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Title: NUCLEIC ACIDS ASSOCIATED WITH MC29 TUMOR VIRUS

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Dr. George S. Beaudreau

Strain MC29 (myelocytomatosis) virus was purified from culture medium of infected CEC fibroblasts and assayed for DNA polymerase activity. The observed activity was dependent on a full complement of deoxyribonucleotide triphosphates as substrates. DNA synthesis was enhanced by the addition of RNA, DNA, or certain RNA:DNA hybrids to the reaction mixture. DNA-directed synthesis by the enzyme from MC29 virus was observed to continue for at least eight hours. Enzyme preparations from culture medium of noninfected CEC fibroblasts and RIF contaminated CEC cultures exhibited no enzyme activity.

MC29 virus particles in supernatant fluids from infected CEC cultures were detectable by either titrating for focus forming units (FFU) or by assaying for DNA polymerase activity. After infection of CEC fibroblasts, newly formed MC29 virus particles were first detected by both methods as early as 10-12 hours after introduction of

the virus. The presence of RIF, a nontransforming leukosis virus in CEC cultures did not contribute to the DNA polymerase activity of strain MC29 virus.

Purification of RNA tumor virus DNA polymerase was accomplished by column chromatography and fractionation in cesium chloride. Enzymes prepared from MC29 virus and avian myeloblastosis virus had sedimentation coefficients of 7.7 and 7.4S, respectively. No difference in the sedimentation rates was detected when either method of purification was employed. Treatment of the enzyme with RNase did not alter its sedimentation rate.

DNA with a buoyant density of 1.74 gm/cm^3 in cesium chloride was found to be present in purified preparations of MC29 virus.

DNA polymerase was also detected in crude homogenates of CEC fibroblasts. However, the use of dC:rG as template for cellular enzyme reactions stimulated DNA product formation only by the extract derived from MC29 virus infected cells, while completely inhibiting any activity from noninfected control cells.

The enzyme activity associated with the virion differed from the normal cell enzyme in size and response to dC:rG template. These two criteria may be useful for separating the enzyme activity normally found in the uninfected cell from that enzyme activity induced by tumor virus infection.

Data concerning the mechanism of DNA synthesis by the DNA polymerase from RNA tumor viruses is discussed in conjunction with proposed models.

Nucleic Acids Associated with MC29 Tumor Virus

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George Harding Weber

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Associate Professor of Biochemistry
in charge of major

Redacted for Privacy

Chairman of the Department of Microbiology

Redacted for Privacy

Dean of Graduate School

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To Mysti, Louie and Margaret.

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NUCLEIC ACIDS ASSOCIATED WITH MC29 TUMOR VIRUS

INTRODUCTION

The viral etiology of malignant transformations was first observed by Ellerman and Bang in 1908. They demonstrated that leukemias of chickens could be transmitted by cell-free filtrates. Soon afterwards, in 1911, Rous showed that avian sarcomas could be similarly transmitted. Additionally, in 1933, Shope found that the papilloma of wild rabbits was virus induced. Other important observations in studies of viral carcinogenesis were made by Bittner in 1936 and Gross in 1951. Bittner discovered that the virus which causes adenocarcinomas in mice is transmitted from mother to offspring by way of the milk, while Gross isolated a murine leukemia virus which induced disease in newborn mice. More recently, Friend (1956, 1957) found another strain of leukosis virus which is rapidly fatal in adult mice.

A significant contribution to investigations of viral induced cancer came from Stewart et al. in 1957. They demonstrated the propagation of a virus, later called polyoma virus, in monkey kidney cell cultures. This virus, when cultured in vitro and then injected into newborn mice, induced malignant tumors similar to those from which the virus was originally isolated.

Of the earliest reported tumor viruses, essentially all have been found to contain RNA. It was only later that DNA tumor viruses were discovered, but of those found only the polyoma, SV40 and human adenoviruses have been studied in detail.

The polyoma virus of mice and the simian virus SV40 are capable of causing either productive infection with virus particle release and cell death, or transformation of the cells with no progeny virus being produced. The type of cell infected appears to play a critical role in what kind of infection results. Cells derived from the permissive host generally result in productive infection, while transformation usually is seen in cells derived from nonpermissive hosts. For example, hamster cells are nonpermissive for polyoma virus and thus result in transformation. Cells from mouse origin (permissive) enable the productive infection of polyoma virus. Likewise, monkey cells allow for the production of progeny SV40 virus, whereas mouse or hamster cells are found to be nonpermissive.

Certain groups of human adenoviruses have been found to be highly oncogenic for newborn rodents (Trentin et al., 1962; Heubner et al., 1962), and it has been shown that they will also transform nonpermissive cells in vitro (McAllister et al., 1969; Strohl et al., 1967; MacBride and Wiener, 1964). However, detection of oncogenecity in cells of human origin resulted in no greater than 1-5% of the cell population being transformed (Todaro and Aaronson, 1968).

RNA Tumor Viruses

The study of RNA tumor viruses for over six decades has resulted in observations that are both intriguing and complex. Unlike their DNA counterparts, RNA tumor viruses coexist in infected cells. Viral infection does not result in cellular death, but alternatively, uncontrolled growth, together with continual virus particle liberation (Beaudreau et al., 1960).

In the leuko-sarcoma complex of RNA tumor viruses the avian myeloblastosis virus (AMV) and the Rous sarcoma virus (RSV) have been two of the most intensively investigated. Of particular importance to all tumor virus studies was the first report of an enumerative dose-response by AMV in vivo (Eckert et al., 1954). A linear relationship was also found between Rous sarcoma virus concentration and focus formation in vitro (Temin and Rubin, 1958). These quantitative methods for viral infectivity became fundamental in succeeding studies dealing with the oncogenicity of tumor viruses.

Particular interest was drawn to the report of Rubin (1960) wherein he demonstrated that the presence of a latent lymphomatosis virus in chicken embryos markedly affected infection of the cells with RSV. This resistance inducing factor (RIF), when present in chick embryo cells (CEC), rendered them at least 40 times more resistant to RSV infection (Rubin, 1961).

Viral interference was also demonstrated between RSV and AMV (Vogt and Rubin, 1963). Avian myeloblastosis virus does not adapt itself well to CEC fibroblasts, so a direct quantitative assay by focus formation is difficult (Moscovici et al., 1969). However, Vogt and Rubin were able to make use of its interference with RSV to devise an in vitro method of titration.

Discovered with the Bryan's high titer strain of RSV (BH-RSV) is another leukosis virus, termed Rous associated virus (RAV) (Rubin and Vogt, 1962). RAV induces visceral lymphomatosis and erythroblastosis in infected chickens, but does not cause transformation of CEC. RAV is present in excess of RSV and therefore is able to be isolated at high dilutions of RSV and RAV mixtures. At least three different isolates (RAV-1, RAV-2, RAV-3) have been found from stocks of the same strain of RSV (Hanafusa, 1968). In any single population of RSV and RAV, the two viruses, with the exception of their transforming ability, are virtually identical in morphological and antigenic properties (Hanafusa et al., 1964a).

The identification of Rous associated virus led to the conclusion that RSV was in actuality a defective virus which requires RAV as a helper, thus explaining the antigenic similarities. This was confirmed by the independent work of Temin (1962, 1963b) and Hanafusa et al. (1963) in which non-virus-producing Rous cells (NP) were isolated. They found that upon superinfection of these cells with different viruses

of the avian leukosis complex, Rous sarcoma virus was produced.

Further clarification of the role of RAV in Rous sarcoma virus infection was brought about by studies of RSV inhibition. It could be shown that when there is antigenic similarity between RSV and RAV the infecting efficiency of RSV is reduced, similar to the effect of RIF. However, only when there is dissimilarity in their antigens is RSV enhanced. The resulting progeny RSV virion has a viral coat antigen corresponding to the particular helper used (Hanafusa et al., 1964b; Vogt, 1964).

Cell cultures obtained from different lines of chickens have been found to exhibit distinct differences in their resistance to infection by viruses of the avian leukosis group (Vogt, 1965; Vogt and Ishisaki, 1965). This genetic resistance of the cells was used for classification of these viruses. It was found that groups of viruses that were antigenically related also elicited a similar interference pattern with a particular host cell type. The interference pattern seen with RSV and antigenically similar viruses may be thought of as a competition of the two viruses for available receptor sites on the cell.

It cannot be excluded that the dependence of RSV upon helper viruses may be reflected in all tumor viruses. Hartly and Rowe (1966) have presented evidence that the mouse sarcoma virus, a member of the murine leukosis complex, is also defective and requires a second virus for focus formation. Whether this defectiveness is common to

all RNA oncogenic viruses is not clear. If it were, and similar to that of RSV, antigenic and morphological similarities would make separation of helper and non-helper viruses difficult. This is especially true if both viruses were present at the same concentration and thus inseparable by dilution.

MC29 Virus

Strain MC29 virus, isolated in 1964 (Ivanov et al.) induces a rather broad spectrum of neoplasms in the chicken. Generally confined to the myeloid hematopoietic tissue, the tumor cells may be diffuse growths, myelocytomatosis, or form solid, localized tumors, myelocytomas (Mladenov et al., 1967). Not infrequently associated with MC29 infection are erythroblastosis, renal, and liver tumors (Heine et al., 1966; Mladenov et al., 1967).

Investigations with MC29 leukosis virus and its effect on chick embryo cells have led to the discovery that MC29 infection results in transformation and virus particle release, similar to that observed with Rous sarcoma virus (Langlois et al., 1967). However, there appears to be a decided difference between MC29 and RSV in the rate of morphological alteration yielding uniformly transformed cells. Although RSV induces a rapid rate of transformation (Manaker and Groupé, 1956), only a small proportion of the exposed cells are altered (Temin and Rubin, 1959). Extended culturing of RSV cells is

needed to yield a population of uniformly transformed cells. In contrast, strain MC29 virus routinely produces uniformly transformed cultures in as little as 74 hours after infection (Bolognesi et al., 1968). Newly formed virus particles, manifested by both titration for focus forming units and by the appearance of virus specific antigen, are evident in culture supernatants as early as 12-18 hours after infection (Langlois et al., 1969a; Fritz et al., 1969).

When other leukosis viruses, RIF (Calnek, 1964), and strain R (Langlois et al., 1967) were examined in reference to cell response to infection, it was found that although virus particle production was rapid, alteration of the cells occurred only after prolonged culturing. Consequently, the in vitro response of MC29 virus, being both brief and complete, is particularly suitable for investigations of the processes of RNA tumor virus infection and transformation.

Replication of RNA Tumor Virus Nucleic Acid

Attempts to discern the biochemical events in the replication of RNA tumor viruses have resulted in diverse lines of investigation. In vitro studies with inhibitors of nucleic acid replication, notably amethopterin and actinomycin D, led to the conclusion that some type of DNA is involved in the early phase of replication (Temin, 1964b). This supported Temin's provirus hypothesis (1964a), which includes a DNA intermediate in the replication of viral RNA. This DNA would

then be available for, or the cause of, cellular transformation.

Temin's provirus hypothesis opposes the probability of an RNA-dependent RNA polymerase for the replication of the viral genome. However, Watson and Beaudreau (1966) have detected RNA-dependent RNA polymerase activity in chick myeloblasts infected with avian myeloblastosis virus. This in vitro activity was found to be stimulated by exogenously added RNA.

Support for the DNA involvement in RNA tumor virus replication was greatly enhanced by the discovery of RNA-dependent DNA polymerase activity associated with RNA tumor viruses (Baltimore, 1970; Temin and Mizutani, 1970). According to Temin's provirus hypothesis, the infecting viral RNA genome could be transcribed by the RNA-dependent DNA polymerase and the product DNA would now be able to become associated with that of the host DNA. Transformation of the infected cell could result and progeny viral RNA would be able to be formed utilizing the DNA-dependent RNA polymerase from the host cell.

However, if both the RNA-dependent RNA polymerase and RNA-dependent DNA polymerase result from RNA tumor virus infection, then the provirus hypothesis may need some modification. It is possible that RNA tumor virus replication utilizes both an RNA-dependent DNA polymerase and an RNA replicase. DNA synthesis from the viral RNA genome may be an essential requirement for

transformation of the host cell, not necessarily for viral RNA synthesis. The RNA genome of the virus could still be replicated in a fashion similar to non-tumor viruses. Since infectious virus is usually detected through its transforming ability, inhibitors of DNA synthesis could prohibit morphological alteration of the cells while possibly still allowing viral RNA replication.

Of extreme importance in deciphering the oncogenic potential of RNA tumor viruses was the discovery of DNA in purified preparations of AMV (Říman and Beaudreau, 1970), RSV (Levinson et al., 1970) and in the MSV-MLV complex (Biswal and Benyesh-Melnick, 1970). This DNA appears to be of two types (Říman and Beaudreau, 1970). One is approximately 7S in size and sediments with the low molecular weight viral nucleic acids. Another DNA, of unknown size and composition, is associated with the high molecular weight viral RNA (Říman, 1971).

In addition to the RNA-dependent DNA polymerase activity associated with RNA tumor viruses, there was also identified a DNA-dependent DNA polymerase activity (Mizutani et al., 1970; Říman and Beaudreau, 1970; Spiegelman et al., 1970b). This, together with the detection of DNA in RNA tumor viruses, immediately raised obvious questions concerning the contribution of each nucleic acid to the RNA-dependent DNA polymerase reaction. Although the degree of participation of the viral DNA and DNA-dependent DNA polymerase activity is

unclear, their presence in viral DNA polymerase preparations is still unrecognized by some investigators.

Some clarification of the enzyme activities in the virus was brought about by Kacian et al. (1971). They demonstrated that both RNA-dependent and DNA-dependent activities could be ascribed to the same enzyme. Yet the function of this enzyme activity in virus infection and the molecular details of the enzyme synthesis still remains an enigma to investigators in the field.

In vitro the initial reaction of the DNA polymerase is one that synthesizes a single strand of DNA using the viral RNA as a template (Garapin et al., 1971; Rokutanda et al., 1970; Spiegelman et al., 1970a; Baltimore, 1970; Temin and Mizutani, 1970; Manly et al., 1971; Faras et al., 1971). This DNA product is then used in turn as a template to form double-stranded DNA (Fanshier et al., 1971; Fuginaga et al., 1970; Manly et al., 1971).

How much of the viral genome that is involved in RNA to DNA transcription is difficult to determine. Duesberg and Canaani (1970) presented evidence that the DNA product from a 90 minute reaction was heterogenous in size (3 to 8S) and that 50-70% of the DNA was hybridizable to viral RNA. The remaining unhybridized DNA was thought to be either double-stranded or DNA with a base sequence similar to the viral RNA. It was postulated that up to 75% of the viral RNA was transcribed to DNA.

Rokutanda et al. (1970), however, analyzed in cesium sulfate the hybrid RNA:DNA product from the DNA polymerase of murine sarcoma virus (MSV). It was found that the buoyant density of the hybrid was very close to that of the viral RNA. They concluded therefore that only small sequences of the viral RNA are involved in hybridization of the viral DNA (found to be 7S). Consequently, whether or not the entire viral RNA genome is transcribed by the DNA polymerase has not been clearly resolved at this time.

The participation of viral DNA in the DNA polymerase reaction has still to be considered. Whether the DNA is serving as a primer, an initiator point, or not involved in DNA synthesis at all, is unknown. If the viral DNA is acting as the sole template for DNA synthesis or as a primer, then Temin's provirus hypothesis necessitates further clarification.

Preliminary investigations concerning the problem of DNA participation in the RNA-dependent DNA polymerase reaction have been presented by Baltimore and Smoler (1971). Utilizing various synthetic homopolymers and homopolymer duplexes, they postulated that the DNA polymerase is incapable of DNA synthesis using RNA alone as a template. Addition of a short complementary segment of DNA to the RNA homopolymer stimulated activity. It was therefore postulated that the RNA is acting as the template, but a segment of DNA, with its free 3'-OH end, is needed as primer.

The results of Baltimore and Smoler present an alternative to other proposed models of viral RNA transcription to DNA, but results based solely on synthetic homopolymers as templates must be viewed with caution. It can be shown that the template activity of seemingly identical homopolymer mixtures has a tendency to differ considerably.

The following study presents evidence that strain MC29 virus contains DNA polymerase activity similar to that found in other tumor viruses (Baltimore, 1970; Green et al., 1970; Hatanaka et al., 1970; Mizutani et al., 1970; Scolnick et al., 1970a; Spiegelman et al., 1970a; Temin and Mizutani, 1970). This enzyme activity, easily detectable from culture supernatants of infected cells, can be used to monitor the appearance of progeny virus after infection. Additionally, MC29 tumor virus infected cells yielded DNA polymerase activity that was similar to that of the infecting virus and distinguishable from the normal cellular DNA polymerase.

Besides the DNA polymerase the MC29 virus was shown to contain DNA in addition to the viral RNA.

MATERIALS AND METHODS

Primary Chick Embryo Cultures

Primary cultures of chick embryo cells were prepared from eggs obtained from Jenk's Hatchery, Tangent, Oregon and from a flock of chickens maintained by R. E. Luginbuhl, University of Connecticut, Storrs, and supplied by the Special Virus Cancer Program, National Cancer Institute. Decapitated and eviscerated 10 or 11-day old embryos were minced and washed three or four times with phosphate buffered saline (PBS) until the supernatant appeared free of RBC's. Tissue fragments were then trypsinized with 0.25% trypsin in PBS for 30 minutes. Cells were filtered through eight layers of sterile gauze into an iced Erlenmyer flask containing five ml of calf serum. Fresh trypsin was then added to the fragments and the procedure repeated as often as necessary. After the last trypsinization the cells were sedimented at 600 x g for five minutes, and resuspended in growth medium. An aliquot of the cell suspension was stained with crystal violet and counted in a hemocytometer. Plastic petri dishes (100 x 20mm, Falcon Plastics, Los Angeles, California) were seeded with 6.0 to 10.0 x 10^6 cells in ten ml of growth medium which consisted of 80% medium 199, 8% newborn calf serum (Microbiological Associates, Albany, California), 10% tryptose phosphate broth (Difco, Detroit, Michigan), penicillin (50 units/ml), streptomycin (50 μg/ml),

and amphotericin B (0.5 μg/ml). The cultures were incubated in a humidified, 5% CO_2 atmosphere at 38.5°C.

Infection with MC29 Virus

Twenty-four hours after seeding primary cultures, the cells were washed with four ml PBS and infected with 0.2 ml of MC29 virus (the original stock of MC29 virus was kindly supplied by J. W. Beard) at a multiplicity of infection of 0.006-0.04 focus forming units (FFU) per cell. Control noninfected cultures were treated with 0.2 ml of medium 199. The virus was allowed to adsorb to the cells for 30 minutes at 38.5°C, after which ten ml of medium was added to the culture and the cells returned to the incubator. Culture medium was routinely replaced 48 hours after infection and thereafter at daily intervals.

Secondary Cultures

The cells were generally subcultured 72 hours after infection. The growth medium was decanted and the cell sheet washed with four ml PBS. Four ml of 0.05% trypsin were added and the cultures incubated for seven minutes at 38.5°C after which the cells were worked off the culture vessel by gentle aspiration with a five ml pipette. The cells were pooled in an iced 50 ml conical centrifuge tube containing one or two ml of calf serum and were sedimented at 600 x g for ten minutes. The cell pellets were resuspended in growth medium,

enumerated in a hemocytometer, and seeded in petri dishes at a cell concentration of 0.5×10^6 cells per ml. Growth medium was usually replaced at daily intervals after subculturing.

Preparation of Stock MC29

For the present studies, three separate stocks of MC29 were prepared in the following way. Primary CEC cultures were prepared, infected and subcultures as above. Growth medium was replaced 48 and 72 hours after subculturing. During the following day a six hour virus harvest was collected, pooled and centrifuged at 5,000 x g for ten minutes to remove the cell debris. The clarified culture medium was then frozen at -70°C for storage. Culture fluid harvested in this manner routinely contained approximately 0.2 to 1.0×10^6 FFU/ml of tissue culture fluid.

Titration of MC29 Virus

The methods of titration for FFU were essentially those of Langlois and Beard (1967) with modifications kindly supplied by A. J. Langlois (1969). Monolayers of primary CEC cultures were removed from the petri dish with 0.05% trypsin and harvested as for secondary cultures. The resuspended cells were filtered through four layers of sterile gauze and diluted to contain 2.0×10^5 cells per ml of growth medium. Petri dishes (60 x 15mm) were seeded with five ml of the

cell suspension. After incubation for 24 hours the medium was decanted and the non-confluent cell layer washed with four ml of PBS. One-tenth ml aliquots of serial dilutions of virus were added to each of four replicate plates and allowed to adsorb for 30 minutes after which five ml of growth medium were added. During the following morning the culture medium was decanted, the cells were washed with four ml of PBS, and four ml of growth medium containing 0.45% Nobel's Agar (Difco, Detroit, Michigan) were added. Three days after adding the virus, three ml of growth medium were added to each culture. The fluid and agar were removed seven days after infection. The cultures were washed with five ml PBS, and three ml of cold methanol were added for five minutes. The alcohol was decanted and the cells stained for ten minutes with five ml of May-Grünwald stain (Allied Chemical, Morriston, New Jersey). This was removed and five ml of Giemsa stain (Allied Chemical, Morriston, New Jersey) added for ten minutes. The cultures were then rinsed with distilled water and allowed to air dry. When necessary the cells were destained with four ml of 0.001 N HCl followed by five ml PBS. Foci of transformed cells were counted under low power magnification.

Enzyme Titration

Culture fluids to be titered for infectious virus by the focus forming unit assay were also collected for the determination of DNA

polymerase activity associated with the virions. At different times after infection, culture medium from six plates (60 ml) was pooled, and two ml aliquots for titration were removed, centrifuged at 1,640 x g for ten minutes, and stored at -70°C. The remaining pool of culture medium was centrifuged at 5,000 x g for ten minutes and the supernatant fluid stored for enzyme assay by freezing at -70°C. Fifty ml samples of culture fluid were prepared for enzyme assay as described below.

Collection and Storage of Avian Myeloblastosis Virus, BAI Strain A

Intravenous injections of 7×10^{10} virus particles were given to one or two day old chicks. Within 10 to 14 days those birds showing acute myeloblastic leukemia were bled via cardiac puncture into heparinized tubes. The blood was centrifuged at 1,400 x g for 15 minutes to separate the cells from the plasma. The plasma was removed, treated with kieselguhr, centrifuged at 1,000 x g and stored at -70°C. Titrations of the plasma virus were made by measuring ATPase activity (Beaudreau and Becker, 1958).

Determination of Cell Growth

Cell growth was monitored by washing duplicate plates with four ml of PBS, adding four ml of trypsin media, pH 7.2 (0.02% trypsin;

0.68 mM ethylenediaminetetraacetic acid; 0.14 M NaCl; 5.0 mM KCl; 6.0 mM glucose; 4.0 mM $NaHCO_3$; 5 x 10-5% phenol red) and incubating at 38.5°C for 15 minutes. The cells were mixed, diluted with crystal violet and counted in a hemocytometer.

Collection of MC29 Infected and Noninfected CEC Cells

MC29 cells and control noninfected cells were harvested by decanting the growth medium, washing the cell sheet with four ml PBS and adding one ml of fresh culture medium to each petri dish. The cells were removed with a rubber policeman, divided into pools from two plates and centrifuged at 1,640 x g for ten minutes. The medium was then decanted and the cell pellets frozen at -70°C.

DNA Polymerase Procedures

Preparation of DNA Polymerase from MC29 Infected and Noninfected CEC Culture Fluids

When a direct comparison was made between infected and control CEC culture fluids, all steps in preparation of the enzyme from both sets of cultures were carried out in an identical manner. Culture fluids were thawed rapidly and centrifuged at 5,000 x g for ten minutes if not done so before freezing. The supernatant was mixed with approximately 0.5 gm kieselguhr and centrifuged again at 1,000 x g for 15 minutes. The virus was pelleted from the clarified culture fluids

by centrifugation at 25,000 rpm in either a Spinco SW 25.1 or SW 25.2 rotor for 60 minutes. The pellets were resuspended in 0.2 ml of 2x Buffer 0 (0.05 M Tris, pH 8.3; 0.02 M $MgCl_2$; 0.01 M reduced glutathione; 0.001 M EDTA; 0.30 M KCl; and 20% glycerol) and mixed with 0.2 ml of 2x Nonidet stock (0.5% Nonidet, Shell P40, plus 0.13 M dithiothreitol). This mixture was stored in an ice bath overnight before assaying for enzyme activity.

DNA Polymerase from Infected and Noninfected CEC Culture Homogenates

The frozen cells from two petri dishes were thawed, resuspended in one ml Buffer RSB (0.01 M Tris, pH 8.3; 0.01 M KCl; 0.005 M $MgCl_2$; 0.005 M EDTA; 0.001 M reduced glutathione; and 10% glycerol) and let stand with periodic mixing in ice for two hours. The cells were disrupted using a tight fitting Dounce homogenizer (about 30 strokes). Cell breakage was followed by microscopic examination during homogenization. Nuclei and cell debris were pelleted by centrifugation for ten minutes at 1,640 x g. Protein determinations were made on the supernatant fluids by the methods of Warburg and Christian (1942). The partially clarified cell homogenates were then detergent treated by adding an equal volume of 2x Nonidet stock and allowed to stand overnight at 0°C before assaying for enzyme activity.

DNA Polymerase Assay

A standard reaction mixture contained, in a volume of 0.1 ml: Tris-hydrochloride buffer (pH 8.3), 4 μmoles; $MgCl_2$, 0.8 μmoles; NaCl, 6 μmoles; reduced glutathione, 0.37 μmoles; unlabeled deoxyribonucleoside triphosphates, 0.02 μmoles; ^3H-labeled deoxyribonucleoside triphosphate (Amersham Searle; specific activity: nine curies per millimole), 1 μc (counts per minute per picomole are reported in legends). DNA, synthetic homopolymers and enzyme were added in amounts indicated in each experiment. All assays were performed at 37°C for 60 minutes and the reactions terminated by chilling in ice and adding either 0.5 ml of 100% trichloroacetic acid or 0.5 ml TCA mixture (1:1:1 of saturated Na_2HPO_4; saturated NaH_2PO_4; 100% TCA). The precipitate was collected on nitrocellulose membrane filters (B6, Schleicher and Schuel, Keene, New Hampshire), washed ten times with four ml of cold 10% w/v TCA, dried at least 15 minutes at 80°C, and the radioactivity measured in a liquid scintillation fluid (0.4% w/v BBOT (2, 5-bis-[2-(5-tert-butybenzoxazolyl)]-thiophene, Packard Instrument Company, Downers Grove, Illinois) with toluene in a liquid scintillation spectrometer. In some instances, the reaction mixtures were reduced to 0.05 ml.

Purification of MC29 Virus DNA Polymerase

Six to eight hour virus harvests totalling approximately 750 ml were obtained from six day infected CEC cultures. After clarification by centrifugation at 5, 000 x g for ten minutes, followed by treatment with kieselguhr, the virus was pelleted by centrifugation at 25, 000 rpm for 60 minutes in a Spinco SW 25.2 rotor. The pellets were resuspended to a volume of ten ml in TNED (0.01 M Tris, pH 8.0; 0.15 M NaCl; 0.001 M EDTA; 0.005 M dithiothreitol) and carefully layered onto a 60-40-20% sucrose gradient made in TNED (eight ml 60% w/w sucrose, ρ =1.287 gm/cm^3; five ml 40% w/w sucrose ρ=1.176 gm/cm^3; five ml 20% sucrose, ρ =1.081 gm/cm^3). The gradient was centrifuged for 120 minutes at 25, 000 rpm and one ml fractions were collected. The visible interface fractions were pooled, resuspended with TNED to a volume of approximately 4.5 ml and centrifuged for 30 minutes at 50, 000 rpm in a Spinco SW 50 rotor. The virus pellet was resuspended in 0.5 ml of 2x Buffer 0, mixed with an equal volume of 2x Nonidet stock and let stand in ice with occasional stirring for 60 minutes. The virus homogenate was then layered on top of four ml of CsCl (2.25 ml saturated CsCl plus 3 ml 1x Buffer 0 made 0.065 M dithiothreitol; ρ =1.4 gm/cm^3) and centrifuged for 30 hours at 50, 000 rpm. Fractions (0.2 ml) were collected and samples taken for protein determination, refractive index and enzyme activity.

Protein estimations were made by a turbidity method using a bovine serum albumin (BSA) standard curve for comparison. Samples of BSA in 0.4 ml water were precipitated with 0.6 ml 5% TCA and allowed to stand for ten minutes after which the optical density at 400 nm was read. Samples from the CsCl fractionation (0.02 ml) were treated in an identical manner.

Isolation of MC29 Nucleic Acid

The pellet fractions after CsCl centrifugation from the above enzyme preparation were resuspended with the first two gradient fractions and diluted to one ml with water. The nucleic acid was precipitated by adding two volumes of ethanol and stored at -20°C for at least two hours. The precipitate was removed by centrifugation at 13,000 rpm for 60 minutes and resuspended in 0.8 ml water. Alcohol precipitation was repeated and the suspension stored at -20°C. The nucleic acid was centrifuged as before, the pellet dried in a vacuum, resuspended in 0.1 ml water and mixed with an equal volume of 1% sodium dodecyl sulfate. The sample was brought to 0.6 ml with 0.28 M EDTA, mixed with 2.7 ml of saturated CsCl and centrifuged at 50,000 rpm in a Spinco SW 65 rotor for 48 hours at room temperature. Fractions of 0.2 ml were collected and refractive indices read. The samples were then transferred to heat sterilized corex tubes and the collection tubes washed with 0.2 ml water followed by 0.8 ml ethanol.

Each fraction was ethanol precipitated and stored overnight at -20°C. Virus DNA polymerase, which was free of endogenous template, was used to assay the samples.

Estimation of Sedimentation Rate of Viral DNA Polymerases

Preformed sucrose gradients (5-20% w/w) were prepared in Buffer 0 made 0.065 M dithiothreitol. Each was carefully layered with a 0.50 ml sample containing 0.20 mg human hemoglobin, 0.80 mg human gamma-globulin and 0.25 ml of either MC29 or AMV template free enzyme. The gradients were centrifuged at 50,000 rpm in a Spinco SW 50 rotor for 12 hours after which 0.2 - 0.3 ml fractions were collected. Protein determinations were made by the TCA-precipitation method. Each fraction was assayed for DNA polymerase activity as was previously explained.

Partial Purification of AMV DNA Polymerase

The following procedure for DNA polymerase purification from AMV was kindly supplied by K. F. Watson. Avian myeloblastosis virus from approximately one liter of blood plasma was prepared according to the methods of Carnegie et al. (1969). Kieselguhr-treated plasma was filtered through Schleicher and Schuell black ribbon filter paper and centrifuged against a ten ml glycerol pad at 75,000 x g for one hour in a Spinco SW 25.2 rotor. The supernatant

plasma was removed, plasma that contained virus was layered on top of the virus band, and the centrifugation procedure repeated until all the virus was collected. The glycerol interfaces, containing the virus, were removed, made 50% glycerol and stored at -20°C. Twenty-eight ml of AMV (five mg/ml in TNE) were disrupted in ten ml quantities by the addition of one ml 100% Nonidet P40, one ml 10% deoxycholate and three ml of 4 M KCl. The suspension was kept at 0°C for at least 15 minutes.

The disrupted virus suspension, diluted to ten times its volume with 0.01 M potassium phosphate (pH 7.2), 0.02 M dithiothreitol, 10% glycerol, was applied to a 2.2 x 11.0 cm column of DEAE-Cellulose (Bio-Rad, Richmond, California) previously equilibrated with the same buffer. The column was washed overnight with 150 ml of 0.05 M potassium phosphate buffer (pH 7.2) and eluted with 50 ml of 0.3 M potassium phosphate buffer (pH 7.2). Samples of 0.01 ml from each fraction were assayed for DNA polymerase activity in a standard enzyme reaction assay using M. lysodeikticus DNA as template. Those fractions showing most enzyme activity were pooled and diluted to three times their volume with 0.01 M potassium phosphate buffer (pH 8.0).

The pooled and diluted enzyme fractions from the DEAE column were applied to a 1.0 x 8.0 cm CM-Sephadex (Pharmacia Fine Chemicals, Piscataway, New Jersey) column previously equilibrated with

0.01 M potassium phosphate buffer (pH 8.0). The column was washed with 15 ml of 0.1 M potassium phosphate buffer (pH 8.0) and eluted with 20 ml of 0.3 M potassium phosphate buffer (pH 8.0). Samples of each fraction were assayed for DNA polymerase activity. Those fractions showing the most enzyme activity were made 50% glycerol and stored at -20°C.

Preparation of Labeled MC29 Virus Particles

CEC cultures were infected with MC29 virus and subcultured as previously explained. Six days after infection the medium from four cultures was replaced with growth medium containing ^{3}H-uridine (New England Nuclear; specific activity: 37 curies per millimole). Each culture received two ml of medium containing 250 μc of radioactive label. Uptake of radioactive label was monitored at hourly intervals by spotting five μl samples of the culture medium on membrane filters and counting the radioactivity in the scintillation counter. Two hours after the addition of label, the cells were twice washed with five ml of growth medium. Ten ml of fresh medium was then added and the plates incubated at 38.5°C. After six hours the medium was decanted, centrifuged at 5,000 rpm for ten minutes, frozen in liquid nitrogen and stored at -70°C. Fresh growth medium was added to the cells and two additional harvests were taken, each six hours.

Isolation and Purification of Labeled Virus Particles

Labeled MC29 particles were isolated by banding the virus at a density of 1.16 gm/cm^3 in a sucrose gradient made in Buffer 1 (0.01 M Tris, pH 8.0; 0.10 M NaCl; 0.01 M $MgCl_2$; 0.002 M EDTA; and 0.005 M dithiothreitol). Fractions were collected and samples taken, diluted to one ml with water, precipitated with 0.1 ml 100% TCA and counted. Fractions showing most activity were pooled, resuspended in PBS and centrifuged at 45,000 rpm for 45 minutes in a Spinco SW 50 rotor. Pelleted virus was either layered onto a continuous gradient or resuspended in growth medium and used for infecting 24 hour CEC cultures.

RESULTS

DNA Polymerase from MC29 Infected and Noninfected CEC Culture Fluids

Deoxyribonucleoside Triphosphate Requirements

Detection of DNA polymerase activity associated with RNA tumor viruses by Baltimore (1970) and Temin and Mizutani (1970) prompted an investigation of the DNA polymerizing activity in strain MC29 tumor virus. DNA polymerase activity was found to be present. Both the endogenous reaction and DNA-directed synthesis of DNA by enzymes from tumor viruses have been shown to be dependent on the presence of a complete complement of deoxyribonucleoside triphosphates (Baltimore, 1970; Spiegelman et al., 1970a; Temin and Mizutani, 1970), and the DNA polymerase associated with the MC29 virus is no exception. Results presented in Table 1 show that the DNA-directed reaction had about a ten-fold rate of ^{3}H-TTP incorporation over the endogenous reaction. The omission of one deoxyribonucleoside triphosphate strongly depressed DNA polymer synthesis in both the presence and absence of DNA. A possible exception can be noted by the omission of dGTP from the endogenous reaction. In the presence of DNA the requirements for dGTP appear to be greater.

Table 1. Deoxyribonucleoside triphosphate requirement for the synthesis of DNA polymer (*).

Condition	Amt (pmoles) of ^{3}H-TTP incorporated	
	With DNA	Without DNA
Complete	3.3	0.39
Minus dATP	0.5	0.15
Minus dGTP	0.3	0.23
Minus dCTP	0.2	0.13

(*) The reaction conditions were the same as reported in Methods except that the amount of materials added to the reaction mixture was reduced to 0.05 ml. DNA primer from Micrococcus lysodeikticus (0.4 μg) was added to each assay. The reactions were incubated at 37°C for 30 minutes. ^{3}H-TTP had a specific activity of 1,000 counts per minute per pmole in the reaction mixture.

Enzyme Activity from Culture Medium of Late Infected Cells

Culture medium from cells that had been infected with MC29 virus for at least six days were assayed for the presence of enzyme activity. Table 2 shows the results when different collections of culture medium containing different amounts of infectious virus were assayed. Six hour harvests of culture medium from infected cells were taken in all cases. From these experiments the greater sensitivity of the FFU assay is obvious. On the average there was required 100 FFU to give an enzyme assay that incorporated five cpm of ^{3}H-dGTP into DNA polymer (at 10% counting efficiency).

Table 2. DNA polymerase activity from MC29 virus.

Experiment*	Enzyme activity**	FFU assayed***	Enzyme activity/FFU
CB-1 (13)	1,743	6.8×10^4	0.03
CB-8 (6)	1,473	1.8×10^4	0.08
CB-17 (6)	1,065	2.2×10^4	0.05
CB-19 (6)	17,859	7.5×10^5	0.02
CB-12 (6)	13,310	4.4×10^5	0.03

* Numbers in parentheses indicate number of days after infection when the collection was made for assay.

** Cpm of ^{3}H-dGTP incorporated into DNA product after 60 minute incubation at 37°C (2,000 cpm = one pmole). DNA primer from M. lysodeikticus (2μg) was added to each assay.

*** Amount of infectious virus added to reaction mixture for enzyme determination.

Kinetics of DNA Polymerase Activity from MC29 Virus

The data in Figure 1 were obtained from cultures six days after infection, with their respective control cultures. It was found that the enzyme fraction from virus-infected cultures had DNA polymerase activity that was strongly stimulated by the addition of DNA and also showed a detectable endogenous reaction. Non-infected control cultures displayed no enzyme activity. In this experiment, synthesis by the enzyme from virus of infected cells in the presence of DNA did not continue beyond 40 minutes. This was the only instance in which short term synthesis was observed. Linear kinetics were typical of DNA synthesis by the enzyme from MC29 virus (Figure 2).

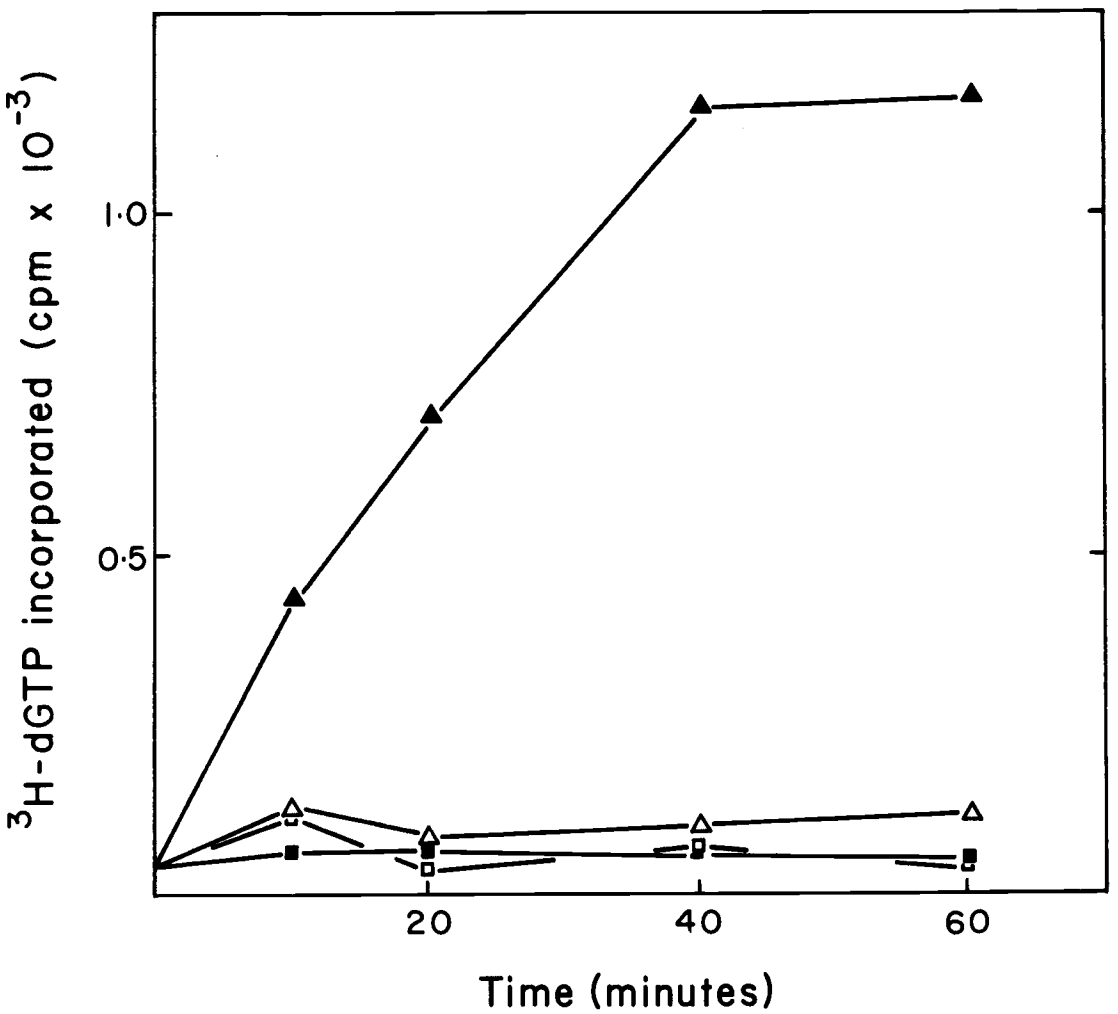


Figure 1. Kinetics of ^{3}H-dGTP incorporation into DNA polymer with enzyme from culture fluids of cells infected with MC29 virus for six days. The preparation of enzyme from culture fluids from infected cells was assayed in the presence of M. lysodeikticus DNA (▲) and its absence (△). Culture fluids from noninfected control cultures were treated the same and assayed for enzyme activity in the presence of DNA (■) and without DNA (□). Both preparations were made from eight ml of culture fluid. The culture fluid from infected cells contained 3.1×10^3 FFU/ml and each assay had 1.5×10^3 FFU. DNA was added at a level of 0.7 μg/assay and one pmole of dGTP was equivalent to 2,000 cpm. The reported values for ^{3}H-dGTP incorporation were from 0.05 ml reaction volume.

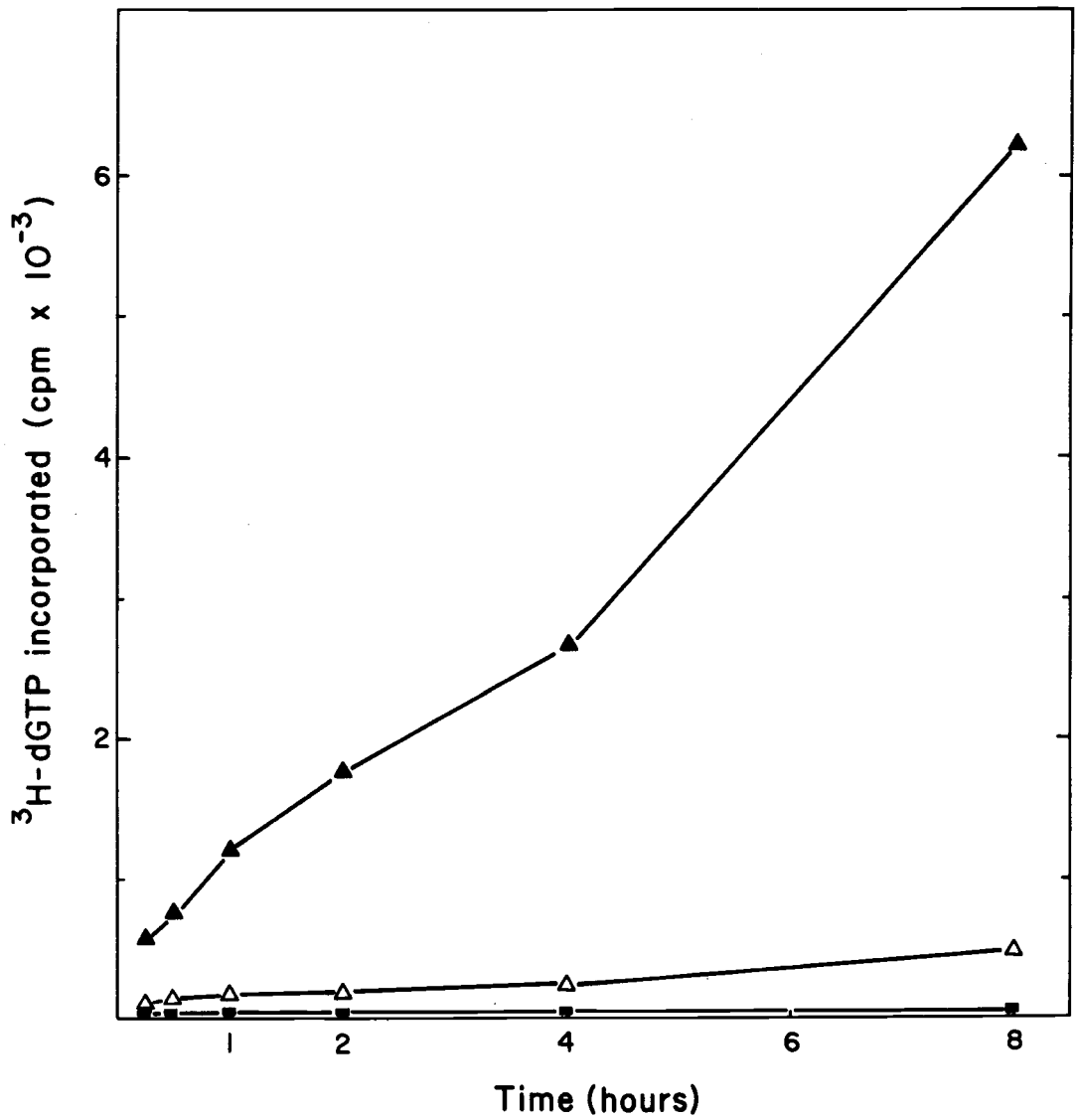


Figure 2. Kinetics of ^{3}H-dGTP incorporated into DNA polymer with enzyme from culture fluids of cells infected with MC29 virus for 72 hours. Reaction conditions were identical to those presented in Figure 1. Enzyme from culture fluids from infected cells was assayed in the presence (▲) and absence (△) of M. lysodeikticus DNA. Noninfected control cell culture fluid was also assayed in the presence (■) and absence (not shown) of DNA. Preparations were made from 50 ml of culture fluid. The culture fluids from infected cells contained 2.4×10^4 FFU/ml and each assay had 7.5×10^4 FFU.

It is important that the particulate fraction concentrated from 50 ml of culture fluid from noninfected cells showed no evidence of DNA polymerase. This demonstrated that there was no enzyme activity from normal cells contaminating the virus fraction under the conditions used for enzyme preparation. These results suggest that relatively simple methods of concentrating particles from tissue culture fluid may be useful in looking for tumor virus from cells in cultures that are suspected of being neoplastic.

Labeled MC29 Virus Particles

To determine optimum conditions for the purification of intact MC29 virus and its associated DNA polymerase, ^{3}H-uridine labeled MC29 virus particles were prepared and purified in either glycerol or sucrose. The following results showed that various methods of purification affected the apparent integrity of the virus particles.

Details of the labeling conditions and determination of uptake are presented in Methods. Figure 3 shows the percent uptake by the cells of ^{3}H-uridine from culture medium containing 250 μc of the radioactive label. Regardless of the conditions used, 75-80% of the radioactive label present in the culture medium was taken up by the cells in four hours. When the ^{3}H-uridine was present in two ml of culture medium (Figure 3A), there was a more rapid rate of uptake in the initial two hours than when the same amount of label was

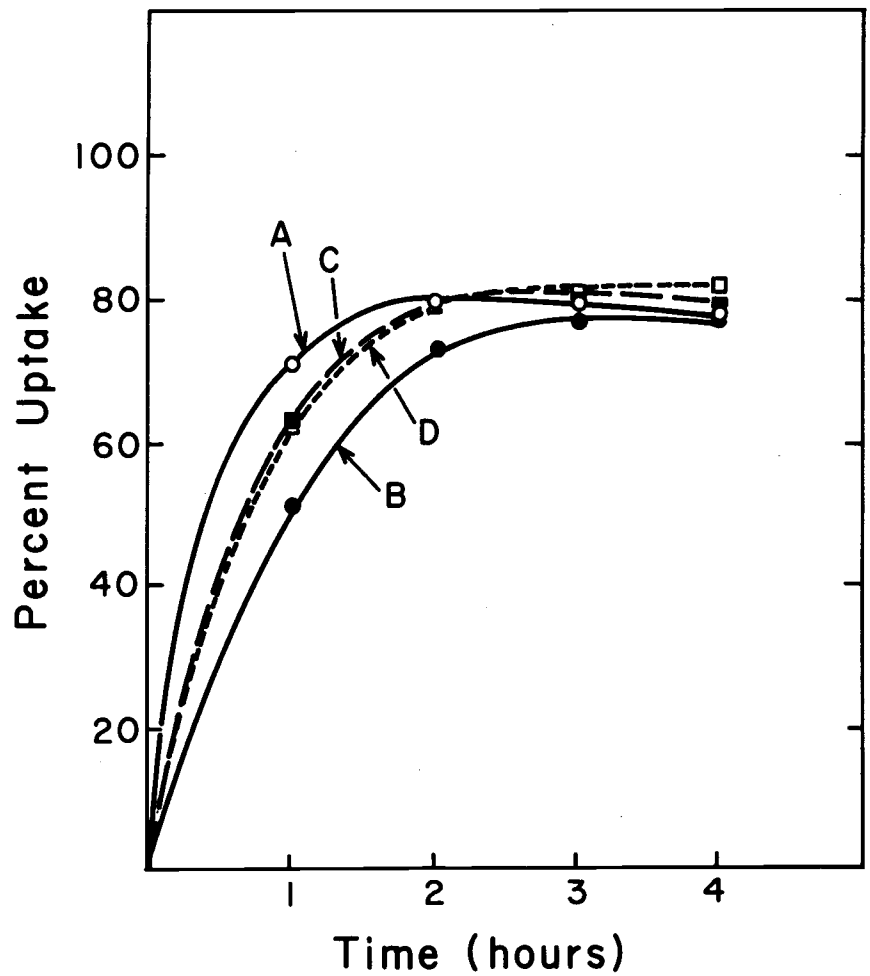


Figure 3. Uptake of ^{3}H-uridine by MC29 infected cells. Culture medium of cells infected for six days with MC29 virus was replaced with medium containing 250 μc of radioactive label (specific activity: 36.8 curies per millimole) in: (A) 2.0 ml 199 medium; (B) 5.0 ml 199 medium; (C) 2.0 ml 199 medium made 1% DMSO; and (D) 2.0 ml MEM culture medium. Uptake of radioactive label was monitored as described in Methods.

present in five ml of culture medium (Figure 3B). The presence of DMSO in the medium (Figure 3C) resulted in no significant change in the efficiency of uptake of ^{3}H-uridine by the cells.

The influence of uracil, present in medium 199 (0.30 mg/liter), on the uptake of ^{3}H-uridine was investigated by utilizing MEM culture medium which contains no uracil. These cells were allowed to grow for five hours in the presence of MEM culture medium before addition of the radioactivity labeled uridine. No improvement in uptake of the label by these cells could be demonstrated (Figure 3D).

Purification of Labeled Virus Particles

Procedures routinely used in this laboratory for the purification of AMV particles include the initial pelleting of the plasma virus onto a 100% glycerol pad. Initial attempts at purifying ^{3}H-uridine labeled MC29 virus were taken from this method.

Glycerol Gradient Centrifugation. Labeled MC29 virus was taken directly from the cell cultures and pelleted against a 0.25 ml pad of 100% glycerol in a five ml nitrocellulose centrifuge tube. After centrifugation, the supernatant culture medium above the glycerol was removed and an 85-35% v/v continuous glycerol gradient in Buffer 1 was constructed directly over the glycerol pad. Following centrifugation at 50,000 rpm for six hours, essentially all of the radioactivity was found in the pellet fraction. When the pellet was resuspended in PBS

and layered onto a preformed 85-35% v/v glycerol gradient, the results in Figure 4 were obtained. Labeled virus from non-frozen culture medium banded at a density of 1.17 gm/cm^3 in glycerol.

The 0.25 ml glycerol pad used in the initial pelleting was found to be of insufficient volume. Consequently, 100-15% v/v discontinuous glycerol gradients were prepared and layered with culture medium that had been previously frozen in liquid nitrogen and stored at -70°C. After centrifugation for two hours at 25,000 rpm, samples from each collected fraction were precipitated and counted. Those fractions showing most activity were then applied to a preformed 85-35% v/v continuous glycerol gradient and centrifuged as reported in Figure 4. Figure 5 shows the results routinely found when frozen virus particles were purified by these procedures in glycerol. Essentially all of the radioactivity was seen to be centered around a density of 1.22 gm/cm^3. No demonstrable activity could be found at the characteristic density of undamaged virus particles (1.17 gm/cm^3). Isolation of intact MC29 virus by way of glycerol appeared to be unsatisfactory.

Sucrose Gradient Centrifugation. As an alternative to the glycerol gradients used above, a 60-40-20% w/w discontinuous sucrose gradient in Buffer 1 was prepared and layered with a labeled virus particle suspension that had been previously frozen. The results are shown in Figure 6. Most of the activity from the labeled virus was found at a density indicative of intact particles (1.16 gm/cm^3). Little

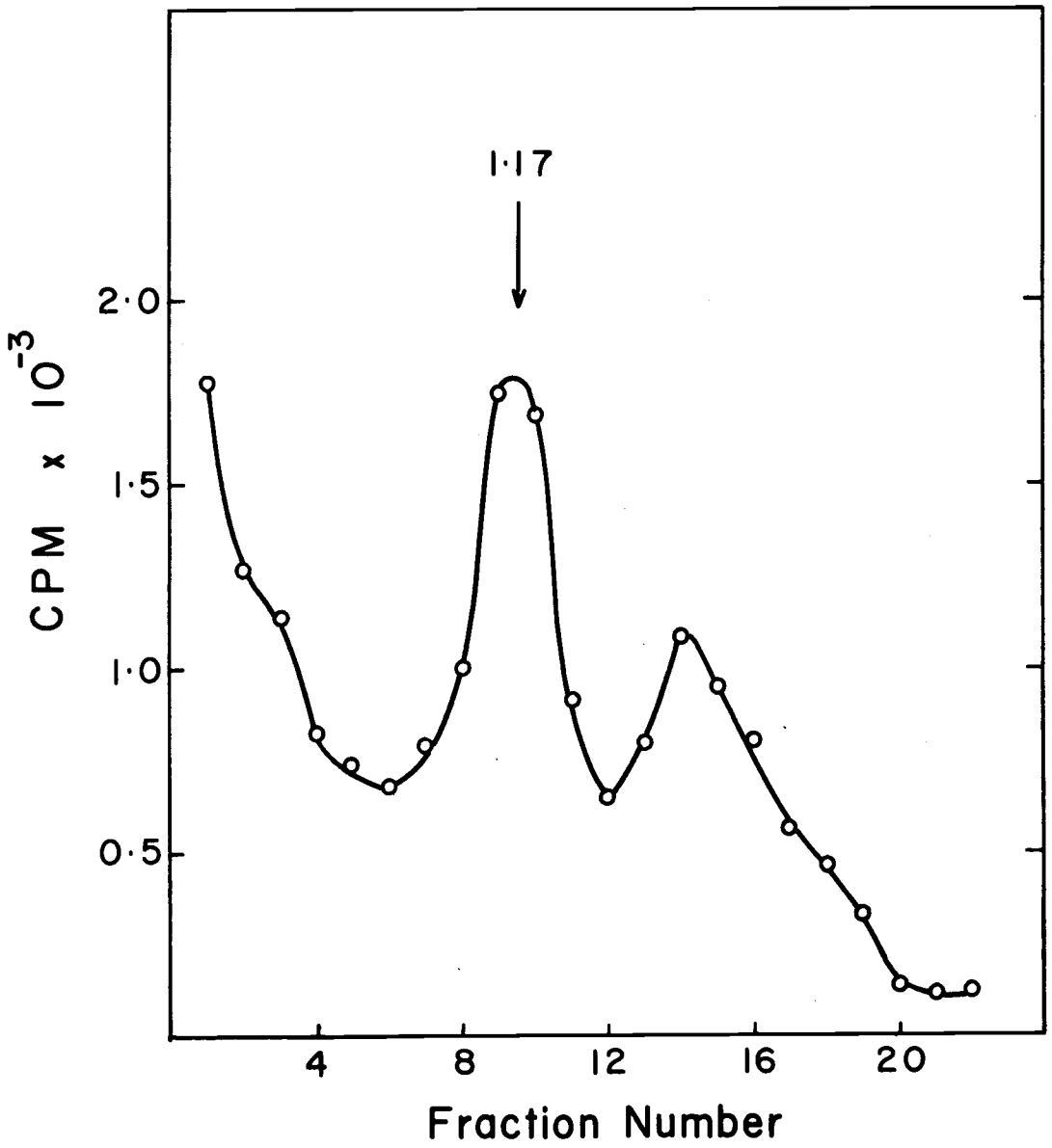


Figure 4. Isopycnic centrifugation in glycerol of ^{3}H-uridine labeled MC29 virus. Radioactivity labeled MC29 virus was isolated from fresh, nonfrozen culture medium as described in Results. The virus was then applied to an 85-35% v/v glycerol gradient made in Buffer 1. Centrifugation was at 50, 000 rpm for 14 hours in a Spinco SW 50 rotor. Approximately 0.2 ml fractions were taken and refractive indices read. Each fraction was then precipitated with 4.0 ml cold 10% TCA, plated and counted as described in Methods.

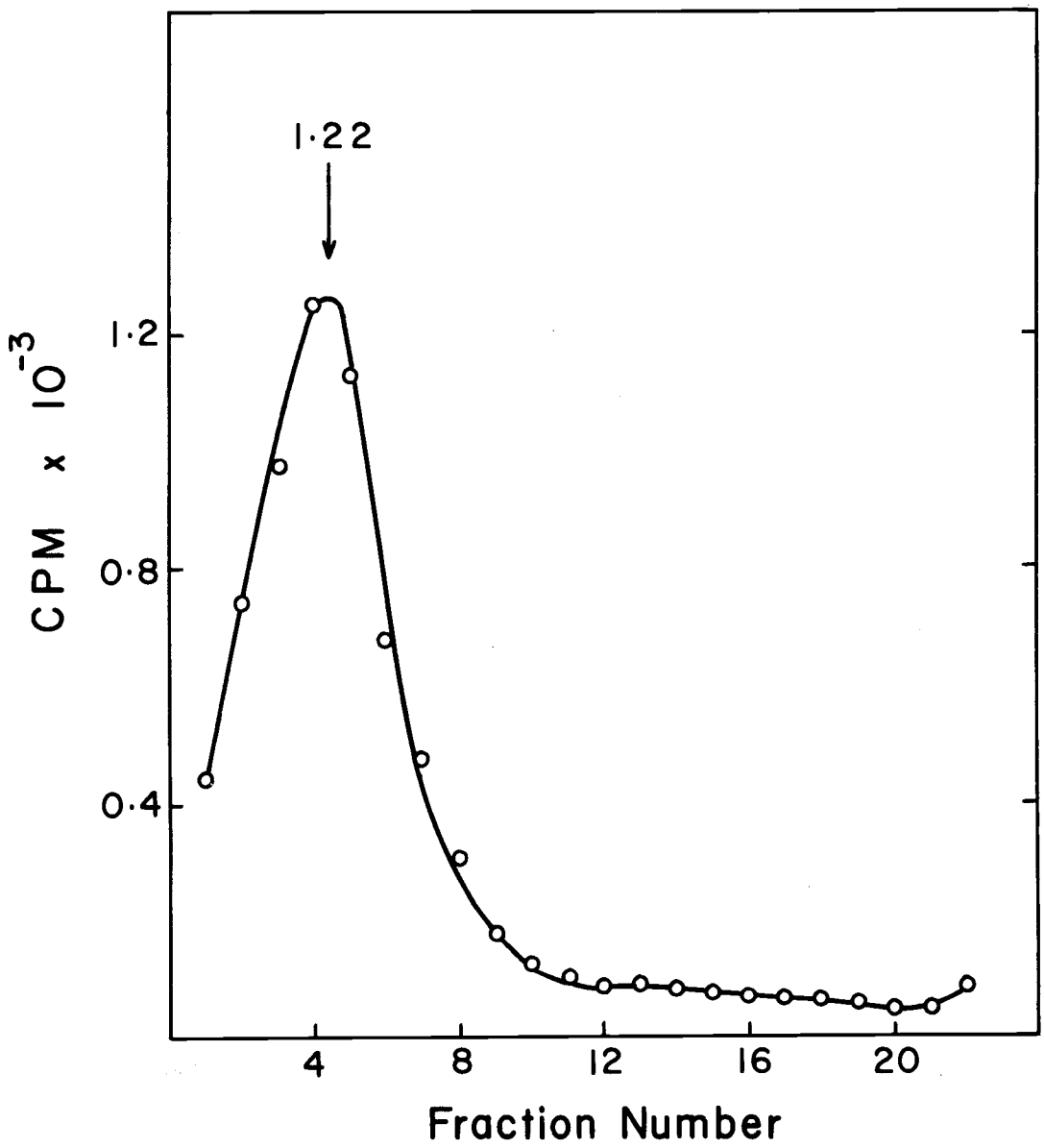


Figure 5. Isopycnic centrifugation in glycerol of ^{3}H-uridine labeled MC29 virus from frozen culture medium. Conditions for the experiment were those reported in Figure 4.

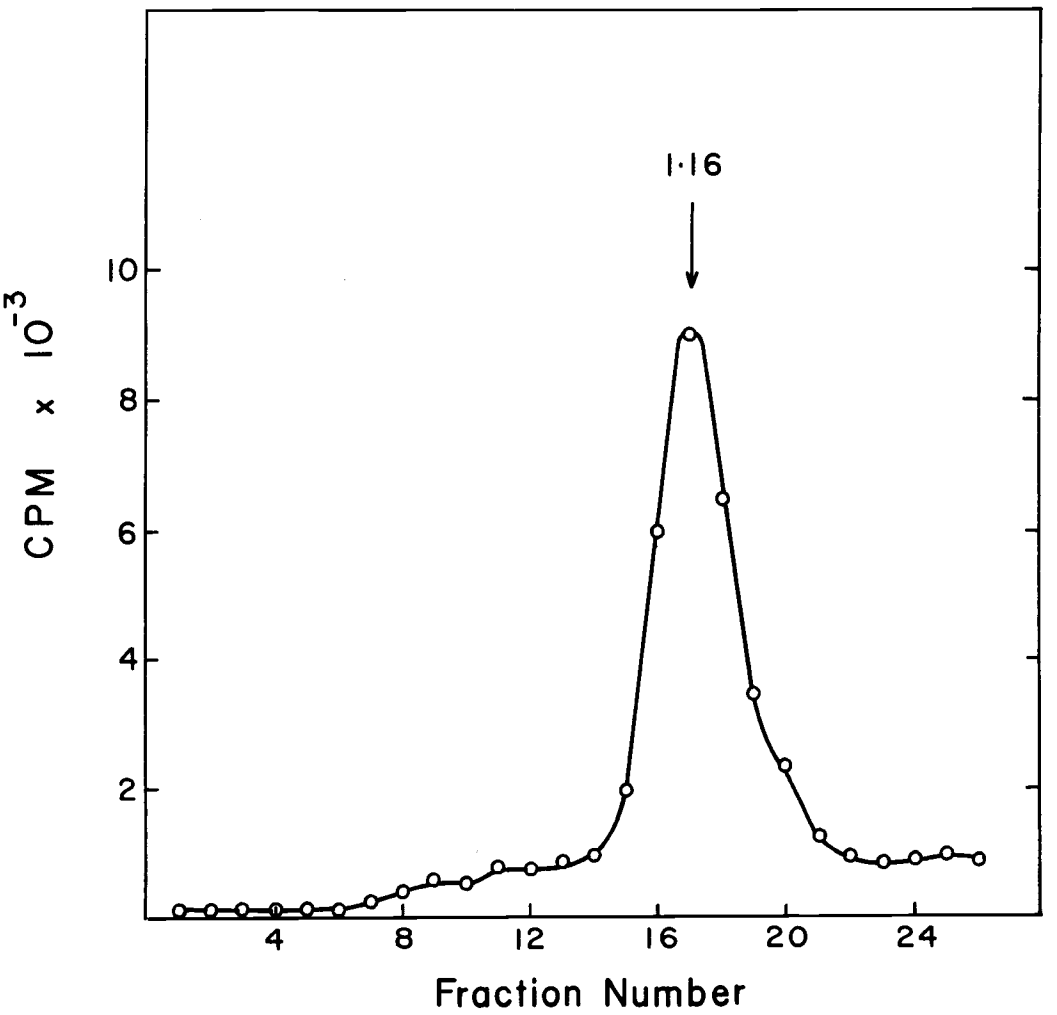


Figure 6. Isopycnic centrifugation in sucrose of ^{3}H-uridine labeled MC29 virus from frozen culture medium. A 60-40-20% w/w sucrose gradient in Buffer 1 was layered with a labeled virus particle suspension and centrifuged for two hours in a Spinco SW 25.1 rotor at 25,000 rpm. One ml fractions were collected, refractive indices read and the samples precipitated and counted as described in Figure 4.

or no activity was present throughout the remainder of the gradient.

The effect of glycerol on the purification of virus particles was again demonstrated using a frozen culture harvest of labeled MC29 virus. The virus was first banded at a density of 1.16 gm/cm^3 in a discontinuous sucrose gradient as previously explained. Virus from those fractions showing most activity were pelleted by centrifugation for 45 minutes at 45,000 rpm in a Spinco SW 50 rotor. After resuspending the pellet in PBS, the virus suspension was then divided into two samples. Part was layered onto an 85-35% v/v preformed glycerol gradient, and the remaining part layered onto a 60-20% w/w linear sucrose gradient. Centrifugation was for 14 hours at 50,000 rpm. Results of both gradients are shown in Figure 7. The difference between the sucrose and glycerol preparations is striking. Labeled virus that had been frozen, banded at a density of 1.23 gm/cm^3 in glycerol, whereas the same virus preparation banded in the density range of 1.17 gm/cm^3 in sucrose. It appears that frozen and thawed virus particles may have lost their outermost membrane or lipid components of the virus membrane when exposed to glycerol. This would result in viral "cores" which have been shown to have a density greater than that of intact virus (Bader et al., 1970).

Purification of frozen MC29 virus in glycerol results in what seems to be a partial fractionation of the particles without the use of detergents. This may be of value in determining the localization of

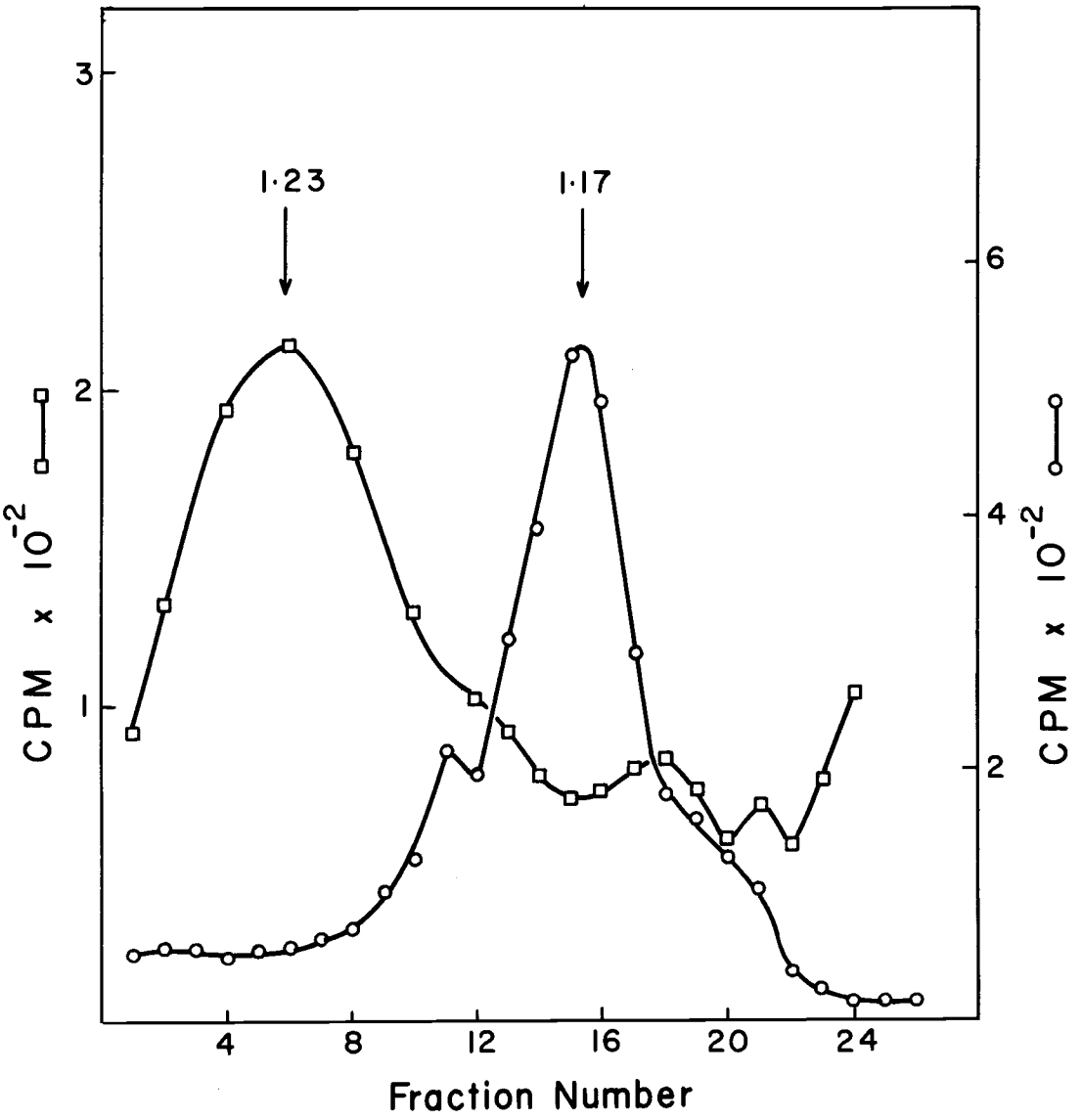


Figure 7. Isopycnic centrifugation in sucrose (O) and glycerol (□) of ^{3}H-uridine labeled MC29 virus from frozen culture medium. Preparation of the virus was presented in Methods. Approximately 0.2 ml fractions were collected and refractive indices read. Each fraction was then precipitated with 4.0 ml cold 10% TCA, plated and counted as described in Methods.

various enzyme activities within, or associated with, the virion. DNA polymerase activity, for example, has been found to be present in glycerol prepared "cores" of MC29 virus (Kiessling, 1971).

In contrast to the effect of glycerol, preparations in sucrose allow for the purification of intact MC29 virus.

Isopycnic Centrifugation of MC29 Virus and Associated DNA Polymerase

Approximately 1,000 ml of tissue culture medium from MC29 infected cells were centrifuged to pellet the virus particles. These pellets were resuspended in TNE and layered on an 80-25% w/w continuous sucrose gradient and centrifuged for 14.5 hours at 50,000 rpm in a Spinco SW 50 rotor. Protein was estimated from each fraction collected. Samples from each fraction were treated with Nonidet-P40, allowed to stand overnight and then assayed for DNA polymerase activity. Figure 8 shows that most enzyme activity occurred at a density of 1.17 gm/cm^3 in sucrose, characteristic for RNA tumor viruses. The protein concentration in the gradient fractions exhibited no correspondence to the virus enzyme activity. It is apparent that protein estimations under these circumstances do not reflect the amount of virus present. When identical conditions were repeated with noninfected control cell culture medium (Figure 9), the resultant protein profile was essentially identical to that found with the MC29 culture

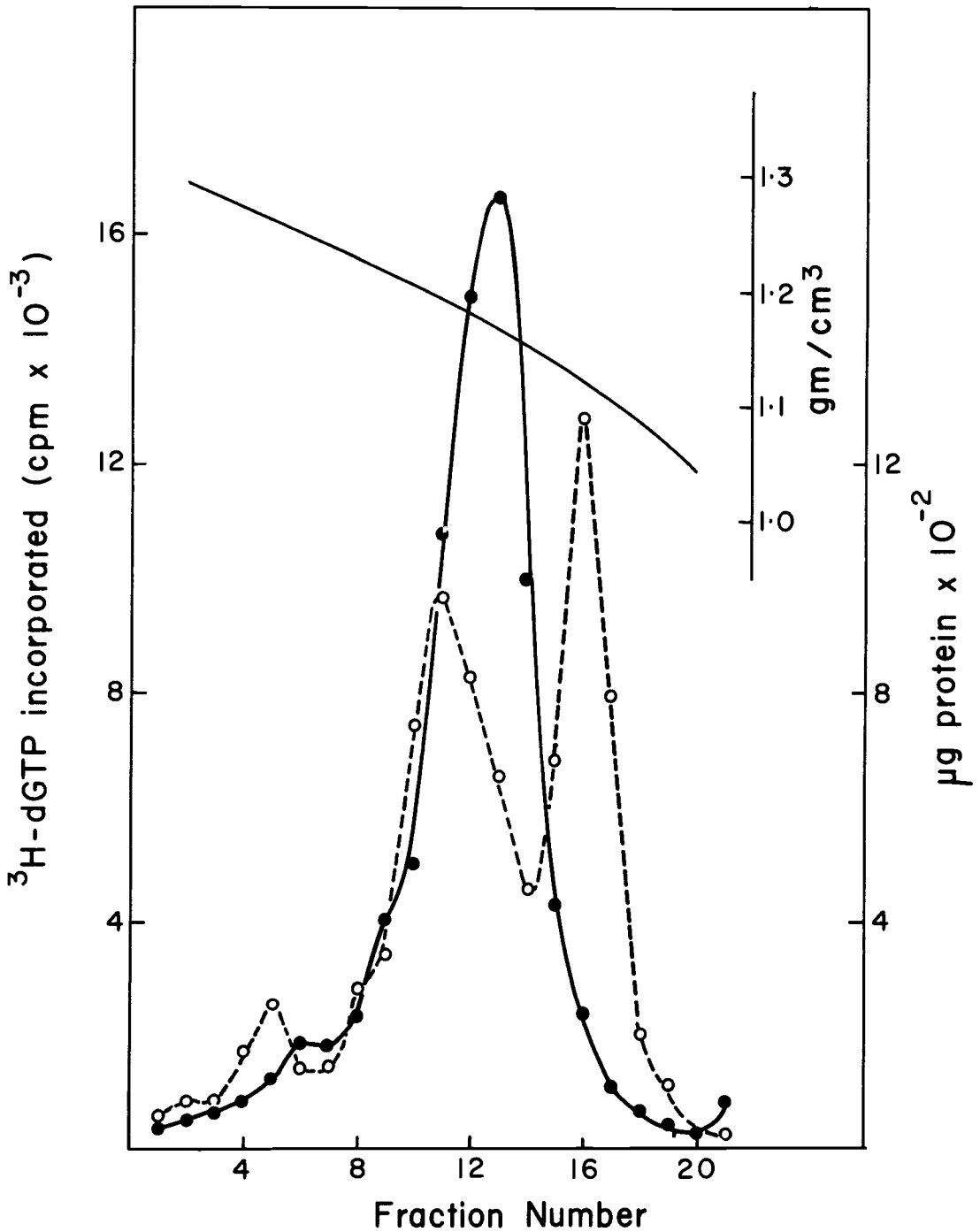


Figure 8. Isopycnic centrifugation of MC29 virus and associated DNA polymerase. Pelleted virus particles from approximately 1, 000 ml of culture medium were centrifuged to equilibrium in an 80-25% w/w continuous sucrose gradient. Protein (O) was estimated from each fraction as described in Methods. Samples were detergent treated and 0. 01 ml assayed for DNA polymerase activity (●) in the presence of 2 μg myeloblast cell DNA. One pmole of dGTP was equivalent to 2, 000 counts per minute.

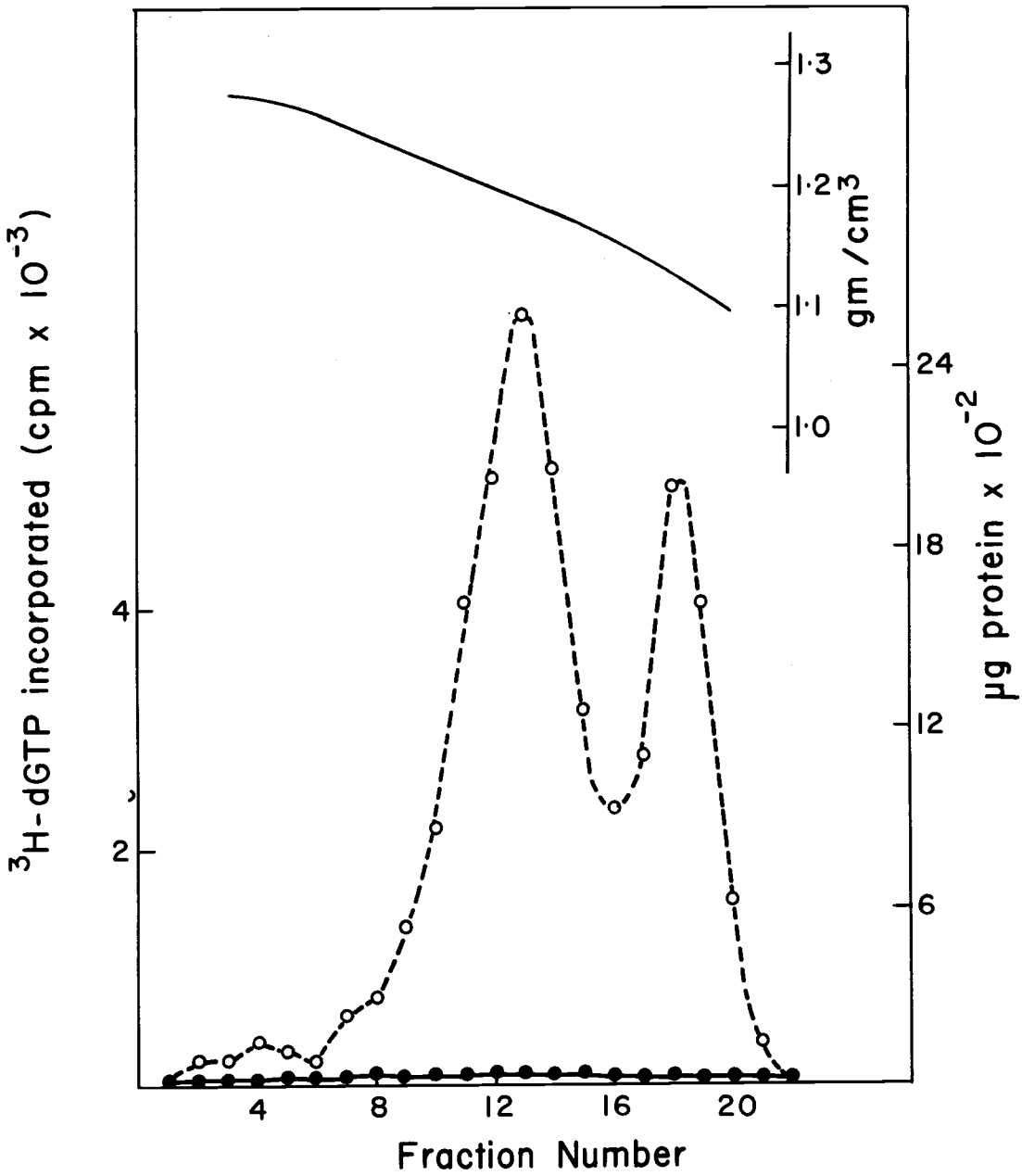


Figure 9. Isopycnic centrifugation of control culture supernatant. Conditions for the experiment were exactly as those explained in Figure 8.

medium; however, no enzyme activity was found. As previously shown (Figure 7), the density of ^{3}H-uridine labeled MC29 virus in sucrose was confirmed here (Figure 8) when DNA polymerase activity was used to detect the virus.

Primer Response and Effect of RNase and DNase

An MC29 viral enzyme preparation was made from a pool of fractions 10 through 15 of Figure 8. Control preparations were made from fractions of a similar density region (see Figure 9). An AMV enzyme, prepared by column chromatography (see Methods) was used as a comparison to the MC29 enzyme. Table 3 demonstrates the effect of added RNA and DNA to the enzyme assays. In some instances, nucleases were preincubated with the reaction mixture prior to addition of the enzyme. A stimulation of DNA product synthesized was found when either Qβ RNA or AMV RNA was added to the enzyme reaction. The marked depression of synthesis observed when the RNA template was preincubated with RNase substantiated the RNA dependency of the enzyme.

Added DNA likewise stimulated DNA production. As might be expected, preincubation of M. lysodeikticus DNA with DNase resulted in a 96% reduction in DNA formed by the MC29 enzyme and a 99% reduction by the AMV enzyme. However, there appeared to be either no effect or even a stimulation of DNA synthesis when DNase was first

Table 3. Primer response of AMV and MC29 DNA polymerase.

Assay Conditions	Enzyme activity (cpm) from: MC29	AMV	Control*
No added DNA	98	--	36
No added DNA + RNase	86	--	39
No added DNA + DNase	62	--	--
Qβ RNA (10 μg)	172	456	--
Qβ RNA + RNase	90	84	--
Qβ RNA + DNase	70	200	--
AMV RNA (20 μg)	526	3,691	43
AMV RNA + RNase	126	590	42
AMV RNA + DNase	3,047	5,202	--
+ Myeloblast cell DNA (2.2 μg)	10,805	100,652	615
dC:rG (0.5 μg)	10,077	129,235	66
dC:rG + DNase	18,048	108,756	--
dC (0.5 μg)	7,863	13,741	221
dC + DNase	14,243	119,877	--
M. lysodeikticus DNA (2.0 μg) (2,098)	949	43,938	63
M. lysodeikticus + RNase (--)	1,004	24,565	--
M. lysodeikticus + DNase (82)	--	590	--
* Myeloblast cell DNA - No enzyme			141
* Myeloblast cell DNA - 40 μg BSA			170
* BSA (40 μg)			56

Where indicated, 10 μg of RNase or DNase was preincubated in the reaction mixture for five minutes at 37°C before addition of the enzyme. MC29 reactions contained 0.02 ml of the detergent treated pool, while 0.01 ml of enzyme was added to the AMV reactions. Incubation was for 60 minutes. Control reactions (*) of 0.2 ml contained 0.02 ml of the control preparation and were incubated for four hours.

+ Kindly supplied by A. A. Kiessling.

preincubated with the synthetic homopolymers. It is interesting to note that even in the presence of DNase, a substantial amount of DNA product was found to be resistant to the nuclease.

Control assays contained twice the amount of protein (24 μg) and were incubated for four hours. The only noticeable activity observed was when myeloblast cell DNA was added. To check for the possibility of some form of endogenous activity associated with this DNA (e.g. microbial contamination), additional reactions were incubated for four hours with myeloblast cell DNA in the presence and absence of 40 μg of bovine serum albumin. The level of activity found in these controls suggests that the culture supernatant from noninfected cells does contain a trace of polymerase activity, possibly as a result of lysis of the uninfected control cells.

MC29 virus was purified from culture medium of infected cells by isopycnic centrifugation in sucrose. DNA polymerase activity was found to be associated with the purified virus particles and clearly distinguishable from control cell preparations. MC29 virus DNA polymerase activity was stimulated by the addition of either RNA or DNA to the reaction mixture; however, maximum enzyme activity was consistently obtained by the addition of either DNA or RNA:DNA homopolymers.

Contamination of CEC Cultures with a Latent Avian Leukosis Virus

A common occurrence of almost all laying flocks of chickens is the presence of a latent leukosis virus. Originally detected in tissue culture as imparting a resistance to Rous sarcoma virus infection (Rubin, 1960), this resistance inducing factor (RIF) was later observed to be extensively widespread in chick embryos.

Preliminary cell cultures in this laboratory were obtained from chick embryos (Jenks Hatchery, Tangent, Oregon) which contained substantial amounts of RIF. Direct counts of virus particles with the electron microscope and RIF interference tests were kindly done by A. J. Langlois of the Duke University Medical Center. A 72-hour uninfected control culture supernatant contained a maximum of 3.9×10^8 virus particles per ml. "This is a significant level since noninfected CEC fluids have counts of non-virus particles ranging from 0 in some instances to as high as $1\text{-}2 \times 10^7$ virus particles per ml, which are considered negative. Particles observed at this level are not virus but amorphous material somewhat resembling virus" (A. J. Langlois, 1971). When RIF negative cultures were exposed to this 72-hour culture medium and in three days challenged with RSV, approximately 95% of the RSV foci failed to appear.

To determine what effect RIF contributes to DNA polymerase assays, medium from a 17-day old RIF positive culture was used to infect

primary CEC cells. At daily intervals for one week, 50 ml of culture fluid were collected from five representative petri dishes. The culture medium was not changed during this time period. Untreated control cultures were prepared in the same manner. Enzyme preparations were made and in no instance could DNA polymerase activity be detected in either the RIF treated or untreated cell culture supernatants.

Titration of MC29 Virus on RIF Positive and RIF Negative CEC Cultures

The effect of RIF positive cells on MC29 focus formation was determined by titrations of stock MC29-419 (grown in RIF negative cultures) on both RIF positive and RIF negative cells. Secondary cultures were prepared from RIF positive and RIF negative cells cultured for six days, and the stock MC29 titered as usual. The use of RIF negative cultures resulted in a titer of 3.7×10^6 FFU/ml while the same virus, when titered on RIF positive cells, resulted in 2.6×10^5 FFU/ml. A 14-fold reduction in the titer on RIF positive cells was indicative of substantial interference.

Within 72 hours after culturing RIF positive cells, significant amounts of RIF were able to be detected by both electron microscopy and RSV interference. This latent leukosis virus was also shown to interfere with MC29 focus formation; however, DNA polymerase activity was absent in culture medium which contained substantial levels

of RIF. Enzyme activity could only be detected in culture medium from cells that had been infected with MC29 virus.

Forty-eight and Seventy-two Hour Culture Media

DNA polymerase activity found in culture medium from MC29 infected cells now appeared to offer an index of the amount of MC29 virus present. Figure 10 shows the results found when 55 ml of culture medium from cells infected for 48 and 72 hours were prepared and assayed under identical conditions. As expected, a significantly greater amount of activity could be detected from the enzyme derived from 72 hours culture medium than from the culture medium of 48 hour infected cells. This difference in enzyme activity, although greatly enhanced by the addition of M. lysodeikticus DNA to the reaction mixture, could still be detected in the endogenous reactions.

Culture fluids from noninfected control cultures were treated in an identical manner and assayed for enzyme activity with and without added DNA. No activity was observed (not shown).

Release of MC29 Virus and DNA Polymerase Activity from Infected CEC Cultures

Medium from MC29 infected and noninfected control CEC cultures were assayed for FFU and DNA polymerase activity at different times after infection to determine the relationship between the viral

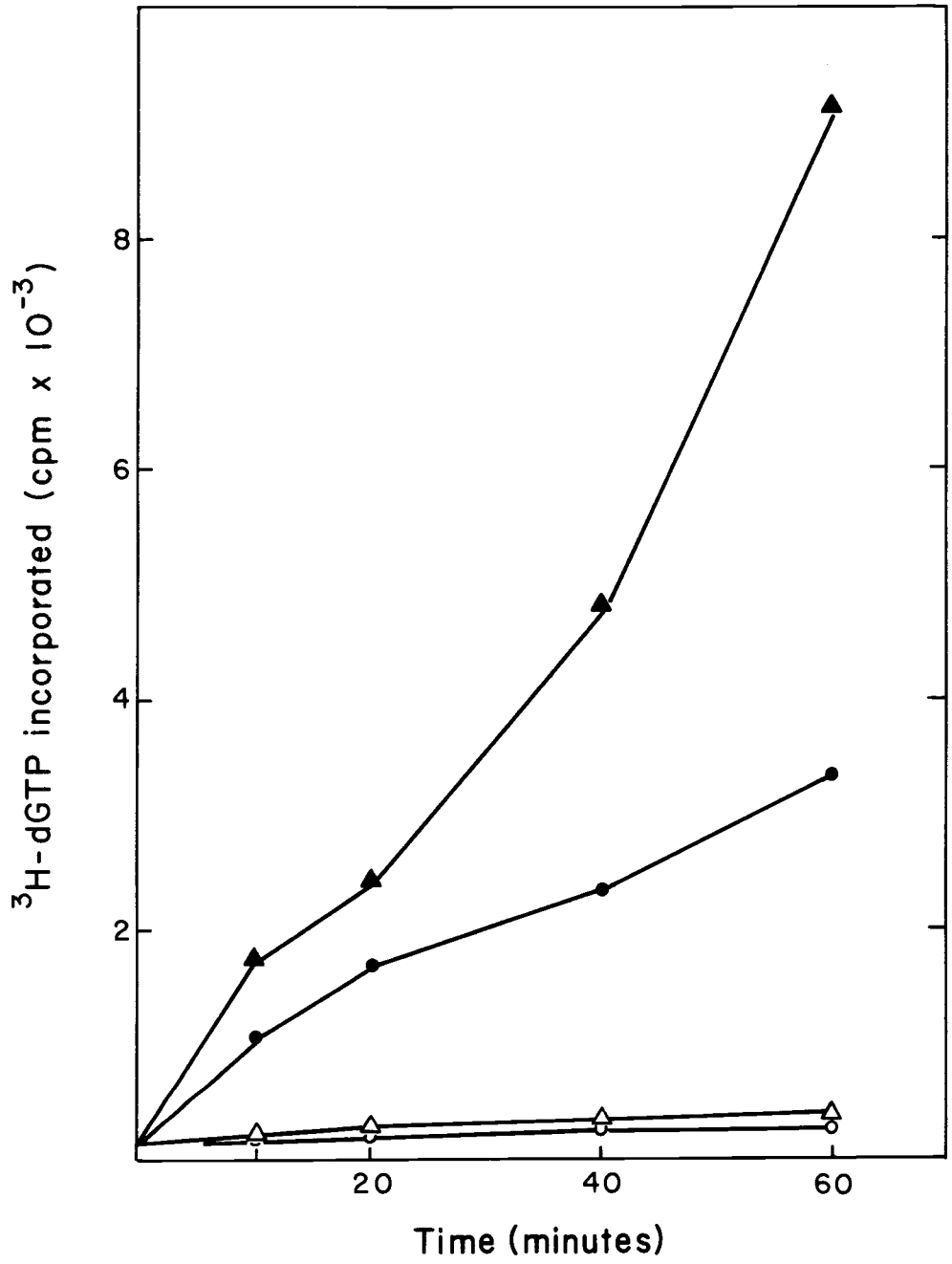


Figure 10. Kinetics of ^{3}H-deoxyguanosine triphosphate (dGTP) incorporation into DNA polymer with the enzyme from culture fluids of cells infected with MC29 virus for 48 (circles) and 72 (triangles) hours. The preparation of enzyme from culture fluids from infected cells was assayed in 0.05 ml reactions in the presence of M. lysodeikticus DNA (solid symbols) and in its absence (open symbols). For each determination enzyme preparations were made from 55 ml of culture fluid. DNA was added at 1.5 μg per assay, and one pmole of dGTP was equivalent to 2,000 counts per minute.

DNA polymerase and newly released MC29 virus particles. Figure 11 shows the results obtained when incomplete CEC monolayers were exposed to an 0.2 ml inoculum of MC29 virus for 30 minutes, after which fresh culture medium was added directly to each culture. The cells were not washed after the adsorption period nor was the culture medium changed for the 72 hours studied. Noninfected control cell culture medium (not shown) showed no FFU or enzyme activity. Both the FFU titrations and the DNA polymerase assays from the MC29 culture medium followed the same general trend, but the first appearance of progeny virus was difficult to estimate, apparently due to the presence of the original inoculum on each plate. Additional experiments (see below) have shown that approximations of the appearance of new virus particles can be confirmed by both methods of assay.

Stability of MC29 Virus and DNA Polymerase Activity Under Simulated Culture Conditions

It might be expected that virus particles would lose infectivity at a greater rate than enzyme activity. Since the virus growth curves were made over a 72 hour period without a change in culture medium, an attempt was made to determine the effect of simulated culture conditions on infectious virus and its associated DNA polymerase activity. Figure 12 represents the decay of MC29 virus infectivity and DNA polymerase when kept at 38.5°C in a humidified, CO_2 incubator.

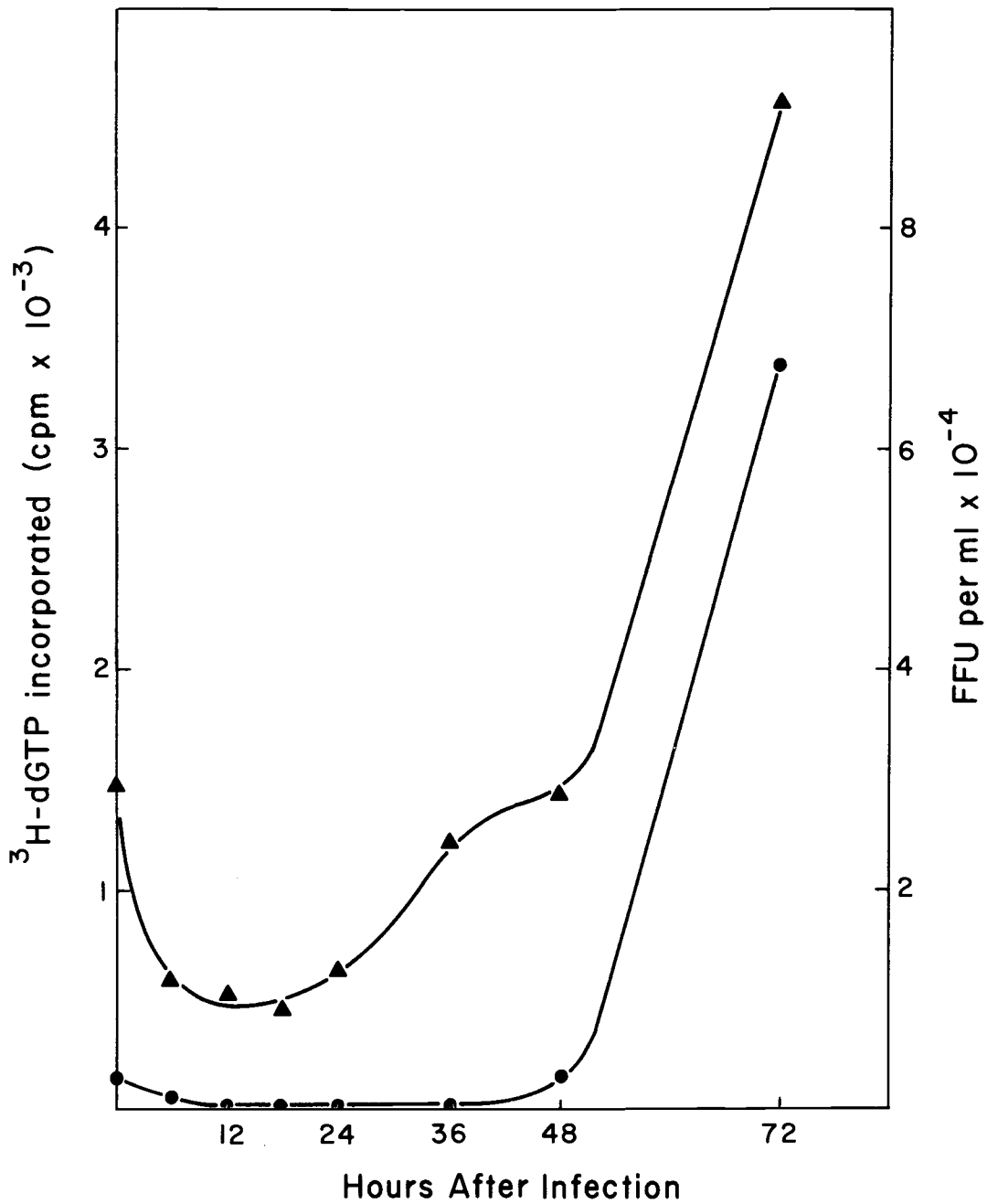
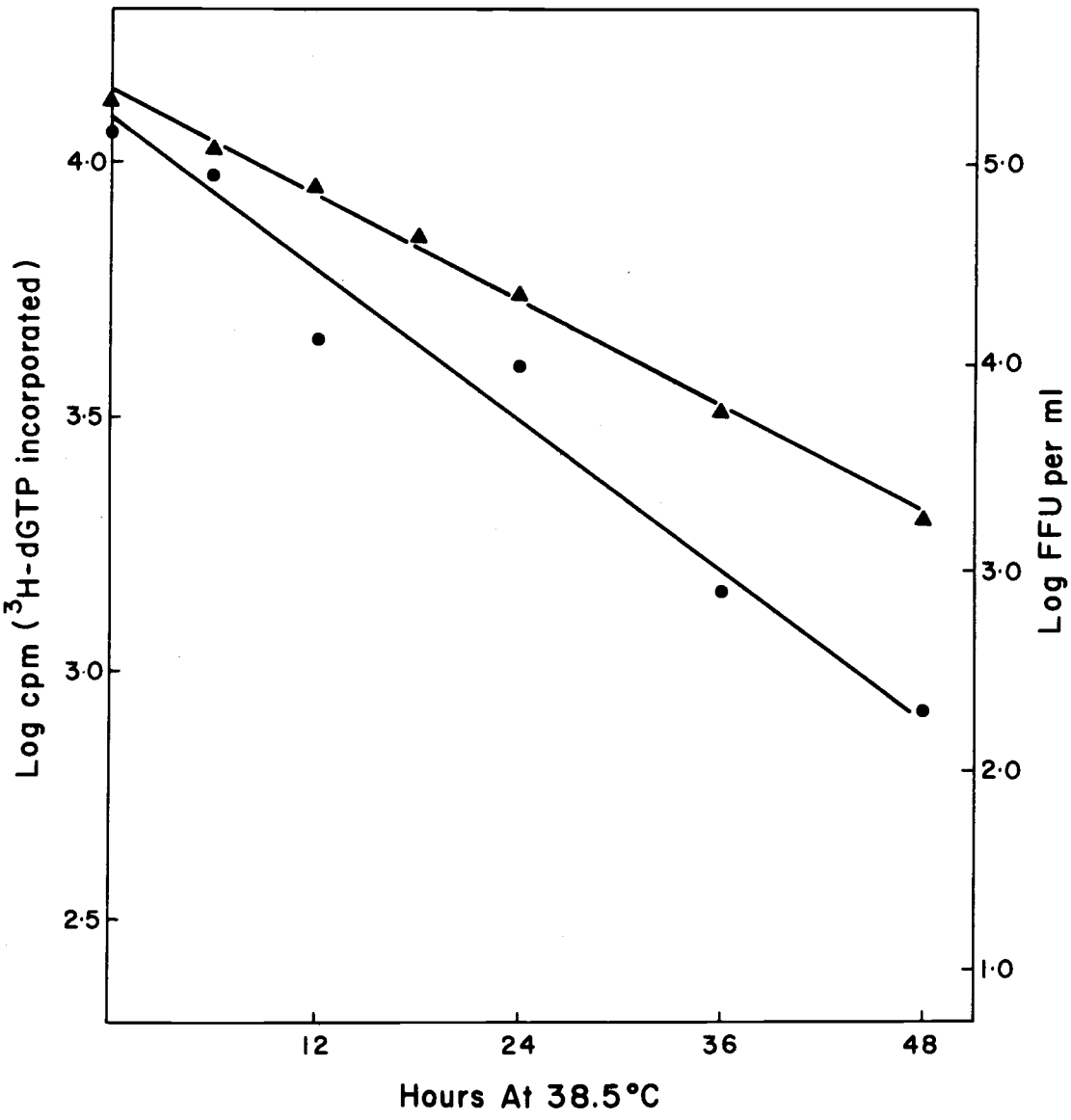


Figure 11. Appearance of infectious virus (●) and virus DNA polymerase (▲) from MC29 infected CEC fibroblasts. The cells were not washed after the 30 minute adsorption period nor was the culture medium changed for the 72 hours studied. Culture supernatants from six representative plates were used for each virus enzyme preparation (see Methods). A standard 0.1 ml reaction was used containing 2 μg of M. lysodeikticus DNA and 0.05 ml of the detergent treated virus suspension. One pmole of dGTP was equivalent to 2,000 counts per minute. Noninfected control cell culture medium (not shown) showed no FFU or enzyme activity.

Figure 12. Stability of infectivity of MC29 virus (●) and DNA polymerase activity (▲) under simulated culture conditions. 30 ml aliquots of a six hour harvest of infected cells were incubated in loosely sealed tubes at 38.5°C in a humidified, CO_2 incubator for the times indicated. Representative samples were taken for determination of focus forming units. Virus DNA polymerase activity is indicated by the incorporation of ^{3}H-dGTP (specific activity of 2,000 cpm/pmole) in 60 minutes in a standard 0.10 ml reaction containing 0.05 ml enzyme and 2 μg of M. lysodeikticus DNA.


Log cpm (^{3}H-dGTP incorporated)
4·0
3·5
3·0
2·5
5·0
4·0
3·0
2·0
1·0
Log FFU per ml
12
24
36
48
Hours At 38.5°C

The specific reaction rates (k) calculated from the slopes of each line gave values of 6.6×10^{-4} min^{-1} for the DNA polymerase and 2.0×10^{-3} min^{-1} for infectious MC29 virus. The calculated half-life of the virus infectivity was 5.8 hours, while that of the polymerase was 17.6 hours.

Template Free Viral DNA Polymerase

DNA polymerase from MC29 virus normally obtained from detergent treated virus pellets usually exhibited endogenous activity. To obtain an enzyme that was free from all endogenous template activity, the methods of Beaudreau (unpublished data) and Kacian et al. (1971) were used to prepare DNA polymerase from both MC29 and AMV.

Cesium Chloride Preparation of MC29 DNA Polymerase

Fractions obtained from the centrifugation of detergent treated virus particles in cesium chloride (1.4 gm/cm^3) were assayed for enzyme activity with and without DNA template. The results from a typical preparation are shown in Figure 13. Enzyme activity was maximum about a density of 1.27 gm/cm^3 while the viral nucleic acids were pelleted to the bottom of the centrifuge tube. Without nucleic acid template added to the assay mixture the enzyme had no activity. Those enzyme fractions showing most activity were pooled and dialyzed one-half hour against Buffer 0 made 0.065 M dithiothreitol. MC29

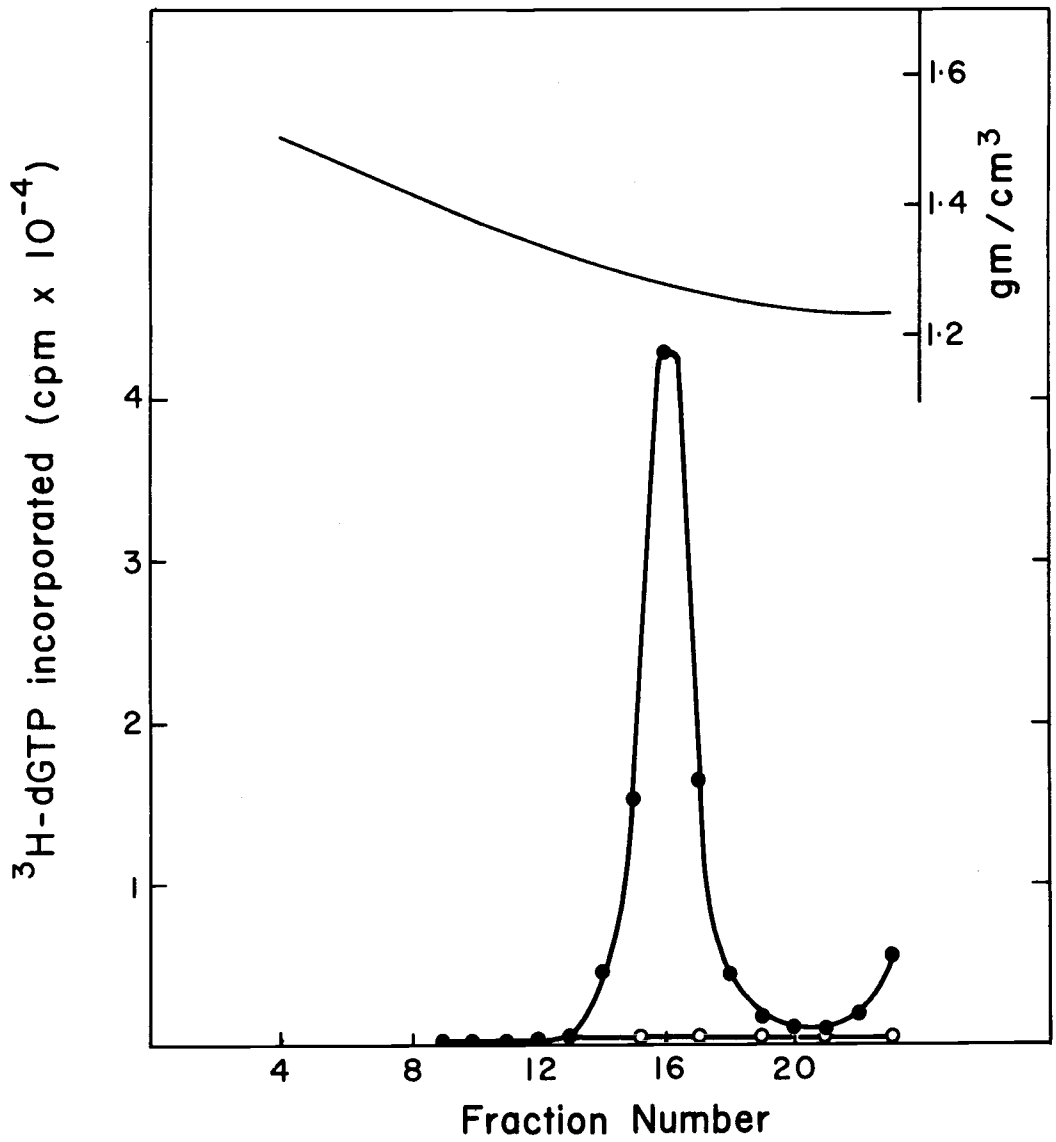


Figure 13. Cesium chloride preparation of MC29 DNA polymerase. Detergent treated virus was centrifuged at 50, 000 rpm for 30 hours as described in Methods. Each fraction was assayed for enzyme activity in the presence (•) and absence (o) of 0.4 μg of myeloblast cell DNA. One pmole of dGTP was equal to 3, 000 counts per minute.

enzyme prepared in this manner, together with a similarly prepared AMV enzyme, was used in sedimentation velocity studies that are described later.

Detection of DNA Associated with MC29 Virus

The pellet fraction from a cesium chloride preparation of the MC29 virus enzyme contained the viral nucleic acids. This was alcohol precipitated and added to cesium chloride as described in Methods. After centrifugation, the collected fractions were assayed for priming activity using virus DNA polymerase which showed no endogenous activity. Figure 14 shows the results that were obtained. The observed maximum activity suggests the presence of DNA at a density of 1.74 gm/cm^3 in cesium chloride. This is not unlike that found with AMV preparations (Beaudreau, unpublished data).

When fractions from a similar preparation were treated with DNase prior to the enzyme assay the results in Figure 15 were obtained. No synthesized DNA product was found in the DNase treated samples, while the untreated assays again showed maximum activity at 1.74 gm/cm^3. Whether the action of the nuclease was on the DNA template or the synthesized product is not known; however, similar results were previously obtained only when DNA served as a template (Table 3).

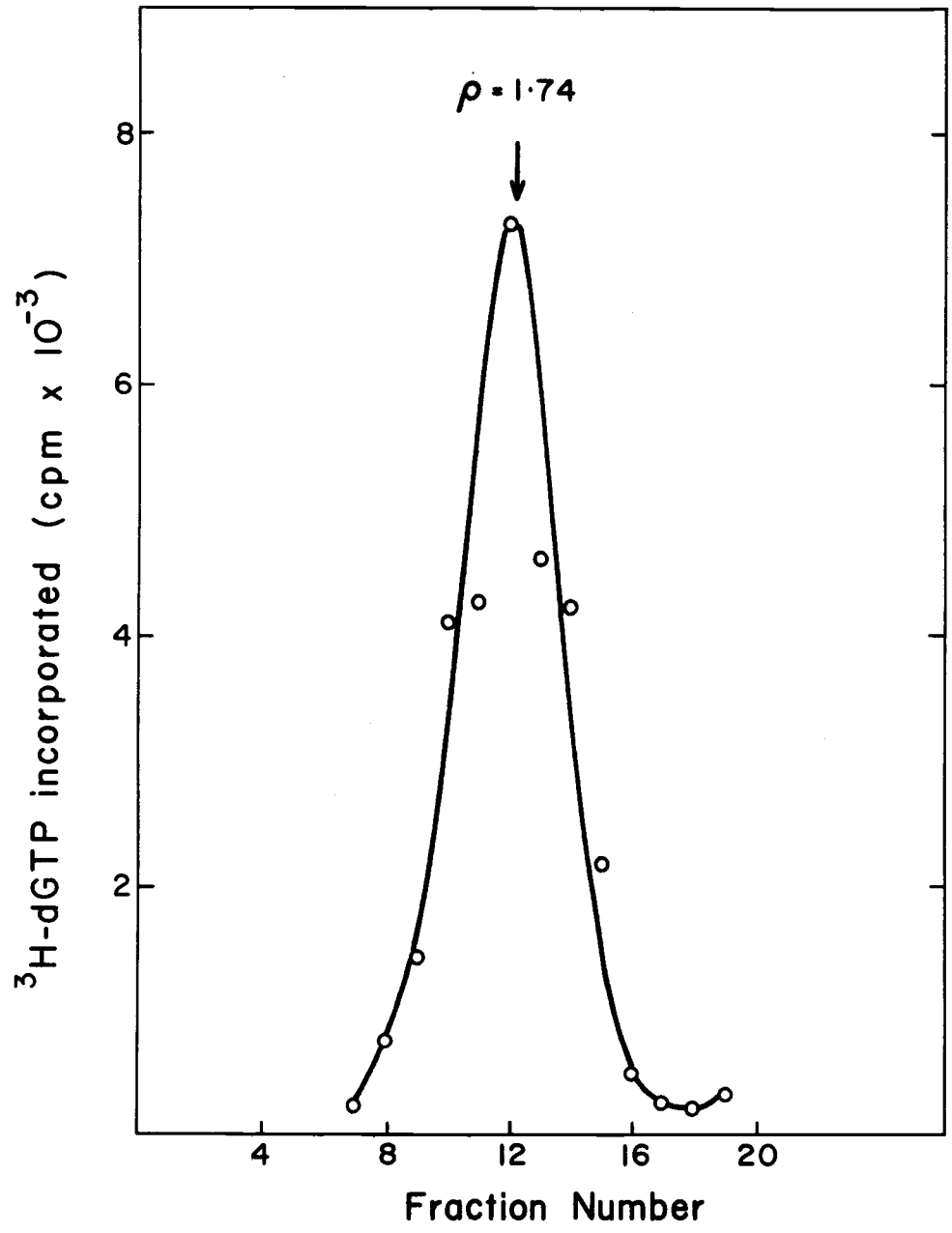


Figure 14. Buoyant density in cesium chloride of DNA from MC29 tumor virus. The pellet fraction from a cesium chloride preparation of DNA polymerase was alcohol precipitated and centrifuged as described in Methods. Each fraction was ethanol precipitated and assayed for template activity with viral DNA polymerase which was free of endogenous template. Two-tenths ml reaction volumes were used. One pmole of dGTP was equivalent to 2, 000 counts per minute.

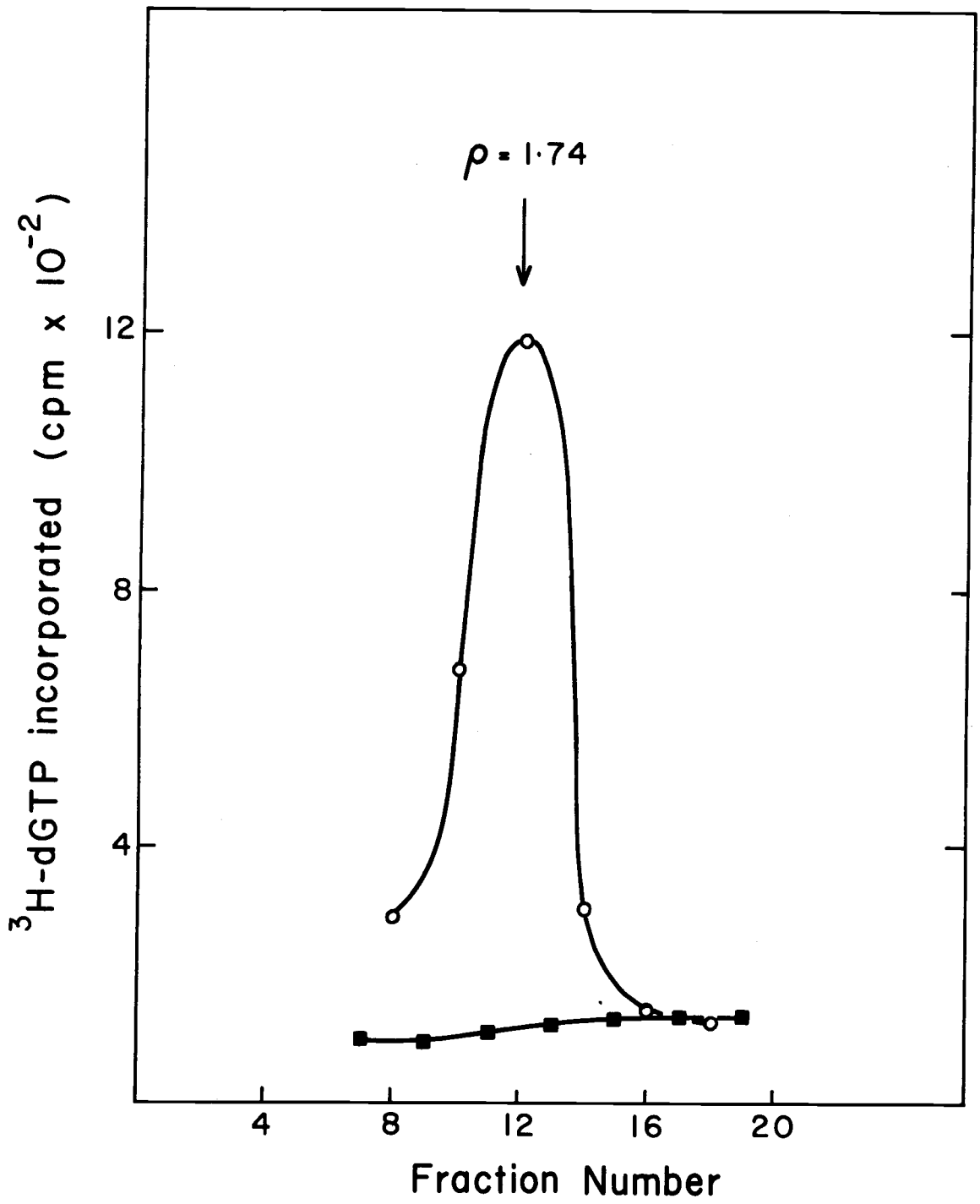


Figure 15. Effect of DNase on the template activity of MC29 viral DNA. Procedures were the same as those reported in Figure 14. Reaction assays were preincubated for ten minutes at 37°C in the presence (■) and absence (o) of 20 μg DNase prior to addition of the enzyme.

Purification of AMV DNA Polymerase by Column Chromatography

As an alternative to the cesium chloride method of purifying the viral DNA polymerase, the procedures of Kacian et al. (1971) were used to purify the AMV DNA polymerase. Detergent treated AMV particles were applied to a DEAE-Cellulose column and the DNA polymerase eluted as described in Methods. Figure 16 shows the results obtained when each fraction was assayed for enzyme activity using M. lysodeikticus DNA as primer. When fractions 12 through 24 were pooled, applied to a CM-Sephadex column and eluted, a sharp profile of both protein and enzyme activity resulted (Figure 17). Assays without added DNA template showed no activity. Enzyme prepared in this fashion has lost no activity for four months when stored in 50% glycerol at -20°C.

Estimation of Sedimentation Rate of Viral DNA Polymerase

It has been previously reported that the sedimentation coefficient for purified virus DNA polymerase is approximately 6S (Duesberg et al., 1971; Kacian et al., 1971). A higher sedimentation coefficient (8S), found with a partially purified enzyme, reportedly was diminished to 6S when the enzyme was preincubated with RNase (Duesberg et al., 1971). Apparently, RNA associated with the enzyme contributed to the high sedimentation rate. Figures 18 and 19 show the results of sucrose

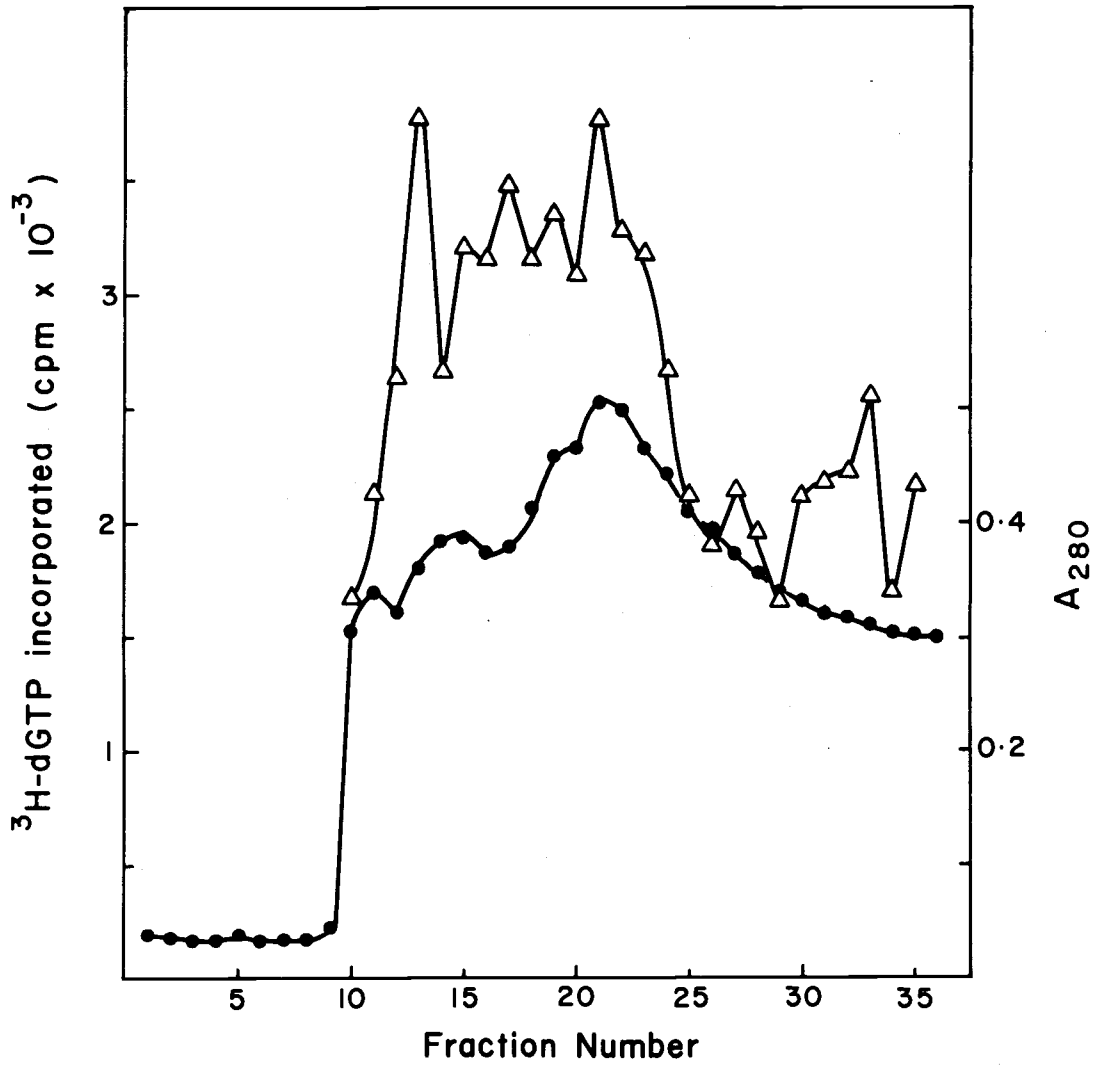


Figure 16. DEAE-Cellulose column chromatography of AMV DNA polymerase. AMV polymerase was prepared and chromatographed as described in Methods. Absorbancy at 280 nm (●) was determined for each fraction. Samples of 0.01 ml from each fraction were assayed for DNA polymerase activity (△) in a standard enzyme reaction using 2.0 μg M. lysodeikticus DNA as primer. Specific activity of ^{3}H-dGTP: 880 counts per pmole.

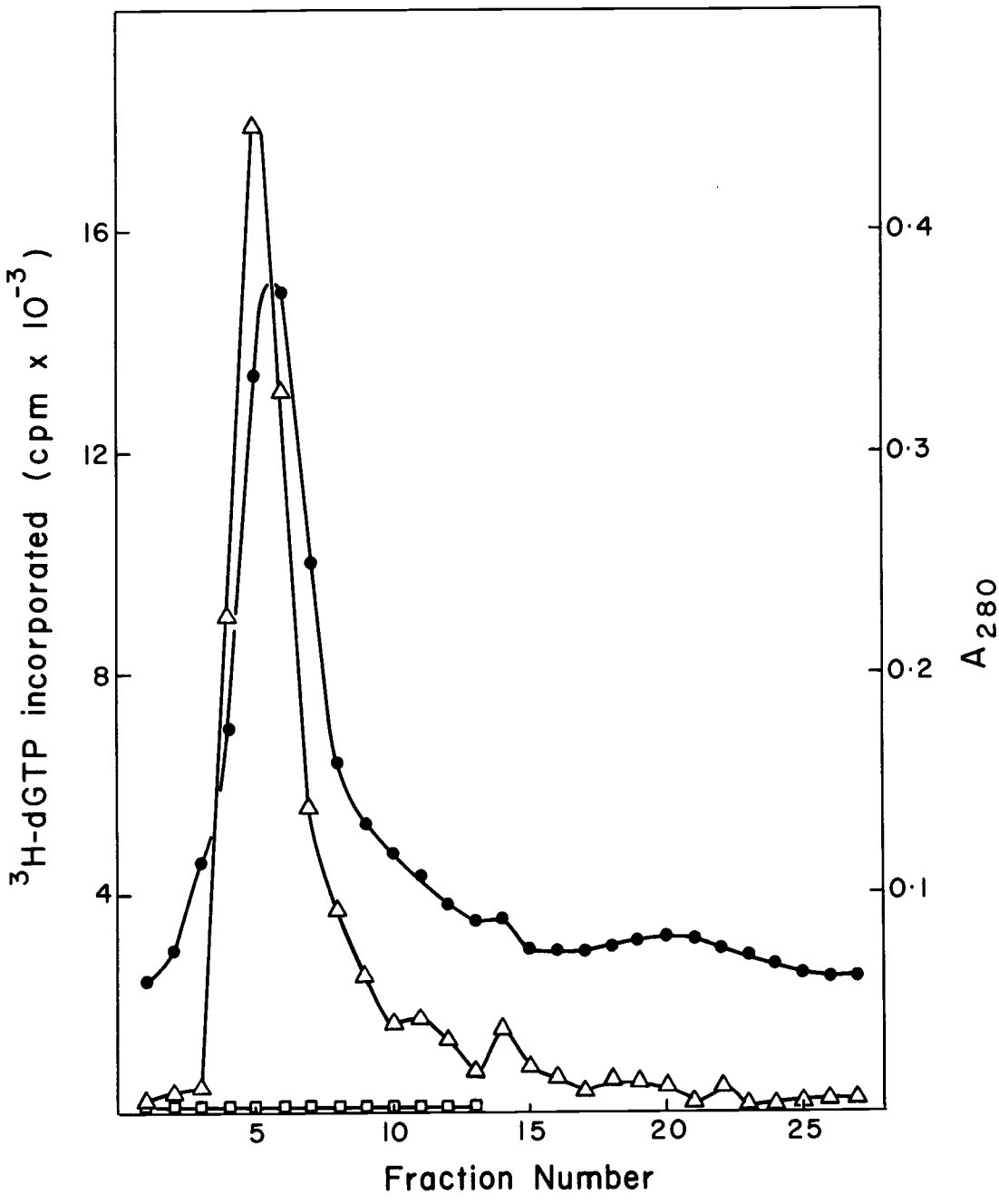


Figure 17. CM-Sephadex column chromatography of AMV DNA polymerase. Fractions 12 through 24 of Figure 16 were pooled and chromatographed as described in Methods. Absorbancy at 280 nm (●) was determined for each fraction, assay conditions for DNA polymerase activity (△) were the same as those of Figure 16. When assayed without added DNA primer (□), no activity was found.

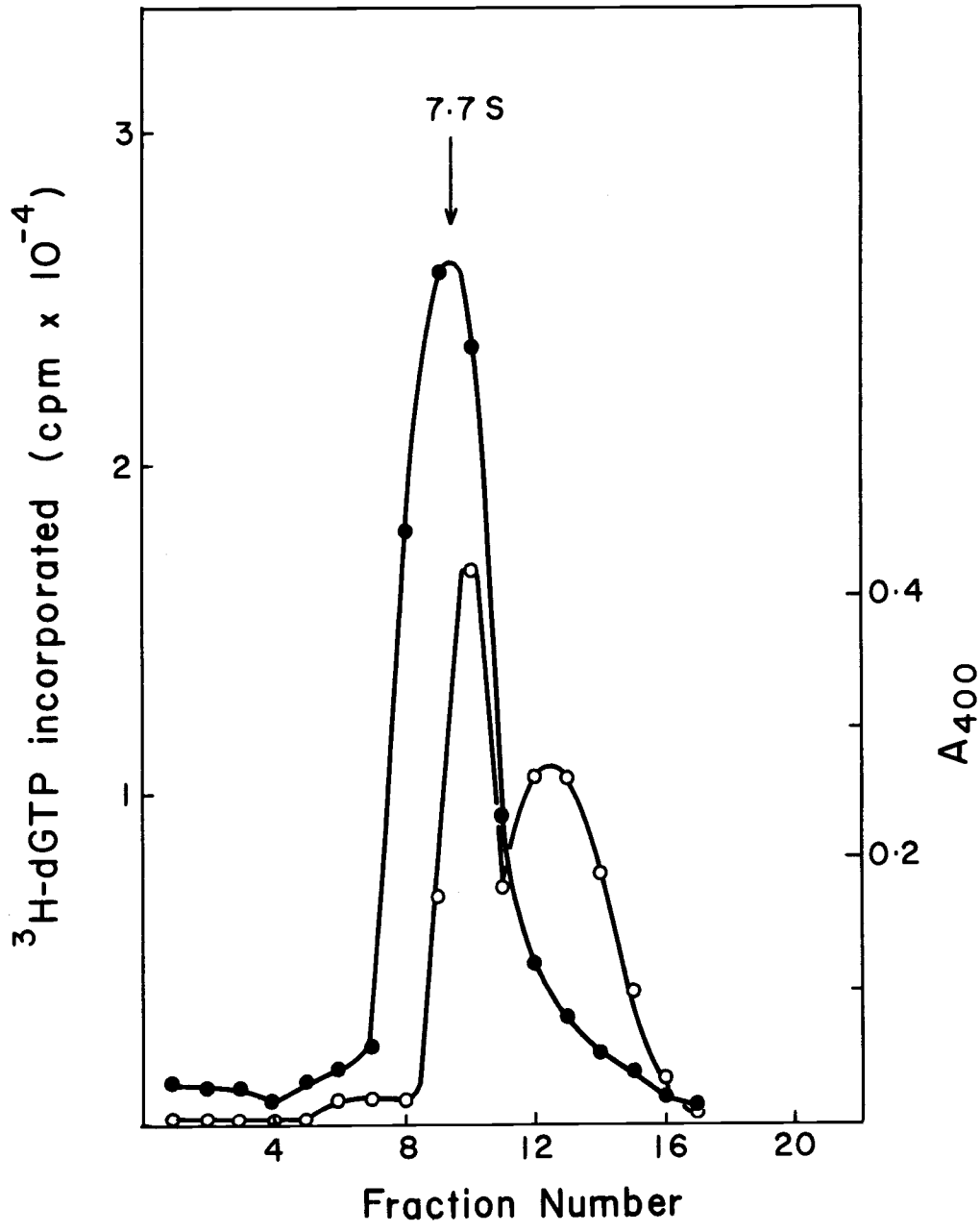


Figure 18. Sedimentation analysis of the MC29 DNA polymerase prepared by cesium chloride fractionation. Centrifugation was performed as described in Methods with gamma-globulin (7.2S) and hemoglobin (4.5S) protein standards. Protein was estimated by precipitation of a sample from each fraction with 3% trichloroacetic acid and measuring the turbidity at 400 nm (O). Each fraction was assayed for enzyme activity (●) using the synthetic homopolymer dC as template. Specific activity of ^{3}H-dGTP: 2,000 counts per minute per pmole.

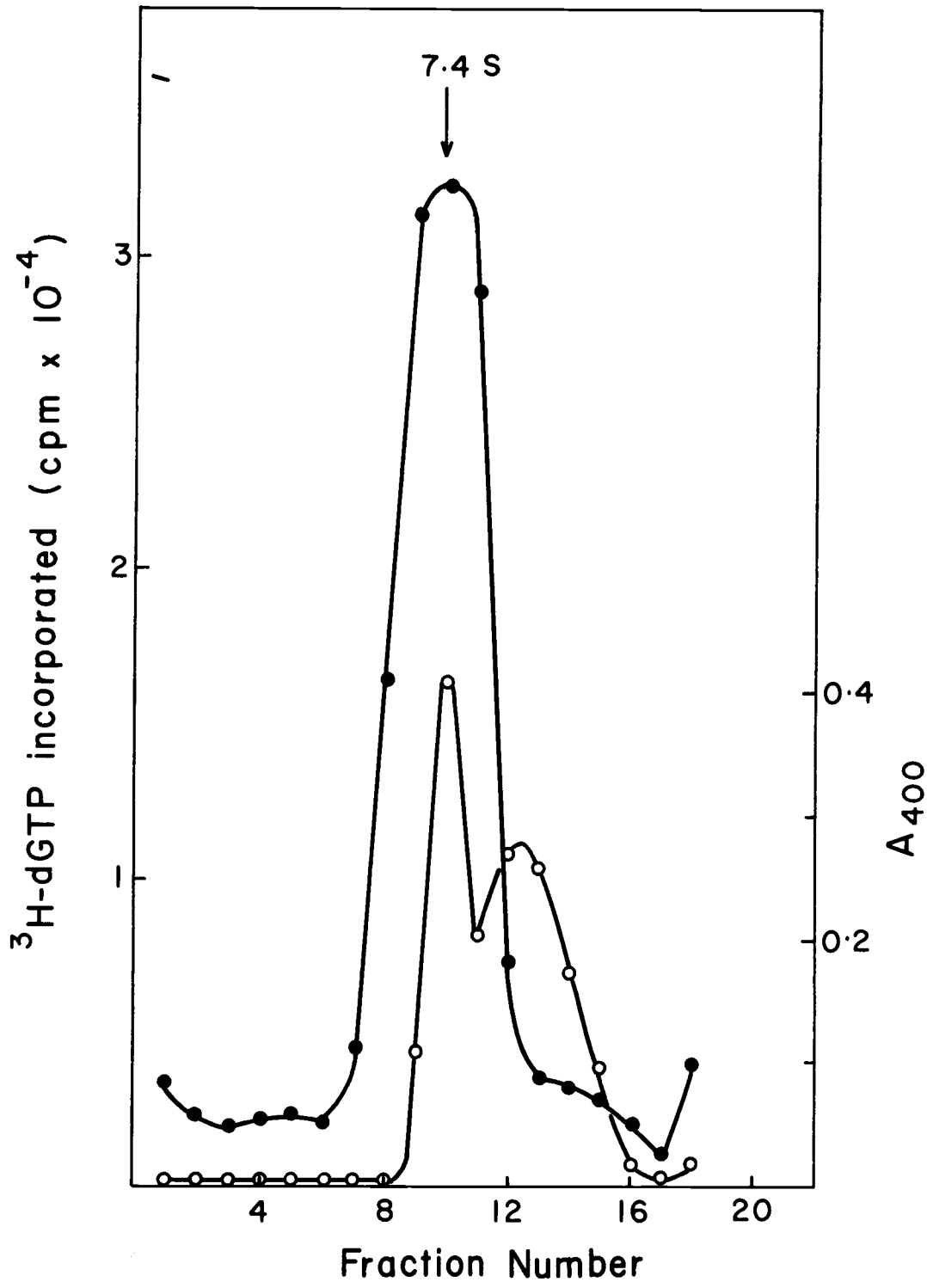


Figure 19. Sedimentation analysis of the AMV DNA polymerase prepared by cesium chloride fractionation. Procedures used were those described in Figure 18.

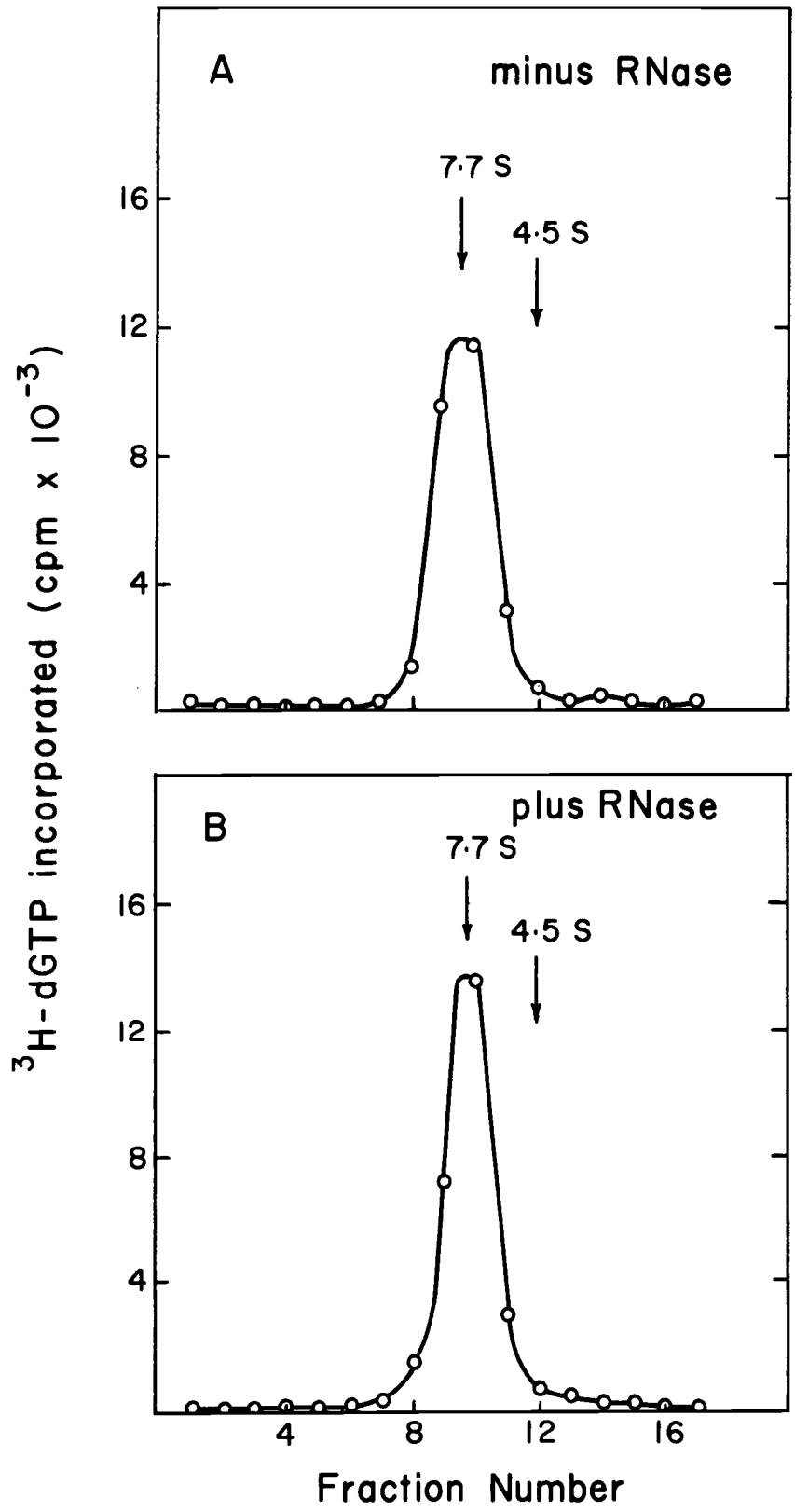
gradient centrifugation of both the MC29 and AMV DNA polymerases. Both enzymes were prepared by the cesium chloride procedure mentioned above. The MC29 and AMV enzymes sedimented at a greater rate than the 7.2S gamma-globulin marker (7.7S and 7.4S, respectively). However, unlike that reported, preincubation of the enzyme with RNase resulted in no change in its sedimentation rate (Figure 20).

A sample of AMV enzyme, purified by column chromatography, was also analyzed by sucrose gradient centrifugation to compare the two procedures of DNA polymerase purification. In this experiment human cytochrome c (2.1S) was substituted for the human hemoglobin (4.5S) marker previously used. Figure 21 shows that the AMV polymerase sedimented at the same rate as was demonstrated in Figure 19 (7.4S). In all preparations there was no significant deviation from these values regardless of the method used to purify the enzyme or the template used to detect the enzyme in velocity gradients. Molecular weight estimations of the MC29 and AMV DNA polymerase correspond to approximately 180,000 and 175,000 daltons, respectively.

DNA Polymerase in Homogenates of MC29 Infected Cells and Noninfected Cells

By necessity, the MC29 viral DNA polymerase must at some time be present in infected cells. To demonstrate that this viral enzyme was present in infected cells, studies were initiated to determine

Figure 20. Effect of RNase on the sedimentation rate of DNA polymerase from MC29 virus. Centrifugation was performed as described in Methods with gamma-globulin and hemoglobin (arrow) protein markers. Prior to centrifugation enzyme samples (0.2 ml) were incubated at 37°C for five minutes in the presence (B) and absence (A) of one μg RNase. Each fraction was assayed for enzyme activity as described in Methods in the presence of 0.2 μg myeloblast cell DNA. Specific activity of ^{3}H-dGTP: 880 counts per minute per picomole.


A
minus RNase
7·7 S
4·5 S
B
plus RNase
7·7 S
4·5 S
16
12
8
4
4
8
12
16
^{3}H-dGTP incorporated (cpm x 10^{-3})
Fraction Number

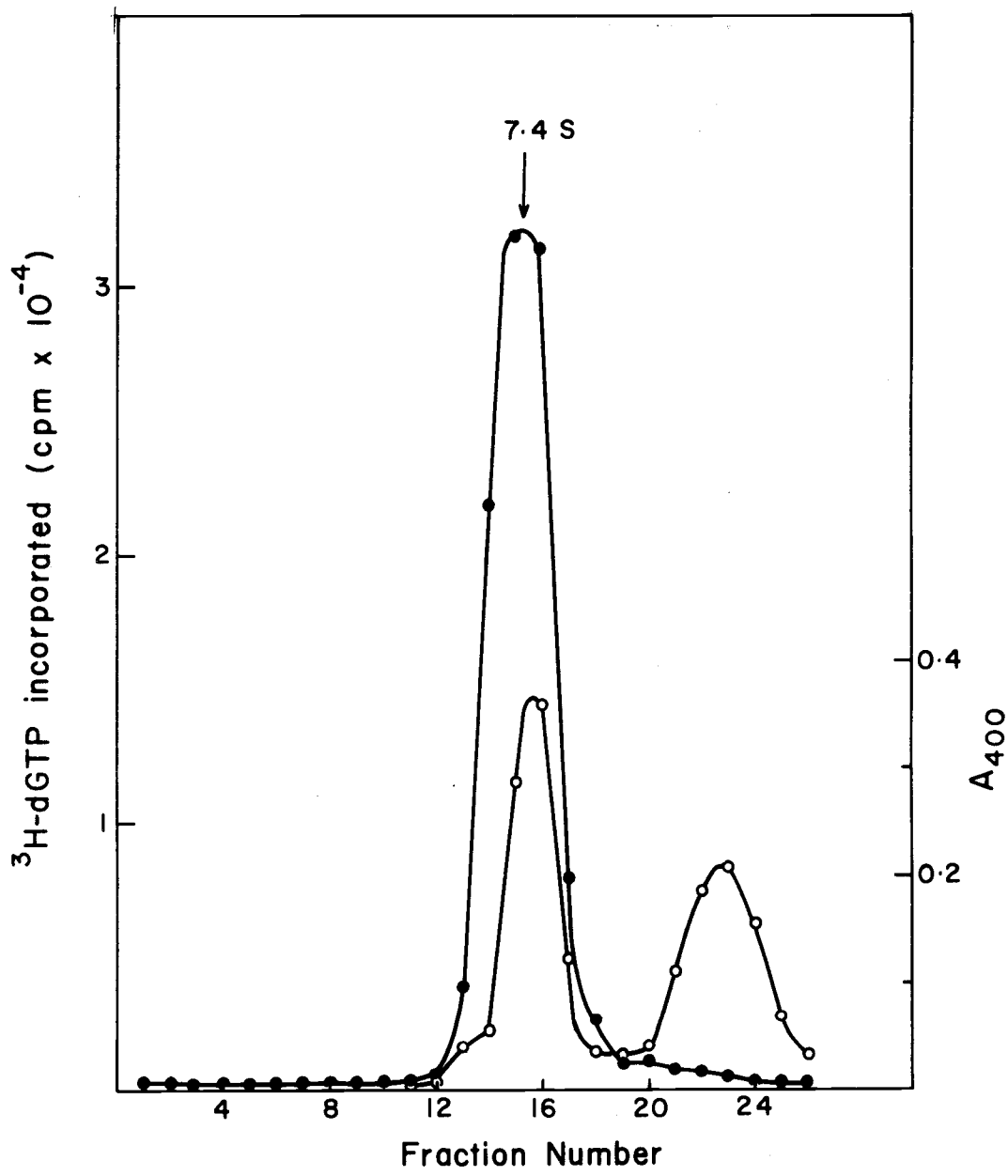


Figure 21. Sedimentation analysis of DNA polymerase from AMV prepared by column chromatography. A 0.04 ml sample of DNA polymerase with protein markers of gamma-globulin (7.2S) and cytochrome c (2.1S) were centrifuged as described in Methods. Protein (o) was estimated as described in Figure 18. Each fraction was assayed for enzyme activity (•) in the presence of 0.4 μg myeloblast cell DNA. Specific activity of ^{3}H-dGTP: 2,000 counts per minute per pmole.

whether the virus DNA polymerase could be distinguished from normal cellular DNA polymerase activity.

Primer Response

Studies of the DNA polymerase activity in homogenates from noninfected cells showed that the enzyme was present. DNA polymerase in the noninfected cells was stimulated slightly by DNA from salmon sperm, Cl. perfringens, B. subtilis, and calf thymus (Table 4). DNA from M. lysodeikticus and from avian myeloblastosis virus provided the greatest amount of DNA synthesis. No stimulation of enzyme activity was obtained with either of the hybrids from synthetic homopolymers (dC:rG and rI:rC).

A comparison of DNA synthesis by polymerase from infected cells is made in the same table. The most important result demonstrated that, in contrast to the control cell preparations, the dC:rG hybrid gave a three-fold stimulation of DNA synthesis. The template activity of the synthetic DNA:RNA hybrid implied the presence of a second DNA polymerase activity appearing in the cell after infection with MC29 tumor virus and provided a method of assay for this new DNA polymerase activity in the presence of polymerase enzyme normally found in cells.

It seems unlikely that the DNA:RNA hybrid functioned by stimulating endogenous DNA synthesis since the ^{3}H-TMP did not, and would

Table 4. DNA polymerase from MC29 virus, infected cells and from control cells.

DNA Source	Enzyme activity (cpm) from: Control Cells ^{3}H-dGMP	Control Cells ^{3}H-TMP	Infected Cells ^{3}H-dGMP	Infected Cells ^{3}H-TMP	Virus ^{3}H-dGMP	Virus ^{3}H-TMP
dC:rG	70	56	1,837	212	10,172	153
M. lysodeikticus	750	90	3,921	1,698	4,396	2,926
Avian myeloblastosis virus	600	450	4,670	3,360	12,200	6,303
Salmon sperm	94	91	2,401	1,573	2,572	2,227
Cl. perfringens	236	247	2,613	2,767	5,356	4,019
B. subtilis	169	111	1,914	1,481	856	553
Calf thymus	166	102	2,127	1,777	5,427	4,731
Human leukemic lymphocytes	63	120	333	209	467	329
Human placenta	66	108	979	921	1,264	735
rI:rC	96	63	336	283	537	218
None	88	65	529	438	311	652

Chick embryo cell cultures were infected with strain MC29 virus. The cells were collected for enzyme preparation five days after infection and corresponding noninfected control cells were collected at the same time. Enzyme activity was measured by the incorporation of ^{3}H-dGMP and ^{3}H-TMP into acid insoluble radioactive material. The enzyme fraction was added at a level of 50 μg of protein for each assay both in the infected and control preparations. ^{3}H-dGTP was added to the reaction at a specific activity of 2,000 cpm/pmole dGTP and ^{3}H-TTP was added at a specific activity of 2,600 cpm/pmole TTP. DNA was added at 2 μg/0.1 ml assay volume. Reaction mixtures were incubated at 37°C for 60 minutes.

not be expected to, incorporate into DNA product when dC:rG was used as template. In addition, it was apparent that DNA polymerase activity in the infected cell preparation greatly exceeded the activity found in the noninfected cell preparations with all effective DNA primers. Preliminary results suggests that the increase in enzyme activity is of both viral and cellular origin.

The virus DNA polymerase also utilized the dC:rG template and incorporated only ^{3}H-dGTP into polymer product. Thus the appearance of the new polymerase activity found associated with cell extract was identified as an activity similar to that found in the MC29 virus.

Enzyme Saturation

A pool of two cultures that were infected with MC29 virus for five days was prepared for DNA polymerase assay as outlined in Methods. Figure 22 shows enzyme saturation data when varying concentrations of cell extract were assayed in a standard 0.10 ml reaction containing 2 μg dC:rG. Control cell homogenates, when assayed under identical conditions, exhibited no DNA polymerase activity. When the amount of MC29 cellular protein added to the reaction mixture was in excess of 50 μg there was no further stimulation in the formation of product. The decrease in product formed when excess protein was added may be a reflection of the presence of substantial amounts

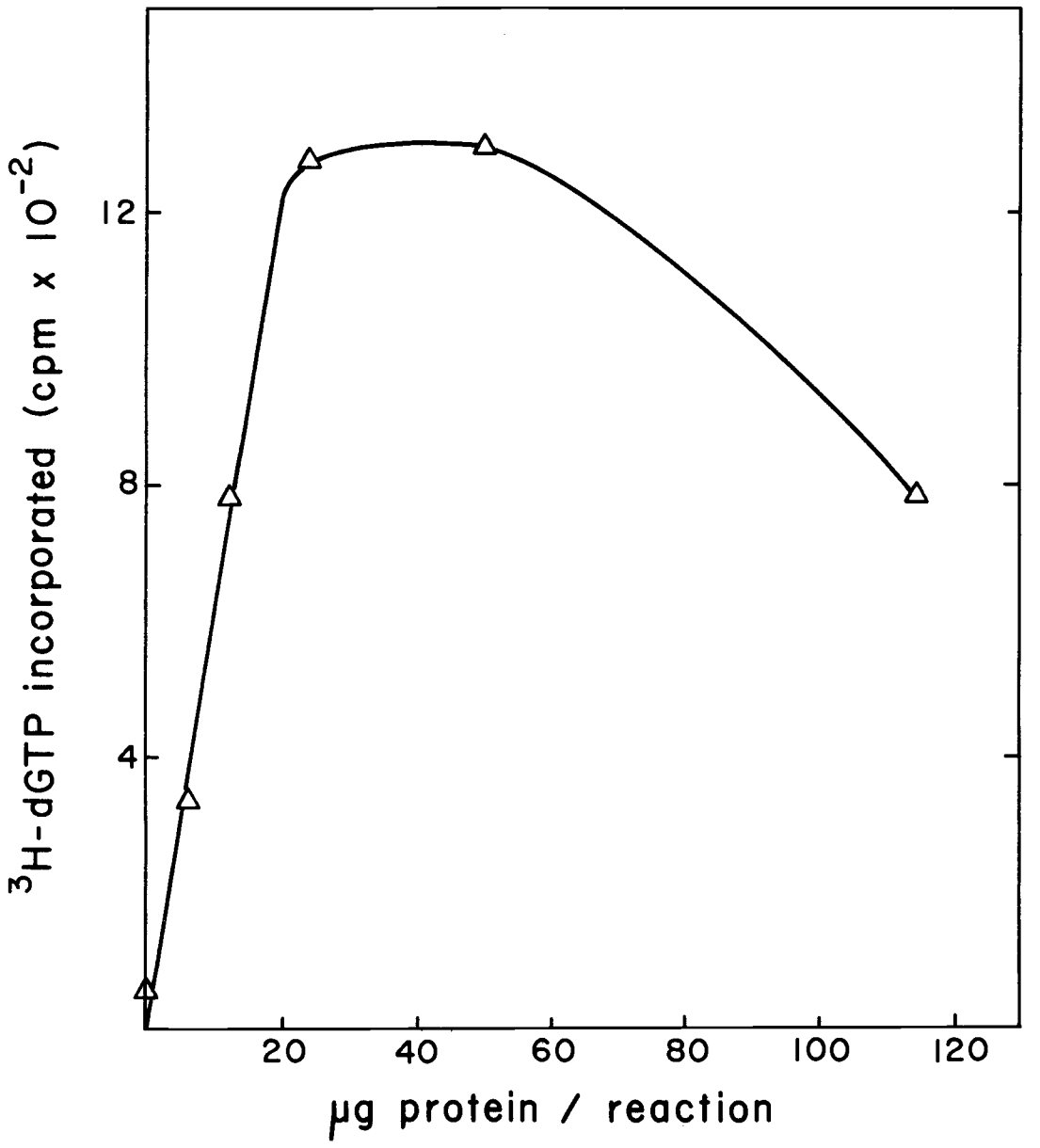


Figure 22. Enzyme saturation of dC:rG template with the DNA polymerase from MC29 infected cells. A two plate pool of cells infected with MC29 virus for five days was prepared for DNA polymerase assay as described in Methods. Standard reactions containing 2.0 µg of dC:rG and varying amounts of cell extract were incubated for 30 minutes. One pmole of ^{3}H-dGTP was equivalent to 2,000 counts per minute.

of cellular DNase present in the crude homogenates.

Primer Saturation

The response of the DNA polymerase from cell homogenates to increasing concentrations of dC:rG is shown in Figure 23. Each of the assays contained 50 μg of cellular protein. It could be demonstrated that 1.0 μg of dC:rG was sufficient to exhibit optimum activity while completely suppressing any uninfected control cell homogenate DNA polymerase activity.

Kinetics

Figure 24 summarizes a comparison of enzyme activity in noninfected cell cultures with enzyme activity in infected cell cultures. These cell homogenates had no detergent treatment and therefore represent enzyme from the cell that was free to participate in the reaction. The data showed that the noninfected cells contained a detectable polymerase (Figure 24A) that is stimulated by M. lysodeikticus DNA primer (Figure 24C) but not by the synthetic hybrid dC:rG (Figure 24B).

Addition of detergent to the cell homogenate stimulated enzyme activity when DNA template was added to both the infected and noninfected cell homogenates (Figure 25C, D). The dC:rG hybrid functioned as template only in the infected cell homogenate (Figure 25B).

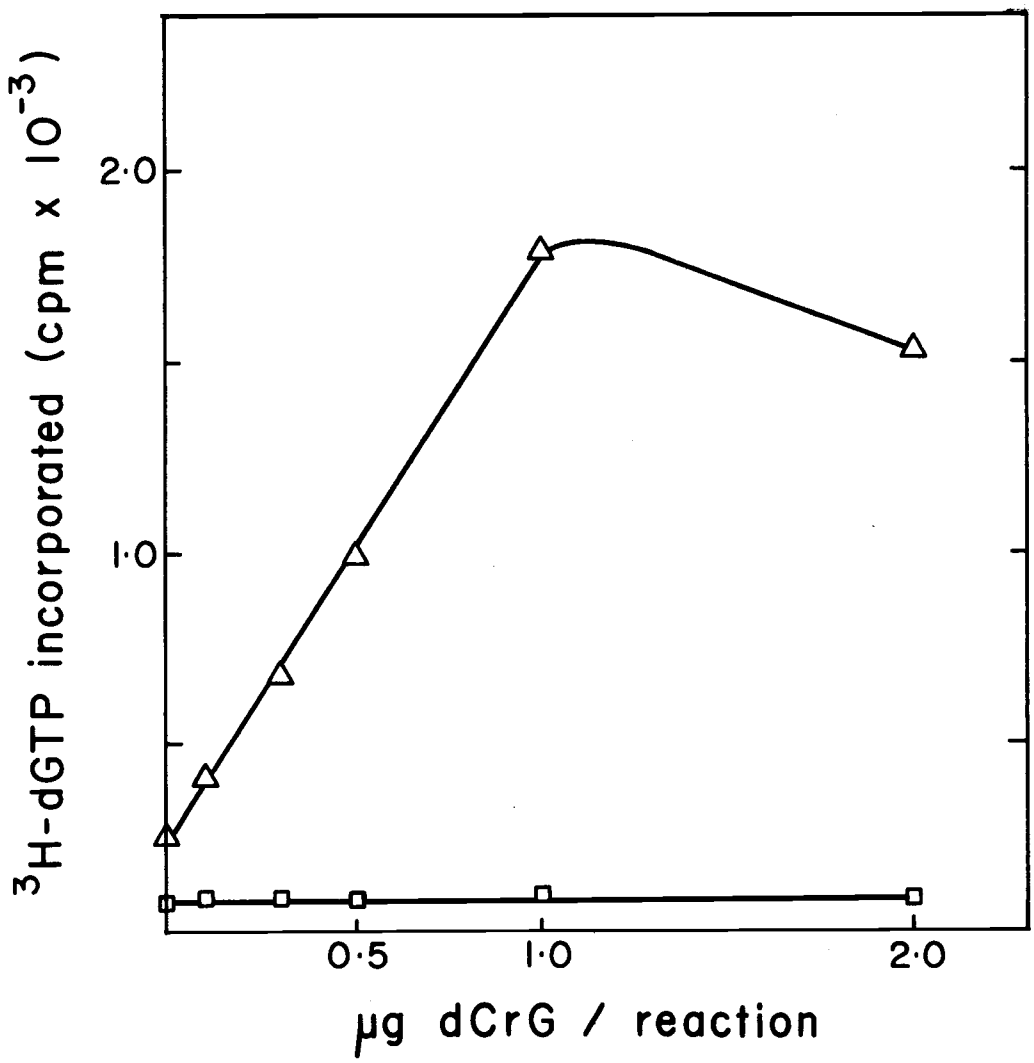


Figure 23. Template saturation of the DNA polymerase from MC29 infected cells (△) and noninfected cells (□) with dC:rG. Preparation of the cell extract was described in Figure 22. Standard 0.10 ml reactions containing 50 μg of cell extract were incubated for 30 minutes in the presence of dC:rG template. One pmole of ^{3}H-dGTP was equivalent to 2,000 counts per minute.

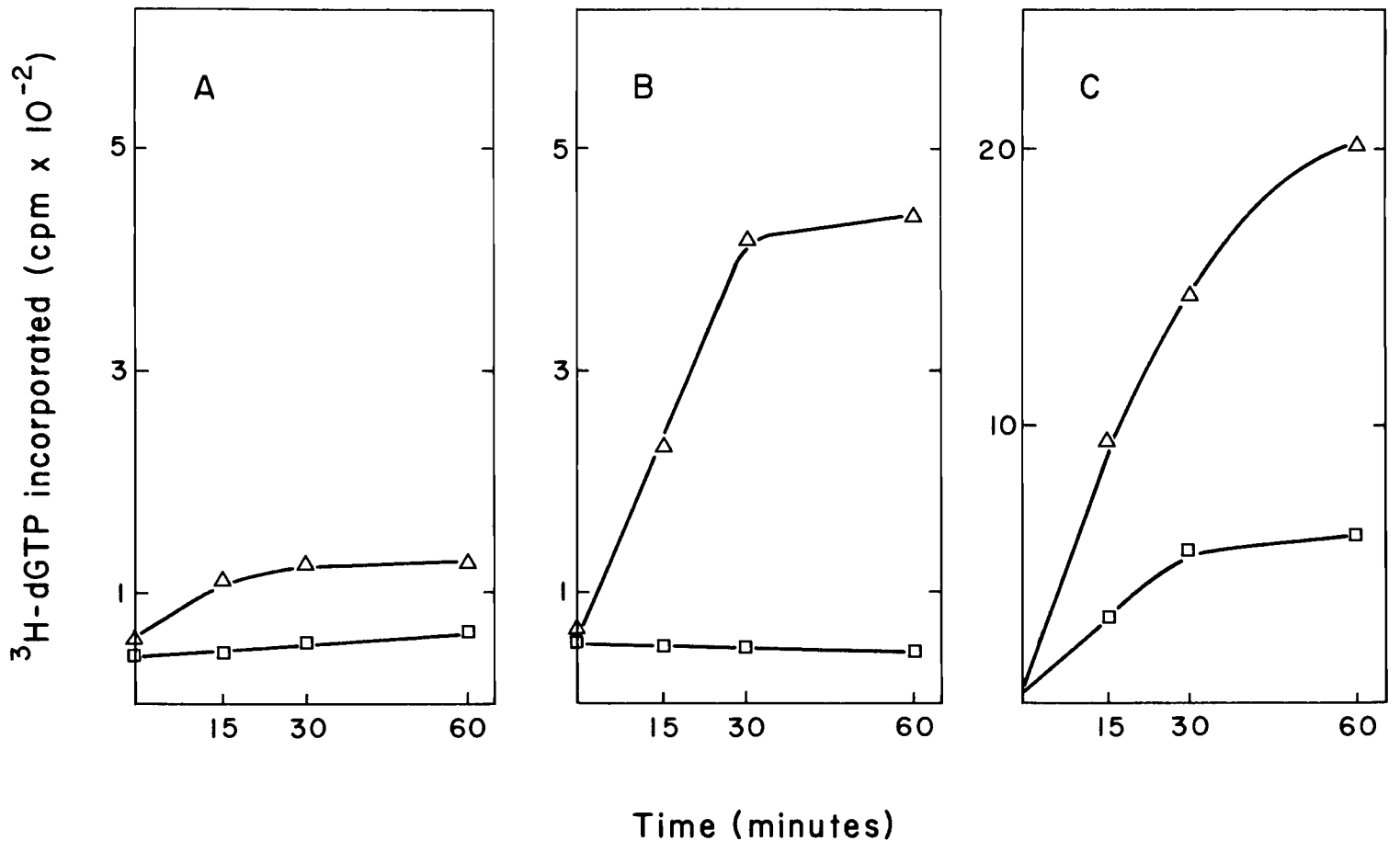
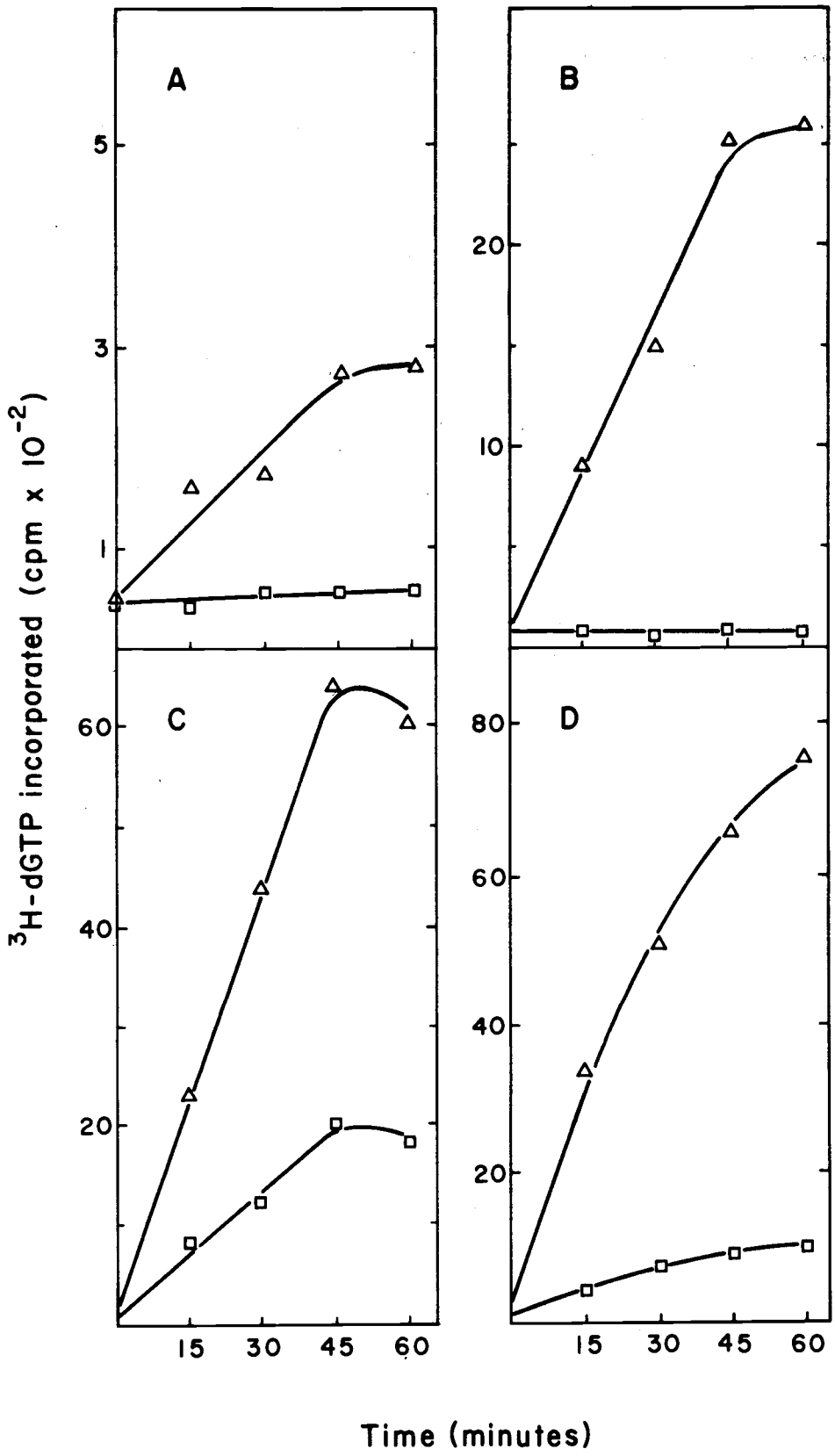


Figure 24. Kinetics of ^{3}H-dGTP incorporation by DNA polymerase from MC2^9 infected cells (triangles) and noninfected cells (squares). The cell homogenates in A, B, and C were not detergent treated. 50 μg of cellular protein were used per 0.1 ml assay. Figure A shows the incorporation of ^{3}H-dGTP with no added primer. B indicates that activity when dC:rG (2 μg/assay) was added, and C when M. lysodeikticus DNA (2 μg/assay) was added. For comparison with the results in Figure 25 the homogenates were allowed to stand 24 hours at 0°C prior to assay. Specific activity of ^{3}H-dGTP: 2,000 counts per minute per pmole

Figure 25. Kinetics of ^{3}H-dGTP incorporation by the DNA polymerase from detergent treated cell extracts in the absence of added DNA (A); with dC:rG (B); M. lysodeikticus DNA (C); and AMV DNA (D) added as primers. The assay conditions were the same as those cited in Figure 24 with the exception that the cell supernatants were pretreated with equal volumes of 2X Nonidet stock and allowed to stand 24 hours at 0°C before assaying for enzyme activity.


A
B
C
D
5
3
1
20
10
60
40
20
80
60
40
20
15
30
45
60
^{3}H-dGTP incorporated (cpm x 10^{-2})
Time (minutes)

Apparently this offers a good method for distinguishing the DNA polymerase that results from tumor virus infection. DNA from AMV (Říman and Beaudreau, 1970) was an excellent primer for the DNA polymerase from MC29 infected cells (Figure 25D). DNA from AMV also stimulated the DNA polymerase of the control cell enzyme. DNA synthesis by enzymes from cell homogenates decreased rapidly after 45 minutes and it is likely that the cell homogenates contained sufficient nuclease activity to give these results.

Response of Chick Embryo Cells to Infection with MC29 Virus

An understanding of the early events in MC29 infection of CEC cultures necessitates a more detailed examination of the processes of virus adsorption and progeny virus release.

Adsorption of MC29 Virus to CEC Cultures

Standard procedures for the infection of CEC cultures with MC29 virus include a one-half hour adsorption period of the inoculum on an incomplete monolayer of cells. In a determination of the efficiency of adsorption of infectious units of MC29 virus, 24 hour primary cultures (6.1×10^6 cells per petri dish) were utilized. The cells were allowed to adsorb 4.0×10^4 FFU of MC29 virus in 0.2 ml medium. After 30 minutes the cells were washed with PBS and an aliquot of the wash titered for FFU. It was found that approximately 80% of the infectious

units had adsorbed to the cells in 30 minutes.

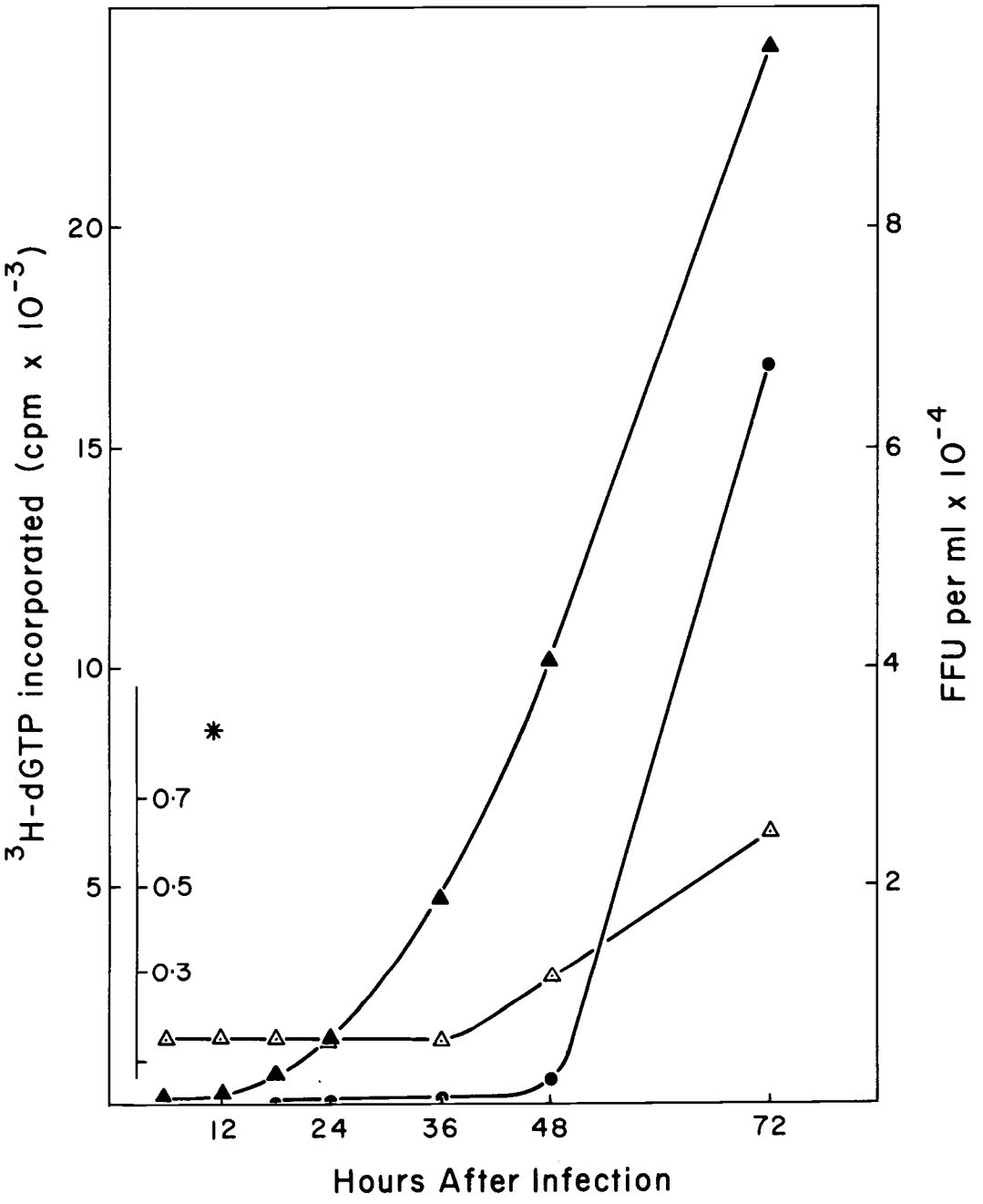
The preparation of ^{3}H-uridine labeled MC29 virus particles suitable for infection of cells provided an alternative method to monitor the adsorption of MC29 virus on CEC cultures. On the average, 58% of the radioactivity labeled virus particles added to a 24 hour CEC culture were adsorbed in 30 minutes.

Appearance of Viral Polymerase and Infectious MC29 Virus

As has been shown, an analysis of culture supernatants from infected and noninfected CEC fibroblasts indicated that the detection of MC29 virus DNA polymerase activity is closely associated with the presence of infectious MC29 virus. In the following experiment the appearance of virus in the culture medium after virus infection was assayed by FFU and by DNA polymerase activity in the particulate fraction of the culture fluid. The cellular DNA polymerase activity that was virus induced was assayed from four plate pools of infected cells. To minimize any effect on the enzyme preparation or FFU assays, the free virus was washed from the culture with four ml of PBS after the one-half hour adsorption period. The culture medium was not changed for the 72 hours studied.

At appropriate times after infection the culture medium from six plates (60 ml) was pooled, an aliquot taken for titration and the remaining used for enzyme preparations. Figure 26 shows the

Figure 26. Appearance of infectious MC29 (●), virus DNA polymerase (▲), and virus specific cellular DNA polymerase (△) in infected CEC fibroblasts. Culture medium was not changed for the 72 hours studied. Culture supernatants from six representative plates were used for each virus enzyme preparation (see Methods). A standard 0.1 ml reaction was used containing 2 μg of M. lysodeikticus DNA and 0.05 ml of the detergent treated virus suspension. Cellular DNA polymerase activity was measured by incorporation of label for 45 minutes in a standard reaction containing 50 μg of cellular protein and 1 μg of dC:rG as template. One pmole of dGTP was equivalent to 2,000 counts per minute. (A separate vertical axis was used in the figure for ^{3}H-dGTP incorporated by cellular DNA polymerase. This axis was marked with an asterisk in the figure.)


^{3}H-dGTP incorporated (cpm x 10^{-3})
FFU per ml x 10^{-4}
20
15
10
5
8
6
4
2
0·7
0·5
0·3
*
12
24
36
48
72
Hours After Infection

results obtained after the cultures were infected at a multiplicity of infection of approximately 0.006-0.007 FFU per cell. Extrapolation of the data from the figure established that detectable viral DNA polymerase appeared in the culture medium 10-12 hours after infection. Infectious MC29 (FFU) could also be seen 10-12 hours after introduction of the virus. However, virus specific DNA polymerase was not detectable in cell homogenates until 36-48 hours following infection. In all cases noninfected control culture supernatants and cell homogenates were tested, but no DNA polymerase activity was found.

The growth of the virus population in the culture medium following the first appearance of virus was slow during the initial period, but an accelerated rate of growth was observed throughout the period of study. Table 5 shows that the general virus growth pattern was similar using either of the two determinations, but greater enzyme activity per FFU was found in the initial period of virus release than at later periods when transformation was complete. Values of cpm per FFU observed from culture fluid of 72 hour infected cells approached those from six day infected cells (see Table 2).

Growth of MC29 Infected and Noninfected Control Cells

Infection of CEC fibroblasts with MC29 virus results in transformation of the infected cells. Differences in the rate of cell growth

Table 5. DNA polymerase activity from MC29 virus released by CEC.

Hours Post-infection	Enzyme activity**	FFU assayed***	Enzyme activity/FFU
0 time*	1,015	22,000	0.05
6	89	56	1.6
12	112	84	1.3
18	570	250	2.3
24	1,447	800	1.8
36	4,614	1,800	2.6
48	10,085	12,000	0.8
72	24,059	380,000	0.06

* 0 time values represent those obtained by assaying the inoculum used to infect six plates of CEC fibroblasts.

** Cpm of ^{3}H-dGTP incorporated into DNA product after 60 minute incubation at 37°C (2,000 cpm = one pmole). Reaction conditions were those reported in Figure 26. Blank (50 cpm) was subtracted from the observed values.

*** Amounts of infectious virus added to enzyme determination.

between infected and noninfected cells were determined from cell counts of duplicate plates of cells. Figure 27 shows the growth of both the MC29 infected and the noninfected control cells used in the experiment presented in Figure 26. Extrapolation of the data from the MC29 cells indicates that the onset of a difference in the rate of cell growth observed with MC29 infection is approximately 12-18 hours after introduction of the virus; that is, shortly after infectious virus are first detected in the culture medium. The population doubling time of the control cells stayed constant throughout the study at about 96 hours, whereas once transformation had begun the MC29 converted cultures attained a population doubling time of 54 hours.

Evidence presented here has shown that detection of MC29 virus in culture medium from infected cells was accomplished by both FFU titrations and assays for virus-associated DNA polymerase activity. Strain MC29 tumor virus infection of CEC fibroblasts resulted in progeny virus production as early as 10-12 hours after introduction of the virus. Shortly after the onset of virus particle liberation, MC29 infected cells began to grow at a rate more rapid than the noninfected control cells.

Although DNA polymerase activity was found in culture medium from MC29 infected cells at early times after infection, virus specific DNA polymerase activity from homogenates of infected cells could only be detected at later times after infection. However, the enzyme

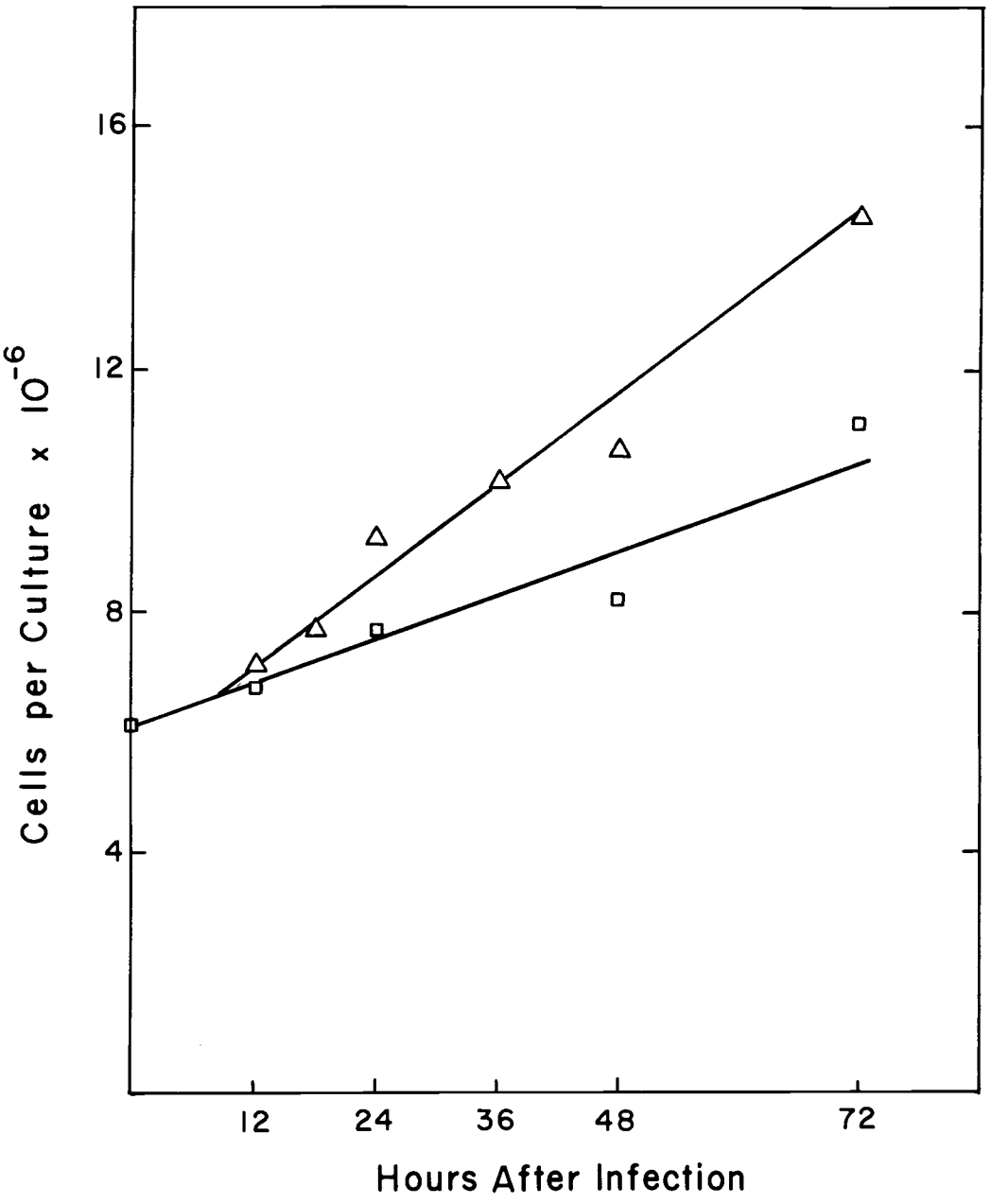


Figure 27. Growth of MC29 infected (△) and noninfected (□) CEC cells. Culture conditions were those reported in Figure 26. Cells from representative cultures were enumerated as described in Methods. Each point represents the mean count of two plates.

activity associated with the virion differs from the normal cell enzyme in size and response to dC:rG template. These two criteria may be useful for separating the enzyme activity normally found in the uninfected cell from that enzyme activity induced by tumor virus infection.

DISCUSSION

The DNA Polymerase from RNA Tumor Viruses

Strain MC29 tumor virus, like other RNA tumor viruses, has DNA polymerase (Baltimore, 1970; Green et al., 1970; Hatanaka et al., 1970; Mizutani et al., 1970; Scolnick et al., 1970a; Spiegelman et al., 1970a; Temin and Mizutani, 1970). Similar to AMV, the MC29 virus contains enzyme that has a low synthesis of DNA in the absence of added nucleic acid template. Enzyme activity was greatly stimulated by the addition of DNA to the incubation mixture. All enzyme preparations from the MC29 virus made throughout this study showed a decided preference for DNA as template rather than RNA.

Investigations with regard to the size of the RNA tumor virus DNA polymerase have been previously reported. Duesberg et al. (1971) examined the sedimentation constant of the Rous sarcoma virus DNA polymerase. Enzyme purified from AMV has been studied by Kacian et al. (1971). Both investigations concluded that the sedimentation constant of the purified virus enzyme was 6S. Contradictory results were found in this study. The MC29 viral DNA polymerase purified by cesium chloride fractionation consistently sedimentated at a rate of 7.7S. Preincubation of the enzyme with RNase had no effect on the sedimentation characteristics. When the DNA polymerase from AMV was purified using cesium chloride, it was found to have a

sedimentation constant of 7.4S.

The AMV DNA polymerase was also purified according to the procedures of Kacian et al. (1971) to determine whether the discrepancy in the sedimentation coefficients was due to a difference in the method of purification of the enzyme. As before, the purified DNA polymerase from AMV sedimentated at a rate of 7.4S.

The estimated molecular weights of the MC29 and AMV enzymes (180,000 and 175,000 daltons, respectively) are more in agreement with the results obtained by Kacian et al. (1971) who analyzed their purified 6S AMV DNA polymerase by gel electrophoresis. They found two subunits of molecular weights 110,000 and 69,000 daltons. The total molecular weight of the two subunits corresponds to the 7.4S values obtained here for the AMV DNA polymerase.

An accurate determination of the size of the RNA tumor virus DNA polymerase may become of significant importance in deciphering any relationship that may exist between the subunit structure of the enzyme and its response to various templates, notably the synthetic homopolymers. It would also become especially important in studies concerned with the synthesis of a fully active viral DNA polymerase in infected cells and its relation, if any, to the host cell DNA polymerase.

The DNA Produced by Viral DNA Polymerases

The products of endogenous reactions of polymerases isolated from different viruses have been analyzed by a variety of investigators. It is commonly held by most that the initial reaction of the DNA polymerase is the synthesis of a single strand of DNA utilizing the high molecular weight RNA of the viral genome as template (Garapin et al., 1971; Rokutanda et al., 1970; Spiegelman et al., 1970a; Baltimore, 1970; Temin and Mizutani, 1970; Manly et al., 1971; Faras et al., 1971). The single-stranded DNA product is then used as template for the subsequent reaction (Garapin et al., 1971; Spiegelman et al., 1970c), the formation of double-stranded DNA (Fanshier et al., 1971; Fuginaga et al., 1970; Manly et al., 1971).

A variety of techniques have been used to distinguish the above two mentioned reactions. Spiegelman et al. (1970a) banded a 20 minute product of the Rauscher murine leukemia virus (RLV) polymerase in cesium sulfate. The product was found associated with RNA as indicated by its density in the gradient. When the product was denatured with formamide, a density characteristic of DNA was observed. In addition, when a two hour reaction product was treated in an identical manner, a series of hybrid structures were observed between the densities of RNA and DNA. Denaturation again removed the DNA from all hybrid structures. Fanshier et al. (1971) used the polymerase

from Rous sarcoma virus (RSV) and extended the cesium sulfate study to include a four hour reaction product. Their results also showed a shift in the product density from the RNA region to the DNA region of the gradient when the reaction was incubated for various times. That the early product was an RNA:DNA hybrid and that the later product consisted of double-stranded DNA was additionally suggested by analysis of the cesium sulfate fractions on hydroxyapatite. Utilizing the same virus DNA polymerase, Garapin et al. (1971) were likewise able to fractionate the single and double-stranded DNA products with hydroxyapatite. They found additionally that virtually all of the early product DNA (single-stranded) could be annealed to the 70S viral RNA, whereas approximately 50% of the double-stranded product (longer reaction times) annealed to the viral RNA. This would suggest, as they mentioned, that the double-stranded product contains sequences complementary to the viral RNA, and also sequences which presumably are identical to regions in the viral genome.

The forms of DNA produced by the DNA polymerase from Maloney mouse leukemia virus were analyzed by Manly et al. (1971). Using ribonuclease digestion and cesium sulfate centrifugation, they were able to detect three species of DNA: single-stranded, double-stranded and hybrid DNA:RNA. The product from an early (20 minute) reaction consisted almost exclusively of single-stranded and hybrid RNA:DNA, whereas longer reactions (120 minutes) resulted in a

marked increase in the percentage of double-stranded DNA. In the presence of actinomycin D longer reactions showed an increase in the percentage of single-stranded DNA together with an inhibition of double-stranded DNA formation. This would suggest that actinomycin affects the conversion of single-stranded DNA to its double-stranded form.

The product from a 60-minute endogenous MC29 reaction has been previously analyzed in a cesium chloride gradient (Kiessling, 1971). It exhibited a buoyant density that was characteristic of DNA (1.70 gm/cm^3). Investigation of the products of reactions primed by DNA revealed that M. lysodeikticus DNA directed the synthesis of a product having the same buoyant density as its primer, but the product synthesized under the direction of calf thymus DNA had a buoyant density different from that of its primer. Apparently the enzyme is capable of making a choice from the multiple DNA species that exist in calf thymus DNA. If this is the case, the selection of the enzyme from MC29 appears to favor the DNA primer with the high density. It may also be possible that the enzyme is only selecting regions in the primer that are rich in guanine and cytosine. The preference of DNA polymerase from AMV for DNA rich in guanine and cytosine has also been observed and DNA with a high guanine plus cytosine content was found in the AMV virus (Řík man and Beaudreau, 1970) and here in the MC29 virus.

Viral DNA and RNA

Because of the previously reported detection of DNA in three RNA tumor viruses (AMV: Řím̌an and Beaudreau, 1970; RSV: Levinson et al., 1970; and the MSV-MLV complex: Biswal and Benyesh-Melnick, 1970) and here in strain MC29, it should be of great interest to determine what role this DNA plays in viral infection and cellular transformation. Although unclear at the present time, the possibility cannot be excluded that these small DNA sequences may contribute in a major way to infection and transformation.

Both Řím̌an and Beaudreau (1970) and Levinson et al. (1970) concluded that most of the viral DNA was approximately 7S in size. In addition it was found that some DNA cosedimented in sucrose gradients with the high molecular weight viral RNA (Řím̌an, 1971; Levinson et al., 1970). Isopycnic centrifugation in cesium chloride of ^{3}H-thymidine labeled material from AMV resulted in a delineation of two kinds of DNA (Řím̌an and Beaudreau, 1970). One banded in a density indicative of a rich G+C content (1.72) while the other banded in a less G+C region (1.69). Results presented here have shown MC29 viral DNA to have a buoyant density in cesium chloride of 1.74 gm/cm^{3}.

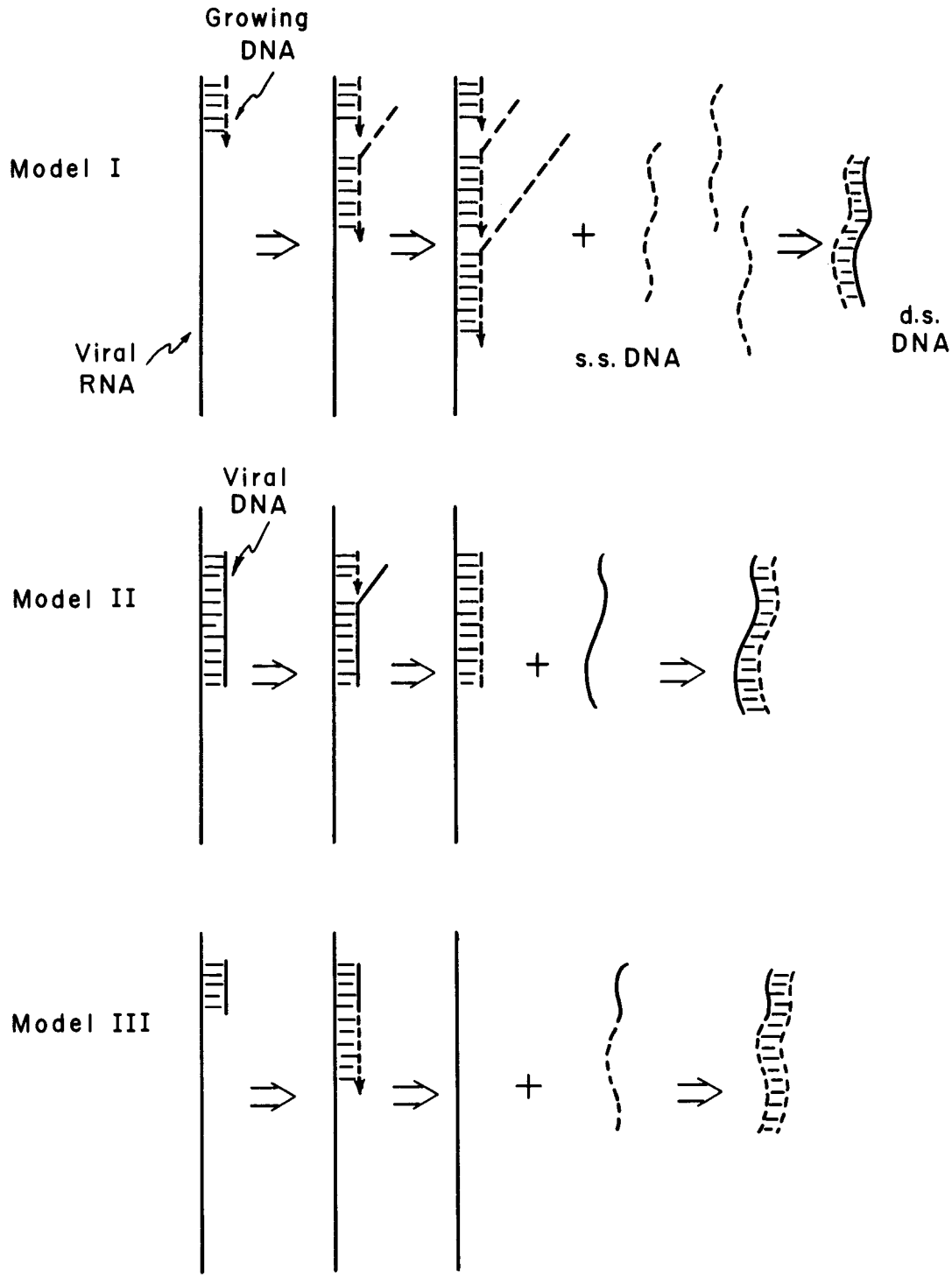
The participation of viral DNA in the DNA polymerase reactions still must be considered. The DNA-dependent DNA polymerase activity associated with AMV virus prefers as template DNA that is rich

in G+C, the kind found in the virus particle. This DNA-dependent enzyme activity apparently cannot be separated from the RNA-dependent enzyme activity as shown by Kacian et al. (1971). Their purest preparation, containing only two polypeptides, responded to both DNA and RNA. It thus appears that the DNA and RNA-dependent polymerase activities can be ascribed to one enzyme.

Whether the viral RNA or its associated DNA is normally used as a template for DNA synthesis by the DNA polymerase has not yet been conclusively shown. Three attractive possibilities may be considered: first, that all or a portion of the viral RNA genome acts exclusively as the template; second, that in actuality small segments of RNA complementary to viral DNA are being copied; or finally, some form of RNA:DNA hybrid serves as an initiator point (primer) for DNA synthesis. These three alternatives diagramed below collectively encompass the majority of observations obtained with the viral DNA polymerase.

Regardless of the mechanisms involved in DNA synthesis, it should be emphasized that few studies of the virus DNA polymerase have been made with a solubilized enzyme, free from any endogenous polymerizing or nuclease activity (Duesberg et al., 1971; Kacian et al., 1971; Baltimore and Smoler, 1971).

The first model, the provirus theory, is that of Temin (1964a). Extensive reviews have been presented (Montagnier, 1969, 1971;


Growing
DNA
Model I
Viral
RNA
s.s. DNA
d.s.
DNA
Viral
DNA
Model II
Model III

Temin, 1967) so the hypothesis will not be discussed in detail here. Generally, it represents the formation of a DNA product using the entire viral genome as template. Although many single and double-stranded DNA products are shown to be synthesized, only one DNA sequence complementary to the viral 65-70S RNA is required for the provirus theory. To date this has been difficult to demonstrate. Studies in determining the size of the early DNA product have resulted in isolation of segments ranging from 3 to 8S (Duesberg and Canaani, 1970; Rokutanda et al., 1970). However, 50-100% of that DNA was able to be hybridized to the viral RNA (Duesberg and Canaani, 1970; Garapin et al., 1971). If the viral RNA is completely transcribed to DNA by the polymerase, detection of a 70S DNA molecule may be hindered by the presence of contaminating nucleases. Nevertheless, identification of DNA larger than 8S has not been reported.

For clarity, models II and III will be discussed together. As shown, virus-associated DNA is an integral part of both schemes, its presence being essential for DNA synthesis to occur. DNA synthesis results first in a hybrid RNA:DNA structure. This is followed by continued transcription to achieve double-stranded DNA. Both of these models (II and III) can account for the following reported observations concerning the viral DNA polymerase:

1. DNA synthesis from an RNA template.

2. Residual DNA synthesis unaffected when enzyme plus endogenous templates are preincubated with RNase (McDonnell et al., 1970).

3. Identification of an RNA:DNA hybrid formed early in DNA synthesis (Spiegelman et al., 1970a; Fanshier et al., 1971).

4. Appearance of single (early reaction) and double-stranded (longer reaction) DNA products (Mizutani et al., 1970; Garapin et al., 1971).

5. Presence of ^{3}H-thymidine labeled material with 64S AMV RNA (Říman, 1971), and RSV RNA (Levinson et al., 1970).

6. Response of enzyme to various templates:

   a. 150-fold greater enzyme activity with dT:rA than AMV RNA (Kacian et al., 1971).

   b. Generally poor enzyme activity with RNA:RNA duplexes (Table 4; Spiegelman et al., 1970c).

   c. Good enzyme activity with RNA:DNA hybrids (Table 3), dC, AMV DNA and nicked double-stranded DNA (myeloblast DNA, Table 4).

   d. Early copying on homopolymer duplexes is asymmetric. (Spiegelman et al., 1970c).

7. All of early product annealable to genome RNA; 50% of later product annealable (Garapin et al., 1971).

8. Delineation of two reactions with actinomycin D treatment (McDonnell et al., 1970; Manly et al., 1971):

a. Formation of RNA:DNA hybrid unaffected.

b. Synthesis of double-stranded DNA inhibited.

Recently Baltimore and Smoler (1971) have reported results using various synthetic polyribonucleotides and polydeoxyribonucleotides as templates for the DNA polymerase from AMV and mouse leukemia virus (MLV). They found that DNA polymerase activity was low when polyribonucleotides alone were provided as templates. When short segments of complementary deoxyribonucleotides were added to the ribose polymers, DNA synthesis was stimulated. It was concluded that both an RNA template and a DNA primer are necessary for stimulation of the DNA polymerase (see model III) and that the enzyme is incapable of initiating DNA synthesis de novo on a single-stranded template. It was found here, on the contrary, that the deoxyribonucleotide homopolymer dC stimulated the incorporation of ^{3}H-dGTP by the purified DNA polymerase and that activity was enhanced when DNase was added. This would seem to indicate that at least the DNA-dependent synthesis can be initiated on single-stranded DNA and that it is the number of available sequences, not the free 3'-OH ends, that is the critical factor.

Whether the viral DNA is serving as a point of initiation for DNA synthesis as shown in model II or is actually serving as a primer for DNA synthesis on the RNA genome (III) is not known at the present time. However, a primary distinction between these hypotheses is

that model II allows for repeated synthesis of single-stranded DNA, whereas model III apparently would not. If the viral DNA associated with the RNA genome is actually needed as a primer with a free 3'-OH end (III), then synthesis of one segment of DNA would eliminate the point of reinitiation on the viral RNA. This could be overcome by a specific endonuclease capable of cleaving the newly synthesized DNA segment from its viral DNA primer. However, nuclease activity may not be necessary, for a single functional segment of virus-specific DNA may be all that is needed to induce transformation in the cell. Therefore, by this reasoning one cannot validly differentiate between the models presented.

Experimentally it may be possible to distinguish between models II and III. Viral DNA, associated with the RNA genome, can be radioactively labeled with ^{3}H-thymidine using the procedures of Říman (1971). The labeled virus particles would then be used to prepare a crude viral DNA polymerase as outlined in Methods. A short term endogenous reaction for approximately 30 minutes with ^{32}P-labeled substrates would then allow single-stranded DNA to be synthesized. The product from this reaction would then be applied to an appropriate cesium sulfate gradient. If either model II or III is correct, both ^{3}H-viral DNA and ^{32}P-product DNA should be able to be isolated in the region of viral RNA (Spiegelman et al., 1970a; Fanshier et al., 1971). Formamide treatment of the peak cesium sulfate fractions will

dissociate the RNA from the product DNA (Spiegelman et al., 1970a). If this were then applied to a sedimentation velocity gradient and compared to unreacted ^{3}H-viral DNA, one could expect:

Model II: That the short term reaction allowed for the formation of ^{32}P-product DNA which was smaller and not covalently bonded to the native viral DNA. Untreated viral DNA should sediment at the same rate as the ^{3}H-DNA used in the reaction mixture.

Model III: ^{32}P-product DNA should not be able to be separated from the ^{3}H-labeled primer DNA. If synthesis had occurred using the viral DNA as primer, one should be able to detect a shift in both labels to a higher sedimentation value. Any nonparticipating viral DNA should sediment as the unreacted DNA.

It may be that viral DNA is not involved at all in initiating DNA synthesis. It is possible that a folded end of the viral RNA genome or a short complementary segment of RNA could supply both a suitable template for DNA synthesis together with a free 3'-OH group which could serve as a primer. If this were the case then one would expect to find a nuclease specific for the RNA-DNA covalent bond formed at the onset of DNA synthesis. Nuclease activity of this type has not yet been reported.

A direct comparison between the proposed models and virus production can be made with previously reported studies using actinomycin. Temin (1964b) reported that actinomycin was capable of inhibiting RSV production in tissue culture. Actinomycin was also used by McDonnell et al. (1970) and Manly et al. (1971) to show an inhibition in the replication of double-stranded DNA by the viral DNA polymerase. It is then possible that the actinomycin sensitive step observed by Temin in tissue culture infected cells may be the synthesis of double-stranded viral DNA. However, Temin's experiments have been difficult to confirm. When his culture conditions were reproduced here with MC29 infected cells, the addition of actinomycin at the lowest reported concentration resulted in cell death within 24 hours.

Bader and Bader (1970) have used the thymidine analogue 5-bromo-deoxyuridine (BUdR) with RSV infected CEC cultures. 5-Bromo-deoxyuridine is incorporated as a substitute for thymidine during DNA synthesis without destroying its capacity for replication (Hayes, 1968). Transcription can proceed but periodic mistakes in base pairing may occur which could result in incorrectly coded RNA. BUdR treatment of cells before infection with RSV had no effect on infectious virus particle production. The same result was found when the cells were treated 24 hours after infection. However, when the cells were treated with BUdR coincident with infection, there was a 50-90% reduction in the number of infectious virus particles produced.

Attempts were made to determine what effect BUdR treatment of the infected cells had on the production of infectious and noninfectious virus particles. Cells were infected and exposed to ^{3}H-uridine after BUdR treatment and the labeled virus particles isolated. Disruption of the virus, followed by electrophoresis in a polyacrylamide-agarose mixed gel, resulted in similar recovery of the total radioactivity in the viral RNA regions from both the BUdR-treated and untreated preparations. This suggested that BUdR treatment did not markedly affect the production of virus particles. Focus forming assays, however, showed that the BUdR treated sample was 15 times less infectious than the non-treated control. It was consequently postulated that, in accord with Temin's provirus theory, RNA to DNA transcriptional mistakes occurred in the presence of BUdR. Upon further transcription of the viral DNA to form progeny viral RNA, virus was produced, but the number of particles possessing the capacity to transform cells was diminished, apparently as a result of BUdR induced mutations.

5-Bromo-deoxyuridine has also been used to study the transforming ability of the avian sarcoma virus B77 (Boettiger and Temin, 1970) and RSV (Balduzzi and Morgan, 1970). These investigations utilized the technique devised by Puck and Kao (1967) to select nutritionally deficient mutants of mammalian cells. The procedure involves incorporation of BUdR into DNA followed by exposure of the cells to ultraviolet or short wavelength visible light. This substituted DNA is

now more sensitive to inactivation by the light. Both reports demonstrated that infection of cells followed by BUdR treatment and exposure to light resulted in an inhibition of transformation. This suggested that the proviral DNA that was synthesized upon infection (Temin's provirus theory) was destroyed. As a consequence, the infected cell failed to become transformed.

In addition to Temin's provirus theory (model I), models II and III presented here can account for the results obtained with BUdR. With all of the models the addition of BUdR could result in the production of incorrectly coded DNA. If DNA were needed for transformation, but not necessarily for virus particle production, we could expect in models II and III the results obtained by Bader and Bader (1970): little effect on virus particle production, but a significant decrease in infectious virus particles. Also, if virus specific DNA is synthesized after infection and is necessary for transformation, then inactivation of BUdR substituted DNA with light would prohibit transformation in the infected cell.

Cell Response to Infection with MC29 Virus

Appearance of Virus and Viral DNA Polymerase Activity After Infection

DNA polymerase preparations from non-infected culture medium showed no activity over a 60-minute period in the presence or absence

of DNA template. Such unambiguous distinction in the level of enzyme activity from culture medium permits a close examination of the origin of the enzyme in the virus-infected cells.

It was possible in this study to distinguish clearly enzyme activity in culture fluids having extremely low levels of virus and to demonstrate that the background activity of the noninfected tissues is not confusing to the interpretation of the results. Newly-formed virus particles, manifested by both titration for focus-forming units and by the appearance of virus DNA polymerase activity, were evident in culture supernatants as early as 10-12 hours after infection. This is not unlike that observed by Langlois et al. (1969a) and Fritz et al. (1969) when assaying for the appearance of both infectious virus particles (FFU) and virus specific antigen. Either enzyme activity or infectivity assays gave a similar picture of the growth of the virus population in tissue culture medium. However, the enzyme activity per FFU during the early infection period was greater than the values calculated from infected cultures that were established for a longer time. In late infection periods (36-72 hours) enzyme activity per infectious particle approached those ratios obtained for virus from older producing cultures.

It might be expected that virus particles would lose infectivity at a greater rate than enzyme activity. Decay studies of MC29 virus infectivity and its associated DNA polymerase activity showed that

virus particles do indeed lose their infectivity at a greater rate.

It is then apparent from these studies that the changes of cpm per FFU during the 72 hours studied (Table 5) cannot be explained by DNA polymerase inactivation that is greater than infectivity loss. These results suggest that either a greater number of enzyme molecules or greater enzyme activity is found in each infectious particle early in infection or that in the early infection period there was a greater production of another tumor virus that does not produce foci when assayed on CEC. If the latter explanation is valid then our results would indicate that virus production by infected cultures that have been established for a long time would contain a constant amount of both types of virus.

Confirmation of an MC29 associated virus which does not transform CEC fibroblasts has come from Langlois et al. (1971). Isolated from terminal dilution clones of MC29, this virus behaves as a leukosis virus in its morphological characteristics, in the activation of the Rous sarcoma virus non-producer cells, and in interference with RSV infection of CEC.

It now appears that strain MC29 virus may be quite analogous to RSV and RAV, each system being composed of both leukosis and sarcoma viruses. Recognition of this dual virus relationship as common to all RNA sarcoma viruses has still to be determined.

Experiments presented here have also shown that culture medium which contained substantial amounts of RIF did not exhibit DNA polymerase activity. The absence of an RNA-dependent DNA polymerase in this agent may be the reason that RIF does not induce cell transformation. Theoretically, virus specific DNA, necessary for transformation, is not able to be synthesized.

Intercellular DNA Polymerase

Detection of virus specific DNA polymerase activity in infected cells has been facilitated by the use of the synthetic homopolymer dC:rG. This hybrid served as template only in the infected cell homogenates.

The relative amounts of dGMP and TMP incorporated into DNA by the DNA polymerase from infected cells were very close to the expected values when M. lysodeikticus template was used as a primer. The mole fraction (dGMP/dGMP + TMP) of labeled substrate incorporated with M. lysodeikticus template was 0.70 (Table 4) which is reasonably close to 0.71, the mole fraction of (G + C) in the template molecule (Szybalski, 1968). On the other hand, similar calculations on products from the other DNA templates produced values greater than the mole fraction (G + C) in the template molecule. This may reflect that the product of early synthesis has a high G content, or that copy was from selected regions in the template which are high in G.

Studies with calf thymus DNA template have confirmed that labeled product DNA from the virus polymerase had a higher buoyant density (greater G content) than the bulk of the calf thymus DNA, suggesting that only a part of the DNA was being copied (Kiessling, 1971). Thus the appearance of the new polymerase activity found in the cell extract from infected cells was identified as an enzyme activity similar to that found in the MC29 virus.

The detection of intercellular DNA polymerase was possible only in the late infected cells (Figure 20). This may be the result of the presence of nuclease in the cell homogenate that prohibits detection of low levels of enzyme. It is also possible that the response of the cellular enzyme to the homopolymer dC:rG is a reflection of the presence of trapped or cell-associated virus. This would then imply that the viral DNA polymerase may be slightly different than the virus-induced cellular DNA polymerase, and that DNA polymerase activity stimulated by dC:rG may be found only in the virus.

Preliminary experiments have been able to demonstrate the presence of an 8S DNA polymerase in the soluble fraction of homogenates from MC29 infected cells. A similar preparation from control noninfected cells did not show this enzyme activity. However, the virus-induced cellular enzyme was not stimulated by the heteropolymer dC:rG. Apparently the MC29 cellular enzyme, by some unknown mechanism, acquires the potential to utilize dC:rG as a template since

the DNA polymerase isolated from virus particles is enhanced by its presence.

The detection of DNA polymerase activity associated with RNA tumor viruses has resulted in new lines of investigation dealing with the processes of virus infection and cellular transformation. Currently this enzyme now provides a means for transcription of viral RNA stretches into DNA copies, DNA copying and possibly the repair of DNA. These reactions have been seen to be associated with the MC29 tumor virus.

Detection of virus-induced DNA polymerase in homogenates from MC29 infected cells now allows for a more detailed examination of the role this enzyme plays in cellular transformation. The contribution of viral DNA both in the virus and produced by the viral DNA polymerase, has not yet been clarified. Yet most indications now seem to favor a critical function for these previously undetected segments of nucleic acids.

BIBLIOGRAPHY

Aaronson, S. A. and G. Todaro. 1969. Human diploid cell transformation by DNA extracted from the tumor virus SV40. Science 165:390-391.

Bader, J. P. and A. V. Bader. 1970. Evidence for a DNA replicative genome for RNA-containing tumor viruses. Proceedings of the National Academy of Sciences. 67:843-850.

Bader, J. P., N. R. Brown and A. V. Bader. 1970. Characteristics of cores of avian leuko-sarcoma viruses. Virology. 41:718-728.

Balduzzi, P. and H. R. Morgan. 1970. Mechanism of oncogenic transformation by Rous sarcoma virus. I. Intracellular inactivation of cell-transforming ability of Rous sarcoma virus by 5-bromodeoxyuridine and light. Journal of Virology 5:470-477.

Baltimore, D. 1970. Viral RNA-dependent DNA polymerase. Nature 226:1209-1211.

Baltimore, D., A. S. Huang and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proceedings of the National Academy of Sciences 66:572-576.

Baltimore, D. and D. Smoler. 1971. Primer requirement and template specificity of the DNA polymerase of RNA tumor viruses. Proceedings of the National Academy of Sciences 68:1507-1511.

Baluda, M. A. and D. P. Nayak. 1970. DNA complementary to viral RNA in leukemic cells induced by avian myeloblastosis virus. Proceedings of the National Academy of Sciences 66:329-336.

Banerjee, A. K., C. H. Kuo, and J. T. August. 1969. Replication of RNA virus. VIII. Direction of chain growth in the QB RNA polymerase reaction. Journal of Molecular Biology 40:445-455.

Beaudreau, G. S. and C. Becker. 1958. Virus of avian myeloblastosis. X. Photometric microdetermination of adenosinetriphosphatase activity. Journal of the National Cancer Institute 20: 339-349.

Beaudreau, G. S., C. Becker, T. Stim, A. M. Wallbank and J. W. Beard. 1960. Virus of avian myeloblastosis. XVI. Kinetics of cell growth and liberation of virus in cultures of myeloblasts. National Cancer Institute Monograph 4:167-187.

Biswal, N. and M. Benyesh-Melnick. 1969. Complementary nuclear RNA's of murine sarcoma-leukemia virus complex in transformed cells. Proceedings of the National Academy of Sciences 64:1372-1379.

_____ 1970. Replication of murine sarcoma-leukemia virus complex. Abstract, "The Second Lepetit Colloqium on Biology of Oncogenic Viruses", Paris, France.

Biswal, N., M. B. Grizzard, R. M. McCombs and M. Benyesh-Melnick. 1968. Characterization of intracellular ribonucleic acid specific for the murine sarcoma-leukemia virus complex. Journal of Virology 2:1346-1352.

Bittner, J. J. 1936. Some possible effects of nursing on the mammary gland tumor incidence in mice. Science 84:162.

Boettiger, D. and H. M. Temin. 1970. Light inactivation of focus formation by chick embryo fibroblasts infected with avian sarcoma virus in the presence of 5-bromodeoxyuridine. Nature 228:622-624.

Bolognesi, D. P., A. J. Langlois, L. Sverak, R. A. Bonar and J. W. Beard. 1968. In-vitro chick embryo cell response to strain MC29 avian leukosis virus. Journal of Virology 2:576-586.

Bonar, R. A., D. Beard, G. S. Beaudreau, D. G. Sharp and J. W. Beard. 1957. Virus of avian erythroblastosis. IV. pH and thermal stability. Journal of the National Cancer Institute 18:831-842.

Calnek, B. W. 1964. Morphological alteration of RIF-infected chick embryo fibroblasts. National Cancer Institute Monograph 17: 425-447.

Carnegie, J. W., A. O'C. Deeney, K. C. Olson, and G. S. Beaudreau. 1969. An RNA fraction from avian myeloblastosis virus having properties similar to transfer RNA. Biochimica et Biophysica Acta. 190:274-284.

Cavalieri, L. F. and E. Carroll. 1970. RNA as a template with E. coli DNA polymerase. Biochemical and Biophysical Research Communications 41:1055-1060.

Chabot, J. F., D. Beard, A. J. Langlois and J. W. Beard. 1970. Mesotheliomas of peritoneum, epicardium, and pericardium induced by strain MC29 avian leukosis virus. Cancer Research 30:1287-1308.

Coffin, J. M. and H. Temin. 1971. Comparison of Rous sarcoma virus-specific deoxyribonucleic acid polymerases in virions of Rous sarcoma virus and in Rous sarcoma virus-infected chicken cells. Journal of Virology 7:625-634.

Duesberg, P. H. and E. Canaani. 1970. Complementarity between Rous sarcoma virus (RSV) RNA and the in vitro-synthesized DNA of the virus-associated DNA polymerase. Virology 42:783-788.

Duesberg, P., K. V. D. Helm and E. Canaani. 1971. Properties of a soluble DNA polymerase isolated from Rous sarcoma virus. Proceedings of the National Academy of Sciences 68:747-751.

Duesberg, P. H. and P. K. Vogt. 1970. Differences between the ribonucleic acids of transforming and non-transforming avian tumor viruses. Proceedings of the National Academy of Sciences 67:1673-1680.

Eckert, E. A., D. Beard and J. W. Beard. 1954. Dose-response relations in experimental transmission of avian erythromyeloblastic leukosis. III. Titration of the virus. Journal of the National Cancer Institute 14:1055-1066.

Eckert, E. A., I. Green, D. G. Sharp, D. Beard and J. W. Beard. 1955. Virus of avian erythromyeloblastic leukosis. VII. Thermal stability of virus infectivity; of the virus particle; and of the enzyme dephosphorylating adenosine-triphosphate. Journal of the National Cancer Institute 16:153-161.

Ellerman, V. and O. Bang. 1908. Experimentelle Leukamie bei Huhnern. Centralblatt fur Bakteriologie, Parasitenkunds und Infectionskrankheiten. 46:595-609.

Fanshier, L., A. Garapin, J. McDonnell, A. Faras, W. Levinson and J. M. Bishop. 1971. Deoxyribonucleic acid polymerase associated with avian tumor viruses: Secondary structure of the deoxyribonucleic acid product. Journal of Virology 7:77-86.

Faras, A., L. Fanshier, A. Garapin, W. Levinson and J. M. Bishop. 1971. Deoxyribonucleic acid polymerase of Rous sarcoma virus: Studies on the mechanism of double-stranded deoxyribonucleic acid synthesis. Journal of Virology 7:539-548.

Freeman, A. E., P. H. Black, E. A. Vanderpool, P. H. Henry, J. B. Austin and R. J. Heubner. 1967. Transformation of primary rat embryo cells by adenovirus type 2. Proceedings of the National Academy of Sciences 58:1205-1212.

Friend, C. 1956. The isolation of a virus causing a malignant disease of the hematopoietic system in adult swiss mice. Proceedings of the American Association for Cancer Research 2:106.

_____ 1957. Leukemia of adult mice caused by a transmissible agent. Annals of the New York Academy of Science 68:522-532.

Fritz, R. B., A. J. Langlois, D. Beard and J. W. Beard. 1968. Strain MC29 avian leukosis virus: immunologic relationships to other avian tumor viruses. The Journal of Immunology 101:1199-1206.

Fritz, R. B., A. J. Langlois and J. W. Beard. 1969. Reaction of chick embryo cells infected with leukosis strain MC29 virus with fluorescein-labeled antibody. Journal of Virology 4:372-379.

Fuginaga, K., J. T. Parsons, J. W. Beard, D. Beard and M. Green. 1970. Mechanism of carcinogenesis by RNA tumor viruses. III. Formation of RNA·DNA complex and duplex DNA molecules by the DNA polymerase(s) of avian myeloblastosis virus. Proceedings of the National Academy of Sciences 67:1432-1439.

Gallo, R. C., S. S. Yang and R. C. Ting. 1970. RNA dependent DNA polymerase of human acute leukemic cells. Nature 228:927-929.

Garapin, A., L. Fanshier, J. Leong, J. Jackson, W. Levinson and J. M. Bishop. 1971. Deoxyribonucleic acid polymerases of Rous sarcoma virus: Kinetics of deoxyribonucleic acid synthesis and specificity of the products. Journal of Virology 7:227-232.

Garapin, A., J. P. McDonnell, W. Levinson, N. Quintrell, L. Fanshier and J. M. Bishop. 1970. Deoxyribonucleic acid polymerase associated with Rous sarcoma virus and avian myeloblastosis virus: Properties of the enzyme and its product. Journal of Virology 6:589-598.

Gerwin, B., G. J. Todaro, V. Zeve, E. M. Scolnick and S. A. Aaronson. 1970. Separation of RNA-dependent DNA polymerase activity from the murine leukemia virion. Nature 228:435-438.

Green, M. 1970. Oncogenic viruses. Annual Review of Biochemistry 39:701-756.

Green, M., M. Rokutanda, K. Fujinagna, R. K. Ray, H. Rokutanda and C. Gurgo. 1970. Mechanism of carcinogenesis by RNA tumor viruses, I. An RNA-dependent DNA polymerase in murine sarcoma viruses. Proceedings of the National Academy of Sciences 67:385-393.

Gross, L. 1951. "Spontaneous" leukemia developing in C3H mice following inoculation in infancy, with AK-leukemic extracts, or AK-embryos. Proceedings of the Society for Experimental Biology and Medicine. 76:27-32.

Hanafusa, H. 1968. Methods for the study of defective viruses. In: Methods in Virology. Ed. by K. Maramorosch and H. Koprowski. Academic Press, New York. p. 321-350.

Hanafusa, T., H. Hanafusa and T. Miyamoto. 1970. Recovery of a new virus from apparently normal chick cells by infection with avian tumor viruses. Proceedings of the National Academy of Sciences 67:1797-1803.

Hanafusa, H., T. Hanafusa and H. Rubin. 1963. The defectiveness of Rous sarcoma virus. Proceedings of the National Academy of Sciences 49:572-580.

Hanafusa, H., T. Hanafusa and H. Rubin. 1964a. Analysis of the defectiveness of Rous sarcoma virus. I. Characterization of the helper virus. Virology 22:591-601.

_____1964b. Analysis of the defectiveness of Rous sarcoma virus. II. Specification of RSV antigenicity by helper virus. Proceedings of the National Academy of Sciences 51:41-48.

Harel, J., L. Harel and G. Frezouls. 1970. Common sequences between RNA from oncogenic viruses and cellular DNA. Bulletin du Cancer 57:283-300.

Hartly, J. W. and W. P. Rowe. 1966. Production of altered cell foci in tissue culture by defective Moloney sarcoma virus particles. Proceedings of the National Academy of Sciences 55:780-786.

Hatanaka, M., R. J. Heubner and R. V. Gilden. 1970. DNA polymerase activity associated with RNA tumor viruses. Proceedings of the National Academy of Sciences 67:143-147.

_____ 1971. Specificity of the DNA product of the C-type virus RNA-dependent DNA polymerase. Proceedings of the National Academy of Sciences 68:10-12.

Hayes, W. 1968. The genetics of bacteria and their viruses. New York, John Wiley and Sons. p. 303.

Heine, U., D. Beard and J. W. Beard. 1968. Ultracytochemical studies on aberrant structures in avian tumor cells. Cancer Research 28:585-594.

Heine, U., A. J. Langlois, R. Říman and J. W. Beard. 1969. Ultrastructure of chick embryo cells altered by strain MC29 avian leukosis virus. Cancer Research 29:442-458.

Heine, U., Z. Mladenov, D. Beard and J. W. Beard. 1966. Morphology of hepatoma induced by strain MC29 avian leukosis virus. In Program of the 24th Annual Meeting of the Electron Microscopy Society of America, B-12. San Francisco, August 22 to 25.

Heubner, R. J., W. P. Rowe and W. T. Lane. 1962. Oncogenic effects in hamsters of human adenovirus types 12 and 18. Proceedings of the National Academy of Sciences 48:2051-2058.

Ivanov, X., Z. Mlanenov, S. Nedyalkov, T. G. Todorov and M. Yakimov. 1964. Experimental investigations into avian leukoses. V. Transmission, haematology and morphology of avian myelocytomatosis. Bulletin de l'Institut de Pathologie Comparee des Animaux Domestiques. Academic des Sciences de Bulgarie. 10:5-38.

Kacian, D. L., K. F. Watson, A. Burny and S. Spiegelman. 1971. Purification of the RNA-directed DNA polymerase of the avian myeloblastosis virus. (In Press).

Kiessling, A. A. 1971. Nucleic acid polymerases associated with neoplasma. Doctoral dissertation. Corvallis, Oregon State University. 161 numb. leaves.

Langlois, A. J. 1969, 1971. Ph. D., Department of Surgery, Duke University Medical Center. Personal Communication. Durham, North Carolina.

Langlois, A. J. and J. W. Beard. 1967. Converted-cell focus formation in culture by strain MC29 avian leukosis virus. Proceedings of the Society for Experimental Biology and Medicine 126:718-722.

Langlois, A. J., D. P. Bolognesi, R. B. Fritz and J. W. Beard. 1969a. Strain MC29 avian leukosis virus release by chick embryo cells infected with the agent. Proceedings of the Society for Experimental Biology and Medicine. 131:138-143.

Langlois, A. J., R. B. Fritz, U. Heine, D. Beard, D. P. Bolognesi and J. W. Beard. 1969b. Response of bone marrow to MC29 avian leukosis virus in vitro. Cancer Research 29:2056-2074.

Langlois, A. J., S. Sankaran, P. L. Hsuing and J. W. Beard. 1967. Massive direct conversion of chick embryo cells by strain MC29 avian leukosis virus. Journal of Virology. 1:1082-1084.

Langlois, A. J., L. Veprek, D. Beard, R. B. Fritz and J. W. Beard. 1971. Isolation of a non-focus-forming agent from strain MC29 avian leukosis virus. Cancer Research 31:1010-1018.

Levinson, W., J. M. Bishop, N. Quintrell and J. Jackson. 1970. Presence of DNA in Rous sarcoma virus. Nature 227:1023-1025.

Lin, F. H. and H. Thorman. 1970. Ribonucleic acid-dependent deoxyribonucleic acid polymerase in visna virus. Journal of Virology 6:702-704.

MacBride, W. D. and A. Wiener. 1964. In vitro transformation of hamster kidney cells by human adenovirus type 12. Proceedings of the Society for Experimental Biology and Medicine 115: 870-874.

Manaker, R. A. and V. Groupe. 1956. Discrete foci of altered chicken embryo cells associated with Rous sarcoma virus in tissue culture. Virology 2:838-840.

Manly, K. F., D. F. Smoler, E. Bromfeld and D. Baltimore. 1971. Forms of deoxyribonucleic acid produced by virions of the ribonucleic acid tumor viruses. Journal of Virology 7:106-111.

McAllister, R. M., M. O. Nicolson, G. Reed, J. Kern, R. V. Gilden and R. J. Heubner. 1969. Transformation of rodent cells by adenovirus 19 and other group D adenoviruses. Journal of the National Cancer Institute 43:917-923.

McDonnell, J. P., A. Garapin, W. E. Levinson, N. Quintrell, L. Fanshier and J. M. Bishop. 1970. DNA polymerase of Rous sarcoma virus: Delineation of two reactions with actinomycin. Nature 228:433-435.

Mizutani, S. D. Boettiger and H. Temin. 1970. A DNA-dependent DNA polymerase and a DNA endonuclease in virions of Rous sarcoma virus. Nature 228:424-427.

Mladenov, K., U. Heine, D. Beard and J. W. Beard. 1967. Strain MC29 avian leukosis virus. Myelocytoma, endothelioma and renal growths: Pathomorphological and ultrastructural aspects. Journal of the National Cancer Institute 38:251-285.

Mommaerts, E. B., D. G. Sharp, E. A. Eckert, D. Beard and J. W. Beard. 1954. Virus of avian erythromyeloblastic leukosis. I. Relation of specific plasma particles to the dephosphorylation of adenosinetriphosphate. Journal of the National Cancer Institute 14:1011-1024.

Montagnier, L. 1968. The replication of viral RNA. In: The Molecular Biology of Viruses. Ed. by L. V. Crawford and M. G. P. Stoker. Cambridge University Press, London. p. 125-150.

_____ 1969. Replication and action of oncogenic viruses. In: Comparative Leukemia Research. Bibl. haemat., No. 36. Ed. by R. M. Dutcher. Karger Basel, New York. p. 45-61.

_____ 1971. Replication of oncornaviruses. Text given at the Symposium "Oncogenic RNA Viruses and Host Cell Genome". Amsterdam. North Holland. (In press)

Moscovici, C., M. G. Moscovici and M. Zanetti. 1969. Transformation of chick fibroblast cultures with avian myeloblastosis virus. Journal of Cellular Physiology 73:105-108.

Parks, W. P., G. J. Todaro, E. M. Scolnick and S. A. Aaronson. 1971. RNA dependent DNA polymerase in primate syncytium-foaming (foamy) viruses. Nature 229:258-260.

Puck, T. T. and F. Kao. 1967. Genetics of somatic mammalian cells. V. Treatment with 5-bromodeoxyuridine and visible light for isolation of nutritionally deficient mutants. Proceedings of the National Academy of Sciences 58:1227-1234.

Quintrell, N., L. Fanshier, B. Evans, W. Levinson and J. M. Bishop. 1971. Deoxyeibonucleic acid polymerase(s) of Rous sarcoma virus: Effects of virion-associated endonuclease on the enzymatic product. Journal of Virology 8:17-27.

Říman, J. 1971. Professor, Institute of Organic Chemistry and Biochemistry. Personal communication. Praha, Czechoslovakia.

Říman, J. and G. S. Beaudreau. 1970. Viral DNA-dependent DNA polymerase and the properties of thymidine labelled material in virions of an oncogenic RNA virus. Nature 228:427-430.

Říman, J., L. Sverak, A. J. Langlois, R. A. Bonar and J. W. Beard. 1969. Influence of toyocamycin on RNA synthesis in chick embryo cells noninfected and infected with strain MC29 avian leukosis virus. Cancer Research 29:1707-1716.

Robinson, W. S. and M. Baluda. 1965. The nucleic acid from avian myeloblastosis virus compared with the RNA from the Bryan strain of Rous sarcoma virus. Proceedings of the National Academy of Sciences 54:1686-1692.

Rokutanda, M., H. Rokutanda, M. Green, K. Fujinaga, R. K. Ray and C. Gurgo. 1970. Formation of viral RNA-DNA hybrid molecules by the DNA polymerase of sarcoma-leukemia viruses. Nature 227:1026-1028.

Rous, P. 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. Journal of Experimental Medicine 13:397-411.

Rubin, H. 1960. A virus in chick embryos which induces resistance in vitro to infection with Rous sarcoma virus. Proceedings of the National Academy of Sciences 46:1105-1119.

_____ 1961. The nature of a virus-induced cellular resistance to Rous sarcoma virus. Virology 13:200-206.

Rubin, H. and P. K. Vogt. 1962. An avian leukosis virus associated with stocks of Rous sarcoma virus. Virology 17:184-194.

Schlom, J. D. H. Harter, A. Burney and S. Spiegelman. 1971. DNA polymerase activities in virions of visna virus, a causative agent of a "slow" neurological disease. Proceedings of the National Academy of Sciences 68:182-186.

Scolnick, E. M., S. A. Aaronson and G. J. Todaro. 1970a. DNA synthesis by RNA-containing tumor viruses. Proceedings of the National Academy of Sciences 67:1034-1041.

Scolnick, E. M., S. A. Aaronson, G. J. Todaro and W. P. Parks. 1971. RNA dependent DNA polymerase activity in mammalian cells. Nature 229:318-321.

Scolnick, E. M., E. Rands, S. A. Aaronson and G. J. Todaro. 1970b. RNA-dependent DNA polymerase activity in five RNA viruses: divalent cation requirements. Proceedings of the National Academy of Sciences 67:1789-1796.

Shope, R. E. 1933. Infectious papillomatosis of rabbits. With a note on the histopathology by E. W. Hurst. Journal of Experimental Medicine 58:607-624.

Spiegelman, S., A. Burney, M. R. Das, J. Keydar, J. Schlom, M. Travnicek and K. Watson. 1970a. Characterization of the products of RNA directed DNA polymerases in oncogenic RNA viruses. Nature 227:563-567.

_____ 1970b. DNA-directed DNA polymerase activity in oncogenic RNA viruses. Nature 227:1029-1031.

_____ 1970c. Synthetic DNA-RNA hybrids and RNA-RNA duplexes as templates for the polymerases of the oncogenic RNA viruses. Nature 228:430-432.

Stewart, S. E., B. E. Eddy, A. M. Gochenour, N. G. Borgese and G. E. Grubbs. 1957. The induction of neoplasma with a substance released by mouse tumors in tissue culture. Virology 3:380-400.

Stone, L. B., E. Scolnick, K. Takemoto and S. A. Aaronson. 1971. Visna virus: A slow virus with an RNA-dependent DNA polymerase. Nature 229:257-258.

Strohl, W. A., A. S. Rabson and H. Rouse. 1967. Adenovirus tumorigenesis: Role of the viral genome in determining tumor morphology. Science 156:1631-1633.

Svoboda, J. 1968. Dependence among RNA-containing animal viruses. In: The Molecular Biology of Viruses. Ed. by L. V. Crawford and M. G. P. Stoker. Cambridge University Press, London. p. 249-271.

Szybalski, W. 1968. Equilibrium sedimentation of viruses, nucleic acids, and other macromolecules in density gradients. Fractions 1:1-25.

Temin, H. M. 1962. Separation of morphological conversion and virus production in Rous sarcoma virus infection. Cold Spring Harbor Symposia on Quantitative Biology 27:407-414.

_____ 1963a. The effect of actinomycin D on growth of Rous sarcoma virus in vitro. Virology 20:577-582.

_____ 1963b. Further evidence for a converted, non-virus-producing state of Rous sarcoma virus-infected cells. Virology 20:235-245.

Temin, H. M. 1964a. Malignant transformation by viruses in vitro. Health Laboratory Science 1:79-83.

_____ 1964b. The participation of DNA in Rous sarcoma virus production. Virology 23:486-494.

_____ 1967. Studies on carcinogenesis by avian sarcoma viruses IV. In: The Molecular Biology of Viruses. Ed. by Colter and Paranchych. Academic Press, New York. p. 707-715.

Temin, H. and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature 226:1211-1213.

Temin, H. and H. Rubin. 1958. Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture. Virology 6:669-688.

Temin, H. M. and H. Rubin. 1959. A kinetic study of infection of chick embryo cells in vitro by Rous sarcoma virus. Virology 8:209-222.

Todaro, G. J. and S. A. Aaronson. 1968. Human cell strains susceptible to focus formation by human adenovirus type 12. Proceedings of the National Academy of Sciences 61:1272-1278.

Trentin, J. J., Y. Yabe and G. Taylor. 1962. The quest for human cancer viruses. Science 137:835-841.

Vogt, P. K. 1964. Fluorescence microscopic observations on the defectiveness of Rous sarcoma virus. National Cancer Institute Monograph 17:523-541.

_____ 1965. A heterogenity of Rous sarcoma virus revealed by selectively resistant chick embryo cells. Virology 25:237-247.

Vogt, P. K. and R. Ishisaki. 1965. Reciprocal patterns of genetic resistance to avian tumor viruses in two lines of chickens. Virology 26:664-672.

Vogt, P. K. and H. Rubin. 1963. Studies in the assay and multiplication of avian myeloblastosis virus. Virology 19:92-104.

Warburg, O. and W. Christian. 1942. Isolierung und kristallisation des Garungsferments enolase. Biochemische Zeitschrift 310: 384-421.

Watson, K. F. 1971. Ph. D. Institute for Cancer Research, College of Physicians and Surgeons, Columbia University College of Medicine. Personal communication. New York.

Watson, K. and G. S. Beaudreau. 1969. Isolation of an RNA-dependent RNA polymerase from virus infected myeloblasts. Biochemical and Biophysical Research Communications 37:925-932.

Wollmann, R. L. and W. H. Kirsten. 1968. Cellular origin of a mouse leukemia viral ribonucleic acid. Journal of Virology 2:1241-1248.

Yoshikawa-Fukada, M. and J. D. Ebert. 1969. Hybridization of RNA from Rous sarcoma virus with cellular and viral DNA's. Proceedings of the National Academy of Sciences 64:870-877.

_____ 1971. Further characterization of the cellular DNA hybridizing with the RNA of Rous sarcoma virus. Proceedings of the National Academy of Sciences 68:743-746.