DNA-DEPENDENT RNA POLYMERASE FROM NUCLEI OF ERYTHROBLASTS OF REGENERATING CHICKEN BONE MARROW

A THESIS PRESENTED TO
THE DEPARTMENT OF BIOCHEMISTRY
THE FACULTY OF GRADUATE STUDIES AND RESEARCH
OF THE
UNIVERSITY OF MANITOBA

SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By
MICHAEL LAWRENCE LEROUX
1967

To my wife

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. Esther V.W. Yamada for her excellent instruction.

I would also like to thank Miss Joanne Campbell for her skill and patience in typing this thesis.

TABLE OF CONTENTS

INTRODUCTION

Prior to 1955 the biosynthesis of nucleic acids was studied by following the rate of incorporation of radioactive precursors such as inorganic phosphate-P^{32}(1), adenine-8-C^{14}(2) and orotic acid-C^{14}(3) into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). It was not until 1955 that an enzyme, polynucleotide phosphorylase, capable of catalyzing the formation of RNA-like polymers from nucleoside-5'-diphosphates was discovered (4). Later, however, it became apparent that the physiological role of this enzyme in living cells was a degradative role rather than a biosynthetic role for the following reasons: (a) DNA was not required as a template for this enzyme and hence this enzyme could not function in the transcription of genetic information from DNA to RNA (5); (b) the composition of the product of the reaction catalyzed by this enzyme varied with the concentration of the substrates and no template-copying mechanism was involved (5, 6); (c) the equilibrium constant of the reaction and the high concentration of inorganic phosphate usually present in cells made it apparent that <u>in vivo</u> the reaction probably proceeds in the direction of the phosphorolysis of RNA (7). In addition the affinity of the nucleoside diphosphates for this enzyme was very low (Km's 0.02M) (8).

In 1959, Weiss and Gladstone (9) reported an RNA-synthesizing enzyme in rat liver that required adenosine-5'-triphosphate (ATP), cytosine-5'-triphosphate (CTP), uridine-5'-triphosphate (UTP) and guanosine-5'-triphosphate (GTP), and the presence of magnesium ions for maximal activity; it was inhibited by pyrophosphate but not by orthophosphate. The product was susceptible to hydrolysis by alkali or in the presence of pancreatic ribonuclease (RNAase I). Weiss (9, 10) further showed that deoxyribonuclease (DNAase), as well as RNAase, inhibited the reaction and the ribonucleoside triphosphates were incorporated internally throughout the polynucleotide chain. Similar findings were reported by Hurwitz et al (11) and Stevens (12) for enzyme preparations from cell-free extracts of E. coli and by Bonner et al (13) for an enzyme preparation from pea embryos. The name DNA-dependent RNA polymerase was given to this enzyme when it was found to lose all activity when DNA was omitted (14, 15) from the incubation medium and, when DNA was included in the incubation medium, to catalyze the synthesis of RNA which resembled DNA in base composition (14, 15) and nearest-neighbour base frequencies (16, 17), even when many types of DNA, from both mammalian and bacterial sources, were used. It was apparent from these experiments that DNA was acting in the role of a template. This was

confirmed when hybridization experiments (18, 19) showed the product RNA to have long sequences of nucleotides complementary to both strands of its template DNA. This evidence and the similarity in the nearest-neighbour base frequencies of the RNA product and DNA template indicated that both DNA strands were copied under these in vitro conditions (17, 18). However, RNA synthesized after infection of bacteria with ØxI74 bacteriophage (20) or bacteriophage α (21) was found to hybridize with only one strand of bacteriophage DNA, indicating that only one strand of DNA was transcribed into RNA in vivo. Later, RNA synthesized in the presence of a partially-purified preparation of RNA polymerase was found to be single stranded when intact circular DNA (22) or DNA in its native conformation (23) were used as templates. Other evidence indicated that the nature of the product RNA does not depend on the state of purification of the enzyme but rather on the size and secondary structure (24, 25) of the template DNA. Double-stranded native DNA was showed to act catalytically (17, 26).

The concept of messenger RNA (mRNA) was introduced in 1961 (27). Jacob and Monod postulated, from induction-repression experiments with E. coli, that a specific polyribonucleotide (mRNA), with a rapid turnover rate, was transcribed from a DNA

template and carried the information for protein synthesis to the 70 S ribosomes. Their hypothesis was supported by the experiments of Brenner et al (28) who showed that phage-induced RNA appeared on stable, pre-existing ribosomes. A rapidly labeled RNA fraction, in low concentrations, that differed in base composition from ribosomal RNA but was similar in base composition to DNA was isolated from normal microorganisms (29). Further convincing support was provided by Nirenberg and Matthaei (30) who demonstrated a stimulation of protein synthesis when crude preparations of RNA, presumably containing mRNA, or even synthetic polyribonucleotides were added to ribosomes pretreated to remove endogeneous mRNA. Wood and Berg (31) and Ning et al (32) showed a stimulation of protein synthesis when double-stranded native DNA and RNA polymerase were added to a cell-free system that was capable of catalyzing protein synthesis. When denatured DNA replaced native DNA (31) a non-functional RNA product of low molecular weight was formed which did not stimulate protein synthesis.

Hybridization experiments (33-36) have shown that all intracellular RNA is probably synthesized on DNA templates. That intracellular RNA does not serve as a template for RNA synthesis was demonstrated by several workers (38-40) studying template

specificity in *in vitro* systems. These workers found that synthetic RNA's could serve as templates to some limited extent (38-40). However, studies of initial velocities showed that natural and synthetic polyribonucleotides inhibit transcription of RNA from DNA *in vitro* (39, 26, 42) and DNA inhibits transcription of RNA from polyribonucleotide templates (39, 43). Since increasing concentrations of DNA overcame the inhibition by RNA and vice versa, it was suggested that RNA and DNA were competing for the same binding sites on the enzyme and hence were competitive inhibitors (42, 43, 44). However, Gumport, Fox and Weiss in subsequent work (44) demonstrated that the order of addition of DNA, RNA and RNA polymerase to the incubation mixture determined the degree of inhibition. RNA polymerase was showed to form an essentially irreversible complex with either DNA or RNA. If the enzyme was preincubated with DNA and then RNA added, no inhibition resulted. If RNA was present along with DNA and then RNA polymerase was added some inhibition resulted, whereas if RNA was preincubated with enzyme, little RNA was synthesized when DNA and cofacters were added. Since the initial rate of RNA synthesis was unaffected if RNA was added to the incubation medium immediately after RNA synthesis had begun, Fox, Gumport and Weiss concluded

that it was unlikely that the endogeneous RNA could compete with DNA for enzyme-binding sites. However, they did not exclude the possibility that, in vitro, failure of the RNA product to dissociate from the DNA-cRNA-enzyme*complex might be related to the decreased rate of RNA synthesis with time with resultant deviations from linear rates. Berg et al (45) also concluded that RNA polymerase forms a poorly dissociable complex with DNA since this complex would not serve as a template for replication by DNA polymerase or as a substrate for exonucleases I, II or III. They further concluded that since a relatively poorly dissociating complex was formed "pseudo-competitive inhibition" rather than competitive inhibition was occurring between DNA and RNA for the enzyme. Krakow (46) investigated the problem further using nucleoside triphosphates labeled with P^{32} in the γ -phosphate group and followed RNA synthesis from a DNA template by measuring the release of radioactive pyrophosphate. He found that if RNAase I was added to the incubation mixture after initial velocities were obtained and then fell off, RNA synthesis began again and approached linearity. He interpreted this to indicate that the RNA product did in fact inhibit the reaction and postulated that the inhibition resulted from the RNA chain increasing in length until it occupied

---

* cRNA-enzyme - complementary RNA

two binding sites on the enzyme, an RNA-synthesizing site and a template DNA binding site, at which point RNA synthesis ceased.

Although early reports indicated the presence in bacterial and plant cells of naturally occurring DNA-RNA hybrids (47, 48), it was not possible to isolate such a complex from RNA polymerase systems in vitro until in 1964, Bremer and Konrad (49) showed that such a complex could be isolated if the incubation mixture containing DNA,RNA polymerase and RNA product was not deproteinized prior to sedimentation in sucrose gradients. They postulated that the DNA-RNA complex was held together by RNA polymerase and showed that the RNA chain lengthened while on the DNA template.

It was found in all experiments in which the nature of the template was investigated that the RNA product resembled the template in such a way that a hydrogen-bonding, base-pairing mechanism for template copying, similar to that indicated by Kornberg et al (50) for DNA synthesis was indicated. Support for this came from the work of Kahan and Hurwitz (51) who substituted base analogues for the natural substrates and found them to be incorporated as long as the 6- keto or amino groups, the groups involved in hydrogen bonding, were left intact. The fact that homopolymers were formed when only

one nucleoside triphosphate was included in the incubation medium (26, 37, 53) was disturbing. However, Chamberlin and Berg (52) presented good evidence that homopolymer formation occurred by a mechanism of reiterative replication in which the template was a short sequence of one base in DNA and the product was a growing chain containing the complementary base. They reported that homopolymer formation also requires some denaturation, at least at the ends, of helical DNA. The presence of very small concentrations of the other nucleoside triphosphosphates ($< 2 \times 10^{-6}$M) inhibited homopolymer formation.

Since its discovery in 1959 in rat liver (9), although DNA-dependent RNA polymerase has been detected in a variety of tissues (11, 13, 53), only the enzyme from bacteria has been purified and characterized extensively (52, 54, 55). In 1964, two DNA-dependent RNA polymerase activities were reported to be present in animal tissues (56) and were thought to represent the activities of a "soluble" (57) and an "aggregate" enzyme (44, 58). The activity of the "aggregate" enzyme of rat liver nuclei was unmasked when ammonium sulphate or detergents were included in the reaction medium (44, 56, 10, 69). A much greater and more consistent stimulation of the "aggregate" enzyme was obtained

by increasing the ionic strength of the reaction mixture to between 0.7 and 1.1 by the addition of either KCl or ammonium sulfate (AmS) (69). On the other hand, "soluble" enzyme activity is inhibited at these high ionic strengths (57, 70) but at least one preparation of soluble enzyme is stimulated by ionic strengths of between 0.1 and 0.3 (70).

The "soluble" enzyme of rat liver nuclei catalyzes the synthesis of RNA whose base composition resembles that of DNA (56, 58), whereas the aggregate enzyme, in a reaction medium of low ionic strength, catalyzes the synthesis of RNA of the high GC-type (ribosomal RNA) but in a reaction medium of high ionic strength, the product RNA is again shifted to the DNA-like type in base composition. There is now, however, good evidence that the "soluble" and "aggregate" enzymes are in fact two forms of the one enzyme (58). The soluble enzyme of E. coli or of rat liver nuclei was showed to be readily converted into the "aggregate" form by the addition of deoxynucleohistones to the reaction mixture and there was a corresponding change in the base composition of product RNA from the DNA-like type to the high GC-type. Furthermore, in reaction medium of high ionic strength this enzyme-deoxynucleohistone complex catalyzed the formation of product RNA of the DNA-like type.

In this thesis studies of the activity of RNA polymerase of immature red blood cells of regenerating avian bone marrow are described. The immature red blood cells were separated by centrifugation in discontinuous dextran gradients. The fractions obtained contained up to 85% erythroblasts. Nuclei from these cells were prepared after homogenization in a French pressure cell. Initial studies of the intact nuclei indicated that they contain both "soluble" and "aggregate" forms of RNA polymerase. Since several workers have used detergents to lyse isolated nuclei and nuclear fractions (59, 69) it was of interest to prepare sodium dodecyl sulfate extracts of the erythroblast nuclei and to characterize the RNA polymerase contained therein. On the basis of kinetic studies and the effects of activators it is concluded that the RNA polymerase of the sodium dodecyl sulfate (SDS) extracts is mainly if not entirely in the "aggregate" form. This method of preparing the "aggregate" enzyme is much less time consuming and tedious than that outlined by Weiss (44) and Goldberg (69).

EXPERIMENTAL PROCEDURE

Materials

Labeled ribonucleoside triphosphates were obtained from Schwarz BioResearch and unlabeled ribonucleoside triphosphates from Sigma Chemical Company. Highly-polymerized calf thymus DNA and deoxyribonuclease I (ribonuclease-free) and ribonuclease I·A were purchased from Mann Research Laboratories. Highly-refined dextran (average molecular weight 80,000) was obtained from either Mann Research Laboratories or Pharmacia of Canada. BBOT (2, 5-bis-(2-(5-tert-Butylbenzoxazolyl))-Thiophene) was purchased from the Packard Instrument Company. Glass membrane filters (984 H ultrafilters) were obtained from H. Reeve Angel and Company.

Separation of Erythroblasts

To obtain regenerating bone marrow, white Leghorn chickens (line 15, East Lansing) were treated with phenylhydrazine as described previously (61). The chickens were decapitated and the bone marrow removed from the leg bones and weighed. All subsequent operations were carried out at 2°. The bone marrow was washed with calcium-free Hank's balanced salt solution and suspended in 4 volumes of Hank's + 10% chicken serum. The bone marrow cells were dispersed by forcing the suspension through a

syringe, then through a stainless wire gauze (No. 60 mesh) and were then filtered through 2 layers of cotton gauze. Density gradients were prepared in 50 ml centrifuge tubes.

In the course of the work both continuous density gradients, linear from 5% to 15% (w/w) dextran and discontinuous density gradients of dextran dissolved in calcium-free Hank's solution were prepared. The latter were prepared by the successive layering of 10 ml of 17.5% (w/w) dextran, 10 ml of 15% (w/w) dextran and 20 ml of 5% (w/w) dextran. Four ml of the bone marrow suspension were then layered on top of each gradient and the tubes were centrifuged in a swing-out bucket rotor (HB4) in a Servall refrigerated centrifuge for 5 minutes at 750 r.p.m. (120 x g) and then for 10 minutes at 3000 r.p.m. (1465 x g). Four layers of cells were visible after centrifugation in linear density gradients and three layers in the discontinuous density gradients. The cell composition of these layers from typical separations are given in Table I. It is apparent from this Table that separation of cells by discontinuous gradients gives cell fractions containing up to 85% erythroblasts whereas separation by linear density gradients did not achieve this purity. In other experiments separation techniques used by others (62, 63) were

tried but with limited success and poorer separation than that reported in Table I.

Thus, in all subsequent experiments the bone marrow cells were separated by the discontinuous density gradient technique. After centrifugation the bottom two layers from the gradients were aspirated and diluted with several volumes of calcium-free Hank's solution. The suspension was centrifuged at 2700 r.p.m. (900 x g) for 10 minutes at 0° and the pellet was washed once with a large volume of Hank's solution. The cells were then suspended in 2 volumes of 0.25 M sucrose containing 0.001 M $MgCl_2$ and 5 m M β-mercaptoethanol and were precipitated by spinning at 2700 r.p.m. (900 x g) for 5 minutes.

Although a purity of 85% erythroblasts has been achieved in our preparations they still contain a significant number of mature erythrocytes. It has been reported, however, that nuclei of mature erythrocytes contain little or no RNA polymerase activity (64) and so the presence of these cells in our preparations will affect the values for specific activity but not for total activity.

Nuclear Enzyme Preparations

The cell fractions obtained from the dextran gradients were resuspended in 10 volumes of homogenizing solution (0.25M sucrose containing 0.001M

Mg^{++} acetate+0.005 M β-mercaptoethanol) and broken in a French pressure cell at 800 lbs/in^2. The nuclei were spun down at 900 x g for 5 minutes washed once with the homogenizing solution and suspended in a solution of 0.05 M TRIS-HCl pH 7.0 + 0.005 M β-mercaptoethanol. The protein concentration was determined and the volume of the nuclear fraction adjusted to contain 10 mg protein/ml. Sodium dodecyl sulphate (SDS) was then added and the preparation allowed to stand for 10 minutes. The detergent-nuclear fraction was spun at 12,000 x g for 10 minutes and the supernatant fraction removed and used as the source of enzyme.

Several experiments were done to determine the concentration at which greatest solubilization of the enzyme from intact nuclei was achieved. To aliquots of the enzyme preparation, equal volumes of detergent containing graded concentrations of SDS were added. As can be seen from Table II little enzyme activity is released until final concentrations of 0.5mg and 1.0mg of SDS/ml are reached. Beyond this range a gelatinous solution was obtained that could not be centrifugated or pipetted. Maximal recovery of the enzyme activity was 64% although the specific activity was usually increased 3-4 fold. When nuclei were lysed by freezing overnight and then treated with SDS, similar results were obtained (Figure 1). The SDS-enzyme preparation was stored

at -15°. The activity fell gradually and after storage for 10 days became negligible.

Some nuclei were prepared by homogenizing the cells with 6-10 strokes of a Teflon pestle in a Potter-Elvehjem apparatus. However, nuclei prepared after homogenization in a French pressure cell were found to be much cleaner of cytoplasm.

A method of preparing nuclei by lysing the cells with saponin as described by Hammel and Bessman (65) for avian erythrocytes, was also experimented with. A solution of 1% saponin dissolved in 0.25M sucrose plus 0.004M $MgCl_2$ was added to the cell preparation in the ratio of 1 ml saponin solution/ 1 ml packed cells. The cells were then gently re-suspended and allowed to sit in the detergent for 5 minutes. Treatment of the cells by this method resulted in incomplete cell lysis and this method was not used further.

Assay of RNA Polymerase Activity

Enzyme activity was assayed by determining the rate of incorporation of ATP-H^3 or ATP-8-C^{14} into acid-insoluble RNA. The reaction mixture contained the following in a final volume of 0.25ml; Tris buffer, pH 7.4, 25 μmoles; pyruvate kinase, 2.5μg; phosphoenol pyruvate, 2.5 μmoles; DNA, 25 μg; magnesium acetate,

1.25 μmoles; UTP, CTP and GTP, 30 mμmoles of each; β-mercaptoethanol, 1.25 μmoles; ATP-H^3, 25 mμmoles (1.10×10^6 disintegrations per minute, 15.5% efficiency) or ATP-8-C^{14} (2.22×10^5 disintegrations per minute, 70% efficiency) and enzyme. The final pH of the reaction mixture was 7.5. The reaction was started by the addition of nucleoside triphosphates. Unless otherwise stated the incubation time was 5 minutes at 37° and the reaction was stopped by the addition of 2 volumes of 10% trichloroacetic acid (TCA, w/w) and carrier albumin (1mg/ml) to bring the protein concentration per tube to between 1 and 2 mg. The precipitate was collected on glass membrane filters and was washed 10 times with 5% TCA containing 0.02 M tetrasodium pyrophosphate and once with 95% ethanol. All tests were run in duplicate along with zero time controls to which the radioactive ATP was added just prior to the addition of TCA. The filters were placed in vials containing 15 ml of BBOT scintillation solution* and the radioactivity was measured in a Packard Tri-carb liquid scintillation spectrometer. A unit of enzyme activity is defined as that amount of enzyme that catalyzes the incorporation of 1 μμmole of radioactive ATP into RNA per hour. All counts of radioactivity were corrected to 100%

---

*4 g of BBOT, 400 ml of methylcellosolve, 80 g of naphthalene and 600 ml of Xylene.

efficiency. Specific activity is defined as the enzyme units/mg of protein. Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (66). Glass membrane filters were found to give better results than Gelman or Millipore filters. Bovine serum albumin was found to serve as a better carrier of acid-insoluble RNA than RNA or RNA plus albumin (Table III).

RESULTS

Time Relationship

Figure 2 illustrates the time-course of the reaction when whole nuclei, either fresh or frozen overnight, were used as the source of the enzyme and KCl, AmS and spermine were omitted from the incubation medium. It is apparent that initial rates obtain only for the first 5 minutes of incubation. The time-course of the reaction when KCl and AmS are included in the incubation medium is given in Figure 3. The rate of RNA synthesis is again linear for the first 5 minutes of incubation but, unlike the findings shown in Figure 2, does not level off at this time but rather another linear rate is established. These data are consistent with those of Tata (56) for time studies of rat liver nuclei and indicate that both "soluble" and "aggregate" forms of RNA polymerase are present in nuclei of erythroblasts.

The time-course of the reaction when SDS-extracts were used as the source of the enzyme without the addition of KCl, AmS or spermine are given in Figure 4. Again one enzyme activity is apparent which reaches a maximum at 8-10 minutes. When KCl and AmS are included in the incubation medium (Figure 5) a similar time-course of the

reaction appears although the high ionic strength of the reaction medium has stimulated the rate of RNA synthesis 3-4-fold. The time studies with the SDS-extracts resemble those found by Weiss (44) and Goldberg (69) for aggregate enzyme preparations of rat liver nuclei and HeLa cell nuclei, respectively, in the presence or absence of salts. With the SDS-extracts, however, after incubation for 10 and 15 minutes enzyme activity decreased markedly and product RNA was lost. To see whether bentonite, an inhibitor of nuclease activity (60) could prevent this loss of product RNA, several concentrations of bentonite, prepared by the procedure of Singer et al (67) were included in the reaction mixture. No significant change in the rate of incorporation was detected in the presence of bentonite for although total radioactivity of the product appeared to increase, the radioactivity of the zero time controls also increased. Moreover, the duplicates were inconsistent (Table IV). These results might possibly be explained by the finding of Richmond and Cherry (68) who reported that bentonite binds some of the acid-soluble, labeled product, thus producing an artifact which appears to be an increase in RNA synthesis. Thus bentonite was not used.

Activity and Enzyme Concentration

Figure 6 shows that there is a linear relationship between enzyme activity and protein concentration up to 240 enzyme units (1.7 mg of protein) when whole nuclei were used as the source of the enzyme. When SDS-extracts of these nuclei were tested the linear relationship, shown in Figure 7 obtained up to 482 units of enzyme (0.12mg of protein). These results are in line with those of Tsukada and Lieberman (59) who reported that linear rates obtained for a deoxycholate extract of rat liver nuclei up to 0.11 mg of protein.

Extraction of RNA Polymerase with Sodium Deoxycholate

In some experiments erythroblast nuclei were treated with graded concentrations of sodium deoxycholate (69). The sodium deoxycholate-extracts(DOC) obtained after centrifuging at 12,000 x g for 10 minutes were then tested for RNA polymerase activity. The results are given in Table V and show that it is possible to obtain DOC-preparations in which the specific activity is increased more than 2-fold. However, higher concentrations of DOC than SDS are required to achieve this purification. Furthermore, in our hands, the enzyme activity after DOC extraction was variable. Thus in all subsequent experiments the erythroblast nuclei were extracted with SDS and the

properties of the SDS-extracts are described.

Substrates and Inhibitors

The enzyme activity was DNA-dependent for when DNAase I was included in the reaction mixture, activity was greatly reduced (Table VI). When RNAase I was included in the reaction mixture there was again a marked reduction in measurable acid insoluble product. The concentrations of DNAase and RNAase required to inhibit the activity of the SDS-extracts are 1/4 to 1/10 that required to inhibit the activity of liver nuclei (71) or that of deoxycholate extracts of rat liver nuclei (59). On the other hand they are approximately 8 times those required to inhibit the activity of "soluble" RNA polymerase of rat testis (70).

Actinomycin D, an inhibitor of DNA-dependent RNA polymerase (72) completely inhibited the activity of the enzyme from erythroblasts at a final concentration of 0.1 μmolar (Table VI).

As can be seen from Figure 8 the addition of ribonucleoside triphosphates resulted in little stimulation of activity and thus a K_m could not be determined. Attempts to remove endogeneous nucleoside triphosphates by dialysis resulted in the loss of most of the enzyme activity.

Addition of highly-polymerized calf thymus DNA

to the incubation medium resulted in some slight stimulation of enzyme activity (Table VII). Others have reported that the aggregate form of RNA polymerase of rat liver nuclei is not stimulated by the addition of DNA to the reaction medium (10, 59) while the reverse is true for the soluble form of the enzyme (57).

Activation by Mg^{+2} and Mn^{+2}

When magnesium ions, in a final concentration of 5 mM, were included in the reaction mixture enzyme activity was increased almost 3-fold (Figure 9). Addition of manganese ions resulted in a 2-fold increase in enzyme activity with maximal stimulation at a concentration of 2 mM (Figure 10). Thus magnesium ions caused a greater stimulation of enzyme activity than did manganese ions. This finding was verified in several other experiments. Addition of magnesium and manganese ions together, at optimal concentrations of each, resulted in inhibition of enzyme activity. These studies were carried out in reaction medium lacking KCl, AmS or polyamines. Lineweaver - Burk plots of these data are given in Figure II. Because of the inhibitory effect of both Mg^{+2} and Mn^{+2} at high concentrations, it was necessary to extrapolate the plots to give the intercepts on the Y-axis. From these data the Michaelis-Menten dissociation constants (Km's) were calculated

by using Δv values (i.e. initial velocities with Mg^{+2} present - initial velocities without Mg^{+2}) since the enzyme preparations contain some Mg^{+2} and it was not possible to show an absolute dependence of enzyme activity on the divalent cation added to the incubation mixture. Thus Km values were found to be 5.0×10^{-3}M for magnesium ions and 1.7×10^{-3}M for manganese ions. These values can be compared to those of 4.8×10^{-3}M and 2.0×10^{-3}M found for magnesium ions and manganese ions, respectively, with "soluble" RNA polymerase purified from bacteria (73).

Effect of Polyamines and Ionic Strength

Between 2 and 3-fold stimulation of enzyme activity was obtained at spermidine concentrations of 1 mM (Figure 12). Addition of KCl alone also resulted in 2-fold stimulation of enzyme activity (Figure 13). At optimal concentrations of KCl (0.3M) both ammonium sulfate or spermine (Table VIII) increased the activity further, AmS causing a 1.5-fold and spermine a 3.5-fold increase in activity. When KCl and AmS were both present in optimal concentrations the ionic strength of the reaction mixture was 0.93.

pH Optimum

Optimal enzyme activity was found at pH 7.5-7.6 (Figure 14). It is rather difficult to compare this optimum pH with those in the literature since others have used a variety of pH's and in many instances pH-activity curves were not described. With purified preparations of RNA polymerase (soluble form) from E. coli, Chamberlin and Berg assayed enzyme activity at pH 7.9 (37), Stevens and Henry, at pH 8.5 (74), and Hurwitz et al at pH 7.5 (17). Furthermore, a number of workers (75, 76) assayed RNA polymerase activity (soluble form) of Micrococcus lysodeikticus at pH 7.5 whereas Ballard and Williams-Ashman (70) used a pH of 7.7 to assay the soluble RNA polymerase of rat testis. When testing the activity of the aggregate form of the enzyme Weiss (9) used a pH of 8.0 and Tata a pH of 8.5 (56).

More recent findings indicate that the pH optimum of RNA polymerase will be close to 7.5 when reaction mixtures of high ionic strength are used but will be greater than 7.5 when reaction mixtures of low ionic strength are used (77). Thus in assays of soluble RNA polymerase a wide pH optimum is found in the range of 6.8 to 7.9 and losses of activity at the extremes of the optimum pH range only amount to 20%. Widnell and Tata (56), in assays of aggregate RNA polymerase in a reaction mixture of high ionic strength, reported that the pH optimum is close to 7.5.

DISCUSSION

What may be considered to be one disadvantage of working with SDS-extracts is the finding that the time-course of the reaction after it reaches a plateau falls off quite rapidly after 15 minutes of incubation whereas when aggregate enzyme preparations (44,69) are used this decrease is not found. However, Tsukada and Lieberman (59) and Burdon and Smellie (78) reported similar activity-time curves when DOC-extracts of rat liver nuclei and crude preparations of RNA polymerase from Ehrlich ascites carcinoma cells, respectively, were used. The latter attributed the observed decrease in enzyme activity with time to the action of nucleoside di- and triphosphatases and nucleases. It is not likely that the phosphatases are causing the observed decrease in incorporation of radioactive substrate into RNA in our studies since saturating amounts of the nucleoside triphosphates are being used and an ATP-regenerating system is also included in the reaction medium. Since our experiments with bentonite were inconclusive it is not possible to say at this point whether the product RNA is being lost because of release of nucleases during the incubation or because of phosphodiesterases, which are known to be active in avian bone marrow cells and to be present

both in the cytoplasmic and nuclear fractions (61).

The concentration of SDS required for optimal release of the enzyme from erythroblast nuclei is approximately the same as that reported by Goldberg to effect optimal stimulation of aggregate enzyme of HeLa cell nuclei when added to the incubation medium (69). Goldberg (69) postulated that detergents may act to stimulate RNA formation by changing the physical state of the enzyme-nucleoprotein complex to a form more suitable for reaction and/or by stabilization of this system or the product of the reaction by inhibiting the activity of degradative enzymes. Although some evidence was cited in favour of the first hypothesis, the results are still equivocal and the actual role of detergents in stimulating RNA synthesis by the aggregate form of the enzyme is not known. However, in our studies, a more likely role of detergents is probably in lysing nuclear membranes and releasing aggregate enzyme while denaturing some of the non-enzyme protein. It is apparent from our studies that such lysis is incomplete at the optimal concentrations of SDS possible and further lysis could not be achieved because of the formation of very viscous gels of deoxyribonucleoprotein.

Since addition of DNA resulted in little stimulation of the activity of SDS-extracts of

erythroblast nuclei it is probable that the endogenous DNA is present in saturating concentrations, probably as a result of solubilization of the deoxynucleoprotein by detergent, both in our preparations and in those studied by Tsukada and Lieberman (59).

The activity of SDS-extracts of RNA polymerase was greater in the presence of Mg^{+2} than Mn^{+2} in reaction medium of low ionic strength. In general Mn^{+2} has been found to be more effective than Mg^{+2} in the activation of highly-purified RNA polymerase of bacteria. However, in initial studies of the aggregate form of the enzyme of rat liver, Weiss found Mg^{+2} to stimulate activity more than Mn^{+2} and our results are in line with those of Weiss, indicating the presence of aggregate enzyme in our SDS-extracts. On the other hand, Goldberg (69) reported that Mn^{+2} was about 4 times more effective than Mg^{+2} in stimulating the activity of the aggregate form of RNA polymerase of HeLa cell nuclei. These differences were later resolved when Widnell and Tata (56) reported that in reaction media of low ionic strength i.e. lacking ammonium sulfate or KCl, Mg^{+2} was more effective than Mn^{+2} in stimulating enzyme activity of liver nuclei whereas in reaction media of high ionic strength, Mn^{+2} is the divalent cation of choice. These results agree with those reported by Pogo, Allfrey and Mirsky (71). If, as is

suggested by the work of Chambon, Ramuz and Doly (58), the presence of salts in high ionic strength removes the inhibitory histones from the DNA-RNA polymerase-cRNA* complex to form the soluble enzyme form, one would expect that when soluble enzyme preparations are used then Mn^{+2} would be a better activator than Mg^{+2}. This conclusion is borne out by the findings with highly-purified, soluble RNA polymerases of bacteria (3741). In addition, Ballard and Williams-Ashman (70) have reported that Mn^{+2} is the better activator of soluble RNA polymerase of rat testicular tissue and similar findings were reported by Furth and Ho in studies of the soluble enzyme of bovine lymphosarcoma (53). Many workers still, however, routinely add both Mn^{+2} and Mg^{+2} to incubation media even if the soluble form of the enzyme is under study. The rationale for this is presumably that in some preparations denatured, or sheared double-stranded DNA templates could be present and it is known that with such templates Mn^{+2} stimulates the incorporation of ribonucleoside triphosphates whereas Mg^{+2} is inhibitory. Such incorporation in the presence of Mn^{+2} could lead to some homopolymer formation (79). Moreover it has been found that Mn^{+2} is a better activator of RNA polymerase in the formation of polyribonucleotides

---

*cRNA- complementary RNA

from RNA templates (42).

The polyamines, spermidine and spermine, have been found in many tissues, including liver (80, 81) and have been shown to have the ability to stimulate protein (82) and RNA synthesis in vitro (83). Polyamines are also thought to play a role in stabilizing cellular organelles, membranes, nucleic acids, and nucleoprotein particles (84). In regenerating rat liver there is a direct correlation between the rate of synthesis of RNA and the concentration of polyamines (83). A direct correlation between the extent of accumulation of polyamines and the rate of synthesis of nucleic acids has also been reported for the developing chick embryo (85).

All the evidence so far gathered indicates that polyamines exert their effect on protein and RNA metabolism mainly by stimulating the activity of RNA polymerase. The studies of Fox, Gumport and Weiss (44) with purified enzyme from Micrococcus lysodeikticus showed that polyamines either prevent the inhibition of RNA polymerase by endogeneous RNA by preventing the binding of the enzyme to RNA or else cause the release of inhibitory RNA from the DNA-RNA polymerase complex. Krakow (46), who studied the properties of a purified RNA polymerase of Azotobacter vinelandii is in favour of the former view. His results indicate that product RNA which accumulates

and causes a decrease in the rate of synthesis of further RNA in a medium lacking polyamines, does not become bound to the DNA-RNA polymerase complex and does not inhibit the rate of reaction in a medium containing polyamines. That product RNA in the presence of polyamines does not become bound to and inhibit the enzyme complex was showed in experiments in which the release of radioactive pyrophosphate from ATP-γ-P^{32} was measured in the presence or absence of polyamine with or without the addition of pancreatic RNAase. Pancreatic RNAase stimulated the release of PP^{32} in the absence of polyamine indicating that if inhibitory product RNA is removed the reaction can proceed further at initial rates. Addition of polyamines had an effect similar to that of RNAase but little added stimulation was found when RNAase in addition to polyamines was also included in the incubation medium. In addition Fox and Weiss (26) showed that polyamines inhibit homopolymer formation and the synthesis of RNA-directed polyribonucleotides (42). Since all the above studies of the effect of polyamines on RNA polymerase were carried out with soluble enzyme preparations it is probable that polyamines may have additional effects on the aggregate form of the enzyme. In this regard it has been reported that polyamines reverse the inhibition of RNA polymerase

by histones (96).

Stimulation of RNA synthesis by KCl and AmS has been reported by a number of workers. Goldberg (69) reported stimulation of RNA synthesis by an aggregate enzyme of HeLa cell nuclei when KCl was added at an ionic strength of approximately 0.7 or AmS, at an ionic strength of 1.1. He suggested that detergents and high ionic strength act to stimulate RNA synthesis by the same mechanism. In our studies maximal stimulation of activity was obtained at an ionic strength of 0.93. This value is close to that found by Goldberg for his preparation of aggregate enzyme and more than three times the 0.1 to 0.3 ionic strengths found to stimulate soluble RNA polymerase of rat testis (70). The activity of this and other soluble forms of RNA polymerase are inhibited by ionic strengths of 0.7 to 1.1 (70).

The mode of action of ammonium sulfate , KCl and polyamines in stimulating the activity of SDS-extracts of erythroblast nuclei cannot be deduced from our experiments. Other workers (44, 69) investigating the effect of polyamines and salts on DNA-dependent RNA synthesis did not study the combined effects of these chemicals. Although Lieberman and Tsukada (59), in their detergent-solubilized preparations included both KCl and ammonium sulfate in their incubation medium, they gave no reason for doing so. However, it does not appear that ammonium sulfate and KCl or

KCl and polyamines are performing the same function since ammonium sulfate and spermine increased enzyme activity further at optimal KCl concentration. Thus optimal stimulation was given by KCl at an ionic strength of 0.3 and higher concentrations were inhibitory. However AmS at an ionic strength of 0.63 (total ionic strength KCl + AmS, 0.93) further increased activity by 1.5-fold. The effect of spermine was even more striking. At an ionic strength of KCl of 0.3, 0.25mM spermine gave an additional 3.5-fold increase in enzyme activity. The possibility that there is some form of cooperative action can not be excluded. The stimulation may be a combination of reduced RNA inhibition and increased template DNA availability.

Widnell and Tata (56) demonstrated a stimulation of RNA synthesis by rat liver nuclei by the addition of AmS. They concluded initially that AmS was unmasking a second enzyme for RNA synthesis. However, in subsequent work (77) and in experiments by Huang et al (86) and by Chambon, Ramuz and Doly (58) it was showed that AmS stimulated RNA synthesis by removing nucleohistones from the DNA-RNA polymerase complex. Widnell and Tata presented some arguments against this postulated mode of action of AmS by asking "why nuclei which presumably were capable of synthesizing DNA-like RNA in the intact cell should apparently lose this capacity except in the presence

of ammonium sulfate during the process of isolation". There is evidence, however, that repressed or "heterochromatin" (representing as much as 75-80% of the total DNA of thymus lymphocytes) is inactive in the synthesis of RNA, whereas extended or "euchromatin" is active (41). The possibility that histones enhance the coiling of chromosomes and thereby

repress their activity cannot be overlooked (87) and there is evidence that histones bind chromatin threads of thymus lymphocytes into dense clumps in cell-free systems (88).

Recently Liau, Hnilica and Hurlbert (89) reported that the synthesis of RNA in vitro by nucleoli isolated from Novikoff ascites rat tumour cells was inhibited up to 90% by the addition of calf thymus histone and the composition of RNA formed in the presence of the added histone was altered in the direction of higher GC content. These nucleoli contained initially 32% histone, the rest of the protein being of the acidic type. When the nucleoli were pretreated with trypsin to remove the histones and other proteins the composition of the RNA synthesized shifted to approach the composition of DNA. Again, when histone was added, the RNA synthesized in the presence of the added histone approached the composition of ribosomal RNA. These results, taken in conjunction with those of Ramuz et al (58) and Tata and Widnell (77) would indicate that histones do have a role in determining the type and amount of RNA that can be synthesized from DNA templates by RNA polymerase in the cell.

Likely candidates for the physiological "derepressors" of template activity of repressed chromatin are the polyamines, (see previously), hormones

(95) and acidic nuclear proteins ("residual proteins") (90). Acidic proteins have been found in histone-deficient "euchromatin" of calf thymus nuclei (91). In in vitro systems it is apparent that polyamines, as well as ammonium sulfate and KCl dissociate the nucleohistone from the DNA-RNA polymerase complex.

That RNA synthesis catalyzed by DNA-dependent RNA polymerase occurs in three steps has been indicated by the work of Anthony et al (92) who suggested the following mechanism:

(1) Association: DNA + enzyme $\longrightarrow$ (DNA-enzyme)

(2) Initiation: (DNA-enzyme) + purine nucleotide $\longrightarrow$ (DNA-enzyme-purine nucleotide)

(3) Polymerization: (DNA-enzyme-purine nucleotide) + NTP $\longrightarrow$ (DNA-enzyme-oligoribonucleotide) + PPi

The formation of the DNA-enzyme complex of Step 1 can be inhibited in media of high ionic strength, in fact the purified enzyme itself is dissociated into subunits in such media (93). After initiation has occurred, a different enzyme-DNA complex is formed which is not easily dissociated by a high concentration of ions. Hence with soluble enzyme preparations inhibition by ammonium sulfate and KCl (57, 70) is probably due to the inhibition of the formation of the initial DNA-enzyme complex. That purine nucleotides in the nucleoside triphosphate are the principal initiators of RNA synthesis has been showed by Bremer and Konrad (54) and Hurwitz (94).

SUMMARY

The isolation of cell fractions containing 85% erythroblasts from regenerating bone marrow of chickens is described. Intact nuclei from these cells were prepared and their activity in catalyzing the incorporation of radioactive-ATP into acid-insoluble RNA was studied. Time studies in the absence and presence of AmS and KCl indicated that two forms of RNA polymerase (RNA nucleotidyl transferase, E.C. 2.7.7.6.) are present in the intact nuclei. Treatment of the nuclei with sodium dodecyl sulfate resulted in the solubilization of the nuclei and release of about 64% of the enzyme activity into a supernatant fraction. The specific activity of this fraction was three times greater than that of the intact nuclei. With this fraction linear incorporation of radioactive ATP into RNA obtained for five minutes in the presence of up to 0.12 mg of enzyme protein. The activity was DNA-dependent and little or no RNA was formed when RNAase I was included in the incubation medium. Activity was stimulated 3-fold and 2-fold by the addition of Mg^{+2} (5mM) or Mn^{+2} (2mM), respectively, to the reaction medium. KCl at an optimal concentration of 0.3M (ionic strength 0.3) stimulated the rate of incorporation 2-fold. Further increases of 1.5-fold and 3.5-fold were found when AmS (0.21 M, ionic strength 0.63) or spermine (0.25mM), respectively, were included along with KCl

in the incubation medium. Endogeneous DNA or nucleoside triphosphates caused little or no stimulation of enzyme activity. In reaction medium of ionic strength of 0.93, the pH optimum of the reaction was 7.5-7.6. These studies indicate that the SDS-supernatant fraction contains a DNA-dependent RNA polymerase which is mainly, if not entirely, in the "aggregate" form.

REFERENCES

1. Hull, W. and Kirk, P.L., J. Gen. Physiol. 33, 335 (1950).

2. Furst, S.S., Roll, P.M. and Brown, G.B., J. Biol. Chem. 183, 251 (1950).

3. Hurlbert, R.B. and Potter, V.R., J. Biol. Chem. 195, 257 (1952).

4. Grunberg-Manago, M. and Ochoa, S., J. Am. Chem. Soc. 77, 3165 (1955).

5. Ochoa, S. and Mii, S., J. Biol. Chem. 236, 3303 (1961).

6. Ortiz, P.J. and Ochoa, S., J. Biol. Chem. 234, 1208 (1959).

7. Lehman, I.R., Progress Nucleic Acid Research Vol. II, pg. 87 (1963).

8. Grunberg-Manago, M., Progress Nucleic Acid Research Vol. I, pg. 116 (1963).

9. Weiss, S.B. and Gladstone, L., J. Am. Chem. Soc. 81, 4118 (1959).

10. Weiss, S.B., Proc. Natl. Acad. Sci. U.S. 46, 1020 (1960).

11. Hurwitz, J., Bresler, A. and Diringer, R., Biochem. Biophys. Res. Comm. 3, 15 (1960).

12. Stevens, A., Biochem. Biophys. Res. Comm. 3, 92 (1960).

13. Bonner, J., Huang, R.C., Maheshwari, N., Biochem. Biophys. Res. Comm. 3, 689 (1960).

14. Stevens A., J. Biol. Chem. 236, P.C. 43 (1961).

15. Hurwitz, J., Furth, J.J. and Goldman, M., Biochem. Biophys. Res. Comm. 4, 362 (1961).

16. Weiss, S.B. and Nakamoto, T., Proc, Natl. Acad. Sci. 47, 1400 (1961).

17. Hurwitz, J., Furth, J.J., Anders, M. and Evans, A., J. Biol. Chem. 237, 3752 (1962).

18. Geiduschek, P., Nakamoto, T. and Weiss, S.B., Proc. Natl. Acad. Sci. 47, 1405 (1961).

19. Geiduschek, P., Moohr, J.W. and Weiss, S.B., Proc. Natl. Acad. Sci. 48, 1078 (1962).

20. Hayashi, M., Hayashi, M.N. and Spiegelman, S., Proc. Natl. Acad. Sci. 50, 664 (1963).

21. Tocchini-Valentini, G.P., Stodolsky, M., Geiduschek, E.P., Sarnat, M. and Weiss, S.B., Proc. Natl. Acad. Sci. 50, 935 (1963).

22. Hayashi, M., Hayashi, M.N. and Spiegelman, S., Proc. Natl. Acad. Sci. 51, 351 (1964).

23. Geiduschek, E.P., Tocchini-Valentini, G.P. and Sarnat, M.T., Proc. Natl. Acad. Sci. 52, 486 (1964).

24. Hayashi, M., Hayashi, M.N. and Spiegelman, S., Proc. Natl. Acad. Sci. 50, 664 (1964).

25. Green, M.H., Proc. Natl. Acad. Sci. 52, 1388 (1964).

26. Fox, C.F. and Weiss, S.B., J. Biol. Chem. 239, 175 (1964).

27. Jacob, F. and Monod, J., J. Mol. Biol. 3, 318 (1961).

28. Brenner, S., Jacob, F. and Meselson, M., Nature 190, 576 (1961).

29. Gros, F., Gilbert, W., Hiatt, H., Kurland, C.G., Risebrough, R.W. and Watson, J.D., Nature 190, 576 (1961).

30. Nirenberg, M.W., and Matthaei, J.H., Proc. Natl. Acad. Sci. 47, 1588 (1961).

31. Wood, W.B. and Berg, P., Proc. Natl. Acad. Sci. 48, 94 (1962).

32. Ning, C. and Stevens, A., J. Mol. Biol. 5, 650 (1962).

33. Yankofsky, S.A. and Spiegelman, S., Proc. Natl. Acad. Sci. 48, 1466 (1962).

34. Goodman, H.M. and Rich, A., Proc. Natl. Acad. Sci. 48, 2101 (1962).

35. Robinson, W.S., Hsu, W.T., Fox, C.F. and Weiss, S.B., J. Biol. Chem. 9, 2944 (1964).

36. Gros, F., Gilbert, W., Hiat, H.H., Attardi, B., Spahr, P.F. and Watson, J.D., Cold Spring Harbor Symp. Quant. Biol. Vol. 26, pg.111 (1961).

37. Chamberlin, M. and Berg, P., Proc. Natl. Acad. Sci. 48, 81 (1962).

38. Krakow, J.S. and Ochoa, S., Proc. Natl. Acad. Sci. 49, 88 (1963).

39. Niyogi, S.K. and Stevens, A., J. Biol. Chem. 240, 2587 (1965).

40. Tissieres, A., Bourgeois, S. and Gros, F., J. Mol. Biol. 7, 100 (1963).

41. Frenster, J.H., Allfrey, V.G. and Mirsky, A.E., Proc. Natl. Acad. Sci. 50, 1026 (1963).

42. Fox, C.F., Robinson, W.S., Haselkorn, R. and Weiss, S.B., J. Biol. Chem. 239, 186 (1964).

43. Wood, W.B. and Berg, P., J. Mol. Biol. 9, 452 (1964).

44. Fox, C.F., Gumport, R.I. and Weiss, S.B., J. Biol. Chem. 240, 2101 (1965).

45. Berg, P., Kornberg, R.D., Fancher, H. and Dieckmann, M., Biochem. Biophys. Res. Comm. 18, 932 (1965).

46. Krakow, J.S., J. Biol. Chem. 241, 1830 (1966).

47. Bonner, J., Huang, R.C. and Maheshwari, N., Proc. Natl. Acad. Sci. 47, 1548 (1961).

48. Spiegelman, S., Hall, B.D. and Storck, R., Proc. Natl. Acad. Sci. 47, 1135 (1961).

49. Bremer, H. and Konrad, M.W., Proc. Natl. Acad. Sci. 51, 801 (1964).

50. Kornberg, A., Josse, J. and Kaiser, A.D., J. Biol. Chem. 236, 864 (1961).

51. Kahan, F.M. and Hurwitz, J., J. Biol. Chem. 237, 3778 (1962).

52. Chamberlin, M. and Berg, P., J. Mol. Biol. 8, 708 (1964).

53. Furth, J.J. and Ho, P., J. Biol. Chem. 240, 2602 (1965).

54. Bremer, H., Konrad, M.W., Gaines, K. and Stent, G.S., J. Mol. Biol. 13, 540 (1965).

55. Colville, A.J., Van Bruggen, E.F.J. and Fernandez-Moran, H., J. Mol. Biol. 17, 302 (1966).

56. Widnell, C.C. and Tata, J.R., Biochim. Biophys. Acta 87, 531 (1964).

57. Ramuz, M., Doly, J., Mandel, P., and Chambon, P., Biochem. Biophys. Res. Comm. 19, 114 (1965).

58. Chambon, P., Ramuz, M. and Doly, J., Biochem. Biophys. Res. Comm. 21, 156 (1965).

59. Tsukada, K. and Lieberman, I., J. Biol. Chem. 239, 2952 (1964).

60. Brownhill, T.J., Jones, A.S. and Stacey, M., Biochem. J. 73, 434 (1959).

61. Bristow, G.K. and Yamada, E.W., Can. J. Biochem. 43, 1319 (1965).

62. Goodman, J.W., Exp. Cell Res. 21, 88 (1960).

63. Leif, R.C. and Vinograd, J., Proc. Natl. Acad. Sci. U.S. 51, 520 (1964).

64. Dingman, C.W., and Sporn, M.B., J. Biol. Chem. 239, 3483 (1964).

65. Hammel, C.L. and Bessman, S.P., J. Biol. Chem. 239, 2228 (1964).

66. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem. 193, 265 (1951).

67. Singer, B., Fraenkel-Conrat, H. and Tsugita, A., Virology 14, 54 (1961).

68. Richmond, A.E. and Cherry, J.H., Biochim. Biophys. Acta 91, 330 (1964).

69. Goldberg, I.H., Biochim. Biophys. Acta 51, 201 (1961).

70. Ballard, P.L. and Williams-Ashman, H.G., J. Biol. Chem. 241, 1602 (1966).

71. Pogo, A.E., Allfrey, V.G. and Mirsky, A.E., Proc. Natl. Acad. Sci. 56, 550 (1966).

72. Kahan, E., Kahan, F.M. and Hurwitz, J., J. Biol. Chem. 238, 2491 (1963).

73. Hurwitz, J., Furth, J.J. and Anders, M., J. Biol. Chem. 237, 2611 (1962).

74. Stevens, A. and Henry, J., J. Biol. Chem. 239, 196 (1964).

75. Fox, C.F., Nakamoto, T. and Weiss, S.B., J. Biol. Chem. 239, 175 (1964).

76. Weiss, S.B. and Nakamoto, T., J. Biol. Chem. 236, P.C. 18 (1961).

77. Widnell, C.C. and Tata, J.R., Biochim, Biophys. Acta 123, 478 (1966).

78. Burdon, R.H. and Smellie, R.M.S., Biochim. Biophys. Acta 51, 153 (1961).

79. Stevens, A., J. Biol. Chem. 239, 204 (1964).

80. Rosenthal, S.M. and Tabor, C.W., J. Pharmacol. Exper. Therap. 116, 131 (1956).

81. Martin, R.G. and Ames, B.N., Proc. Natl. Acad. Sci. 48, 2171 (1962).

82. Hershko, A., Amoz, S. and Mager, J., Biochem. Biophys. Res. Comm. 5, 46 (1961).

83. Dykstra, W.G. Jr. and Herbst, E.J., Science 149, 428 (1965).

84. Raina, A. and Cohen, S.S., Proc. Natl. Acad. Sci. 55, 1587 (1966).

85. Caldarera, C.M., Barbiroli, B. and Moruzzi, G., Biochem. J. 97, 84 (1965).

86. Huang, R.C.C., Bonner, J. and Murray, K., J. Mol. Biol. 8, 54 (1964).

87. Busch, H., Histones and Other Nuclear Proteins, pg. 111 (Academic Press, 1965).

88. Littau, V.C., Burdick, C.J., Allfrey, V.G. and Mirsky, A.E., Proc. Natl. Acad. Sci. 54, 1204 (1965).

89. Liau, M.C., Hnilica, L.S. and Hurlbert, R.B., Proc. Natl. Acad. Sci. 53, 626 (1965).

90. Littau, V.C., Allfrey, V.G., Frenster, J.A. and Mirsky, A.E., Proc. Natl. Acad. Sci. 52, 93 (1964).

91. Kleinsmith, L.J., Allfrey, V.G. and Mirsky, A.E., Proc. Natl. Acad. Sci. 55, 1182 (1966).

92. Anthony, D.D., Zeszotek, E. and Goldthwait, D.A., Proc. Natl. Acad. Sci. 56, 1026 (1966).

93. Richardson, J.P., Proc. Natl. Acad. Sci. 55, 1616 (1966).

94. Hurwitz, J., Proc. Natl. Acad. Sci. 54, 815 (1966).

95. Tata, J.R., Progress Nucleic Acid Research Vol. V, pg. 207 (1966).

96. Skalka, A., Fowler, A.V. and Hurwitz, J., J. Biol. Chem. 241, 588 (1966).

Figures and Tables

Figure 1: Effect of sodium dodecyl sulfate on RNA polymerase of lysed nuclear preparations. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles and enzyme fraction (0.1mg of protein). Incubation was carried out at 37° for 5 minutes.

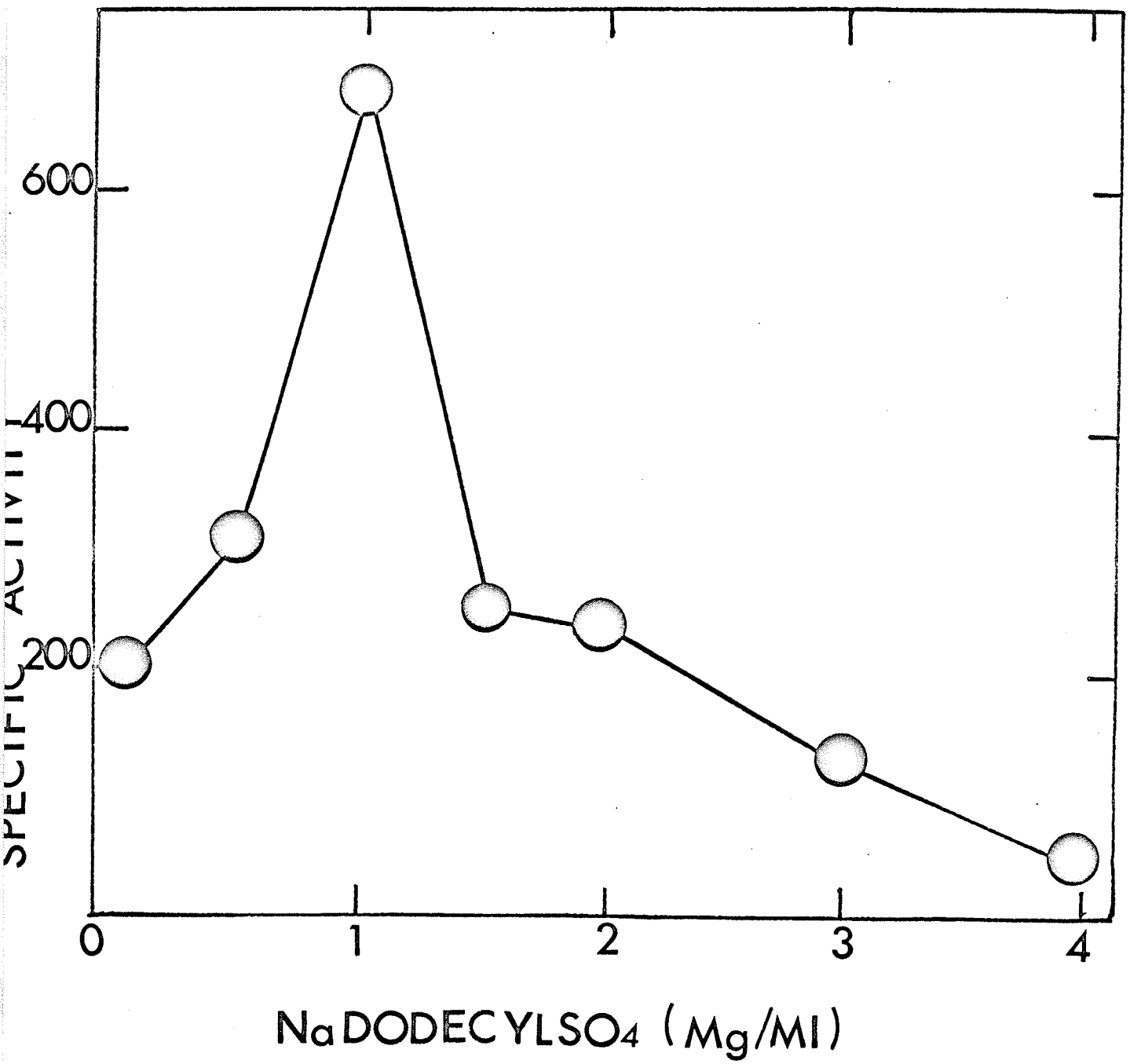
600
400
200
0
1
2
3
4
NaDODECYLSO4 (Mg/MI)

Figure 2: Activity of RNA polymerase with time in intact nuclei. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-C^{14}, 25 mµmoles and enzyme fraction (0.9mg of protein). Incubation was carried out at 37°.

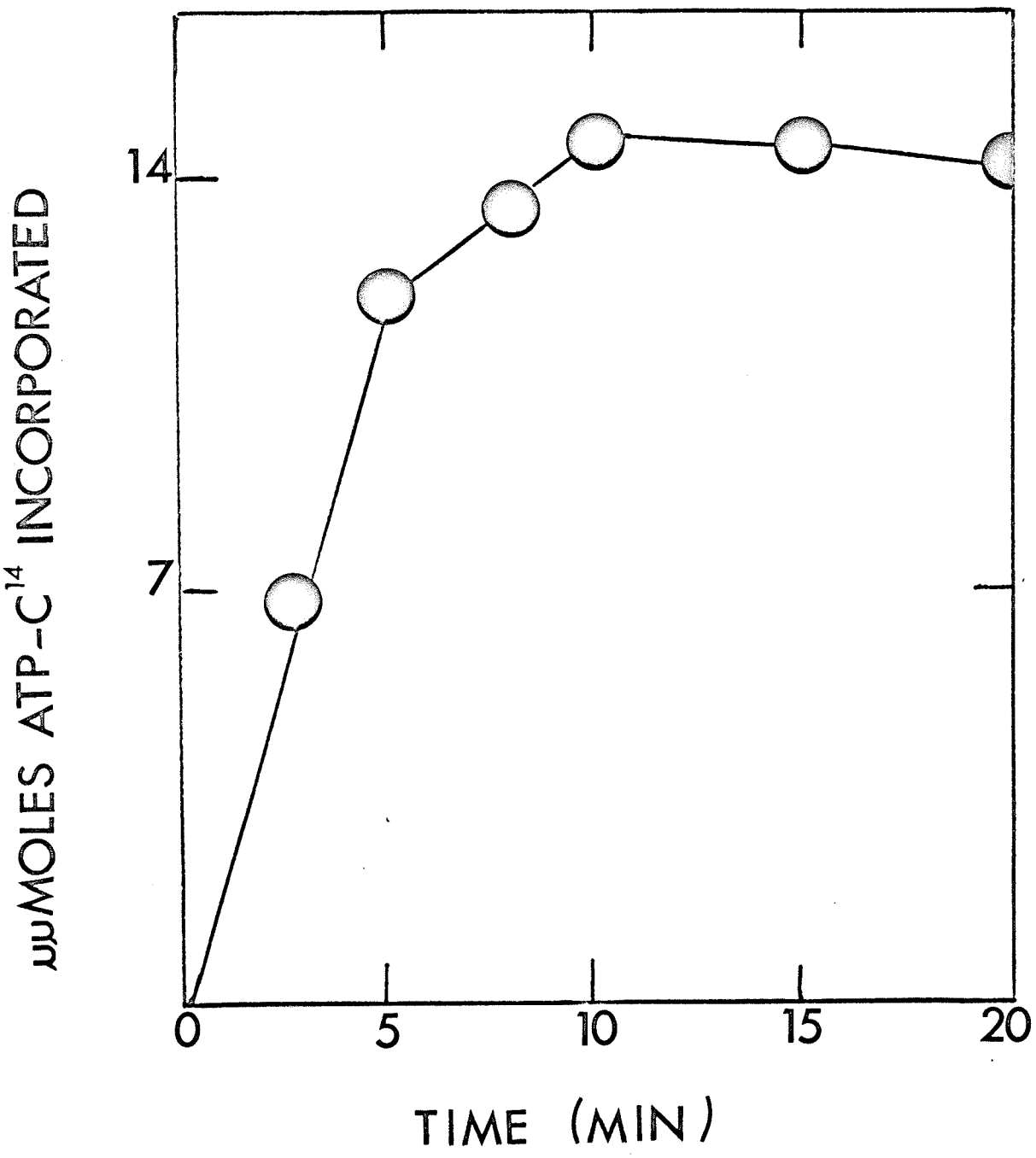
μμMOLES ATP-C14 INCORPORATED
14
7
0
5
10
15
20
TIME (MIN)

Figure 3: Activity of RNA polymerase with time in intact nuclei in the presence of salts. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-C^{14}, 25 mµmoles; KCl, 77 µmoles; AmS, 52.5 µmoles and enzyme fraction (0.9mg of protein). Incubation was carried out at 37°.

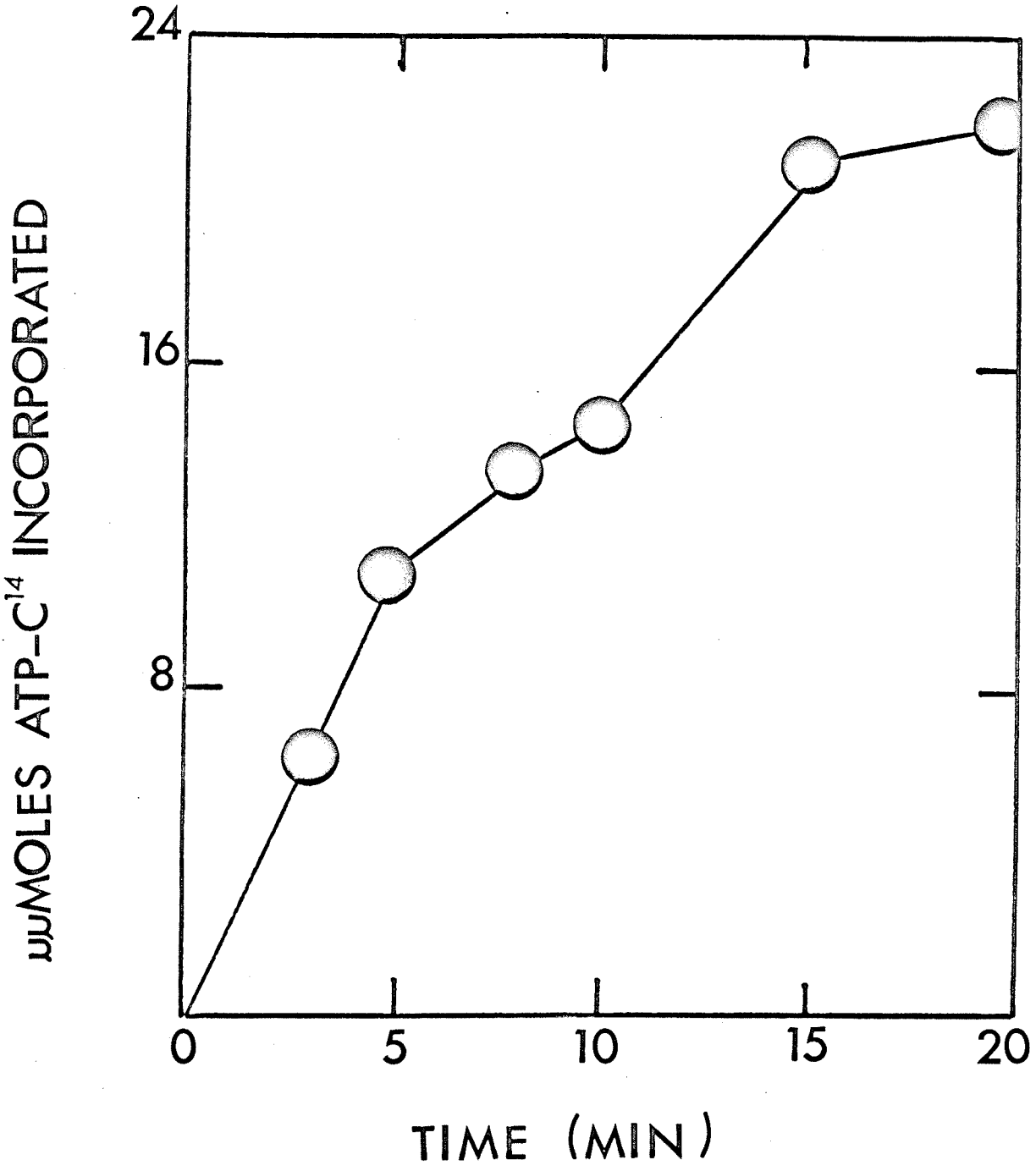
24
16
8
0
5
10
15
20
μμMOLES ATP-C14 INCORPORATED
TIME (MIN)

Figure 4: Activity of RNA polymerase with time in SDS-solubilized nuclear preparations. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles and enzyme fraction (0.1mg of protein). Incubation was carried out at 37°.

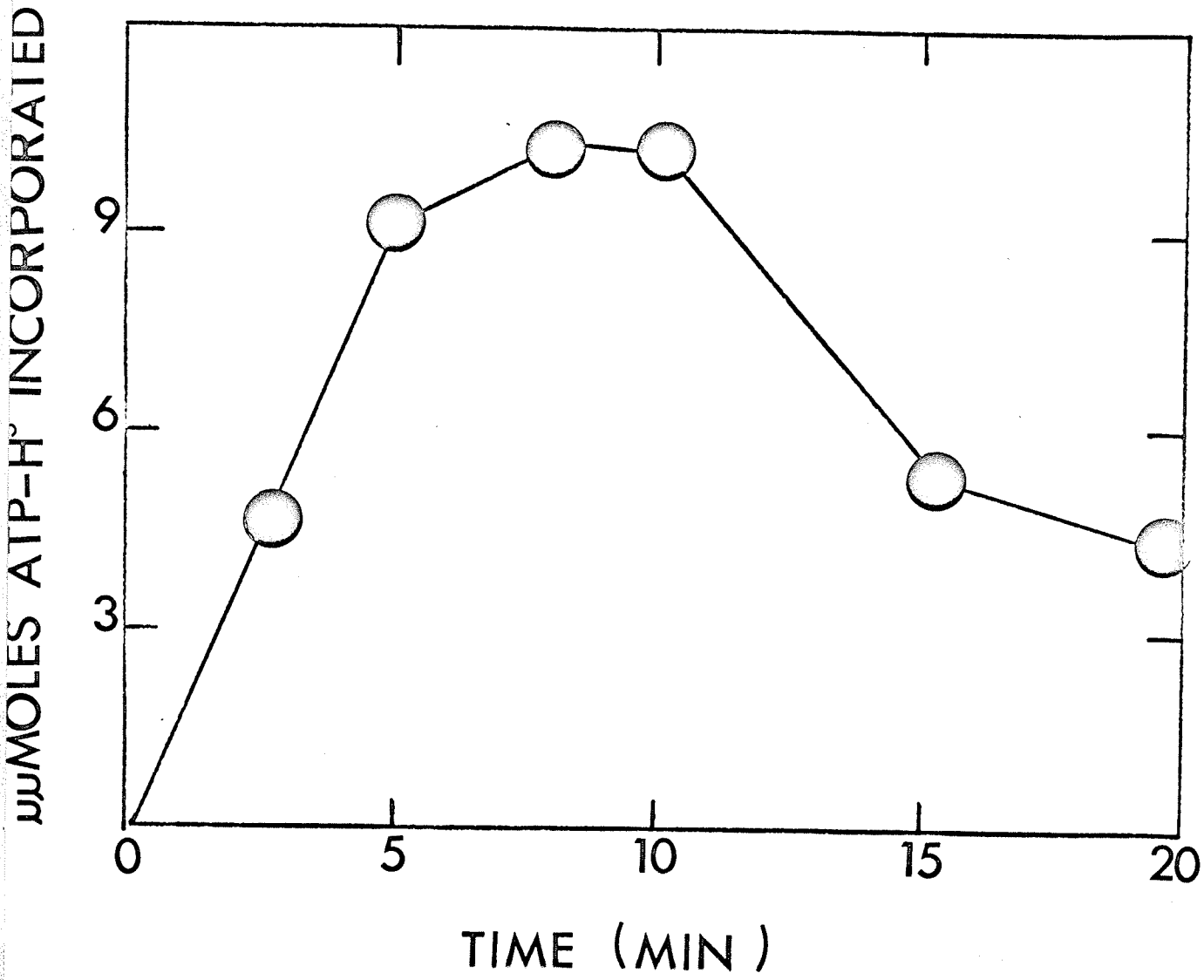
μμMOLES ATP-H° INCORPORATED
9
6
3
0
5
10
15
20
TIME (MIN)

Figure 5: Activity of RNA polymerase with time in SDS-solubilized nuclear preparations in the presence of salts. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles; KCl, 77 µmoles; AmS, 52.5 µmoles and enzyme fraction (0.1mg of protein). Incubation was carried out at 37°.

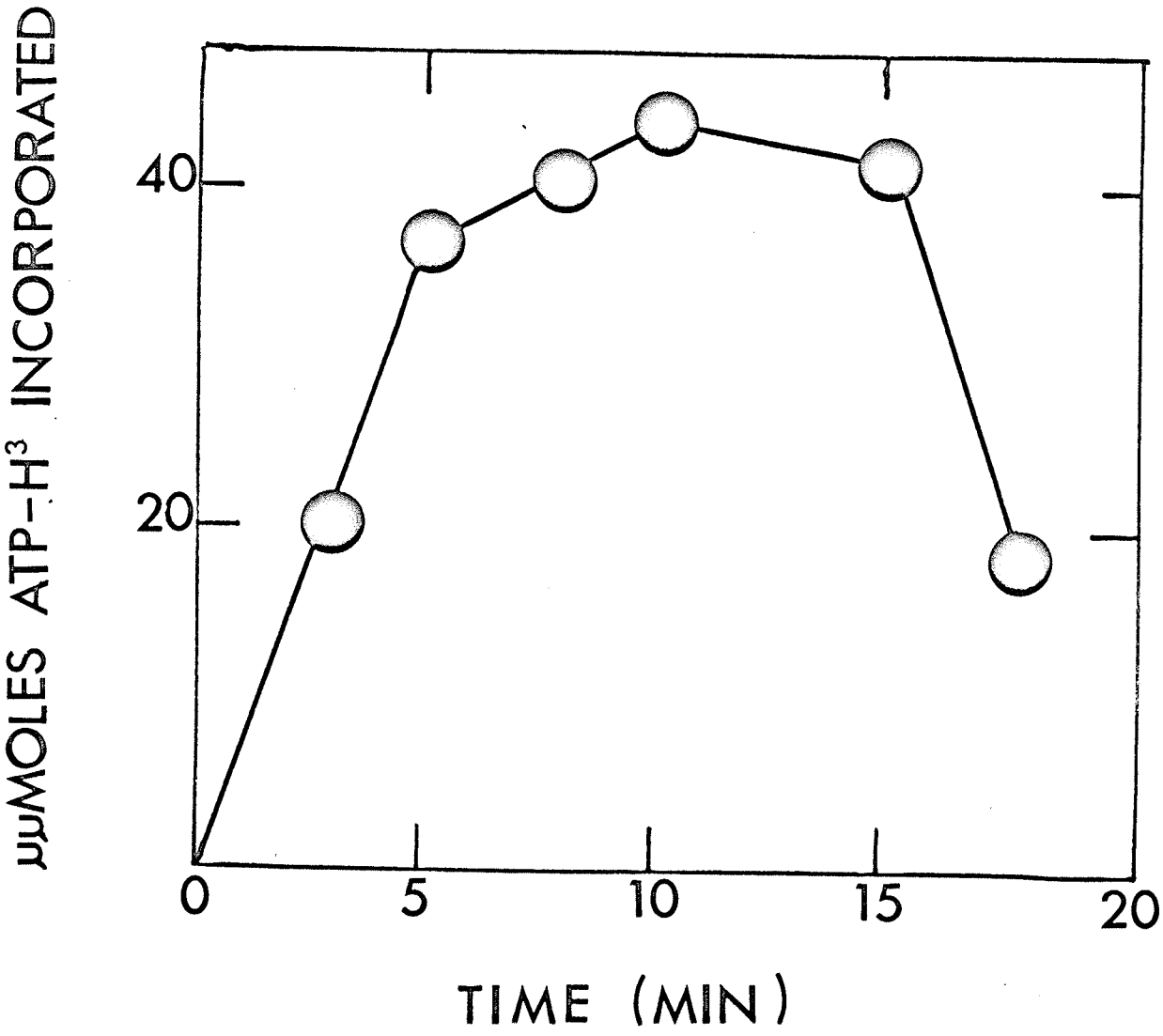
μμMOLES ATP-H³ INCORPORATED
40
20
0
5
10
15
20
TIME (MIN)

Figure 6: Activity of RNA polymerase in intact nuclei with enzyme concentration. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-C^{14}, 25 mµmoles and enzyme fraction. Incubation was carried out for 5 minutes at 37°.

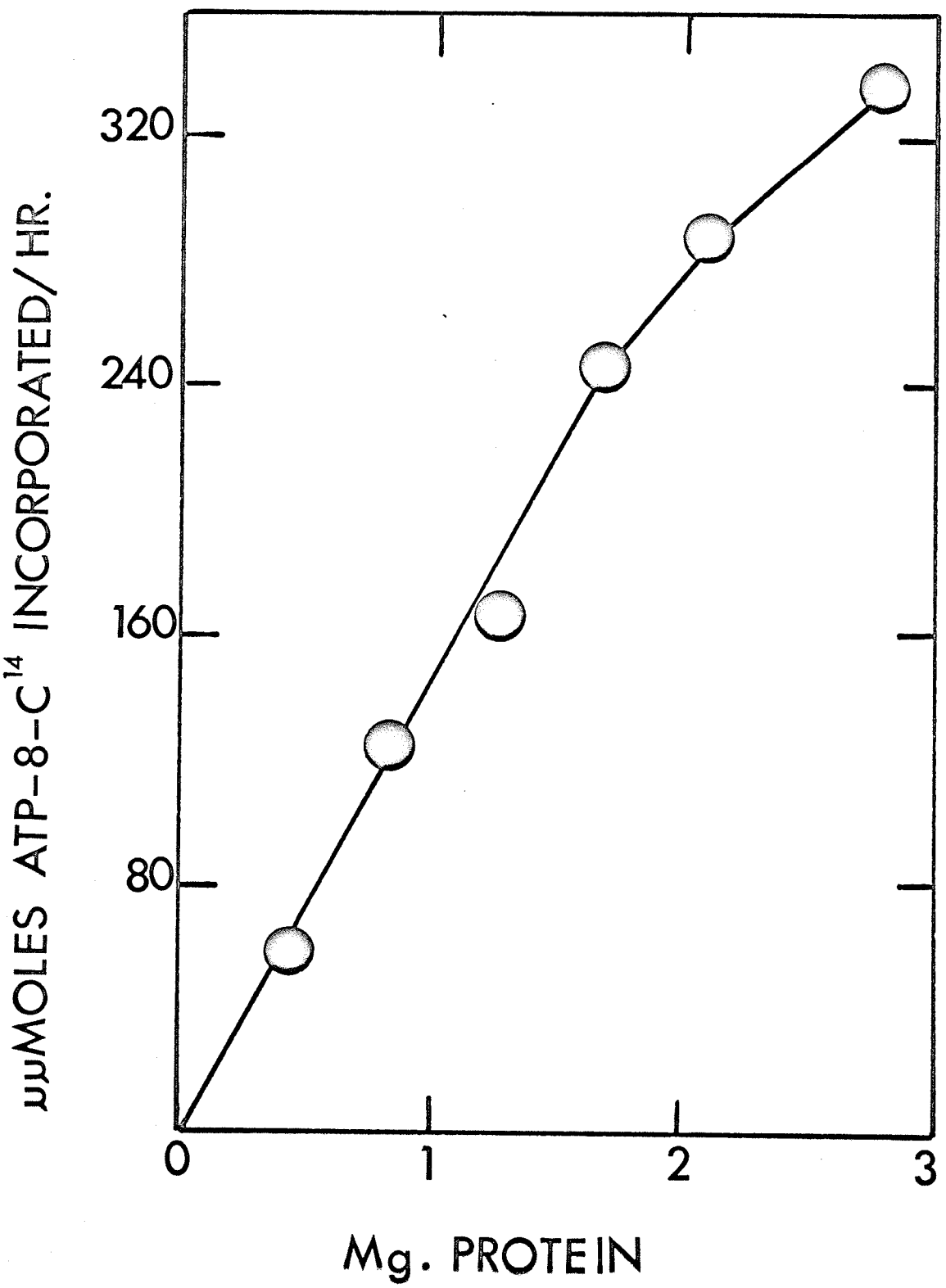
μμMOLES ATP-8-C14 INCORPORATED/ HR.
320
240
160
80
0
1
2
3
Mg. PROTEIN

Figure 7: Activity of RNA polymerase with enzyme concentration in SDS-solubilized nuclear preparations. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles, KCl, 77 µmoles; AmS, 52.5 µmoles and enzyme fraction. Incubation was carried out for 5 minutes at 37°.

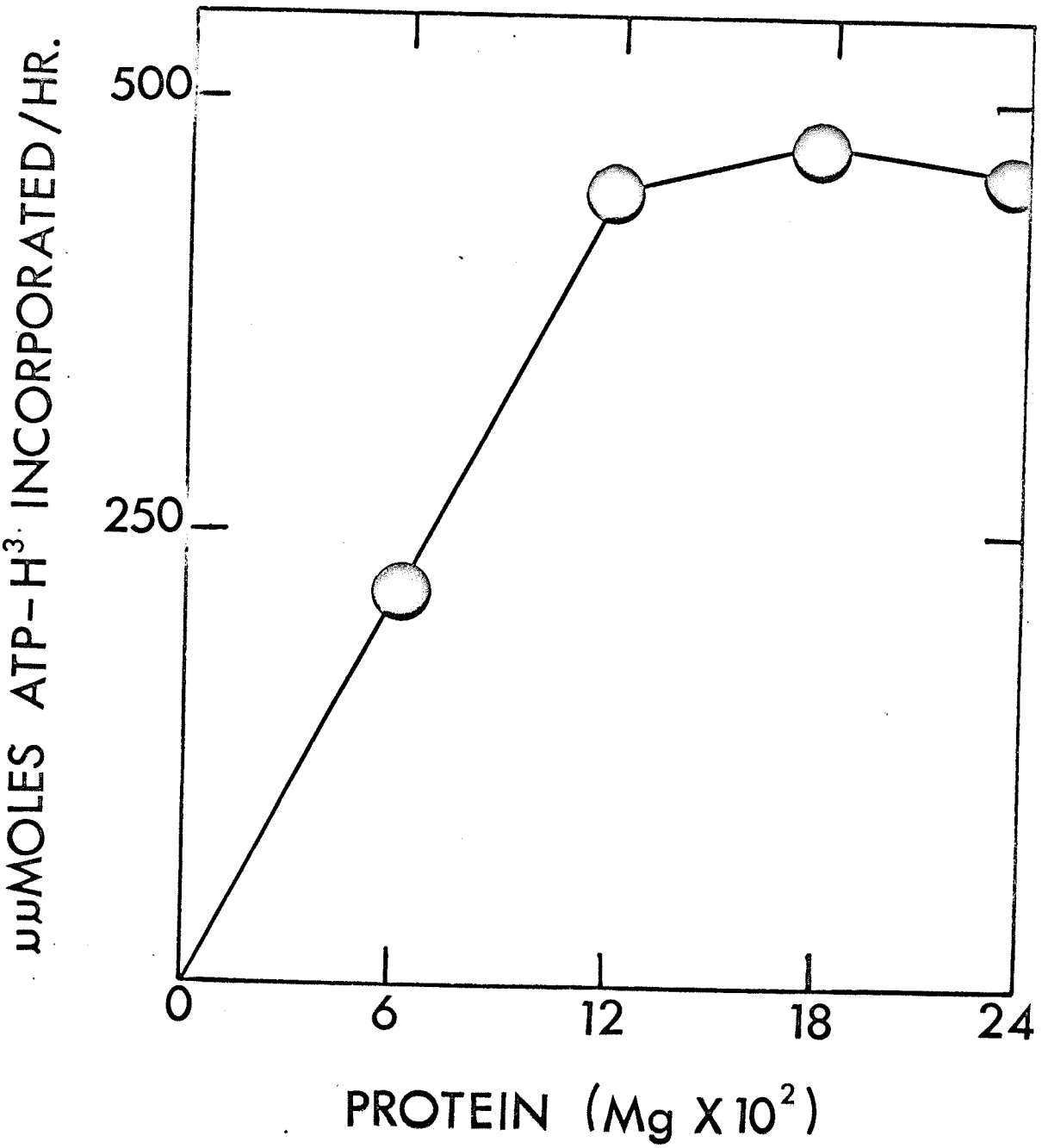
μμMOLES ATP-H³ INCORPORATED/HR.
500
250
0
6
12
18
24
PROTEIN (Mg X 10²)

Figure 8: Effect of nucleoside triphosphates on RNA polymerase activity. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4 umoles; pyruvate kinase, 2.5ug; phosphoenol pyruvate, 2.5 umoles; DNA, 25ug; magnesium acetate, 1.25 umoles; UTP, CTP and GTP; β-mercaptoethanol, 1.25 umoles; ATP-H^3, 25 mumoles and enzyme fraction (0.1mg of protein). Incubation was carried out for 5 minutes at 37°.

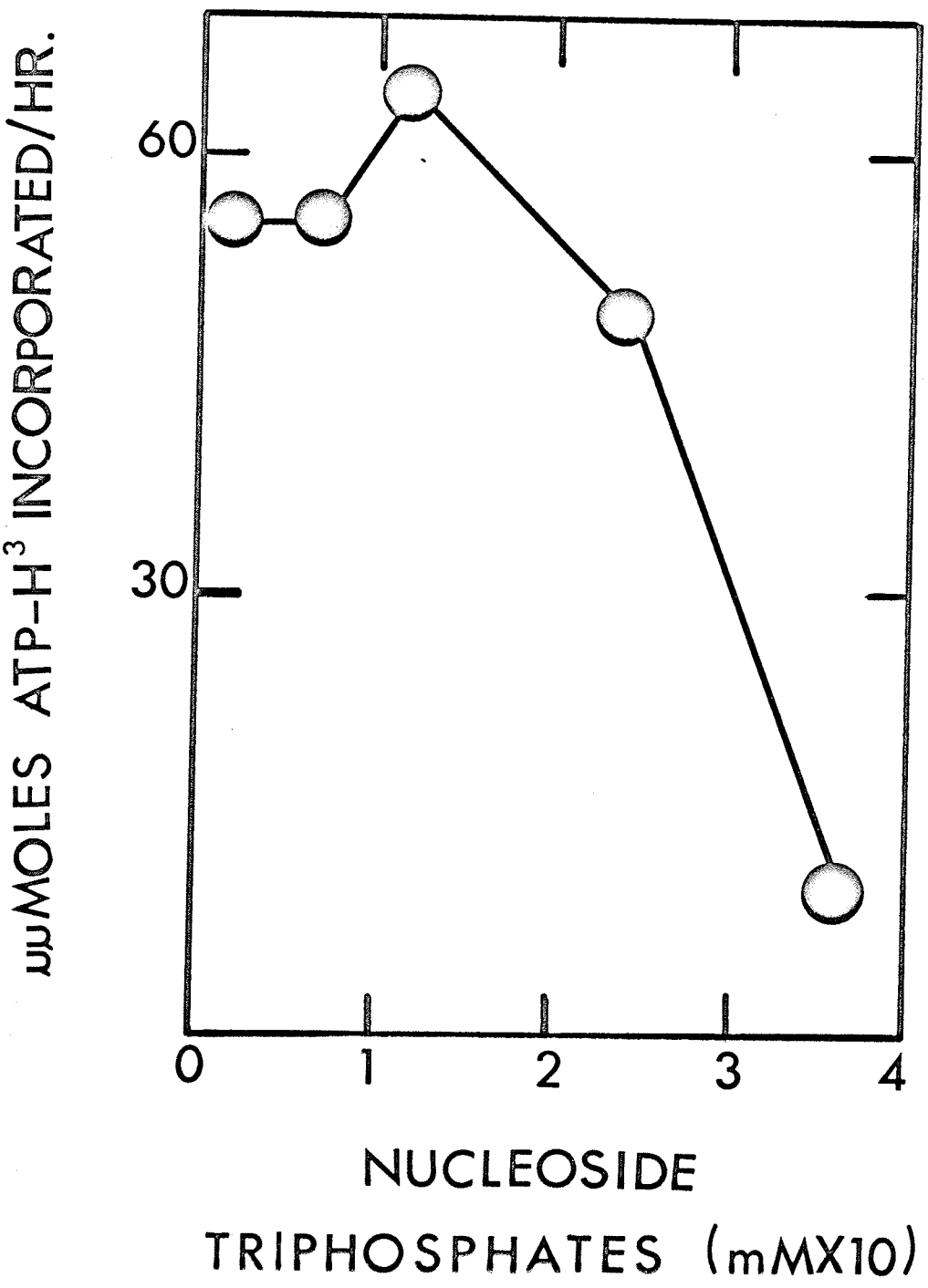
μμMOLES ATP-H3 INCORPORATED/HR.
60
30
0
1
2
3
4
NUCLEOSIDE
TRIPHOSPHATES (mMX10)

Figure 9: Effect of Mg^{+2} concentration on enzyme activity. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles, ATP-H^3, 25 mµmoles and enzyme fraction (0.1mg of protein). Incubation was carried out for 5 minutes at 37°.

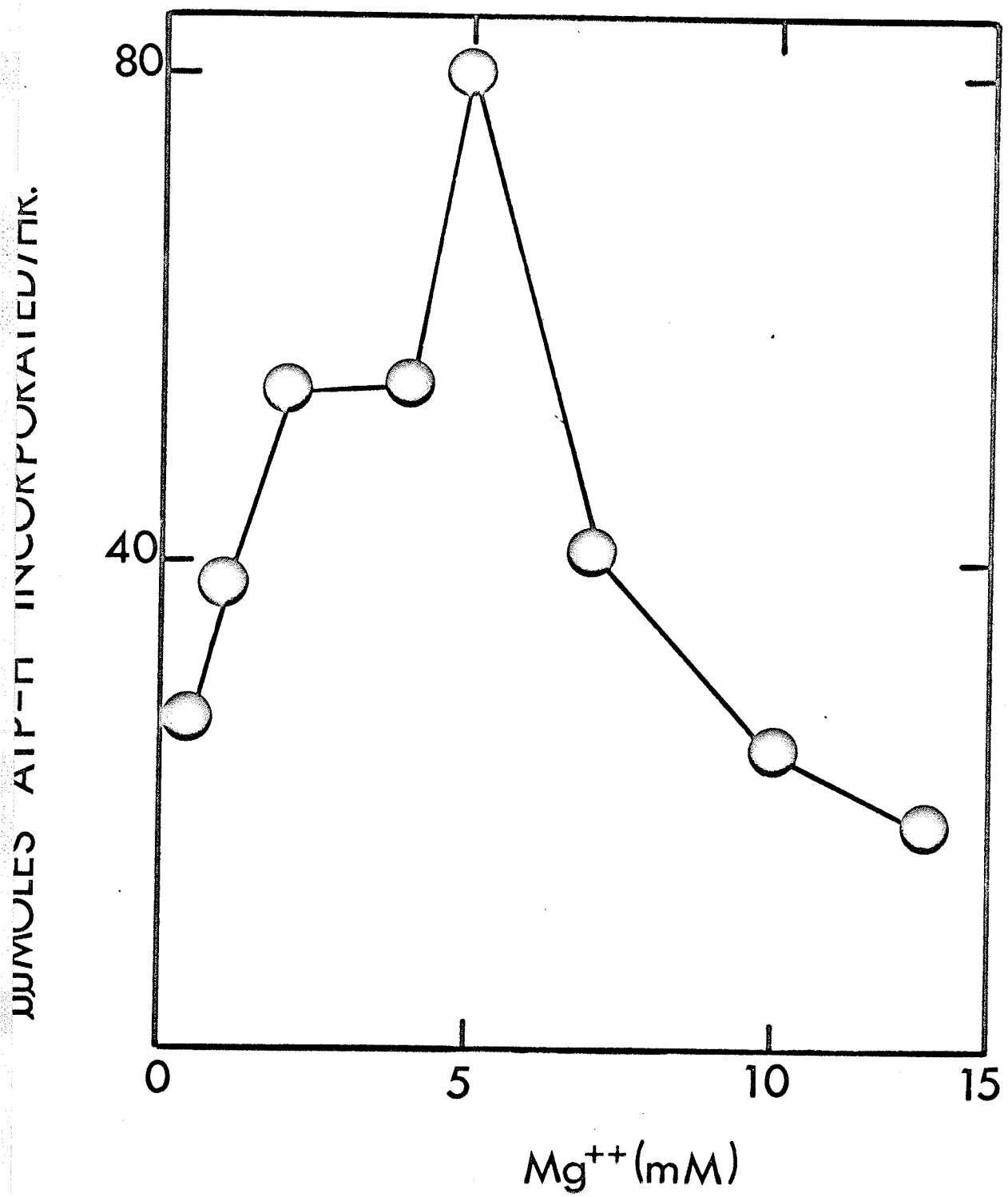
μμMOLES ATP-P INCORPORATED/MK.
80
40
0
5
10
15
Mg++ (mM)

Figure 10: Effect of Mn^{++} concentration on enzyme activity. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; Mn acetate; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles and enzyme fraction (0.1mg of protein). Incubation was carried out for 5 minutes at 37°.

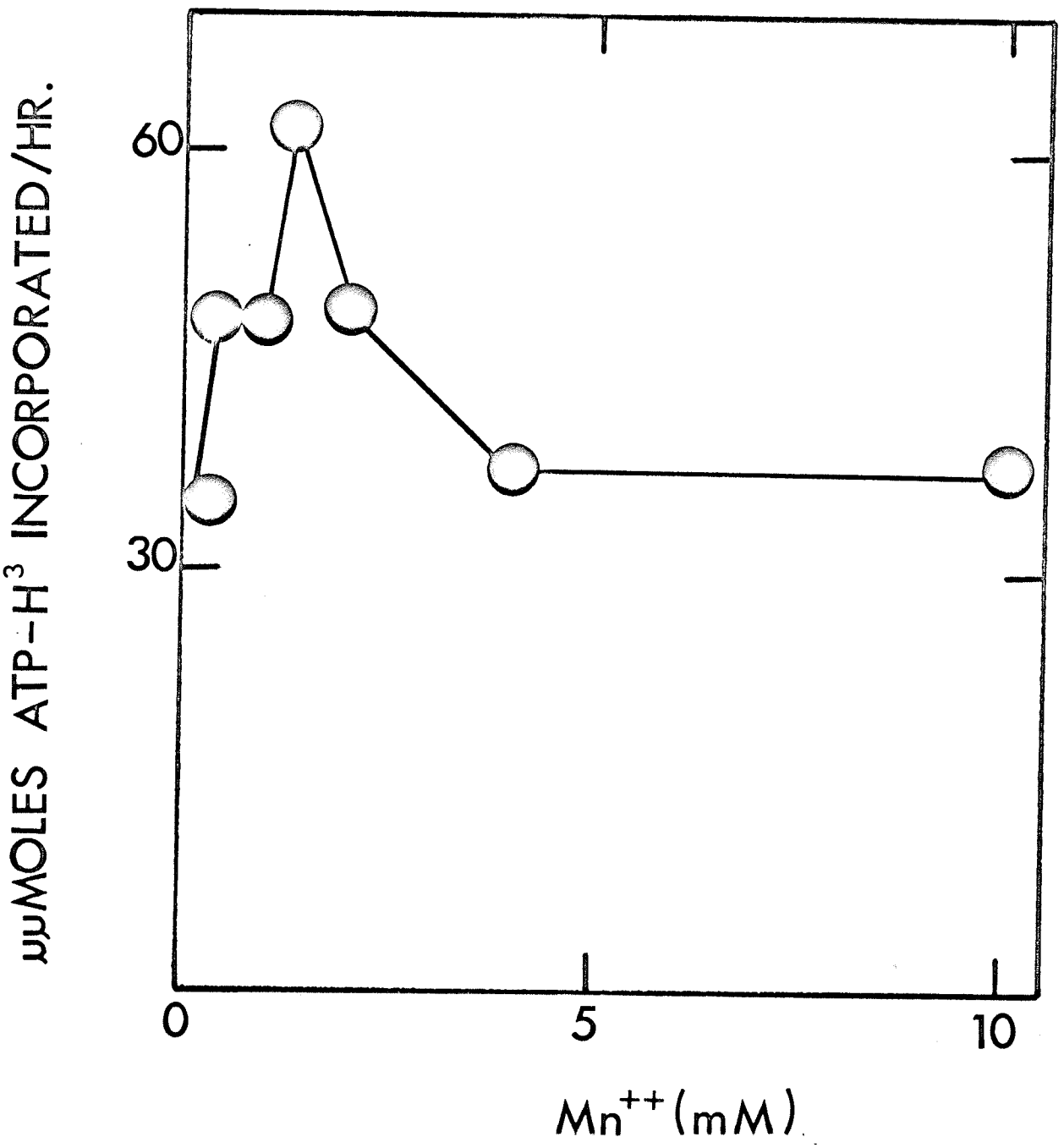
μμMOLES ATP-H3 INCORPORATED/HR.
60
30
0
5
10
Mn++ (mM)

Figure 11: Lineweaver-Burk double reciprocal plots for Mg^{+2} and Mn^{+2}.

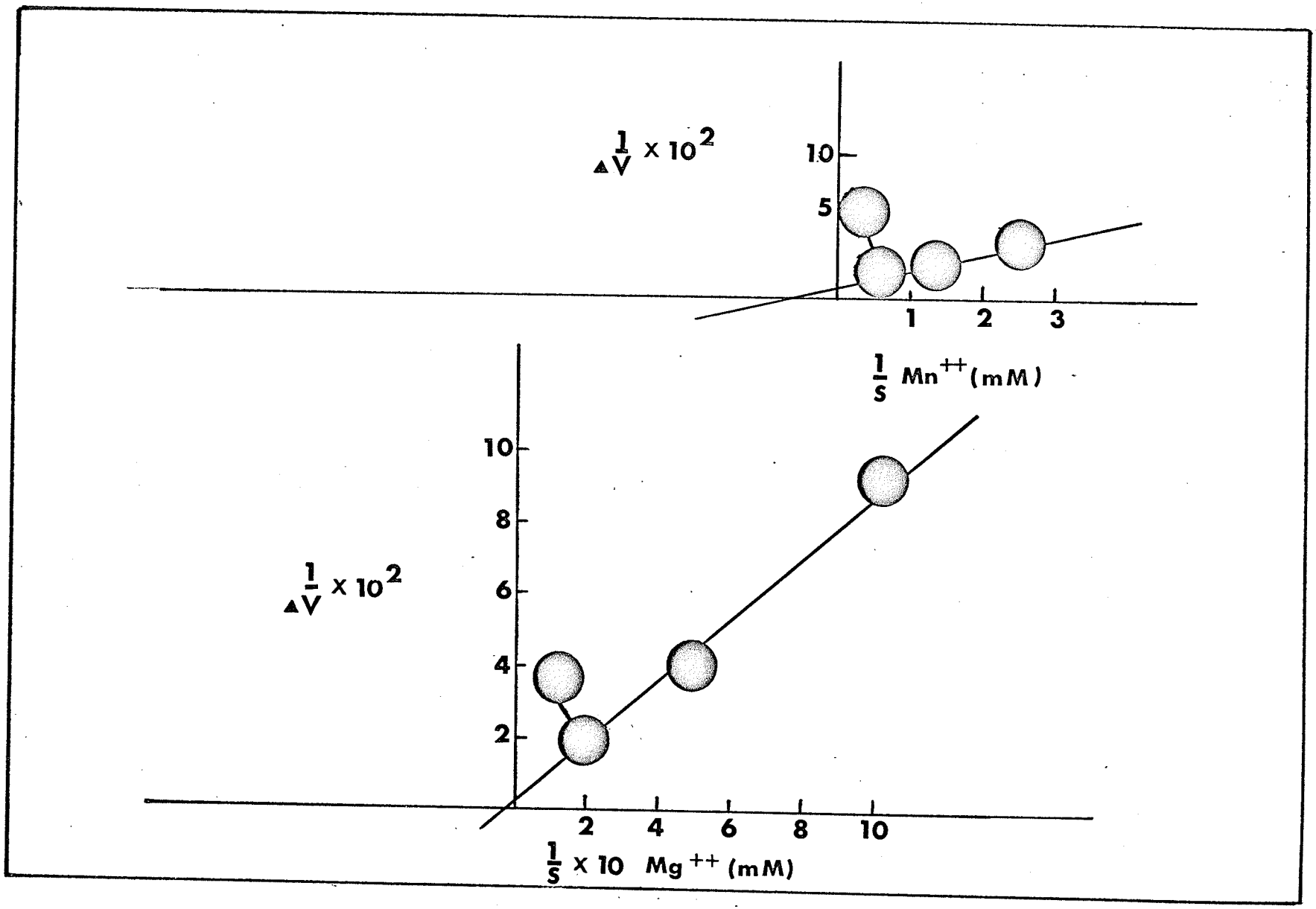
$\frac{1}{\Delta V} \times 10^2$
10
5
1
2
3
$\frac{1}{S}$ Mn^{++} (mM)
$\frac{1}{\Delta V} \times 10^2$
10
8
6
4
2
2
4
6
8
10
$\frac{1}{S} \times 10$ Mg^{++} (mM)

Figure 12: Effect of spermidine on RNA polymerase activity. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles; spermidine, pH 7.0 and enzyme fraction (0.1mg of protein). Incubation was carried out for 5 minutes at 37°.

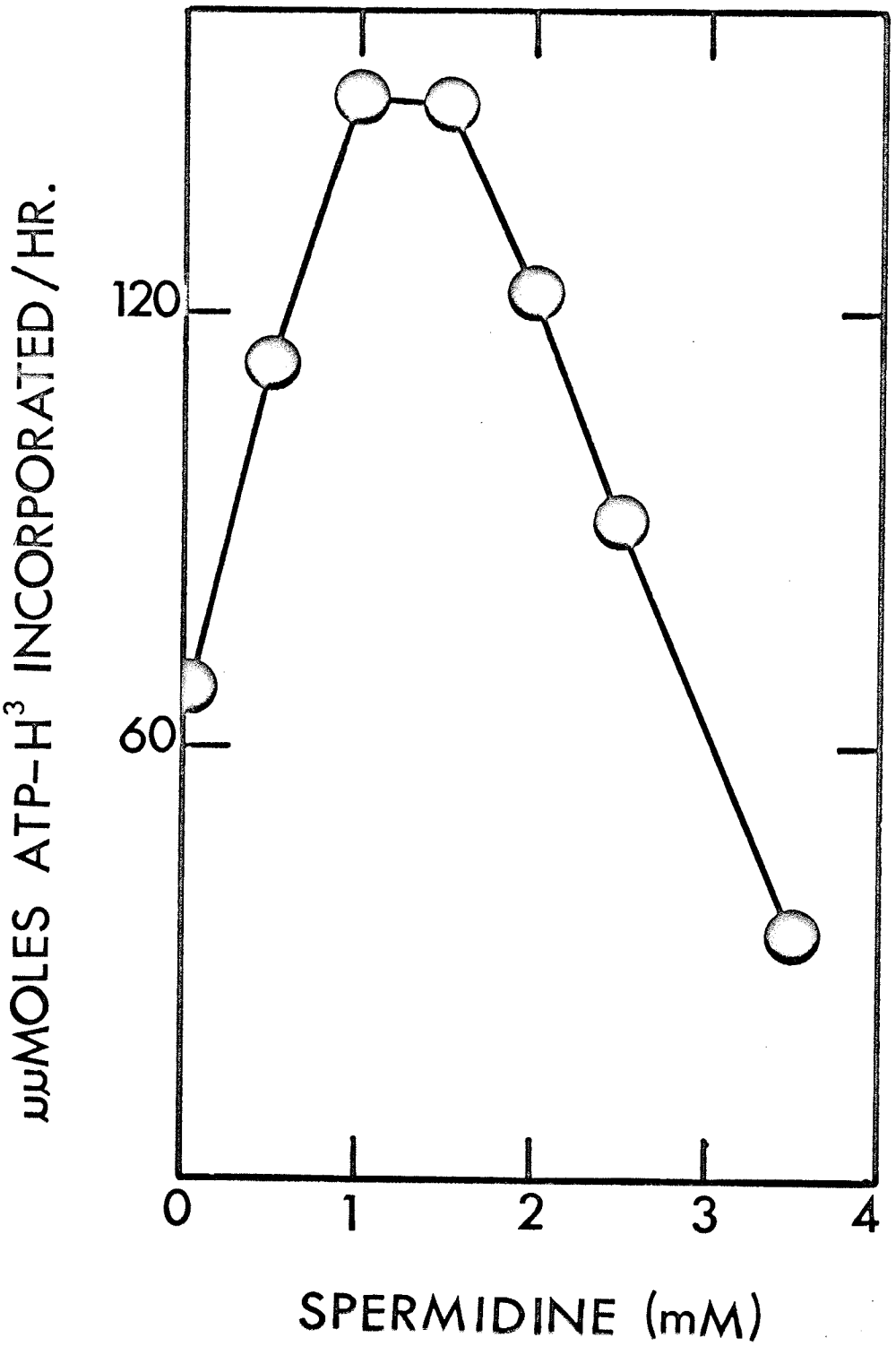
μμMOLES ATP-H³ INCORPORATED/HR.
120
60
0
1
2
3
4
SPERMIDINE (mM)

Figure 13: Effect of KCl on RNA polymerase activity. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles; KCl and enzyme fraction (0.1mg of protein). Incubation was carried out for 5 minutes at 37°.

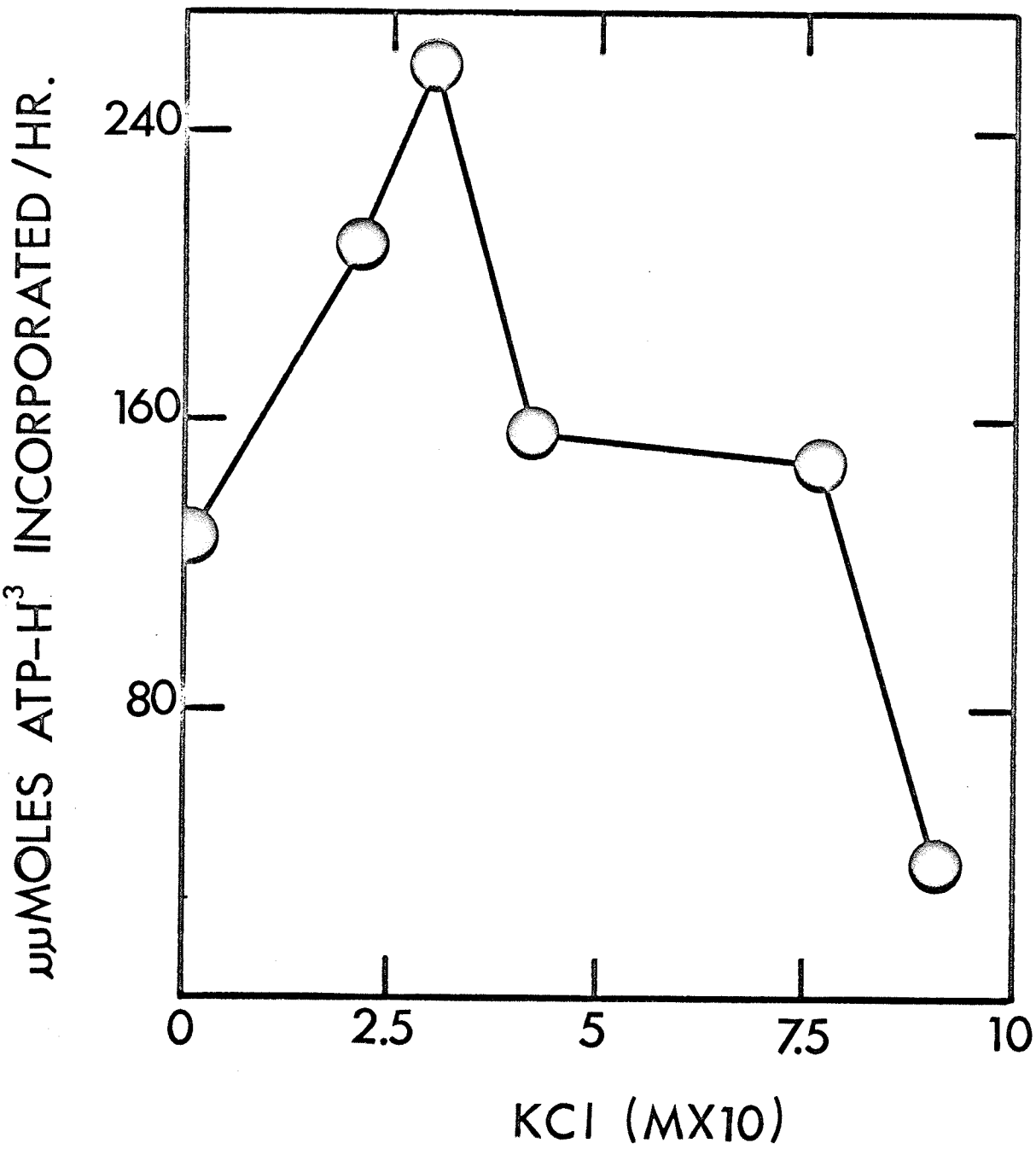
μμMOLES ATP-H3 INCORPORATED / HR.
240
160
80
0
2.5
5
7.5
10
KCl (MX10)

Figure 14: Effect of pH on RNA polymerase activity. The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercapto-ethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles; KCl, 77 µmoles; AmS, 52.5 µmoles and enzyme fraction (0.1 mg of protein). Incubation was carried out for 5 minutes at 37°.

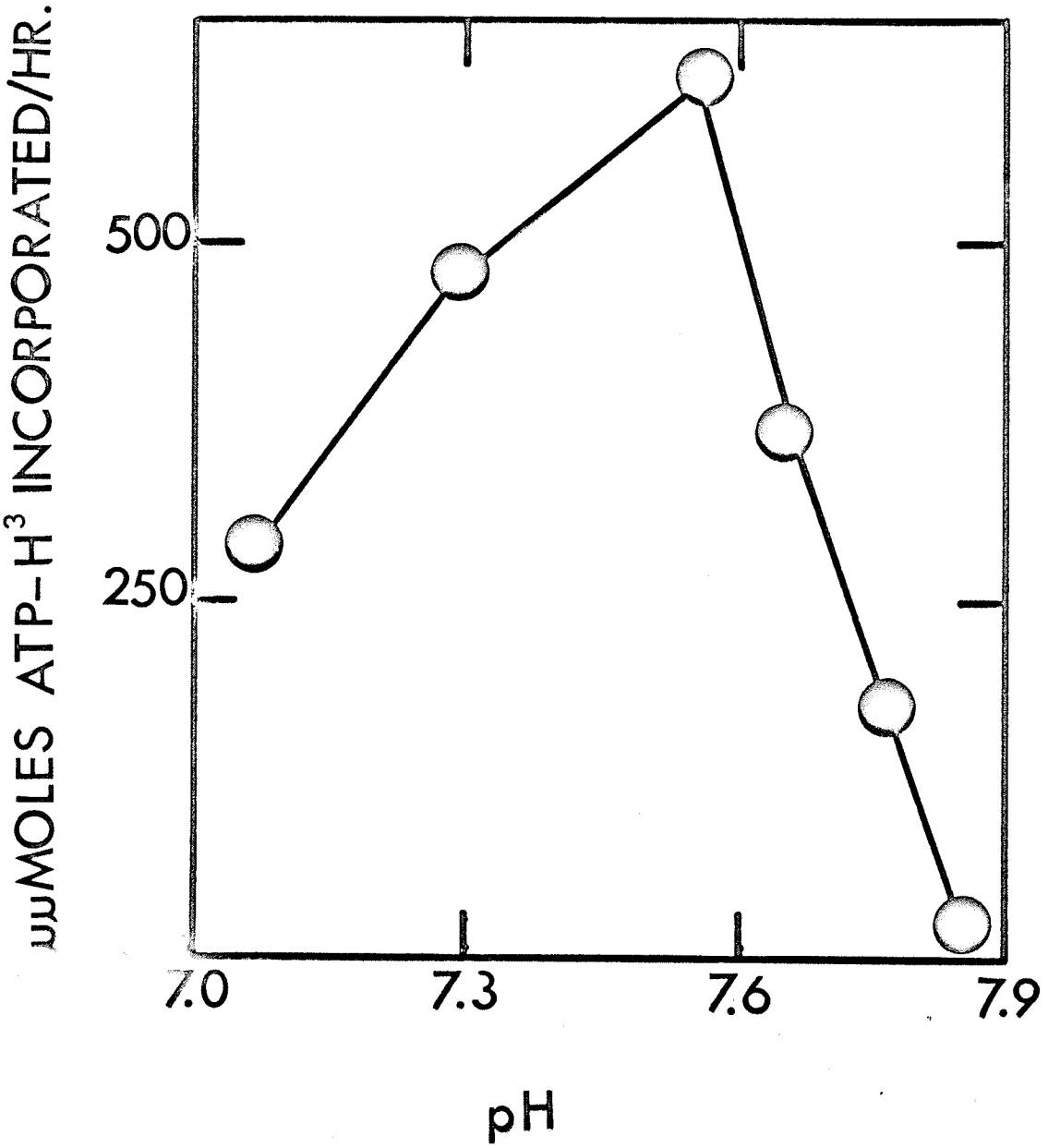
μμMOLES ATP-H³ INCORPORATED/HR.
500
250
7.0
7.3
7.6
7.9
pH

Table I

Separation of Bone Marrow Cells in Dextran Gradients

Linear Gradient

raction	Total Cell Count	Differential Cell Counts (%)* Erythrocytes	Erythroblasts	Leucocytes	Lymphocytes
ıole bone marrow	8,880,000	66	30	1	2
ayer 1	489,600	56	1	6	37
ayer 2	1,188,600	89	7		4
ayer 3	3,398,851	75	25	1	
ayer 4	864,000	50	48	1	1
ıole bone marrow	8,400,000	49	43	4	4
ayer 1	2,050,000	60	34	2	4
ayer 2	4,175,000	60	35		
ayer 3	270,000	45	49	2	4

I Discontinuous Gradient

ıole bone marrow	20,960,000	40	51	4	5
ayer 1 Top)		cell debris, erythrocytes, few erythroblasts, myeloblasts and white blood cells			
ayer 2 Middle)	5,015,000	36	58	1	5
ayer 3 Bottom)	8,704,000	11	85	2	2

Reticulocytes and other immature forms of the red blood cells are ıcluded in the erythroblast fraction. Myeloblasts and similar forms re included in the leucocyte counts.

Table II

Effect of SDS on Solubilization of
RNA Polymerase of Intact Nuclei

SDS (mg/ml)	CPM/ml/hr*	Specific Activity	% Recovery
0	5350	200.0	100
0.25	267	209.5	5.0
0.50	2970	311.5	55.6
1.00	3421	675.5	64.0

The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles and enzyme fraction. Incubation was carried out for 5 minutes at 37°.

---

*CPM/ml/hr - counts per minute per ml of enzyme fraction per hour incubation time.

Table III

Comparison of Carriers

Carrier	Incorporation of Radio-active precursor into RNA (CPM/ml/hr)*
0	5220 4540
RNA	4830 5200
Albumin	5470 5290
RNA Albumin	5500 5010

The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles and enzyme fraction (1.5mg of protein). Incubation was carried out at 37° for 5 minutes. Carrier, 0.5mg of each, was added.

*CPM/ml/hr - counts per minute per ml of enzyme fraction per hour incubation time.

Table IV

Effect of Bentonite

Tube	Bentonite (mg/ml)	Incorporation of Radioactive precursor into RNA (CPM/ml/hr)*
zero time	0	1611
1	0	6500
2	0	8160
zero time	0.5	16090
1	0.5	18520
2	0.5	29200

The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles; KCl, 77 µmoles; AmS, 52.5 µmoles; bentonite and enzyme fraction (0.1mg of protein). Incubation was carried out at 37° for 5 minutes.

---

*CPM/ml/hr - counts per minute per ml of enzyme fraction per hour incubation time.

Table V

Effect of DOC on Solubilization of RNA Polymerase of Intact Nuclei

DOC(mg/ml)	Experiment 1 Specific Activity	Experiment 2 Specific Activity
0	221.0	142.0
1.0	358.5	106.3
2.0	372.0	52.8
4.0	438.1	140.0
7.0	470.5	99.8
10.0	330.0	37.5

The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; DNA, 25µg; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles and enzyme fraction (0.1mg of protein). Incubation was carried out for 5 minutes at 37°.

Table VI

Effect of Inhibitors on RNA Polymerase Activity

Reaction Mixture	Incorporation of Radioactive precursor into RNA (CPM/ml/hr)**	% Incorporation
Complete*	3140	100
DNAase (10μg)	806	25
RNAase (10μg)	449	14.0
Actinomycin D (0.1μM)	0	0

*The complete reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 μmoles; pyruvate kinase, 2.5μg; phosphoenol pyruvate, 2.5 μmoles; magnesium acetate, 1.25 μmoles; UTP, CTP and GTP, 30 mμmoles of each; β-mercaptoethanol, 1.25 μmoles; DNA, 25μg; ATP-H^3, 25 mμmoles; and enzyme fraction (0.1mg of protein).

**CPM/ml/hr - counts per minute per ml of enzyme fraction per hour incubation time.

Table VII

Effect of DNA on RNA Polymerase Activity

Reaction Mixture	DNA (µg)	Incorporation of Radioactive precursor into RNA (CPM/ml/hr)**
Complete*	0	5920
	12.5	6270
	25	6700
	50	3290

* The complete reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; ATP-H^3, 25 mµmoles; and enzyme fraction (0.12mg of protein).

**CPM/ml/hr - counts per minute per ml of enzyme fraction per hour incubation time.

Table VIII

Effect of Ammonium Sulfate and Spermine on RNA Polymerase in the Presence of Optimal Concentration of Potassium Chloride

Reaction Mixture			Incorporation of Radioactive precursor into RNA (CPM/ml/hr) *
0.3 M KCl	0	spermine	4860
0.3 M KCl	0.1mM	spermine	13900
0.3 M KCl	0.25	spermine	15600
0.3 M KCl	0.5	spermine	11000
0.3 M KCl	1.0mM	spermine	2840
0.3 M KCl	0	AmS	7960
0.3 M KCl	0.07M	AmS	11310
0.3 M KCl	0.14M	AmS	11310
0.3 M KCl	0.21M	AmS	12210
0.3 M KCl	0.3 M	AmS	7370

The reaction mixture contained the following in a final volume of 0.25ml; TRIS buffer, pH 7.4, 25 µmoles; pyruvate kinase, 2.5µg; phosphoenol pyruvate, 2.5 µmoles; magnesium acetate, 1.25 µmoles; UTP, CTP and GTP, 30 mµmoles of each; β-mercaptoethanol, 1.25 µmoles; KCl, 77 µmoles; ATP-H^3, 25 mµmoles, enzyme fraction (0.1mg of protein) and AmS or spermine.

*CPM/ml/hr - counts per minute per ml of enzyme fraction per hour incubation time.