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RNA tumor viruses, such as avian myeloblastosis virus (AMV) and MC29 virus, cause malignant transformation of the cells they infect. Transformed cells are able to continually produce progeny virus particles.

Evidence is given that RNA-dependent RNA polymerase is present in virus infected cells. RNA-dependent RNA polymerase isolated from AMV-infected myeloblasts had an absolute requirement for heteropolymer RNA, and 64S RNA from AMV was the preferred primer for RNA synthesis. Under assay conditions where ribonuclease activity was inhibited by polyvinylsulfate, the RNA-dependent RNA polymerase synthesized RNA *in vitro* which had the velocity sedimentation properties of 64S RNA from AMV.

RNA-dependent RNA polymerase was separated by CM-Sephadex chromatography into two polymerase activities which did not incorporate all four ribonucleoside monophosphates equally into RNA

product. In particular, a polymerase fraction was obtained which incorporated only UMP into RNA product, but maintained the specificity for high molecular weight RNA as primer.

An RNA polymerase activity was also obtained by detergent solubilization procedures which synthesized RNA from a template associated with the enzyme fraction. The activity was largely resistant to deoxyribonuclease. Under appropriate assay conditions the detergent-solubilized RNA polymerase also synthesized RNA product which had velocity sedimentation properties of 64S RNA from AMV.

RNA tumor virus particles contain DNA polymerase. Evidence is presented that both RNA and DNA will serve as templates for DNA synthesis by DNA polymerase from AMV and MC29 virus. AMV particles and MC29 virus were banded isopycnically in preformed glycerol density gradients. DNA polymerase activity was located at a buoyant density (1.16 gm/cc) characteristic of tumor virus particles.

Virus-associated DNA polymerase was also isolated from chick myeloblasts infected with AMV and from chick embryo culture (CEC) cells infected with MC29 virus. The virus-associated DNA polymerase banded isopycnically at a buoyant density of 1.16 gm/cc. RNA polymerase also banded at the same buoyant density. RNA polymerase activity was stimulated by both DNA and RNA primers.

Human leukemia blood samples were examined for DNA polymerase activity which would fit the criteria for tumor virus DNA

polymerase. Blood samples from patients with chronic lymphocytic leukemia, acute lymphocytic leukemia, and chronic myelogenous leukemia contained DNA polymerase in particulates which were obtained from the blood plasmas. Plasmas from healthy individuals and from a patient with nonmalignant lymphocytosis did not contain DNA polymerase. DNA polymerase from chronic lymphocytic leukemia banded isopycnically at a buoyant density similar to RNA tumor virus nucleocapsids.

Preliminary studies on chronic lymphocytic leukemia lymphocytes revealed DNA polymerase activity in postnuclear supernatant fractions with specific activity and buoyant density characteristics very similar to the virus-associated DNA polymerase obtained from myeloblasts and MC 29 infected CEC.

Possible mechanisms for RNA tumor virus replication are discussed. Evidence for human tumor virus etiology of human leukemia is presented.

Nucleic Acid Polymerases Associated with Neoplasms

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Typed by Donna L. Olson for Ann Drue Kiessling

We shall not cease from
exploration
And the end of all our
exploring
Will be to arrive where
we started
And know the place for
the first time

....T.S. Eliot

To Pete, for not losing his sense of humor, and for maintaining mine.

And to my parents, for their understanding and encouragement.

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INTRODUCTION

Tumor viruses induce a cellular pathology which is expressed as invasive, uncontrollable growth of the cell population. Malignant processes that are brought about by successful virus infection involve an alteration of cell morphology, permanent transformation of the cell system into a neoplastic state and replication of the virion. There are two general classes of tumor viruses: those containing predominantly desoxyribonucleic acid (DNA), and those containing predominantly ribonucleic acid (RNA) as viral genetic material.

The DNA viruses present a widely divergent spectrum of virus-cell interactions and oncogenicity. Only two of the group have actually been related to tumors which occur spontaneously in nature. These are the papilloma virus (Shope, 1933), which is associated strictly with papillomas and does not lend itself to tissue culture studies; and the polyoma virus (Gross, 1953; Stewart, 1953) which induces a variety of tumors in several species. The herpesviruses are associated with several malignancies (Lucké, 1934; Biggs, 1968; Epstein, 1964) but have not been demonstrated conclusively to be the oncogenic agent. The adenoviruses have been extensively studied since they induce transformation in tissue culture cells, but they have

not been found to be oncogenic to their natural hosts (Green, 1970).

The natural host response to adenovirus infection is the production of progeny virus particles and cell death. A permissive host, which is transformed but not destroyed by adenovirus, expresses virus specific functions but rarely produces progeny virus particles (Green, 1970).

Polyoma virus induces an initial infective response in susceptible cells during which time progeny virus particles appear. Transformation of the infected cells follows, accompanied by termination of virus particle production (Eckhart, 1968).

In contrast to the DNA tumor viruses, RNA tumor viruses are oncogenic to their natural hosts as well as to permissive hosts. Transformed cells are induced to produce progeny virus particles without reducing the viability of the cells (Beard, 1963). The RNA tumor virus system therefore represents a complex picture of viral genome replication concomitant with cell transformation and compatible with cell life.

Most of the studies on the RNA tumor virus-cell interactions have been done with two groups of oncogenic RNA viruses: the avian leukemia-sarcoma viruses isolated from chickens (Ellerman and Bang, 1909; Rous, 1911) and the murine leukemia-sarcoma viruses isolated from mice (Gross, 1951). The studies presented in this report were further investigations on two of the oncogenic avian viruses,

the avian myeloblastosis virus and the MC29 virus.

The avian tumor viruses are a group of pathogens with very similar chemical composition and morphology (Bernhard, 1959; Bonar and Beard, 1959; Crawford and Crawford, 1961). Collectively they induce a wide variety of neoplastic disorders in their natural host (Beard, 1963). That they are all related is evidenced by the presence of a group specific antigen (Huebner et al., 1964) in addition to the antigen which characterizes each subgroup or virus strain (Vogt, 1965).

The principal virus system studied in this report was avian myeloblastosis virus (AMV) BAI strain A which causes an acute myeloblastic leukemia in chicks (Eckert et al., 1955). Electron microscope studies reveal the virus is typical of the avian leukemia-sarcoma group. AMV is approximately 100 millimicrons in diameter. It has an outer membrane derived from the myeloblast membrane, an inner membrane, and a dense nucleoid core (Bonar et al., 1963). It is composed of 60% w/w protein, 38% w/w lipid, and 2.2% w/w nucleic acid (Bonar and Beard, 1959). The nucleic acid is composed of several species: 1) a high molecular weight RNA with a sedimentation coefficient of 64 to 70S and a molecular weight of approximately 1×10^7 daltons, 2) low molecular weight (LMW) RNA species with sedimentation coefficients of 4 to 5S (Robinson and Baluda, 1965), and 3) a DNA species with a sedimentation value of approximately 7S

(Říman and Beaudreau, 1970).

Intimately associated with AMV particles is an adenosine triphosphatase activity (Mommaerts et al., 1952). The study of this enzyme activity lead to the discovery of the mechanism of RNA tumor virus release from transformed cells (De Thé, et al., 1963). The electron microscope studies of De Thé revealed AMV particles matured at the cell membrane of the leukemic myeloblasts. The maturing particles could be seen protruding through the cell membrane and finally budding off the surface of the cell, enveloped in the cell membrane, thus giving rise to the outer membrane of the virus particle. This evidence supported earlier evidence that the intracellular site of AMV replication was in the cytoplasm of the myeloblast (Bonar et al., 1960).

The second virus system investigated was MC29 avian leukosis virus which was recently isolated (Ivanov et al., 1964, 1965). This virus induces primarily myelocytomatosis in chicks with some incidence of primary renal and hepatic tumors (Mladenov et al., 1967). MC29 virus promises to be of particular usefulness in the molecular biology of tumor viruses since it can be successfully grown in tissue culture where it induces complete transformation of chick embryo culture cells (CEC) within 72 hours postinfection (Langlois et al., 1967; Bolognesi et al., 1968), and virus particle release is evident by 12 hours postinfection (Langlois et al., 1969; Weber, 1970). The

morphology and composition of this virus have not as yet been published, but it has been shown to contain the avian leukosis virus group specific antigen (Fritz *et al.*, 1968).

RNA Virus Genome Replication

One of the basic questions pertinent to the oncogenicity of the RNA tumor viruses is how the RNA genome of the virus particles is replicated inside infected cells. Elucidation of the replication process of viral RNA began with the RNA viruses which infect bacteria.

Bacteriophage

Investigation of the RNA replication process of RNA phages demonstrated the existence of an RNA-dependent RNA polymerase in *Eschericia coli* cells infected with MS2 (Haruna *et al.*, 1963). This work was further substantiated by the discovery of a similar enzyme, Qβ-replicase, in *E. coli* cells infected with Qβ-phage (Haruna and Spiegelman, 1965). Neither of these specific RNA-dependent RNA polymerases could be isolated from uninfected *E. coli* cells. Purified Qβ-replicase exhibited a striking preference for RNA isolated from the phage particles as template for *in vitro* synthesis of RNA. Subsequent investigations yielded proof that the *in vitro* Qβ-replicase system was synthesizing RNA strands of the same molecular weight as the RNA isolated from Qβ-phage, and the newly synthesized RNA

strands would infect *E. coli* (Spiegelman *et al.*, 1965).

RNA Viruses

Studies on the RNA genome replication of nontumor RNA viruses which infect higher organisms have only just begun. However, a number of virus-induced RNA-dependent RNA polymerases from cells infected with RNA viruses have now been reported. Among these are the poliovirus (Baltimore and Franklin, 1963), foot-and-mouth disease virus (Polatnick and Arlinghaus, 1967); influenza virus (Scholtissek and Rott, 1969a); Newcastle disease virus (Scholtissek and Rott, 1969b); mengovirus (Plagemann and Swim, 1966); and reovirus (Gomatos, 1968). In addition to the RNA-dependent RNA polymerase in infected cells, reovirus and vesicular stomatitis virus each have an RNA-dependent RNA polymerase associated with the virion itself (Banerjee and Shatkin, 1970; Baltimore *et al.*, 1970).

None of these RNA-dependent RNA polymerases have been extensively purified from infected cells, and little is known about primer specificity. RNA product is synthesized *in vitro* using endogenous RNA template which is associated with the RNA polymerase. This *in vitro* synthesis is not sensitive to actinomycin D which provides strong evidence that the observed RNA-dependent RNA polymerase has an enzyme activity unique from the cell DNA-dependent RNA polymerase. Further evidence that the RNA-dependent RNA

polymerase is actually responsible for replication of the virus RNA genome by a base complementary mechanism, stems from the ability of the *in vitro* synthesized RNA to form hybrids with RNA isolated from the infecting virus particles (Caliguiri and Tamm, 1969; Arlinghaus and Polatnick, 1969; Scholtissek, 1969; Scholtissek and Rott, 1969b; Plagemann and Swim, 1968; Banerjee and Shatkin, 1970; Baltimore *et al.*, 1970). From the results to date on these enzyme systems it appears as though nontumor animal RNA viruses may replicate by a mechanism very similar to the Qβ -phage system.

RNA Tumor Virus Replication

Information on the RNA replication process for RNA tumor viruses is notably lacking. It seems reasonable to theorize that RNA tumor virus genome replication could be mediated by an RNA-dependent RNA polymerase similar to the ones described for the other RNA viruses. An attempt to detect RNA polymerase activity ascribable to Rous avian sarcoma virus infection of CEC cells was unsuccessful with the procedures used to isolate the RNA-dependent RNA polymerase from cells infected with vesicular stomatitis virus (Wilson and Bader, 1965). However, a subsequent report on mouse spleens infected with murine leukemia virus described an RNA polymerase activity not found in normal spleens (Lin and Rich, 1968). The RNA polymerase was located principally in cell nuclei and synthesized RNA *in vitro*

from endogenous template. *In vitro* RNA synthesis was strongly depressed by actinomycin D and deoxyribonuclease, suggesting the endogenous template was DNA. More recent investigations in this laboratory (Watson and Beaudreau, 1969) have revealed the presence of a high level of RNA polymerase activity in chick myeloblasts infected with avian myeloblastosis virus. The polymerase activity was dependent on RNA primer. Heterogenous high molecular weight virus RNA was the preferred template for *in vitro* RNA synthesis, but studies on the product RNA synthesized by the myeloblast RNA polymerase were inconclusive.

From these results it would appear that nature has engineered a replication process for the genetic information of RNA tumor viruses which is not as easily discernible as the corresponding process for nontumor RNA viruses. Indeed, the differences noted could be the essence of oncogenesis. Inhibitor studies on RNA tumor virus infection and transformation processes have given rise to additional theories regarding the replication cycle of the tumor virus RNA.

Low levels of actinomycin D, which were found to inhibit myeloblast cell RNA synthesis up to 90%, were not found to inhibit the production of AMV particles (Zischka *et al.*, 1964). However, in Rous sarcoma virus (RSV) infected CEC, a higher level of actinomycin D which inhibited the chick embryo cellular RNA synthesis up to 99%, also inhibited the appearance of infective RSV up to 99% (Bader, 1964;

Temin, 1963), and the reduction of infectious virus was due to an inhibition of synthesis of RSV nucleic acid (Temin, 1964a). These actinomycin D inhibitor studies were interpreted as evidence for a step in tumor virus RNA synthesis which involved DNA-dependent RNA synthesis.

Additional inhibitor experiments indicated that DNA synthesis was necessary at the time of RSV infection for cell transformation and production of infective progeny virus particles. However, DNA synthesis was not required for production of progeny virus particles by cells already transformed by RSV (Bader, 1964; Temin, 1964a).

The apparent role of DNA-dependent RNA synthesis in the production of RSV nucleic acid, and the requirement for DNA synthesis at the time of RSV infection, prompted Temin to postulate that tumor virus RNA was transcribed into DNA in infected cells, and the new DNA species (the "provirus") served as template for replication of the tumor virus RNA (Temin, 1964a and 1964b). This theory requires an RNA-dependent DNA polymerase to transcribe the viral RNA into the cellular DNA provirus form, and predicts that a DNA species would exist in RNA tumor virus infected cells which would have a base sequence complementary to tumor virus RNA.

Hybridization experiments designed to detect a DNA species in tumor virus infected cells which was homologous to RNA isolated from the virus particles were subsequently conducted in several

laboratories. Most of the studies revealed tumor virus RNA would form hybrids with DNA from a variety of plant and animal sources (Harel et al., 1967; Wilson and Bauer, 1967; Rokutanda et al., 1970; Yoshikawa-Fukada and Ebert, 1969) with no significant differences between DNA from tumor virus-infected cells and DNA from control cells. Two notable exceptions are a report stating only the low molecular weight RNA from murine erythroblastosis virus was homologous to cellular DNA (Wollman and Kirsten, 1968) and a more recent report that infected myeloblast DNA hybridized nearly twice as much 64S RNA (highly radioactive) from AMV particles as did normal chicken DNA (Baluda and Nayak, 1970). An interesting aspect of the AMV-RNA involved in the RNA: DNA hybrid was that it was only 4 to 5% of the total viral RNA sequences and was found to contain 60% adenine (Harel et al., 1967). This is in striking contrast to the adenine content of the total AMV viral RNA which is 23% (Bonar and Beard, 1959; Robinson and Baluda, 1965). A similar analysis of the RSV-RNA involved in the DNA: RNA hybrids revealed it had a sedimentation constant of only 3S and was high in adenine and guanine in comparison to the base composition of the whole RSV-RNA molecule (Yoshikawa-Fukada and Ebert, 1969).

The hybridization studies to date have shown a small but significant number of tumor virus RNA base sequences to be complementary to a variety of sources of DNA. Baluda and Nayak are the only

investigators to report an increase in AMV-RNA hybridization to infected myeloblast DNA as compared to normal chicken DNA. They estimate 100 nucleotides of the 64S RNA from AMV are involved in the hybrid with myeloblast DNA (Baluda and Nayak, 1970). Unfortunately, the base composition of the 100 nucleotide sequence was not reported, but in this context it should be pointed out that 100 nucleotides are only 3% of the total nucleotides in the 64S RNA. The hybridization studies suggest that if DNA is transcribed from tumor virus RNA, it is not a complete transcription of the entire virus genome.

A different series of hybridization experiments revealed nuclear RNA species unique to rat embryo fibroblasts which were infected with murine sarcoma-leukemia virus. These RNA species (31-36S and 18-22S) were complementary in base sequence to 69S RNA isolated from murine sarcoma-leukemia virus particles, as determined by the formation of ribonuclease resistant RNA:RNA hybrids (Biswal and Benyesh-Melnick, 1969). These studies suggest that intranuclear RNA was transcribed from tumor virus RNA by a mechanism similar to the replication process of the nontumor RNA viruses. The inference then, is that an RNA-dependent polymerase is involved in tumor virus RNA replication, and the expressed need for DNA synthesis and DNA-dependent RNA synthesis could be a requirement for the product(s) of cellular gene expression in tumor virus replication.

Hence, current evidence on the molecular biology of RNA tumor

virus production does not provide a straight-forward RNA-dependent RNA polymerase system of replication, nor does it provide a "provirus" which is a complete DNA replica of the RNA genome contained in tumor viruses.

The recent exciting discovery of RNA-dependent DNA polymerase activity in the virions of two RNA tumor viruses (Baltimore, 1970; Temin, 1970) provided considerable support for the "provirus" theory. RNA-dependent DNA polymerase activity has now been reported in a number of tumor viruses (Spiegelman *et al.*, 1970a; Green *et al.*, 1970; Scolnick *et al.*, 1970; Gerwin *et al.*, 1970). The DNA product synthesized *in vitro* in the presence of endogenous virus template will hybridize to the virus RNA, but it has been characterized as a low molecular weight (7S) double stranded molecule (Spiegelman *et al.*, 1970a; Mizutani *et al.*, 1970; McDonnell *et al.*, 1970; Fujinaga *et al.*, 1970). The size of the *in vitro* DNA product is remarkably similar to the size of the DNA species recently reported in the virions of two of the RNA tumor viruses, AMV (Říman and Beaudreau, 1970) and murine sarcoma-leukemia virus (Biswal and Benyesh-Melnick, 1970). A 7S DNA species would not qualify as the "provirus" replica of the high molecular weight RNA in tumor virus particles, but with approximately 500 nucleotides per strand, the 7S DNA is approaching the size of the DNA isolated from tumor virus infected cells that Baluda estimated to be complementary

to the high molecular weight RNA from AMV (Baluda and Nayak, 1970).

A DNA-dependent DNA polymerase activity is also associated with the virions of RNA tumor viruses which will use DNA from a variety of sources as template for in vitro DNA synthesis (Spiegelman et al., 1970b, 1970c; Mizutani et al., 1970; Říman and Beaudreau, 1970; McDonnell et al., 1970).

Four enzyme systems are now implicated as having a possible role in RNA tumor virus replication: RNA-dependent RNA polymerase, DNA-dependent RNA polymerase, RNA-dependent DNA polymerase and/or DNA-dependent DNA polymerase. It is obvious that complete elucidation of the nature of the mechanism of tumor virus RNA replication would require isolation and characterization of virus specific replication complexes inside tumor virus infected cells, and a description of the nucleic acid polymerases and nucleic acid intermediates involved. The experimental results presented in this paper provide additional information on the nucleic acid polymerases associated with two types of avian tumor viruses, AMV and MC29, and the cell systems they infect.

The discovery of one of the corollaries to the DNA "provirus" theory of Temin (1964b), the RNA-dependent DNA polymerase, is of extreme significance to the field of RNA tumor virus investigation, and has heralded a new experimental direction for the study of oncogenesis. RNA tumor viruses could be the responsible agent in human

malignancies just as they are in the animal systems described here. Radioassays of RNA-dependent DNA polymerase activity could provide a more sensitive tool for detection of tumor viruses than the antigenic and electron microscopic methods heretofore employed. Studies presented in this paper provide evidence that a DNA polymerase very similar to the tumor virus DNA polymerase is present in human leukemic blood.

METHODS AND MATERIALS

Harvest of Virus Particles and Virus-Infected Cells

Avian Myeloblastosis BAI Strain A System

One to three day old chicks were inoculated intravenously with approximately 1×10^{11} virus particles. This inoculum resulted in an 85% incidence of acute myeloblastic leukemia and associated viremia within 10 to 14 days. Blood was collected via cardiac puncture into heparinized conical tubes and centrifuged at 1400 x g for 15 minutes to separate red cells and white cells from the plasma.

Virus Particles. Plasma which contained avian myeloblastosis virus (AMV) particles was removed from the centrifuged blood samples, treated with kieselguhr, clarified by centrifuging at 1000 x g, and stored at -20°C or -70°C. Plasma aliquots were titered for virus particles by measuring ATPase activity (Beaudreau and Becker, 1958).

Myeloblasts. Washed myeloblasts were obtained by suspending the white cell layer in a solution of 30% v/v chick serum in dilute medium 199 (Microbiological Associates, Albany, California). The cell suspension was then centrifuged at 2500 x g, the supernatant fluid discarded and the cells (approximately 95% virus infected myeloblasts) stored at -20°C or -70°C

MC29 Chick Embryo Cell Cultures

Ten-day old chick embryos were decapitated, eviscerated, minced, trypsinized, suspended in growth medium and cultured in 100 mm petri dishes by essentially the procedure of Langlois (Bolognesi *et al.*, 1968). Incomplete monolayers of chick embryo cells (CEC) were exposed to stock virus for 30 minutes, the virus medium washed off with phosphate buffered saline, and fresh culture medium replaced. Control cells were treated similarly with the omission of the virus particles.

MC29 Virus Particles. Tissue culture medium containing MC29 virus particles was decanted from the monolayers at the designated times, treated with kieselguhr, and clarified by centrifuging at 10,000 x g. The resulting supernatant medium containing virus particles was quick frozen in liquid nitrogen and stored at -70°C. Virus particle titer was determined by assaying serial dilutions of the culture medium for focus forming units (FFU) (Langlois *et al.*, 1969).

Chick Embryo Culture Cells. Infected and control non-infected CEC consisted mainly of fibroblasts and were harvested in fresh medium with a rubber policeman. The cell suspension was centrifuged at 1640 x g for 15 minutes, the supernatant medium discarded, and the cell pellets stored at -70°C.

All MC29 infected CEC, control CEC, and MC29 virus particles

were supplied for this study by George Weber of this laboratory.

Isolation and Purification of Nucleic Acids

All glassware employed in the isolation of RNA was heat sterilized at 190°C for at least one hour. All buffers, as well as the ethanol used for precipitations, were filtered through B6 nitrocellulose membrane filters (Schleicher and Schuell, Keene, New Hampshire), and the phenol (Phenol Liquified A. R., Mallinckrodt, Saint Louis, Missouri) used for protein extraction was distilled and equilibrated with approximately three volumes 0.01 M Tris ((Tris(hydroxymethyl) aminomethane) Trizma Base, Sigma Chemical Co., St. Louis, Missouri) buffer, pH 11.0.

RNA from Myeloblasts

Whole myeloblasts, or the myeloblast membrane fraction obtained during the enzyme solubilization procedure, were suspended in an appropriate volume of TNE ((0.01 M Tris-HCl, pH 8.5; 0.1 M NaCl; 1.0 mM EDTA (ethylenediaminetetraacetic acid)), made 0.5% sodium dodecyl sulfate (SDS), and allowed to clarify at 0°C for approximately 20 minutes. The mixture was centrifuged at 10,000 x g for 15 minutes and the supernatant fluid extracted with an equal volume of buffer-equilibrated phenol for 15 to 30 minutes. Following centrifugation at 10,000 x g for 15 minutes to separate the layers, the phenol

layer was reextracted with TNE, and the pooled aqueous layers adjusted to 0.4 M acetate with 3.4 M acetate buffer, pH 4.8, and the RNA precipitated by two volumes of ethanol at -20°C for two hours.

The ethanol-precipitated RNA pellet was obtained by centrifugation at 10,000 x g for 20 minutes, resuspended in an appropriate volume of TNE, and treated with 20 μg/ml DNAse ((deoxyribonuclease) 3.1.4.5 "RNAse free", Worthington, Freehold, New Jersey) at 0°C for approximately one hour. Following the DNAse treatment the phenol extraction procedure was repeated and the RNA again precipitated with ethanol as before. The ethanol precipitate was again suspended in TNE at a concentration of 5 to 10 mg/ml, made 1.0 M NaCl, and allowed to precipitate at 0°C for one hour. The salt-fractionated RNA pellet was resuspended in TNE, examined on a glycerol density gradient, and stored under ethanol at -20°C or in solution at -70°C (1 mg RNA/ml TNE).

Nucleic Acids from AMV Particles

Virus particle purification and nucleic acid extraction were performed for this study by Dr. Anne O'C. Deeney of this laboratory (Deeney *et al.*, 1969). An outline of the procedure is as follows. Plasmas containing AMV particles were thawed, treated with kieselguhr, and clarified by centrifuging at 1700 x g for 10 minutes. The clarified plasma was then vacuum filtered consecutively through a

Black Ribbon filter and a White Ribbon filter (Schleicher and Schuell). Virus particles were harvested from the filtered plasmas by centrifugation against a 100% glycerol pad at 75,000 x g for one hour, and washed twice by suspending the virus particle layer atop the glycerol pad in TNE and repeating the centrifugation. Nucleic acid extraction from the purified virus particles was performed essentially as described above for RNA extraction from myeloblasts. However, in the virus particle extraction the DNAse treatment was omitted, the salt fractionation step was not always included and 0.2 M NaCl was substituted for 0.4 M acetate, pH 4.8.

Separation of AMV nucleic acids was accomplished on 10% to 30% glycerol density gradients by centrifuging at 64,000 x g for eight hours at 2°C (sample load: 1 mg nucleic acid/30 ml gradient). The high molecular weight RNA (64S AMV RNA) was obtained by pooling the 70S fractions from the gradient, and the low molecular weight nucleic acid species (LMW AMV RNA and AMV DNA) were obtained by pooling the 7S to 4S fractions from the gradient. Further separation of the LMW AMV RNA from the AMV DNA was accomplished by preparative electrophoresis through a 10 ml 7.5% polyacrylamide gel (Busch et al., 1968). The LMW RNA had a much greater mobility through the gel than the 7S DNA and both appeared as sharp bands. The 7S DNA was further identified by its resistance to RNAse ((ribonuclease) 2.7.7.16 "DNase free" Worthington, Freehold, New

Jersey) treatment and alkali degradation (Deeney, unpublished results).

Enzyme Assay Procedures

All protein estimations were calculated from the ratio of absorbance at 280 and 260 nm for each protein fraction according to the procedure of Warburg and Christian (Warburg and Christian, 1942). RNA concentrations in solution were calculated from the absorbance at 260 nm using an extinction coefficient of 20 absorbance units per mg of RNA. DNA concentrations were estimated either from a known dry weight of purified DNA or a known extinction coefficient for the DNA species.

RNA Polymerase

RNA synthesis was assayed *in vitro* by following the incorporation of radioactively labeled ribonucleoside monophosphates into trichloroacetic acid (TCA) insoluble material. Tritium-labeled ribonucleoside triphosphates (adenosine-5'-triphosphate, ATP; cytosine-5'-triphosphate, CTP; guanosine-5'-triphosphate, GTP; uridine-5'-triphosphate, UTP) were obtained from Schwarz BioResearch or New England Nuclear at specific activities of 1 to 15 curies per millimole. α-^{32}P-UTP was synthesized at specific activities of 3 to 6 curies per millimole according to the procedure of Haruna (Haruna *et al.*, 1963) using carrier-free ^{32}P-phosphoric acid obtained from International

Chemical and Nuclear (Irvine, California), pyruvate kinase from Sigma Chemical Company (Saint Louis, Missouri), and uridine monophosphate kinase from E. coli supplied by Dr. S. Spiegelman.

Incubation mixtures for radioassay were 40 mM Tris-HCl, pH 8.5 at 37°C; 4 to 8 mM $MgCl_2$; 0 to 50 mM KCl or NaCl, 0 to 3 mM reduced glutathione (GSH); 0.2 mM in unlabeled ribonucleoside triphosphates, and 0.02 to 0.2 mM in the labeled ribonucleoside triphosphate at 4 to 8 microcuries per ml; 0 to 100 μg per ml RNA or DNA primer, and 100 to 1000 μg per ml protein fraction being assayed for enzyme activity. Unless otherwise stated the reaction mixture volume was 0.20 ml and was incubated at 37°C in a water bath. The reaction was terminated at 0°C by adding 0.4 ml of TCA mixture (equal volumes of 100% w/v TCA, saturated solution of sodium orthophosphate, and saturated solution of sodium pyrophosphate). Following a minimum of 15 minutes precipitation time at 0°C, the TCA insoluble material was collected on a nitrocellulose membrane filter (B6), washed 12 times with 4 ml of cold 10% w/v TCA, dried at least 20 minutes at 170°C, and radioactivity measured in a liquid scintillation system of 0.4% w/v BBOT (2, 5-bis-[2-(5-tert-Butylbenzoxazolyl)]-thiophene, Packard, Instrument Co., Downers Grove, Illinois) in toluene with a Packard Tri-Carb liquid scintillation spectrometer.

DNA Polymerase

The *in vitro* synthesis of DNA was radioassayed in the same manner as RNA synthesis. Unlabeled deoxyribonucleoside triphosphates (deoxyadenosine-5'-triphosphate, dATP; deoxycytosine-5'-triphosphate, dCTP; deoxyguanosine-5'-triphosphate, dGTP; and thymidine-5'-triphosphate, TTP) were obtained from P. L. Biochemicals (Milwaukee, Wisconsin). Tritium-labeled dGTP was obtained from Amersham/Searle (Chicago, Illinois) at a specific activity of 9.1 curies per millimole. All other tritium labeled deoxyribonucleoside triphosphates were obtained from Schwarz Bioresearch (Orangeburg, New York) at specific activities of 5-15 curies per millimole. Incubation mixtures for radioassay were 40 mM Tris-HCl, pH 8.0 to 8.5; 6 to 8 mM $MgCl_2$; 50 mM KCl, or 60 mM NaCl; 3 mM GSH; 0.2 mM in the unlabeled deoxyribonucleoside triphosphates; and 0.001 to 0.05 mM in the labeled primer, and 100 to 500 μg per ml protein being assayed for enzyme activity. Some virus particle enzyme preparations were assayed on the basis of numbers of virus particles or focus forming units rather than protein concentration. The human plasmas examined for DNA polymerase activity were assayed with respect to particulates obtained from a given volume of plasma instead of protein concentration. The standard reaction volume was 0.20 ml although many assays, as indicated, were 1/2 or 1/4 this amount.

Incubation, termination, and radioactivity measurement were as described for the RNA polymerase assays.

Ribonuclease

Enzymatic degradation of RNA was assayed by two methods: 1) loss of TCA-precipitable ^{14}C-uridine labeled myeloblast RNA, ^{14}C-polyadenylic acid (Poly A), ^{3}H-polycytidylic acid (Poly C), ^{3}H-polyguanylic acid (Poly G), or ^{3}H-polyuridylic acid (Poly U), or (2) by changes in sedimentation velocity constants of RNA species incubated with enzyme as determined by glycerol density gradient analysis.

Soluble RNA Polymerase Activity

Myeloblast RNA Polymerase

All enzyme isolation procedures carried out at 0 to 4°C. Soluble RNA polymerase fractions were obtained and partially purified essentially according to the procedure of Watson (Watson and Beaudreau, 1969). Twenty to 100 ml of pelleted myeloblasts were suspended in an equal volume of Buffer 0 (0.1 M Tris-HCl, pH 7.7 at 0°C; 0.01 M $MgCl_2$; 0.001 M GSH; 10% glycerol) and homogenized in a VirTis-45 blender on high speed for one minute. The homogenate was centrifuged at 28,000 x g for 30 minutes and the supernatant fluid (Sup 0) carefully removed. The membrane layer atop the pellet was

removed separately and stored at -20°C for myeloblast RNA extraction. Sup 0 was adjusted to 0.04 mg protamine sulfate per unit of absorbance at 260 nm and allowed to stand at 0°C for 20 minutes. Centrifugation of the resulting suspension at 23,500 x g for 20 minutes yielded a clear supernatant fluid (Sup 1) and a firm, protamine sulfate-precipitated pellet. Sup 1 was decanted and concentrated in dialysis tubing (pre-treated by boiling in dilute EDTA solution) surrounded with dry polyethylene glycol (PEG) 6000. The concentration was carried out at 0°C with frequent changes of PEG until approximately a three-fold increase in protein concentration was achieved.

CM-Sephadex Chromatography. The concentrated protein solution (Sup 2) was then applied to a C-50 (Pharmacia, Piscataway, New Jersey) column (2.5 x 20 cm for a 100 ml myeloblast prep) which had been previously swelled, poured, and equilibrated in 0.01 M potassium phosphate buffer, pH 8.0; 5% glycerol, and 0.001 M GSH. In some experiments 0.01 M Tris-HCl, pH 8.0, was substituted for potassium phosphate. Following sample application, the column was washed with the equilibration buffer overnight or until the absorbance at 260 nm was less than 0.1 units. RNA polymerase activity was then eluted with higher concentrations of phosphate buffer or 0.01 m Tris-HCl plus NaCl as described in Results.

Hydroxylapatite Chromatography. Hydroxylapatite columns were prepared by combining 25 grams Bio-Gel HT (Bio-Rad, Richmond,

California) with 2 grams Sephadex G-25 Coarse as a slurry in 0.01 M phosphate buffer, pH 8.0; 5% v/v glycerol, and 0.001 M dithiothreitol (DTT). The slurry was poured into a 2.5 x 20 cm column all at one time and the column bed thoroughly equilibrated with the same buffer. Protein fractions containing RNA polymerase activity were then applied to the hydroxylapatite column, and enzyme eluted stepwise, or by a continuous gradient with potassium phosphate concentrations in the equilibration buffer of 0.1 M to 0.4 M. If the protein fraction to be applied to the hydroxylapatite column was an eluate from a CM-Sephadex column, the sample was diluted with equilibration buffer to an ionic strength less than the ionic strength of 0.1 M potassium phosphate buffer.

Column Gel Filtration. Molecular sieve chromatography was carried out on Bio-Gel or Sephadex columns of the appropriate gel size: Sephadex G-25 for desalting procedures, Bio-Gel P-150 or Sephadex G-200 for protein fractionation procedures. All column beds were thoroughly equilibrated in the same buffer employed for protein elution.

Salt Fractionation. Ammonium sulfate fractionation was achieved by adding saturated ammonium sulfate (pH 7.0 to 7.5, saturated at $4^{o}C$) to the protein solution with stirring until the desired ammonium sulfate concentration was reached. The mixture was allowed to stand in ice 15 minutes prior to centrifugation at 12,000 x g

for 20 minutes. The supernatant solutions were reserved and the pellets allowed to drain before suspension in an appropriate buffer. Where indicated the pellet suspensions were dialyzed for 60 minutes against frequent buffer changes, or if concentrated protein solutions were desired, the pellets were scooped directly into dialysis tubing and the proteins dissolved during the dialysis procedure.

RNA Polymerase From Infected Spleen, Kidney, and Pancreas

Spleens, kidneys and pancreases of AMV infected chicks were removed at the time of exsanguination and stored at -70°C. Each organ was examined for soluble RNA polymerase activity by essentially the same methods employed for myeloblasts with the exception of the initial homogenization. Organ tissue was placed in liquid nitrogen in a mortar and ground with glass beads until substantial cell disruption was observed as determined by suspending a small amount of the powder in Buffer 0 and examining it under the microscope. When grinding was considered complete the powdered organ tissue was suspended in approximately one volume of Buffer 0 and centrifuged at 2,000 x g for five minutes to remove the glass beads and large debris. The pellet was washed once with a small volume of Buffer 0, recentrifuged, and the supernatant fluids combined and centrifuged at 27,000 x g for 30 minutes. The resulting supernatant protein solution

(Sup 0) was carefully removed, treated with protamine sulfate, and placed on a CM-Sephadex column as in the procedure for myeloblasts. No PEG-6000 concentration step was performed on Sup 1 from organ tissues.

Detergent-Solubilized RNA Polymerase

Myeloblast Enzyme

Myeloblasts were suspended in 1 to 3 volumes of homogenization buffer (HBD: 0.05 M Tris-HCl, pH 8.5; 0.3 to 0.4 M sucrose; 5 mM $MgCl_2$; 0 to 0.1 M KCl; 0.5 mM DTT; 0.2 mM EDTA) plus 10 to 30 μg per ml polyvinylsulfate (PVS) in some experiments and stirred at $0^{o}C$ for 15 to 30 minutes. The suspension was made 0.2 to 0.5% sodium desoxycholate (DOC) and/or 0.2 to 0.7% Triton X-100 (Rohm and Haas, Philadelphia, Penn.), and stirred an additional five minutes. The detergent treated cells were then homogenized with 5 to 10 strokes of a Potter-Elvehjem homogenizer and the homogenate centrifuged at 2,000 to 5,000 x g for 15 minutes to remove nuclei and cellular debris. The resulting supernatant fluid (Sup A) was carefully removed and reserved for glycerol density gradient centrifugation and molecular seive chromatography.

Glycerol Density Gradients. All glycerol solutions were made up in Suspension Buffer (SB: 0.01 M Tris-HCl, pH 8.5; 5 mM $MgCl_2$;

0.1 M KCl, 0.1 mM EDTA; 0.5 to 1.0 mM DTT) plus 5 to 10 μg per ml $PVSO_4$, and/or 0.1% Triton X-100 in some experiments. The density gradients were preformed in the concentration ranges indicated for each experiment in Results. Protein solutions were layered on the top of the gradient at approximately 30 mg protein per 5 ml of gradient solution and centrifuged as indicated.

Protein Concentration. Protein solutions were concentrated with Sephadex G-25 Coarse by making a slurry with 1 gm dry Sephadex for every 2.5 ml buffer to be absorbed. After swelling at $0^{o}C$ for 20 to 30 minutes the slurry was poured into 10 ml plastic syringes which had been perforated at the bottom and stoppered well with glass wool plugs. The syringes were then placed into 30 ml Corex centrifuge tubes and centrifuged at 2000 rpm for ten minutes. The concentrated protein solution could be recovered from the bottom of the centrifuge tube and the process repeated if additional concentration was necessary. A two-fold concentration of protein per operation was found to be the most convenient level of Sephadex slurry to handle. This operation did not change the buffer concentration, only the concentration of molecules with a molecular weight greater than 2500 daltons.

Column Gel Filtration. Molecular sieve chromatography was performed on 500 ml Sephadex G-200 columns which were equilibrated in SB plus in some experiments 0.5% DOC and/or 2 to 10 μg/ml $PVSO_4$.

Detergent equilibration of the column required at least 5 void volumes of buffer.

MC29 Infected CEC and Control CEC Enzyme

Virus-infected CEC and non-infected CEC were each suspended in approximately 3 volumes buffer "H" (0.10 M Tris-HCl, pH 8.0; 20% glycerol; 0.01 M NaCl; 0.2 mM EDTA; 0.5 mM DTT; 10 μg/ml $PVSO_4$), allowed to shake at 0^oC for ten minutes and then made 0.2% DOC. The detergent suspension was gently homogenized with a Ten-Broeck homogenizer and fractionated via differential centrifugation. A postnuclear supernatant was obtained by centrifugation at 1000 x g for ten minutes; a postmitochondrial supernatant by centrifugation at 11,000 x g for ten minutes, and a postmicrosomal supernatant by centrifugation at 30,000 x g for 30 minutes.

Particulate RNA Polymerase Activity

Myeloblasts from chicks exhibiting an early fulminating leukemia, MC29 infected CEC, and control CEC were each suspended in 4 volumes HB (0.05 M Tris-HCl, pH 8.3; 0.3 M sucrose, 0.05 M KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT) and swirled at 0^oC for 30 minutes. Each cell suspension was then homogenized with 20 strokes of a tight-fitting Dounce homogenizer and centrifuged at 1400 x g for five minutes. The supernatant solutions were carefully

removed and each pellet resuspended in 2 original volumes HB and homogenized again with 10 strokes of the Dounce homogenizer. The pellet homogenate was centrifuged at 1400 x g for five minutes and each supernatant solution pooled with the original. The combined supernatant solutions were rehomogenized with the Dounce homogenizer followed by a final centrifugation at 1400 x g for five minutes. The final supernatant fractions (Sup A) were reserved and further fractionated on either continuous or discontinuous glycerol density gradients.

Discontinuous Glycerol Gradients

All glycerol solutions were made up in SB. Three-phase density gradients were constructed from the following glycerol solutions: 1.0 ml 91% v/v glycerol (ρ = 1.24 gm/cc^3), 1.5 ml 75% v/v glycerol (ρ = 1.21 gm/cc^3) and 1.5 ml 45% v/v glycerol (ρ = 1.13 gm/cc^3). Thirty mg of sample protein was layered onto each gradient and centrifuged at 200,000 x g for two hours. Fractions were collected by carefully removing the visible bands with a Pasteur pipet. Aliquots from each fraction were adjusted to 0.5% Nonidet (Shell NP40), 1% DTT and incubated at 0°C for at least six hours prior to assay.

Continuous Glycerol Gradients

Preformed 40 to 95% v/v glycerol gradients were overlayed

with 30 mg protein sample per 4 ml of gradient solution. Mineral oil was added where necessary to fill the centrifuge tube. Sample components were centrifuged to equilibrium at 200,000 x g for ten hours, or 64,000 x g for 16 hours. Fractions were collected dropwise from the bottom of the tube and the density of each sample was determined from the refractive index. Each fraction was diluted with an equal volume of SB, and protein estimations were made as previously described in Methods. Aliquots of each diluted fraction were then made 0.25% Nonidet and 1% DTT by addition of one/tenth volume of a 10X stock Nonidet solution (2.5% Nonidet, 10% DTT), and stored at $0^{o}C$ for at least six hours prior to assay.

DNA Polymerase Activity

Enzyme From Virus Particles

All plasmas and tissue culture fluids were treated with kieselguhr and clarified by centrifuging at 5,000 rpm for ten minutes. Particulate DNA polymerase was then either pelleted directly or banded in a continuous glycerol density gradient.

DNA Polymerase from Pelleted Virus Particles. Virus particles were pelleted from the clarified plasmas or tissue culture fluids by centrifugation at 200,000 x g for one hour, or at 64,000 x g for two hours. Supernatant plasma was discarded, and the pellet allowed to

drain before resuspension in 2X Buffer 0 (0.05 M Tris-HCl, pH 8.3; 0.02 M $MgCl_2$; 20% v/v glycerol, 0.01 M glutathione; 1 mM EDTA; 0.3 M KCl). The pellet suspensions were then diluted with an equal volume of 2X Nonidet solution (0.5% Nonidet, 2% DTT) and allowed to stand at $0^{o}C$ for a minimum of three hours prior to assay. Human plasma pellets were obtained and treated in the same manner as the AMV particles and the MC29 virus particles.

Isopycnic Banding of Virus Particle DNA Polymerase. Equilibrium density centrifugation was performed on continuous glycerol gradients in the following manner. Virus particles were centrifuged from clarified plasmas and tissue culture fluids against a 95% glycerol pad at 200,000 x g for two hours. A continuous gradient of 35 to 85% glycerol was then poured directly onto the pad after careful removal of the supernatant plasma or culture fluid. Particulate activity was banded isopycnically by centrifugation at 200,000 x g for two hours. The gradients were fractionated by puncturing the bottom of the centrifuge tubes and collecting 0.2 ml fractions dropwise. Refractive indices were obtained to determine fraction densities, and each fraction was treated with an equal volume of 2X Nonidet solution as previously described and incubated at $0^{o}C$ for at least three hours prior to assay. Human plasma particulate DNA polymerase activity was banded, fractionated, and detergent treated in the same manner.

DNA Polymerase From Cells

Particulate DNA polymerase from myeloblasts, MC29 infected CEC, control CEC, and human chronic lymphocytic leukemia lymphocytes (CLL) was obtained by the same homogenization procedures and glycerol density gradients previously described in Methods for particulate RNA polymerase activity. Each gradient was examined as shown in Results for both RNA polymerase and DNA polymerase activities.

Product Studies

Ribonucleic Acid Product

The product RNA was examined for size by sedimentation velocity in glycerol gradients and for base composition by nearest-neighbor analyses.

Velocity Sedimentation Analysis. RNA synthesis was allowed to proceed in a standard assay as previously described. The reaction was terminated by the addition of an equal volume of cold water and made 0.4% v/v diethylpyrocarbonate (DEPC). Following vigorous shaking and standing at room temperature for two to five minutes the mixture was centrifuged in a clinical centrifuge for ten minutes to remove the DEPC-precipitated protein. The supernatant solution containing the RNA product was layered over a 10% to 30% v/v

preformed glycerol gradient (0.01 M Tris-HCl, pH 7.7, 0.1 M NaCl, 0.001 EDTA) and centrifuged at 200,000 x g (0.5°C) for the time periods indicated in Results. Control primer gradient profiles were obtained by following all steps with the omission of enzyme from the incubation mixture. Fractions were collected from the bottom of the tube and absorbance at 260 nm determined, if indicated. Each fraction was then made 10% w/v TCA and precipitated at 0°C for a minimum of 20 minutes prior to collection on nitrocellulose filters as previously described. Radioactivity was determined for each sample in the liquid scintillation system.

Nearest-Neighbor Frequency Analysis. Nearest-neighbor analyses were performed on routine RNA synthesis assays using α-^{32}P-labeled nucleoside triphosphate substrate. Carrier RNA was added when the assays were terminated with TCA mixture and each sample was collected on nitrocellulose filters. The precipitated RNA was washed off the filters with 0.3 M KOH and the suspension incubated overnight at 37°C to insure complete hydrolysis of the RNA. The hydrolysate was then chromatographed on a Dowex-formate column to separate the four nucleoside monophosphates and the separation was followed by absorbance at 260 nm (Hayashi and Spiegelman, 1961). Nearest-neighbor frequencies were calculated as the percent of radioactivity occurring in each nucleoside monophosphate peak.

Deoxyribonucleic Acid Product

Buoyant densities of product DNA were determined in CsCl gradients. A standard assay for DNA synthesis was terminated by dilution with cold water and 0.4% v/v DEPC. After vigorous shaking and standing at room temperature for two to five minutes the mixture was quick-frozen in an acetone bath at -70°C. The mixture was then thawed and added to a CsCl solution, the concentration adjusted to the median CsCl density desired for a 5 ml gradient and the gradient topped off with mineral oil. The density gradient was formed by centrifugation at 160,000 x g for 40 to 44 hours at 22°C, and fractions were collected dropwise from the bottom of the centrifuge tubes. Densities were determined from refractive indices of every fifth fraction and the absorbance at 260 nm obtained to locate the marker DNA peak. Each fraction was then made 10% TCA and collected on nitrocellulose filters as previously described in Methods for determination of radioactivity in the liquid scintillation system.

RESULTS

Soluble RNA Polymerase Activity

Previous studies in this laboratory by K. F. Watson on the RNA-polymerase which can be isolated from virus infected myeloblasts provided techniques for purifying and characterizing the enzyme activity. Conditions which were used for homogenization yielded most of the RNA polymerase in the soluble fraction from the myeloblasts. The following fractionation scheme is essentially that of Watson (Watson and Beaudreau, 1969) and the details are outlined in Methods.

Homogenization

| centrifugation

Sup 0

| protamine sulfate fractionation
| centrifugation

Sup 1

| concentration with polyethylene glycol

Sup 2

| chromatography on CM-Sephadex column
| pool peak enzyme activities

Sup 3

The soluble enzyme fractions obtained by this procedure had a dependence on exogenous RNA primer for *in vitro* RNA synthesis which increased with each purification step. Table 1 shows the specific activity of the RNA-dependent RNA polymerase from each of the

above fractions.

Table 1. Specific Activity of RNA-Dependent RNA Polymerase During Purification.

	pmoles incorp.*/60 μg protein/10 minutes			
Fraction	AMP	CMP	GMP	UMP
Sup 0	23	31	9	31
Sup 1	88	69	35	55
Sup 3	102	88	34	130

*Each assay (0.25 ml) contained 40 μg of myeloblast RNA primer. Acid-precipitable radioactivity from assays with no added RNA primer was subtracted from each value as background. Other conditions for assay are given in Methods.

CM 50-Sephadex Chromatography

Figure 1 shows the elution profile of RNA-dependent RNA polymerase from the cation exchange Sephadex column with 0.3 M phosphate buffer. Essentially the same elution profile from this type of column was obtained with 0.01 M Tris-HCl plus 0.5 M NaCl. This elution profile indicated that either the RNA-dependent RNA polymerase was composed of separate ribonucleoside monophosphate incorporating activities, or the activity of the polymerase was altered during elution of the column. Fractionation of the RNA-dependent RNA polymerase was achieved by a step-wise elution of the CM-Sephadex column. As shown in Figure 2 a polymerase fraction which incorporated primarily CMP into acid-precipitable product in the

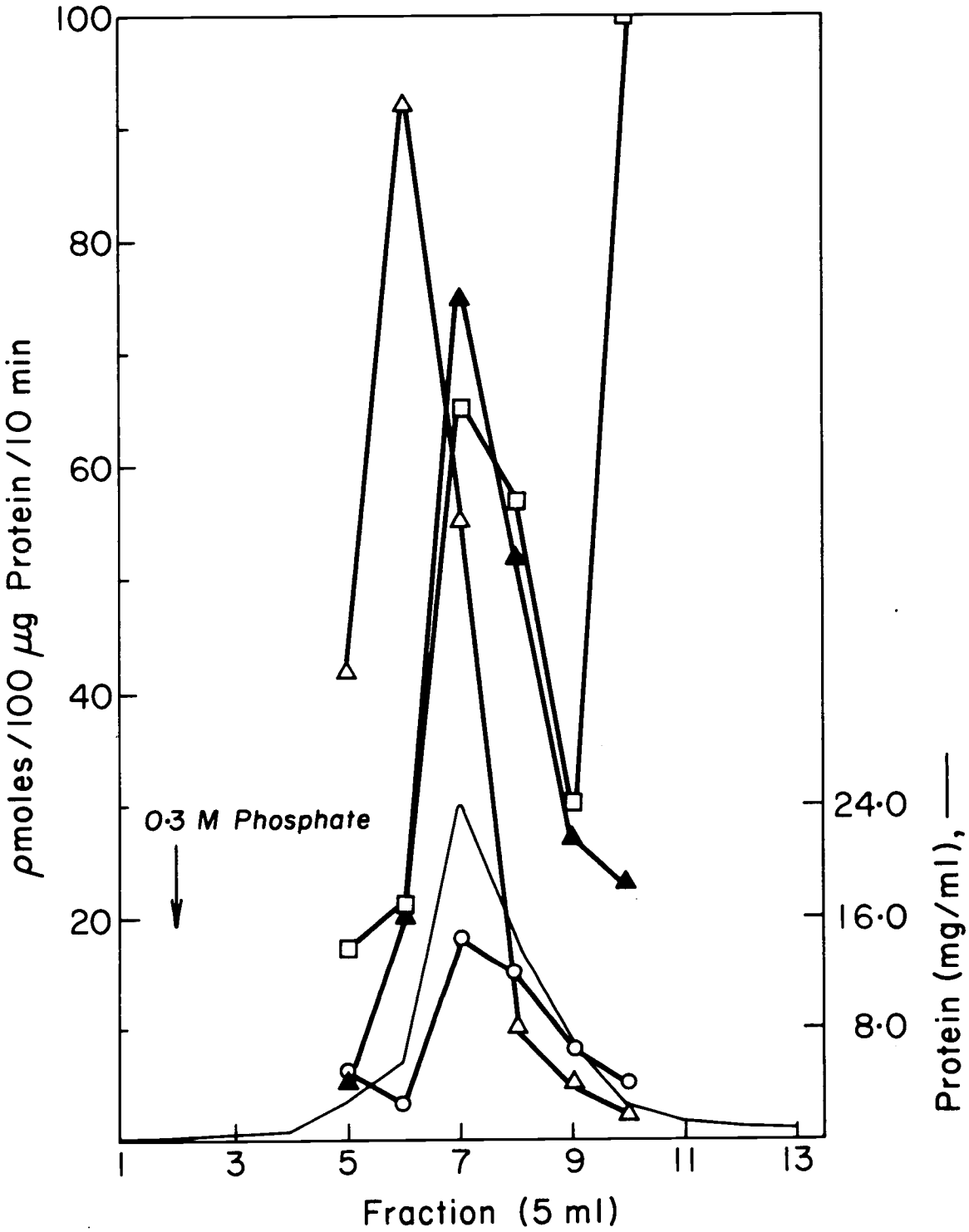


Figure 1. CM 50-Sephadex chromatography of RNA-dependent RNA polymerase. Each assay (0.25 ml) contained 40 μg myeloblast RNA primer, and was performed as described in Methods. Acid-precipitable radioactivity from assays containing no added primer was subtracted as background from each value. Specific activity of ^{3}H-ATP (▲—▲), ^{3}H-CTP (△—△), ^{3}H-GTP (o—o): 5.0 cpm/ρmole, and ^{32}P-UTP (□—□): 8.0 cpm/ρmole.

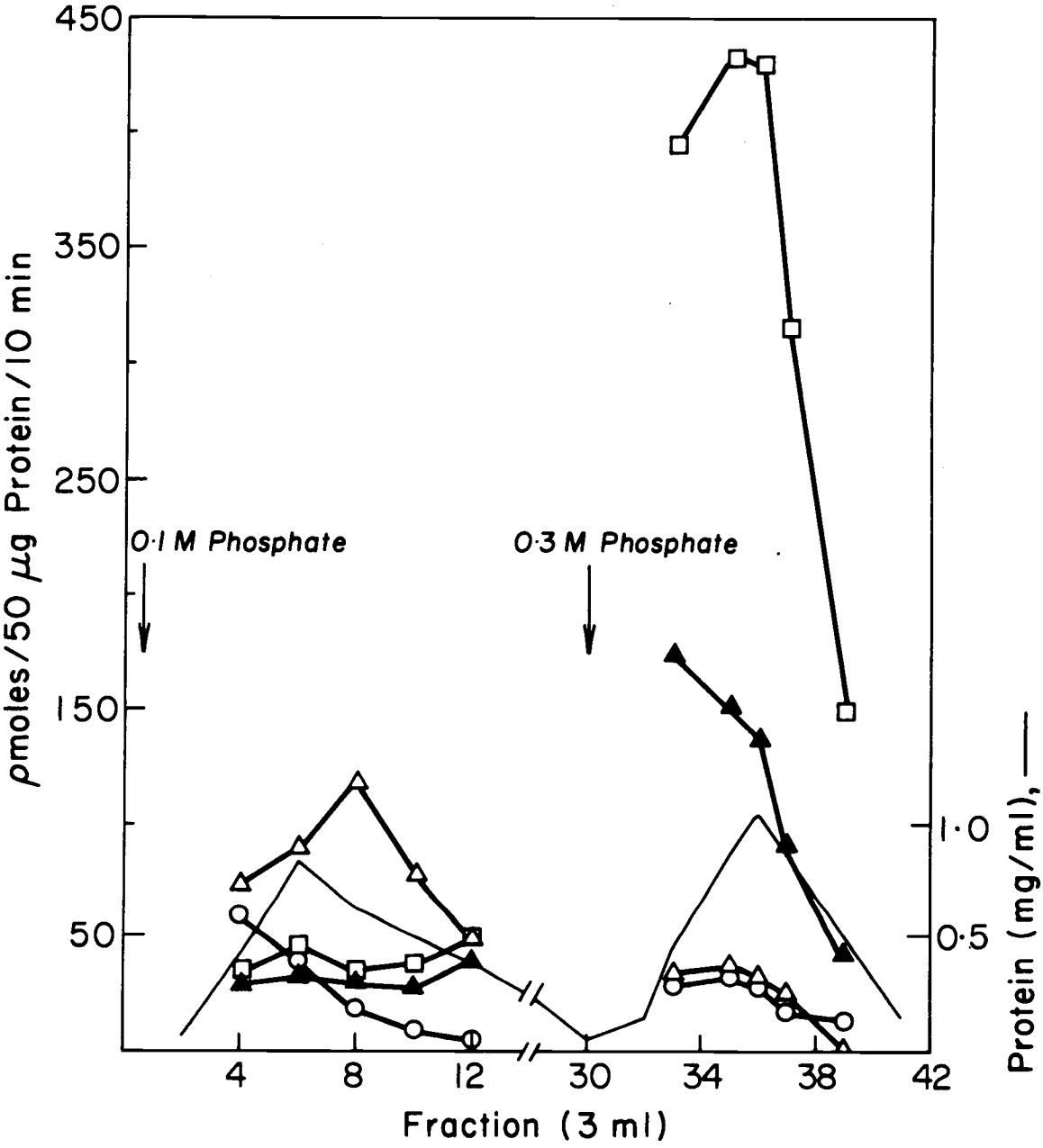


Figure 2. Step-wise elution of RNA-dependent RNA polymerase from CM 50-Sephadex. Assay conditions were the same as in Figure 1. Specific activity of ^{3}H-ATP (▲—▲): 2.5 cpm/pmole, ^{3}H-CTP (△—△) and ^{3}H-GTP (o—o): 3.6 cpm/pmole, ^{32}P-UTP (□—□): 3.1 cpm/pmole.

presence of myeloblast RNA primer (termed High C-polymerase) was eluted with 0.1 M phosphate buffer, and a second polymerase fraction which incorporated primarily UMP into acid-precipitable product in the presence of myeloblast RNA primer (termed High U-polymerase) was eluted with 0.3 M phosphate buffer.

Hydroxylapatite Chromatography

The fractionation of the RNA-dependent RNA polymerase which was observed on the CM-Sephadex column was also possible with hydroxylapatite columns as shown in Figure 3. Enzyme elution with a continuous buffer gradient of potassium phosphate (0.1 M to 0.4 M, pH 8.0) resulted in similar peaks of RNA-dependent RNA polymerase activity in the presence of myeloblast RNA primer: an early peak at 0.15 M phosphate which incorporated primarily CMP (High C-polymerase), and another peak at 0.26 M phosphate which incorporated primarily UMP (High U-polymerase) into acid-precipitable product.

Ammonium Sulfate Fractionation

The separation of the ribonucleoside monophosphate incorporating activities could be further demonstrated by ammonium sulfate fractionation. Table 2 shows the precipitation of polymerase activities from Sup 3 with increasing levels of ammonium sulfate.

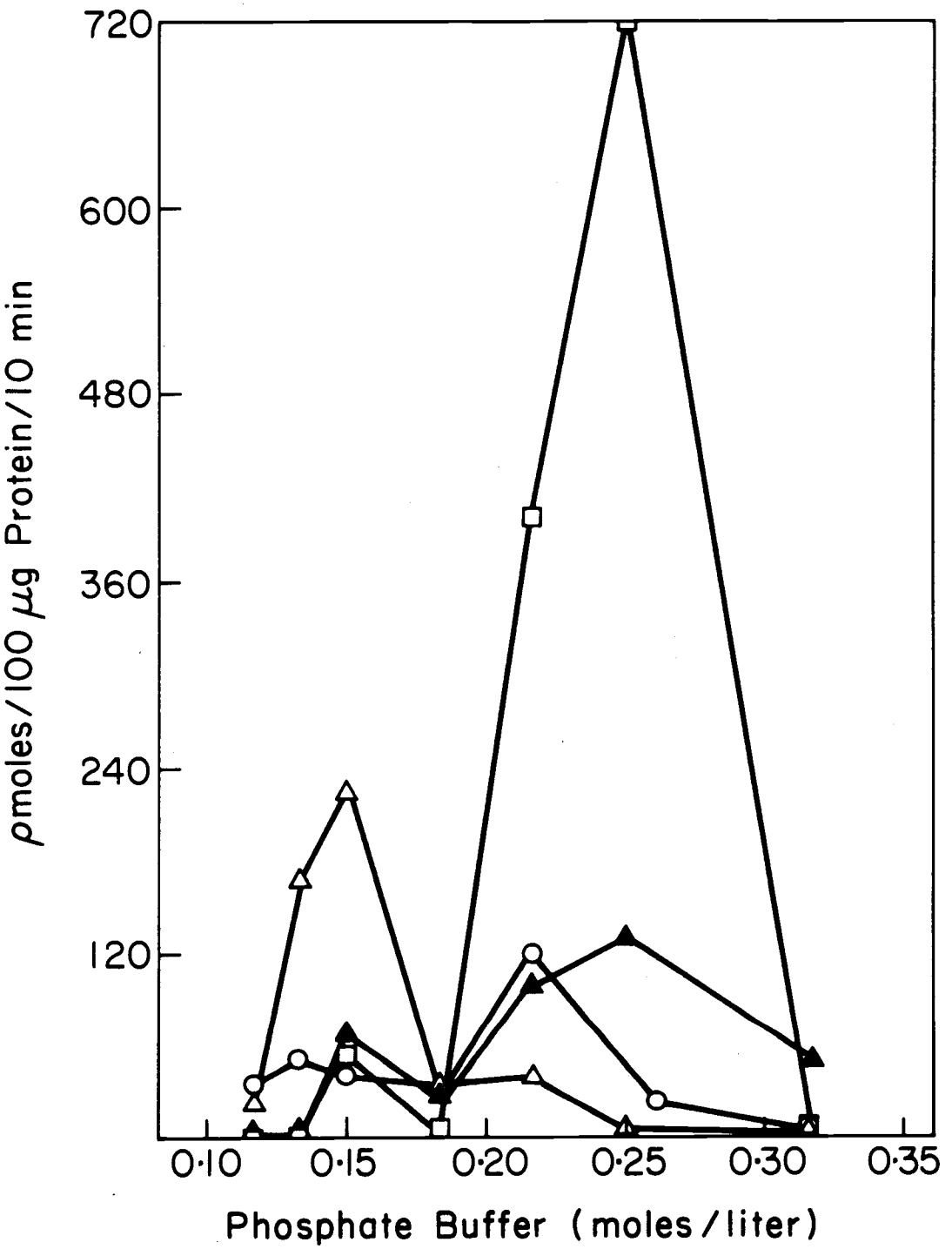


Figure 3. Hydroxylapatite chromatography of RNA-dependent RNA polymerase. Enzyme was eluted with a continuous phosphate gradient as shown. Assay conditions were the same as in Figure 1. Specific activity of ^{3}H-ATP (▲—▲): 7.0 cpm/ρmole, ^{3}H-CTP (△—△): 4.5 cpm/ρmole, ^{3}H-GTP (o—o): 5.5 cpm/ρmole, ^{32}P-UTP (□—□): 12.8 cpm/ρmole.

Table 2. RNA-Dependent RNA Polymerase Precipitation by Ammonium Sulfate.

	pmoles incorp.**/70 μg protein/10 min.			
Fraction*	AMP	CMP	GMP	UMP
Sup 3	112	70	32	107
Pellet fraction from:				
40% ammonium sulfate	173	38	58	27
58% ammonium sulfate	180	64	37	142
75% ammonium sulfate	25	13	12	245

*Each pellet from ammonium sulfate precipitation was resuspended in the buffer of Sup 3.

**Each assay (0.25 ml) contained 40 μg myeloblast RNA primer and radioactivity from assays with no added primer was subtracted as background.

As can be seen from the above table there is a High U-polymerase fraction which does not precipitate with the polymerase fractions incorporating all four ribonucleoside monophosphates into acid-precipitable product RNA.

Gel Filtration

The High-U-polymerase fraction from the CM-Sephadex column was further analyzed on a Bio-Gel P-150 column. Figure 4 shows the elution profile of the High U-polymerase and the AMP incorporating activity associated with it. The elution volume of the High U-polymerase peak indicated a molecular weight in the range of 40,000 to 50,000

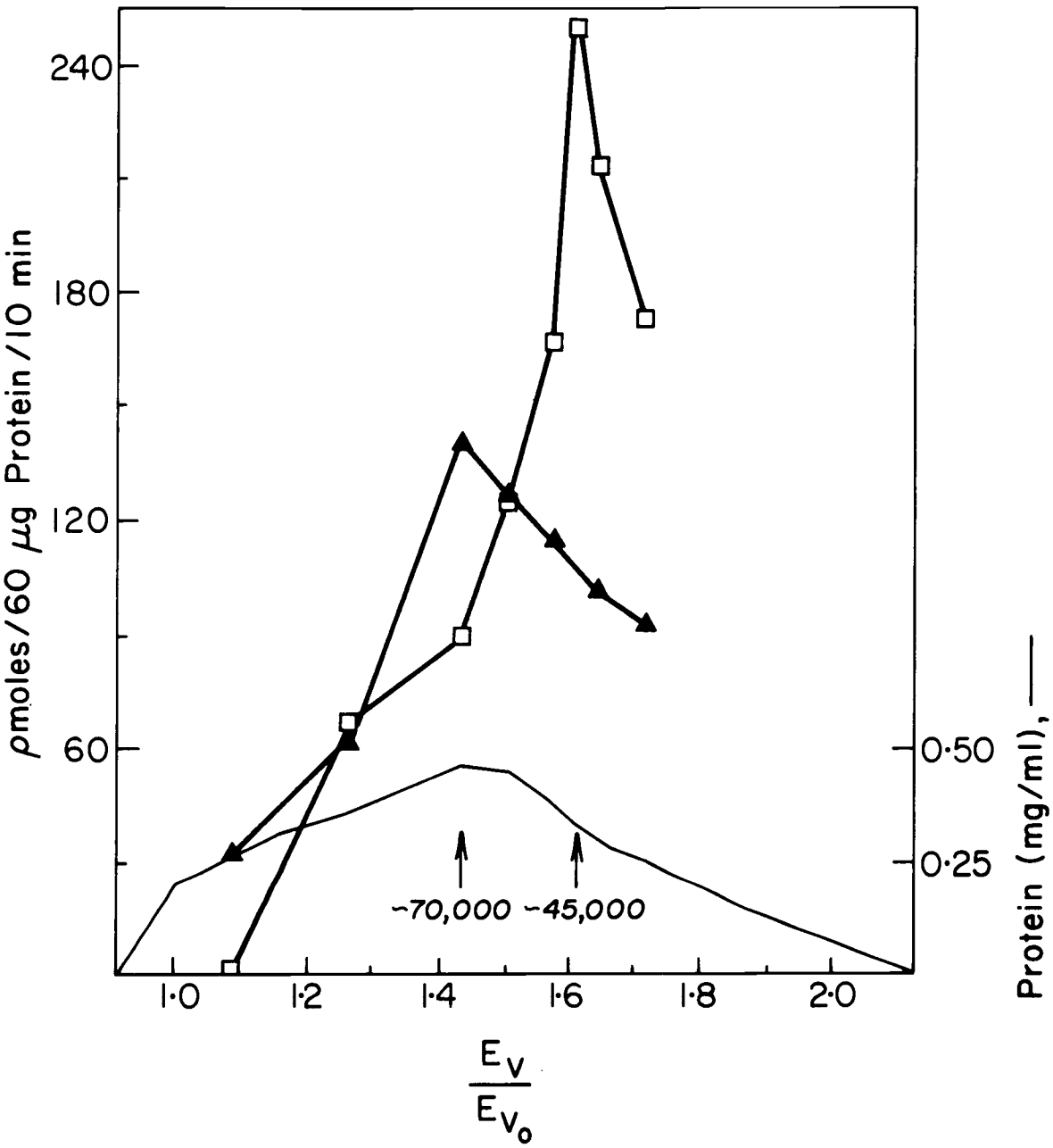


Figure 4. Gel filtration of HighU-polymerase. Chromatography was performed on Bio-Gel P-150 column (V_0 = 56 ml) as described in Methods. Each fraction (2 ml) was assayed as in Figure 1. Specific activity of ^{3}H-ATP (▲—▲): 3.1 cpm/pmole, ^{32}P-UTP (□—□): 22.0 cpm/pmole.

daltons. The AMP incorporating activity eluted in a rather broad molecular weight range with the peak activity corresponding to a molecular weight of approximately 70,000 daltons.

Ribonuclease Activity

Sup 3 contained ribonuclease activity as determined by loss of acid-precipitable ^{14}C-myeloblast RNA. This nuclease activity was also found to degrade all four synthetic ribonucleoside homopolymers: Poly A, Poly G, Poly C, Poly U. The ribonuclease activity co-chromatographed with RNA-dependent RNA polymerase on CM-Sephadex and gel filtration columns.

Primer Response

It was important to distinguish the RNA-dependent RNA polymerase from normal cellular DNA-dependent RNA polymerase. Therefore, the observed RNA stimulated RNA polymerase was challenged with various primers to see if polymers other than heterogeneous RNA would stimulate RNA synthesis. Table 3 lists the primer responses of the RNA-dependent RNA polymerase from two enzyme preparations. One fraction is unfractionated RNA polymerase in Sup 3, and the other is High U-polymerase obtained by step-wise elution of the CM-Sephadex column.

Table 3. Primer Response of RNA-Dependent RNA Polymerase.

	pmoles incorp./60 μg protein**/10 min.					
	AMP		CMP		UMP	
Primer*	Sup 3	High-U polymerase	Sup 3	High-U polymerase	Sup 3	High-U polymerase
DNA (calf thymus)	57	--	28	--	4	--
Myeloblast RNA	258	73	209	38	54	567
Poly A	210	60	--	--	3	94
Poly C	--	--	17	--	--	--
Poly G	--	--	16	--	--	--
Poly U	0	1	--	--	1	0
Poly AC	5	28	14	--	28	34
Poly AG	90	40	34	--	54	472
Poly AU	20	5	--	--	27	2

*All activities are with 20 μg primer except the myeloblast RNA which was used at a level of 40 μg.
**Acid-precipitable radioactivity with no added primer in an assay was subtracted as background for each value. Specific activity of ^{3}H-ATP and ^{3}H-CTP was 5 cpm/pmole, ^{32}P-UTP was 12.2 cpm/pmole.

It should be noted that the amount of poly A primed AMP incorporating activity could be reduced in Sup 3 by extensive washing of the CM-Sephadex column with 0.01 M phosphate buffer prior to elution with 0.3 M phosphate buffer. Poly A polymerase activity has been demonstrated to be a normal cell component of rat liver and developing sea urchin embryos (Roeder and Rutter, 1969).

The above studies demonstrate that RNA heteropolymer was found to be the best primer for RNA synthesis. The synthetic polymers which did stimulate UMP incorporation imply a complementary base pair copying mechanism for the polymerase. This is indicated by the stimulation of UMP incorporation observed with the polymers complementary to U, Poly A and Poly AG, whereas, Poly U and Poly AU showed little if any priming effect. CMP incorporation is also stimulated by complementary Poly AG twofold over the Poly AC stimulation. These findings are not supported, however, by the primers which stimulated the incorporation of AMP by the RNA polymerase. Poly U, complementary to A, did not serve as primer for AMP incorporation and Poly AU had less stimulation than Poly AG for AMP incorporation. The ribonuclease activity present in this enzyme fraction would significantly influence the apparent primer response of the RNA polymerase. The important observations from these experiments were that DNA was not an effective primer for *in vitro* RNA synthesis, and that heteropolymer RNA was the most effective primer for *in vitro*

RNA synthesis.

Kinetics

Watson observed that the RNA-dependent RNA polymerase preferred high molecular weight (64S) RNA from AMV particles as primer for RNA synthesis (Watson and Beaudreau, 1969). This observation was supported by the kinetics of RNA synthesis shown in Figure 5. These results compare myeloblast RNA and 64S RNA from AMV as primers for the RNA-dependent RNA polymerase. Ten μg of 64S AMV RNA (approximately 6×10^{11} strands of RNA) stimulates RNA synthesis to a higher level and for a longer time period than 40 μg of myeloblast RNA primer (approximately 120×10^{11} strands of RNA). The level of GMP incorporation remains low for both primers.

Ribonucleoside Triphosphate Requirement for RNA Synthesis

The RNA-dependent RNA polymerase did not require all four ribonucleoside triphosphates to synthesize acid-precipitable RNA product. Table 4 illustrates the incorporation of UMP into acid precipitable product in the absence of other ribonucleoside triphosphates from the assay mixture.

Figure 6 illustrates the kinetics of RNA synthesis by the RNA-dependent-RNA polymerase in the presence and absence of combinations of the four ribonucleoside triphosphates. These results indicate

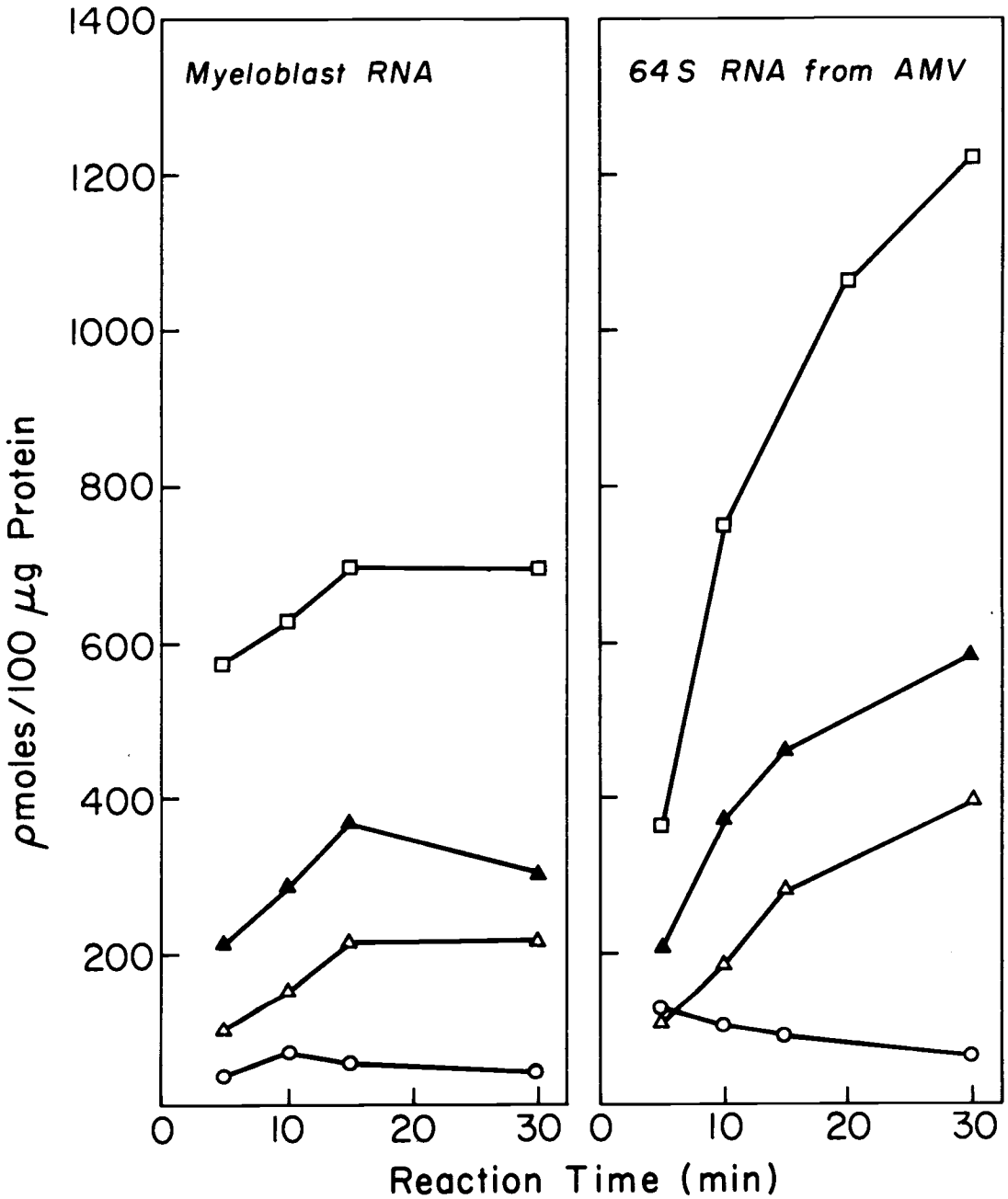


Figure 5. Kinetics of RNA synthesis with myeloblast RNA primer and 64S RNA primer from AMV. Assays of enzyme in Sup 3 contained either 40 μg myeloblast RNA primer, or 10 μg 64S RNA primer from AMV, and the conditions were as in Figure 1. Specific activity of ^{3}H-ATP (▲—▲) and ^{3}H-CTP (△—△): 5.0 cpm/pmole, ^{3}H-GTP (o—o) 6.2 cpm/pmole, ^{32}P-UTP (□—□) 9.2 cpm/pmole.

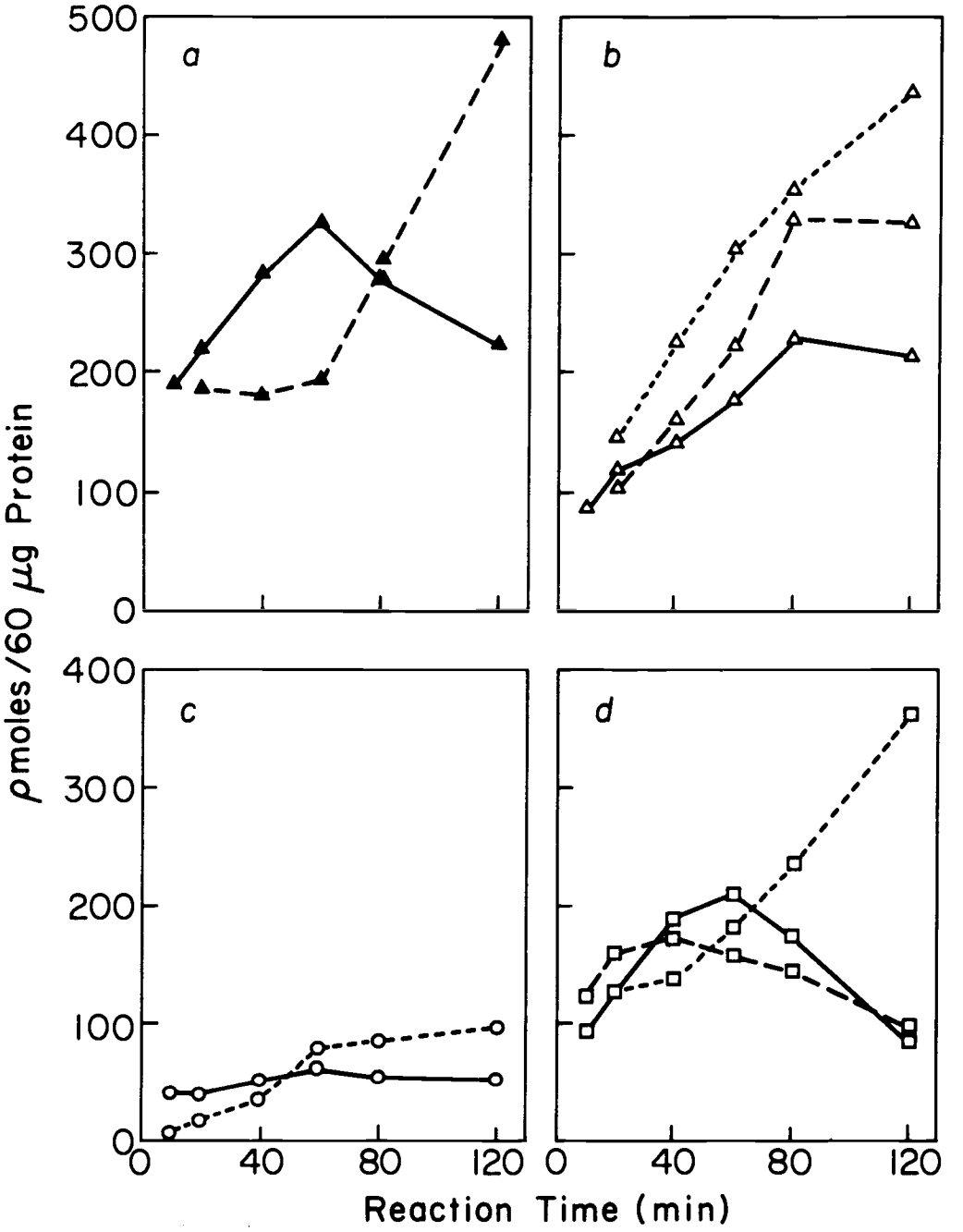


Figure 6. Effect of omission of ribonucleoside triphosphates on RNA synthesis. Each assay contained enzyme from Sup 3 and 40 μg myeloblast RNA primer. Assay conditions were the same as in Figure 1 except ribonucleoside triphosphates were omitted in some assays: (a) ^{3}H-ATP, specific activity = 3.6 cpm/pmole, (▲—▲) complete, (▲- -▲) minus UTP; (b) ^{3}H-CTP, specific activity = 3.5 cpm/pmole, (Δ—Δ), complete, (Δ- -Δ) minus UTP, (Δ···Δ) minus GTP; (c) ^{3}H-GTP, specific activity = 4.5 cpm/pmole, (o—o) complete, (o- - o) minus UTP or minus CTP; (d) ^{32}P-UTP, specific activity = 12.2 cpm/pmole, (□—□) complete, (□- -□) minus CTP, (□····□ minus CTP and GTP.

more than one type of reaction may be catalyzed according to the ribonucleoside triphosphate complement available to the RNA polymerase. This is consistent with Watson's observations on the RNA product synthesized by RNA-dependent RNA polymerase with 64S RNA from AMV as primer in the presence and absence of one or more of the ribonucleoside triphosphates (Watson and Beaudreau, 1969). In the presence of all four ribonucleoside triphosphates RNA polymerase from Sup 3 synthesized product which sedimented with the primer to the high molecular weight region of a glycerol density gradient. If one or more of the ribonucleoside triphosphates were omitted from the assay the 64S RNA primer from AMV was considerably degraded even at short (five minute) incubation times and the product synthesized by the RNA polymerase was low molecular weight

Table 4. RNA-Dependent RNA Polymerase Dependence on Ribonucleoside Triphosphates.

Assay system	pmoles ^{32}P-UMP incorp.*/10 min.
Complete	124
minus GTP	112
minus GTP and CTP	111
minus GTP, CTP and ATP	115

*Each assay contained 40 μg protein from Sup 3 plus 40 μg myeloblast RNA. Acid precipitable radioactivity in assays containing no myeloblast RNA was subtracted as background from each value. Specific activity of ^{32}P-UTP was 20.1 cpm/pmole.

Enzyme Fraction Incorporating
Only UMP (UMP-Enzyme)

An enzyme fraction was obtained which incorporated only UMP into acid-precipitable product as detected by the standard assay method. The UMP-enzyme was obtained by chromatographing the High U-polymerase fractions from the CM-Sephadex column on an hydroxylapatite column which was eluted with a shallow phosphate gradient as shown in Figure 7; the peak UMP-enzyme fractions were subsequently desalted on a Sephadex G-25 column. The resulting UMP-enzyme was associated with little if any ribonuclease activity, and was further characterized by product studies and nearest neighbor analyses as will be discussed later. (It should be noted that the High C-polymerase obtained in the 0.1 M phosphate fraction from the CM-Sephadex column was purified in the same manner as the UMP-enzyme, but the procedure did not appreciably decrease the amount of ribonuclease activity associated with the High C-polymerase.)

RNA Product Studies

Polyvinylsulfate inhibits ribonuclease activity (Shinozawa *et al.*, 1968). Low levels of polyvinylsulfate in assays of RNA-dependent RNA polymerase activity were found to inhibit the degradation of RNA primers. Polyvinylsulfate also had an effect on the RNA products synthesized by RNA-dependent RNA polymerase, as discussed below.

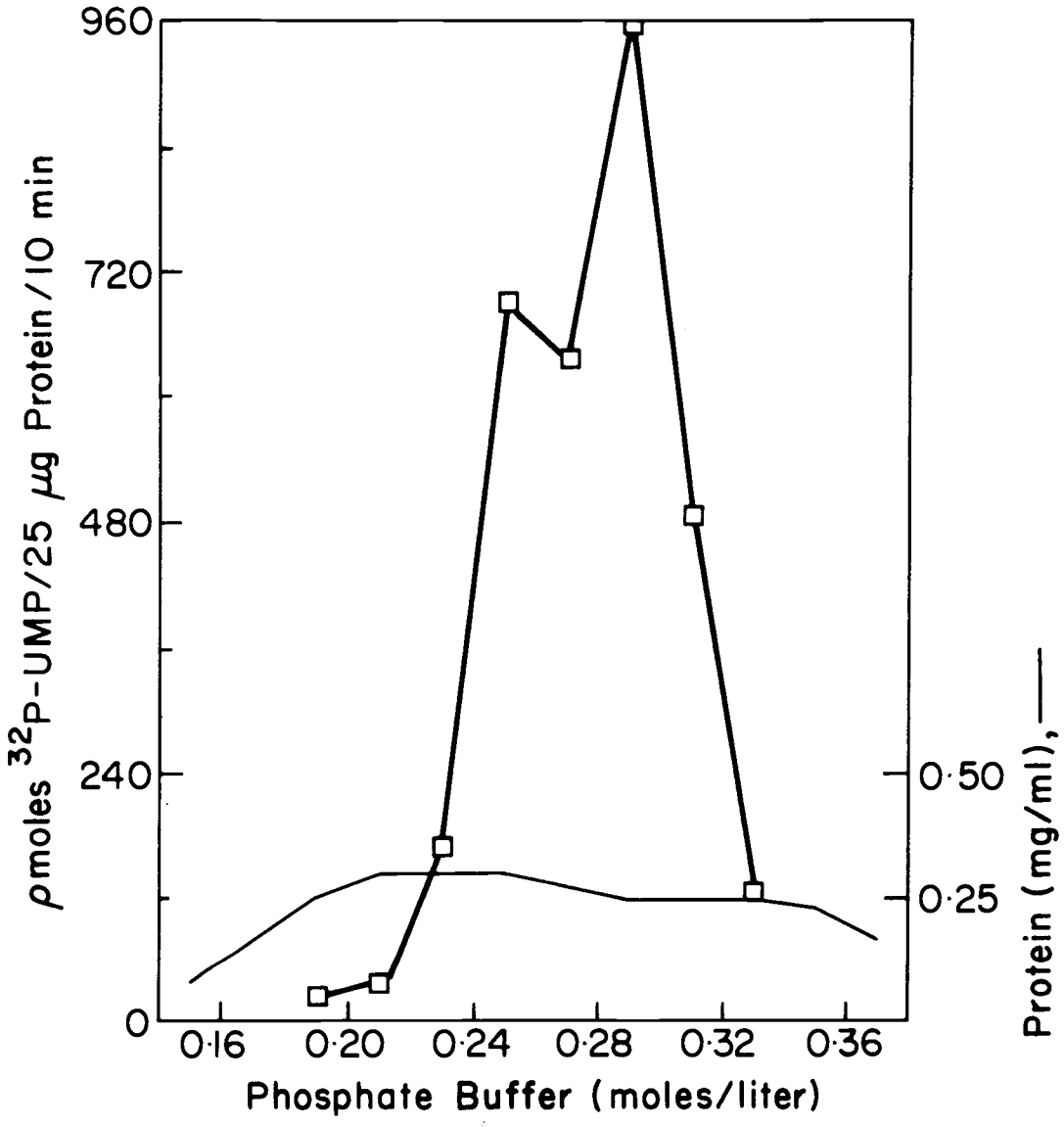


Figure 7. Hydroxylapatite chromatography of UMP-enzyme. Enzyme was eluted in a continuous phosphate gradient as shown and each fraction was assayed as described in Figure 1. Specific activity of ^{32}P-UTP : 11.5 cpm/ρmole.

Velocity Sedimentation Analysis. Figure 8 shows the velocity sedimentation profiles of myeloblast RNA primer and radioactive RNA product from two RNA-dependent RNA polymerase assays containing different levels of polyvinylsulfate. The assays yielded comparable total amounts of acid-precipitable RNA product (approximately 640 pmoles) but with the higher level of polyvinylsulfate an increased fraction of both primer and product sedimented into the high molecular weight region of the velocity gradient.

Figure 9 shows the velocity sedimentation profiles of 64S RNA primer from AMV and RNA product containing both ^{32}P-UMP and ^{3}H-GMP from two RNA polymerase assays at different levels of polyvinylsulfate. No appreciable degradation of 64S primer RNA was observed in either assay, but the level of polyvinylsulfate present had a significant effect on the RNA product synthesized.

The incorporation of UMP into product which sedimented as a high molecular weight species was the same at both levels of polyvinylsulfate. However, the amount of UMP incorporated into RNA product which sedimented as a low molecular weight species was greatly reduced by the higher level of polyvinylsulfate.

The opposite response was observed for the incorporation of GMP into RNA product. The amount of GMP observed in product sedimenting as a low molecule weight RNA species was the same at both levels of polyvinylsulfate, but the GMP detected in product which

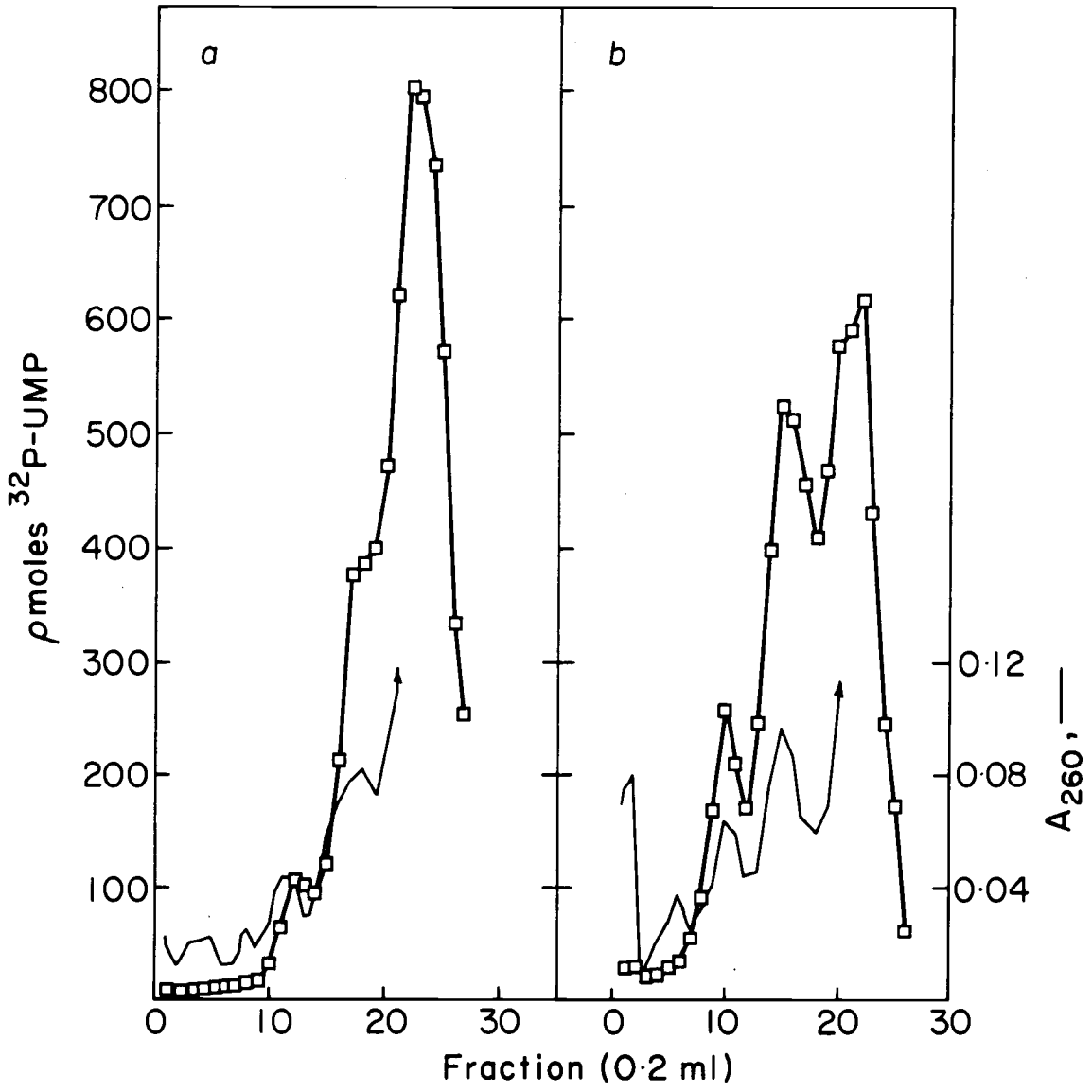


Figure 8. Velocity sedimentation characteristics of myeloblast RNA and RNA product synthesized in the presence of polyvinylsulfate. Two assays containing 100 μg protein (Sup 3) and 40 μg myeloblast RNA were incubated for 10 minutes under the conditions described in Methods, (a) contained 0.5 μg polyvinylsulfate, and (b) contained 1.0 μg polyvinylsulfate. The assays were terminated and velocity sedimentation analysis performed as described in Methods. Specific activity of ^{32}P-UTP (□—□) : 8.8 cpm/pmole.

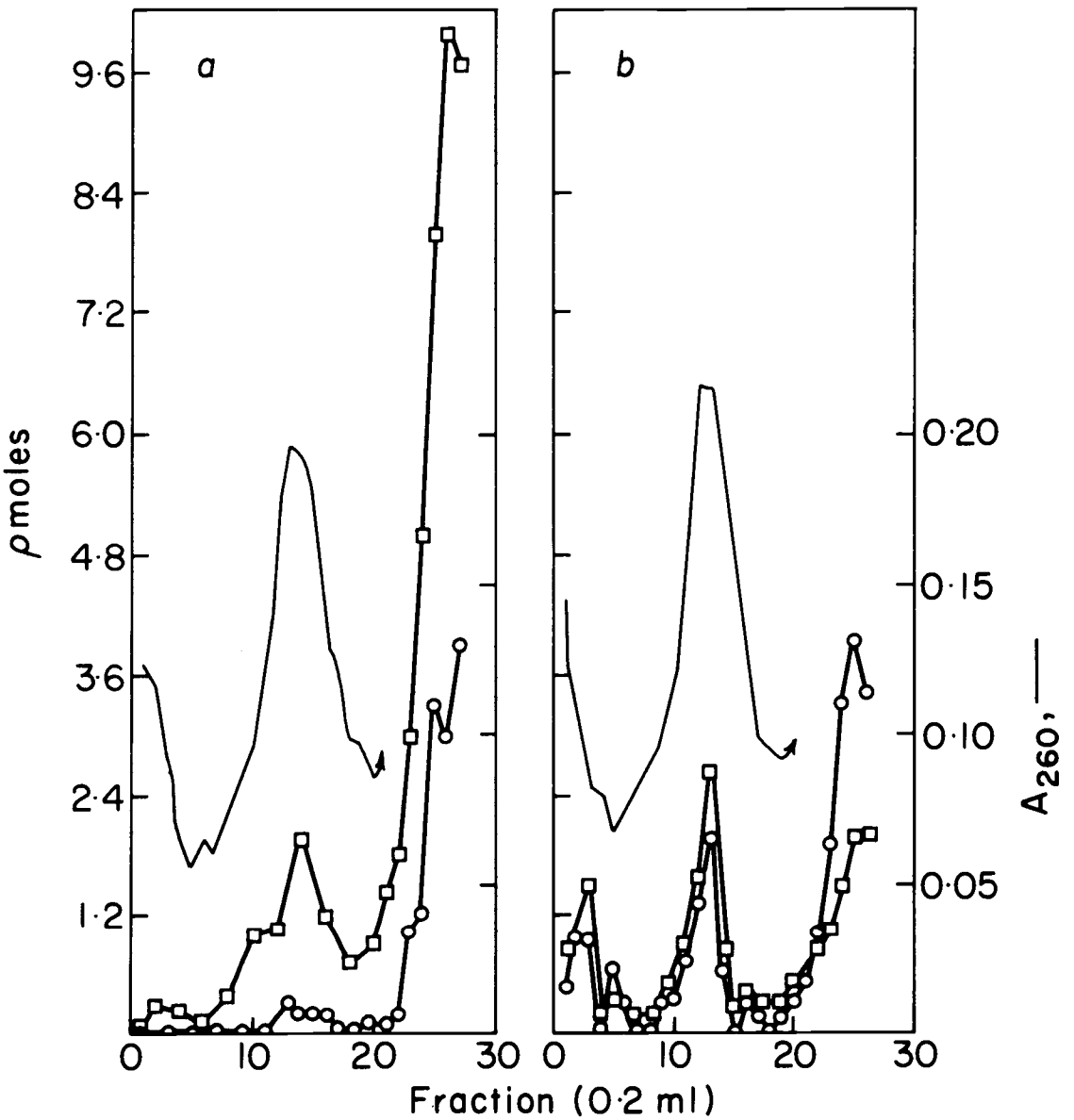


Figure 9. Velocity sedimentation analysis of 64S RNA from AMV and RNA product synthesized in the presence of polyvinylsulfate. Two assays containing 240 μg protein (Sup 3) and 40 μg 64S RNA from AMV were incubated for 5 minutes under the conditions described in Methods, (a) contained 0.75 μg polyvinylsulfate, and (b) contained 1.0 μg polyvinylsulfate. Velocity sedimentation analysis was performed as described in Methods. Specific activity of ^{3}H-GTP (o—o): 30 cpm/ρmole, ^{32}P-UTP (□—□): 22 cpm/ρmole.

sedimented with the 64S primer RNA was markedly increased at the higher level of polyvinylsulfate.

These data suggested that the inhibition of UMP incorporation into low molecular weight RNA product occurred concomitantly with a net synthesis of high molecular weight RNA product containing GMP. Under assay conditions which allowed the incorporation of GMP into RNA product sedimenting at 64S, another RNA product peak appeared which sedimented at greater than 64S.

Interpretation of these results suggests several possibilities. One is that the polyvinylsulfate inhibited ribonuclease degradation of GMP regions of the RNA product which sedimented with the 64S primer. Another possibility is that GMP incorporation into high molecular weight RNA product is dependent upon the integrity of the 64S RNA primer, and nicks introduced into the primer by ribonuclease prevented the synthesis of high molecular weight product containing GMP. A third possibility is that the polyvinylsulfate inhibited a UMP-enzyme which synthesizes poly U and competes with the RNA-dependent RNA polymerase for 64S RNA primer. This third possibility will be further discussed in the following section.

Polyvinyl sulfate was found to inhibit the UMP-enzyme as shown in Figure 10. Figure 10a is the velocity sedimentation profile of 64S RNA primer from AMV and the product synthesized by the UMP-enzyme. No detectable primer breakdown was noted on this gradient

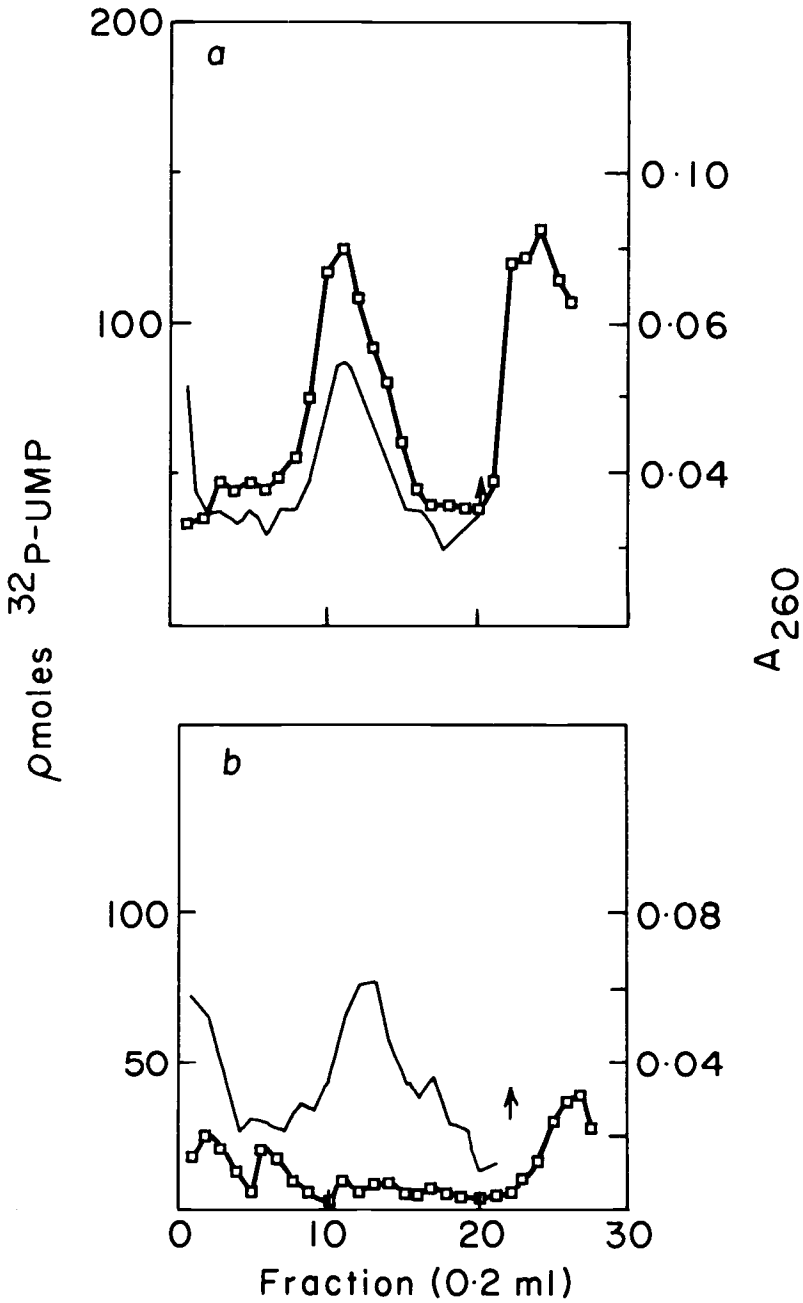


Figure 10. Effect of polyvinylsulfate on UMP-enzyme activity. Two assays containing 30 μg protein and 15 μg 64S RNA from AMV were incubated for 5 minutes under the conditions described in Methods, (a) contained no polyvinylsulfate (b) contained 0.25 μg polyvinylsulfate. Velocity sedimentation analyses were performed as described in Methods. Specific activity of ^{32}P-UTP (□—□) : 33 cpm/ρmole.

in the absence of polyvinyl sulfate. Figure 10b illustrates the marked inhibition of the UMP-enzyme activity by a low level of polyvinylsulfate in the presence of 64S AMV-RNA primer. It seems reasonable to postulate polyvinylsulfate was a competitive inhibitor of UMP-enzyme binding to RNA primer. The UMP-enzyme was found to be a relatively low molecular weight molecule which was highly positively charged at pH 8.0, and hence could be expected to bind to polyvinylsulfate which is a polyanion.

These product studies indicated that under carefully controlled assay conditions RNA product could be detected in the high molecular weight region of velocity sedimentation gradients. Polyvinylsulfate was found to inhibit the ribonuclease activity thus protecting the RNA primer and the newly synthesized RNA product. In addition, polyvinylsulfate also inhibited the UMP-enzyme and the evidence suggests this may have been necessary for the RNA-dependent RNA polymerase to synthesize heteropolymer RNA product which sedimented with the 64S RNA primer.

Nearest Neighbor Frequencies. Table 5 lists the nearest neighbor frequencies of RNA product synthesized by two different enzyme fractions. One fraction was High U-Polymerase, obtained by the step-wise elution of the CM-Sephadex column, which incorporated UMP into product ten-fold higher than AMP or CMP and 30-fold higher than GMP with 64S RNA primer from AMV. The other fraction was

the UMP-enzyme which incorporated no detectable CMP, AMP, or GMP into RNA product in the presence of 64S RNA primer from AMV.

Table 5. Nearest Neighbor Frequencies of *in vitro* RNA Product.

	Percent of Total	
Couplet	High U-polymerase	UMP-enzyme
ApU	21.5	21.5
CpU	13.4	9.0
GpU	10.9	7.4
UpU	54.2	62.2

As would be expected from the high level of UMP incorporated by each of these polymerase fractions, UpU was the predominant couplet in the product RNA. What was not expected was the fact that the frequency of AMP next to UMP remained high in the products of both polymerase reactions even though there was no detectable AMP incorporation with the UMP-enzyme. These results together with the appearance of acid-precipitable UMP product when any one or all of the other three ribonucleoside triphosphates were omitted from an assay, indicated that some of the observed UMP incorporating activity was poly U synthesis at the ends of the RNA primers. The above nearest neighbor analyses strongly suggest adenosine is the terminal residue at the 3'-hydroxyl end of the 64S RNA from AMV.

Watson observed nearest neighbor frequencies on the product synthesized by RNA-dependent RNA polymerase in unfractionated

Sup 3 more consistent with heteropolymer synthesis (Watson and Beaudreau, 1969). Thus, fractionating the RNA-dependent RNA polymerase may select for an enzyme which adds UMP onto the ends of primer molecules, or actually confer such end-addition activity on the polymerase.

Spleen, Kidney, and Pancreas

The cells of these organs from chicks with avian myeloblastosis contain AMV particles (De Thé, 1964). Each of these organs was obtained from AMV infected chicks and investigated for RNA-dependent RNA polymerase activity.

Figure 11 shows the elution profile from a CM-Sephadex column of RNA-dependent RNA polymerase from infected kidneys. This profile was the same as the activity profile found in the infected myeloblasts (Figure 1).

Similar results were obtained with infected spleens, but the specific activity of the RNA polymerase was somewhat lower. Essentially no RNA polymerase activity was detected in the infected pancreases, but this could be due to the very high level of ribonuclease activity found in all fractions of the pancreas.

Summary of Soluble RNA-Dependent RNA Polymerase

Myeloblasts, spleens, and kidneys from chicks infected with

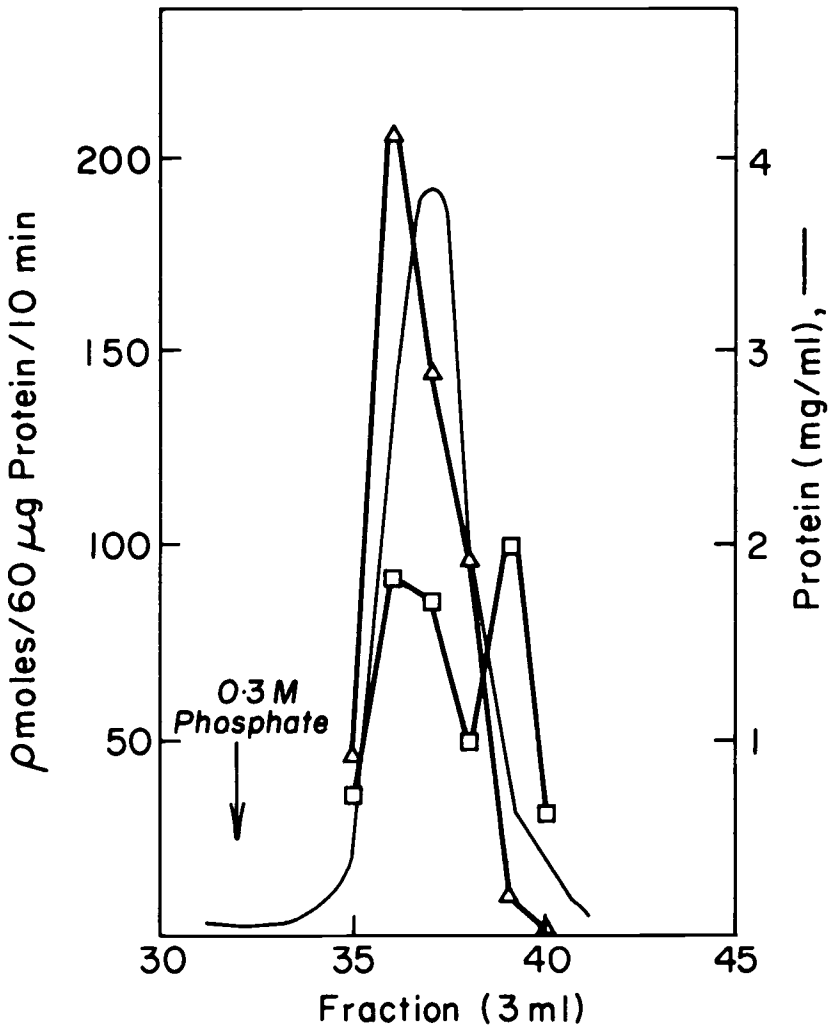


Figure 11. CM 50-Sephadex chromatography of RNA-dependent RNA polymerase from virus-infected kidney cells. Each fraction was assayed as described in Figure 1. Specific activity of ^{3}H-CTP (Δ—Δ) : 5.5 cpm/ρ mole, ^{32}P-UTP (□—□) : 47.8 cpm/ρ mole.

AMV contain a RNA-dependent RNA polymerase which was isolated and partially purified. Heteropolymer RNA, particularly 64S RNA from AMV, served as the best primer for RNA synthesis. The RNA-dependent RNA polymerase appeared to be a complex of more than one protein since it could be salt fractionated into separate nucleoside monophosphate incorporating activities. Ribonuclease activity was closely associated with the RNA polymerase and was notably dissociated only from the UMP-enzyme.

Several lines of evidence indicated the RNA-dependent RNA polymerase incorporated ribonucleoside monophosphates into RNA product by more than one mechanism. The following data suggested an end-addition mechanism for the RNA polymerase: UMP was incorporated into acid-precipitable product in the absence of the other three ribonucleoside monophosphates; the frequency of AMP next to UMP was 21% in the product from a reaction in which UMP was the only detectable ribonucleoside monophosphate incorporated.

A strict end-addition mechanism was not supported, however, by the information that 64S RNA from AMV served as the best primer for RNA synthesis, neither Poly U nor Poly AU stimulated the incorporation of UMP into acid-precipitable product; and under the appropriate assay conditions UMP and GMP were incorporated in a 1:1 ratio into two RNA product species which sedimented into the high molecular weight region of glycerol velocity gradients.

Detergent Solubilized RNA Polymerase

The low level of GMP incorporation by the RNA-dependent RNA polymerase from the soluble fractions of the myeloblasts, prompted studies searching for RNA polymerase activity with levels of GMP incorporation into RNA product which were closer to the incorporation of the other three nucleoside monophosphates. For this effort the efficacy of detergent treatment of the myeloblasts was explored.

The following is the general fractionation scheme for the detergent solubilized RNA polymerase fractions from myeloblasts. The details are given in Methods.

Homogenization
| centrifugation
Sup A
| glycerol density gradient
| pool peak activities
Sup B
| Sephadex G-200 chromatography
| pool peak activities
Sup C

A low level of detergent (0.2% DOC or 0.3% Triton X-100) during homogenization of the myeloblasts was found to solubilize an increased level of RNA polymerase activity as shown in Table 6. Care was taken during the homogenization procedure to solubilize as much RNA polymerase activity as possible with minimal disruption to the myeloblast

nuclei. Early detergent solubilization procedures were followed with frequent microscopic examination of the myeloblasts and the liberated nuclei.

Table 6. Increase in RNA Polymerase Activity by Detergent Treatment of Myeloblasts.

	pmoles/170 μg protein*/10^1			
	No detergent		With detergent**	
Enzyme Fraction	GMP	UMP	GMP	UMP
Sup A	24	46	43	97
Sup B	40	85	78	129

*Assays (0.25 ml) contained 40 μg myeloblast RNA. Background of 20 cpm subtracted. (^{3}H-GTP: 25 cpm/pmole, ^{32}P-UTP: 20 cpm/pmole).
**Homogenization buffer contained 0.2% DOC. All other details of assay in Methods.

Glycerol Density Gradients

Figure 12 is the RNA polymerase activity of each fraction from a 20 to 40% glycerol density gradient showing both UMP and GMP incorporation into RNA product. The enzyme profile shown was endogenous RNA polymerase activity with no RNA primer added to the assays. Myeloblast RNA primer in the assays of the gradient fractions stimulated the incorporation of UMP into product RNA to a higher level than GMP incorporation.

The peak RNA polymerase fractions were treated with a high

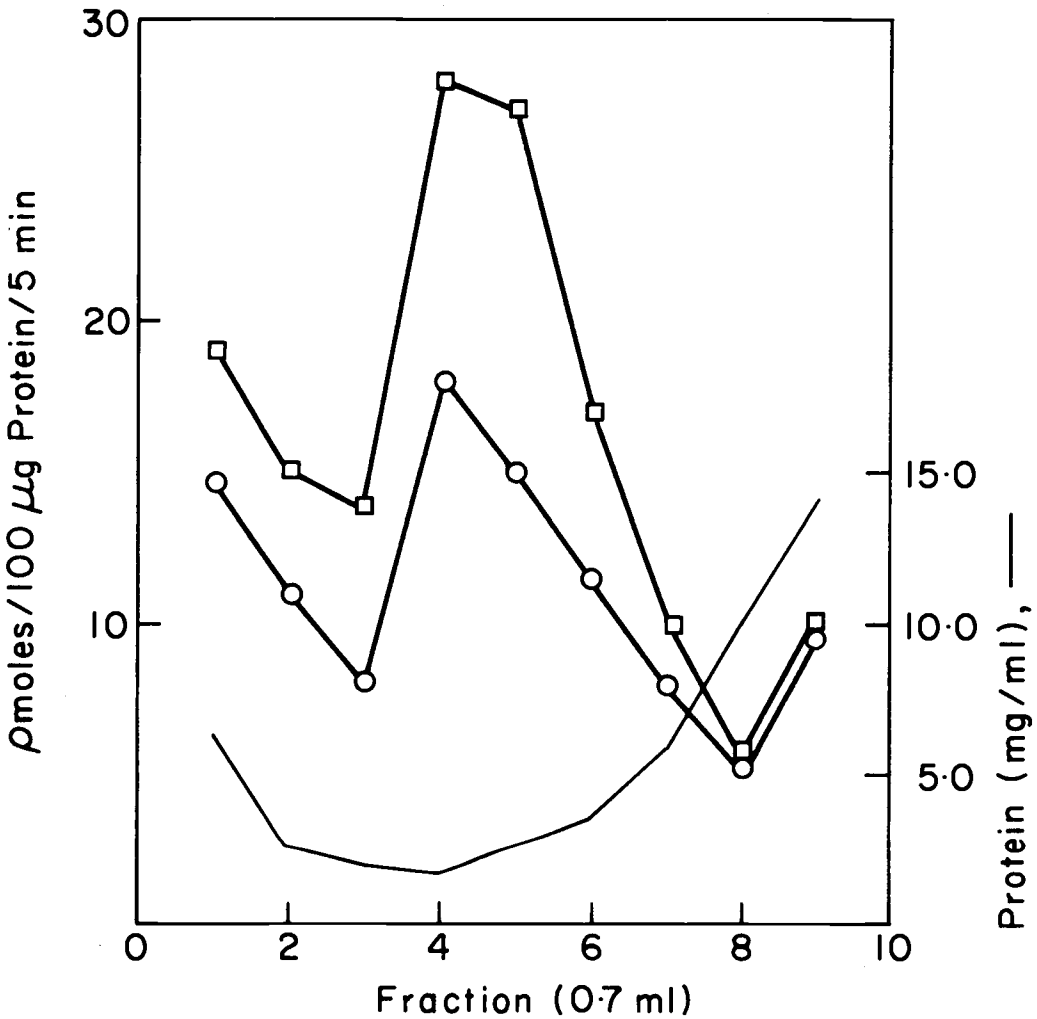


Figure 12. Velocity sedimentation profile of detergent solubilized RNA polymerase activity. Sup A was fractionated on 20 to 40% glycerol density gradient as described in Methods. Each fraction was assayed with no RNA primer added as described in Methods. Background radioactivity was not subtracted from values shown. Specific activity ^{3}H-GTP (o—o) : 27 cpm/ρ mole, ^{32}P-UTP (□—□) : 19 cpm/ρ mole.

level of detergent (2.0% Triton X-100) and fractionated again on a glycerol density gradient under the same conditions as shown in Figure 13a. The additional detergent treatment produced a RNA polymerase fraction with a greater velocity sedimentation constant and a higher specific activity than was originally observed, although some RNA polymerase remained in the middle of the gradient. The RNA polymerase fractions which sedimented to the middle of the second gradient were pooled and again treated with the high level of Triton X-100. Figure 13b shows the RNA polymerase profile of the fractions treated with detergent the second time and subjected to the same gradient conditions as before. As can be seen, still more RNA polymerase sedimented close to the bottom of the gradient with a second activity peak remaining in the middle of the gradient.

The RNA polymerase which sedimented to the middle of each of the three glycerol density gradients was contained in fractions which were turbid until treated with detergent which clarified them. This series of experiments suggested the RNA polymerase was tightly bound to membrane fractions from the myeloblasts.

Sephadex Chromatography

Sup B was further fractionated on 500 ml Sephadex G-200 columns. Due to the membrane association of the RNA polymerase in Sup B it was of interest to determine the effect of adding detergent to

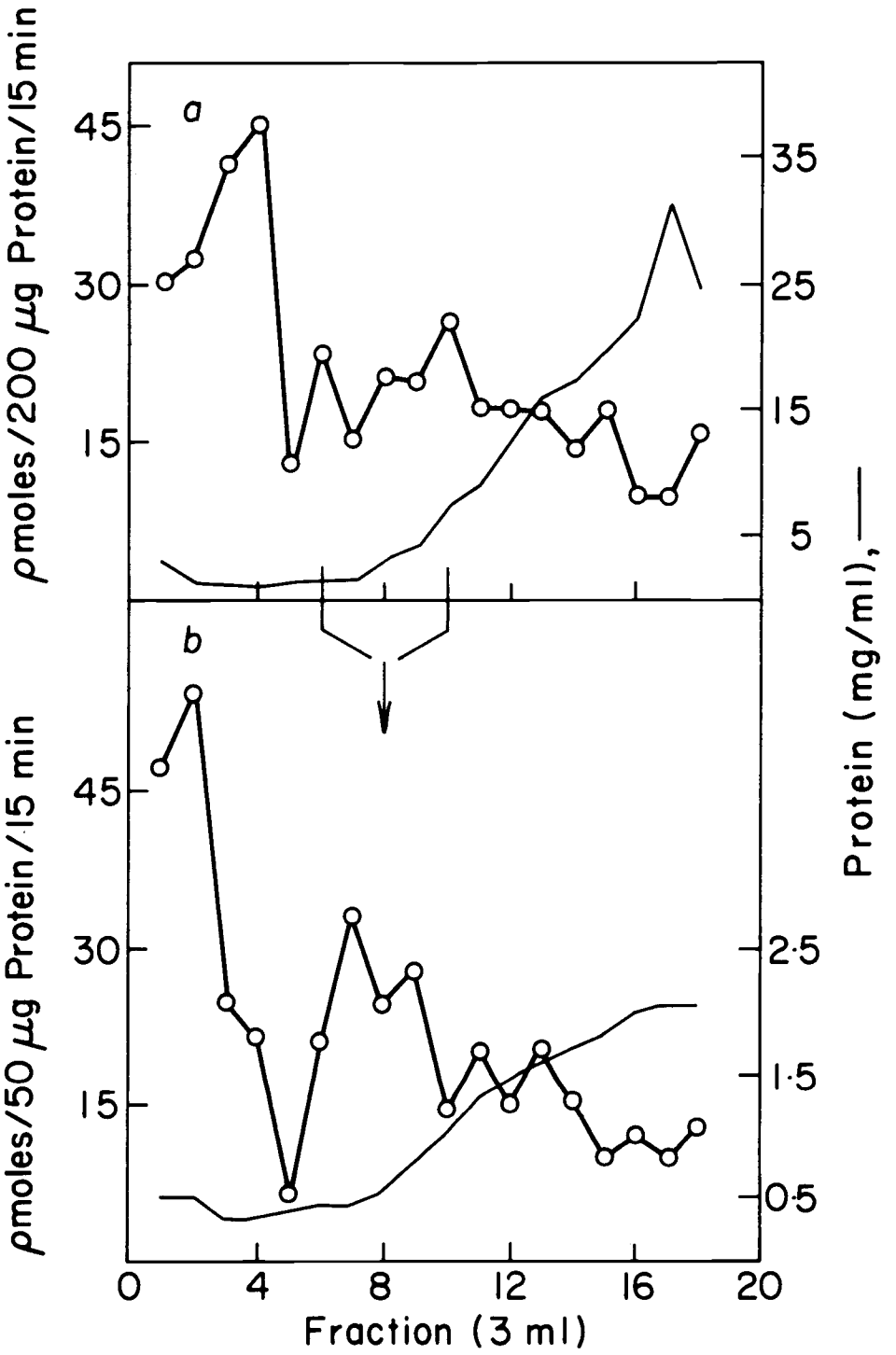


Figure 13. Velocity sedimentation profile of RNA polymerase activity following treatment with 2.0% Triton X-100. (a) Sup B was made 2% with respect to Triton X-100 and fractionated on 20 to 40% glycerol density gradient as described in Figure 12. (b) the fractions indicated by the bracket were pooled and treated again as described for Sup B. Each fraction was assayed as described in Figure 12. Specific activity ^{3}H-GTP (o—o) : 27 cpm/ρ mole.

the Sephadex G-200 column elution buffer. Figure 14a is the RNA polymerase elution profile of a Sephadex G-200 column equilibrated with 0.5% DOC in the elution buffer, and Figure 14b is the same column procedure without DOC in the elution buffer. As can be seen, there was little if any difference in the two RNA polymerase elution profiles.

The three peaks of RNA polymerase corresponded to approximate molecular weights in the ranges of $\geq$ 800,000, 450,000, and 300,000 daltons respectively. The fractions eluting with the column void volume ($\geq$ 800,000 daltons) appeared to be membrane associated since these fractions were turbid and could be clarified by detergent treatment.

Assay Optima

The pH and Mg^{++} optima for GMP and UMP incorporation were found to be very similar for the detergent solubilized RNA polymerase as for the soluble RNA polymerase activity. Figure 15 shows the pH and Mg^{++} optima profiles for the RNA polymerase with 64S RNA primer from AMV.

Kinetics

The RNA polymerase isolated by this procedure did not need added RNA primer to synthesize RNA. However, added primer was

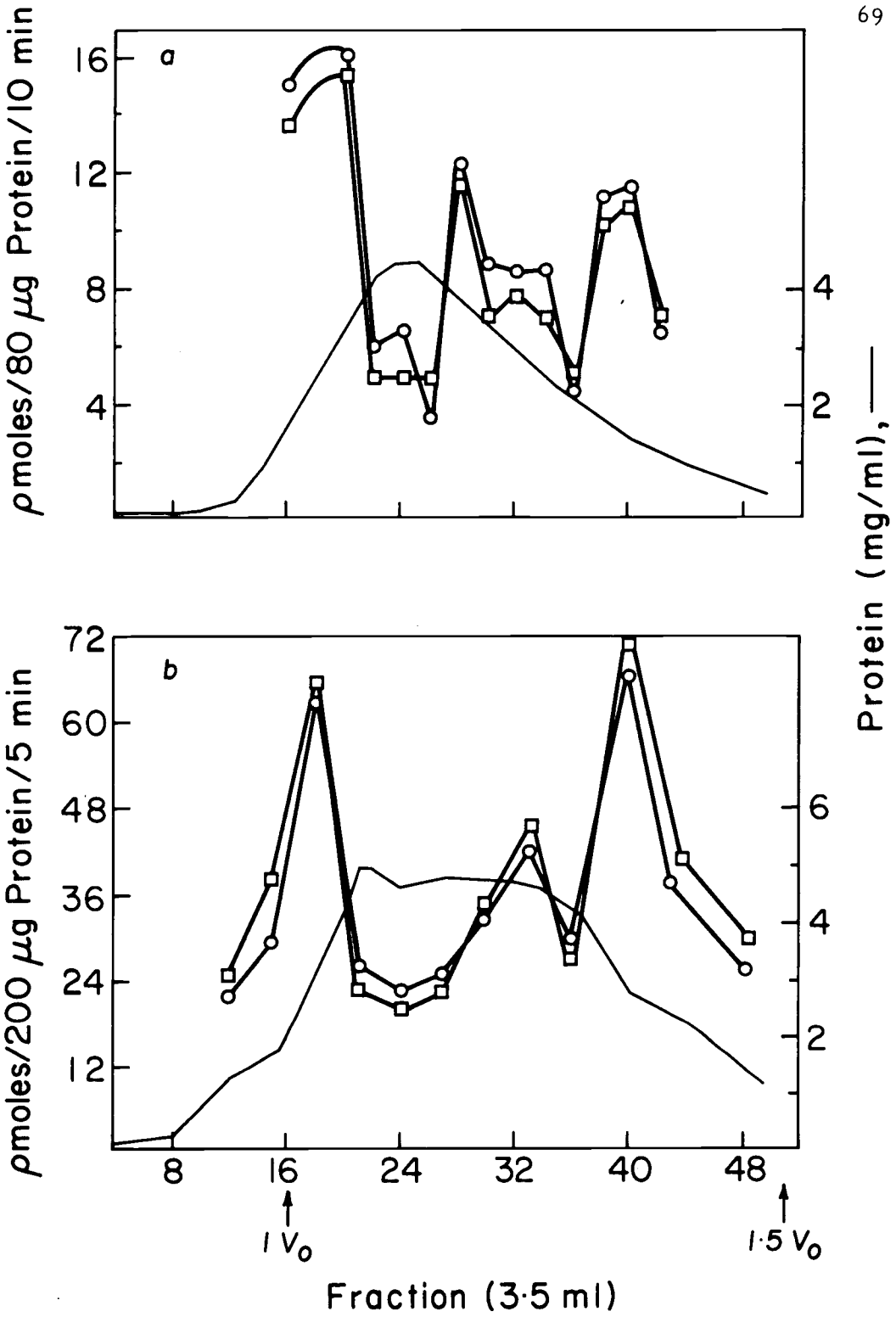


Figure 14. Gel filtration of detergent solubilized RNA polymerase. Sup B was chromatographed on Sephadex G-200 as described in Methods. (a) Elution buffer contained 0.5% DOC, (b) elution buffer contained no DOC. Each fraction was assayed as described in Figure 12. Specific activity ^{3}H-GTP (o—o) : 22 cpm/ρ mole, ^{32}P-UTP (□—□) : 10 cpm/ρ mole.

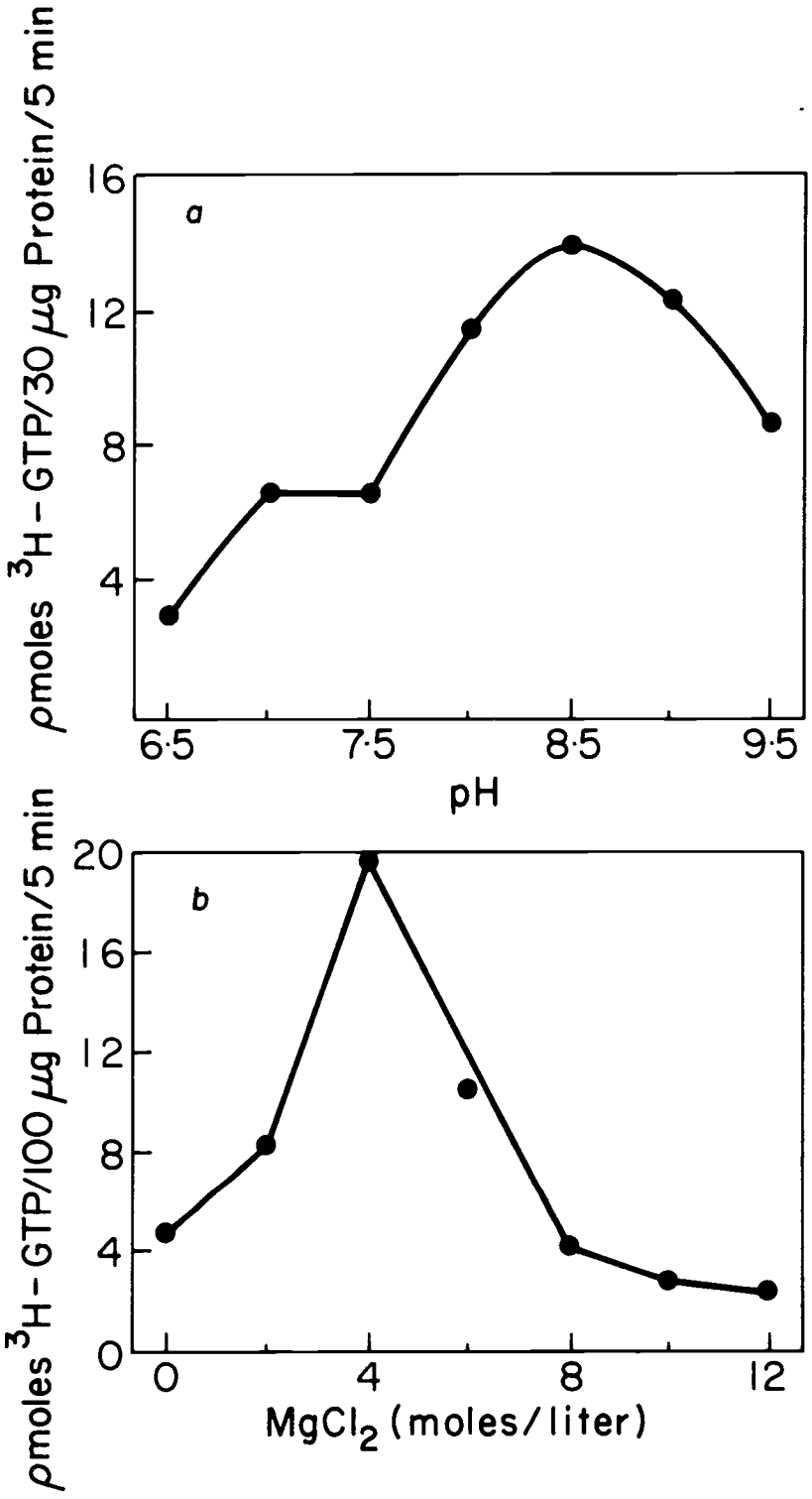


Figure 15. Optimal pH and $MgCl_2$ concentration for RNA synthesis by detergent solubilized RNA polymerase. Each assay contained enzyme from Sup C plus 20 μg 64S RNA from AMV. Assays were performed as described in Figure 12 except in (a) the pH was varied as shown and in (b) the $MgCl_2$ concentration was varied as shown. Specific activity of ^{3}H-GTP : 32 cpm/ρ mole.

important to extend the reaction time, and 64S RNA from AMV was a much better primer than myeloblast RNA. Figure 16 illustrates the kinetics of RNA synthesis by RNA polymerase in Sup C with no added primer, with myeloblast RNA primer, and with 64S RNA primer from AMV. The UMP incorporation into RNA product by this RNA polymerase fraction was at the same level as the GMP incorporation except in the presence of myeloblast RNA primer which stimulated the UMP incorporation to a higher level than the GMP incorporation.

Deoxyribonuclease Effect

The level of RNA polymerase activity in Sup A with no primer added to an assay was depressed 10% to 20% when the enzyme fraction was preincubated with deoxyribonuclease, or when deoxyribonuclease was added directly to the assay. However, addition of deoxyribonuclease to Sup A produced no noted difference in the level of endogenous RNA polymerase activity in Sups B or C.

Nucleoside Triphosphate Requirements

The RNA polymerase activity of Sup B was found to be depressed by the omission of one ribonucleoside triphosphate from the assay. Figure 17 illustrates the kinetics of UMP and GMP incorporation with and without CTP in the assay mixture which contained no added RNA primer.

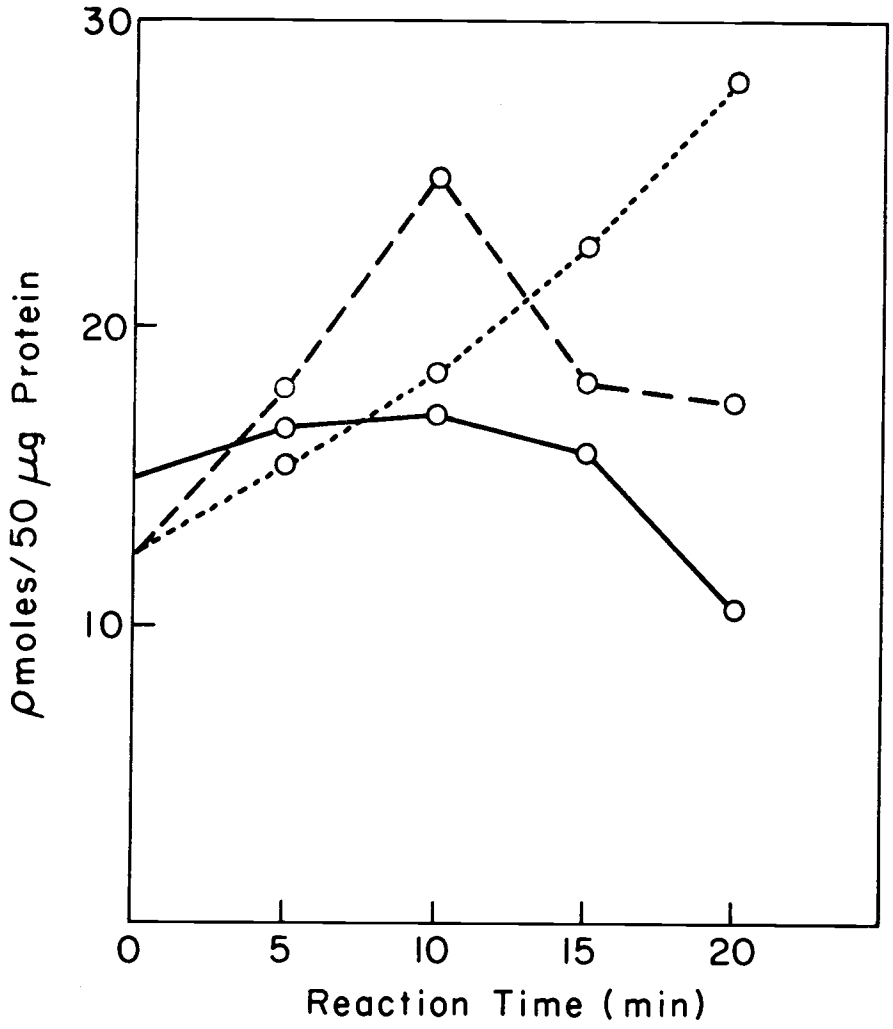


Figure 16. Primer response of detergent solubilized RNA polymerase. Enzyme from Sup C was incubated with no added primer (o- -o), with 20 μg 64S RNA from AMV (o ··· o), or with 40 μg myeloblast RNA primer (o—o) in assay conditions as described in Figure 12. Specific activity of ^{3}H-GTP : 18 cpm/ρ mole.

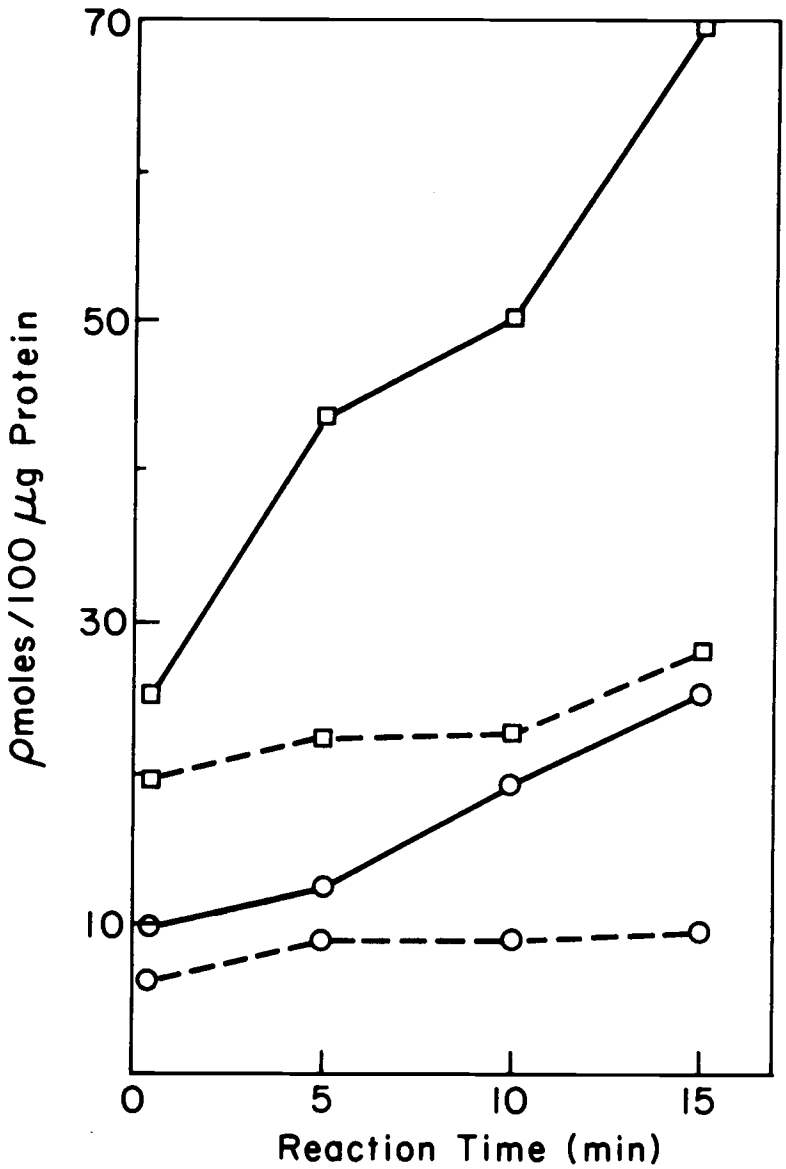


Figure 17. Ribonucleoside triphosphate dependence of detergent solubilized RNA polymerase. Enzyme from Sup B was assayed as described in Figure 12 except that CTP was omitted from some assays. ^{3}H-GTP (o—o) complete, (o- -o) minus CTP, specific activity : 32 cpm/ρ mole; ^{32}P-UTP (□—□) complete, (□- -□) minus CTP, specific activity : 20 cpm/ρmole.

Product Analysis

The RNA polymerase activity eluted from the G-200 Sephadex column (Sup C) was stimulated by 64S RNA from AMV. Very low levels of polyvinyl sulfate in the assay were found to stabilize this primer without completely depressing the RNA polymerase reaction. Figure 18 shows the velocity gradient sedimentation profiles of both primer and product from RNA polymerase assays incubated with 64S RNA from AMV for five minutes and for 15 minutes. As can be seen there was an increase in the amount of RNA product labeled with both GMP and UMP which sedimented to the high molecular weight region of the gradient with increase in incubation time. There was also a low molecular weight RNA product detected at both incubation times.

Summary of Detergent Solubilized RNA Polymerase

Myeloblasts from chicks infected with avian myeloblastosis virus were found to contain a membrane associated RNA polymerase which could be solubilized by detergent treatment. The RNA polymerase had a high level of endogenous activity which was not sensitive to deoxyribonuclease. All four ribonucleoside triphosphates were required for RNA synthesis as measured by the kinetics of GMP and UMP incorporation into acid-precipitable product. In the presence of low levels of polyvinylsulfate and 64S RNA from AMV, the RNA polymerase

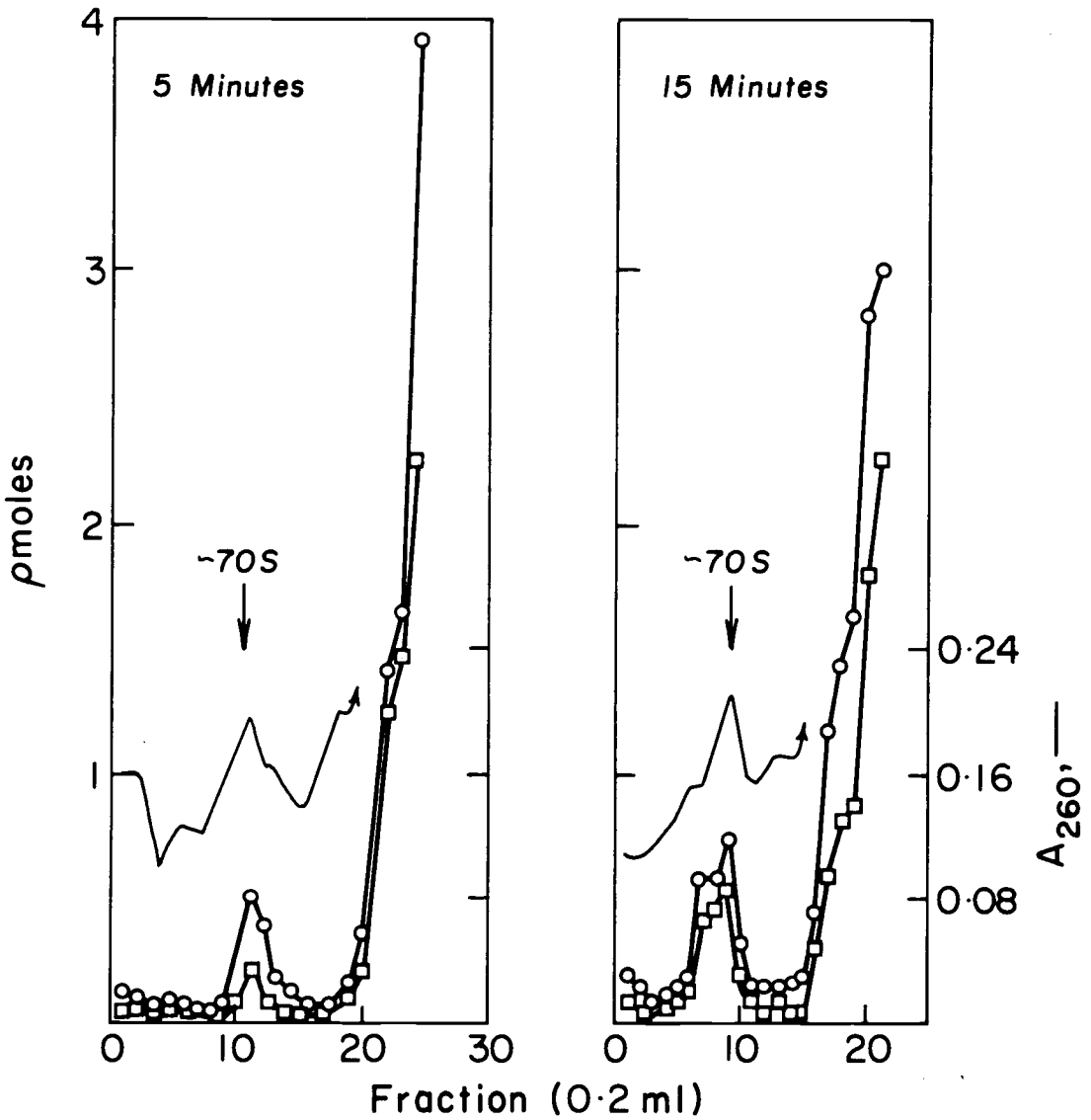


Figure 18. Velocity sedimentation analysis of 64S RNA from AMV and product RNA synthesized by detergent solubilized RNA polymerase. Two assays containing 100 μg protein and 40 μg 64S RNA from AMV were incubated for 5 minutes and 15 minutes and both primer and product analyzed on glycerol density gradients as described in Methods. Specific activity of ^{3}H-GTP (o—o) : 100 cpm/ρ mole, ^{32}P-UTP (□—□) : 120 cpm/ρ mole.

synthesized product which sedimented as a high molecular weight RNA species.

DNA Polymerase in AMV and MC29 Virus

The presence of DNA polymerase in RNA tumor viruses prompted two lines of investigation. The first stemmed from the possibility that radioassay of virus DNA polymerase activity would provide a more sensitive tool for detection of virus particles than was previously available with infectivity assays in tissue culture, direct visualization of particles by electron microscope methods, or detection of virus specific antigens. It would therefore be possible to detect tumor virus at concentrations which have heretofore been undetected by conventional methods. This presented the exciting possibility of demonstrating tumor viruses in other neoplasms, such as human leukemia.

The second line of investigation was to attempt to demonstrate the viral DNA polymerase in myeloblasts infected with AMV and CEC infected with MC29. Assay of viral DNA polymerase in infected cells would provide valuable information on the intracellular location of virus-directed functions, as well as provide another means for identifying and studying tumor virus involvement in human leukemia. Of additional interest to this study was determining a relationship (intracellular location or primer response) between the virus DNA polymerase and the RNA polymerase activity previously presented.

A study of both nucleic acid polymerases simultaneously would provide further information as to the replication process of the virus RNA and DNA species.

Hence a characterization of the DNA polymerases associated with both the AMV and the MC29 virion was conducted to provide criteria for identifying virus specific DNA polymerase.

The tumor virus DNA polymerases were prepared from purified virus particle pellets by a modification of the Temin procedure (Temin, 1970) as outlined in Methods.

Assay Optima

DNA as well as RNA had been shown to serve as primer for DNA polymerase activity associated with tumor viruses (Spiegelman *et al.*, 1970b; Řiman and Beaudreau, 1970). Figure 19 illustrates the kinetics of DNA synthesis for both the AMV and MC29 virus polymerase with *Micrococcus lysodeikticus* DNA primer. DNA polymerase activity was present in both virus preparations with no added primer, but at such a low level as to appear negligible relative to the DNA primed reaction.

DNA synthesis by DNA polymerase from AMV particles was greatly stimulated by $MgCl_2$ as shown in Figure 20b. Monovalent salt, NaCl or KCl, was also important for maximum DNA synthesis in the presence of *M. lysodeikticus* DNA primer as shown in Figure 20a.

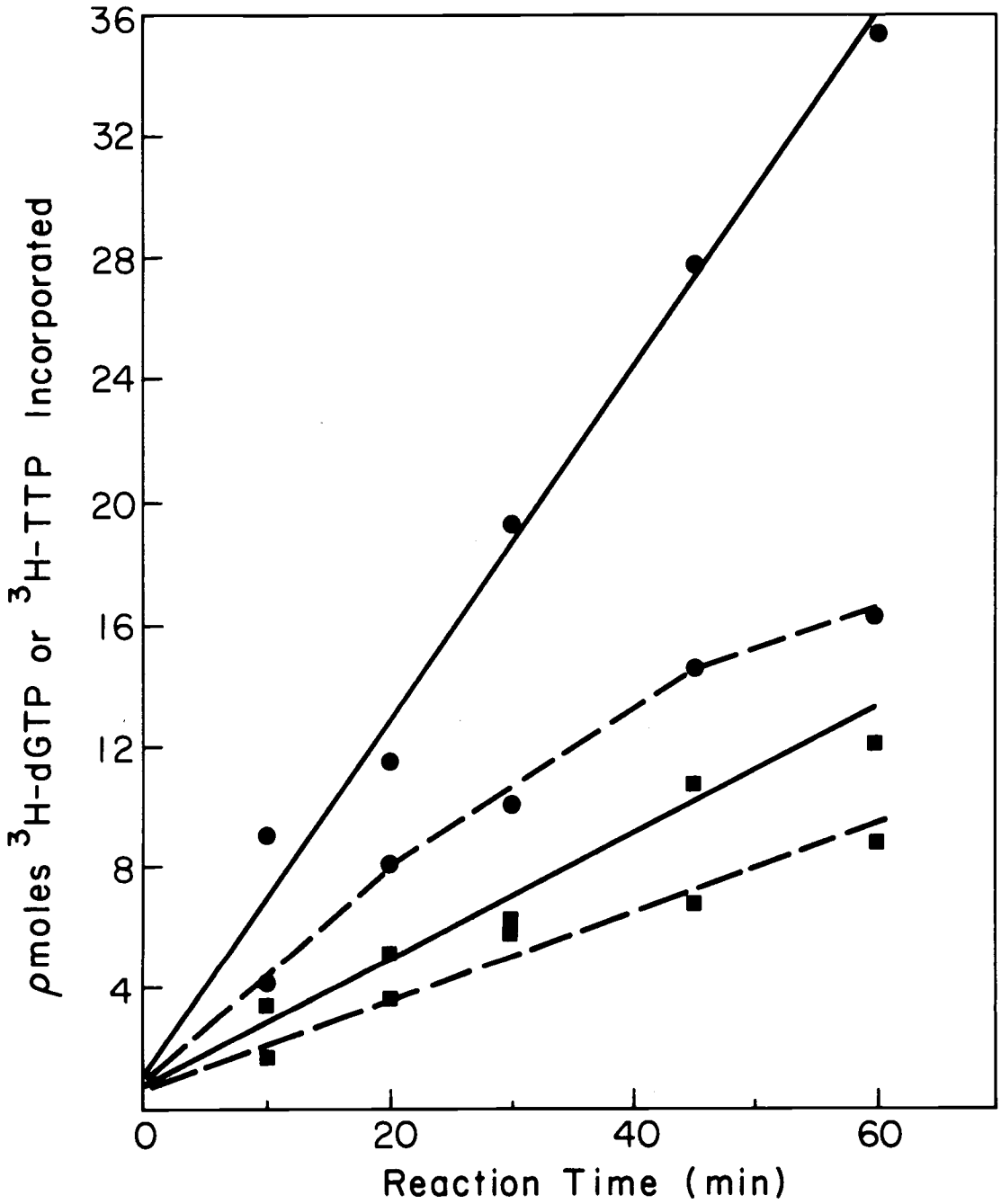
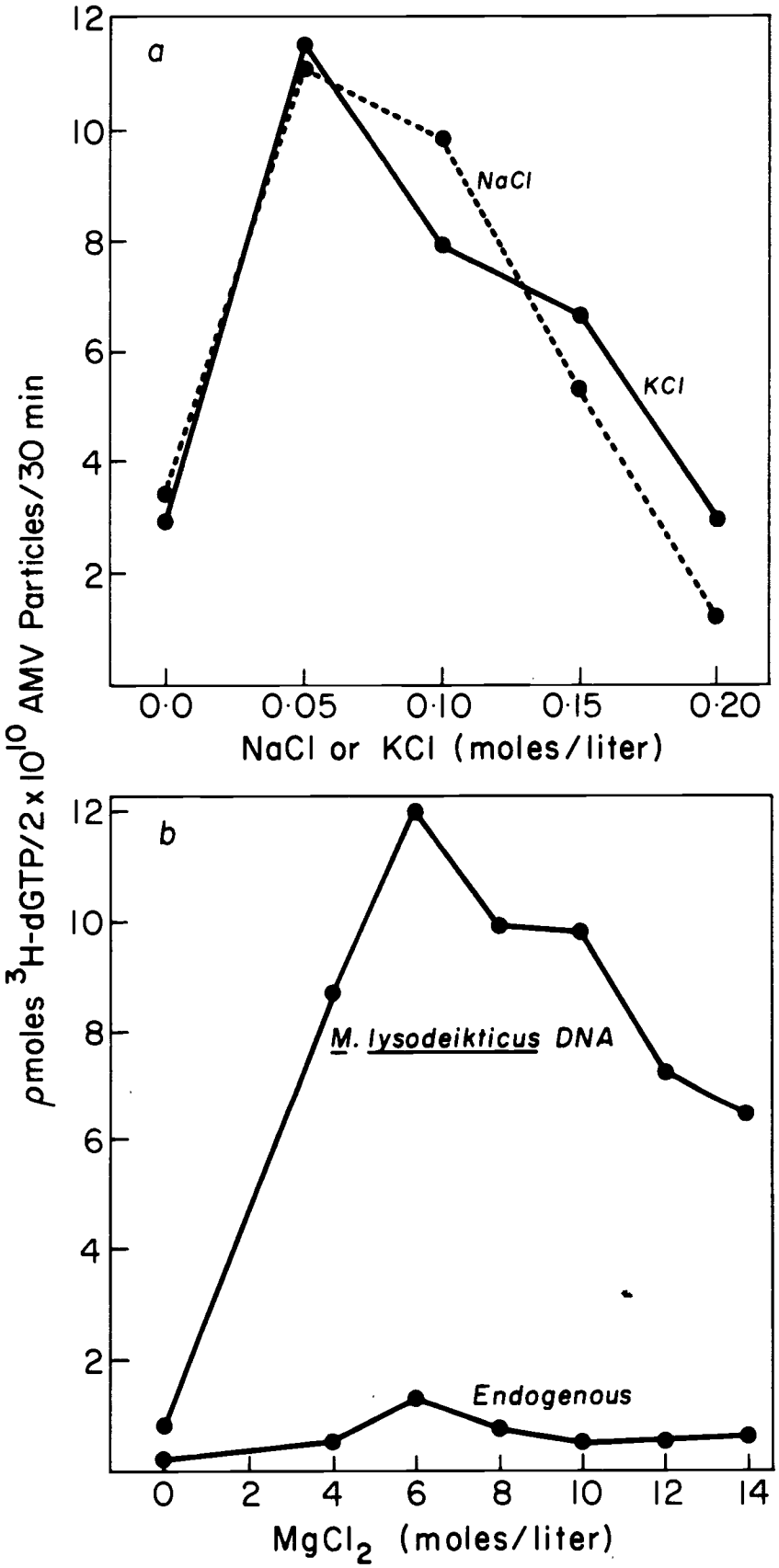


Figure 19. Kinetics of DNA synthesis by DNA polymerase from MC29 virus and AMV. Each assay (0.1 ml) contained 2 μg of M. lysodeikticus DNA and either MC29 particles (dashed lines) equal to 2.4×10^6 ffu, or 1.4×10^{10} AMV particles (solid lines). Assay conditions were as described in Methods, no radioactivity was subtracted as background. Specific activity of ^{3}H-dGTP (●—●) and ^{3}H-TTP (■—■) : 1000 cpm/ρ mole.

Figure 20. Optimal $MgCl_2$ and monovalent salt concentrations for DNA synthesis by DNA polymerase from AMV. Assays (0.1 ml) were performed as described in Figure 19. (a) All assays contained 2 μg of M. lysodeikticus DNA; (b) M. lysodeikticus DNA added as in (a), no template added to endogenous as shown. Specific activity ^{3}HdGTP : 2000 cpm/pmole.


a
NaCl
KCl
12
10
8
6
4
2
0·0
0·05
0·10
0·15
0·20
NaCl or KCl (moles/liter)
pmoles ^{3}H-dGTP/2 x 10^{10} AMV Particles/30 min
b
M. lysodeikticus DNA
Endogenous
0
2
4
6
8
10
12
14
$MgCl_2$ (moles/liter)

Primer Response

Table 7 shows the amount of DNA synthesis by the virus DNA polymerases in the presence of a variety of primers. As can be seen in this table heterogeneous DNA stimulated DNA synthesis to a much higher level than heterogeneous RNA with the notable exception of *Bacillus subtilis* DNA which showed no priming activity for DNA synthesis. The stimulation of DNA synthesis by the synthetic RNA duplex, Poly I: Poly C, was observed with the AMV DNA polymerase, but not with the MC29 DNA polymerase (Weber *et al.*, 1971a). However, the high stimulation of DNA synthesis by the synthetic DNA: RNA hybrid (Poly dC: Poly G) noted with the AMV DNA polymerase was also observed with the MC29 DNA polymerase (Weber *et al.*, 1971b). It should be noted that the two preparations of Poly dC: Poly G did not stimulate DNA synthesis equally well, and that the Poly dC alone was a better template in the second preparation than the Poly dC: Poly G hybrid mixture. The reason for these differences is not known at this time, but probably reflect a variation in the synthetic polymers involved.

Product Studies

It was of interest to determine the buoyant densities of the DNA products synthesized by the virus DNA polymerases for comparison to

Table 7. Response of Virus DNA Polymerases to a Variety of Primers.

Template	Amount (μg)	pmoles ^{3}Hd6MP*/30 minutes	
		AMV	MC 29
Experiment A			
None	--	0.3	0.2
64S AMV RNA	8	3.2	1.1
Qβ RNA	10	1.6	0.8
28S Myeloblast RNA	5	1.0	0.4
B. Subtilis DNP	4	0	0
M. lysodeikticus DNA	2	18.8	2.2
Poly I: Poly C		11.2	--
Experiment B			
None	--	1.3	--
64S AMV RNA	5	5.2	--
AMV DNA	0.5	6.5	--
Poly dC: Poly G[b]	0.5	61.8	--
Poly dC	0.5	19.3	--
Poly G	0.5	0.9	--
Poly dC: Poly G[c]	0.5	3.0	--

*All assays were 0.10 ml total volume. In Experiment A 2×10^{10} AMV particles or virus equivalent to 2×10^7 ffu of MC 29 were added per assay. In Experiment B 1×10^{10} AMV particles were added per assay. Assay conditions were as described in Methods.

[a]300 mg Poly I were mixed with 100 mg Poly C in 0.2 m NaCl. 0.01 ml mixture was added to assay.

[b]Obtained as a gift from Dr. S. Spiegelman.

[c]Prepared by mixing 100 mg Poly dC (gift from K. C. Olsen, Hoffman-LaRoche Laboratories) with 100 mg Poly G in 0.2 M NaCl.

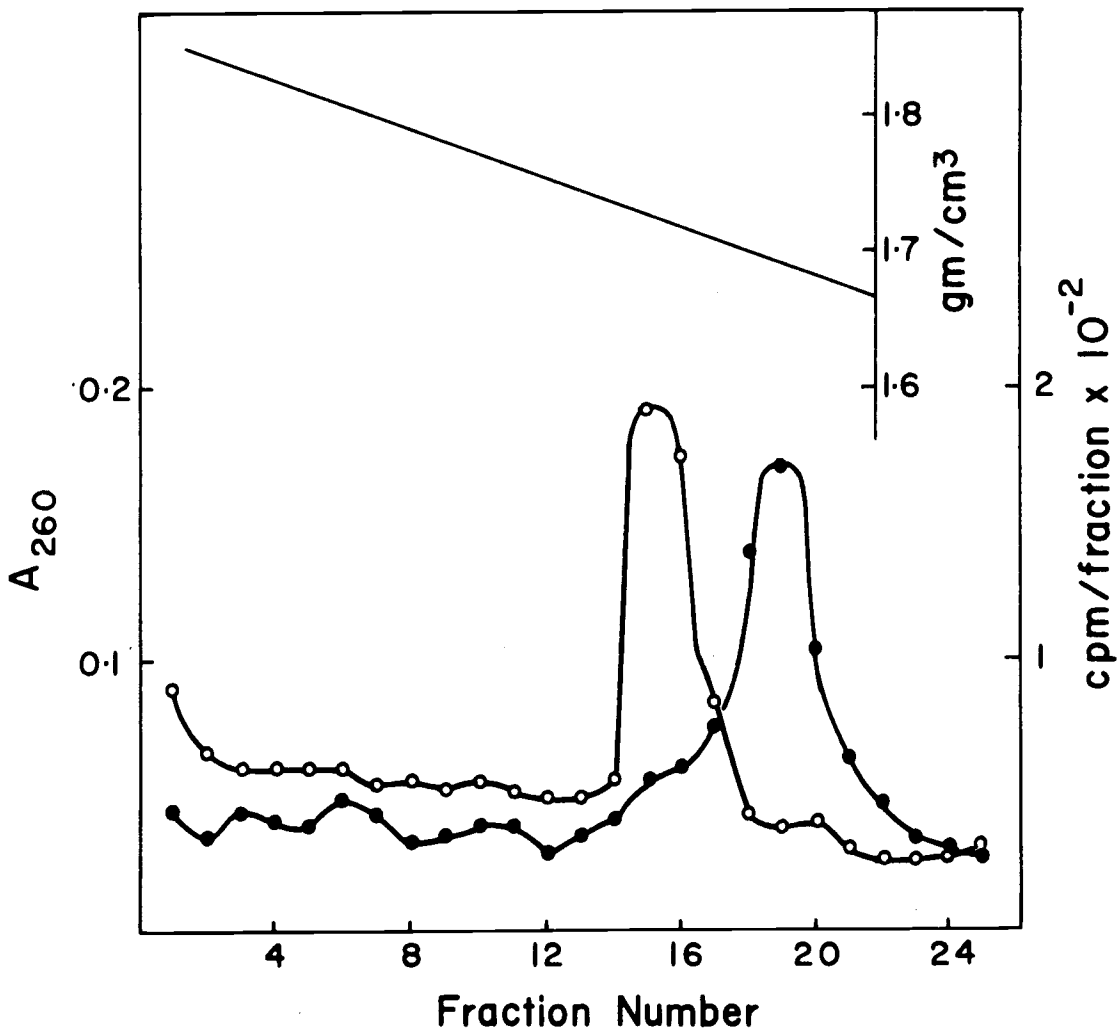


Figure 21. Buoyant density determination in CsCl of DNA product synthesized by DNA polymerase from AMV. No exogenous DNA was added to the assay which was incubated for 60 minutes under the conditions described in Methods. The density gradient was prepared from a mixture of the product DNA and 40 μg M. lysodeikticus DNA used as marker in a solution of CsCl with a final density of 1.77 gm/cc. The CsCl density gradient was formed and fractionated for determination of radioactivity and absorbance at 260 nm as described in Methods. (o—o) absorbance of M. lysodeikticus at 260 nm, (•—•) ^{3}H-dGTP, specific activity : 1000 cpm/pmole.

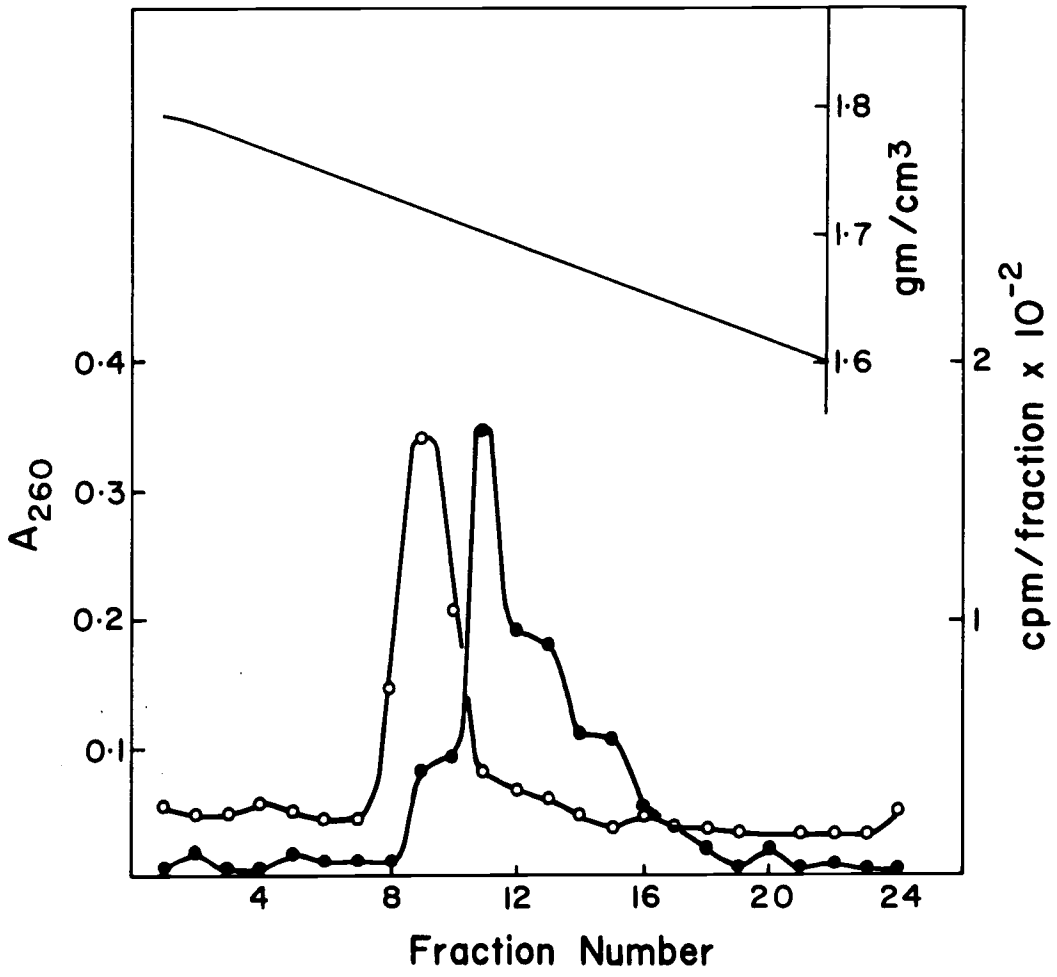


Figure 22. Buoyant density determination in CsCl of DNA product synthesized by DNA polymerase from MC29 particles. Conditions were the same as in Figure 21 except the CsCl solution had a final density of 1.70 gm/cc prior to forming the gradient. (o—o) absorbance at 260 nm of M. lysodeikticus DNA marker, (•—•) ^{3}H-dGTP, specific activity : 1000 cpm/ρ mole.

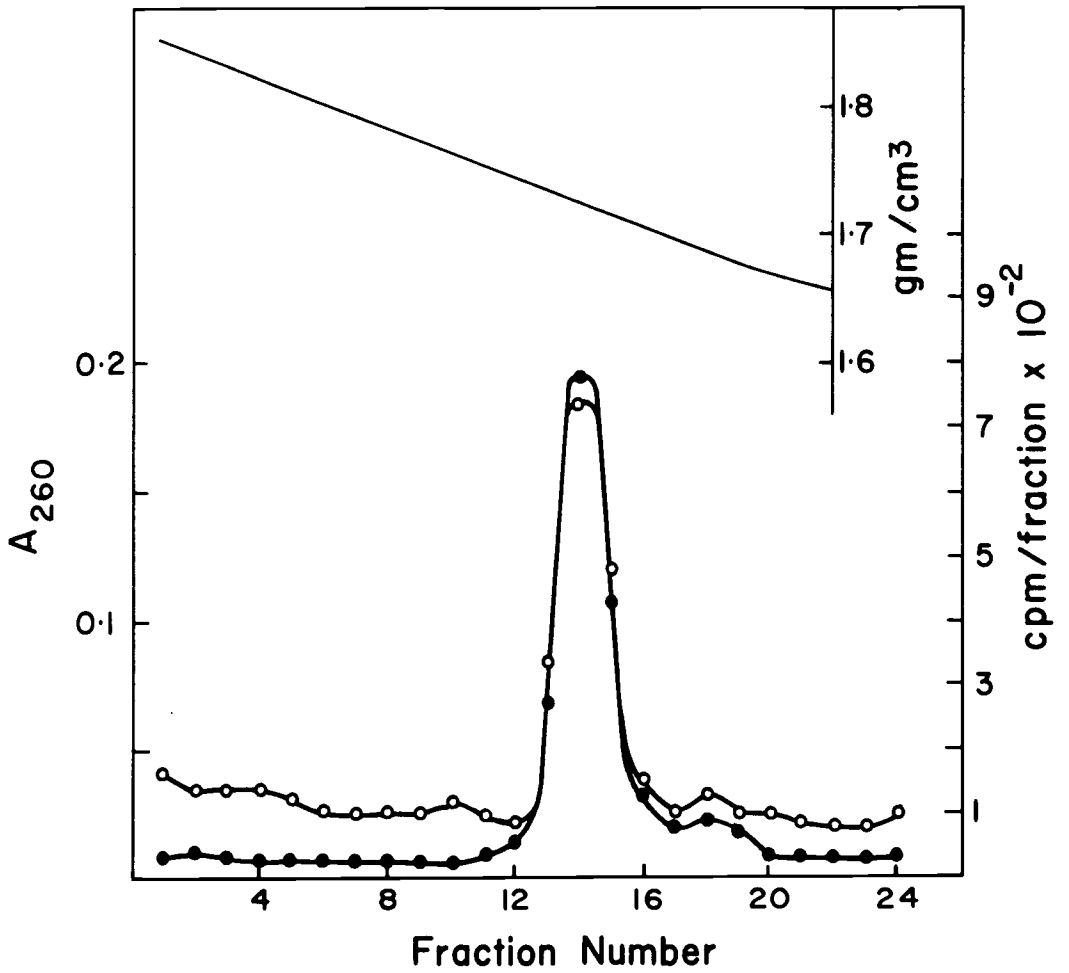


Figure 23. Buoyant density determination in CsCl of DNA product synthesized from M. lysodeikticus DNA template by DNA polymerase from MC29 particles. Conditions were identical to those reported in Figure 21 except 4 g M. lysodeikticus DNA was added to the enzyme reaction mixture. (o—o) absorbance at 260 nm by M. lysodeikticus DNA marker, (•—•) ^{3}H-dGTP, specific activity : 1000 cpm/ρ mole.

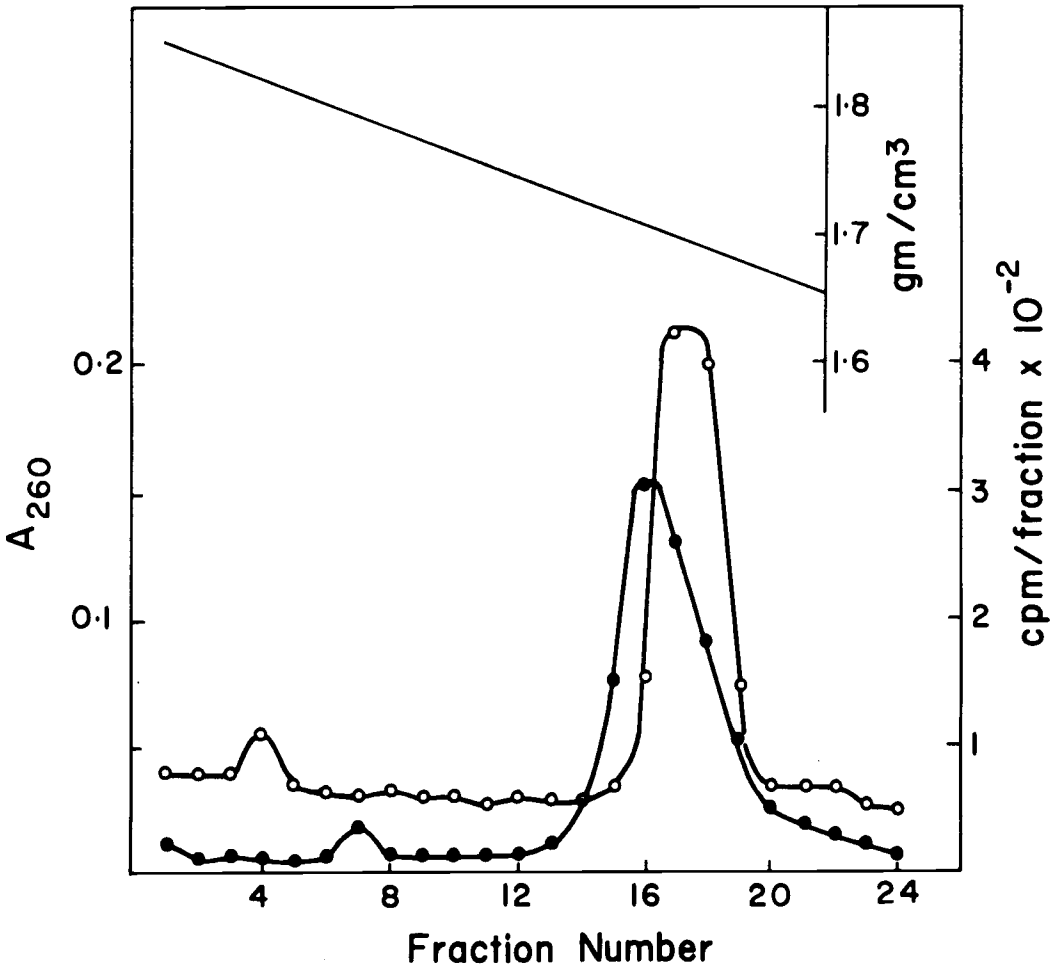


Figure 24. Buoyant density determination in CsCl of DNA product synthesized from calf thymus DNA template by DNA polymerase from MC29 particles. Conditions for product synthesis and gradient analysis were identical to those reported in Figure 23 with the substitution of calf thymus DNA for M. lysodeikticus DNA. (o—o) absorbance at 260 nm by calf thymus DNA, (•—•) ^{3}H-dGTP; specific activity : 1000 cpm/ρ mole.

the buoyant densities of the DNA templates. Figures 21 and 22 show the density gradient profiles of the DNA products synthesized by the DNA polymerases from AMV and MC29, respectively, with no added template (endogenous reaction). The buoyant density (1.69 gm/cc) of the peak DNA product synthesized from endogenous AMV template indicated a G+C content of approximately 35 mole % (Szybalski, 1968). The MC29 endogenous template directed the synthesis of DNA species with four discernible buoyant densities, the two peak areas (1.70 gm/cc and 1.69 gm/cc) banding at densities equivalent to 40 mole % and 30 mole % G+C respectively.

Figures 23 and 24 show the product profiles of MC29-DNA polymerase with M. lysodeikticus, DNA template, and with calf thymus DNA template, respectively. The DNA product from M. lysodeikticus DNA template equilibrated exactly with the template DNA at a buoyant density of 1.72 gm/cc, which corresponds to 60 mole % G+C. However, the calf thymus DNA template directed the synthesis of DNA product equilibrating at a buoyant density of 1.71 gm/cc which was slightly greater than the buoyant density of the DNA template and corresponds to 50 mole % G+C.

It was interesting from these product studies that the DNA synthesized from endogenous virus template had a G+C content of 40 mole % or less, whereas in the presence of exogenous DNA the virus DNA polymerase selectively copied G+C rich areas of the DNA template.

These results were in accord with the high stimulation of DNA synthesis by the viral DNA polymerases in the presence of Poly dC or Poly dC: Poly G template.

64S RNA from AMV and AMV DNA as Primers for Viral DNA Polymerase

The original evidence that the tumor virus DNA polymerase used virus RNA as template for DNA synthesis was the sensitivity of the endogenous reaction to ribonuclease (Baltimore, 1970; Temin, 1970) and the formation of a hybrid between product DNA and viral RNA (Spiegelman *et al.*, 1970). Figure 25 illustrates the effect of ribonuclease on DNA synthesis by AMV DNA polymerase. The kinetics shown are DNA synthesis with endogenous template, added 64S RNA template from AMV, and added AMV-DNA template. These studies indicated that both the viral RNA and viral DNA served as templates for DNA synthesis. Ribonuclease treatment completely depressed RNA stimulated DNA synthesis, but did not completely depress DNA synthesis with endogenous template nor DNA stimulated DNA synthesis.

Figure 26 shows another study of DNA synthesis by DNA polymerase from AMV. The incorporation of both ^{3}HdGMP and ^{3}HTMP into DNA product was followed with time in the presence of endogenous template, added 64S RNA template from AMV, and added AMV-DNA template. These kinetics showed that the TMP:dGMP ratio of the DNA

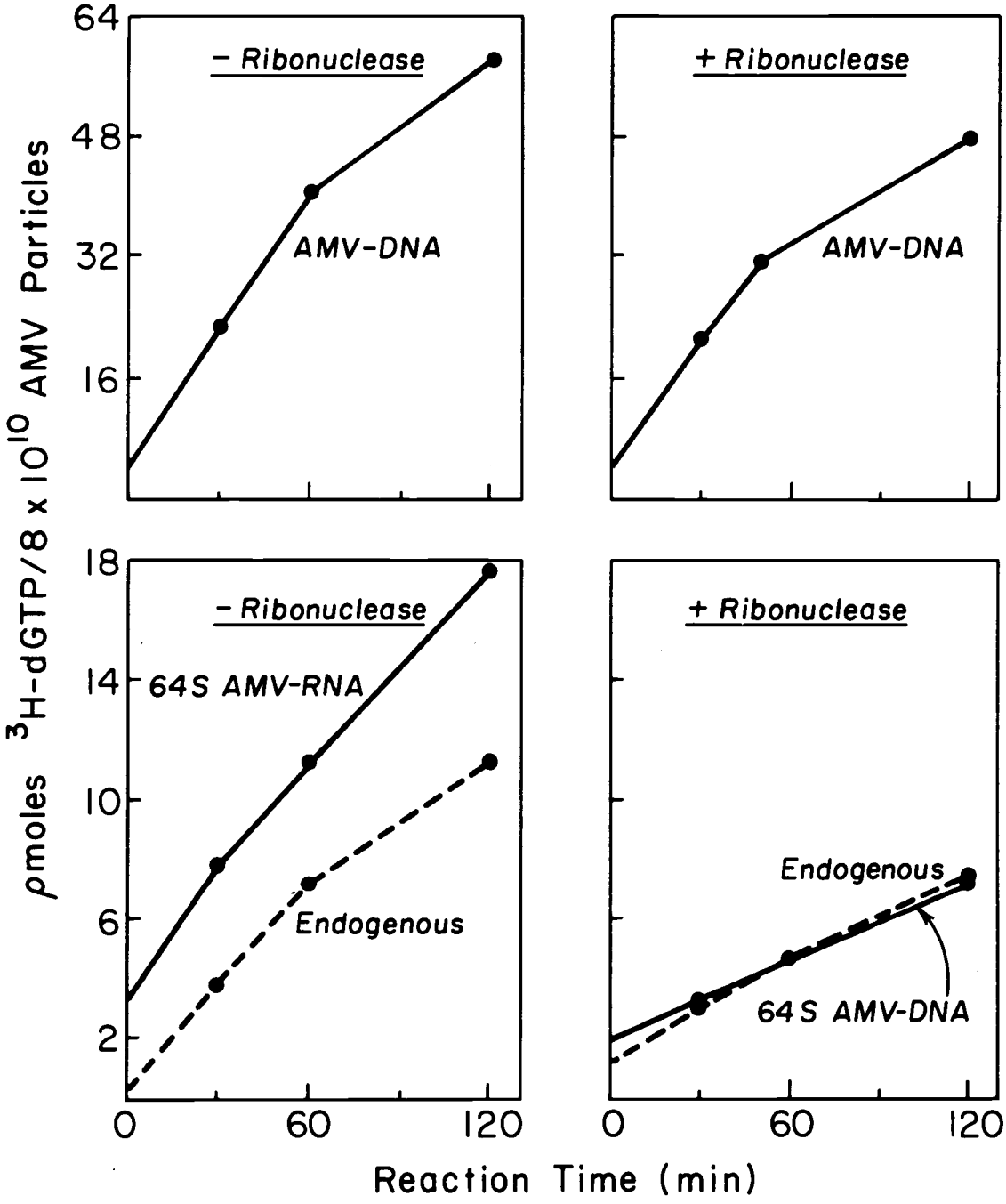
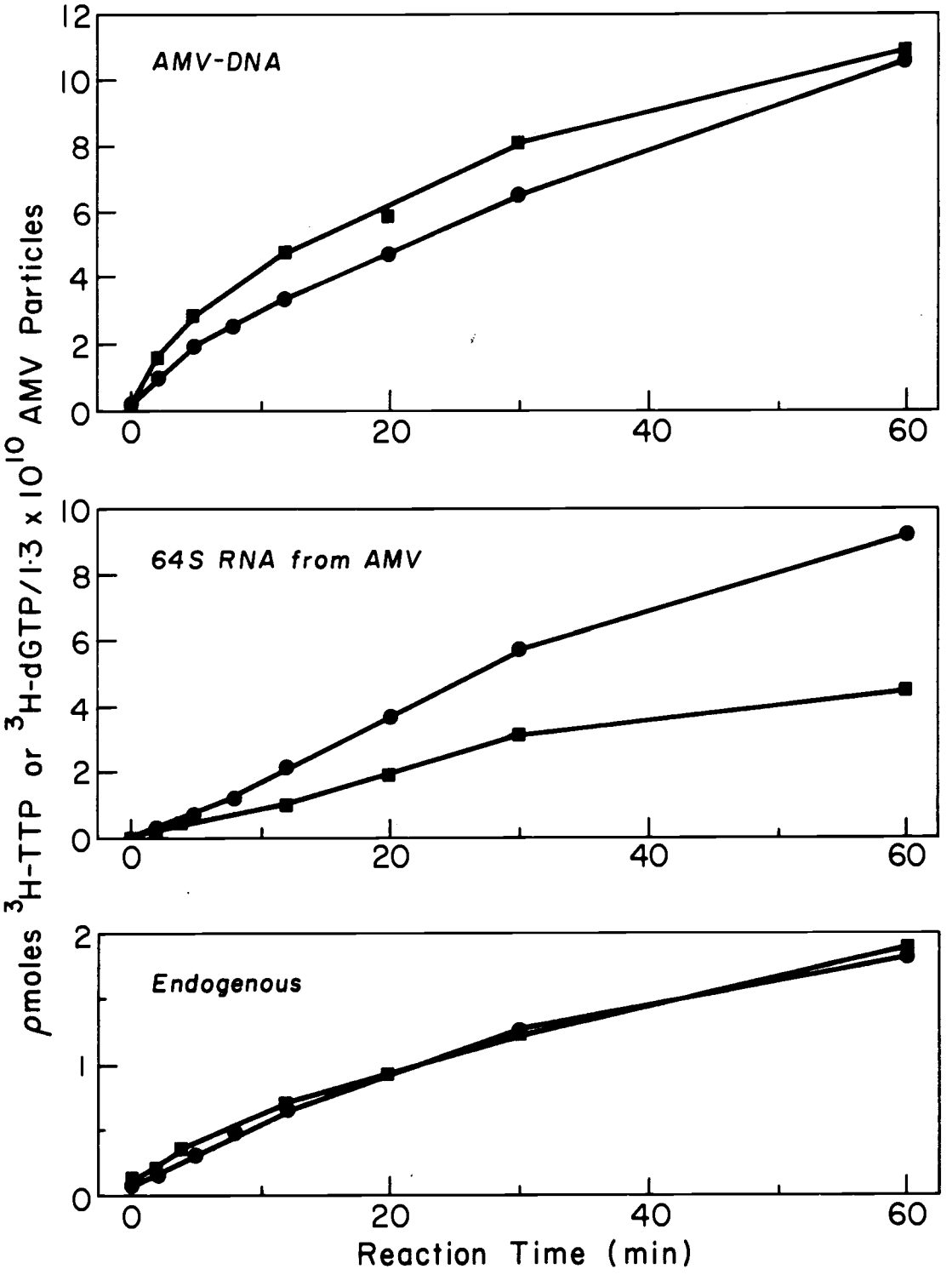


Figure 25. The effect of ribonuclease on DNA synthesis by DNA polymerase from AMV. Assays (0.1 ml) were performed as described in Methods. AMV-DNA was added at a level of 0.4 μg per assay, and 64S RNA from AMV was added at a level of 3 μg per assay. Specific activity of ^{3}H-dGTP : 1000 cpm/ρmole.

Figure 26. Kinetics of DNA synthesis by DNA polymerase from AMV. Assays (1.0 ml) contained 1.3×10^{11} AMV particles and were performed as described in Methods. AMV-DNA was added at a level of 5 μg per ml and 64S RNA from AMV was added at a level of 50 μg per ml. At the indicated times 0.10 ml of the incubation mixtures was removed for determination of radioactivity as described in Methods. Specific activity of ^{3}H-dGTP (●—●) and ^{3}H-TTP (■—■) : 1000 cpm/pmole.


AMV-DNA
64S RNA from AMV
Endogenous
pmoles ³H-TTP or ³H-dGTP/1·3 x 10¹⁰ AMV Particles
Reaction Time (min)
0
20
40
60
0
2
4
6
8
10
12
1
2

product was approximately 1:1 from the endogenous reaction, and approximately 1.3:1 with AMV-DNA template. These TMP:dGMP ratios were consistent with a G+C content $\leq$ 50 mole % in a double-stranded DNA product, which is in good agreement with the observed buoyant density of the DNA product synthesized from endogenous template (Figure 22). In contrast to these findings the DNA product synthesized by the AMV-DNA polymerase in the presence of 64S RNA from AMV particles had a TMP:dGMP ratio of 1:2. This ratio predicted a double-stranded DNA product with G+C content in the range of 60-70 mole %. Such a DNA species would exhibit a buoyant density between 1.72 gm/cc and 1.73 gm/cc (Szybalski, 1968), and no product DNA peak was observed in this density range on the cesium chloride gradient (Figure 22). The strong implication from these results is that the DNA product from the endogenous reaction observed on the cesium chloride gradients is primarily AMV-DNA primed rather than AMV-RNA primed. More rigorous product analyses are necessary to confirm this observation.

It was necessary to determine the saturation levels of the DNA polymerase from AMV with the 64S RNA from AMV and with AMV-DNA in order to obtain the maximum velocity of DNA synthesis with each of the virus templates. Figure 27 is the response of the viral DNA polymerase to increasing levels of 64S RNA from AMV and AMV-DNA. Also shown is the much lower level of priming activity exhibited by

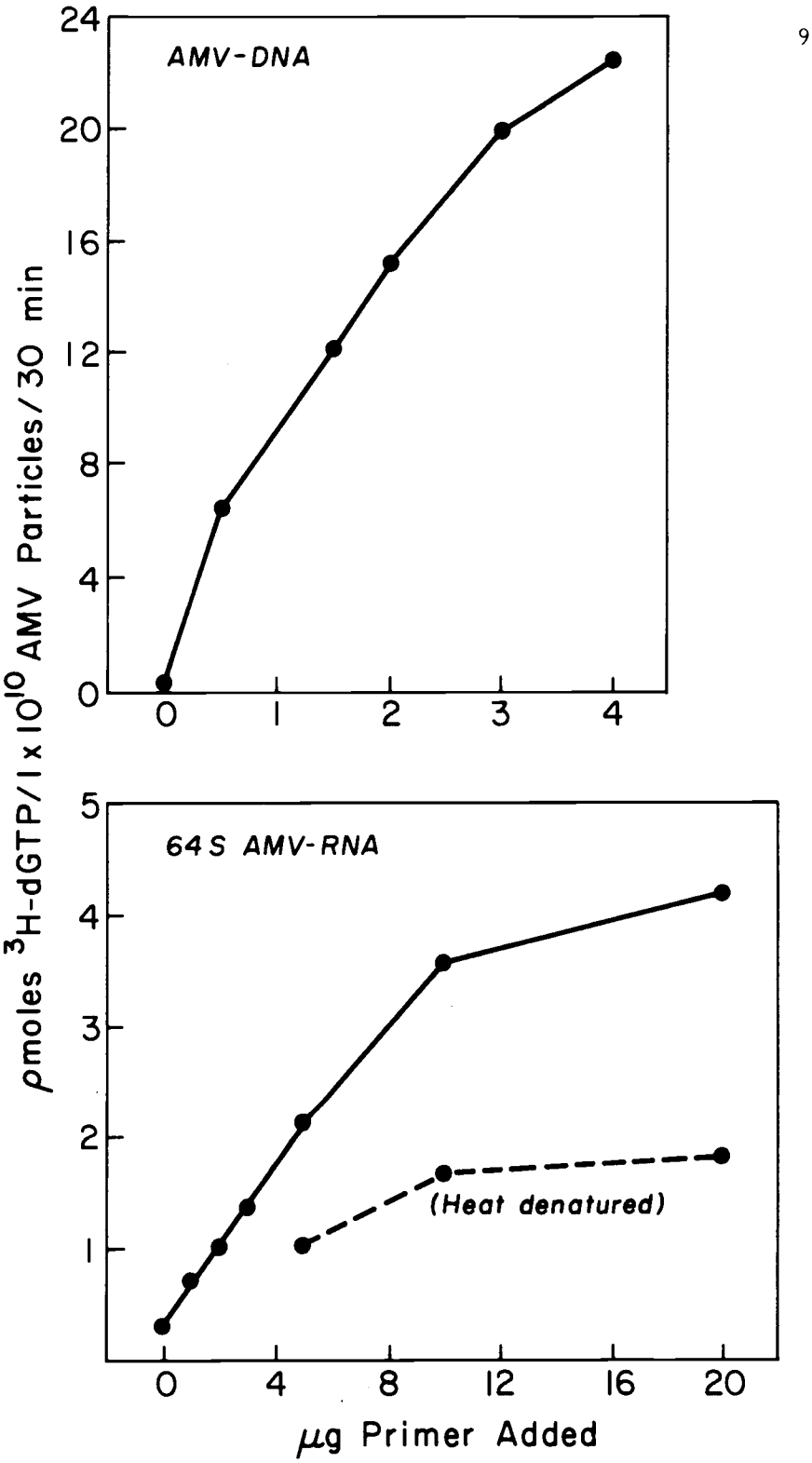


Figure 27. Template saturation of DNA polymerase from AMV. Assays were performed as described in Methods. Specific activity of ^{3}H-dGTP : 1000 cpm/ρmole.

64S RNA from AMV which had been denatured by heating at 92°C for two minutes. The apparent maximum velocity (V_m) of AMV DNA polymerase with the 64S RNA template from AMV and the AMV-DNA template was extrapolated from a Lineweaver-Burke plot of the primer response curves as shown in Figure 28. Although it is difficult to rigorously interpret kinetic constants for the complicated polymerase reaction, the apparent maximum velocities which arise from the Lineweaver-Burke plot are 7.0 pmoles dGMP/30 minutes with 64S RNA template from AMV and 40 pmoles dGMP/30 minutes with AMV-DNA template. The observation that the DNA primed DNA synthesis occurs at a rate six-fold higher than the RNA primed synthesis is in accord with the other data that DNA is a more effective template than RNA for the viral DNA polymerase.

The above studies provided assay optima and template response characteristics for the virus DNA polymerases. An additional parameter available for identifying virus DNA polymerase activity was the fact it was located within the virions themselves. Tumor viruses have a high lipid content and thus a relatively low buoyant density making it possible to band them in glycerol gradients.

Isopycnic Centrifugation

AMV and MC29 virus particles were banded in continuous glycerol gradients as described in Methods. Figures 29 and 30 are the

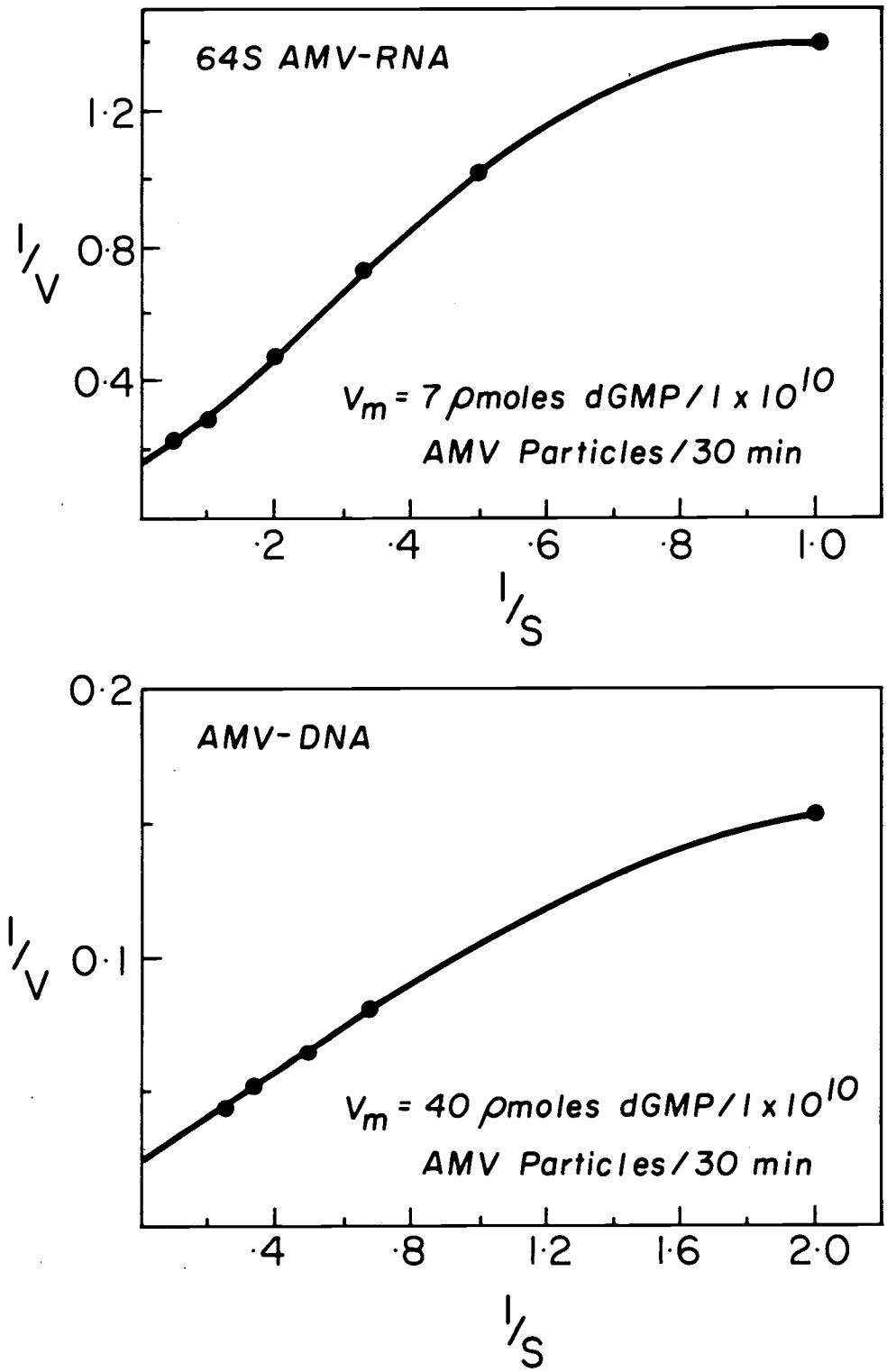


Figure 28. Lineweaver-Burke plot of DNA polymerase activity from AMV. The data shown was obtained from the experiments described in Figure 27. 1/S is in units of μg^{-1} template added.

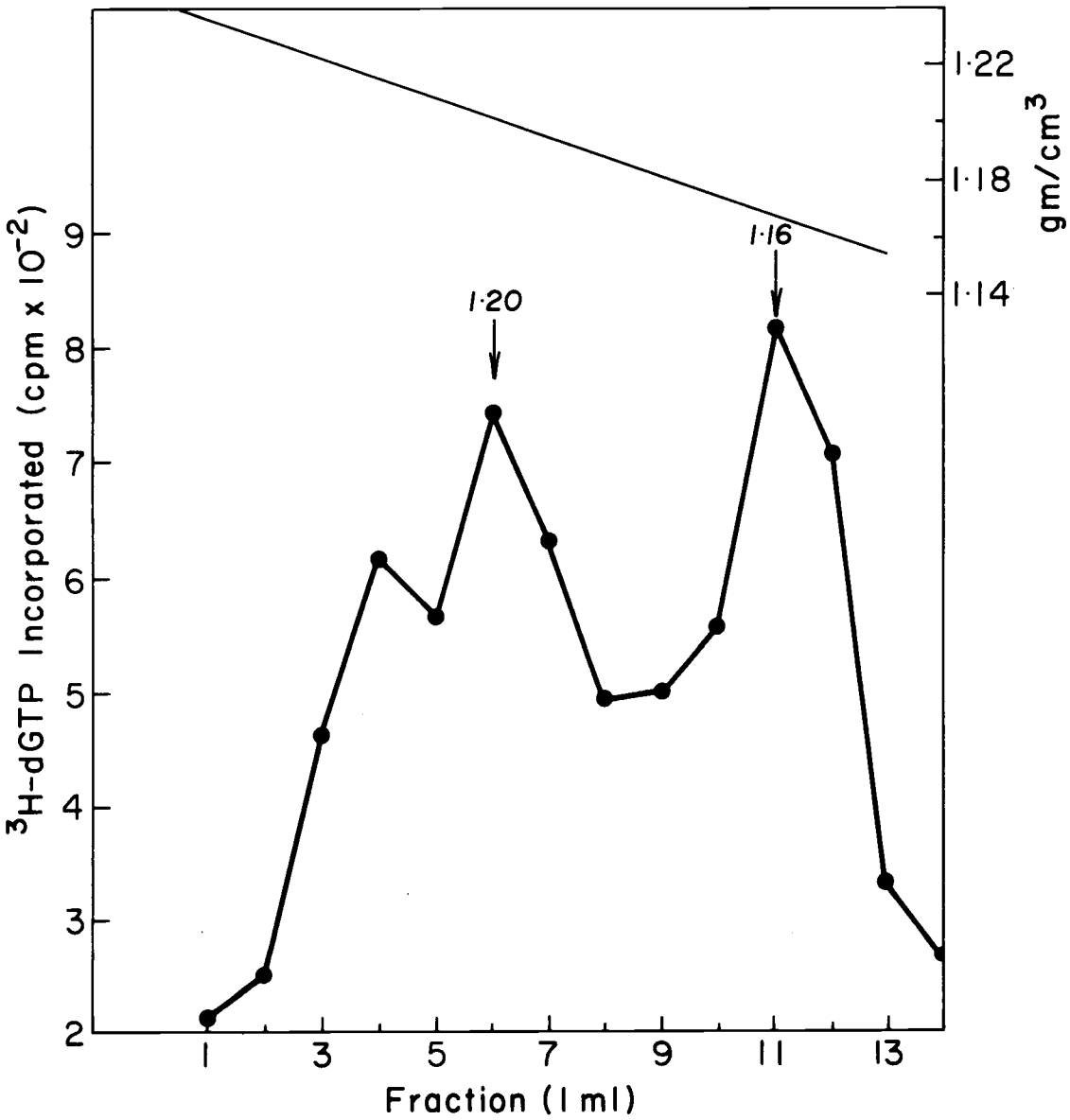


Figure 29. Isopycnic centrifugation of DNA polymerase in AMV particles. AMV from chick plasma was centrifuged to equilibrium in preformed glycerol density gradients, the gradients were fractionated, and each fraction was treated with detergent as described in Methods. Assays (0.2 ml) contained 0.05 ml of detergent treated fractions plus 2 μg M. lysodeikticus DNA, and were performed as described in Methods. No radioactivity was subtracted as background. Specific activity of ^{3}H-dGTP : 1000 cpm/ρ mole.

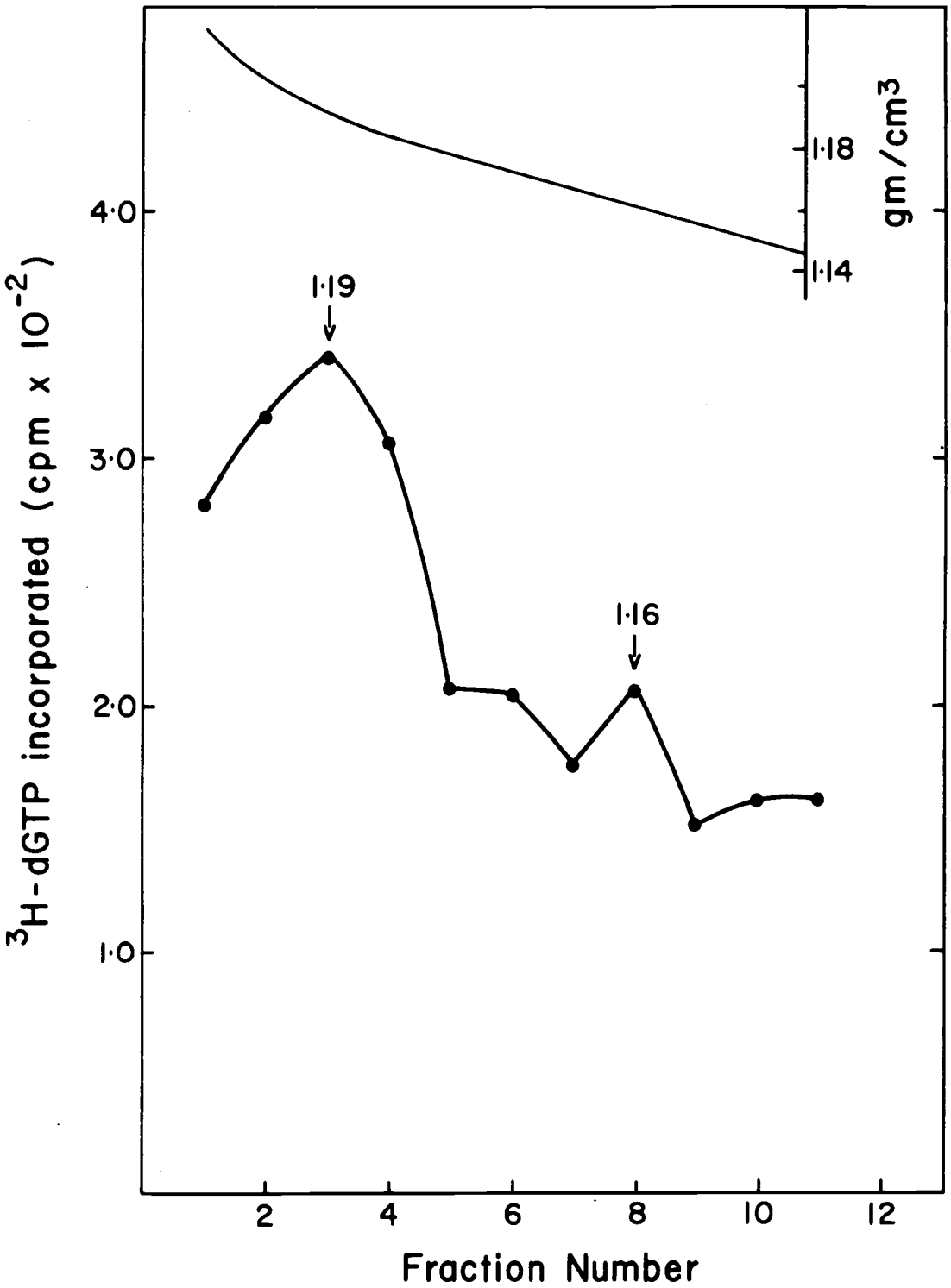


Figure 30. Isopycnic centrifugation of DNA polymerase in MC29 virus. Conditions for the experiment were identical to those reported in Figure 29. Specific activity of ^{3}H-dGTP : 1000 cpm/ρ mole.

glycerol gradient profiles of DNA polymerase activity from AMV and MC 29 virus particles, respectively. Each gradient fraction was detergent treated prior to assay. These gradients were centrifuged for two hours which may not have been sufficient time to obtain sharp density bands. Figure 29 indicates two buoyant density peaks for the AMV DNA polymerase activity: one at a buoyant density of 1.21 gm/cc which is the density of nucleocapsids, core virus particles which have lost their outer membranes, and another at 1.16 gm/cc which is the buoyant density of intact AMV particles (Bader et al., 1970). This plasma had been frozen and thawed, a process which is known to disrupt virus particles. The MC 29 virus particles were obtained from tissue culture fluid which had also been frozen and thawed and two buoyant density peaks of DNA polymerase activity were observed (Figure 30) in the same density regions as the AMV particles.

These gradient results demonstrated it was possible to band virus particles from plasma isopyenically and detect virus DNA polymerase activity.

Particulate Nucleic Acid Polymerases from Virus Transformed Cells

Discontinuous Glycerol Gradients

Preliminary searches for virus-associated DNA polymerase from tumor virus infected cells involved separating cellular

components on discontinuous glycerol gradients. Postnuclear supernatants were obtained from myeloblasts, MC29 infected CEC, and non-infected CEC and further fractionated on discontinuous gradients according to the following diagram. The details of the procedure are in Methods.

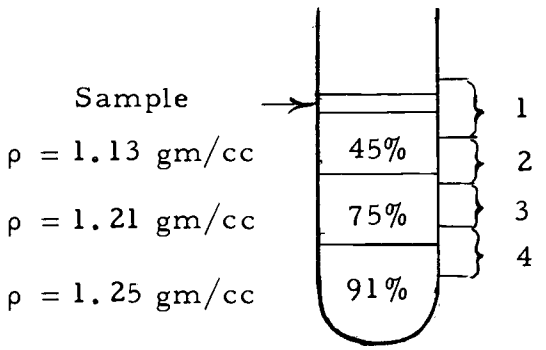


Figure 31 compares the levels of DNA-dependent DNA polymerase activity in each fraction. There was clearly a higher level of DNA polymerase activity in each of the infected cell fractions than in the control CEC fractions. Much of the activity from the virus transformed cells was particulate DNA polymerase activity since it sedimented at densities greater than 1.13 and was stimulated by detergent treatment, as was the case with the virus particle DNA polymerases.

The results in Figure 32 show it was possible to locate the virus associated DNA polymerase in the myeloblast cell fractions by using 64S RNA from AMV and Poly dC: Poly G templates which were previously shown to stimulate DNA synthesis by the DNA polymerases from AMV and MC29 virions. These findings were further supported

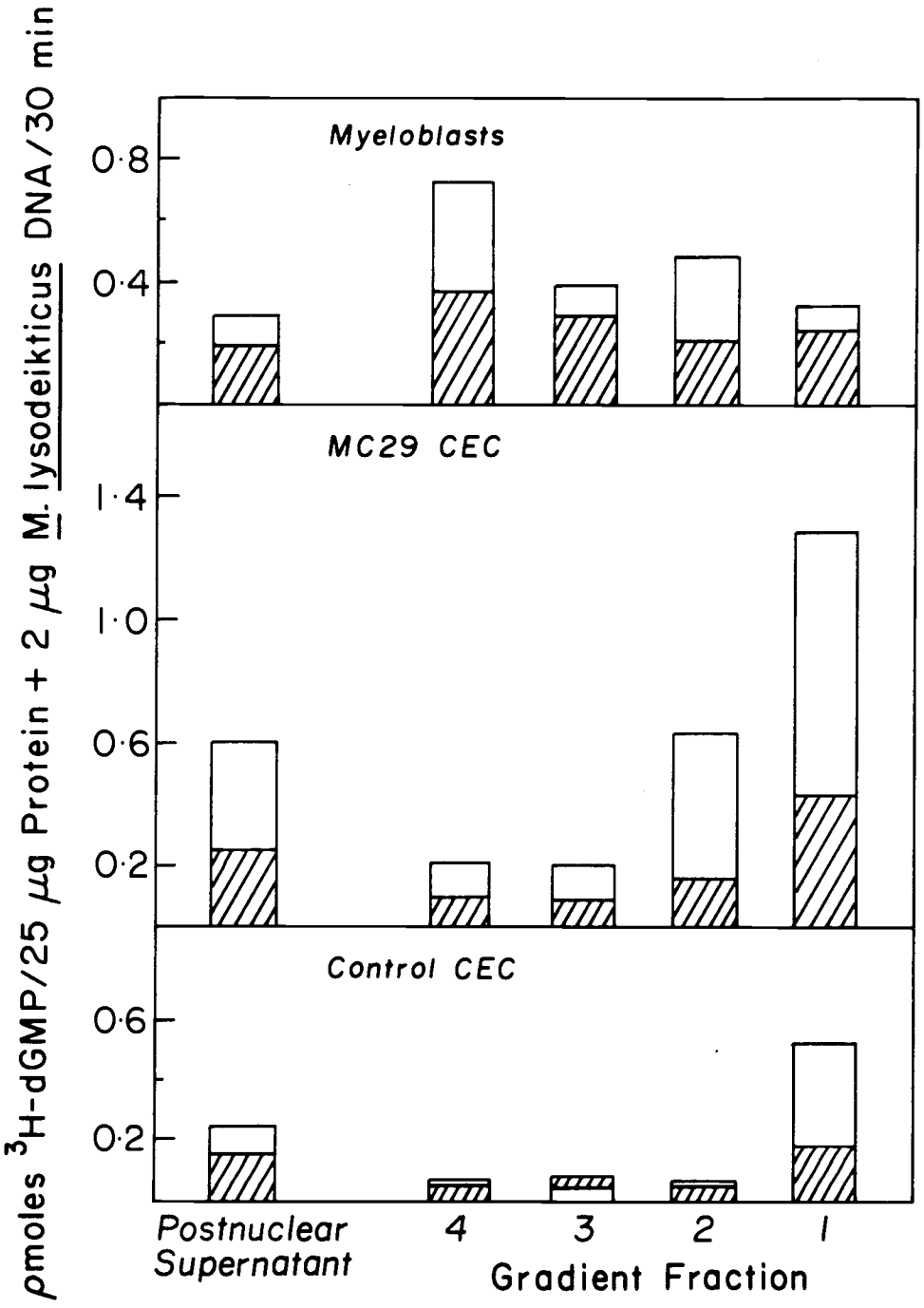


Figure 31. DNA polymerase activity in fractions from virus-infected cells and control cells. Discontinuous glycerol gradient fractionation of postnuclear supernatants from AMV-infected myeloblasts, MC29-infected CEC, and control CEC was performed as described in Methods. Fractions were obtained according to the diagram in the text. Shaded areas represent DNA polymerase activity in each fraction before detergent treatment, nonshaded areas indicate DNA polymerase activity in each fraction following detergent treatment as described in Methods. Assays were performed as described in Figure 29. Specific activity of ^{3}H-dGTP : 1000 cpm/ρ mole.

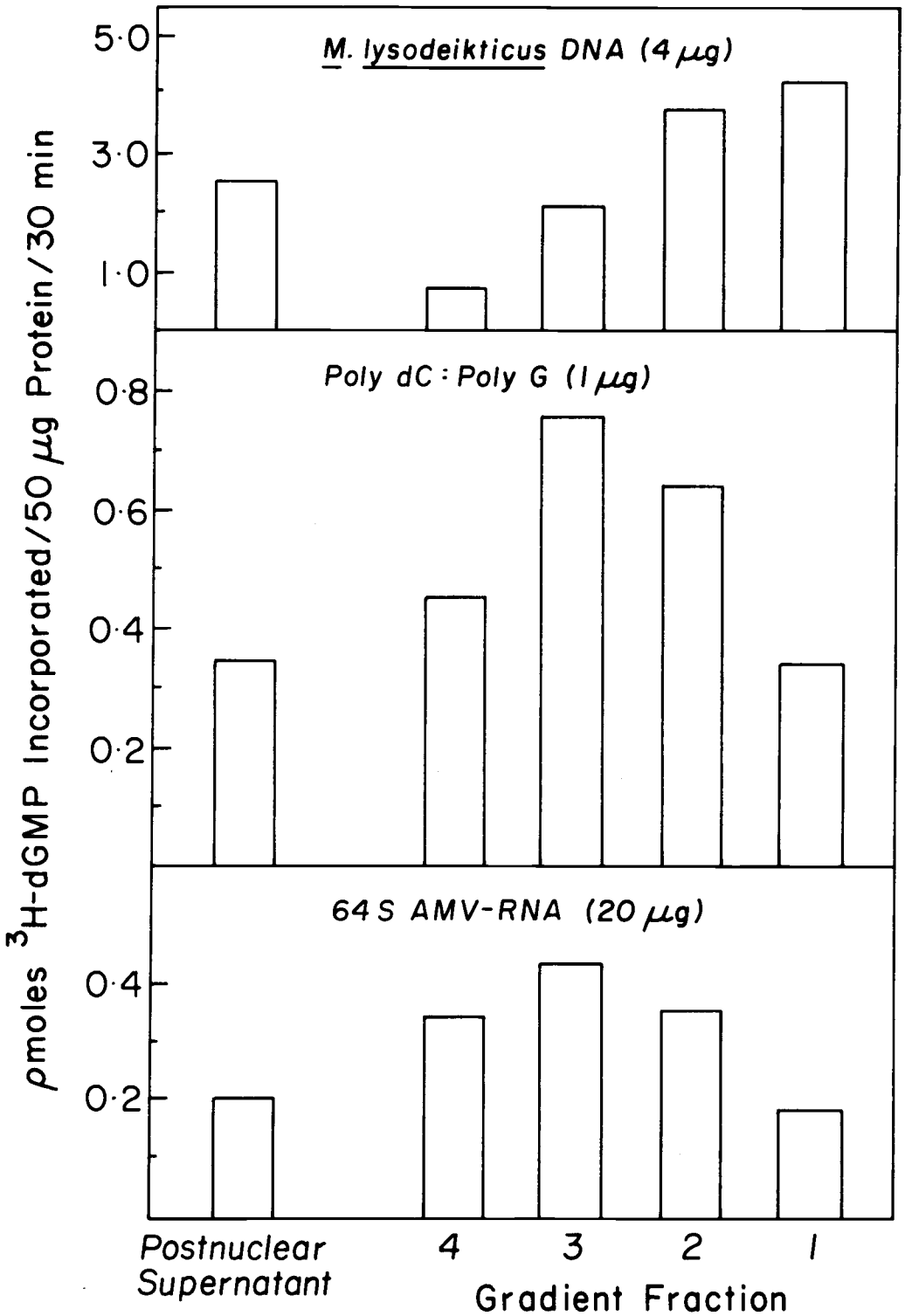


Figure 32. DNA polymerase activity in fractions from AMV-infected myeloblasts. Experimental conditions were identical to those reported in Figure 31.

by the primer response of DNA polymerase in fractions obtained from MC29 infected CEC as shown in Figures 33 and 34. These results illustrated that although a low level of M. lysodeikticus DNA primed DNA polymerase activity was present in control CEC, no DNA synthesis was detected in the same fractions with Poly dC: Poly G primer; whereas both M. lysodeikticus DNA and Poly dC: Poly G served as primers for DNA synthesis in the same fractions from MC29 infected CEC. The data presented in Figures 31 through 34 verify that it was possible to detect DNA polymerase activity in virus infected cells which was not found in control CEC, and further that this DNA polymerase would recognize the same primers for DNA synthesis as the virus particle DNA polymerases.

Isopycnic Glycerol Gradients

More definitive studies on locating the virus-associated DNA polymerase activity in infected cells were performed by isopycnic banding of the enzyme activity. Preliminary studies of the discontinuous glycerol gradients had also revealed levels of DNA primed RNA synthesis and RNA primed RNA synthesis in the virus infected cell enzyme fractions not found in the control CEC fractions. Consequently RNA polymerase activity as well as DNA polymerase activity was investigated in each fraction from isopycnic glycerol gradients of post-nuclear supernatants from myeloblasts, MC29 transformed CEC and

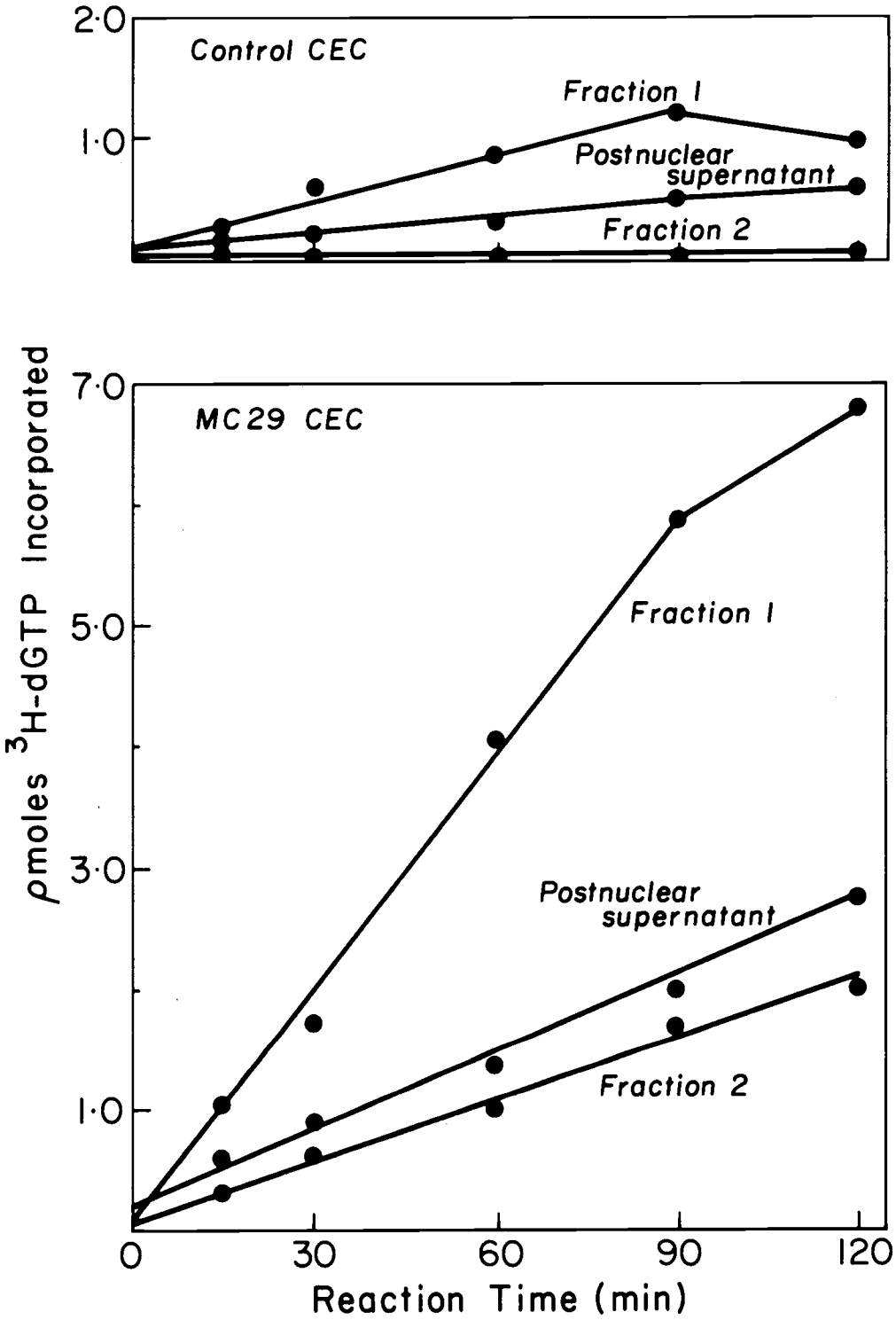


Figure 33. DNA synthesis by fractions from MC29-infected CEC and control CEC with M. lysodeikticus DNA primer. Assays contained 25 μg protein from fractions obtained in the experiment described in Figure 31, plus 2 μg M. lysodeikticus DNA. Assays (0.1 ml) were performed as described in Figure 29. Specific activity of ^{3}H-dGTP : 1000 cpm/ρmole.

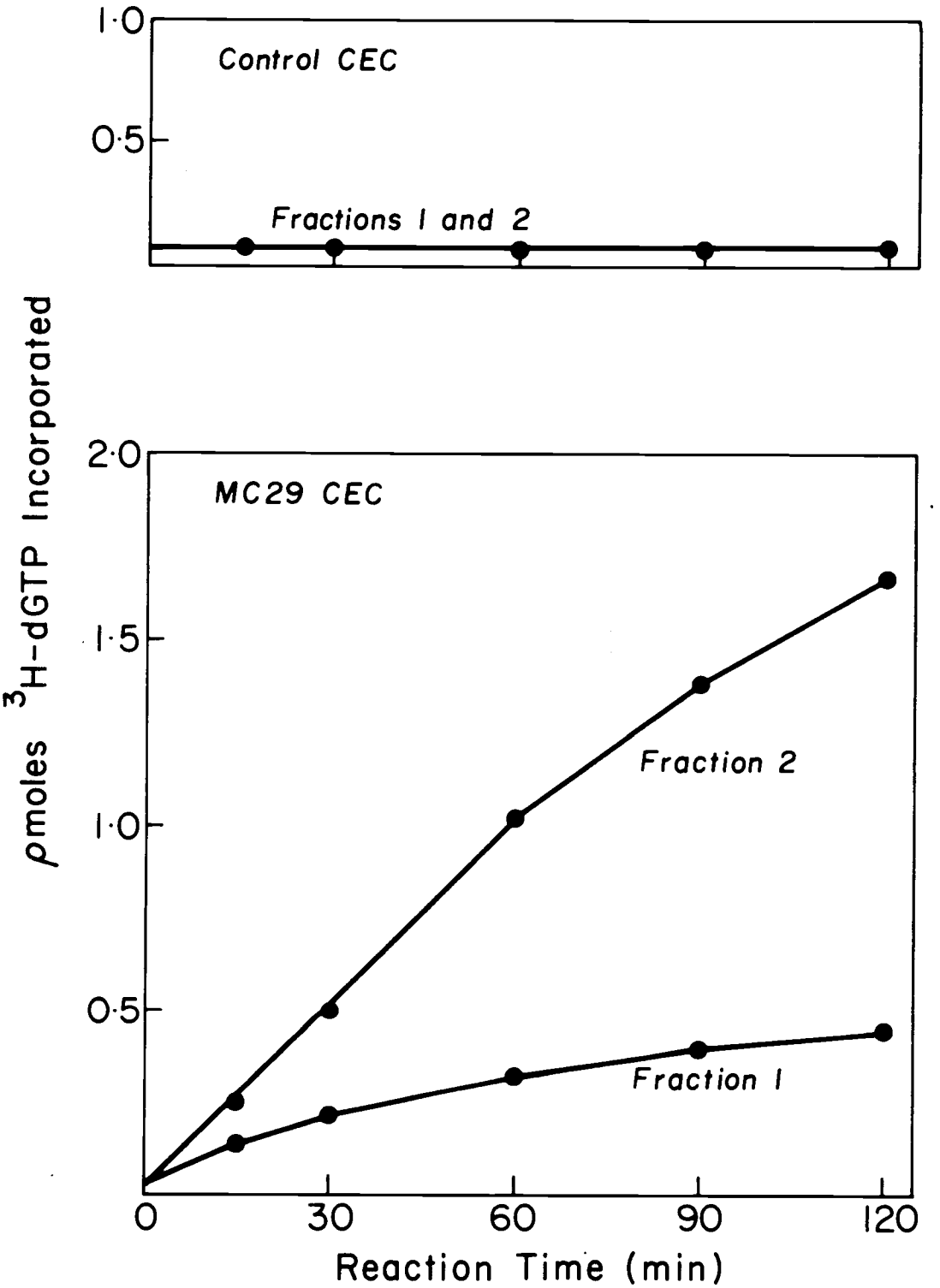


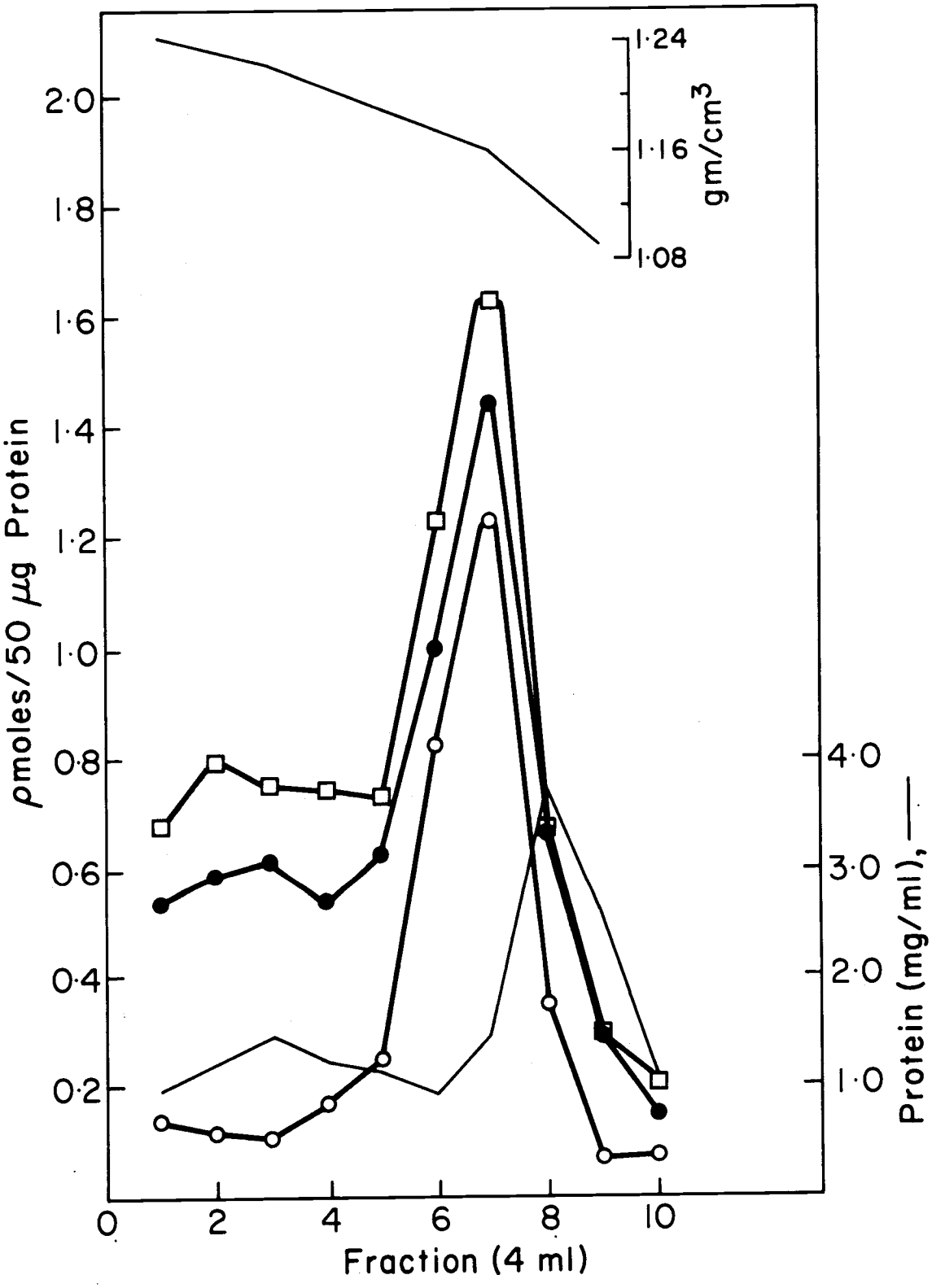
Figure 34. DNA synthesis by fractions from MC29-infected CEC and control CEC with poly dC : poly G primer. Experimental conditions were identical to those described in Figure 33 with the substitution of 2 μg poly dC : poly G for M. lysodeikticus DNA.

control CEC as shown in Figures 35, 36, and 37, respectively. Both DNA primed DNA synthesis and DNA primed RNA synthesis peak at the same buoyant density (1.16 gm/cc) on the virus infected cell gradients. No similar peaks of nucleic acid polymerases were observed on the control CEC gradient. The low level of polymerase activities which did exist on the control CEC gradient peaked at a buoyant density of 1.13 gm/cc. All gradients were assayed following detergent treatment of each gradient fraction. Table 8 shows the enzyme activities observed for the peak activity gradient fractions with and without detergent.

The DNA polymerase from AMV-infected cells was stimulated ten-fold by detergent treatment, whereas the DNA polymerase from MC 29-infected cells was only stimulated two-fold by the detergent treatment. The RNA polymerase from AMV-infected cells was stimulated 3 to 12-fold by the detergent treatment, but the RNA polymerase from MC 29-infected cells was stimulated little if any by the detergent treatment. As can be seen from the data in Table 9, there was little if any nucleic acid polymerase activity in the control CEC fraction.

Table 9 shows the primer responses of the cellular DNA polymerases which were isopycnically banded. In other laboratories, poly dC: poly dG and poly A: poly T were found to be good primers for DNA polymerase from tumor virus (Spiegelman, 1970).

Figure 35. Isopycnic centrifugation of nucleic acid polymerases from AMV-infected myeloblasts. Postnuclear supernatant from myeloblasts was centrifuged to equilibrium in a preformed glycerol density gradient, the gradient fractionated, and each fraction treated with detergent as described in Methods. Each assay contained 2 μg M. lysodeikticus DNA. Assays for RNA synthesis [^{3}H-GTP (o—o) and ^{3}H-UTP (□—□)] were incubated for 15 minutes. Assays for DNA synthesis [^{3}H-dGTP (●—●)] were incubated for 30 minutes. Assay conditions were as described in Methods. No radioactivity was subtracted as background. Specific activity of all ^{3}H-labeled substrates was 1000 cpm/pmole.


2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
pmoles/50 μg Protein
1·24
1·16
1·08
gm/cm3
4·0
3·0
2·0
1·0
Protein (mg/ml), ——
0
2
4
6
8
10
Fraction (4 ml)

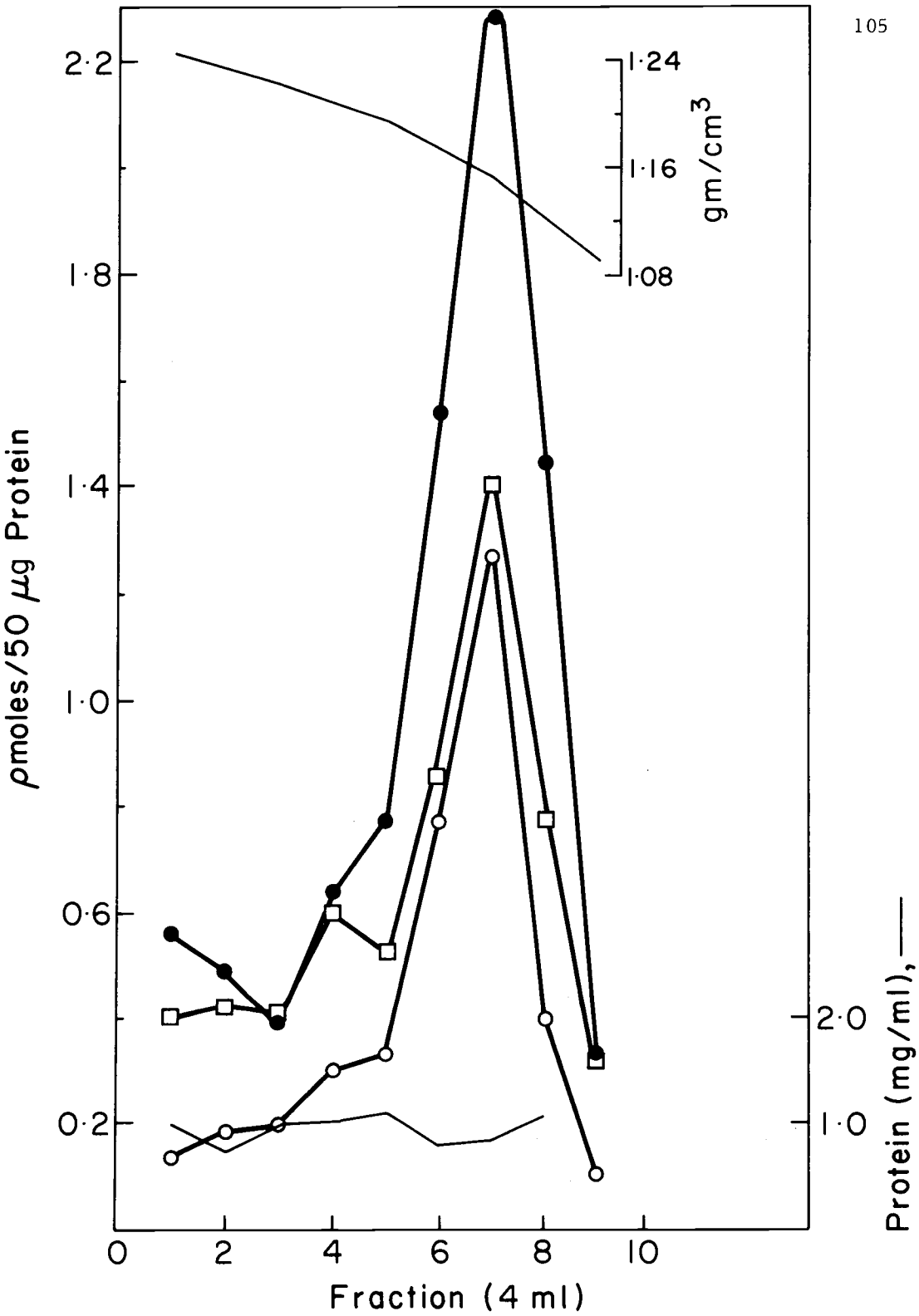


Figure 36. Isopycnic centrifugation of nucleic acid polymerases from MC29-infected CEC. Experimental conditions were identical to those reported in Figure 35.

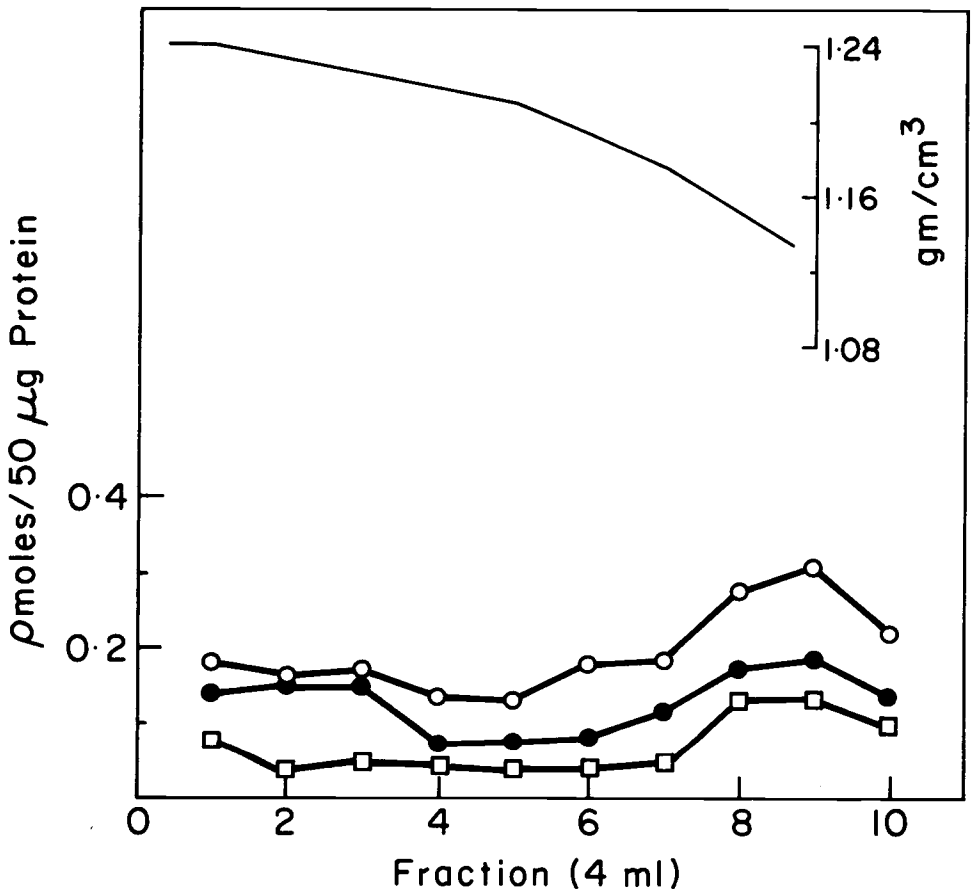


Figure 37. Isopycnic centrifugation of nucleic acid polymerases from uninfected control CEC. Experimental conditions were identical to those described in Figure 35.

Table 8. Detergent Requirement of Peak Isopycnic Gradient Enzyme Activities.

	cpm**/35 μg protein/30 minutes					
	without detergent			with detergent		
Enzyme*	^{3}H-dGMP	^{3}H-GMP	^{3}H-UMP	^{3}H-dGMP	^{3}H-GMP	^{3}H-UMP
Myeloblast	188	28	113	1969	387	367
MC 29 CEC	2615	231	253	3996	258	317
Control CEC	126	17	52	167	22	50

*Enzyme was from the fraction with buoyant density = 1.16 gm/cc from each gradient. The detergent treated fractions were made 0.25% with respect to Nonidet NP-40, and 1% with respect to DTT.

**Assays (0.20 ml) contained 2 μg AMV-DNA template and the conditions were as described in Methods. The specific activity of all tritiated (deoxy)nucleoside triphosphates was 1 pmole - 1000 cpm. Twenty cpm was subtracted from each value as background.

Table 9. Primer Response of DNA Polymerase from Cells.

Primer*	cpm**/25 μg protein/30 minutes					
	Myeloblasts		MC29 CEC		Control CEC	
	^{3}H-dGMP	^{3}H-TMP	^{3}H-dGMP	^{3}H-TMP	^{3}H-dGMP	^{3}H-TMP
None	239	107	319	189	105	77
M. lysodeikticus DNA	1678	--	2296	--	280	--
AMV DNA	1992	1525	3468	2424	372	329
dC[b]	2731	--	13519	--	317	--
dCdG	261	--	--	--	--	--
dCrG[a]	435	--	1158	--	133	--
rG	219	--	--	--	--	--
rA	--	147	--	--	--	--
rAT[a]	--	364	--	1503	--	101
AMV RNA	461	330	591	412	109	101

*Assays contained 20 μg of Poly rG, Poly rA, and 64S RNA from AMV, and 2 μg of all the other primers. Assay conditions were as in Table 8.

**Specific activity of tritiated deoxyribonucleoside triphosphates: 1 pmole = 1000 cpm.

[a]Gift from Dr. S. Spiegelman.

[b]Gift from K. C. Olsen.

From the results shown in Table 9 it was apparent that these intracellular enzymes would recognize the same primers that the virus particle-DNA polymerases recognized, namely, the synthetic DNA: RNA hybrids (Poly dC: Poly G and Poly A: Poly T), the homopolymer dC, and 64S RNA from AMV as well as the AMV-DNA species. It was interesting that both DNA and Poly dC served as more effective templates for the infected cell DNA polymerases than Poly dC: Poly G when the reverse was true for the virus particle DNA polymerases, and that the TMP: dGMP incorporation ratio with 64S RNA from AMV as primer was not 2: 1, but more highly purified cellular enzymes could clarify this discrepancy.

Primer responses of the isopycnically banded RNA polymerase from the virus infected cells were also compared with RNA polymerase from control CEC. Table 10 shows RNA synthesis in response to the primers observed to stimulate DNA synthesis by the virus-associated DNA polymerase.

The DNA-dependent RNA polymerase activity was 5 to 10-fold higher in the enzyme fractions from virus infected cells compared to the enzyme from control CEC. The RNA-dependent RNA polymerase activity was 2 to 4-fold higher in the virus infected cells compared to the control CEC fractions. The synthetic DNA: RNA hybrid polymers stimulated RNA synthesis in the virus infected cell fractions 3 to 8-fold higher than the activity in the same fractions from the

control CEC.

It was of interest to determine if the peak RNA polymerase activities observed on the isopycnic glycerol gradients were related to the RNA-dependent RNA polymerase activities previously isolated from myeloblasts. The response of the RNA-dependent RNA polymerase to various synthetic RNA primers was previously noted (Table 3). RNA synthesis by the polymerase fraction examined in Tables 9 and 10 was also determined in response to the synthetic RNA primers, as shown in Table 11.

The nuclease activity present in these fractions (20% loss in acid-precipitable RNA in 15 minutes) precluded rigorous interpretation of the primer responses of the RNA polymerase activities in the virus infected cells. However, there was a higher level of RNA-dependent RNA synthesis in the virus infected cells than in the control CEC. The UMP incorporation by the myeloblast RNA polymerase fraction was highest with heterogeneous RNA primer and Poly AG primer which was also observed for the soluble RNA polymerase reported in Table 3. There is some RNA polymerase activity in the control CEC which incorporates UMP into acid precipitable product. The most effective primers for the control cell enzyme were Poly AG, Poly A, and Poly AC and not heterogenous RNA; there is little if any GMP incorporation into RNA product by the control cell polymerase activity.

Table 10. Primer Response of RNA Polymerase from Cells.

Primer*	cpm**/25 μg protein***					
	Myeloblasts		MC 29 CEC		Control CEC	
	^{3}H-GMP	^{3}H-UMP	^{3}H-GMP	^{3}H-UMP	^{3}H-GMP	^{3}H-UMP
None	193	793	141	317	87	211
64S RNA from AMV	317	1563	282	992	141	424
M. lysodeikticus DNA	1424	1551	775	1154	187	308
AMV DNA	1426	1593	947	1544	104	355
Poly A: Poly T	--	957	--	639	--	203
Poly dC	444	--	235	--	90	--
Poly dC: Poly dG	48	--	198	--	47	--
Poly dC: Poly G	2191	--	1319	--	288	--

*Assays (0.20 ml) contained 20 μg of 64S RNA from AMV and 2 μg of all other primers

**Specific activity of each ribonucleoside triphosphate: 1 pmole = 1000 cpm. Background of 20 cpm subtracted.

***RNA primed reactions were incubated at 37°C for 15 minutes; all other assays were incubated at 37°C for 30 minutes. Assay conditions were as described in Methods.

Table 11. RNA Polymerase Response to Synthetic RNA Primers.

	cpm** /25 μg protein***					
	Myeloblasts		MC29 CEC		Control CEC	
Primer*	^{3}H-GMP	^{3}H-UMP	^{3}H-GMP	^{3}H-UMP	^{3}H-GMP	^{3}H-UMP
None	193	793	141	317	87	211
Myeloblast RNA	474	2398	--	683	--	272
64S RNA from AMV	317	1563	282	992	141	424
Poly A	--	1618	--	1048	--	598
Poly C	303	--	--	--	--	--
Poly G	276	--	427	--	157	--
Poly U	--	845	--	318	--	209
Poly AC	402	1706	335	608	51	533
Poly AG	161	2434	378	1812	73	777
Poly AU	--	829	--	--	--	--

*Assays (0.20 ml) contained 40 μg myeloblast RNA and 20 μg of all other primers.

**Specific activity of each ribonucleoside triphosphate: 1 pmole = 1000 cpm. Background of 20 cpm subtracted.

***All assays incubated 15 minutes at 37°C. Assay conditions were as in Table 10.

Summary of Virus-Associated Nucleic Acid Polymerases

The above studies have shown the *in vitro* radioassay characteristics of the tumor virus DNA polymerase. The DNA polymerase was shown to be intimately associated with the tumor virus particles on isopycnic glycerol gradients. DNA-dependent as well as RNA-dependent DNA polymerase activity exists in the virus particles. Evidence was presented that AMV-DNA was the principal template for the endogenous synthesis of DNA.

Virus-associated DNA polymerase activity was demonstrated in cells infected with tumor viruses. The virus-associated DNA polymerase from infected cells had a buoyant density of 1.16 gm/cc. There was little if any DNA polymerase activity from control cells with a buoyant density greater than 1.13 gm/cc.

RNA polymerase activity was also observed in the infected cell fractions at a buoyant density of 1.16 gm/cc which was not found in control CEC cell fractions. DNA-dependent as well as RNA-dependent RNA polymerase activity was found in the virus infected cells. The RNA-dependent RNA polymerase had some of the properties of the soluble RNA-dependent RNA polymerase from myeloblasts.

Human Leukemia Studies

Once criteria for DNA polymerase activity in tumor viruses had

been established, it then became possible to examine human leukemias for the presence of tumor virus DNA polymerase activity. Blood samples from leukemic patients were obtained from Dr. Patrick Ragen, Dr. John Huff, and Dr. Peter Kiessling of the Virginia Mason Clinic, and from Dr. Sol Spiegelman, Columbia University. DNA polymerase activity was assayed in pellets obtained from centrifugation of human plasmas, in isopycnic gradients of human plasma material, and in the white blood cells from human leukemic blood samples.

DNA Polymerase Activity in Plasma Pellets from Human Leukemia

Plasmas were obtained from two patients with chronic lymphocytic leukemia (CLL). Blood sample, CLL-V, was from a 67 year old white male with CLL for ten years who had a white cell count of 500,000 cells/mm^3 and had just been started on ^{60}Cobalt therapy to the spleen. Blood sample, CLL-D, was from a 70 year old white female who had just been recently diagnosed with CLL, had a white cell count of 30,000 cells/mm^3, and was hospitalized for blood transfusions at the time the sample was obtained. Plasmas from two individuals with no known diseases were obtained as controls.

Pellets were obtained from each of the CLL plasmas and from the control plasmas as described in Methods. Table 12 lists the DNA polymerase activity in each of the pelleted fractions with and without

Nonidet NP-40 treatment.

Table 12. DNA Polymerase Activity in Human Plasma Pellets.

Enzyme*	Amount (ml)	Primer**	cpm ^{3}HdGMP***/60 min. no detergent	with detergent
CLL-V	0.01	DNA	81	201
CLL-V	0.02	DNA	--	253
CLL-V	0.05	DNA	125	570
CLL-V	0.01	Poly I: Poly C	72	47
CLL-D	0.01	DNA	82	91
CLL-D	0.02	DNA	--	121
CLL-D	0.05	DNA	50	170
CLL-D	0.01	Poly I: Poly C	84	89
Control #1	0.02	DNA	--	83
Control #2	0.02	DNA	--	82

*For each of the CLL 0.01 ml enzyme corresponds to pellet obtained from 0.25 ml plasma, for each of the controls 0.02 ml enzyme corresponds to pellet obtained from 1.0 ml plasma.

**4 μg M. lysodeikticus DNA was added to each assay (0.20 ml), Poly I: Poly C was made as previously described and 0.01 ml added to each assay.

***Specific activity of ^{3}HdGTP: 1 pmole = 2000 cpm. No radioactivity was subtracted as background.

M. lysodeikticus DNA was used as template for the assays shown in Table 12 because maximum sensitivity of DNA polymerase activity was desired, and M. lysodeikticus DNA was an excellent template for DNA synthesis by the DNA polymerases from AMV and MC29 virus. As shown in Table 12 detergent treatment greatly stimulated the DNA polymerase activity in the human plasma pellets. Detergent treatment of AMV and MC29 particles is a requisite for

expression of the viral DNA polymerase activity (Spiegelman et al., 1970a; Weber et al., 1970a). Poly I: Poly C (which stimulates DNA synthesis by the AMV DNA polymerase but not the MC 29 DNA polymerase) showed no stimulation of DNA synthesis.

There was no detectable DNA polymerase activity in the particulates pelleted from the plasmas of the two healthy individuals.

Kinetics of DNA Synthesis. The presence of DNA polymerase in human leukemic plasma pellets was further supported by the kinetics of DNA synthesis shown in Figure 38. No DNA synthesis by the pellet fractions from normal plasma was observed, as shown by the incubation times in Figure 39.

Deoxyribonucleside Triphosphate Requirement. That a DNA heteropolymer is being synthesized by the DNA polymerase from CLL was shown by a requirement for all four deoxyribonucleoside triphosphates in the assay system (Table 13).

Table 13. Response of CLL Enzymes to Omission of Deoxynucleoside Triphosphates from the Assay.

Assay System*	cpm ^{3}H-TMP**/0.04 ml enzyme***/120 min.	
	CLL-V	CLL-D
Complete	672	117
minus dATP	113	94
minus dCTP	148	66
minus dGTP	103	46

*Each assay (0.20 ml) contained 4 μg M. lysodeikticus. DNA primer

**Specific activity ^{3}H TTP: 1 pmole = 2700 cpm. 15 cpm was subtracted as background

***In this experiment 0.04 ml of enzyme corresponds to particulates from 0.50 ml of plasma.

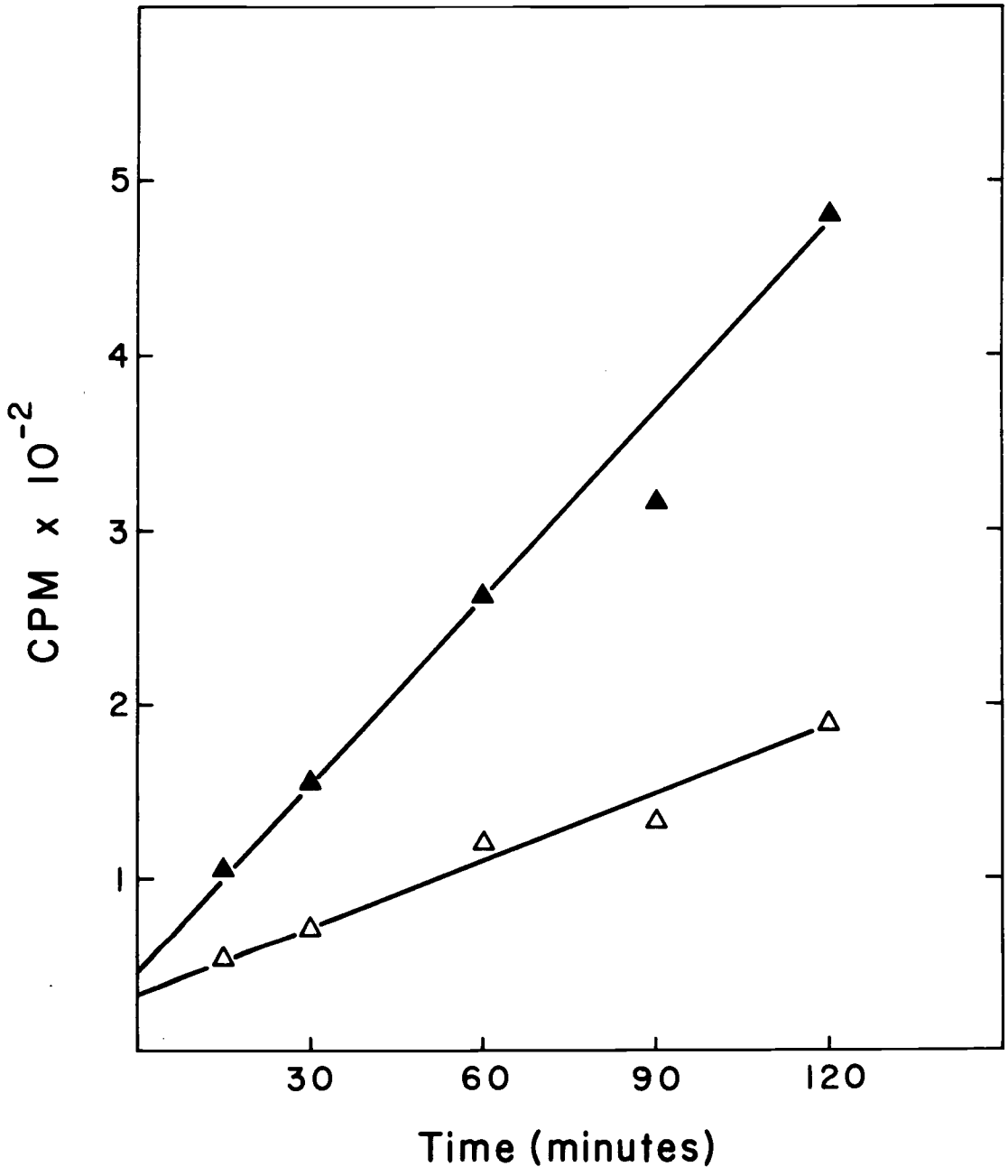


Figure 38. Kinetics of DNA synthesis by DNA polymerase associated with CLL. Assays (0.2 ml) contained particulates from 0.5 ml of plasma plus 4 μg M. lysodeikticus DNA primer and were performed as described in Methods. The ^{3}H-dGTP incorporated into product DNA by CLL-V (▲—▲) and CLL-D (△—△) had a specific activity of 2,000 cpm/ρmole.

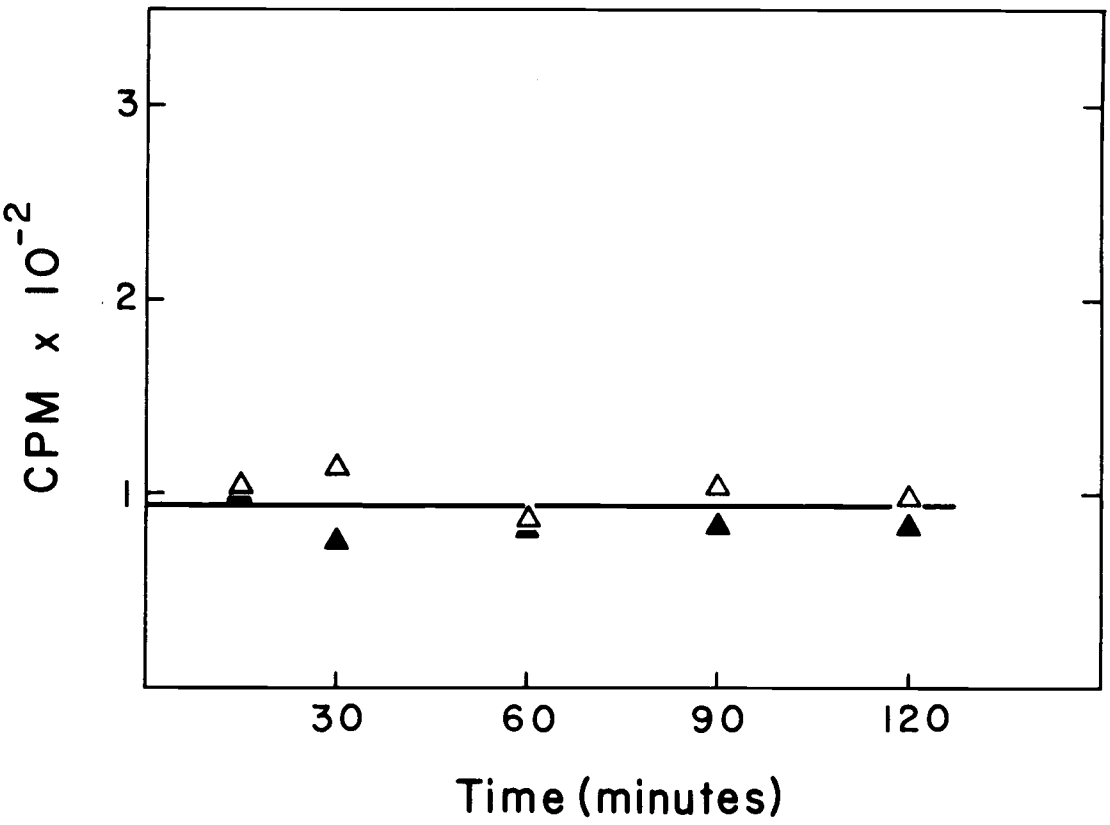


Figure 39. Absence of DNA polymerase activity in non-leukemic plasmas. Assays (0.2 ml) contained particulates from 1.0 ml of plasma from two healthy individuals (open and closed triangles) plus 4 μg M. lysodeikticus DNA and were performed as described in Methods. ^{3}H-dGTP had a specific activity of 2,000 cpm/ρ mole.

As shown in Table 13, the omission of any one of the deoxyribonucleoside triphosphates from the enzyme reaction significantly reduced DNA synthesis by DNA polymerase from CLL-V. The amount of DNA synthesized by DNA polymerase from CLL-D was low in the complete reaction mixture. The omission of dGTP, however, did significantly decrease the amount of DNA synthesized by the polymerase from CLL-D.

Primer Response. It was of interest to determine if the CLL enzymes would use RNA as template for DNA synthesis, as would the DNA polymerases from AMV and MC29 virus. A slight stimulation of DNA synthesis with RNA primer was observed as shown in Table 14.

Table 14. Primer Response of CLL DNA Polymerase.

Primer*	cpm ^{3}HdGMP**/0.035 ml enzyme***/120 min.	
	CLL-V	CLL-D
None	76	48
M. lysodeikticus DNA	525	134
64S AMV-RNA	114	98

*M. l. DNA was used at a level of 4 μg and AMV-RNA was used at a level of 10 μg per assay (0.20 ml).
**Specific activity of ^{3}HdGMP: 1 pmole = 2000 cpm.
***The same enzyme fraction was used here as in Table 11.

Product Studies. The DNA product synthesized by the CLL-V enzyme from *M. lysodeikticus* DNA template was banded isopycnically in a CsCl density gradient as described in Methods. The major

radioactivity peak, representing the DNA product, had the same buoyant density as the primer DNA (1.72 gm/cc). These results are shown in Figure 40 and are very similar to the results obtained with the DNA polymerase from MC29 (Figure 23).

The above studies on the plasma pellet fractions from two patients with chronic lymphocytic leukemia revealed a detergent-activated DNA polymerase activity which was not found in the plasma pellets from two people with no known diseases. The DNA polymerases associated with particulates from CLL plasma synthesized DNA heteropolymer with the same buoyant density as the M. lysodeikticus DNA template. Primer studies for DNA synthesis were limited by the small amount of enzyme available for study, but a slight stimulation of DNA synthesis by 64S RNA from AMV was observed. The characteristics of the DNA polymerases thus far observed presented a striking resemblance to tumor virus DNA polymerase activity. To further ascertain the resemblance to tumor virus activity, the CLL plasma particulates were banded isopycnically and the gradient fractions examined for DNA polymerase activity.

Buoyant Density Determination of DNA Polymerase from Human CLL Plasma

A second blood sample of CLL-V was obtained following more ^{60}Co therapy treatments to his spleen. The patients white blood cell count had dropped to 250,000 cells/mm^3. This plasma was clarified,

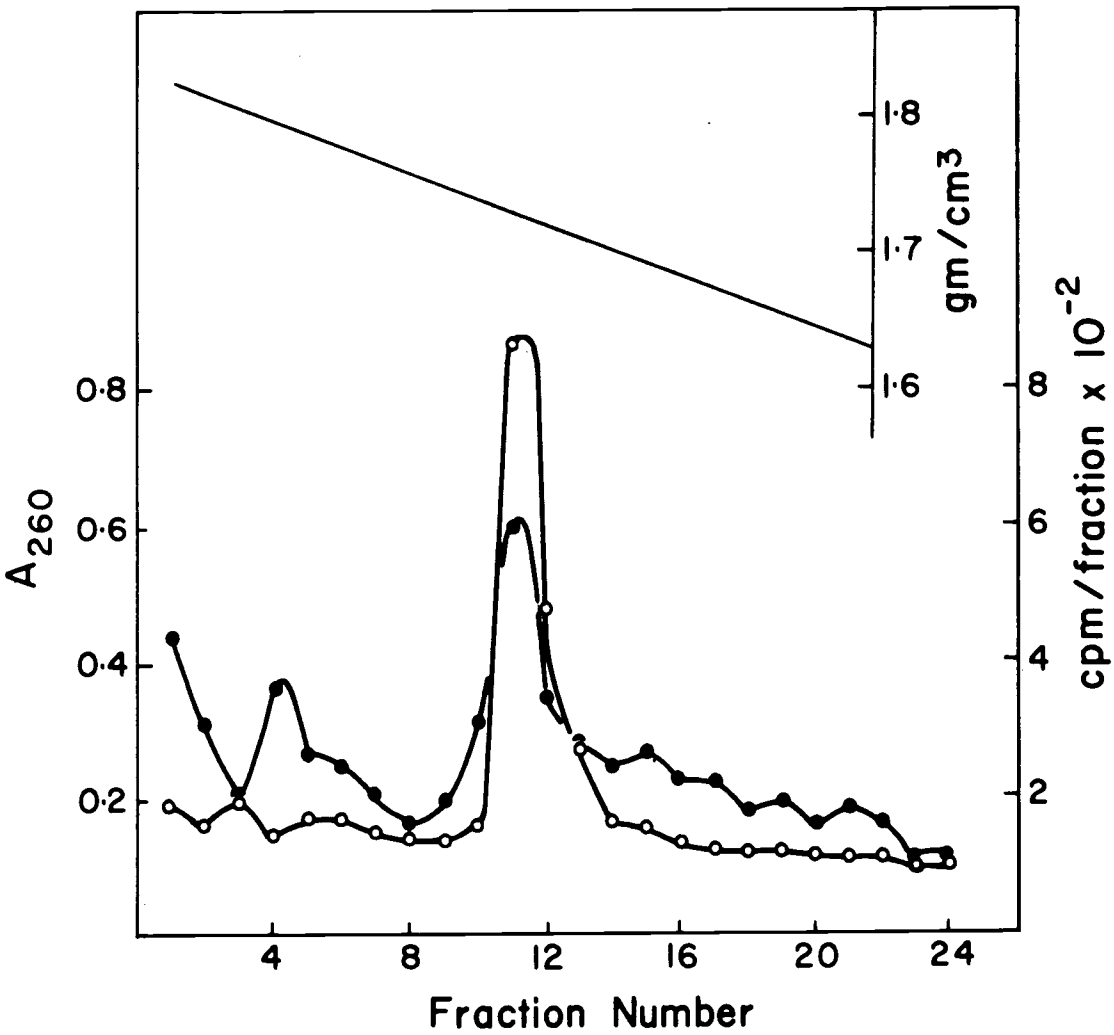


Figure 40. Isopycnic centrifugation of DNA product synthesized by DNA polymerase from CLL-V. ^{3}H-labeled product from a two-fold assay (0.4 ml) and 50 μg of M. lysodeikticus DNA were mixed with CsCl to give a density of 1.70, and a density gradient formed and fractionated as described in Figure 23. (o—o) absorbance of M. lysodeikticus DNA at 260 nm, and (•—•) ^{3}H-dGTP at specific activity of 2,000 cpm/ρ mole.

pelleted against a 95% glycerol pad and banded in an isopycnic glycerol gradient as described in Methods.

Chicken plasma containing AMV particles was treated in the same manner at this time for comparison. Figures 41 and 42 are the DNA polymerase activity profiles for the CLL-V gradient and the AMV gradient, respectively. As can be seen from these two figures, the buoyant density profile of DNA polymerase activity from CLL-V was strikingly similar to the AMV profile and to the MC29 profile (Figure 30) as well. The plasma containing the AMV particles had been frozen and thawed three times and most of the virus particles were in the form of nucleocapsids as was evident from the buoyant density of the AMV DNA polymerase. Assuming the observed DNA polymerase activity from CLL-V was contained in a human tumor virus, by comparison to the AMV particles, the human tumor virus might also be in the form of nucleocapsids.

Additional Plasma Samples from CLL-V

Two more plasma samples were obtained from CLL-V: #1 following the administration of 250 ml packed red blood cells, and #2 following several more ^{60}Co therapy treatments which lowered the white blood cell count to 6,000 cells/mm^3. Neither of these plasmas contained any particulate DNA polymerase activity when analyzed by the same techniques. These negative results indicated that plasma

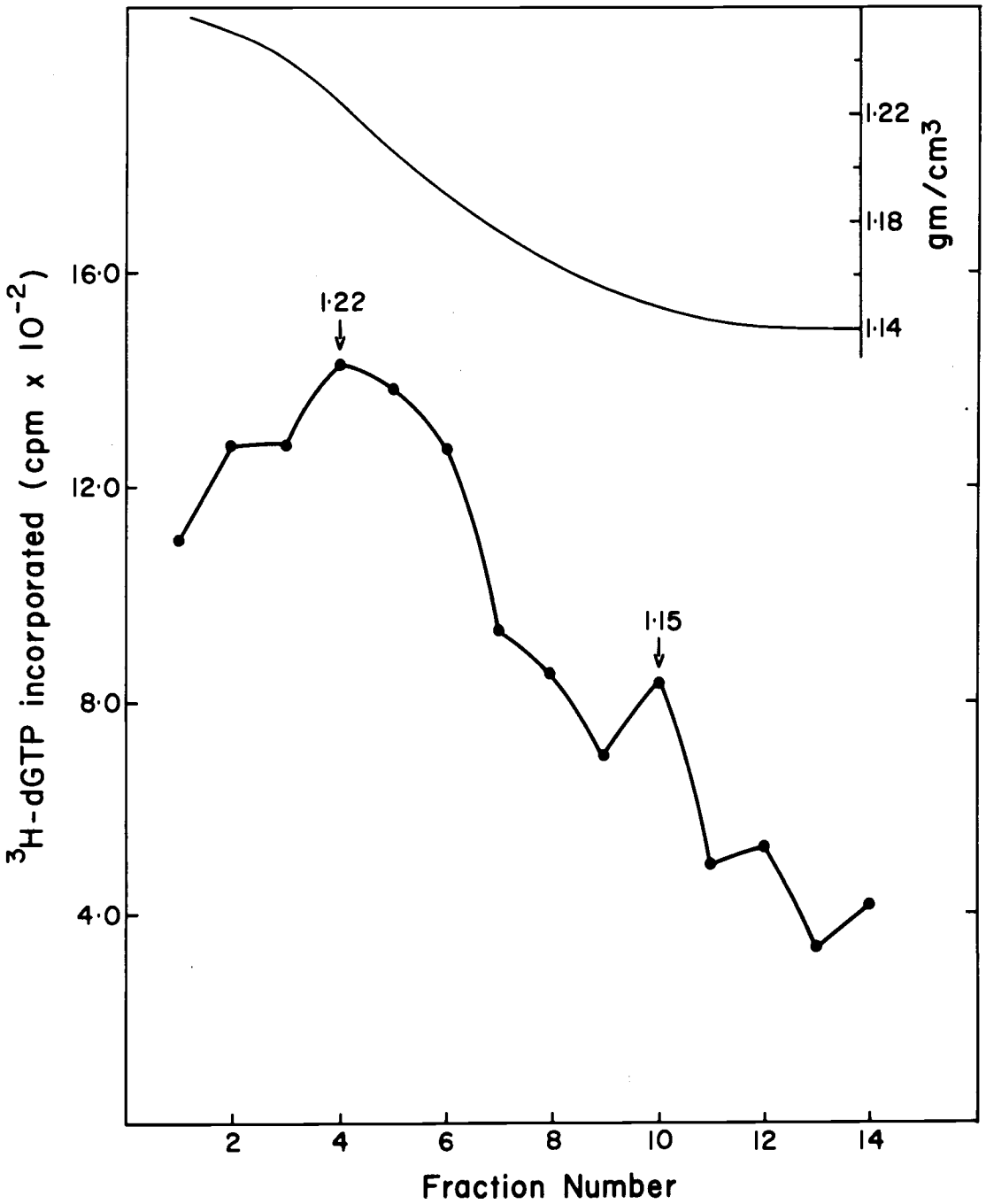


Figure 41. Isopycnic centrifugation of DNA polymerase activity in the particulate fraction from CLL-V plasma. Particulates from CLL-V plasma were centrifuged to equilibrium, the glycerol gradient fractionated, and each fraction treated with detergent as described in Methods. Assays (0.2 ml) contained 0.02 ml of each fraction plus 6 μg M. lysodeikticus DNA and were performed as described in Figure 29 (incubation time : 4 hours). Specific activity of ^{3}H-dGTP : 2,000 cpm/ρmole.

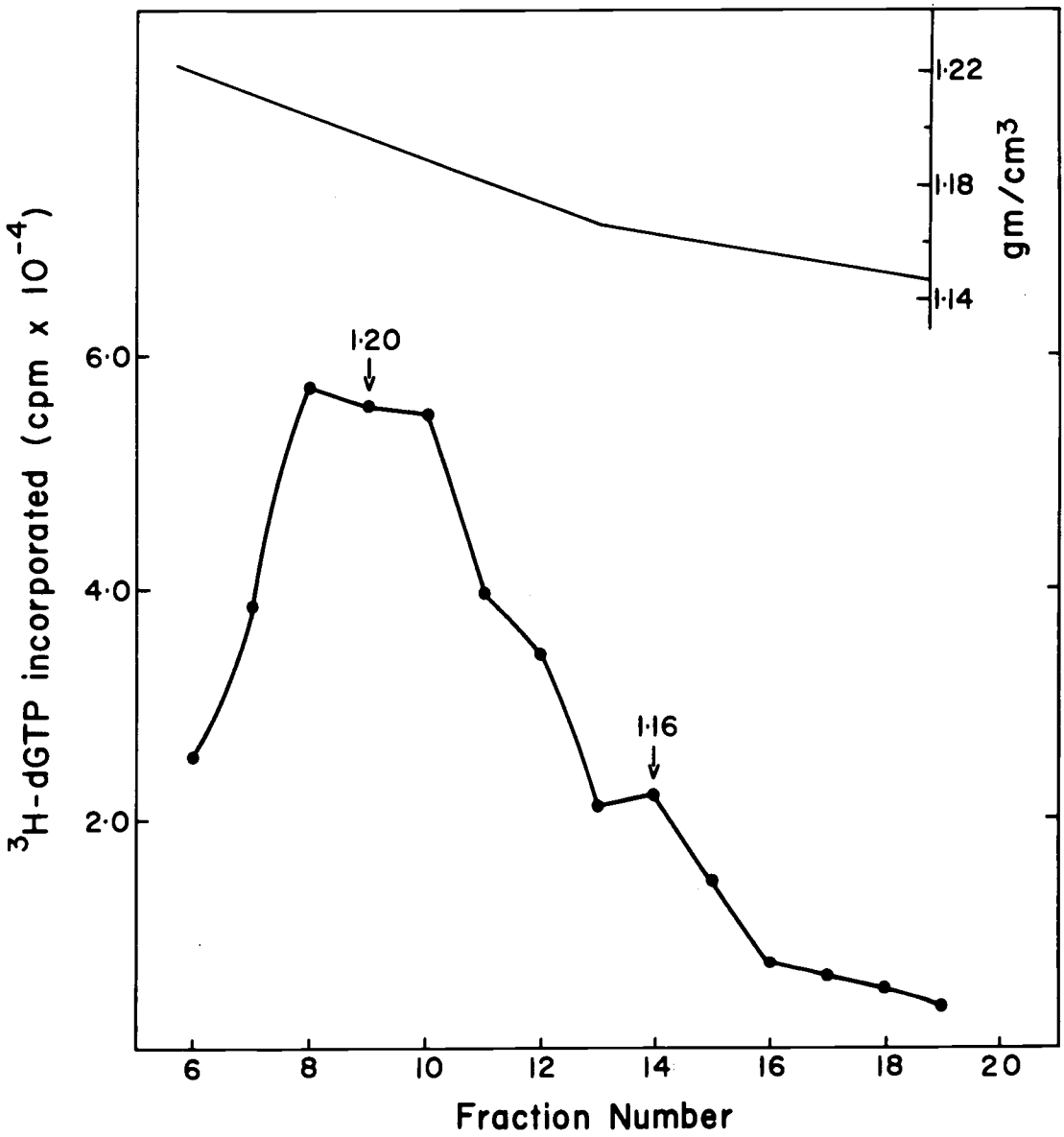


Figure 42. Isopycnic centrifugation DNA polymerase activity in AMV particles. Experimental conditions were identical to those described in Figure 41. AMV particles were from a chick plasma which had been frozen and thawed three times. Assays (0.2 ml) contained 0.025 ml of each gradient fraction plus 4 μg M. lysodeikticus DNA and were incubated for one hour. Specific activity of ^{3}H-dGTP : 2,000 cpm/ρ mole.

pellet DNA polymerase activity was not a normal constituent of CLL-V blood, or was at least present only when his leukemic state was high and untreated.

DNA Polymerase from Other Types of Human Leukemias

Two other human leukemia plasmas were investigated in Dr. Sol Spiegelman's laboratory at Columbia University. One was from a patient with chronic myelocytic leukemia (CML-P) in the blastic phase and the other was from a patient with acute lymphocytic leukemia (ALL-M). A control plasma was also obtained from a patient with nonmalignant lymphocytosis and a white blood cell count of 35,000 cells/mm^3.

The whole blood from each of these patients had been allowed to settle in the cold for at least three days which had not been the case with the other plasmas investigated. The collected plasmas were clarified, and pellets obtained by the same procedures previously employed. Table 14 lists the DNA synthesis activity from these plasma pellets following detergent treatment.

Figure 43 shows the kinetics for DNA synthesis by each of the above enzyme fractions. Thirty minute assays of the leukemia DNA polymerases with no added primer gave the same radioactivity level as the control fraction (130 to 150 cpm).

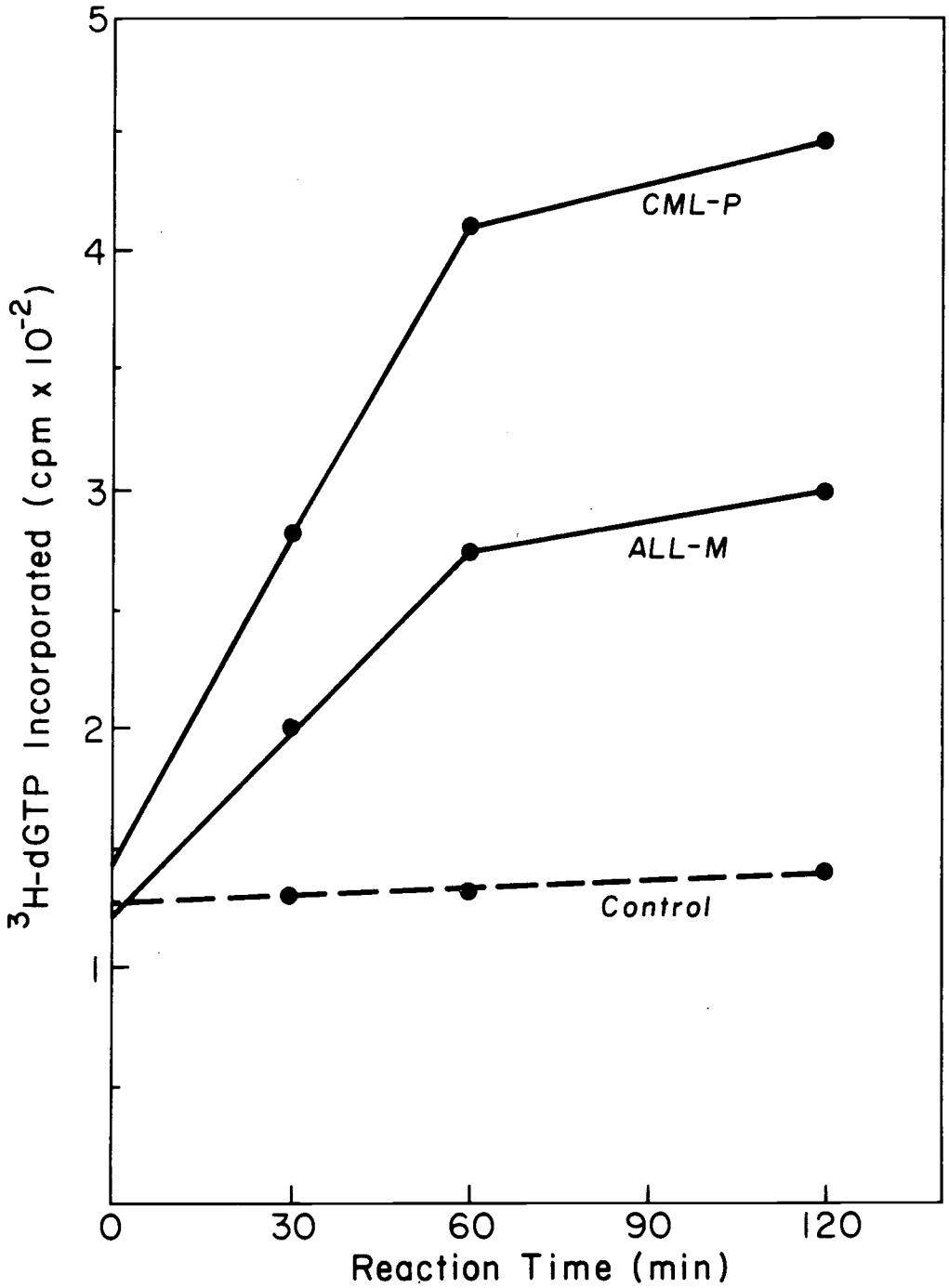


Figure 43. Kinetics of DNA synthesis by particulates from leukemic and nonleukemic human plasmas. Assays (0.2 ml) contained particulates from 0.01 ml of plasma plus 1 μg Poly dC : Poly dG. Assay conditions for CML-P (chronic myelogenous leukemia), ALL-M (acute lymphocytic leukemia), and control (nonleukemic lymphoblastosis) were performed under the conditions described in Methods. No radioactivity was subtracted as background. Specific activity of ^{3}H-dGTP : 100 cpm/pmole.

Table 15. DNA Polymerase Activity in Three Human Plasma Pellets.

Enzyme*	Amount (ml)	Primer**	cpm 3HdGMP***/120 minutes
CML-P	0.03	DNA	239
ALL-M	0.03	DNA	293
Control	0.03	DNA	164
CML-P	0.02	Poly dC: Poly dG	447
ALL-M	0.02	Poly dC: Poly dG	275
Control	0.02	Poly dC: Poly dG	150

*0.01 ml each enzyme suspension corresponded to particulates from 0.01 ml plasma

**Each assay (0.20 ml) contained 2 μg M. lysodeikticus DNA or 1 μg Poly dC: Poly dG primer

***Specific activity of ^{3}HdGMP: 1 pmole = 1000 cpm.

The results cited in Table 15 and Figure 43 show DNA polymerase activity was associated with the plasmas from chronic myelogenous leukemia and acute lymphocytic leukemia, but there was no DNA polymerase activity associated with the plasma from nonmalignant lymphocytosis. These results confirm the earlier findings with CLL-V and CLL-D that DNA polymerase activity could be demonstrated in particulates from leukemic plasma which was not found in nonleukemic plasmas. The high white blood cell count of the control sample with nonmalignant lymphocytoses was not found to induce DNA polymerase activity in the plasma pellet. This indicates that the presence of DNA polymerase in leukemic plasmas was not merely a function of a high level of white blood cells in the circulating blood.

Particulate DNA Polymerase in
Chronic Lymphocytic Leukemia Lymphocytes

The presence of DNA polymerase in the plasma from CLL-V encouraged preliminary investigation of DNA polymerase activity in the leukemic lymphocytes obtained from the blood sample, CLL-V. The buffy coat layer of white blood cells from three normal individuals was pooled for use as control cells. All procedures employed for homogenization, isopycnic centrifugation, and detergent treatment of the fractions from the human cells were identifcal to those used for the myeloblasts and MC29-infected CEC.

The postnuclear supernatants of each of the human cell fractions was examined for DNA polymerase activity as shown in Table 16.

Table 16. DNA Polymerase Activity in Postnuclear Supernatants from Leukemic and Normal Human White Blood Cells.

Enzyme	Primer*	cpm ^{3}H-dGTP**/50 μg protein/30 min.
CLL-V	DNA	5380
CLL-V	Poly dC: Poly G	720
Control	DNA	93
Control	Poly dC: Poly G	73

*Assays (0.2 ml) contained 4 μg M. lysodeikticus DNA or 1 μg Poly dC: Poly G and were performed as described in Methods.
**Specific activity of ^{3}H-dGTP: 1000 cpm/pmole. No radioactivity was subtracted as background.

A high level of DNA polymerase activity was found in the postnuclear supernatant from CLL-V and little if any DNA polymerase activity in the postnuclear supernatant from the control white blood cells. The observed DNA polymerase activity from CLL-V was comparable in amount to that obtained from the postnuclear supernatant of AMV-infected chick myeloblasts.

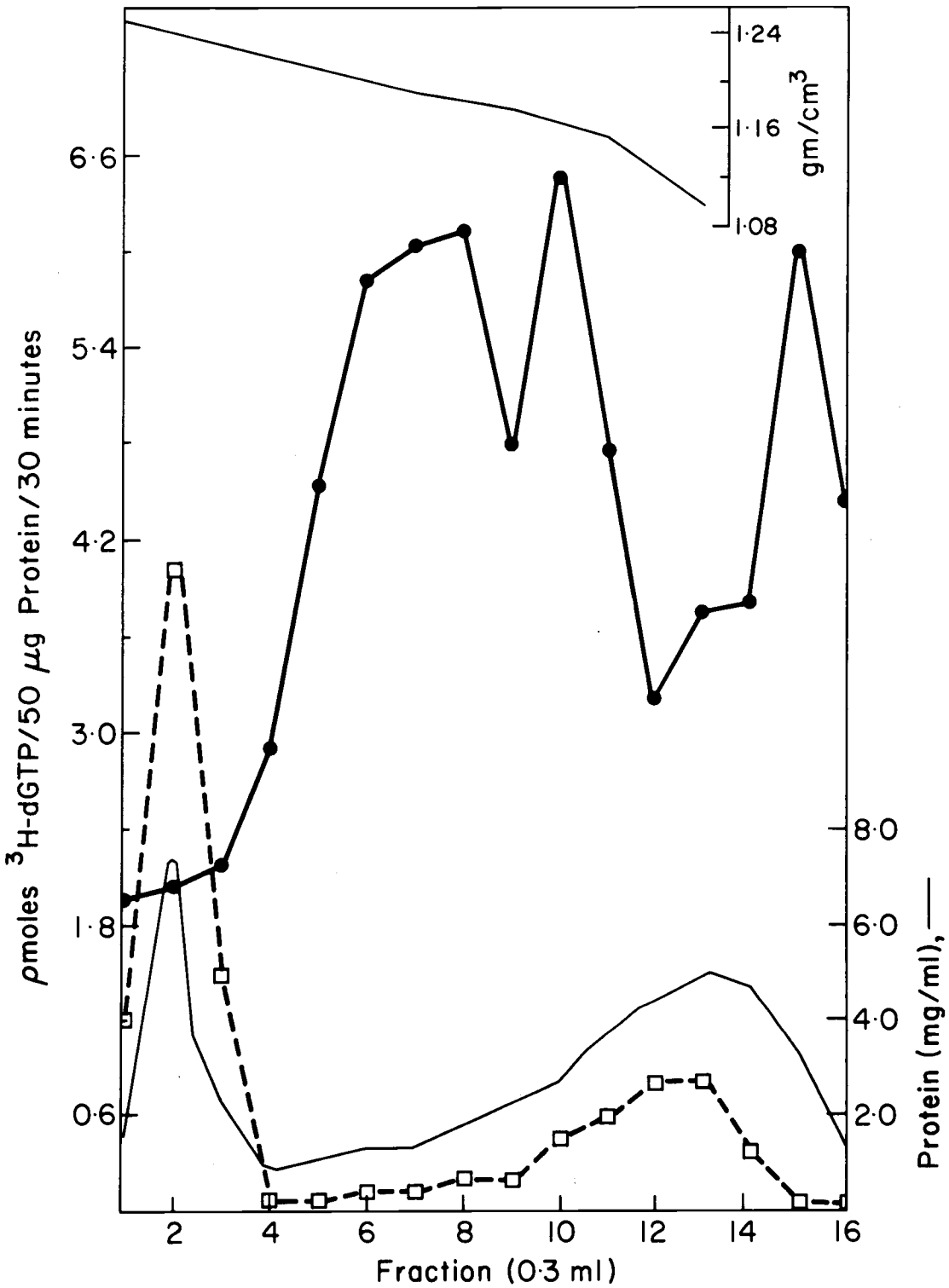
The DNA polymerase activity from CLL-V was further fractionated on a glycerol density gradient as shown in Figure 44. The control cell supernatant was treated in the same manner, but is not shown since little if any DNA polymerase activity was detected in any of the control cell gradient fractions.

Much of the DNA polymerase activity from CLL-V was particulate and exhibited a buoyant density very similar to the DNA polymerase from myeloblasts and MC29-infected CEC. The peak buoyant density for DNA polymerase activity in the presence of Poly dC: Poly G primer was displaced from the peak buoyant densities of DNA polymerase activity in the presence of *M. lysodeikticus* DNA primer (Figure 44) and the reason for the discrepancy is not known at this time.

Summary of the Human Leukemia Studies

Evidence was obtained from four human leukemia blood samples that a DNA polymerase exists in a particulate form in human leukemic

Figure 44. Isopycnic centrifugation of DNA polymerase activity from chronic lymphocytic leukemia lymphocytes. Experimental conditions were identical to those described in Figure 35. Assays (0.2 ml) contained enzyme from each fraction plus 2 μg M. lysodeikticus DNA (●—●) or 1 μg Poly dC : Poly G (□—□) and were performed as described in Figure 43. Specific activity of ^{3}H-dGTP : 100 cpm/ρ/mole.


1·24
1·16
1·08
gm/cm3
6·6
5·4
4·2
3·0
1·8
0·6
pmoles 3H-dGTP/50 μg Protein/30 minutes
8·0
6·0
4·0
2·0
Protein (mg/ml), —
2
4
6
8
10
12
14
16
Fraction (0·3 ml)

plasma that is not found in plasma from healthy individuals, nor in plasma from nonmalignant lymphocytosis. A causal relationship was not demonstrated between the particulate DNA polymerase activity in human leukemic plasma, and the disease state of the patients, but a high level of particulate DNA polymerase activity was demonstrated in a preliminary examination of chronic lymphocytic leukemia lymphocytes.

DNA polymerase in the leukemic plasma, CLL-V_3 was associated with a particulate fraction which exhibited a buoyant density very similar to the buoyant densities of the avian RNA tumor viruses. Further, the DNA polymerase was stimulated by detergent treatment of the particulate fraction; such a response was also very similar to the DNA polymerase in AMV and MC29-virus. These types of experiments do not prove the existence of virus particles in human leukemic plasma, but they do suggest that human tumor viruses might be a causative agent in human leukemia.

DISCUSSION

The genome of avian myeloblastosis virus is replicated, assembled into maturing virus particles, and released with completed virions, which bud off the myeloblast membrane at the rate of 30 particles/cell/hour (Beaudreau et al., 1960). Neither the continual replication of viral nucleic acid, nor the constant expulsion of virus particles appears to be damaging to the myeloblast which is capable of indefinite growth and cell division (Beard, 1962). This apparent symbiotic relationship between virus and cell is definitive for the effect of RNA tumor viruses upon the cells they infect and transform. A direct causal relationship exists between infection with murine leukemia-sarcoma viruses or avian leukemia-sarcoma viruses, and the primitive nature permanently imposed upon the infected cell. How does a virus, whose RNA complement inside infected cells comprises less than 0.4% of the total intracellular RNA (Beaudreau, 1970; Allen, 1966), direct and maintain the obligate neoplastic state of the cell? The recent discovery (Riman and Beaudreau, 1970) of a small piece of DNA within AMV particles casts a new light on the possible malignant mechanism of RNA tumor viruses, but the fact remains that a very large RNA species is faithfully replicated and enclosed within progeny AMV particles.

Replication of Nontumor RNA Viruses

To discern the oncogenicity of AMV (and RNA tumor viruses in general) it is helpful to refer to the replication processes of RNA viruses which do not induce malignant changes in the cells they infect, designated here as nontumor RNA viruses.

Replication of the RNA genome of nontumor viruses and RNA bacteriophage is mediated by a RNA-dependent RNA polymerase which is viral induced in infected cells. The RNA-dependent RNA polymerase transcribes the plus RNA strand contained in the virus particle into a complementary minus RNA strand inside the cell, which is then transcribed again into plus RNA strands for progeny virus particles (Spiegelman et al., 1965; Gomatos, 1968; August, 1969; Baltimore, 1966).

Production of most of the nontumor RNA viruses is not sensitive to the inhibition of DNA-dependent RNA synthesis by actinomycin D. There are, however, at least two notable exceptions: influenza virus (Scholtissek and Rott, 1968) and mengovirus (Plageman and Swim, 1966). Infection of a cell by either influenza virus or mengovirus induces the appearance of an intracellular RNA-dependent RNA polymerase. Treatment of infected cells (with actinomycin D) suppresses the production of virus particles, but it does not suppress the level of intracellular RNA-dependent RNA polymerase activity (Scholtissek

and Rott, 1969). Further, the RNA-dependent RNA polymerase associated with mengovirus or influenza virus infection is not sensitive to actinomycin D *in vitro* (Scholtissek and Rott, 1969; Ho and Walters, 1966; Plageman and Swim, 1968). These two nontumor RNA virus systems therefore, exhibit a dependence upon DNA-dependent RNA synthesis which does not appear to be directly related to the RNA-dependent RNA polymerase activity.

More recently it was shown that in addition to the inhibition of influenza virus by actinomycin D, which acts directly with the DNA template (Hurwitz *et al.*, 1962), inhibition of cellular DNA-dependent RNA polymerase by α-amanitin (Jacob *et al.*, 1970) also suppressed the production of influenza virus particles (Scholtissek and Rott, 1970). The suppression appeared to be due to a decreased synthesis of RNA minus strands, rather than an inability to transcribe RNA plus strands from RNA minus strands.

None of the nontumor RNA viruses studied to date have been sensitive to inhibitors of DNA synthesis (Montagnier, 1968; Baltimore, 1966). Virus particle production proceeds relatively unhindered in the presence of cytosine arabinoside (Bader, 1967), bromodeoxyuridine, or amethopterin (Temin, 1964).

Thus, it appears that nontumor RNA viruses do not require DNA synthesis for successful infection of a cell and the production of progeny virus particles. Most do not even require DNA-dependent RNA

polymerase activity, but in the case of mengovirus and influenza virus, cellular DNA-dependent RNA polymerase activity is necessary for production of progeny virus particles, in addition to the virus-specific RNA-dependent RNA polymerase.

Replication of RNA Tumor Viruses

Actinomycin D has been shown to inhibit the production of AMV from infected CEC cells (Allen, 1966) but not from infected myeloblasts (Zischka *et al.*, 1964). Actinomycin D also inhibits the production of Rous sarcoma virus from infected CEC (Temin, 1963; Bader, 1964). Inhibition of RNA tumor virus production by actinomycin D has been interpreted as showing a requirement for DNA-dependent RNA synthesis in the replication process of RNA tumor viruses. The inhibitor experiments have not been entirely satisfactory however, because of conflicting findings. Pulse-labeling studies have shown that there is a period of 18 to 24 hours between the time RSV-RNA is synthesized intracellularly and the time it appears in progeny virus particles (Temin, 1964; Robinson, 1966). Actinomycin D inhibits the production of progeny virus particles within four hours after treatment of infected cells (Temin, 1963; Bader, 1964). It would appear then, that actinomycin D inhibits the release of virus particles rather than inhibiting the synthesis of viral RNA, but no evidence has been found for accumulation of viral RNA within infected cells following treatment

by actinomycin D (Allen, 1966; Temin, 1964).

Nonetheless, even if DNA-dependent RNA synthesis were necessary for production of RNA tumor viruses, this does not preclude the existence of an RNA-dependent RNA polymerase which mediates transcription of viral RNA, as is the case for mengovirus and influenza virus.

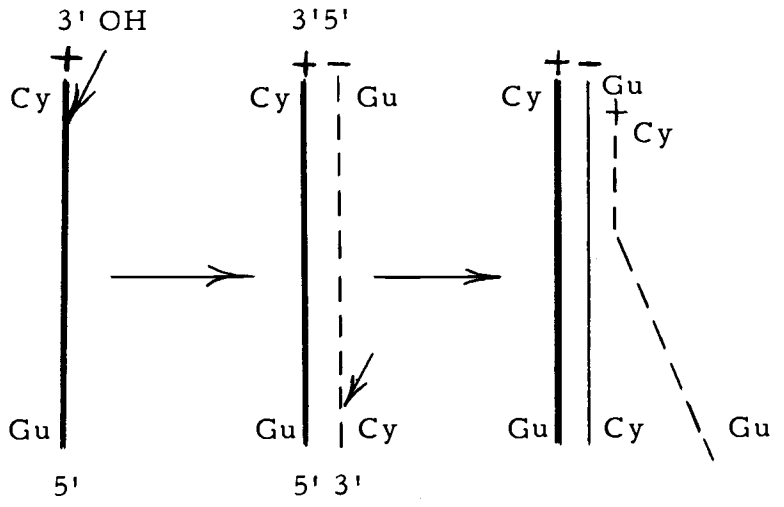
Indeed, an RNA-dependent RNA polymerase was isolated from myeloblasts in this laboratory by Watson (Watson and Beaudreau, 1966) and those findings were further verified by the results presented here.

RNA-Dependent RNA Polymerase

Neither actinomycin D nor deoxyribonuclease inhibit *in vitro* RNA synthesis by soluble RNA-dependent RNA polymerase from myeloblasts (Watson and Beaudreau, 1969). Indeed, the enzyme has many of the characteristics required of an RNA polymerase which specifically replicates viral RNA: DNA is not an effective template for RNA synthesis, heterogeneous RNA (particularly 64S RNA from AMV) is the preferred primer for RNA synthesis, and under the appropriate assay conditions, RNA product is synthesized which sediments to the same position on a velocity gradient as the 64S RNA from AMV. A double-stranded RNA replication intermediate has not been demonstrated however, as would be expected if the RNA-dependent RNA polymerase were transcribing a minus RNA strand from the 64S RNA plus strand.

Such a replicative intermediate has been demonstrated in the Qβ-phage system (Haruna et al., 1963) and in a number of the nontumor RNA virus systems (Plageman and Swim, 1968; Scholtissek, 1969; Penman et al., 1964; Banerjee and Shatkim, 1970; Gomatos, 1968).

Qβ-replicase provides the best model at this time for the replication process of RNA strands. Qβ-replicase exhibits a striking preference for Qβ-phage RNA as template for RNA synthesis (Spiegelman et al., 1965). This implies that there is a specific initiation site on Qβ-phage RNA strands for RNA synthesis. Since the enzyme synthesizes both minus and plus strands of Qβ-phage RNA, the initiation sites on both the minus and plus strands must be very similar. RNA synthesis has been shown to occur from the 3'OH end of each strand (Bishop et al., 1967; August, 1969). This information provides a working model for the mechanism of Qβ-phage replication which is shown below.



(adapted from Montagnier, 1968)

This model obviously predicts that RNA strands which are replicated by an RNA-dependent RNA polymerase have a base at the initiation site on the 3'OH end of the RNA strand which is complementary to the base at the 5' end of the RNA strand. If such were not the case, then the RNA-dependent RNA polymerase would either not demonstrate a high specificity for viral RNA primer, or it would replicate either the RNA minus strand or the RNA plus strand, but not both. In the latter instance, two RNA polymerase species would be required for the synthesis of viral RNA.

The implication from the studies on the inhibition of influenza virus by α-amanitin, was that the virus-induced RNA-dependent RNA polymerase transcribed plus RNA strands from RNA minus strands, but α-amanitin blocked the initial synthesis of minus RNA strands from RNA plus strands.

A similar mechanism could be postulated for the RNA-dependent RNA polymerase from myeloblasts. If the enzyme were specific for synthesizing plus RNA strands from minus strands, it would exhibit a preference for high molecular weight RNA as primer for RNA synthesis, and might possibly be able to replicate certain stretches of RNA plus strands from AMV particles, without being able to synthesize an entire RNA minus strand. The results presented here are consistent with this type of mechanism. The detergent solubilized RNA polymerase from myeloblasts synthesized RNA from endogenous

template which was only slightly deoxyribonuclease sensitive. RNA synthesis was stimulated by the addition of 64S RNA from AMV, but little stimulation of GMP incorporation into RNA product was noted by the addition of myeloblast cell RNA primer. Therefore, both the soluble RNA-dependent RNA polymerase and the detergent solubilized RNA polymerase found in myeloblasts exhibited a preference for 64S RNA from AMV as primer for RNA synthesis. However, the synthesis of double-stranded RNA which would qualify as a replicative intermediate was not demonstrated.

In addition to the high level of RNA polymerase activity which can be obtained from myeloblasts, the preliminary studies on the RNA polymerase activity in CEC cells shown here indicated there was a higher level of RNA polymerase activity in MC29-infected CEC than could be found in noninfected control CEC. This situation will be discussed again in more detail.

Relationship of Tumor Virus Replication to Cell Transformation

Two lines of dissimilarity now emerge between the nontumor RNA viruses and the RNA tumor viruses. One is the obvious fact that tumor viruses are oncogenic to their host cell. The other is the negative line of evidence that RNA-dependent RNA polymerase responsible for replicating viral RNA through a double-stranded RNA

intermediate, is not as easily demonstrable in the tumor virus system as in the nontumor virus systems.

Transformation of the cell does not seem to be necessary for tumor virus RNA replication since avian myeloblastosis virus is produced by CEC cells which do not appear to undergo morphological changes (Bolognesi et al., 1968). Thus, virus genome replication can occur, at least in the case of AMV, without cell transformation.

Rous sarcoma virus can induce transformation of CEC cells without the production of virus particles. The Bryan high titer strain of RSV has been shown to be a defective virus and virus particle production is not observed unless a Rous associated virus (RAV) is also present (Hanafusa, 1968). The failure to produce RSV particles is not, however, due to the failure of the transformed cells to replicate RSV-RNA, but rather to a lack of the ability to synthesize virus specific coat proteins. In some instances transformation has been observed in cells infected with RSV which was not accompanied by detectable viral RNA synthesis, but the transformed cells did contain the RSV genome (Svoboda, 1970). Thus, no evidence to date has shown that transformation occurs without at least the presence of tumor virus genetic information. It would appear then, that there is a step in the replication of tumor virus RNA which may be responsible for transformation rather than being dependent upon transformation of the infected cells.

Successful infection of a cell system by RNA tumor viruses has

an obligate requirement for DNA synthesis at the time of infection. Inhibitors of DNA synthesis, amethopterin, bromodeoxyuridine, cytosine arabinoside, fluorodeoxyuridine, suppress the production of infectious virus particles almost completely if applied at the time of virus infection (Temin, 1964; Bader, 1964, 1966; Nakata and Bader, 1968; Knudsen et al., 1967). The need for DNA synthesis is a transient one however, since the same inhibitors have little effect on production of infectious virus particles from cells which are already transformed.

These results imply that a DNA species is synthesized at the time of infection by RNA tumor viruses which is responsible for cell transformation and related to virus RNA replication.

More recent inhibitor studies with bromodeoxyuridine indicate that cell transformation by RSV is not suppressed if bromodeoxyuridine is added at the time of virus infection, and that the actual numbers of virus particles produced by the transformed cells is not inhibited, but the infectivity of the progeny RSV is greatly reduced (Bader and Bader, 1970). Bromodeoxyuridine is incorporated into DNA and renders the DNA product susceptible to breakage upon exposure to light. If CEC cells are treated with bromodeoxyuridine at the time of infection with RSV, subsequent exposure of the cells to light suppresses the transformation process (Balduzzi and Morgan, 1970; Boettiger and Temin, 1970).

The existence of RNA-dependent DNA polymerase in tumor virus particles supports the findings from the inhibitor studies that there is information transfer from the RNA species of the tumor virus particle to a DNA species inside the cell, the "provirus."

In this context it should be pointed out that the studies presented here show there is DNA-dependent polymerase activity in RNA tumor virus particles which has a maximum velocity six-fold greater with AMV-DNA template than the maximum velocity of RNA-dependent DNA polymerase activity with 64S RNA template from AMV.

It is possible therefore, for tumor virus information transfer to occur from the DNA contained in the virus particle to an intracellular DNA species. However, if the tumor virus DNA were solely responsible for cell transformation, then the addition of bromodeoxyuridine to cells which are producing virus would decrease the infectivity of the subsequent virus particles, and such does not appear to be the case (Bader and Bader, 1970; Boettiger and Temin, 1970).

These experiments impose an inflexible mechanism of information transfer from tumor virus to cell in which a DNA copy must be first expressed. The "provirus" theory predicts that the RNA strands of progeny tumor virus particles would be synthesized from the DNA "provirus" template by a DNA-dependent RNA polymerase.

Nucleic Acid Polymerase Activities in Cells Infected with RNA Tumor Viruses

Results from the experiments presented here on the nucleic acid polymerase activities obtained from RNA tumor virus-infected cells, provided evidence that the DNA polymerase associated with tumor virus particles is active inside transformed cells. The DNA polymerase obtained from homogenates of myeloblasts or MC29-infected CEC exhibited a buoyant density (1.16 gm/cc) which was the same as that of the AMV and MC29 particles. This striking similarity in buoyant densities could be interpreted as being due to trapped virus particles in the infected cells, or the fact that the intracellular DNA polymerase was associated with cell membrane fractions which have the same buoyant density as the virus particles. The latter possibility seems more likely to be the case since the homogenization procedures themselves could convert virus particles to nucleocapsids which have a buoyant density of 1.20 to 1.25 (Bader et al., 1970). Since the RNA tumor viruses appear to mature in the cytoplasm near the cell membrane (Bonar, 1960), and at least one of the virus-particle membranes is derived from the cell (Mommaerts et al., 1952), it seems possible that the tumor virus replication process is associated with a membrane fraction of the same buoyant density as the virus particles themselves.

RNA polymerase activity was also observed from virus-infected cell homogenates which had a buoyant density of 1.16 gm/cc. Both

DNA and RNA would serve as primer for RNA synthesis by the particulate RNA polymerase. The amount of RNA polymerase activity in the infected cell fractions was considerably higher than the RNA polymerase activity in the same fractions from control cell homogenates. The incorporation of UMP into acid-precipitable product was observed, however, in assays of control cell gradient fractions.

The idea that part of the enzyme which synthesizes viral RNA might pre-exist in uninfected cells is consistent with findings in other systems. Qβ-replicase was separated into two protein components, one of which pre-existed in uninfected E. coli cells (Eikhom and Spiegelman, 1967).

The evidence presented here strongly suggests that replication of tumor virus RNA may involve both DNA-dependent RNA synthesis and RNA-dependent RNA synthesis. This evidence is supported by recent findings on the murine sarcoma-leukemia virus system.

RNA from murine sarcoma-leukemia virus dissociates into three subunits when heated at 95°C for two minutes; 37S, 18S and 4S. Each of these subunits will form hybrids with two species of nuclear RNA unique to cells infected with the virus (Biswal and Benyesh-Melnick, 1969). The murine sarcoma-leukemia virus also contains a DNA species with the same sedimentation coefficient as AMV-DNA (7S). Hybridization studies have shown that the DNA from murine sarcoma-leukemia virus is complementary to only the 18S RNA

subunit (Biswal and Benyesh-Melnick, 1970).

These findings are in agreement with observations that only a portion of the 64S RNA from AMV is transcribed into DNA *in vitro* by the DNA polymerase from AMV particles, and the DNA product appears to be the same size (7S) as AMV-DNA isolated from the virions themselves (Spiegelman *et al.*, 1970; Rokutanda *et al.*, 1970).

Therefore, the DNA "provirus" could be transcribed from only a portion of the tumor virus RNA and is responsible for transformation of infected cells. This would predict that replication of a portion of tumor virus RNA would be DNA-dependent, and replication of the remainder of the tumor virus RNA would be RNA-dependent.

RNA Tumor Viruses as the Etiologic Agent in Human Leukemia

The idea that viruses might serve as a principal etiologic agent in neoplasms has only in recent years been publicly embraced by virologists and cancer researchers. Sixty years ago it was shown that avian leukemias and avian sarcomas (Ellerman and Bang, 1909; Rous, 1911) were transmissable by a viral agent. Twenty years passed before two more oncogenic viruses were discovered: the rabbit papilloma virus (Shope, 1933; Rous and Beard, 1935) and the mouse mammary tumor virus (Bittner, 1936). Another twenty years passed during which time the theory of viral oncogenesis gained little support. The known tumor viruses were quite species specific and produced

only a few of the neoplastic disorders observed in each species. The concept of a separate viral agent for each neoplasm of every species was unacceptable to the researchers in the field. However, in the early 1950's a mouse leukemia virus was discovered (Gross, 1951) and a few years later the mouse leukemia virus preparation was shown to contain another tumor virus, polyoma virus (Gross, 1953; Stewart, 1953; Stewart et al., 1957). The polyoma virus was found to induce a variety of neoplasms in several species, and the theory of viral oncogenesis came into serious consideration.

In the past 15 years a number of DNA and RNA tumor viruses have been reported. The studies have been mostly descriptive using the available methods of electron microscopy, animal transmission, serology, and tissue culture infection, to describe the viruses and the neoplastic responses they induce. Only three of these methods are applicable to human neoplasms: tissue culture of malignant cells, electron microscopic examination, and serologic assay of virus-specific proteins. Some studies have been suggestive of a viral involvement in human neoplasms (Epstein et al., 1964; Benyesh-Melnick et al., 1963; Todaro and Martin, 1967; Dmochowski, 1968; Naib et al., 1969) but not conclusive. Even though virus-like particles have been seen occasionally in electron micrographs of malignant human cells, and serologic antigen to virus protein has been detected a direct causal relationship has not been demonstrated between the

presence of the virus and the observed neoplasia.

The studies reported here suggest a new approach to the study of human neoplasms is now available. DNA polymerase activity was detected in particulates from leukemic plasma and in chronic lymphocytic leukemia lymphocytes which exhibited properties very similar to DNA polymerase associated with the avian myeloblastosis system and the MC 29 infected CEC system.

These findings are supported by a recent report of RNA-dependent DNA polymerase activity in acute lymphocytic leukemia lymphocytes which was not demonstrable in phytohemagglutinin induced normal lymphocytes (Gallo _et al._, 1970).

Demonstration of a tumor virus etiology in human neoplasms would be a landmark in medical science, but understanding the malignant process must come from unraveling completely the mystery of tumor virus replication and cell transformation.

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