 9). The enzyme was found to be inactive above about 0.2 M as monovalent cation in both cases.

There exists the possibility that in exposing the enzyme to low ionic strength during preparation, some change in enzyme structure may have occurred. This change may be irreversible so that activity in high salt systems is lost. Therefore, an enzyme preparation was run on a G - 100 Sephadex column packed, equilibrated and eluted with D¹ buffer. In this way the enzyme was never exposed to low ionic strengths. The optical density profile of such an elution is shown in Figure 10.

The void volume was pooled and the enzyme was assayed using both salmon sperm and H. cutirubrum DNA as template. The results of the assay are shown in Table VII (a). Material recovered from the void volume of this column showed no activity under these

FIGURE 10

**ELUTION PROFILE OF AN ENZYME PREPARATION
PASSED THROUGH A G - 100 COLUMN PACKED IN D'**

The G-100 column was packed as described in Materials and Methods with D' as the working buffer. Four mls. of the undialyzed DNase treated "Complex" was layered and eluted. Two ml. fractions were collected and the optical density of the fractions was read at 280 m μ . For information as to the activity of the extract, see text.

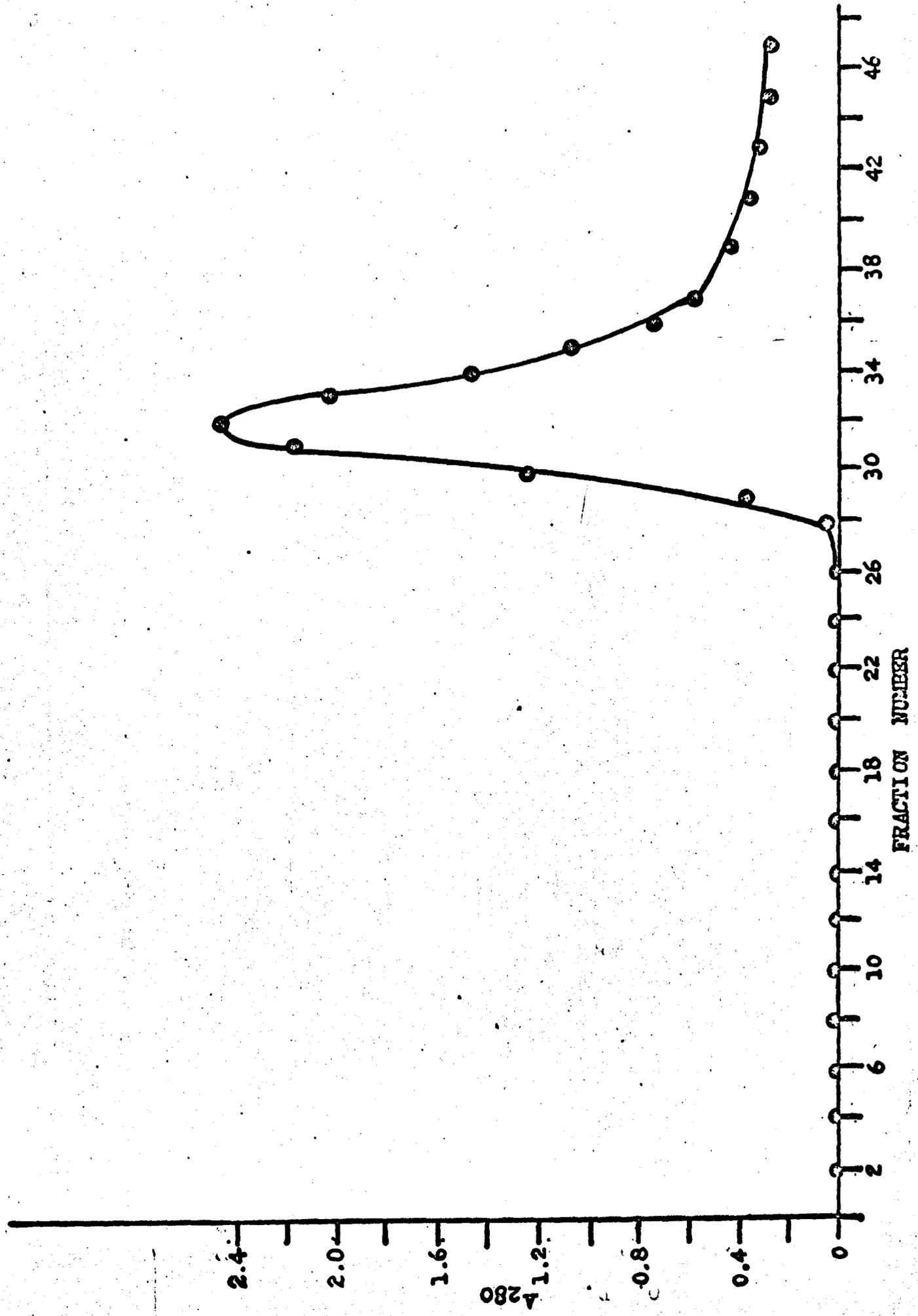


TABLE VII

ACTIVITY AT HIGH SALT CONCENTRATIONS
INCORPORATION OF C¹⁴ UMP INTO A TCA INSOLUBLE PRODUCT

(a) <u>H. cutirubrum</u> DNA	120 (0.1)
<u>H. cutirubrum</u> DNA, - cold NTP's	110 (0.1)
Salmon Sperm DNA	110 (0.1)
Salmon Sperm DNA, - cold NTP's	110 (0.1)

The assay was carried out as described in Materials and Methods, with the addition of dry KCl to bring the final concentration of the reaction mixture to 3.8 M.

(b) Salmon Sperm DNA	18,200 (21.6)
Salmon Sperm DNA, - cold NTP's	1,400 (1.6)

The assay was carried out as above, without the addition of dry KCl.

assay conditions.

The G - 100 void volume was then dialyzed into the low salt working buffer to confirm the presence of the enzyme in the eluant.

The results of a normal low salt enzyme assay are shown in Table VII (b). Enzyme activity is recovered at low ionic strength.

A variety of experiments were done in an attempt to demonstrate activity of the isolated enzyme in high ionic strength.

Incubation mixtures were set up at normal low ionic strengths and were allowed to incubate at 37 C. for several minutes at which point KCl was added either by dialysis or by dry salt addition to bring the ionic strength of the incubation mixture to 3.8 M as KCl. It was found that upon addition of the salt, all RNA synthesis ceased, even though RNA synthesis had taken place prior to the addition of the salt.

A low salt preparation of the isolated enzyme was dialyzed against D¹ and added to a crude extract to attempt to demonstrate a stimulation of RNA synthesis. The background activity was too high to detect any stimulation.

In case the enzyme was lacking a factor which enabled it to function in high salt, the 229,000 x g supernatant was added to an isolated enzyme preparation and the assay was carried out at high ionic strength. Again no activity at high ionic strength could be demonstrated.

To date no activity with isolated enzyme has been demonstrated

in a high salt environment.

In contrast to these results, similar experiments were carried out on the DNA-membrane-protein complex prepared in the course of enzyme extraction. Enzyme activity in the presence of endogenous template was found throughout a large range of salt concentrations. This is shown using KCl as the monovalent cation and plotting enzyme activity as a function of salt concentration in Figure 11.

Again in contrast to the isolated enzyme system where NH_4Cl and KCl have the same effect on enzyme activity (cf. Figures 8 and 9), NH_4Cl will not support enzymic activity at high ionic concentrations to the same extent as KCl. This is also illustrated in Figure 11.

Therefore, in summary, when attached to template the enzyme has a wide range of salt tolerance with a preference for KCl.

When the enzyme is freed from its template, this tolerance is lost. Further speculation on the cause of such results will be reserved for discussion later.

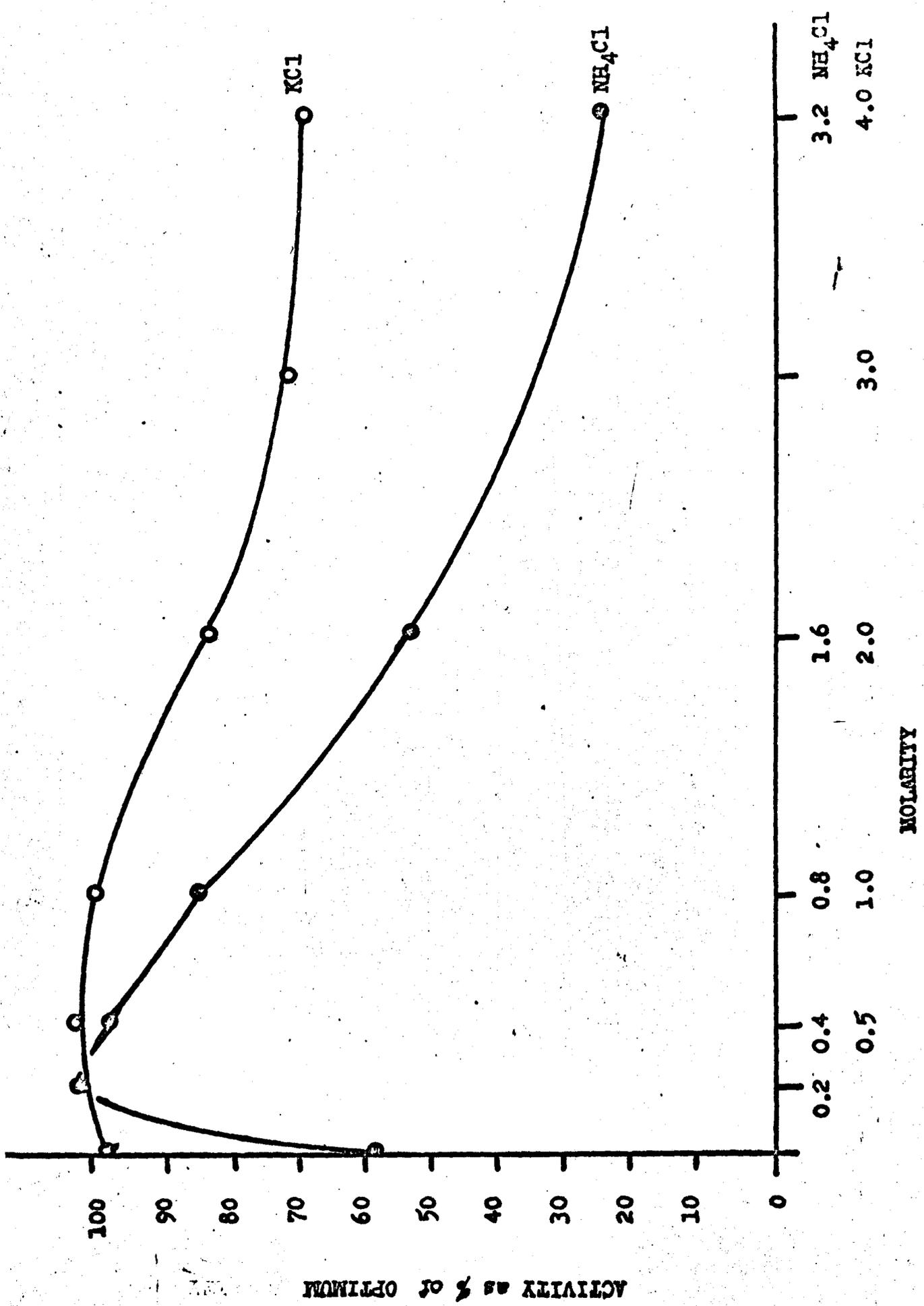
FIGURE 11

ENZYME ACTIVITY OF THE DNA-MEMBRANE-PROTEIN
COMPLEX AS A FUNCTION OF CONCENTRATION OF
MONOVALENT CATION

The assay was carried out as described in Materials and Methods using endogenous DNA as template. Dry KCl or NH_4Cl was added to bring the final incubation mixture to the indicated molarity. The activity was plotted as a percentage of optimum activity.

Open circles represent results with KCl

Closed circles represent results with NH_4Cl



ACTIVITY as % of OPTIMUM

MOLARITY

KCl

NH₄Cl

3.2 NH₄Cl

4.0 KCl

1.6

2.0

0.8

1.0

0.2 0.4

0.5

3.0

V. NATURE OF THE ENZYME REACTION AND PRODUCT

(a) Controls

A number of controls were utilized to confirm the nature of the enzyme activity of the extract.

- (i) The preparation was treated with DNase at different stages of the assay. The enzyme should hydrolyze the template if added before or during the incubation. After the incubation, when the role of the template is over, the DNase should have no effect.
- (ii) The preparation was treated with RNase both during and after incubation. The RNase should decrease the acid precipitable C¹⁴ label at any time of addition.
- (iii) The ribonucleoside triphosphates (unlabelled) were omitted. In the absence of substrate the enzyme should have no activity.
- (iv) In the case of the isolated enzyme, template was omitted. Without template, the enzyme should have no activity.

The results of such experiments on both the complex and isolated enzyme are outlined in Table VIII. Note that the results are as expected with the exception of the decrease in acid precipitable counts when the DNase post treatment is used. This can be ex-

TABLE VIII

CONTROL EXPERIMENTS ON THE
INCORPORATION OF C¹⁴ UMP INTO A TCA INSOLUBLE
PRODUCT

(expressed as a per cent of complete reaction)

	<u>COMPLEX</u>	<u>ISOLATE</u>
Complete mixture ¹	100%	100%
- NTP's	10%	8%
- DNA	-----	12%
DNase, pre-incubation ²	8%	10%
DNase, post-incubation ³	75 %	55 %
RNase, during incubation	10%	11%
RNase, post-incubation ³	10%	10%

The assay was carried out as described in Materials and Methods without the addition of dry KCl. No DNA was added in the assay of the "Complex"

1. C.P.M./mgm protein was 41,200 for the complex, and 20,300 for the isolated enzyme.
2. The extract was pre-incubated with DNase at a concentration of 80 ugm./ml. for 5 minutes at 37 C.
3. As in number 2. above, except RNase or DNase was added after incubation

plained by considering that the DNA template is precipitated along with the product RNA when treated with cold TCA. Small RNA chains which may be precipitated by their attachment to template DNA may become TCA soluble when the template is hydrolyzed by DNase. Therefore the acid precipitable counts are decreased.

For maximum activity, all four ribonucleoside triphosphates must be present. As seen in Table VIII, the omission of the three unlabelled ribonucleoside triphosphates greatly inhibited the enzymic reaction. An experiment was carried out to find the effect of leaving out only one unlabelled ribonucleoside triphosphate at a time. Radioactively labelled UTP and ATP were used in turn so that each different ribonucleoside triphosphate could be omitted. The results of such an experiment are outlined in Table IX. The degree of dependence upon the presence of any ribonucleoside triphosphate varies with the ribonucleoside triphosphate omitted. Omission of purines seems in general to have a greater inhibiting effect than omission of pyrimidines. This could be related to initiation of enzyme activity and RNA synthesis at pyrimidine clusters. By omitting a purine the synthesis of RNA cannot begin, while pyrimidine omission still allows initiation of RNA synthesis, and therefore, would allow short chains of ribonucleotides to be linked together. This will be discussed further in later sections.

TABLE IX

EFFECT OF RIBONUCLEOSIDE TRIPHOSPHATE OMISSION

<u>Ribonucleoside Triphosphates</u>	<u>Label</u>	<u>% Activity</u>
All	C ¹⁴ UTP	100%
- ATP	"	25%
- CTP	"	60%
- GTP	"	16%
- ATP, CTP, GTP	"	12%
All	p ³² ATP	100%
- CTP	"	35%
- GTP	"	21%
- UTP	"	43%
- CTP, GTP, UTP	"	16%

The assay was carried out as described in Materials and Methods without the addition of KCl. Incorporation of UMP into a cold TCA insoluble product was 22,000 C.P.M./mgm. protein. Incorporation of AMP was 41,500 C.P.M./mgm. protein.

(b) Inhibitors

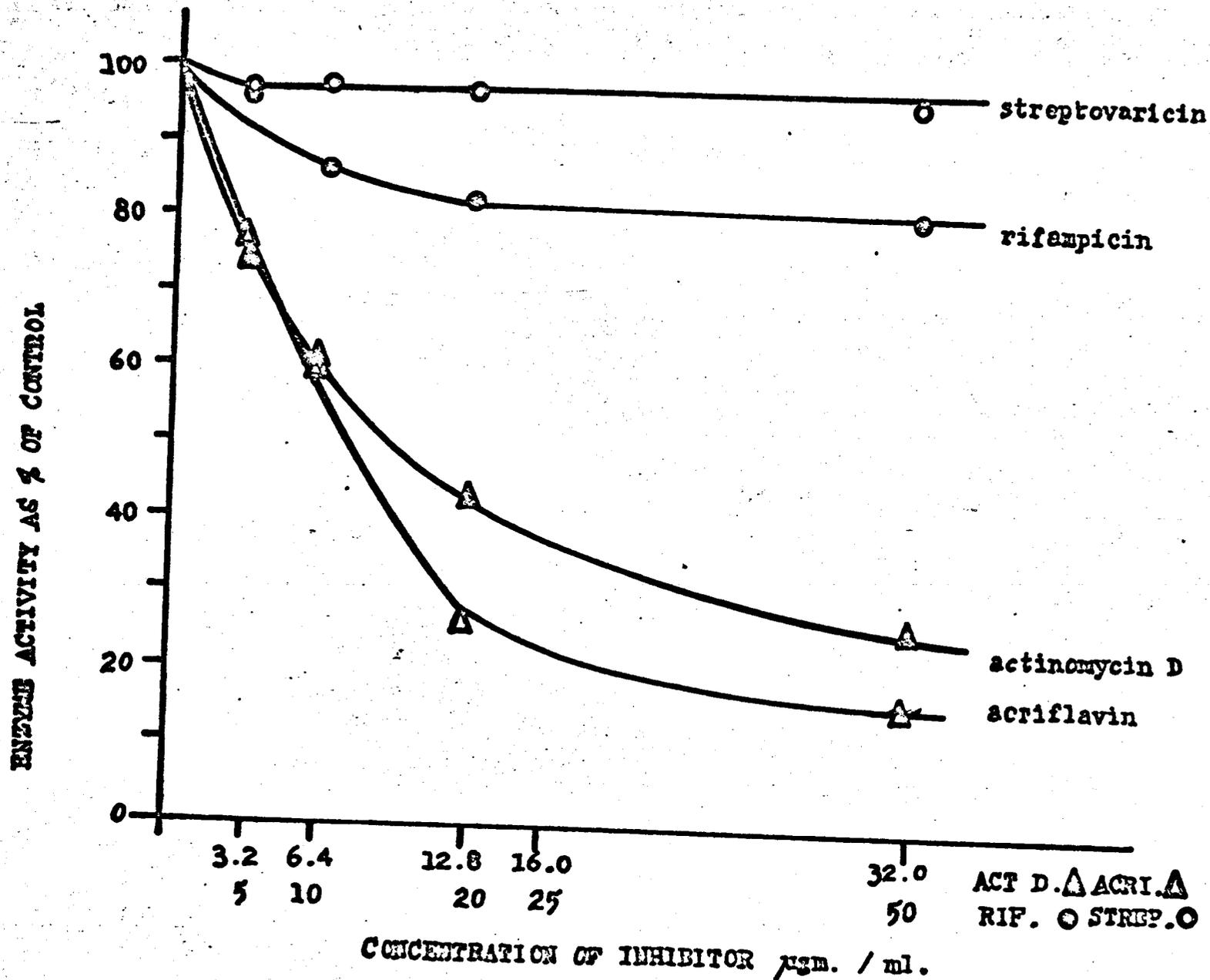
Studies were carried out with inhibitors known to affect RNA synthesis specifically in conventional bacterial systems. Experiments were done to determine their effect on a halophilic RNA synthesizing system, Actinomycin D and acriflavin inhibit RNA synthesis by interacting with the DNA template (Klenow, 1964; Kersten, 1966), while rifampicin and streptovaricin interact with the enzyme itself (Mizuno, 1968; Wehrli, 1968; Wehrli, 1969; Hemphill, 1969; diMauro, 1969). The effect of these inhibitors is illustrated in Figure 12. Note that those inhibitors normally affecting the enzyme are ineffective, in contrast to inhibitors which affect RNA synthesis by binding to the template. The significance of this effect will be discussed in a later section.

It should be noted that the rifampicin and streptovaricin were tested in an E. coli RNA polymerase system to confirm the activity of these inhibitors. The inhibitors were found to be functional.

FIGURE 12

THE EFFECT OF INHIBITORS ON ENZYME ACTIVITY

The assay was carried out as described in Materials and Methods without the addition of dry KCl. Inhibitors were added to give the final concentrations as indicated in the Figure.



(c) Nearest Neighbour Analysis of the RNA Product

The possibility existed that the product of the enzymic reaction was a homopolymer of the labelled ribonucleotide, and that RNA which incorporated all the ribonucleoside triphosphates available was not being produced. To confirm the production of an RNA product having the random distribution of all available ribonucleotides, a nearest neighbour analysis was performed on the in vitro product of the enzyme.

The results of the nearest neighbour analysis are graphically illustrated in Figure 13. A calculation of the average nearest neighbour frequency is presented in Table X.

As the results indicate, all the ribonucleotides available are incorporated into RNA without selectivity for any specific ribonucleotide.

FIGURE 13

RESULTS OF A NEAREST NEIGHBOUR ANALYSIS

The nearest neighbour analysis was carried out as described in Materials and Methods. Identification of peaks was done by co-chromatography using known markers. The results illustrated here are the resultant of three separate experiments.

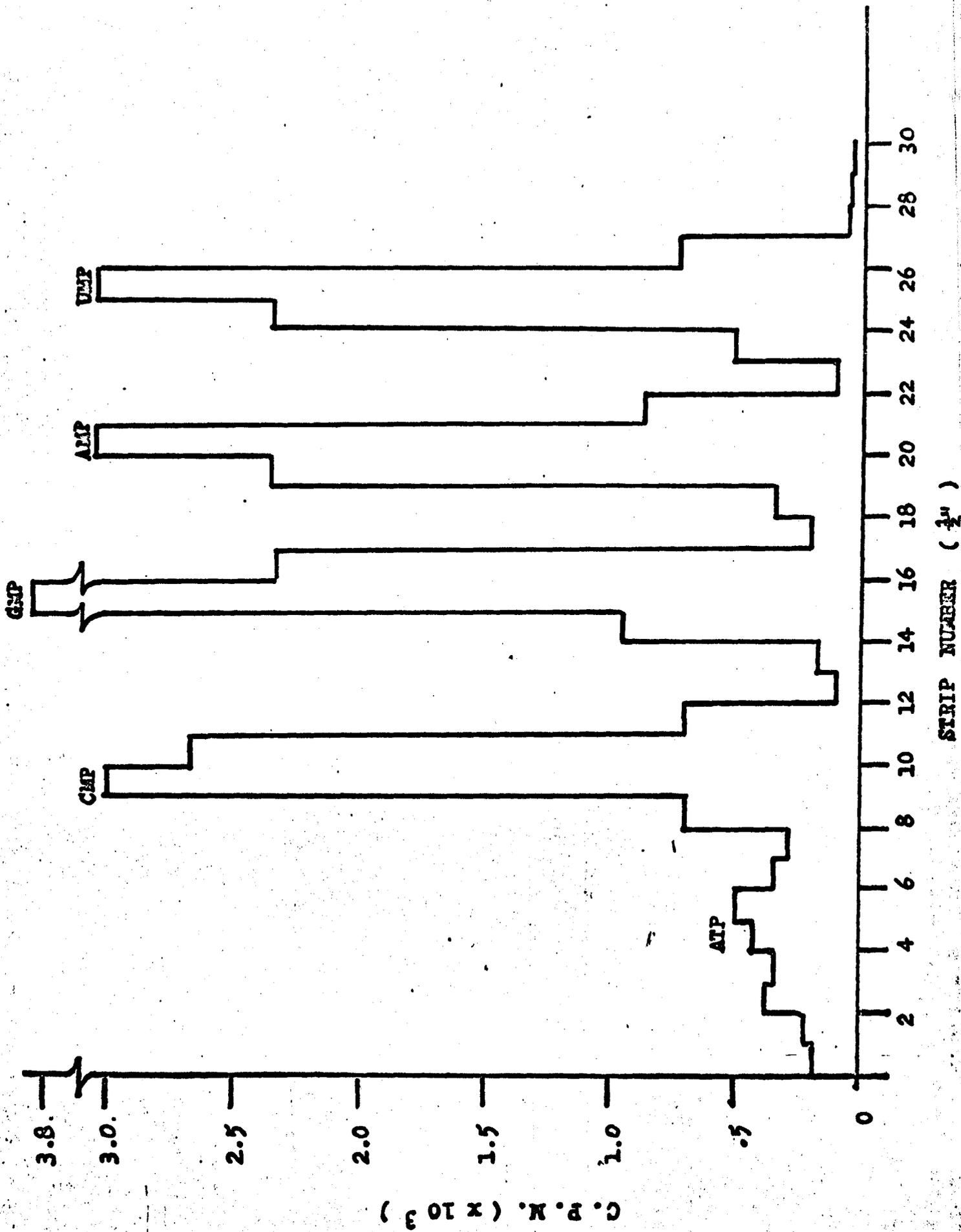


TABLE X

RESULTS OF NEAREST NEIGHBOUR ANALYSIS

Using ATP - alpha - p³²

ApC	26%
ApG	25%
ApA	25%
ApU	24%

The analysis was carried out as described in Materials and Methods. These results represent three independent experiments plus or minus 2% .

VI. MOLECULAR WEIGHT OF THE ENZYME

With the enzyme freed from its template, it is possible to estimate its molecular weight.

In preparing the enzyme, the extract was passed through a G - 100 Sephadex column. The enzyme eluted in the void volume indicating a molecular weight greater than 100,000. This elution profile is shown in Figure 14. When chromatography was carried out at high ionic strength by using a column equilibrated, packed and eluted in D¹ buffer, the enzyme chromatographed in the same position (cf. Figure 10). The finding that the enzyme is of such a high molecular weight is in disagreement with recently published results which indicate the molecular weight of the enzyme isolated from H. cutirubrum to be of the order of 20,000 (Louis and Fitt, 1971 (a); Louis et al., 1971 (b)). These contrasting results will be further discussed in Section VII of this thesis.

The enzyme was then chromatographed on a G - 200 Sephadex column. In this gel, proteins with a molecular weight of 5,000 to 800,000 are fractionated (Sephadex-Gel Filtration in Theory and Practice). The elution profile of such a column is shown in Figure 15. The enzyme elutes in the trailing edge of the void volume. An estimate of the molecular weight from such an elution is not possible due to the non-linearity of the column. Therefore, glycerol gradients

FIGURE 14

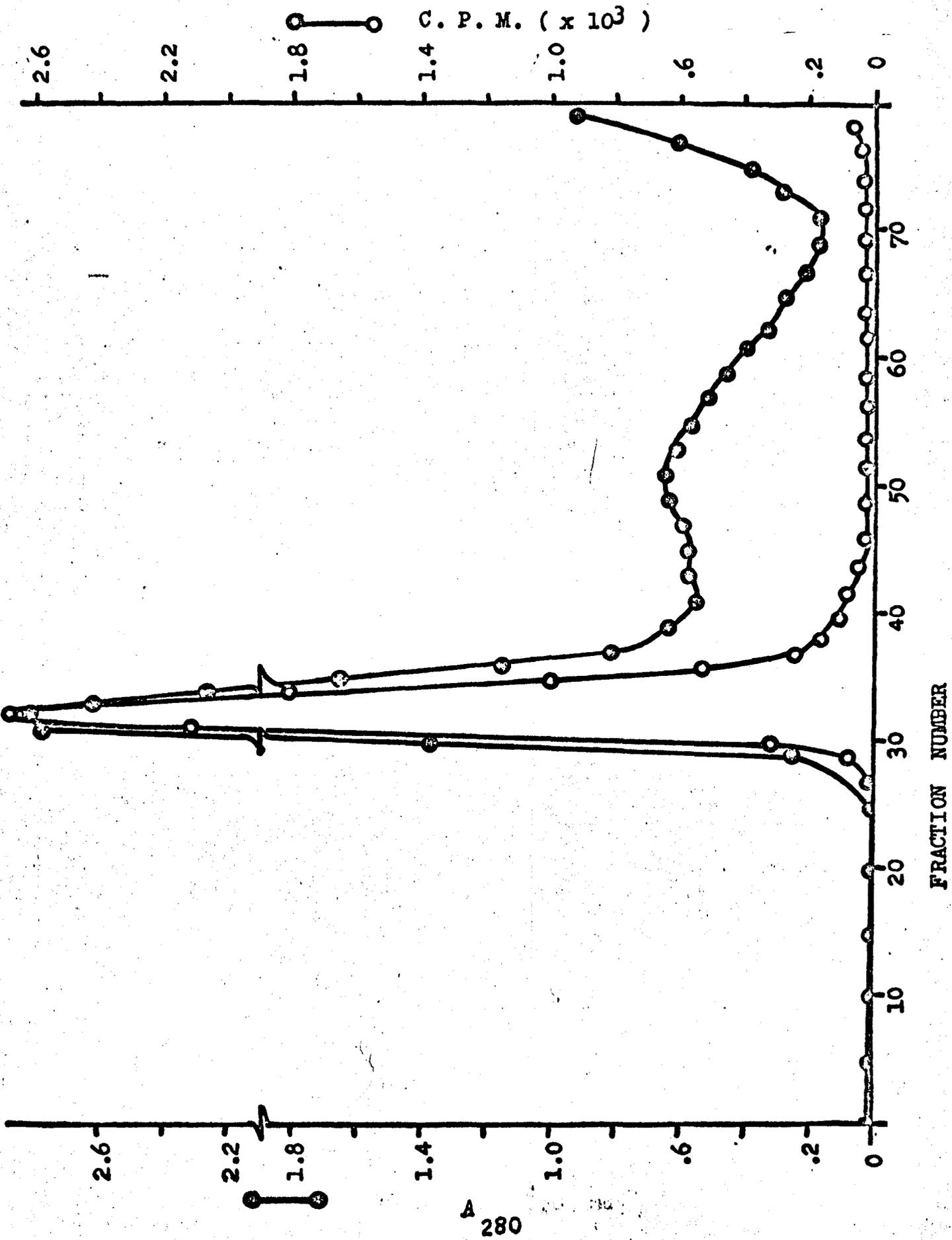
G - 100 ELUTION PROFILE OF AN ENZYME PREPARATION

The column was run as described in Materials and Methods.

The two ml. fractions were assayed for optical density and enzymic activity as described in Materials and Methods.

Open circles represent enzymic activity.

Closed circles represent optical density at 280 m μ .



A 280

FIGURE 15

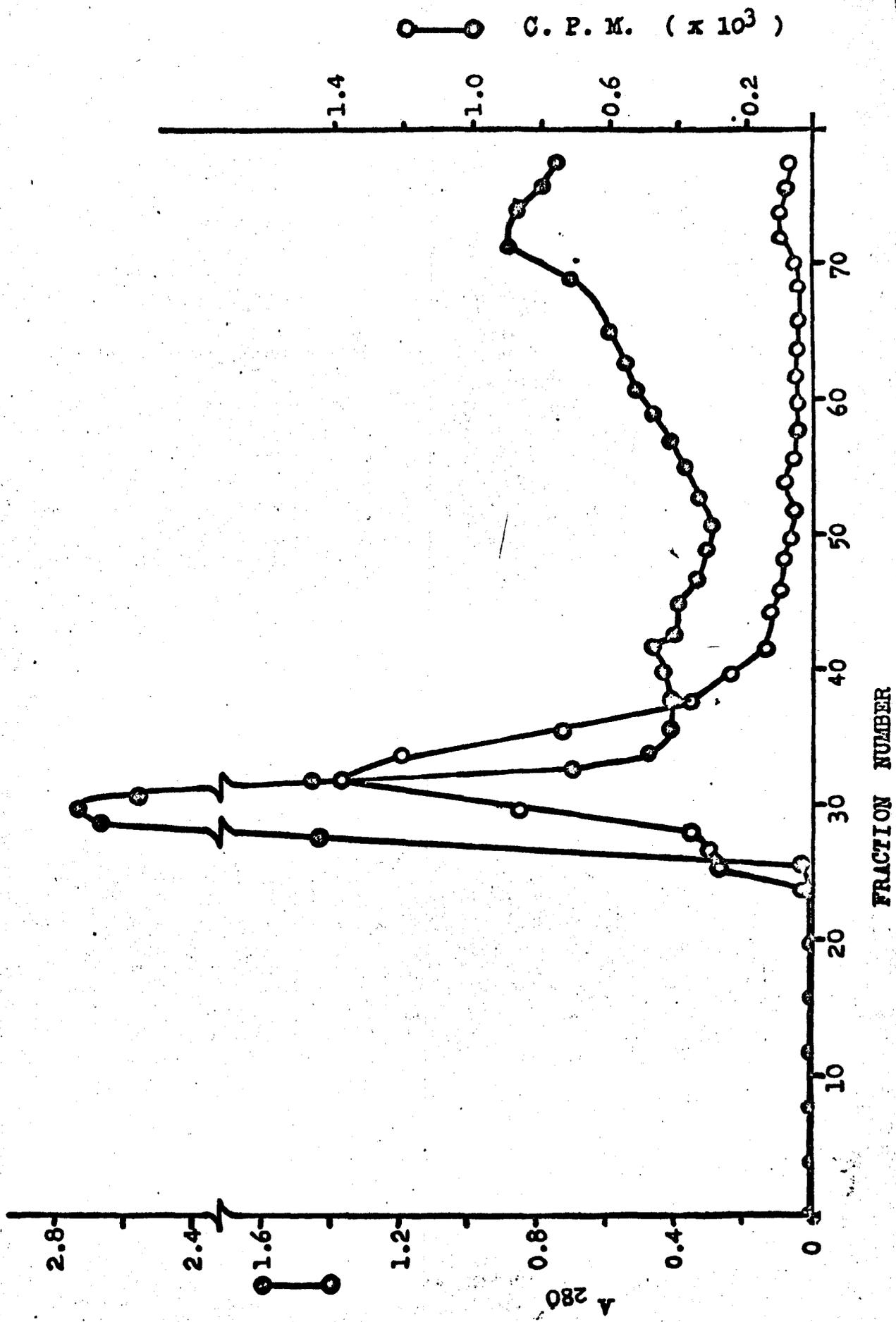
G - 200 ELUTION PROFILE OF AN ENZYME PREPARATION

The column was run as described in Materials and Methods.

The two ml. fractions were assayed for optical density and enzymic activity as described in Materials and Methods.

Open circles represent enzymic activity.

Closed circles represent optical density at 280 m μ .



were run to make a better estimate of the enzyme's molecular weight.

Glycerol gradients were prepared as described in Materials and Methods. Gamma globulin (M.W. 160,000) and catalase (M.W. 240,000) were used as protein markers. Gamma globulin was sedimented through a glycerol gradient having the ionic strength of physiological saline (0.85% NaCl; 0.01 M Tris - HCl pH 7.66) and catalase was sedimented through a glycerol gradient in 0.05 M Na_2HPO_4 pH 7.0 to correct for any difference in sedimentation which may result due to the higher ionic strength of the low salt working buffer. No sedimentation difference was found.

Figure 16 illustrates a composite profile of glycerol gradients carried out on the isolated DNA-dependent RNA polymerase, catalase and gamma globulin. The sedimentation of the DNA-dependent RNA polymerase is more rapid than either gamma globulin or catalase. By extrapolation the molecular weight of the enzyme was estimated to be of the order of 400,000. This is in the same range of molecular weight as determined for conventional E. coli RNA polymerase (Burgess, 1969).

To confirm the similarity in molecular weights between the halophile polymerase and E. coli polymerase, E. coli polymerase and H. cutirubrum polymerase were run on duplicate glycerol gradients. With limited material the E. coli polymerase sedimented at the same rate as the halophile polymerase further confirming the

FIGURE 16

RESULTS OF GLYCEROL GRADIENT CENTRIFUGATIONS

The glycerol gradients were run as described in **Materials and Methods**.

Closed circles represent RNA polymerase activity.

Open circles represent Gamma Globulin optical density.

Closed triangles represent Catalase activity.

RNA POLYMERASE

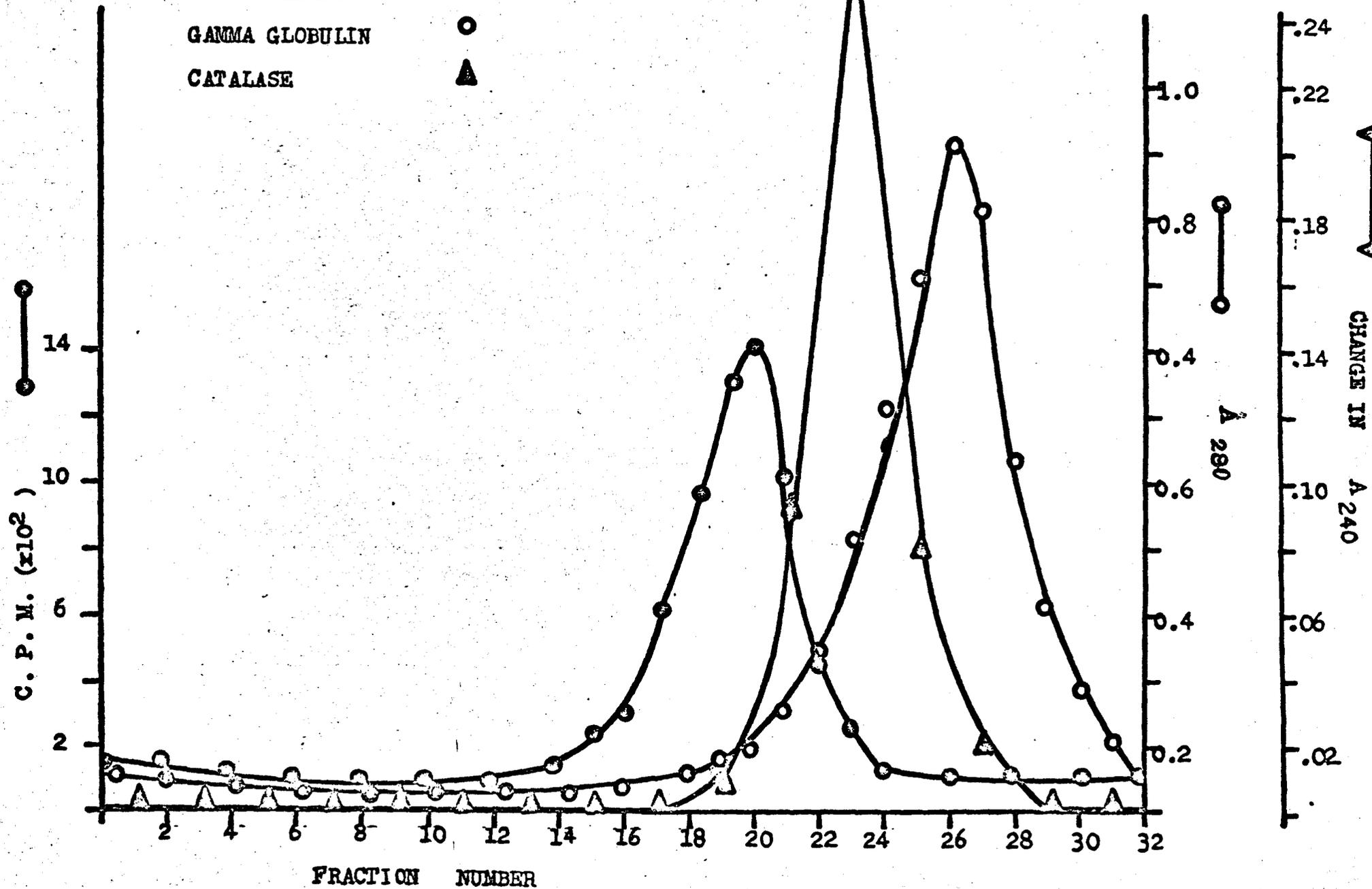
GAMMA GLOBULIN

CATALASE

●

○

▲



same rate as the halophile polymerase further confirming the similar molecular weights.

A lipid analysis was carried out on the isolated enzyme to determine if there was any possibility that the high molecular weight of the halophile polymerase was due to a lipid-protein complex. Lipid analysis of a G - 100 eluant showed the total protein content to be less than 5 μ gm. lipid/mgm. protein, or a lipid to protein ration of 1:200. This lipid content seems too low to form such a lipid-protein complex.

VII. DISCUSSION

The preceding described the isolation of a DNA-dependent RNA polymerase from the extreme halophile H. cutirubrum. Although the enzyme was not purified to homogeneity, it was isolated to a state where some interesting experiments could be done.

The enzyme functioned well at ionic strengths representative of non-halophilic bacteria. This is in contrast to a majority of halophilic enzymes (Baxter, 1956; Baxter, 1959; Holmes, 1963; Holmes, 1965 (a, b, c)). A problem, however arises when an attempt is made to assay the enzyme at high ionic strength. What could some of the reasons for this problem be? As the results have indicated, the DNA-membrane-protein complex is able to function well in high and low ionic strengths. It is only when the enzyme is freed from its endogenous template that the enzyme is no longer active in high salt.

If the enzyme undergoes an irreversible structural change when exposed to a low salt environment allowing it to maintain activity, but rendering it unable to be re-introduced to high ionic strength, then this could mean that the DNA template protects the enzyme from undergoing this structural change. The template bound enzyme is therefore stabilized and made resistant to wide variations in salt concentrations. This, however, does not explain the inability

of the enzyme to function at high ionic strength when never exposed to a low salt buffer as indicated in Section IV, Table VII.

Another consideration which may explain the enzyme's inactivity at high ionic strength is the possible loss of an initiation factor. If a soluble factor is present in halophiles as it is in E. coli, by collecting the DNA-membrane-protein complex and discarding the supernatant, the factor may be lost. However, addition of the 229,000 x g supernatant to a high salt assay did not result in the restoration of activity. The existence of such a factor, at least under present assay conditions cannot be confirmed.

There is a final possibility for the inability to recover enzymic activity at high ionic strength. The DNA of halophiles may be in a complex with a variety of proteins which enables the RNA polymerase to bind to the DNA and initiate protein synthesis. If as found for other halophilic proteins, the DNA-dependent RNA polymerase is acidic in nature, there may be electrostatic repulsion between enzyme and template. The proteins bound to the DNA may serve to cancel these repulsive forces and allow the enzyme and the DNA template to interact. If this speculation is valid, only DNA isolated from halophiles with bound protein intact could be used as a template in a high salt system. When such a DNA was used, background enzyme activity was too high to yield meaningful results. Another interesting feature of the enzyme system is the effect of

purine omission. A number of papers on the nature of the initiation site of RNA synthesis have been published (Bremer, 1965; Nishimura, 1965; Maitra, 1965; Senetec, 1968; Bremer, 1969). These studies indicate that the first ribonucleotide incorporated into an RNA chain is a purine, preferentially ATP. Consequently, the omission of a purine would have a far greater inhibitory effect than the omission of a pyrimidine since a purine could start an RNA chain while a pyrimidine could not. It was interesting to see that our results seemed to bear these data out. Also of interest is the effect of rifampicin and streptovaricin in relation to the effect of actinomycin D and acriflavin on the activity of the enzyme. Rifampicin and streptovaricin are ineffective. In conventional bacterial systems these inhibitors act by binding to the core enzyme to prevent the initiation of RNA synthesis. The inability of these inhibitors to inhibit enzyme activity in the halophilic system seems to indicate a difference in enzyme structure compared to the E. coli enzyme. Actinomycin D and acriflavin act by binding the template and a difference in enzyme structure would not be of significance in their inhibitory effect.

By G - 100 Sephadex chromatography in both low and high ionic strength, the enzyme was found to be of a molecular weight greater than 100,000. By G - 200 Sephadex chromatography, the enzyme was found to have a molecular weight in the region of 400,000 to 500,000. Glycerol gradients confirmed the molecular weight to

be of approximately the same magnitude as the conventional E. coli RNA polymerase, about 400,000 to 500,000. This is not surprising because the enzyme from both systems must carry out the same highly specific well controlled synthesis of RNA.

Recent publications by Louis and Fitt (1971 (a) and (b)) report the isolation of a DNA dependent RNA polymerase from an extreme halophile. The enzyme preparation described here has a number of properties different from the Louis and Fitt isolated enzyme. The Louis and Fitt enzyme has a molecular weight of about 20,000, while the enzyme described here has a molecular weight of about 500,000. This much larger molecular weight is consistent with the molecular weights reported for non-halophilic polymerases. Louis and Fitt sonicated their preparation in the course of their extraction procedure. In the preparation of our extract, it was found that sonication, even for very short periods, decreased enzyme activity by 70 - 80% in a crude cell lysate. With the gentle procedures used in the preparation of our enzyme, such damage is far less likely.

Both enzyme preparations require the addition of DNA for activity. Louis and Fitt could not, however, find enzyme activity when denatured DNA was used as template. This is in contrast to our results which showed denatured DNA to be a more effective template than native DNA (cf. Table IV).

Louis and Fitt also found differences in the enzyme's response to divalent cations. Increasing concentrations of manganese increased enzymic activity to the point where further addition of manganese caused a precipitate in the incubation mixture. In our studies a definite peak in activity was found (cf. Figure 7), and manganese could not replace magnesium as the divalent cation without decreased enzyme activity (cf. Table V).

Unlike the enzyme system described here, where at high concentrations of substrate a plateau in enzyme activity is reached (cf. Figure 5), the activity of Louis and Fitt's enzyme was markedly inhibited by increasing substrate.

It would therefore seem that the enzyme isolated by the methods described here is the more likely candidate for a comprehensive study in comparing the features of halophilic DNA-dependent RNA polymerase to conventional DNA-dependent RNA polymerase.

VIII. CONCLUSION

An enzyme has been isolated and studied from the extreme halophile Halobacterium cutirubrum which catalyzes the synthesis of RNA. The enzyme meets all the criteria for classification as a DNA-dependent RNA polymerase. The enzyme shares many features with its counterparts in non-halophilic bacteria, e.g., molecular weight, substrate requirements and divalent cation requirements. There are some differences displayed by the halophilic enzyme as well, e.g., the effect of specific inhibitors.

What, however, is yet to be investigated, are the factors required to promote enzymic activity in high ionic strength and further purification methods. Such a study may provide a substantial contribution to understanding adaption of enzymes to conditions of high ionic strength.

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