A STUDY OF THE PSEUDOTYPES OF VSV AND MuLV

A STUDY OF THE PSEUDOTYPES OF VESICULAR STOMATITIS VIRUS AND MURINE LEUKEMIA VIRUSES

by

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SCOPE AND CONTENTS:

The research of this thesis was carried out to understand the biology of production of pseudotypes of Vesicular Stomatitis virus (VSV) and murine leukemia viruses (MuLV). An attempt had also been made to use pseudotype production as a tool to study the intracellular events concerned with the production and availability of murine leukemia viral glycoprotein.

ABSTRACT

Superinfection of murine leukemia virus infected cells with VSV produces a certain proportion of pseudotype particles which have the genome of VSV and envelope antigen characteristics of murine leukemia virus, i.e. these particles cannot be neutralized by anti-VSV antiserum. Pseudotypes of VSV and Friend murine leukemia virus have been neutralized by anti-FLV antiserum. These particles do not form plaques on the cells which are already infected with any of Friend-, Moloney-, Rauscher- or Gross murine leukemia viruses. This shows that this interference effect is an envelope mediated phenomenon which is common to all four of these murine leukemia viruses.

At a high MOI of VSV infection the yield of VSV(FLV) pseudotype particles is reduced suggesting that this is due to direct inhibitory effect of VSV on FLV protein synthesis as well as on the host cell protein synthesis.

Pseudotype particles have been shown to possess the same density and probably the same sedimentation coefficient values as normal VSV, but they have a tendency to form aggregates with non-pseudotype material more readily than do VSV(VSV) particles.

In the studies of intracellular events involved in the synthesis of FLV specific glycoproteins, it has been shown that FLV

glycoprotein is available for pseudotype production some 9 hours after FLV infection. The input FLV genome does not appear to act as a m-RNA for the glycoprotein in these experiments. Addition of actinomycin D after VSV superinfection does not reduce the yield of VSV(FLV) pseudotype synthesis significantly showing that concurrent DNA-dependent transcription is not needed for pseudotype formation. Effect of actinomycin D on pseudotype production in chronically infected FN-3T3 cells has shown that either the m-RNA or the glycoprotein itself is stable during 6 hour time prior to VSV infection.

Experiments with glucosamine have shown that the majority of the FLV glycoprotein necessary for pseudotype production in chronically infected cells is synthesized after VSV infection.

An interesting possibility that unintegrated "proviral" DNA may be capable of being transcribed and giving rise to FLV glycoprotein is suggested by experiments with ethidium bromide.

ABBREVIATIONS

Ab	= antibody
Act D	= actinomycin D
AMV	= avian myeloblastosis virus
ASV	= avian sarcoma virus
Ara C	= cytosine arabinoside
Cpm	= counts per minute
CSA	= cell surface antigens
dATP	= deoxyadenosine triphosphate
dCTP	= deoxycytidine triphosphate
dGTP	= deoxyguanosine triphosphate
DNA	= deoxyribonucleic acid
ds	= double stranded
EDTA	= ethylenediaminotetra-acetic acid
FLV	= Friend leukemia virus
FMR-Gi	= Friend-Moloney-Rauscher-Graffi
FN-3T3	= NIH-3T3 cells chronically infected with FLV
G-AKR	= Gross-AKR
GLV	= Gross leukemia virus
G-3T3	= NIH-3T3 cells chronically infected with GLV
LLV	= lymphatic leukemia virus
ME	= mouse embryo cells
MEM	= Eagle's minimum essential monolayer medium
MLV	= Moloney leukemia virus

MOI	= multiplicity of infection
Mol-3T3	= NIH-3T3 cells chronically infected with MLV
m-RNA	= messenger RNA
MuLV	= murine leukemia virus
M.W.	= molecular weight
NBCS	= newborn calf serum
NDV	= Newcastle disease virus
PBS	= phosphate buffered saline
PFU	= plaque forming unit
RLV	= Rauscher leukemia virus
RNA	= ribonucleic acid
RNP	= ribonucleoprotein
RSA-NIH	= NIH-3T3 cells chronically infected with RLV
RSV	= Rous sarcoma virus
S	= sedimentation coefficient in Svedbergs
SFFV	= spleen focus forming virus
SFU	= spleen focus forming unit
TCA	= trichloroacetic acid
tris	= tris(hydroxymethyl) amino-methane
TTP	= thymidine triphosphate
UV	= ultraviolet light
VEA	= virus envelope antigen
VSV	= vesicular stomatitis virus

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INTRODUCTION

Following 1930, with the beginning of an era of molecular biology, viruses became "macromolecules" of interest to chemists, biochemists, cell biologists and geneticists. The reason for this interest lies in the fact that viruses are self-replicating macromolecules which carry the genetic information necessary for the production of progeny viruses in the form of RNA or DNA enclosed in a coat made of protein molecules. Some viruses also contain carbohydrates and lipids as part of their essential structure. Since viruses do not posses enzymes necessary for energy metabolism, they use the synthetic capacity of suitable host cells for the synthesis of molecules necessary for the production of new progeny viruses. Viruses are primarily classified on the basis of the kind of nucleic acid they are carrying. Since in this research work RNA viruses were used, the introduction will consider only this virus class.

Following adsorption to and penetration of a suitable host cell, an RNA virus begins a series of biological activities in the cell, result of which may be the death of the cell and release of progeny viruses (e.g. VSV and NDV) or the state of persistent infection of the cell without leading the cell to death. RNA tumour viruses which fall into the second category allow the integration of the viral genetic material into the cellular gene upon infection.

While small RNA viruses, e.g. picornaviruses or RNA phages, because of their relative simplicity provide excellent system for the study of gene expression and protein synthesis, many large RNA

viruses e.g. RNA tumour viruses are of special interest because they produce tumours in animals. A study of these viruses at cellular and molecular level, it is believed, will provide information about the initiation and maintenance of the oncogenic state in susceptible hosts. As far as the viral etiology of human cancer is concerned, the study of RNA tumour viruses of lower species is very important at this time.

Since RNA tumour viruses do not inhibit host cell metabolism, a study of intracellular macromolecular activity of the virus has been more difficult than with more cytopathic viruses. Although during past few years with improved techniques considerable information of virus replication at the molecular level has been obtained as shall be discussed subsequently in this section, the problem of oncogenecity is still far from clear. Since attention in the membrane of the infected cell as a result of virus infection are thought to be important in the oncogenic state, the examination of virus-induced membrane-associated proteins may be a particularly fruitful research area. In 1972 Zavada has produced a pseudotype virus which has the genome of VSV and envelope glycoprotein of RNA tumour viruses. These pseudotype particles have proven to be particularly useful in defining the basis of the host-range characteristics of RNA tumour viruses as shall be discussed subsequently. In the work of this thesis an attempt has been to use pseudotype production to characterise the synthesis and availability of murine leukemia viral glycoprotein in the infected cell. Since in this study both Friend murine leukemia virus which is an RNA tumour virus

and Vesicular Stomatitis virus which is a non-oncogenic cytocoidal RNA virus have been used, information about the characteristics of these two viruses have been given separately in the next few pages.

Classification, Morphology and Physico-Chemical Properties of Oncornaviruses

The best known groups of RNA tumour viruses are the avian leukosis-sarcoma viruses, the murine sarcoma and leukemia viruses and the mouse mammary tumour viruses. In addition to these, primate, amphibian, bovine, feline and reptile tumour viruses have been isolated (Temin, 1971).

Bernhard (1958, 1960) has classified the RNA tumour viruses into three categories called A-type, B-type and C-type on the basis of thin section morphology in the electronmicroscope. Since then, attempts have been made to understand the biology and the structure of these three kinds of particles (Sarkar and Moore, 1968; Sarkar et al., 1971, 1972). B and C-type particles are observed in extracellular fluid and they are produced by budding through the plasma membrane of the infected host cells. On the other hand, the A-type particles are observed only in intracellular locations. The murine leukemia viruses used in this research are C-type particles. This group possesses a central nucleoid and are 100 nm in diameter. Figure 1 describes the maturation of a C-type particle. The crescent-shaped structure of the nucleocapsid is initiated near the cell membrane and the envelope is acquired as the particle buds through the cell membrane (Sarkar et al., 1971, 1972). Figure 2 shows a photomicrograph and a schematic diagram of the internal structure of the mouse leukemia C-type particle. Nermut et al., (1972) have studied the detail structure of three mouse

FIGURE 1

A Schematic Diagram of the Budding of a C-type Particle

The crescent-shaped nucleocapsid is formed below in site of budding giving rise to a hollow sphere which is ultimately budded-off from the membrane and is transformed into a condensed nucleoid.

The drawing is based on the diagram of Sarkar et al. (1971).

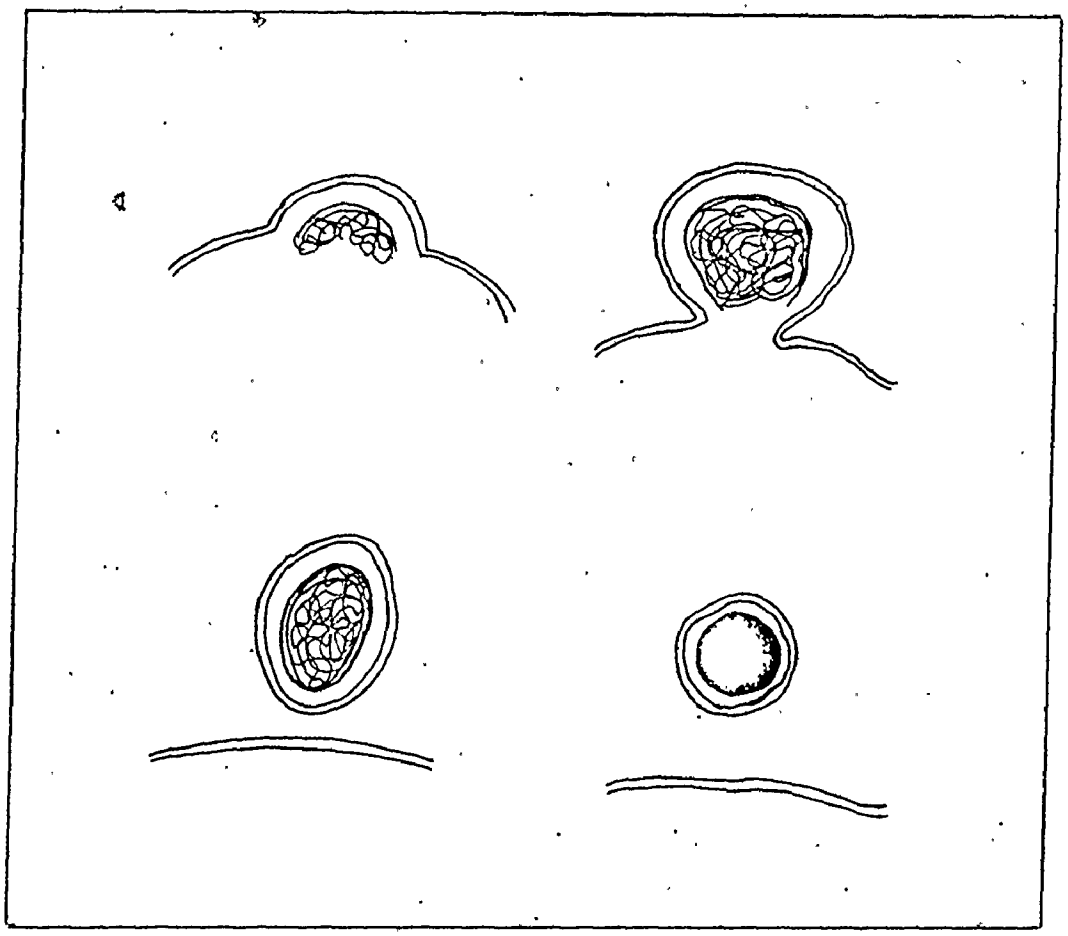


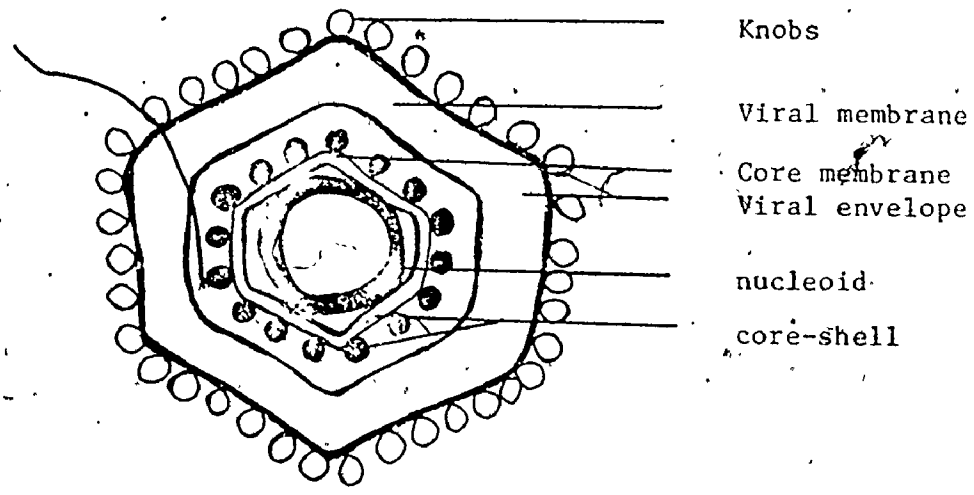
FIGURE 2

Upper Picture: An Electron Micrograph of FLV Particles

The portion of cell demonstrates one mature C-type particle budded-off from the membrane and two more particles budding through cell membrane. Magnification approximately x 150,000. (The electron micrograph is obtained by the courtesy of Mr. Martin Breitman.)

Lower Picture: A Schematic Diagram of Murine Leukemia Virion

The drawing is based on the diagram of Nermut et al. (1972).


Knobs
Viral membrane
Core membrane
Viral envelope
nucleoid
core-shell

leukemia viruses using negative staining, freeze-drying and freeze-etching techniques. A combination of all these methods shows that these particles are roughly spherical and possess knobs at the surface which are weakly bound to the membrane. The knobs have a diameter of about 80 A. The surface is composed of hexagonally arranged subunits. The core shell is made of two components: a smooth membrane layer and another layer of regularly arranged subunits above that. The core appears to be a filamentous structure which may possess helical symmetry.

C-type particles contain about 1% RNA, 60-70% protein, 2% carbohydrate and 20-30% lipids (Beard, 1963; Bonar and Beard, 1959) In addition to RNA, they contain a small amount of DNA (Levinson et al., 1970; Biswal et al., 1971). According to Varmus et al., (1971) this DNA is of cellular origin. The intact virion has a buoyant density of 1.16 - 1.18 gm/ml in equilibrium sucrose density gradient, but the disruption of the lipid-containing envelope by treatment with non-ionic detergent results in the production of the core ribonucleoprotein particle which has a buoyant density of 1.22 - 1.27 gm/ml in the sucrose density gradient (Temin, 1971).

Viral Nucleic Acids

The major RNA of C-type particles as extracted with phenol and run through a neutral sucrose gradient is a 60-70S single stranded RNA with a molecular weight of about 10-12 X 10^6 (Robinson and Baluda, 1965; Duesberg and Robinson, 1966). A 4S RNA species which is referred to as "free" 4S RNA has also been obtained by phenol extraction (Cannani and Duesberg, 1972). Denaturation of the 60-70S RNA gives rise to

RNA species 35S sedimentation coefficient (Duesberg, 1968, 1970) and another 4S RNA species referred to as "associated" 4S RNA (Faras et al., 1973). It has been suggested that the 70S species are made up of two to four 35S species which may be hydrogen-bonded in the larger 60–70S species. While the 70S RNA is the natural template of the virion-associated RNA-dependent DNA polymerase (Baltimore, 1970; Temin and Mizutani, 1970), "associated" 4S RNA probably acts as primer for the synthesis of DNA by RNA-dependent DNA polymerase (Dahlberg et al., 1974; Faras et al., 1974). Both of the 4S species contain modified methylated bases and have amino acid acceptor activity (Rosenthal and Zamnicek, 1973; Elder and Smith, 1974). The "associated" 4S primer RNA has been shown to possess tryptophan-acceptor activity (Sawyer et al., 1974).

Other RNA species with sedimentation coefficients of 7S, 18S and 28S are present in RNA tumour viruses (Bishop et al., 1970a, b). While 28S and 18S RNAs appear to be cellular ribosomal RNA, the role and origin of 7S RNA is totally unknown. DNA of unknown function and probably of cellular origin is also present in the virion (Levinson et al., 1970; Biswal et al., 1971).

Enzymes of the Virion

The most important enzyme present in the virions of the RNA tumour viruses is RNA-dependent DNA polymerase or reverse transcriptase (Temin and Mizutani, 1970; Baltimore, 1970). The reverse transcriptase of avian system consists of two polypeptide chains, α (M.W. 110,000) and β (M.W. 69,000) (Kacian et al., 1971). In murine system, in Rauscher

MuLV, it is made of one polypeptide chain of 70,000 molecular weight (Ross et al., 1971). The significance of the presence of this enzyme will be discussed later. Molling et al., (1971) have detected the presence of RNase H in the virion of AMV which can degrade RNA moiety from a DNA-RNA hybrid. In addition to this enzyme, several other enzymes, e.g. DNA endo- and exonucleases (Mizutani et al., 1970, 1971), DNA ligase (Mizutani et al., 1971), phosphatase, hexokinase, nucleotide kinase, lactate dehydrogenase (Mizutani and Temin, 1971), protein kinase (Strand and August, 1971), t-RNA synthetase (Erikson and Erikson, 1972), RNA methylase (Gantt et al., 1971), nucleotidyl transferase (Nakato and Sakamoto, 1971; Faras et al., 1974) have been detected in the virion of RNA tumour viruses. The biological role of many of these enzymes is still unknown. It has been suggested that some of these enzymes may be of cellular origin trapped in the virion during budding (Tooze, 1973) or they are not part of the virion but of the "membrane-limited vesicles" which gets purified with the virion (Temin and Baltimore, 1972).

Viral Proteins

The polypeptide components of the RNA tumour viruses are conventionally identified by their apparent molecular weights on acrylamide gel. The molecular weights, particular characteristics and the intra-virion location of the MuLV proteins are given in Table 1.

The smallest identified polypeptide, p10, is associated with the viral core and has shown strong group-specific activity (Green et al., 1973; Fleissner and Tress, 1973). Recently, Parks et al., (1975) have observed high degree of type-specificity associated with it. Protein

TABLE 1

Molecular Weight, Characteristics and Localization of MuLV Polypeptides

(Table modified from Bolognesi et al., (1974) (BBA review))

Polypeptide	Molecular Weight	Characteristics (Antigen specificity)	Location
p10	10,000	group	Core interior (RNP)
p12	12,000	type, group	Virion Surface
p15	15,000	type	Virion Core
p15(E)	15,000	group	Virion Surface
p30	30,000	type, group interspecies	Core Surface
Gp69/71	69-71,000	type, group interspecies	Virion Surface
Gp45	45,000	-	Virion Surface

p12, most probably, represents a surface component (Bolognesi et al., 1973). Strong group- and type-specific determinants are present on this polypeptide (Tronick et al., 1973; Stephenson et al., 1974a and b). Lilly and Steeves (1974) have suggested that antibody to this molecule may be involved in virus neutralization. It has been suggested that p15 is present in the core (Bolognesi et al., 1973), but it is not associated with the RNA (Fleissner and Tress, 1973). Recently, Ihle et al., (1975) have shown that a 15,000 molecular weight protein is present on the virion surface. In all probability, this latter envelope protein is the same as the group-specific p15E identified by Ikeda et al., (1975) as a new protein of molecular weight 15,000 which serologically distinct from the type-specific core p15 protein. Protein p30 represents a portion of the core shell (Bolognesi et al., 1973) and constitutes about 30% of the total protein of the virion (Gilden et al., 1971). Multiple antigenicities including type-, group- and interspecies specific determinants have been demonstrated on the molecule (Gilden et al., 1971; Strand and August, 1975).

Glycoprotein 69/71 consists of two antigenically related molecules of molecular weights 69,000 and 70,000 (Strand and August, 1973). This is the major constituent of the surface knobs of MuLV (Schaefer et al., 1972; Witter et al., 1973a and b). It contains type-specific, group-specific and interspecies determinants (Interspec II) (Strand and August, 1973; Hunsmann et al., 1974). It also has the capacity to induce neutralizing antibody, to interfere with murine leukemia viruses and to hemagglutinate (Hunsmann et al., 1974; Steeves et al., 1974).

TABLE 2

Classification of MuLV According to their VEAs

(Table taken from Lilly and Steeves, 1974)

Subgroup	Type	Strains
1		Gross
2	a	LLV-Friend, Graffi, Tennant (B/T-L), SimLV, Rowson-Parr
	b	Moloney, Abelson
	c	LLV-Rauseher, Rich, Breyere-Moloney
	d	Buffett (334c)

Until recently, murine leukemia viruses have been classified on the basis of the cell surface antigens (CSA) of the infected cells. Attempt has been made to classify different mouse leukemia viruses on the basis of the virus envelope antigens (VEA). Table 3 shows some of the members of different classes. Since VEAs are present on the surface of the infected cells at the sites of budding (Lilly and Steeves, 1974), the relationship of VEA and CSA is not clearly understood.

Replication of RNA Tumour Viruses

According to Temin's provirus hypothesis (Temin, 1971), the viral RNA of the RNA tumour viruses in a permissive cell acts as a template for DNA synthesis by the reverse transcriptase enzyme. The ds-DNA ("provirus") transcribed from the viral RNA is integrated at specific sites on the cellular DNA and subsequently transcribed into viral m-RNA which gives rise to virus-specific proteins using cellular protein synthesis machinery. Viral RNA which has the same base sequence as the viral mRNA is synthesized by the transcription of the integrated "provirus". Virus specific glycoproteins modify the infected cell membrane and when the virion cores are formed, viruses are released from the cells by budding. Although, many of the steps involved in the above hypothesis are yet to be proved, there are evidences available for certain steps which will be discussed below.

Inhibitors of DNA synthesis block the replication of the RNA tumour viruses only early after infection while inhibitors of DNA-dependent RNA synthesis block replication at all times during the replicative cycle (Bader, 1964, 1966; Temin, 1963, 1967; Vigier and

Golde, 1964). These findings indirectly suggest that DNA synthesis is an essential step in the early hour of virus infection while continual transcription is needed throughout the viral replicative cycle, and DNA is the template for virus specific RNA synthesis. Also, cells after de novo infection with RNA tumour virus, produce virus only after mitosis (Yoshikura, 1970; Fischinger et al., 1975). Using two temperature sensitive mutants of ASV bearing heat labile reverse transcriptase, Linial and Mason (1973), Baltimore et al., (1974) and Varmus et al., (1975) have produced evidence of virus reverse transcriptase activity inside the infected cell. Takano and Hatanaka (1975a and b) have detected the presence of RNA:DNA covalent complex, the possible product of the reverse transcriptase activity in both cytoplasm and nuclei of the infected cells. The cytoplasmic hybrids are of 4-36S and density 1.56 (RNA:DNA = 1:1) and the nuclear hybrid is of 65S size and density 1.48, close to the density of cell DNA. The RNA moiety carries sequence homologous to the viral RNA and only the nuclear hybrid contains sequence homologous to the host DNA. These experiments have also shown that the labelled input viral RNA is conserved following DNA synthesis and is released by some unknown mechanism since the label can be recovered as RNA species.

Regarding the site of "provirus" synthesis from the input viral RNA, there exists some controversy. Varmus et al., (1975) have detected duplex virus-specific DNA in the cytoplasm within 3 hours after virus infection. This result agrees with the autoradiographic results of Hatanaka et al., (1971). Dales and Hanafusa (1972) using the same technique have failed to see any DNA synthesis in the

cytoplasm, while they have evidence that virus directed DNA synthesis probably occurs in the nucleus. Ali and Baluda (1974) have detected virus-specific DNA within 1 hour after infection. Only part of the newly synthesized DNA gets integrated into the cell DNA while the rest of the DNA is degraded. Green et al., (1975) have found that viral DNA synthesis is maximal around 1 to 4 hours after infection. Their results indicate that DNA synthesis takes place in the cytoplasm and the newly synthesized DNA is transported to the nucleus. The virus-specific duplex DNA probably attains a covalently closed circular form prior to integration into the cellular DNA (Varmus et al., 1975; Guntaka et al., 1975).

Evidence of integration of virus-specific DNA into the cellular DNA have resulted mainly from the work done on avian virus infected systems. Using nucleic acid hybridization techniques, it has been shown that when a virus infects a heterologous host which does not contain DNA sequences homologous to the viral RNA, the infection results in the appearance of virus-specific DNA integrated into the host DNA (Varmus et al., 1972, 1973a and b; Baluda, 1972). In a homologous host where the DNA of the uninfected cell contains sequences partially homologous to the viral RNA, it has been observed that additional viral sequences appear in the host DNA after infection (Baluda, 1972; Shoyab et al., 1974a and b, 1975; Varmus et al., 1975). In mammalian systems, Loni and Green (1975) have shown that mouse and rat cells transformed by Harvey and Moloney strains of MSV(MuLV) have increased the levels of virus-specific DNA sequences compared to

normal cells. After the virus-specific DNA gets integrated into the host DNA, viral RNA is probably synthesized by the modified cellular DNA-dependent RNA polymerase (Temin, 1971).

The presence of virus-specific RNA in the infected avian and murine cells has been identified both in producer (Leong et al., 1972; Green et al., 1971; Salzberg et al., 1973) and in non-producer cells (Coffin and Temin, 1971; Tsuchida and Green, 1974), both in nuclear and cytoplasmic fractions. All of these virus-specific RNAs have the same base sequence as the viral RNA. Parsons et al., (1973) have shown that in RSV-infected cells virus-specific labelled RNA appears first in the nucleus, then in the cytoplasm and later in mature virion which suggests again that viral RNA is synthesized in the nucleus of the infected cells. Intracellular viral RNA is composed of three well defined species of the sizes 70S, 35S and 20S (Fan and Baltimore, 1973; Tsuchida et al., 1972; Tsuchida and Green, 1974). Experiments performed by Cannani et al., (1972) suggest that 60-70S RNA is synthesized by the aggregation of 30-40S RNA and some smaller species. Virus-specific m-RNA of the size of 35S has been found to be associated with polyribosomes of the infected cells (Fan and Baltimore, 1973). The function of 20S RNA has not been explored. Using immunological techniques, Shanmugam et al., (1972) and Vecchio et al., (1973) have detected nascent viral polypeptide chains associated with the polyribosomes in cells replicating MSV(MuLV).

Expression of Viral Genes

As Strand and August (1975) have discussed, viral genes are expressed in three different ways: (1) virus structural proteins; these virion proteins have been discussed earlier. (2) transformation factor(s): the existence of this factor in molecular form is unexplored. (3) virus-specific cell surface antigens: this aspect of viral gene expression is discussed below.

Expression of Viral Antigens

After infection, MuLV induces new cell surface antigens (CSA) which are virus coded and have initially been detected mainly by cytotoxic and immunofluorscence tests and recently by immunoelectron microscopy and radioimmune assay (Klein and Klein, 1964; Old et al., 1964, 1966; Geering et al., 1966; Aoki et al., 1970; Tronick et al., 1973). In virus-induced leukemic mice, it has been observed that the leukemic cells acquire new surface antigens which are absent on the surface of the virus particles (Pasternak, 1967; Steeves, 1968). On the basis of the cell surface antigens murine leukemia viruses are divided into two serological categories: G(Gross)-AKR type and FMR-Gi (Friend-Moloney-Rauscher-Graffi) type (Old et al., 1964; Aoki et al., 1966). Both Gross and FMR cell surface antigens have been found in soluble form in the plasma of the infected mice (Old et al., 1965; Aoki et al., 1968). G-AKR antigens are found in soluble form in the sera of normal C57BL mice or in pre-leukemic AKR mice (Aoki et al., 1966, 1968; Hartley et al., 1969).

The question that whether the cell surface antigens (CSA) are the same as the virus envelope antigens (VEA) has not been yet solved. The cytotoxic inhibition test using intact virions shows that the virion surface does not contain antigens equivalent to CSA (Pasternak, 1967; Steeves, 1968; Lilly and Nathenson, 1969), but Friedman et al., (in review by Lilly and Steeves, 1974) have observed that disrupted virions release antigens which share a common specificity with CSA. Steeves et al., (1970) have also found that they could induce immunity to Friend tumour colony-forming cells by using disrupted Friend virus particles. Since monospecific antibodies to p15 virion protein are cytotoxic to FMR assay, Strand and August (1975) have concluded that FMR-CSA antigen is the same as the p15 virion protein. By using immunoelectron microscopy, Aoki et al., (1970) have shown that in Gross leukemic cells virus-budding takes place in specific regions which are distinctly different from regions anchoring G-CSA or H-2 cell surface antigents. These studies probably indicate that the virion acquires CSA (= p15) prior to budding.

Host-Range of MuLV

Hartley et al. (1970) have observed that MuLV can be divided into three host-range groups namely, "N-tropic", "B-tropic" and "NB-tropic". N-tropic viruses grow more efficiently in NIH-Swiss mouse embryo cells than in Balb-C mouse embryo cells while B-tropic viruses show the reverse pattern. NB-tropic viruses (e.g. Moloney leukemia virus) grow in both cell types with equal efficiency. Further experiments have shown that this resistance and susceptibility

are genetically controlled (Pincus et al., 1971; Ware and Axelrad, 1972).

Interference Between Different MuLV

According to Sarma et al., (1967), murine leukemia viruses, i.e. Gross, Friend, Moloney and Rauscher belong to a single interference group because preinfection of cells with any of these viruses induce strong resistance to infection with others.

Vesicular Stomatitis Virus

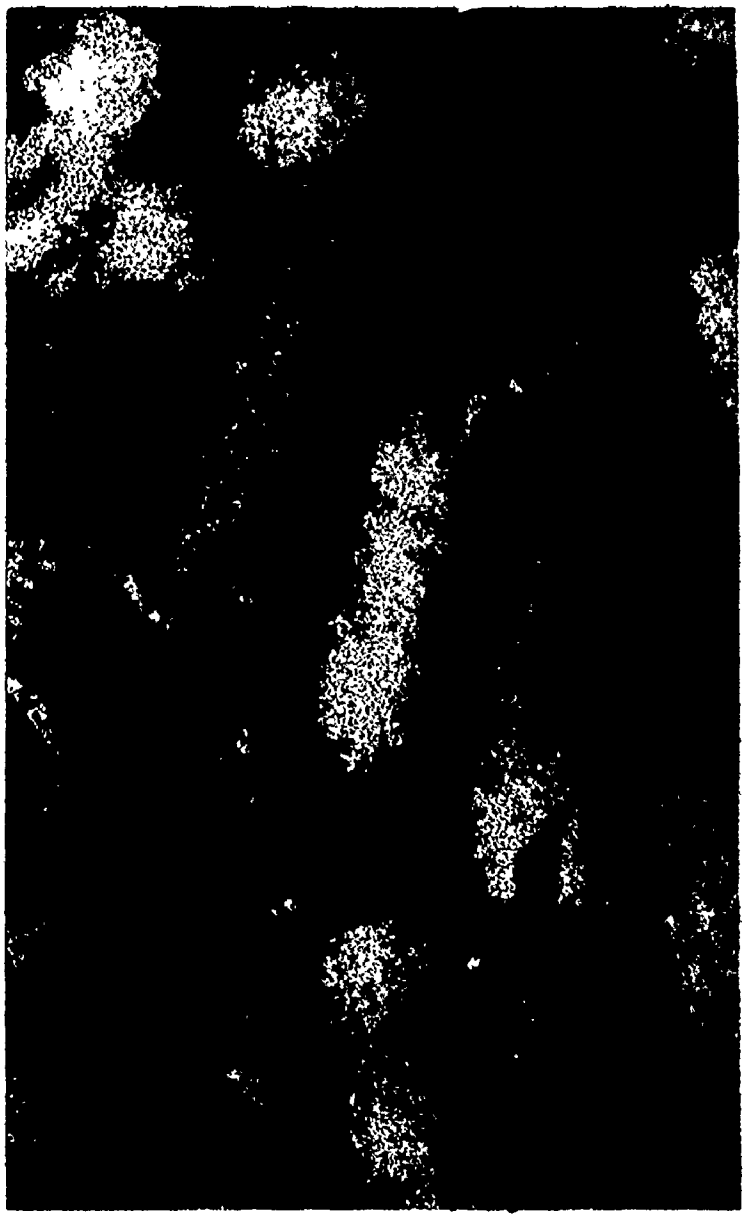
Morphology and Physico-Chemical Properties

Vesicular Stomatitis virus (VSV) is a bullet-shaped virus which belongs to the rhabdovirus group and consists of an internally wound nucleocapsid enclosed in a lipid bilayer envelope (Howatson, 1970). The infectious particle ('B' particle) is 170-175 nm in length and 65 nm in diameter containing a single-stranded RNA genome (Prevec and Whitmore, 1963) of molecular weight 3-4 X 10^6 (Huang and Wagner, 1966) and carries a virion-associated transcriptase (Baltimore et al., 1970). The viruses are released from the infected cells by budding from cell membranes. In L cells the plasma membrane is the maturation site while in chick embryo fibroblasts and in a pig kidney cell line VSV buds from the membranes of intra-cytoplasmic vesicles (Hackett et al., 1968; Zee et al., 1970). In figure 3, a photomicrograph of VSV is presented.

FIGURE 3

An Electron Micrograph of VSV Particles

Magnification approximately x 300,000.



The VS virion contains five major polypeptides which are also found in the infected cells (Kang and Prevec, 1969; Mudd and Summers, 1970; Wagner et al., 1970). These peptides are designated as L (large), N (nucleocapsid), M (matrix), G (glycoprotein) and NS (a nucleocapsid associated phosphoprotein) of molecular weights 190,000, 50,000, 29,000, 69,000, and 45,000 respectively (Wagner et al., 1972). The glycoprotein G which comprises the peplomers of the virion envelope (Cartwright et al., 1970a and b; McSharry et al., 1971) is the major antigenic determinant of intact VSV and is responsible for the production of and reaction with the neutralizing antibody (Kelley et al., 1972; Cartwright et al., 1970a; Kang and Prevec, 1970). G and M proteins are not needed for the virion-associated transcriptase activity while N, NS and L proteins are believed to be required for the transcriptase activity (Emerson and Wagner, 1972, 1973).

Effect of VSV on Host Macromolecular Synthesis

The effect of VSV on the cellular DNA, RNA and protein synthesis appears to depend on three factors: (1) the multiplicity of infection, (2) the cell type used and (3) the type of virus used, e.g. wild type or plaque variant (Bablanian, 1975).

The Indiana serotype of VSV inhibits cellular RNA synthesis in Krebs-2 cells at a rate which is dependent on the multiplicity of infection (Huang and Wagner, 1965; Wagner and Huang, 1966). This inhibitory effect cannot be prevented by irradiating the infecting virus with UV or infecting the cells in the presence of puromycin. Similar inhibition of RNA synthesis by UV-irradiated

New Jersey strain of VSV in primary chick embryo fibroblasts (Yaoi et al., 1970) and by Indiana strain of VSV in primary rabbit kidney and L cells (Yamazaki and Wagner, 1970; Wertz and Younger, 1970) has been observed.

VSV inhibits host protein synthesis significantly in a variety of cell types. The speed of the inhibition of protein synthesis may not be related to the virus-induced inhibition of RNA synthesis (Bablanian, 1975). Yamazaki and Wagner (1970) have observed 80% inhibition of cellular protein synthesis in primary rabbit kidney cells 2 hours after infection with VSV (Indiana type) at an MOI of 100 PFU/cell and 40% inhibition after infection with 10 PFU/cell. Almost similar inhibition has been observed in HeLa and L cells (Mudd and Summers, 1970; Wertz and Younger, 1970).

Huang and Wagner (1966) and Yaoi et al., (1970) have shown that VSV infection inhibits DNA synthesis in Krebs-2 cells and chick embryo fibroblasts. This inhibition is insensitive to UV-irradiation of the virus prior to the infection. Yaoi and Amano (1970) have shown that there is selective inhibition of the flow of G_1 cells to S phase after the infection with VSV. In contrast to the inhibitory effects of most VSV strains, Farmilo and Stanners (1972) have reported that infection with VSV-ts 1026 temperature sensitive mutant allows host DNA synthesis and cell division concurrent with the synthesis of large amounts of viral RNA.

Replication of VSV in Infected Cells

The replication of VSV is independent of any nuclear function as seen best by the fact that VSV is capable of replication in enucleated cells (Follett et al., 1974). In the infected cells VSV produces m-RNA species of size-classes 13-15S and 28S all of which are complementary to viral RNA (Huang et al., 1970; Mudd and Summers, 1970; Schincariol and Howatson, 1972). In a cell-free system transcription of viral RNA by the virion-associated polymerase produces similar RNA species of the sizes 12-18S and 31S (Moyer and Banerjee, 1975). The heterogeneous 12-18S RNA have further been resolved into 17S, 14.5S and 12S species. Morrison et al., (1974) have shown that the 28S species code for the largest protein L. The heterogeneous RNA contains the m-RNAs which codes for the remaining proteins. The 17S RNA codes for a protein of M.W. 63,000 which is probably the non-glycosylated form of G, 14.5S codes for N while 12S codes for both NS and M (Both et al., 1975; Knipe et al., 1975). The 17S species which codes for the glycoprotein G is present exclusively in membrane-bound polysomes while the other messenger species are present in the remaining cytoplasm (Grubman et al., 1974, 1975; Both et al., 1975).

Wagner et al., (1970) in their study with the kinetic of VSV specific protein synthesis in the infected cells have observed that proteins N and NS appear first in soluble form while G and M have never been found in free soluble form, probably they are inserted into the plasma membrane as soon as they are synthesized. On the other hand, Kang and Prevec (1971) have observed that G and N proteins have longer delay while M and L proteins are incorporated into the virion soon after

synthesis. In the final stage, the ribonucleoprotein (RNA and the proteins N, L and NS) probably accumulates in the cytoplasm and then attaches to the cytoplasmic membrane which harbor G and M proteins and then buds out as completed virion acquiring the envelope containing the G and M proteins.

Pseudotypes of 'Vesicular' Stomatitis Virus and RNA tumour Viruses

It has been reported by Zavada (1972) that superinfection of murine leukemia virus- or avian myeloblastosis virus-infected cells with VSV results in the production of pseudotype particles representing a small fraction (0.1 - 1%) of the total wild type VSV yield. These pseudotype particles contain the genome of VSV but have the envelope antigens characteristics of MuLV or AMV. Hence, these particles cannot be neutralized by anti-VSV antiserum but are susceptible to corresponding anti-tumour virus antiserum. Also, because of their envelope properties these pseudotype particles are restricted from infection in the corresponding tumour virus-infected cells.

Zavada (1972) has utilized the thermolabile envelope of the VSV temperature sensitive mutant, ts-0-45, to select for pseudotype particles. Due to a mutation in the glycoprotein (G), viruses containing this protein are inactivated by heating at 45°C (Deutsch and Berkaloff, 1971). Avian myeloblastosis virus-infected cells superinfected with ts-0-45 mutant produce a population of virus yielding VSV plaques after heating at 45°C. The heat resistant population is sensitive to anti-AMV antiserum but totally resistant anti-VSV antiserum showing that pseudotype particles carry the VSV nucleocapsid with an AMV envelope.

Examination of the progeny of pseudotype infection reveal that the host-range properties and susceptibility to antiserum are completely identical to that of original wild type VSV showing that the original VSV genotype is indeed present in the pseudotype particles (Zavada, 1972).

Zavada et al., (1972) have produced pseudovirions of VSV using the MaTu cell line which is derived from human mammary carcinoma. Attempts to demonstrate the presence of any virus particle in MaTu cells have not been successful. The pseudotype particles produced are resistent to anti-VSV antiserum and do not plaque well on MaTu or human HeLa cells. Of a large number of sera obtained from both normal individuals and those with tumours including carcinoma, the pseudotypes have been neutralized only by the sera of two mammary carcinoma patients. Zavada et al., (1975) have also obtained pseudotypes with two more human tumour cell lines. One human cell line (PCA) which probably sheds some C type particles has not produced any anti-VSV antiserum resistant pseudotypes, instead it has produced pseudotypes with VSV genome covered with an envelope containing varying proportions of antigens of both VSV and PCA virus. All these experiments suggest a possibility of detecting latent virus particles or the expression of viral antigens in human tumour cells.

Since Zavada's first report on pseudotypes of VSV and RNA tumour viruses, several laboratories have attempted to use pseudotype production as a tool to study certain phenomenon such as host range specificity of mouse leukemia viruses (Huang et al., 1973; Koentris et al., 1973) and of avian tumour viruses (Boettiger et al., 1975). Using pseudotypes of VSV of both N- and B-tropic

murine leukemia viruses, Huang et al., (1973) and Koentris et al., (1973) have shown that host restriction of murine leukemia viruses is an intracellular phenomenon, since pseudotypes of VSV containing the envelope of either N- or B-tropic MuLV grow equally well on NIH-3T3 or Balb-3T3. Love and Weiss (1974) have shown that VSV forms pseudotypes with chick cell-associated helper factor (chf). Boettiger et al., (1975) have reported that mammalian cells are susceptible to the pseudotypes of VSV and avian tumour virus of subgroup D and avain sarcoma virus B77, but are resistant to the pseudotypes formed with avian viruses of other strains. The efficiency of penetration of VSV pseudotypes on mammalian cells has been found to be higher than the efficiency of transformation by the corresponding avian tumour virus on the same cells. This result indicates that block in the transformation expression in the mammalian cells is not penetration, but a post-penetration intracellular step.

Purpose of the Study

As has been mentioned, RNA tumour viruses do not inhibit host cell macrmolecular events, thus making the virus-induced intracellular events rather difficult to study. The phenotypic mixing of VSV with RNA tumour viruses in which VSV nucleocapsids acquire the envelope glycoproteins and hence the neutralizing antigens of MuLV, provides a system to study the course of events regarding the synthesis and availability of the MuLV glycoprotein inside the infected cells. Furthermore, as Weiss et al., (1975) have suggested, pseudotypes can be an excellent model to study virus assembly and

the structural and functional relationship of the envelopes of different enveloped viruses. Pseudotypes of VSV applied to the detection of latent virus or viral antigenic expression in the tumour cells have considerable potential in the study of the viral aetiology of human cancer. To explore all these possibilities, it was first necessary to define the biology of pseudotype virus. In the work of this thesis an attempt has been made to detect and characterize VSV(MuLV) pseudotype particles and to use them to characterize some of the intracellular events in the synthesis of MuLV glycoproteins.

MATERIALS AND METHODS

1. Media and Reagents for the Growth and Maintenance of Tissue Culture

All tissue culture media and reagents were purchased from Grand Island Biological Company, Grand Island, N.Y. (GIBCO), unless otherwise stated. Since different cell lines have somewhat different cultural and media requirements, three types of tissue culture media were employed during the course of this work.

a. Eagle's Minimum Essential Medium (MEM) with Earle's balanced salt solution was supplemented with 10% (v/v) heat-inactivated (56°C for 30 mins) newborn calf serum (NBCS), and 100 units/ml of penicillin and 100 mcg/ml of streptomycin.

b. McCoy's 5a medium supplemented with 10% NBCS and penicillin-streptomycin of the concentration noted above was used for the growth and maintenance of chronically infected and uninfected 3T3 mouse lines.

c. Joklik - modified MEM supplemented with 5% NBCS was used for the growth of mouse L cells in suspension culture.

For routine passage of monolayer cultures, cells were detached from the glass surface after removing growth medium by one wash with prewarmed PBS (see below) followed by 2 ml of trypsin - EDTA solution (0.05% trypsin and 0.02% EDTA in PBS). After incubation at 37°C for approximately 5 minutes the detached cells were diluted in medium supplemented with 10% NBCS, counted in a haemocytometer and appropriate volume transferred to new culture bottles.

For separation of embryo cells from tissues, minced tissues were dispersed with 5% (w/v) bacto-trypsin in PBS. The bacto-trypsin was purchased from DIFCO Laboratories, Detroit, Michigan.

2. Other Chemicals and Solutions

A. Phosphate Buffer Saline (PBS) was prepared without calcium and magnesium (Dulbecco and Vogt, 1954). 1000 ml PBS (pH 7.5) contains:

Sodium Chloride	8000 mgm
Potassium Chloride	200 mgm
Na_2HPO_4	1150 mgm
KH_2PO_4	200 mgm

B. Sodium Citrate Saline

Potassium Chloride	1.0 gm
Sodium Citrate	0.44 gm

made upto 100 ml with glass distilled water.

C. STE-Buffer

NaCl	0.1 M
Tris(hydroxymethyl) Aminomethane. HCl	0.01M, pH 7.0
EDTA	0.001 M

D. Bouin's Fluid

Picric acid (1.4 g./100 ml H_2O)	75 ml
Formalin (40% Formaldehyde)	25 ml
Glacial Acetic Acid	5 ml

E. Toulene Based Scintillation Fluid

2,5-Diphenyloxazole (PPO)	4 gm
1,4-Bis(2-(5-Phenyloxazolyl))-Benzene (POPOP)	0.3 gm
Toulene	1000 ml.

All chemicals were purchased from Fischer Scientific Company and were all reagent grade.

3. Growth and Maintenance of Tissue Cultures

A. Mouse Embryo Cells (ME Cells)

Approximately 14 day-old mouse embryos were used to prepare primary ME cultures. The embryos were minced in a sterile petri dish (60 mm) in PBS. The minced tissues were then transferred to a sterile Erlenmeyer flask and suspended in 50 ml of 0.5% of Bactotrypsin solution prepared with sodium citrate saline. The suspension was stirred on a magnetic stirrer at 37°C for 10 - 15 mins. The larger cell aggregates were allowed to settle and the supernatant was decanted. Another 50 ml volume of 0.125% bacto-trypsin solution was then added to the remaining tissue. The suspension was again stirred at 37°C for another 20 mins. and then passed through sterile gauze. The filtrate was centrifuged at 500 g in a clinical centrifuge for about 15 mins. and the pelleted cells were resuspended in MEM containing 10% NBCS. About 5×10^6 cells were seeded in 32 oz. Brockway bottles. After 4-5 days secondary cultures were prepared by subculturing the cells using 3-4 ml of trypsin-EDTA solution to resuspend the cells. These cells were seeded in 32 oz Brockway bottles at 5×10^6 cells/bottle and the secondary

cultures were used when the subconfluent monolayers were formed.

B. 3T3 Cells

Continuous cell lines of swiss NIH/3T3 (Todaro and Green, 1963) and Balb/3T3 (Aaronson and Todaro, 1968) mouse embryo cells were obtained from Dr. C. Pringle, Virology Institute, Glasgow. Both cell lines were grown in monolayer cultures in McCoy's 5a medium supplemented with 10% NBCS in 32 oz Brockway Bottles. Subculturing was routinely done twice weekly.

C. Chronically Infected 3T3 Lines

Swiss NIH/3T3 cells were infected with the Friend leukemia virus to produce chronically infected cell line. Some 2.5×10^6 NIH/3T3 cells were seeded in 32 oz Brockway bottle. The next day the cells were washed with PBS, then incubated with 10 ml of 10 µg/ml of Polybrene (obtained from Aldrich Chemicals) solution at 37°C for 1 hour and then the cells were infected with Friend leukemia virus at an MOI of 10 XC focus-forming units/ml. The infected cells were grown in McCoy's medium containing 10% NBCS with regular subculturing when confluency was attained. The resulting cell line sheds Friend leukemia virus continuously and served both as a virus source and test cell line in experiements described in this thesis. This cell line is designated FN-3T3 (Friend-NIH-3T3).

Lines of NIH-3T3 cells chronically infected with either Moloney leukemia virus or Gross leukemia virus were produced identically

as described for the FN-3T3 line except that Moloney leukemia virus or Gross leukemia virus was used to infect the NIH-3T3 cell line. These cell lines are designated as Mol-3T3 or G-3T3 respectively.

An NIH-3T3 cell line chronically infected with Rauscher leukemia virus was obtained from Dr. C. R. Pringle, Institute of Virology, Glasgow. This line has properties similar to those of FN-3T3 and continuously produce Rauscher leukemia virus.

D. XC Cells

This is a continuous cell line obtained from a tumour induced in a rat by Rous sarcoma virus (Svoboda, 1961). The cells show a transformed morphology but do not shed infectious virus particles. The cells were grown in monolayer cultures in Eagle's MEM containing 10% NBCS in 32 oz Brockway bottles and routinely subcultured twice weekly.

E. L Cells

This is a subline, L-60, of Earle's continuous mouse L cell line (Earle, 1943). The cells were grown in suspension culture at 37°C in Joklik modified MEM supplemented with 5% NBCS. The cells were kept in constant exponential growth phase by daily dilution.

4. Source and Growth of the Viruses

A. Friend Leukemia Virus (FLV)

The N-tropic strain of FLV(F-S) was obtained from Dr. A. Axelrad, University of Toronto as mouse spleen extract. The virus was grown both

in vitro tissue culture and *in vivo* in NIH swiss mice. To grow the virus *in vitro*, ME or NIH/3T3 cells were infected with the virus as described in section 3(c). At least after a week of subculturing the infected cells, the virus was harvested by collecting the supernatant of the confluent cell cultures (FN-3T3) and then centrifuging the supernatant at 500 g for 10 - 15 mins. This final supernatant was stored at -70°C.

To grow stock virus *in vivo* 0.5 ml volume of the virus *in vivo*, appropriately diluted in saline was infected into the tail vein of mice. Three weeks after injection the enlarged spleens were collected and homogenised using a sterile mortar and pestle with sufficient precooled PBS to produce a 25% (weight/volume) suspension. After homogenization the suspension was centrifuged at 500 g for 15 mins to remove the cell debris. The supernatant was then collected and centrifuged again at 7000 g for 15 minutes for further clarification of the virus pool. This final supernatant was stored as virus stock at -70°C.

B. Moloney Leukemia Virus (MLV)

The N-B tropic Moloney virus was obtained from Dr. A. O. McCarter, University of Western Ontario. The virus was routinely obtained by culturing the supernatant from the Mol-3T3 cells just when they attain confluency. Cells and debris were removed by centrifuging at 500 g for 15 mins and the supernatant was stored at -70°C.

C. Rauscher Leukemia Virus (RLV)

The N-B tropic Rauscher leukemia virus was obtained from supernatant harvests of RSA-NIH cells identically as described for Moloney leukemia virus.

D. Vesicular Stomatitis Virus (VSV)

(i) HR-LT strain of Indiana serotype of VSV used in most of the studies was originally obtained from Dr. Howatson, Ontario Cancer Institute, Toronto (Nakai and Howatson, 1969).

To grow the virus, L cells from a growing suspension culture were collected by centrifuging at 400 g for 10 minutes and then resuspended to a concentration of 10^7 cells/ml in MEM containing 2% NBCS. This concentrated cell suspension was infected with HR-LT strain of VSV (Ind.) at an MOI of 0.1 PFU/cell and the virus was adsorbed at 37°C for 45 minutes. After the adsorption period sufficient MEM containing 2% NBCS was added to bring the concentration of cells to 10^6/ml. This cell suspension was incubated at 37°C for about 18 hours and then the suspension was centrifuged at 400 g for 15 mins to remove the cells and the cell debris. The supernatant containing the virus was stored at -70°C.

(ii) A temperature sensitive mutant of the Indiana serotype of VSV (ts-0-45) was obtained from Dr. Pringle, Glasgow. A stock of ts-0-45 virus was produced by infecting L cells essentially as described for the HR-LT strain above except that virus growth was carried out at 32°C for 24 hours.

5. Preparation of Concentrated Virus Stocks

The same procedure was followed for concentrating either VSV or mouse leukemia viruses. VSV grown in L cells on leukemia viruses grown in ME or NIH/3T3 cells as described in section 4 were first clarified by centrifuging at 400 g for 15 mins. The supernatant, free of large cell debris, was centrifuged at 34,000 g for 165 mins at 4°C in a Spinco type 19 rotor to pellet the virus. The virus pellet was resuspended in PBS-0.002M EDTA in 1/100th of the original volume and the concentrated virus stock was stored at -70°C.

6. Assay of the Viruses

A. VSV Plaque Assay

To determine the infectivity of the VSV stock, 0.1 ml of the appropriately diluted virus stock was added directly on a full monolayer of susceptible cells, usually seeded one day before, in Falcon petri dishes (60 mm x 15 mm). Viruses were allowed to adsorb to the cells for 45 mins at 37°C, after which the cell sheets were overlaid with 5 ml of solution containing MEM, 5% NBCS and 0.9% of Noble Agar (DIFCO). After 20 - 24 hours at incubation at 37°C or in some cases at 32°C in an humid atmosphere of 5% CO_2 in air plaques could be observed. Cell sheets were then fixed with Carnoy's fluid (Ethanol: Glacial Acetic Acid = 3:1) and after removing the agar overlay the plaques were scored. The titer was expressed as plaque forming unit /ml (PFU/ml).

B. Assay of the Mouse Leukemia Viruses

(i) XC Assay: This assay used by Rowe et al., (1970) is based on the observation made by Klement et al., (1969) that when XC cells come in contact with the mouse leukemia virus infected cells, the XC cells fuse with each other and form syncytia. This produced a visible plaque-like area in a cell monolayer. The following standardized method was used in this work. Some 2×10^5 ME or 3T3 cells were seeded in Falcon petri dishes (60 mm x 15 mm) in 4 ml medium. The next day the medium was removed and the cells were washed with 2 - 3 ml of PBS. After washing, the cells were incubated with 2 ml of 10 µg/ml of Polybrene (Aldrich chemicals) solution at 37°C for 45 - 60 mins. After the incubation, 0.1 ml of appropriately diluted virus was added to the cells and the cells were incubated again in the presence of the virus for 45 - 60 mins at 37°C. During this period, the petri dishes were rotated 2 or 3 times to allow maximum adsorption of the virus. After the adsorption period 4 ml of fresh medium was added to the petri dishes. This medium was changed after 3 days of infection. On the 5th day of infection, the medium was removed and the cells were exposed for 30 seconds to UV irradiation at approximately 67 ergs/mm^2/sec. Approximately 1×10^6 XC cells were then added to each petri dish in 4 ml medium. After 1 or 2 days the medium was changed and fresh medium was added. After 3 - 4 days, the cells were fixed with 70% Methanol for 30 minutes and stained then with 1:20 times diluted Giemsa stain solution of Fischer Scientific Company. The areas where XC cells formed syncytia appeared as transparent plaques. Under the microscope it was observed that

these plaques were surrounded by giant cells and this way they could be distinguished from non-specific holes on the cell sheet. The number of plaques on the dishes were counted. The titer was expressed as plaque forming unit/ml (PFU/ml).

(ii) Spleen Focus Assay: This assay was used to titer the FLV (grown in vivo) only. It had been observed by Axelrad and Steeves (1964) that when the Friend virus stocks were injected in susceptible mice intravenously in different dilution, macroscopic foci were formed on the spleens. The number of foci formed on the spleen were dose dependent. This dose dependency was the basis of this assay.

In this study 0.5 ml of appropriately diluted Friend virus stocks were injected intravenously into mice. After 9 days, the spleens were removed and fixed in Bouin's fluid. After fixation the foci on the spleens were counted and the titer of spleen focus forming activity of the virus stock was determined. The titer was expressed as spleen focus-forming unit/ml (SFU/ml).

(iii) Reverse Transcriptase Assay: The enzyme assay method followed in this work was based on the method of Baltimore and Smoler (1971). Table 3 shows the standard reaction mixture with concentrations of the reactants. Warburg and Christian's formula (1941) given below was employed to determine the protein concentration: $1.54\ A_{280} - 0.76\ A_{260}$ = protein concentration in mg/ml, where A_{280} is the absorbancy at 280 nm and A_{260} is the absorbancy at 260 nm respectively.

TABLE 3

Reaction Condition of the RNA-Dependent DNA Polymerase of FLV

Tris-HCl (pH 7.8)	0.05 M
NaCl	0.06 M
Dithiothreitol	0.02 M
$MnCl_2$	0.05 mM
TTP	0.02 mM
Triton-X-100	0.28 %
Poly A	420 pmoles
$(dT)_{12-18}$	170 pmoles.
^3H-TTP	3.3 nmoles (220 dpm/pmole)
Viral protein	9.5 - 10.0 µg

Reactions were performed in a 0.1 ml volume. The reaction mixture was incubated for 45 mins, unless otherwise mentioned, at 37°C. The reaction was terminated by the addition of 0.5 ml of 0.08 M sodium pyrophosphate and 0.5 ml of 25% TCA. The precipitate formed was then filtered through membrane filters. The filters were washed a few times with 10% TCA and then dried and counted in a scintillation counter.

7. Preparation and Analysis of Sucrose Gradients

A. Buoyant Density Analysis

Linear gradients of 15 - 60% sucrose dissolved in PBS -.002M EDTA were prepared in 5 ml tubes using a Buchler gradient maker. Tubes were then placed in SW 50 rotor buckets. Some 0.2 ml of virus preparation was layered on the top of the gradient. Centrifugation was carried out at 100,000 g for 3 hours at 4°C in a L2-65B Beckman Ultracentrifuge. The gradient fractions were collected by puncturing the bottom of the tube. The density of the samples was determined by weighing known volumes of samples.

B. Sedimentation Velocity Analysis

Linear gradients of 5 - 40% sucrose dissolved in PBS-0.002M EDTA were prepared in 18 ml volumes using a Buchler gradient maker. 0.5 ml of virus preparation was layered on the top of the gradients. Tubes were then placed in the buckets of SW 27.1 rotor and the centrifugation was carried out at 81,000 g for 35 mins at 4°C. Fractions were collected by puncturing the bottom of the tubes.

8. Preparation and Analysis of Potassium Tartrate Density Gradients

Thirty-five percent solution of potassium tartrate (Fischer Chemicals) with 0.002M merceptoethanol was used. Five ml of the solution was taken in the centrifuge tubes of the SW 50 rotor and 0.2 ml of the virus material was layered on the top of the solution. After centrifugation at 100,000 g for 48 hours at 4°C fractions were

collected by puncturing the bottom of the tubes. Fraction densities were determined using refractive indices measured on a refractometer (Bausch and Lomb, Abbe 3L) together with a calibration curve of density against refractive index. The calibration curve prepared by measuring the refractive indices of solutions of known weight and volume is shown in Fig. 4.

9. Preparation of Antisera

A. Anti-VSV Antisera

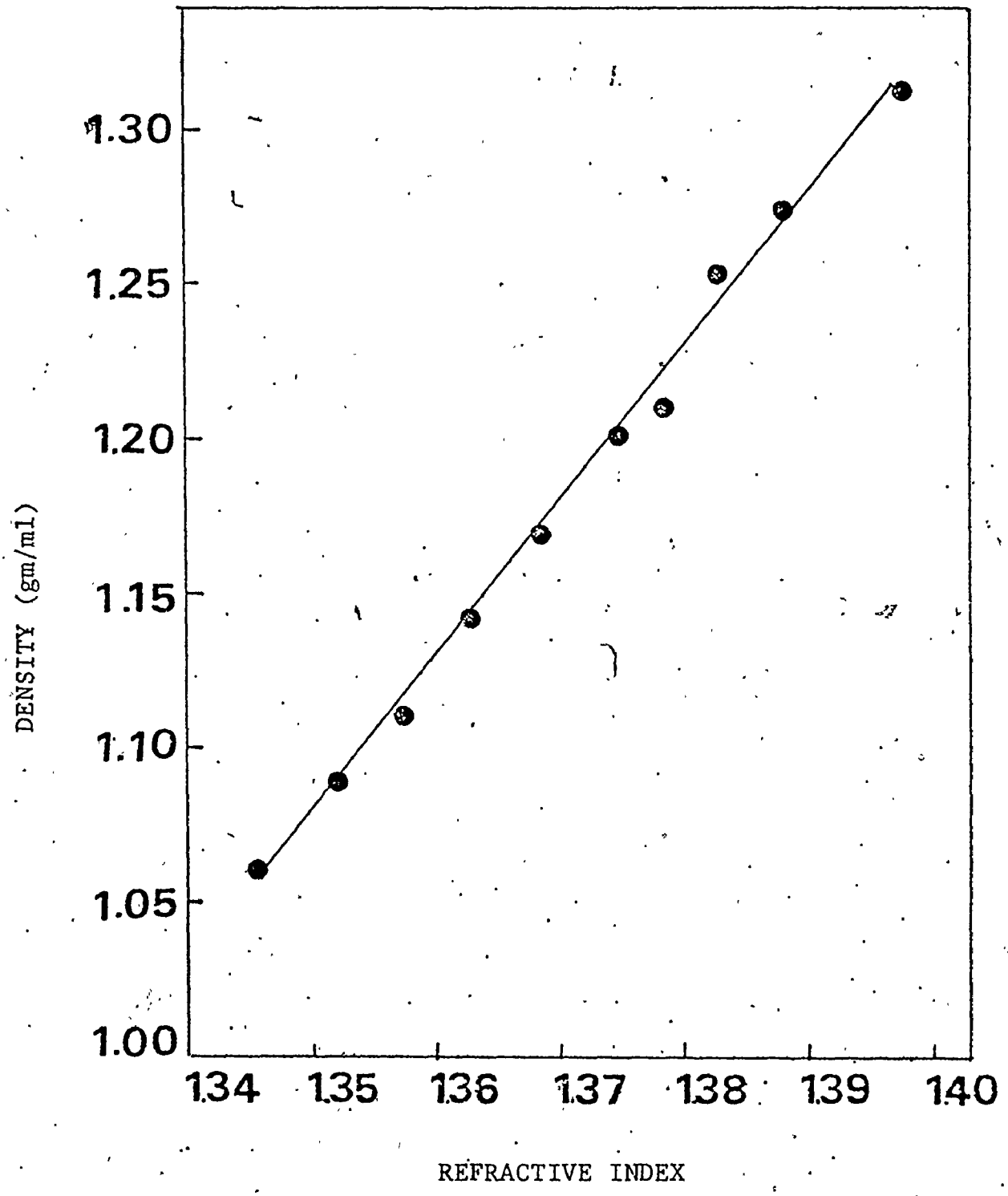
VSV grown in human KB cells, purified by differential centrifugation and rate zonal sedimentation on sucrose gradients was suspended to a concentration of 10^9 PFU/ml. To produce antisera in rabbits the following primary injections were given: 0.1 ml intramuscularly and 0.5 ml with complete Freund adjuvant intraperitioneally. Two weeks after primary injection, three subsequent similar weekly injections were given. These 3 injections were 0.1 ml intramuscular and 0.5 ml with adjuvant intraperitoneal. One week after the last injection the rabbit was bled, the blood was allowed to clot at $4^{\circ}C$ overnight and the sera stored at $-70^{\circ}C$.

B. Anti-Leukemia Virus Antisera

The antisera was made against either the Friend or the Rauscher leukemia viruses. About 200 - 300 spleen focus forming units were injected intraperitoneally in NIH swiss mice. After 6 - 7 weeks, the blood was collected and allowed to clot overnight at $4^{\circ}C$.

FIGURE 4

Calibration Curve of Densities of Potassium Tartrate Solutions Against Refractive Indices


1.30
1.25
1.20
1.15
1.10
1.05
1.00
DENSITY (gm/ml)
1.34
1.35
1.36
1.37
1.38
1.39
1.40
REFRACTIVE INDEX

Sera from several blood samples were pooled and stored at -70°C.

Before use all the antisera were heated at 56°C for 30 minutes.

Source of Other Chemicals Used in this Research

All radioactive chemicals and dATP, dCTP and dGTP were purchased from Schwazn/Mann. Oligo dT was obtained from Collaborative Research, Poly A from P - L Biochemicals, dithiotheritol was from Calbiochem., glucosamine and ethidium bromide from Sigma Company (St. Louis, Mo.), cycloheximide from Upjohn Company, Triton - X-100 from Hartmann-Leddon Corporation. Actinomycin D was a gift from Merck, Sharp and Dohme Company of Montreal.

RESULTS

SECTION I. Biological Studies of FLV, VSV and the Pseudotypes VSV(FLV)

Studies on the Assay Methods of Murine Leukemia Viruses (MuLV)

XC-Assay for MuLV

Klement et al. (1969) observed that XC, a rat tumour cell line, formed syncytia when placed in contact with mouse embryo (ME) cells which were already infected with murine leukemia viruses. This phenomenon was used by Rowe et al. (1970) as the cytopathic end point for titrating these viruses. When the work of this thesis began, this assay method was not as widely used as it is now, so, some preliminary standardization was needed.

(i) Variation in the Virus Concentration and the Effect on Number of Plaques

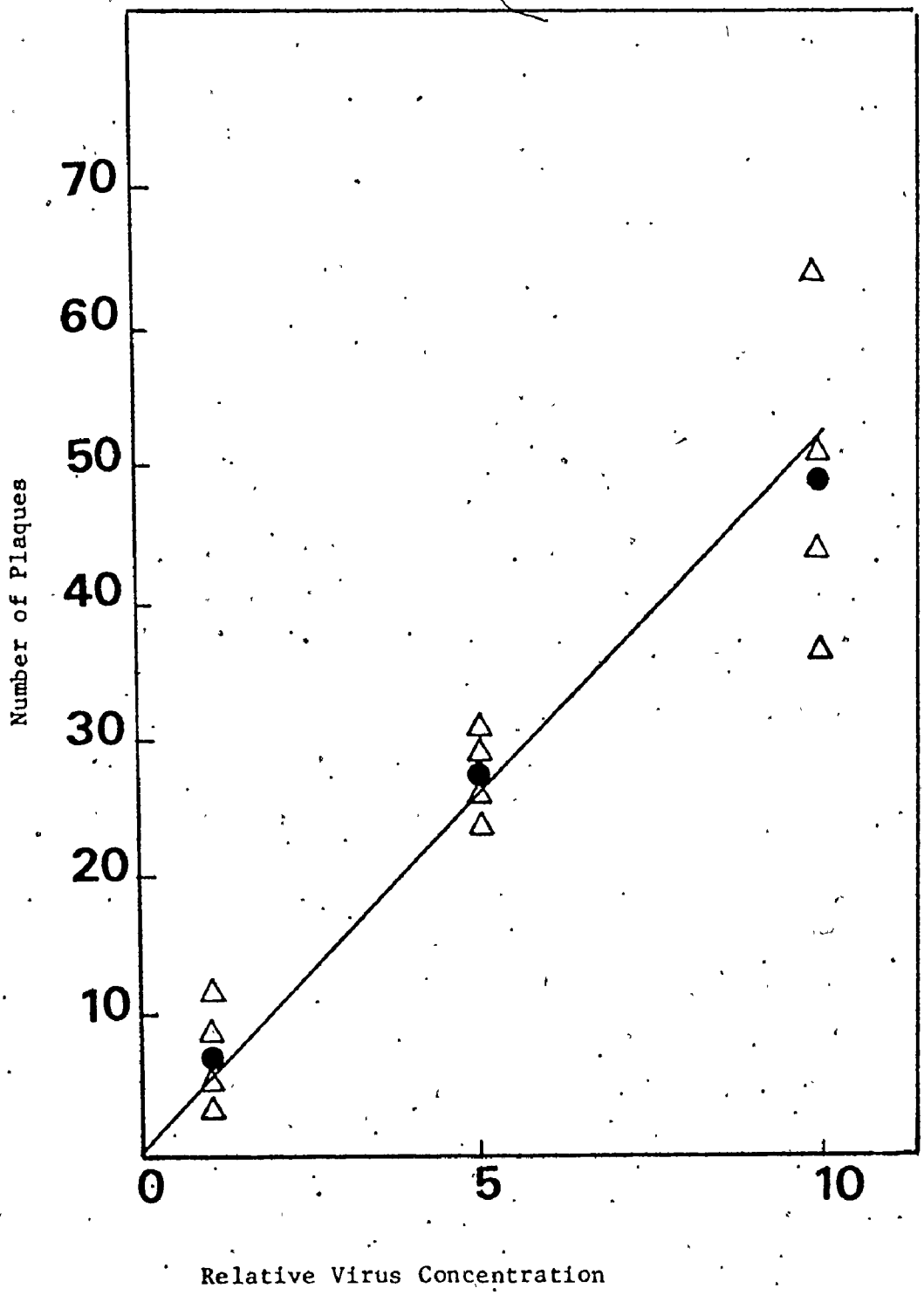
A series of dilutions of the standard Friend leukemia virus (N-tropic) were prepared and assayed by titration on partial monolayer cultures of NIH-Swiss mouse embryo (ME) cells by the XC-assay as described in 'Materials and Methods'. The number of resultant XC plaques as a function of virus dilution are plotted in Fig. 5. Over the concentration range investigated there is a linear relationship between the concentration of the virus used and the number of plaques formed.

FIGURE 5

The Relationship Between the Number of Plaques Formed by XC-assay and Dilution of FLV as Stated in the Graph.

△..... Number of plaques on a single plate

●..... The mean number of plaques formed at different dilutions.


70
60
50
40
30
20
10
0
5
10
Number of Plaques
Relative Virus Concentration

(ii) Effect of Polybrene Concentration on the XC-Assay

Toyoshima and Vogt (1969) showed that the polycation polybrene enhanced the infectivity of those avian sarcoma viruses belonging to subgroup B and C, but not those of subgroup A and D. This shows that the specificity of enhancing capacity of polybrene is similar to that of the polycation DEAE-Dextran. DEAE-Dextran is widely used in enhancing the infectivity of murine leukemia viruses. Stephenson, Reynolds and Aaronson (1973) first used polybrene in their experiments with murine leukemia viruses. The use of polybrene has the advantage over DEAE-Dextran that it is less toxic to the cells and does not need to be washed from cell monolayers after virus adsorption.

The effective polybrene concentration to be used in the XC-assay was determined as follows. Before infection with virus, cells were incubated for one hour in 2 ml of medium containing concentrations of polybrene ranging from 2.5 µg/ml to 25 µg/ml. The medium was then removed and the cells were infected by the addition of 0.1 ml volumes of appropriately diluted virus. After adsorption for 60 mins fresh medium was added and the XC-assay was carried out as described in 'Materials and Methods'. The result presented in Table 4 shows that there is at least a 10-fold increase in observed titer when polybrene was employed in the assay. No significant enhancement of infectivity was observed at polybrene concentrations greater than 10 µg/ml. Since polybrene concentrations above 20 µg/ml caused some cytotoxicity as observed by detachment of

TABLE 4

Effect of Polybrene Concentration on the Infectivity of FLV

Expt.	Polybrene Conc. in µg/ml.	FLV Titer in PFU/ml
	0	1.0×10^6
	2.5	1.2×10^7
	5.0	1.5×10^7
	10.0	5.2×10^7
	15.0	2.3×10^7
	20.0	3.0×10^7
	25.0	3.5×10^7

Different standard stocks of FLV were used for the assays. The FLV titer was determined by the XC-assay as described in the 'Materials and Methods'. Before infection with FLV, cells were incubated for 60 mins at 37°C with 4 ml of McCoy's medium without serum containing the stated amounts of polybrene.

cells from petri dishes, 10 µg/ml of polybrene was routinely employed in the assays in subsequent experiments.

Mouse Spleen Focus-Forming Assay

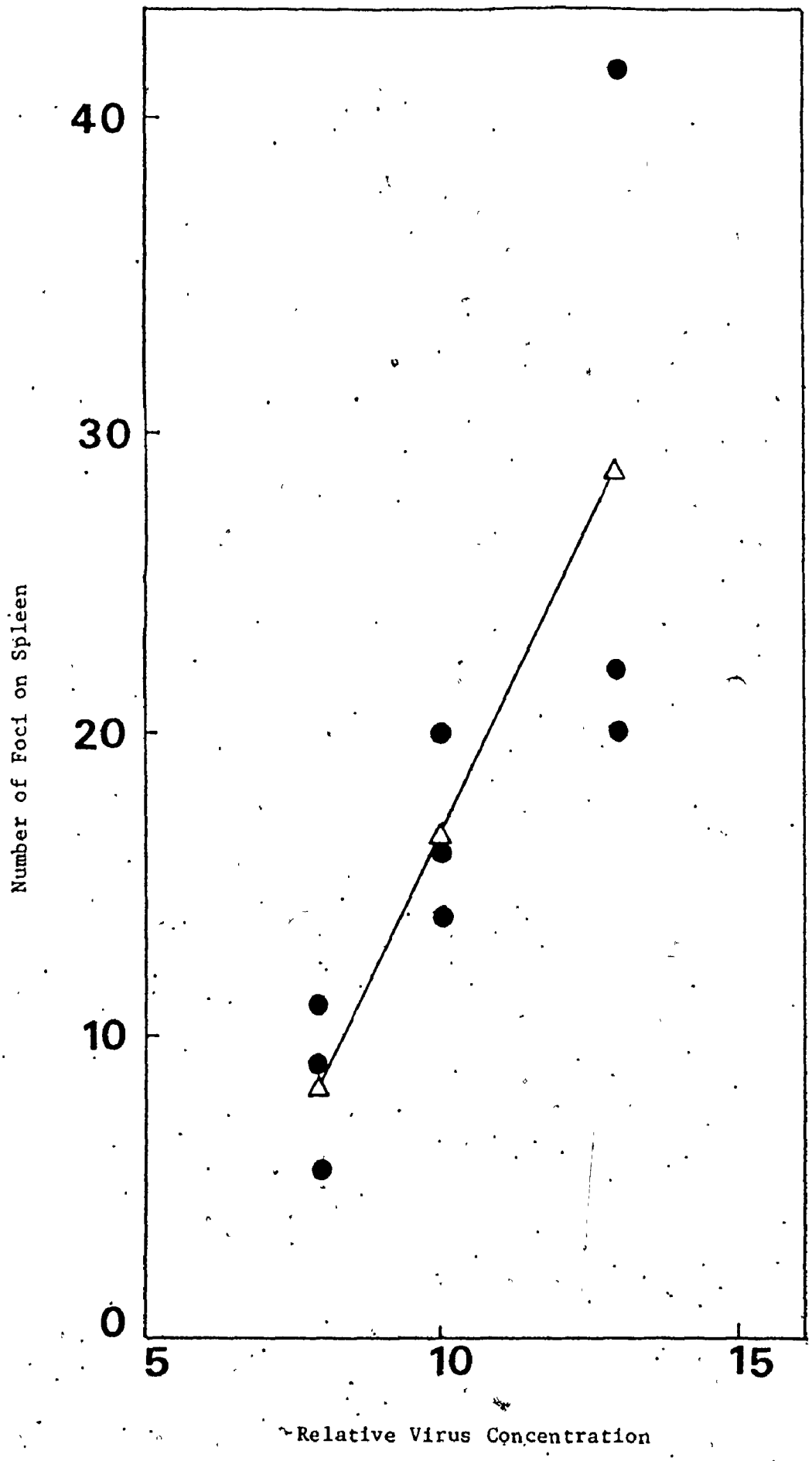
It has been shown that the erythroleukemia-producing virus complex of Friend leukemia virus consists of two components, namely, the spleen focus-forming virus (SFFV) and another agent which will be called lymphatic leukemia virus (LLV) (Dawson et al., 1968; Steeves et al., 1971). SFFV seems to be a defective virus and requires the helper activity of LLV for its replication (Eckner and Steeves, 1971). Since the FLV complex induces localized macroscopic lesions in the spleen of infected mice, Steeves and Axelrad (1963) established an assay based on this observation. Using this spleen focus-forming assay, the SFFV titer was determined in both spleen-grown stock virus and in the Friend leukemia virus harvested from cell lines chronically infected with stock FLV. While the detail of the assay is given in the 'Materials and Methods', it basically involved injection of appropriately diluted virus intravenously into mice, nine days after which the mice were sacrificed and the spleen colonies were counted after fixing and staining. In Fig. 6 the number of foci on the spleen is plotted against the virus dilution. The result is not inconsistent with a linear dose-response. At higher doses, the foci on the spleen were too numerous to be counted. Of more consequence concerning the subsequent work in this thesis is the fact that only the *in vivo* grown FLV stock responded to this assay. The virus which was passed several

FIGURE 6

Relationship Between Number of Foci Formed on a Spleen and Dilution of FLV

..... Number of foci on a single spleen

..... Mean number of foci formed at a particular dilution.


40
30
20
10
0
5
10
15
Number of Foci on Spleen
Relative Virus Concentration

times in fibroblastic tissue culture (ME or NIH-3T3) did not produce any foci on the spleen. This is in agreement with experiments carried out in Dr. Axelrad's laboratory (personal communication) which suggests that only the LLV component of the Friend virus complex replicates in tissue culture.

Reverse Transcriptase Assay

The enzyme assay method followed in this work is given in 'Materials and Methods'. Table 5 shows the incorporation of ^{3}H-TTP into an acid insoluble product catalysed by partially purified *in vitro* grown FLV. The template-primer complex, poly rA.dT was essential for activity as was the presence of $MnCl_2$. Fig. 7 shows that the enzyme-catalysed incorporation was linear with time for at least 45 minutes at 37°C. As seen in Fig. 8, there was a linear relationship between the concentration of viral protein per reaction volume and the amount of ^{3}H-TTP incorporated.

In order to determine the optimum substrate concentration for the crude virus enzyme preparation, the synthesis of product was studied as a function of the TTP concentration. For this experiment the amount of radioactive TTP was kept constant and the specific activity was diluted by the addition of unlabelled TTP to the desired concentration. The amount of product synthesized after 45 minutes was calculated from the counts per minute of ^{3}H-TTP incorporated and the known specific activity. As seen in Fig. 9 the optimum substrate concentration was approximately 0.1 M.

TABLE 5

Incorporation of ^{3}H-TTP by RNA-dependent DNA Polymerase of FLV

Reaction System	cpm Incorporated
Complete reaction mixture	8,363
Complete, without the exogeneous template	83
Complete, without $MnCl_2$	221
Complete, reaction mixture kept at 4°C	250

The reaction condition was the same as described in 'Materials and Methods' section. The reaction mixture was incubated at 37°C for 45 mins. 9 - 10 µg of viral protein was used.

FIGURE 7

Kinetics of Incorporation of ^{3}H-TTP into Acid Insoluble Product by the Reverse Transcriptase Enzyme

Reaction system was the same as described in 'Materials and Methods'. 40 - 50 µg of viral protein was used. Several tubes obtained the same mixture were incubated at 37°C and samples were removed at the indicated times and were precipitated with cold TCA. The mean of triplicate samples are plotted against time.

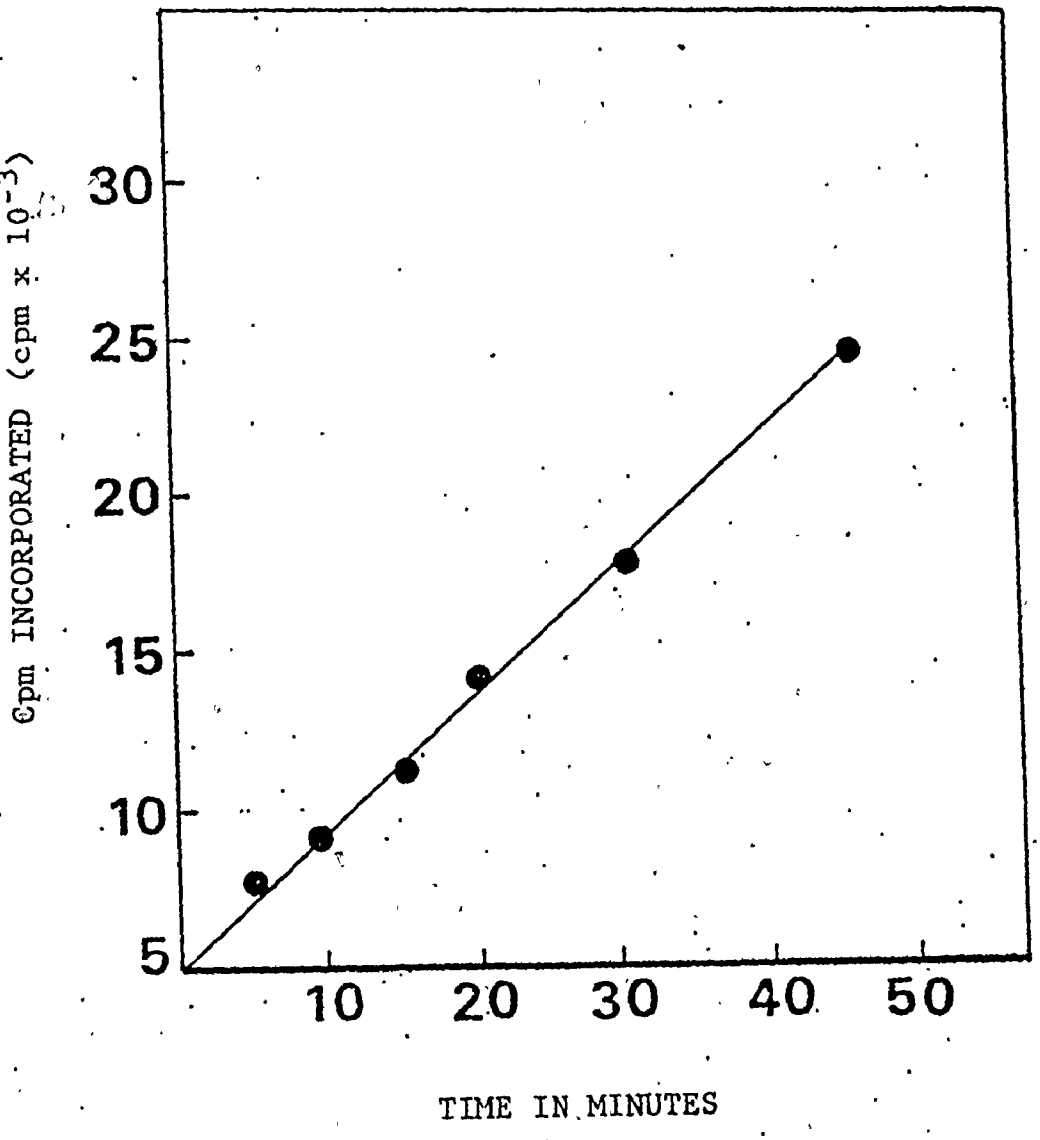

Cpm INCORPORATED (cpm x 10^{-3})
30
25
20
15
10
5
10
20
30
40
50
TIME IN MINUTES

FIGURE 8

Incorporation of ^{3}H-TTP as a Function of Protein Concentration

Reaction system was the same as described in 'Materials and Methods' excepting the protein concentrations. Samples containing different amount of protein were incubated at 37°C for 45 minutes.

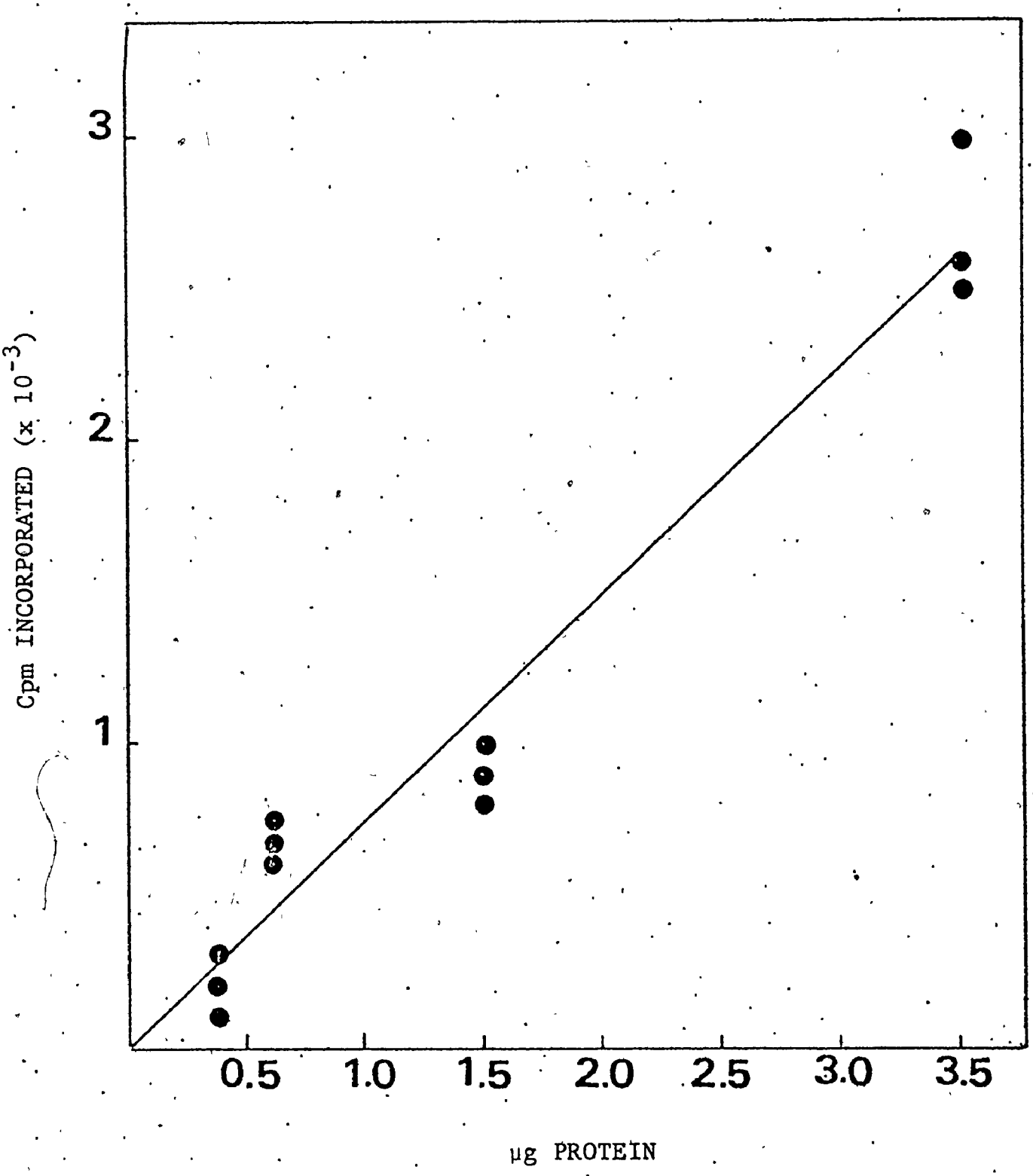
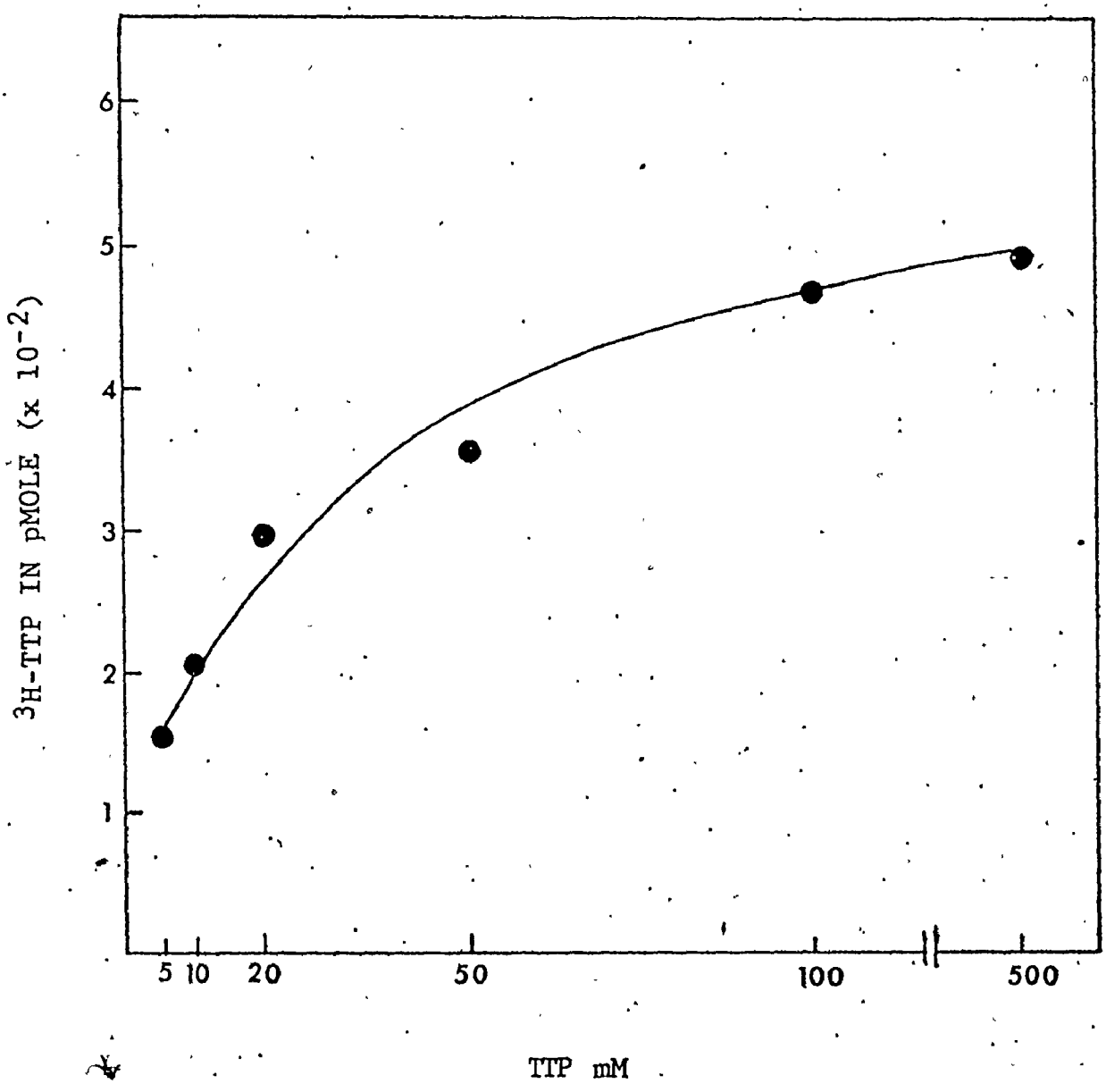

3
2
1
Cpm INCORPORATED (x 10^{-3})
0.5
1.0
1.5
2.0
2.5
3.0
3.5
µg PROTEIN

FIGURE 9

Effect of TTP Concentration on ^{3}H-TTP Incorporation

Reaction system was basically the same as described in 'Materials and Methods', excepting that the concentration of unlabelled TTP was varied to desired concentrations while the concentration of labelled ^{3}H-TTP was the same. Viral protein concentration was 40 - 50 µg per assay. Incubation was done for 45 minutes at 37°C.


6
5
4
3
2
1
^{3}H-TTP IN pMOLE (x 10^{-2})
5 10 20 50 100 500
TTP mM

McDonnell et al., (1971) have found that incorporation of ^{3}H-TTP into an acid insoluble product by the reverse transcriptase enzyme complex is partially sensitive to the inhibitory effect of actinomycin D. By analysing the enzymic product they have concluded that this is due to the inhibition of DNA-dependent DNA polymerase activity of the enzyme complex. The RNA-dependent DNA polymerase activity remains unaffected. In the next experiment, the effect of actinomycin D was observed in the enzyme reaction using both endogeneous and exogeneous templates. Table 6 shows that the exogeneous reaction was unaffed by actinomycin D. Since actinomycin D inhibits by intercalating at G-C regions of double stranded nucleic acids, the lack of effect in this experiment was not unexpected as the template used was poly rA.dT. In the endogeneous reaction (Table 7) the inhibitory action of actinomycin D was observed. The incorporation was inhibited by 50% in the presence of actinomycin D concentration as high as 100 µg/ml. The observed effects of actinomycin D in the exogeneous and endogeneous assays is totally consistent with the hypothesis that the assay is truly measuring viral reverse transcriptase activity.

Growth of FLV

The kinetics of FLV production following infection of mouse embryo cells are of considerable importance to subsequent pseudotype studies. Approximately 1 x 10^{5} ME cells were seeded in 60 mm falcon plastic dishes. After 24 hours, the cells were infected at an MOI of 2 PFU/cell with Friend leukemia virus. Samples were withdrawn from the same duplicate plates every 24 hours for 7 days, 4 ml new

TABLE 6

Effect of Actinomycin D

on the Incorporation of ^{3}H-TTP

by RNA-Dependent DNA Polymerase

in Presence of Exogeneous Template-Primer

Reaction System	cpm Incorporated
Complete	23,312
Complete, at 4°C	1,112
Complete plus 25 µg/ml Act-D	22,822
Complete plus 50 µg/ml Act-D	20,777
Complete plus 100 µg/ml Act-D	22,082

Reaction system was the same as described in 'Materials and Methods'. Exogeneous template-primer, poly rA.dT was used. 40 - 50 µg of viral protein was added in the reaction mixture. Incubation was carried out at 37°C for 45 mins.

TABLE 7

Effect of Actinomycin D on the Incorporation of ^{3}H-TTP by RNA-Dependent DNA Polymerase in Presence of Endogeneous Template

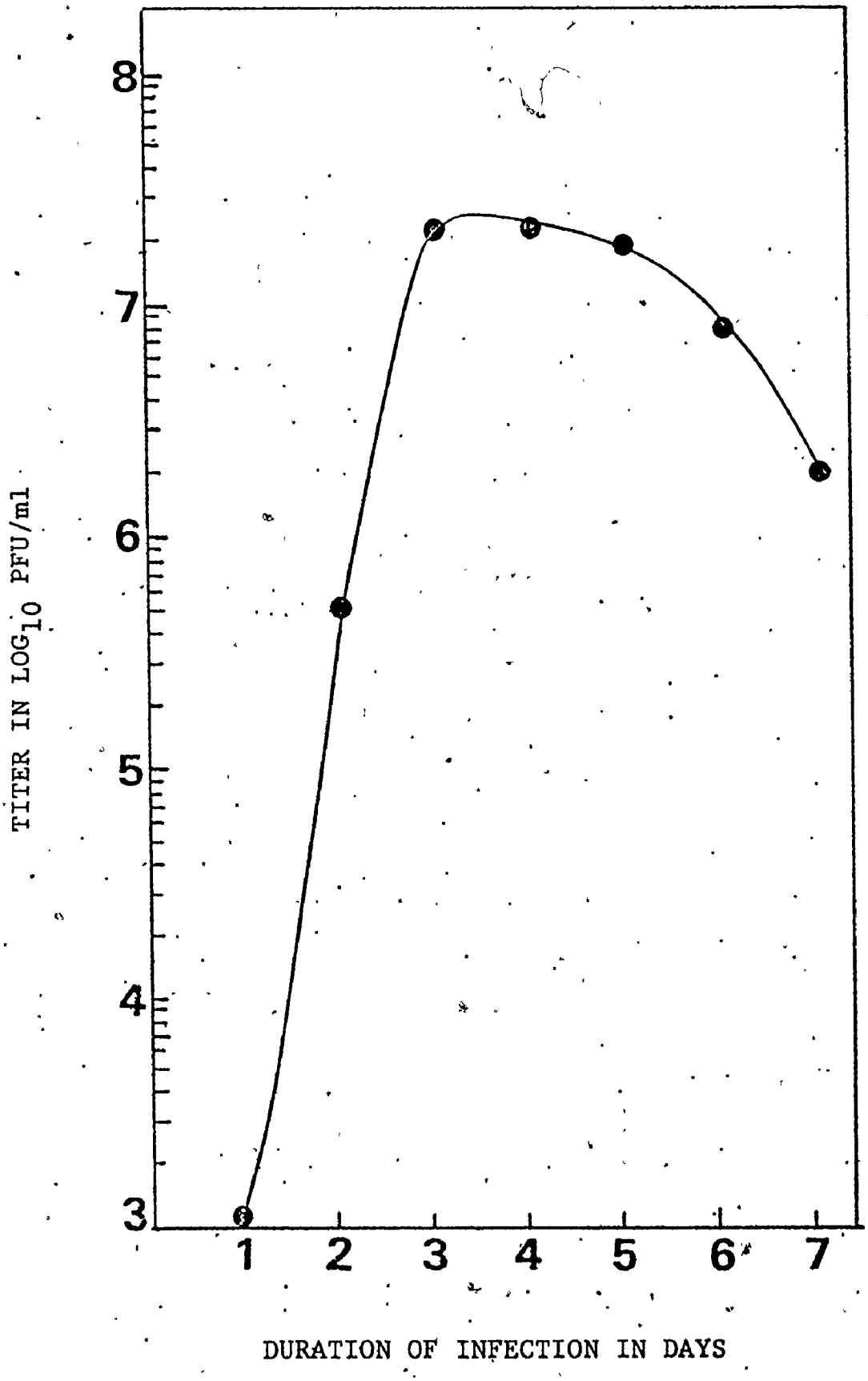
Reaction System	cpm Incorporated
Complete	2,925
Complete, at 4°C	60
Complete plus 25 µg/ml Act-D	1,439
Complete plus 50 µg/ml Act-D	1,332
Complete plus 100 µg/ml Act-D	1,295

The following reaction system was used for this study. Tris-HCl (pH 7.8), 0.05 M; NaCl, 0.06 M; Dithiothreitol, 0.02 M; $MnCl_2$, 2.5 mM; each three triphosphates (dATP, dGTP, dCTP), 0.8 mM; Triton-X-100, 0.14%; ^{3}H-TTP, 3.3 nmoles (220 cpm/pmole); viral protein 40 - 50 µg. Incubation was carried out for 45 mins. at 37°C.

FIGURE 10

Growth Curve of FLV in NIH-3T3 Cells

Some 1×10^5 cells were infected with FLV at an MOI of 2 PFU/cell. Samples were withdrawn from the same duplicate plates every 24 hours for 7 days, 4 ml fresh medium being added after each collection. The titer (PFU/ml) shown in the figure is the mean of duplicate samples.


8
7
6
5
4
3
1
2
3
4
5
6
7
TITER IN LOG10 PFU/ml
DURATION OF INFECTION IN DAYS

medium being added after each collection. Cell debris was removed from the samples by centrifuging at 400 g and the virus titer in the supernatant was determined by the XC-assay as described in 'Materials and Methods'. Figure 10 indicates that relatively little virus was produced during the first 24 hours post infection. An appreciable titer was obtained during the second 24 hour harvest and increased to 1.5×10^7 PFU/ml by the 3rd day. This rate of virus production was observed for two more days, then the 24 hour titer gradually declined. The cells continued dividing until the 4th day by which time they had reached the confluency stage and no more obvious increase in cell number occurred after this time. The resultant stationary phase attained by the cells at this time may account for the decreased titer at later times.

Growth Curves of VSV in L-60 and NIH-3T3 Cells

The growth curves of VSV was determined using L-60 and NIH-3T3 cells. L-60 cells in suspension were infected with VSV at an MOI of 2 PFU/cell. Samples were withdrawn every hour and assayed for plaque-forming activity on L-60 cells. The number of PFU/ml as a function of time is presented in Fig. 11. NIH-3T3 cells seeded in 60 mm falcon petri dishes were infected 24 hours later with VSV at an MOI of 2 PFU/cell. The cell free supernatant was harvested at 2 hour intervals from duplicate plates and the VSV titer was determined by plaque assay on NIH-3T3 cells. Figure 12

FIGURE 11

Growth Curve of VSV in L-60 Cells

L-60 cells were infected in suspension with HR-LT VSV at an MOI of 2 PFU/cell. Samples were withdrawn at indicated times and assayed for plaque forming activity.

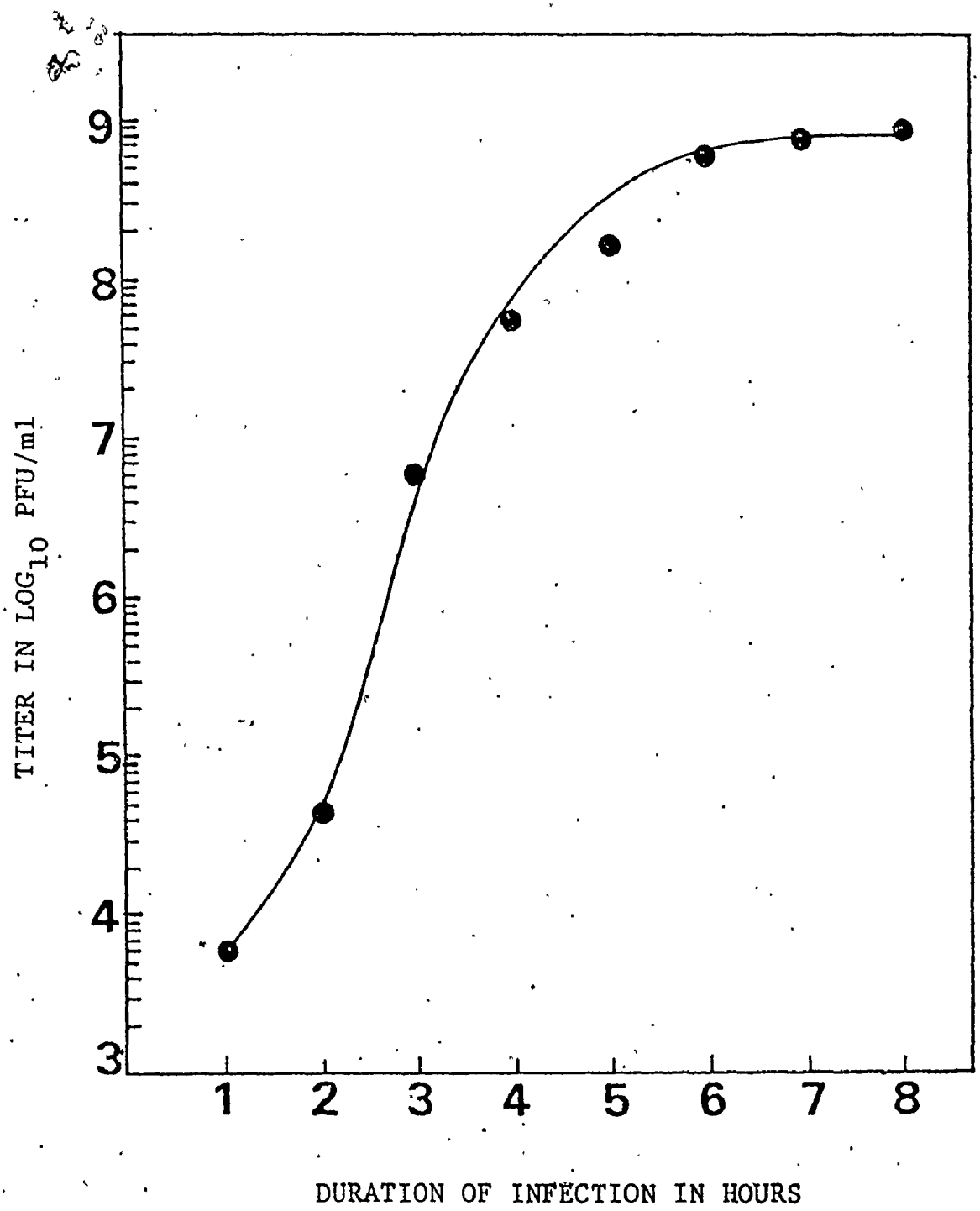
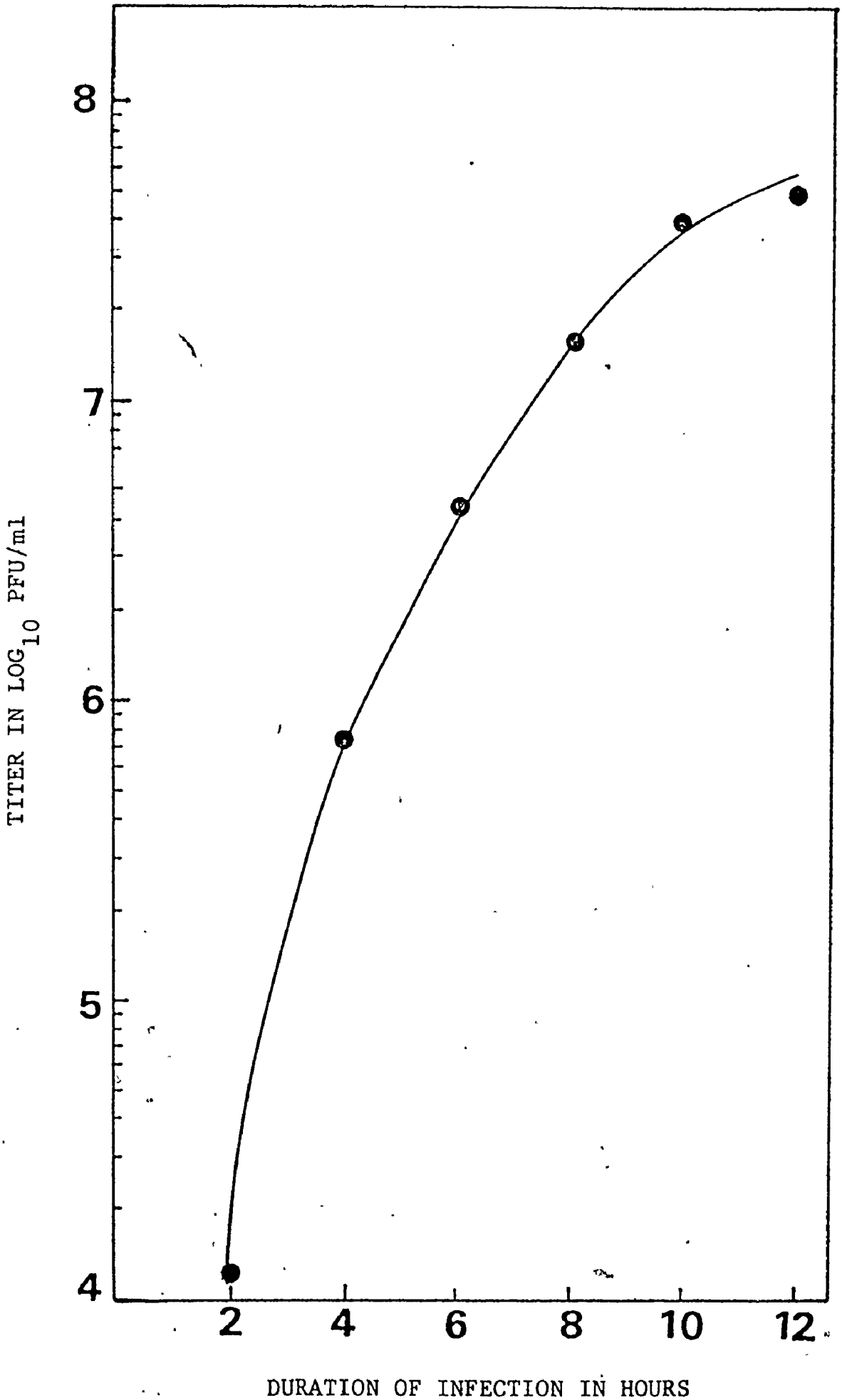

TITER IN LOG10 PFU/ml
9
8
7
6
5
4
3
1
2
3
4
5
6
7
8
DURATION OF INFECTION IN HOURS

FIGURE 12

Growth Curve of VSV in NIH-3T3 Cells

Some 2 x 10^6 NIH-3T3 cells in 60 mm plastic petri dishes were infected with HR-LT VSV at an MOI of 2 PFU/cell. Samples from duplicate plates were collected at indicated times and assayed for plaque forming activity.


8
7
6
5
4
2
4
6
8
10
12
TITER IN LOG10 PFU/ml
DURATION OF INFECTION IN HOURS

shows the growth of VSV on NIH-3T3 cells. The exponential phase of virus release began at approximately 2 hours post-infection and reached a plateau at 6 - 7 hours after infection in case of L-60 and 8 hours in case of NIH-3T3 cells. The maximum titer reached in L-60 cells was 10-fold greater than the maximum titer in NIH-3T3 cells. Also, during the growth of VSV in NIH-3T3 cells the cytopathic effect of the virus was minimal even 12 hours after infection.

Neutralization of FLV by Anti-FLV Antiserum

To determine the potency of anti-FLV antiserum, a standard volume of FLV (tissue culture virus) was mixed with equal volumes of anti-FLV antiserum of different dilutions. The mixtures were then incubated at 37°C for 30 mins. The residual plaque forming activity was determined by XC-plaque forming assay. Figure 13 shows the residual infectivity as a function of antiserum concentration. Eight-fold diluted antiserum was capable of reducing the infectivity by 10 times.

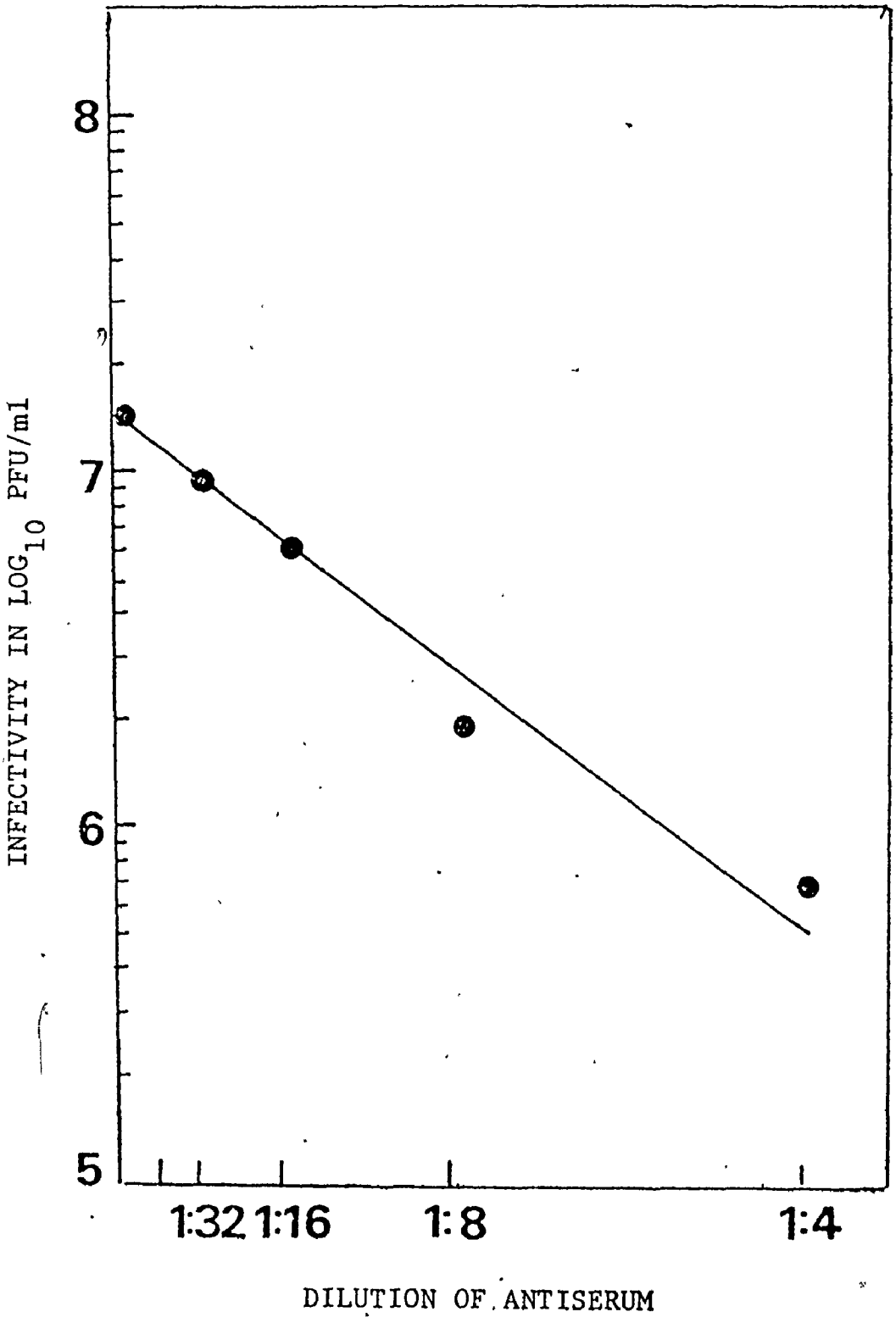
Neutralization of VSV by Anti-VSV Anti-serum

One method of determining the level of pseudotypes in a population of virus involves the use of antiserum to inactivate non-pseudotype particles. In order to be able to detect very low levels of pseudotype particles this procedure requires the use of a

FIGURE 13

Neutralization of FLV by Anti-FLV Antiserum

To serial dilutions of antiserum an equal volume standard FLV stock was added and the mixtures were incubated at 37°C for 30 minutes. The residual infectivity was determined by plaque forming assay.


8
7
6
5
INFECTIVITY IN LOG10 PFU/ml
1:32
1:16
1:8
1:4
DILUTION OF ANTISERUM

high potency neutralizing serum. To determine the potency of anti-VSV antiserum, a standard amount of HR-LT strain of VSV was mixed with serial dilutions of anti-VSV antiserum prepared as described in 'Materials and Methods' section. The mixtures were then incubated at 37°C for 30 mins and the residual plaque-forming activity was then determined. Figure 14 shows the neutralization of VSV by different dilutions of antiserum. As it can be seen in the figure, 50-fold diluted anti-VSV antiserum was capable of reducing the infectivity by 10^4 times.

Figure 15 shows a kinetic study of neutralization of VSV by anti-VSV anti-serum. A series of mixtures of the virus and anti-serum were incubated at 37°C. All of the mixtures contained the same amount of the virus and the anti-serum (1:50 times diluted stock). The samples were withdrawn at 0, 5, 10, 20, 30, 40, 50, 60 minutes and immediately diluted appropriately and tested for the presence of residual plaque forming activity. The residual infectivity is expressed as a function of time in Figure 15. The graph shows that within 10 minutes the titer dropped by approximately 1000-fold and then decreased only slightly over the subsequent 50 minutes.

The above experiment was done with 1:50 times diluted antiserum. By using 1:10 times diluted antiserum, the virus stock containing 10^8 PFU/ml was reduced to less than 10^2 PFU/ml. For the detection of pseudotypes 1:10 times diluted anti-VSV antiserum was used in subsequent experiments.

FIGURE 14

Neutralization of VSV by Anti-VSV Antiserum

To serial dilutions of antiserum an equal volume of standard VSV stock was added and the mixtures were incubated at 37°C for 30 mins. The residual infectivity was determined by plaque forming assay.

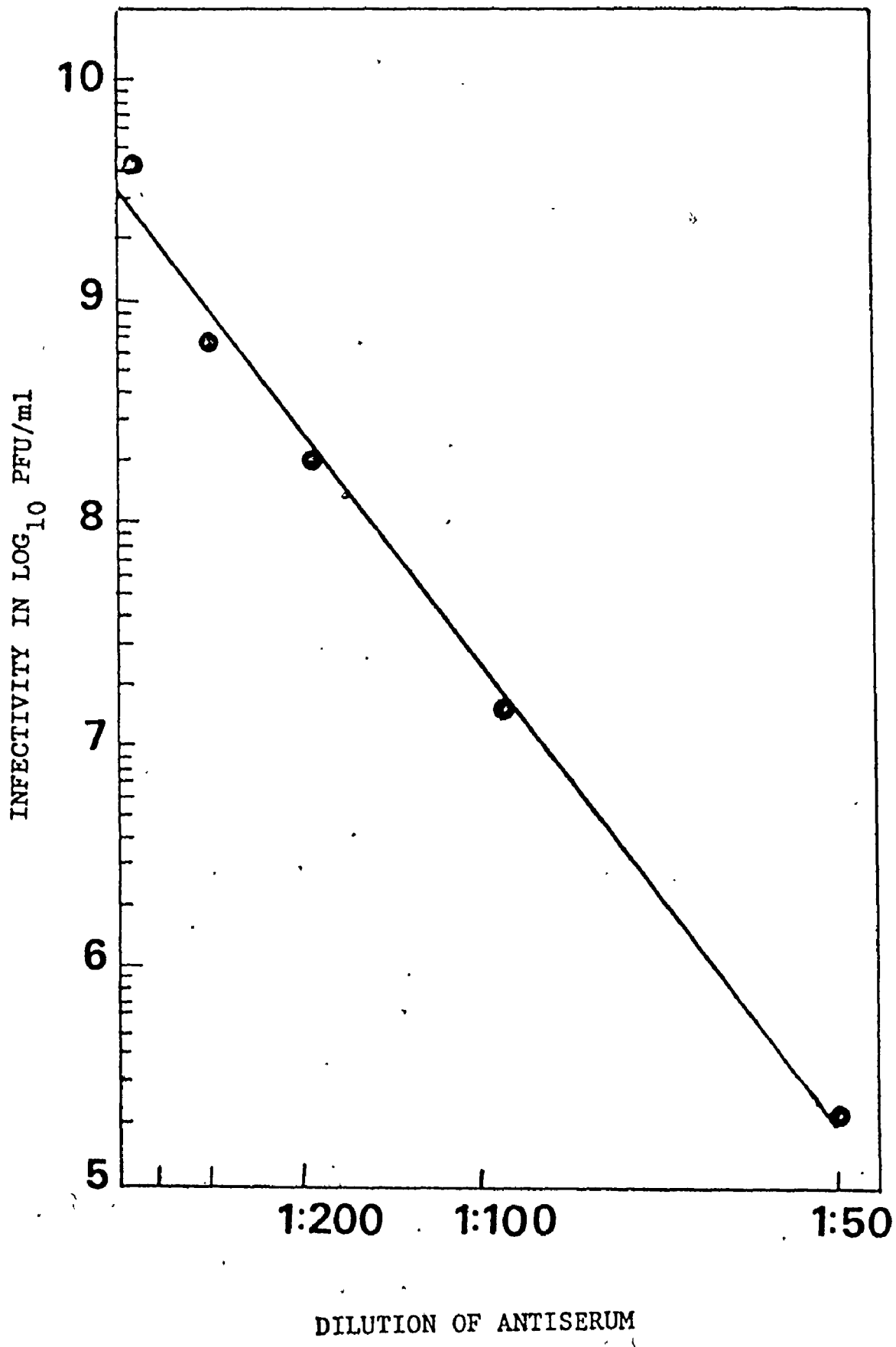
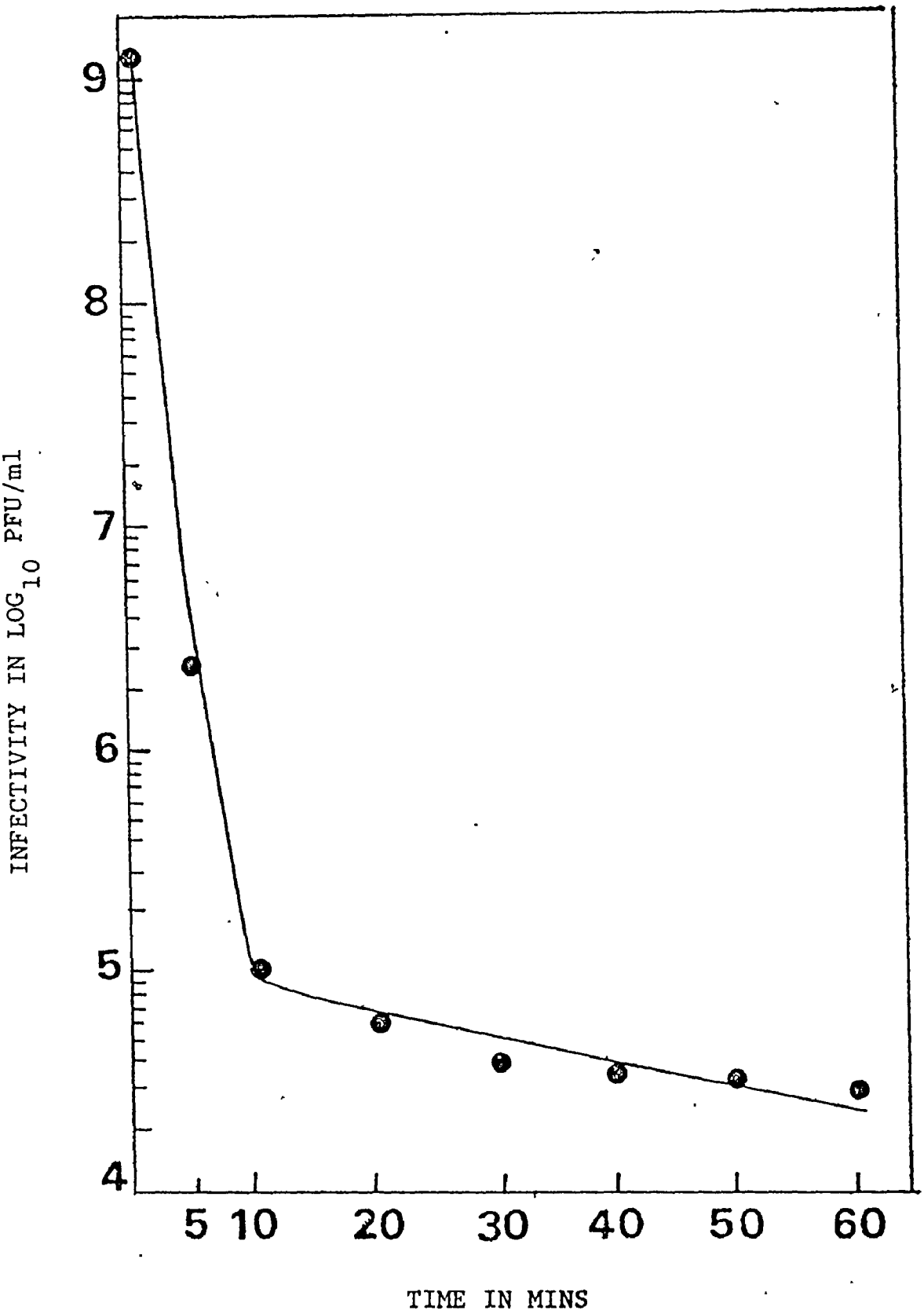

10
9
8
7
6
5
INFECTIVITY IN LOG10 PFU/ml
1:200
1:100
1:50
DILUTION OF ANTISERUM

FIGURE 15

Kinetic of Neutralization of VSV by Anti-VSV Antiserum

A series of mixtures containing the same amount of virus and the antiserum (50-fold diluted antiserum stock) were incubated at 37°C. Samples were removed from the bath at the indicated times and immediately diluted with ice-cold medium and tested for residual infectivity.


INFECTIVITY IN LOG_{10} PFU/ml
9
8
7
6
5
4
5 10 20 30 40 50 60
TIME IN MINS

Study and Characterization of Pseudotypes of VSV and Murine Leukemia Viruses

Production of VSV Pseudotypes

As it has been described in the 'Introduction', murine leukemia virus infected cells produce, when superinfected with VSV, a certain proportion of VSV pseudovirions. These pseudovirus particles contain the genome of VSV but have the evelope antigen characteristics of the leukemia viruses (Zavada, 1972). To produce pseudotypes, 1.0×10^6 FN-3T3 (Friend leukemia virus infected NIH-3T3) cells were seeded in 60 mm plastic petri dishes. One day after seeding, the FN-3T3 cells were superinfected with HR-LT strain of VSV at an MOI 5 PFU/cell. The virus was harvested after 18 hours of infection. Parallel control infection was carried out on NIH-3T3 cells. VSV harvested from each of these cell lines was assayed directly on both NIH-3T3 and FN-3T3 cells to determine the total virus yield and the residual virus titer after treatment with anti-VSV antiserum. Anti-FLV antiserum was also employed in conjunction with anti-VSV antiserum to determine whether the infectious virus surviving anti-VSV antibody treatment could be neutralized by anti-FLV antiserum.

The result presented in Table 8 shows that the total yields of VSV grown in uninfected NIH-3T3 and in FLV infected FN-3T3 cells when assayed either on NIH-3T3 or on FN-3T3 were not significantly different. After treatment with anti-VSV antiserum, the assay on NIH-3T3 cells indicates that the titer of VSV grown

in control NIH-3T3 was reduced from 2.9×10^7 to less than 10^2 PFU/ml. On the other hand, VSV grown in FN-3T3 cells showed residual virus resistant to anti-VSV antiserum but susceptible to anti-FLV antiserum. This residual virus was not detected when assayed on FN-3T3 cells. The result shows that superinfection with VSV of Friend leukemia virus-infected-cells produces pseudotype virus at a concentration approximately 0.1% of the total virus yield. The fact that the virus resistant to anti-VSV antiserum was neutralized by anti-FLV antiserum shows that the pseudotypes had the envelope antigen characteristics of FLV. The level of pseudotypes in this experiment was the same as that observed by Zavada (1972).

To observe the effect of cell density on the titer of pseudotypes produced, FN-3T3 cells were seeded at the stated numbers (Table 9) in 60 mm plastic petri dishes and after 24 hours were infected with HR-LT strain of VSV at an MOI of 5 PFU/cell. The virus was harvested after 18 hours of infection and VSV(VSV) and VSV(FLV) were assayed as in the previous experiment. The result presented in Table 9 shows that while the total yield was reduced at the lowest cell density (2.5×10^5 cells/dish), no significant difference was observed at higher cell densities upto 2×10^6/dish. In subsequent experiments 1×10^6 cells per dish were routinely used to produce pseudotypes.

Since, NIH-3T3 cells exhibit reduced growth due to contact inhibition at high densities (Todaro and Green, 1963), the next experiment was performed to find out if contact inhibition has

TABLE 8

Production of VSV(FLV) Pseudotypes

VSV grown in	Serum	VSV Titer (PFU/ml) on NIH-3T3	VSV Titer (PFU/ml) on FN-3T3
NIH-3T3	0	2.9×10^7	3.0×10^7
	As-VSV	$<10^2$	$<10^2$
	As-VSV+ As-FLV	$<10^2$	$<10^2$
FN-3T3	0	2.0×10^7	2.3×10^7
	As-VSV	2.3×10^4	2.0×10^2
	As-VSV+ As-FLV	$<10^2$	$<10^2$

VSV grown in both the cell lines was assayed for plaque forming units before and after the addition of anti-VSV antiserum alone and also after the addition of both anti-VSV antiserum and anti-FLV antiserum. The virus was incubated with equal volume of antiserum at 37°C for 30 mins. The final dilution of anti-VSV antiserum was 1/10 and anti-FLV antiserum was 1/3. After the incubation with the antiserum-treated virus, the cells on which the assays were performed were washed with PBS. The same procedure was followed in all the later experiments.

TABLE 9

Production of VSV(FLV) Pseudotypes Using Different Number of FN-3T3 Cells

No. of Cells Used	Total Yield of VSV in PFU/ml	Pseudotype Titer in PFU/ml
2.5×10^5	6.5×10^7	7.7×10^3
5.0×10^5	6.2×10^7	2.0×10^4
7.5×10^5	8.1×10^7	1.9×10^4
1.0×10^6	6.6×10^7	2.8×10^4
1.5×10^6	6.9×10^7	2.4×10^4
2.0×10^6	5.7×10^7	2.9×10^4

Cells were superinfected with VSV at an MOI of 5 PFU/cell, 24 hours after seeding in 60 mm plastic tissue culture dishes. Progeny virus was assayed on NIH-3T3 cells before and after the addition of anti-VSV antiserum. In this experiment and in all subsequent experiments, the pseudotype titer means the residual titer of the virus which remained active after the addition of the anti-VSV antiserum, which could be neutralized by anti-FLV antiserum and which did not produce plaques on FN-3T3 cells.

TABLE 10

The Effect of Contact Inhibition on the Production of Pseudotypes

No. of Cells seeded	Total VSV titer in PFU/ml	Pseudotype titer in PFU/ml
1×10^5	4.5×10^7	8.0×10^4
5×10^5	3.6×10^7	1.7×10^4
1×10^6	3.9×10^7	7.5×10^3

FN-3T3 cells were seeded in 60 mm plastic petri dishes in the number as stated in the Table. After 3 days of seeding, cells were infected with VSV at an MOI of 5 PFU/cell. Viruses were harvested 12 hours after infection and plaque-forming assay was performed to determine the total yield of VSV and the pseudotype population.

any effect on the production of pseudotypes. FN-3T3 cells which were derived from NIH-3T3 cells were used. In 60 mm tissue culture dishes 1×10^5, 5×10^5 and 1×10^6 cells were seeded and 3 days after seeding the cells were infected with VSV and the total VSV and pseudotype yield were determined by plaque assay. Plates in which 5×10^5 and 1×10^6 cells were seeded had obtained confluency before VSV infection while the cells seeded at 1×10^5 were still subconfluent. The result in Table 10 shows that in the plate seeded with 1×10^6 cells, the pseudotype yield was 10 times lower than that in the plate seeded with 1×10^5 cells. The yield of normal VSV was not affected. While the addition of fresh medium and serum at the time VSV infection may have released the cells partially from growth inhibition it nontheless seems that pseudotype production and hence presumably murine leukemia virus glycoprotein synthesis may be reduced during confluency.

Use of ts-O-45 in the Production of VSV(FLV) Pseudotypes

Zavada (1972) and Koentris et al., (1973) have used a temperature sensitive mutant of VSV called ts-O-45 to produce VSV (MuLV) pseudotypes. This mutant produces a thermolabile virion envelope glycoprotein (G) which renders virions having this glycoprotein susceptible to thermal inactivation by heating at 45°C. The infectivity remaining after thermal inactivation is, therefore, due either to pseudotype particles which do not possess the mutant glycoprotein or to virus bearing a revertant protein.

In order to test whether ts-0-45 would be useful in the pseudotype assay, the following studies were carried out.

(i) Assay of ts-0-45 and HR-LT Strains of VSV on L and ME cells

Titer of ts-0-45 and HR-LT strains of VSV stocks were determined both on L and ME cells at 32°C. The result in Table 11 shows that when assayed on ME cells, the titer of ts-0-45 was some ten-fold lower than that of HR-LT VSV and some five-fold lower than the titer on L cells.

Table 12 shows the resultant titration of ts-0-45 and HR-LT VSV on L cells at 32° and 39°C. As can be seen, the mutant ts-0-45 shows considerably less capability for plaque formation at 39°C than is seen by the HR-LT strain, nevertheless, plaques were observed at a frequency of approximately $1:10^4$ at this temperature due either to revertants or to "leakiness".

(ii) Thermal Inactivation Kinetics of ts-0-45

Some 2 ml of each of ts-0-45 and HR-LT virus strains of VSV was incubated at 45°C. A 0-time sample was withdrawn from each of the virus samples immediately and further samples were withdrawn at regular intervals during incubation. All sample dilutions were subsequently done at 0°C. Appropriately diluted samples were plated on confluent monolayers of L cells and the VSV plaque assay was performed at 32°C. The surviving virus is plotted as a function of time in Fig. 16. The result shows that

TABLE 11

Titer of ts-0-45 and HR-LT Strains of VSV on L-60 and ME-Cells

Virus Type	Titer in PFU/ml	
	On L-60 cells	On ME-cells
ts-0-45	2.9×10^9	2.6×10^8
HR-LT	2.2×10^9	4.8×10^8

TABLE 12

Titer of ts-0-45 and HR-LT Strains of VSV on L-60 Cells at 32^oC and 39^oC

Virus Type	Titer in PFU/ml at 39^oC	Titer in PFU/ml at 32^oC	Plaque Ratio of $39^oC/32^oC$
ts-0-45	4.2×10^5	2.9×10^9	1.4×10^{-4}
HR-LT	5.7×10^8	2.1×10^9	2.7×10^{-1}

FIGURE 16

Thermal Inactivation Kinetics of ts-0-45 VSV

Approximately 2 ml of ts-0-45 and HR-LT VSV stocks were incubated at 45°C. Samples were withdrawn at 0, 5, 10, 20, 30, 40, 50 and 60 mins and assayed for residual infectivity. All dilutions of the samples were done at 0°C.

● ..... ts-0-45 VSV

▲ .....HR-LT VSV

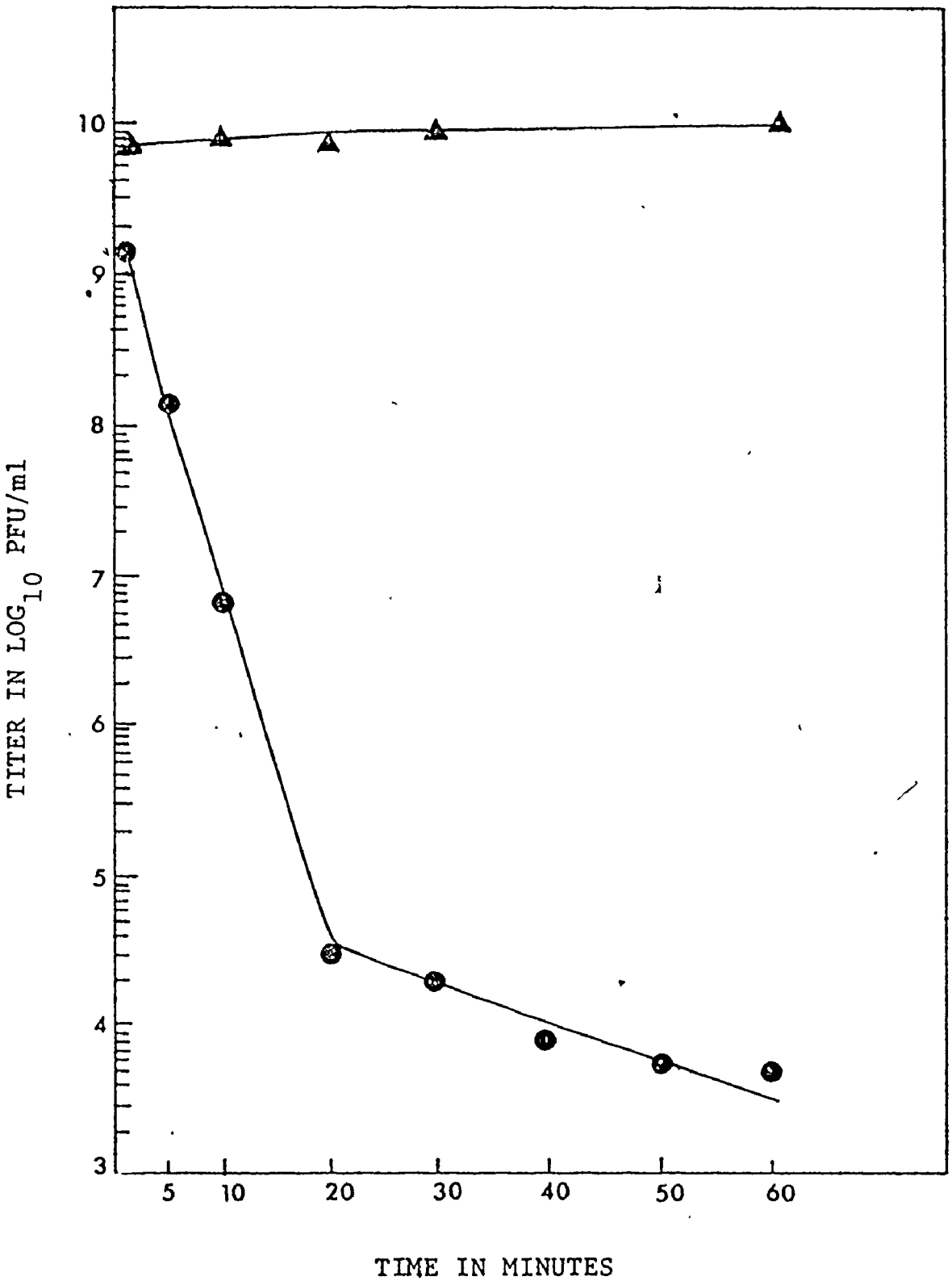

10
9
8
7
6
5
4
3
TITER IN LOG10 PFU/ml
5
10
20
30
40
50
60
TIME IN MINUTES

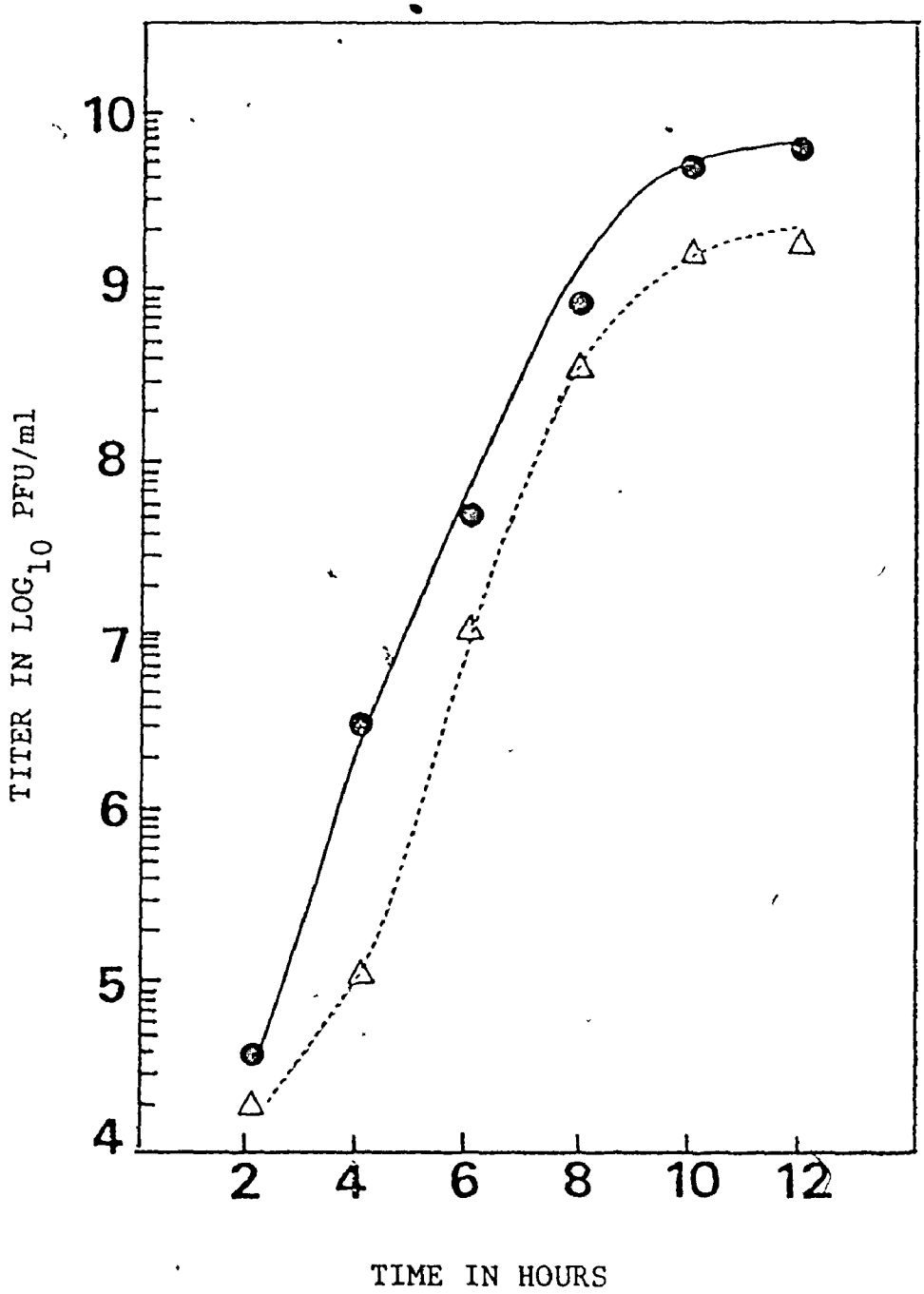
FIGURE 17

Growth Curves of ts-0-45 and HR-LT VSV at 32°C

Some 2 x 10^6 L-60 cells seeded day before, were infected with ts-0-45 and HR-LT VSV at MOI of 5 PFU/cell. Cells were incubated at 32°C and samples were collected at indicated times. The plaque forming assay of the samples were performed at 32°C.

△ ..... ts-0-45 VSV

● ..... HR-LT VSV


10
9
8
7
6
5
4
2
4
6
8
10
12
TITER IN LOG10 PFU/ml
TIME IN HOURS

there was reduction of $10^{4.5}$ times of infectivity in 20 mins and more than 10^5 times within 60 mins when ts-0-45 underwent heat-treatment. The HR-LT VSV showed no significant loss of infectivity even after 60 mins of heat-treatment.

(iii) The growth kinetics of ts-0-45 and HR-LT strains of VSV in L cells was examined to obtain information concerning the time of maximum virus production. Some 2.0×10^6 L cells were seeded in 60 mm plastic petri dishes and incubated at 32°C. After 24 hours, parallel sets of dishes were infected with either ts-0-45 or with HR-LT VSV. In both the cases, the input multiplicity was 5 PFU/cell and the cells were kept at 32°C . Samples were collected at intervals of 2 hours by removing the cell supernatant from 2 dishes at each time point. All samples were then assayed for VSV plaque forming activity at 32°C. The titer in $\log_{10}$ PFU/ml as a function of time is shown in Fig. 17. The result shows that the growth of ts-0-45 almost paralleled the growth of HR-LT VSV. The maximum titer was attained at about 10 hours after infection at these multiplicities of infection.

Production of VSV(FLV) Pseudotypes Using ts-0-45 VSV

In order to use ts-0-45 VSV to produce VSV(FLV) pseudotypes, both FN-3T3 and NIH-3T3 cells were infected with ts-0-45 VSV at an MOI of 1 PFU/cell at 32°C. The progeny viruses were inactivated by heat-treatment at 45°C for 60 mins and a portion was subsequently also incubated with anti-VSV antiserum. The assays

of the total yield and the residual viruses after these treatments were carried out at 32°C. Table 13 shows the result of these treatments. After 60 mins of heat-treatment the titer of ts-0-45 grown in NIH-3T3 cells was reduced from 2.0×10^8 to 4.4×10^3 PFU/ml and the titer of the virus grown in FN-3T3 cells was reduced from 1.8×10^8 to 1.7×10^4 PFU/ml. This shows that ts-0-45 grown in NIH-3T3 cells contained fairly high level of heat-stable virus due perhaps to the presence of wild type revertants. The ts-0-45 grown in FN-3T3 cells which was expected to contain resistant pseudotype particles in addition produced only 4-fold more heat-stable virus. The high background of heat-stable virus makes it difficult to detect the low levels of pseudotype particles produced in infected FN-3T3 cells. On the other hand, when anti-VSV antiserum was used, the residual non-neutralized titer was some 100 times greater in the case of ts-0-45 grown in FN-3T3 cells than that of the virus grown in NIH-3T3 cells. From this experiment, it was concluded that antiserum treatment would be a more effective and hence useful method to inactivate the VSV(VSV) population and allow detection of the VSV(FLV) pseudotypes. In subsequent experiments only the HR-LT strain of VSV was used to produce VSV(FLV) pseudotypes.

Determination of Optimal Conditions for the Production of Pseudotypes

Since it had been found that only 0.1% of the total progeny produced in VSV-infected FN-3T3 cells appeared to be pseudotypes, an effort was made to increase the level of pseudotype production by

TABLE 13

Production of VSV(FLV) Pseudotypes

Using ts-0-45 VSV

Virus	Titer in PFU/ml
ts-0-45-NIH-3T3	2.0×10^8
ts-0-45-NIH-3T3 + Heat*	4.0×10^3
ts-0-45-NIH-3T3 + As-VSV×	$<10^2$
ts-0-45-FN-3T3	1.8×10^8
ts-0-45-FN-3T3 + Heat*	1.7×10^4
ts-0-45-FN-3T3 + As-VSV×	1.4×10^4

ts-0-45-NIH-3T3: ts-0-45 grown in NIH-3T3 cells

ts-0-45-FN-3T3: ts-0-45 grown in FN-3T3 cells

* 0.1 ml of the virus was incubated at 45°C for 60 mins. After that period, the virus was diluted in ice-bath and assayed for VSV plaque forming units.

× The virus was incubated with equal volume of anti-VSV antiserum at 37°C for 30 mins. The final dilution of antiserum in the mixture was 1/10. After the incubation, the appropriately diluted virus was assayed for plaque forming activity.

TABLE 14

The Effect of MOI of FLV on the Production of VSV(FLV) Pseudotypes

MOI of FLV in PFU/cell	Total VSV Titer in PFU/ml	Pseudotype Titer in PFU/ml
0.1	2.3×10^7	1.2×10^3
1.0	3.2×10^7	3.3×10^3
5.0	2.2×10^7	3.5×10^3
10.0	2.5×10^7	2.8×10^3
20.0	1.6×10^7	2.3×10^3

NIH-3T3 cells were infected with FLV at different MOI as noted in the Table. Cells were subcultured for 7 days and then used for the experiment. 1×10^6 cells were seeded in 60 mm plastic tissue culture dishes. After 24 hours, the cells were infected with VSV at an MOI of 5 PFU/cell. Virus was harvested after 20 hours of VSV infection. VSV plaque assay was carried out before and after the addition of anti-VSV antiserum. Pseudotype titer is the residual virus remained after the antiserum treatment and which did not produce any plaque on FN-3T3 cells. Antiserum treatment was done with equal volumes of the virus and antiserum incubated at 37°C for 30 mins. Final dilution of antiserum in the mixture was 1/10.

varying certain parameters in the system under study.

To a first approximation it might be supposed that the level of pseudotype production would be a function both of the concentration or availability of viral glycoprotein on the surface of the FLV-infected cells and concurrently on the level of VSV nucleocapsid produced within that cell. It might also be reasonably supposed that these parameters will be related to the initial multiplicity of infection of the cells both with murine leukemia virus and VSV. To determine this the first few experiments examine the production of pseudotypes as a function of the MOI of the pre-infecting FLV and superinfecting VSV.

(i) The Effect of MOI of FLV on the Production of Pseudotypes

To study the effect of MOI of FLV on the production of VSV(FLV) pseudotypes, control NIH-3T3 cells were infected with FLV at different input multiplicities ranging from 0.1 to 20 PFU/cell. The infected cells were subcultured for about 7 days and were then superinfected with VSV for pseudotype production at an MOI of 5 PFU/cell. The progeny virus was tested for the presence of VSV(VSV) and VSV(FLV) pseudotypes. The result presented to Table 14 shows that under the conditions used there was no significant difference in the levels of pseudotypes produced. This result cannot be used to directly relate pseudotype level with MOI since the FLV-infected cells were subcultured twice over the seven day period and thus allowed for possible selection of faster growing cells. Furthermore,

secondary infection of cells previously uninfected at low MOI during the seven day period might result in a significantly higher proportion of infected cells in the culture than expected from the MOI employed.

(ii) The Effect of MOI of VSV on the Production of Pseudotypes

Approximately 1 x 10^6 chronically infected FN-3T3 cells were seeded in a series of 60 mm petri dishes. After 24 hours, the cells were infected with VSV at MOIs ranging from 0.1 to 100 PFU/cell. The virus was harvested at 20 hours after infection and the titers of VSV(VSV) and VSV(FLV) pseudotypes were determined by plaque assay. As shown in Table 15 the level of pseudotype virus produced decreased as the MOI of VSV increased. At multiplicities of 50 to 100 PFU/cell the pseudotype level was reduced some 10-fold over the yield at 1 PFU/cell while the total VSV yield was reduced by only 2-fold. Since VSV inhibits cellular protein synthesis at high MOI it is possible that the reduction of pseudotype yield is the direct consequence of the inhibition of FLV protein synthesis by VSV. If so, this would suggest that the translation of cellular and murine leukemia virus messenger RNA may not be discriminated by the VSV induced shut-off mechanism.

(iii) Kinetics of Release of Pseudotypes from Infected Cells

As observed in the data previously presented in Figs. 11 & 12, the yield of VSV reaches a plateau between 6 and 7 hours post infection. When VSV infects MuLV-infected cells, MuLV specific

TABLE 15

Effect of MOI of VSV on the Production of VSV(FLV) Pseudotypes

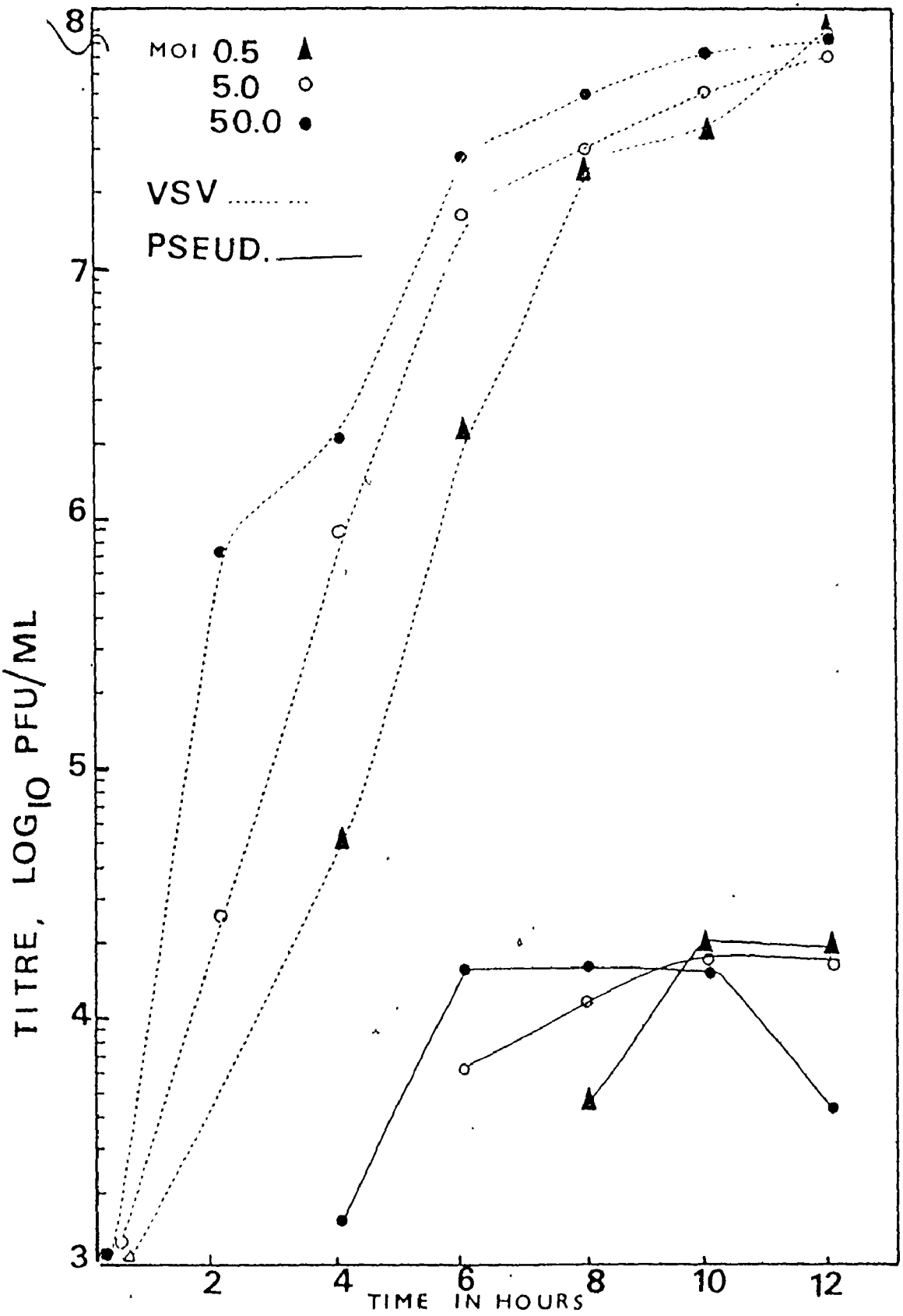
MOI of VSV in PFU/cell	Total VSV Titer in PFU/ml	Pseudotype Titer in PFU/ml
0.1	4.5×10^7	1.0×10^4
0.5	5.0×10^7	5.0×10^3
1.0	5.8×10^7	4.0×10^3
2.5	5.3×10^7	3.7×10^3
5.0	5.2×10^7	2.4×10^3
7.5	4.7×10^7	1.7×10^3
10.0	3.5×10^7	1.6×10^3
25.0	3.7×10^7	1.0×10^3
50.0	2.9×10^7	7.0×10^2
100.0	2.4×10^7	5.0×10^2

1.0×10^6 FN-3T3 cells were seeded in 60 mm plastic tissue culture dishes. Twenty-four hours after seeding, the cells were infected with VSV at MOIs ranged from 0.1 to 100 PFU/cell. After one hour of incubation the cells were washed with PBS and fresh medium was added. Virus was harvested 20 hours after infection. Plaque forming assay was performed before and after the addition of anti-VSV antiserum to determine the total VSV and the pseudotype titers.

FIGURE 18

Growth Curves of VSV(VSV) and VSV(FLV) Pseudotypes at Three Different MOI

Some 1 x 10^6 FN-3T3 cells, seeded day before, were infected with HR-LT VSV at MOIs 0.5, 5 and 50 PFU/cell. Samples were collected at the indicated times and tested for the presence of VSV(VSV) and VSV(FLV) pseudotype plaque forming activities.


MOI 0.5
5.0
50.0
VSV
PSEUD.
TITRE, LOG10 PFU/ML
TIME IN HOURS
8
7
6
5
4
3
2
4
6
8
10
12

glycoproteins are already expressed on the cell surface. Hence, it could well be possible that in VSV infected FN-3T3 cells proportionately more VSV(FLV) pseudotypes are formed in the earlier part of the growth cycle because at that time Friend viral glycoprotein is already available while VSV glycoprotein may not be yet synthesized in adequate quantity.

The following experiment was done to test this hypothesis. The growth of VSV(FLV) pseudotype and the control VSV(VSV) was studied in FN-3T3 cells superinfected with VSV at three different MOI namely 0.5, 5 and 50 PFU/cell. Figure 18 indicates that the kinetics of pseudotype production from the Friend virus infected cells essentially paralleled the growth of control VSV(VSV). No preferential synthesis of pseudotypes was observed at early time, a fact which suggests that the rate limiting factor in pseudotype synthesis is probably the availability of VSV nucleocapsid.

Characterization of the VSV(MuLV) Pseudotypes

(i) Production of Pseudotypes Using Four Different Leukemia Virus Infected Cell Lines

Murine leukemia viruses are divided into two groups, G- and FMR-group, on the basis of the antigens present on the cell surface (Old *et al.*, 1964); on the other hand, they belong to a single group based on their capability to interfere to the infection of each other (Sarma *et al.*, 1967). The next experiment was carried

out to find out whether pseudotypes produced with different murine leukemia viruses exhibit any of these phenomena.

In addition to the Friend virus infected FN-3T3 cells, Rauscher and Moloney leukemia virus infected cells were used to produce pseudotypes in the usual manner. Viruses produced were assayed on NIH-3T3, FN-3T3, Mol-3T3 and RSA-NIH cells. The result is presented in Table 16. The total yield of VSV was almost the same in all cases, irrespective of the cell type on which the assay was carried out. The titer of VSV produced in control NIH-3T3 cells were reduced to 1.0×10^2 PFU/ml after addition of anti-VSV antiserum. The highest titer of pseudotypes was obtained in the Moloney virus infected cells (Mol-3T3) and the least in the Rauscher infected cells when assayed on NIH-3T3 cell monolayers. The relative difference in producing pseudotypes by these three lines may be a function of the passage history of the cells rather than an inherent feature of the particular virus. None of the pseudotypes, i.e., VSV(FLV), VSV(MLV), and VSV(RLV) produced any detectable plaque when assayed on FN-3T3, Mol-3T3 or RSA-NIH cells.

In another similar experiment, pseudotypes were produced using Friend virus infected FN-3T3 and Gross leukemia virus infected (Gross-3T3) cells. The total yield and the pseudotypes were titered on NIH-3T3, Gross-3T3 and FN-3T3 cells. Table 17 shows that total yields of VSV were similar in all the cases, but both the VSV(FLV) and VSV(GLV) pseudotypes were restricted from infection of FN-3T3 and Gross-3T3 cells while NIH-3T3 cells were permissive.

TABLE 16

Assay of Pseudotypes Produced by Three Different Leukemia Virus Infected Cells

Cell type infected with VSV	Ab-VSV	TITER (PFU/ml) IN N-3T3	FN-3T3	Mol-3T3	RSA-NIH
N-3T3	-	6.0×10^7	6.5×10^7	5.5×10^7	7.1×10^7
	+	1.0×10^2	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$
FN-3T3	-	5.5×10^7	4.0×10^7	3.7×10^7	3.0×10^7
	+	7.5×10^3	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$
Mol-3T3	-	5.0×10^7	5.0×10^7	5.6×10^7	4.3×10^7
	+	3.2×10^4	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$
RSA-NIH	-	5.4×10^7	4.5×10^7	3.9×10^7	4.5×10^7
	+	2.0×10^3	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$

1×10^6 cells, either leukemia virus infected or uninfected control, were seeded in 60 mm plastic petri dishes. Next day, the cells were infected with VSV at an MOI of 5 PFU/cell. Viruses were harvested 12 hours after infection. Samples were assayed for plaque forming units before and after addition of anti-VSV antiserum. Assay was performed on NIH-3T3, FN-3T3, Mol-3T3 and RSA-NIH cells.

FN-3T3 - Friend leukemia virus infected cells

Mol-3T3 - Moloney leukemia virus infected cells

RSA-NIH - Rauscher leukemia virus infected cells

TABLE 17

Assay of Pseudotype Produced by Friend and Gross Leukemia Virus Infected Lines

Cell Type Infected with VSV	Ab-VSV	Titer (PFU/ml) in NIH-3T3	FN-3T3	G-3T3
NIH-3T3	-	1.0×10^8	2.8×10^8	1.5×10^8
	+	$<10^2$	$<10^2$	$<10^2$
FN-3T3	-	1.8×10^8	1.9×10^8	1.6×10^8
	+	4.1×10^4	$<10^2$	$<10^2$
G-3T3	-	2.4×10^8	3.4×10^8	1.4×10^8
	+	8.0×10^3	$<10^2$	$<10^2$

The experiment was performed in a similar fashion as described in Table 16.

NIH-3T3 - Uninfected control

FN-3T3 - Friend leukemia virus infected cells

G-3T3 - Gross leukemia virus infected cells

These experiments show that all of these pseudotypes i.e., VSV(FLV), VSV(MLV), VSV(RLV) and VSV(GLV) can form plaques on uninfected NIH-3T3 cells but are restricted from infection of the infected cell lines. This result confirms the finding of Sarma et al., (1967) that murine leukemia viruses belong to a single interference group and further shows that the interference is an viral envelope mediated phenomenon.

(ii) Host-Range of VSV(FLV) Pseudotypes

To determine whether the plaquing efficiency of pseudotype particles in different cell lines was influenced by factors other than the presence of an interfering leukemia virus within these cells, the VSV(FLV) pseudotype titer was compared on five cell lines. The non-pseudotype VSV(VSV) assay on the same lines served as a control for the growth of VSV within these lines. As seen in Table 18, the plaque titer of VSV(VSV) was generally about ten-fold lower in 3T3 cell lines than it was in the XC or L-60 cell lines. The level of pseudotype plaques was also somewhat less in uninfected 3T3 lines than it was in rat XC cell line. As expected from the experiments of Koentris et al., (1973) and Huang et al., (1973) no restriction of the N-tropic FLV-produced pseudotypes was observed in the Balb-3T3 cell line. The XC cell line which contains the genome of the avian Rous sarcoma virus did not exclude pseudotypes of VSV(FLV), in fact the highest titer of both VSV(VSV) and VSV(FLV) was observed in this line. As expected from the previous experiments pseudotypes were completely

TABLE 18

Host-Range Specificity of VSV and VSV(FLV) Pseudotypes

Cell Type Used for Assay	Total VSV Titer in PFU/ml	Pseudotype Titer in PFU/ml
NIH-3T3	9.2×10^7	8.2×10^3
BALB-3T3	1.9×10^8	1.4×10^4
FN-3T3	8.0×10^7	$<10^2$
XC	1.8×10^9	8.9×10^4
L-60	1.4×10^9	1.0×10^3

A stock of VSV grown in FN-3T3 cells was used for the assay. VSV plaque forming assay was performed before and after the addition of anti-VSV antiserum. 0.1 ml of the virus was incubated with equal volume of antiserum at 37°C for 30 mins. The residual virus after the antiserum treatment is referred as the pseudotype titer.

restricted from the FN-3T3 cells and a partial restriction in pseudotype titer was observed in L cells. This latter result in all probability due to the fact that L cells produce a C type murine leukemia virus which partially excludes the Friend group of MuLV. A similar result was observed by Zavada (1972).

(iii) Effect of Polybrene on the Efficiency of the Pseudotype Assay

Since the VSV(FLV) pseudotypes have Friend leukemia virus envelope, it was thought that polycation polybrene would be effective in increasing the infectivity of the pseudotypes. In Table 19 the effect of polybrene on the infectivity of VSV(VSV) and VSV(FLV) is given. It has been shown earlier that there is an increase in FLV infectivity over the range of polybrene concentration of 2.5 - 10 μg/ml. The result here shows that the incubation of the cells with polybrene at different concentration did not increase the pseudotype infectivity. Actually, there was a slight reduction in the pseudotype titer when polybrene was used. It appears that polybrene possesses a differential effect on the infectivity of FLV(FLV) and VSV(FLV). VSV(VSV) titer was also not affected by the presence of polybrene.

(iv) Neutralization of VSV(FLV) Pseudotypes with Anti-FLV Antiserum

As further evidence that the pseudotype particles produced were in fact enveloped with the murine virus coat, VSV(FLV) particles were neutralized by different dilutions of anti-FLV antiserum. Pseudotypes were produced in the usual manner by infecting FN-3T3

TABLE 19

Effect of Polybrene on VSV and VSV(FLV) Pseudotype Assay

Conc. of polybrene in µg/ml	VSV-FN* Total VSV in PFU/ml	VSV-FN* Pseudotype in PFU/ml	VSV-N× in PFU/ml
0	1.0×10^9	2.2×10^4	2.4×10^8
0.5	1.8×10^9	1.3×10^4	1.9×10^9
5.0	2.2×10^9	1.0×10^4	2.1×10^9
10.0	2.0×10^9	9.1×10^3	2.0×10^9
20.0	2.0×10^9	1.1×10^4	1.8×10^9
30.0	1.7×10^9	1.1×10^4	1.5×10^9
40.0	1.6×10^9	8.3×10^3	1.5×10^9
50.0	1.3×10^9	7.8×10^3	1.2×10^9

* VSV-FN: VSV grown in NIH-3T3 cells

× VSV-N: VSV grown in FN-3T3 cells, i.e. it contains VSV(FLV)

1×10^6 seeded 24 hours prior to the infection were incubated with the polybrene solution of the concentrations as mentioned in the Table. After 60 mins of incubation, the cells were infected with the appropriately diluted virus stocks and the plaque forming assay was performed in the usual manner. VSV-FN was assayed before and after incubation with anti-VSV antiserum to determine the total yield of VSV and the VSV(FLV) pseudotype titer.

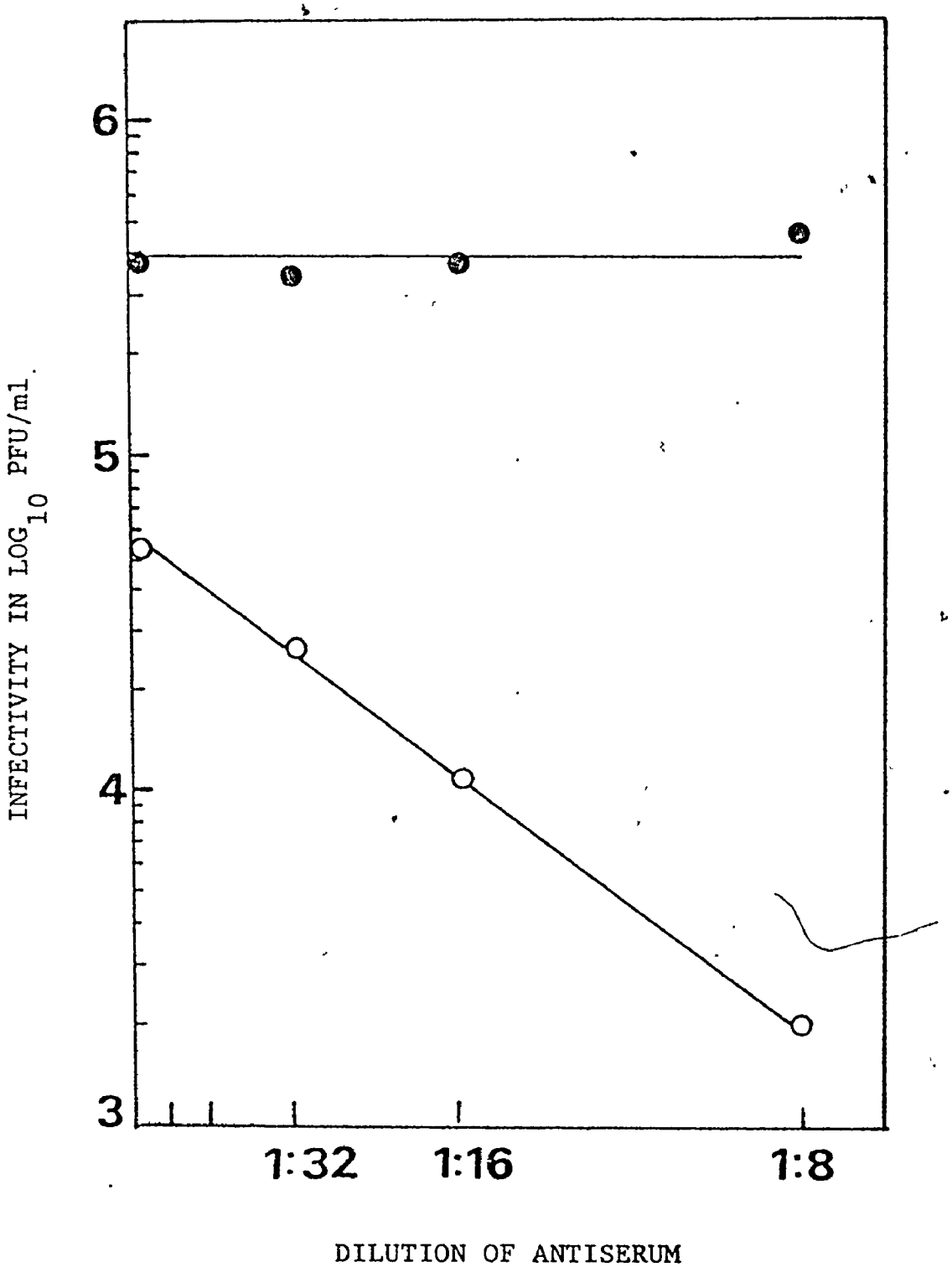
FIGURE 19

Neutralization of VSV(FLV) Pseudotype with Anti-FLV Antiserum

Virus samples containing pseudotypes were mixed with anti-VSV antiserum and anti-FLV antiserum of different dilutions and incubated at 37°C for 30 mins. Residual infectivity was determined by plaque forming assay. A sample stock of VSV (grown in NIH-3T3 cells) was treated with anti-FLV antiserum only in the same manner.

○ ..... VSV(FLV) pseudotypes

● ..... VSV(VSV)


6
5
4
3
INFECTIVITY IN LOG10 PFU/ml
1:32
1:16
1:8
DILUTION OF ANTISERUM

cells with VSV at an MOI of 5 PFU/cell and concentrated by centrifugation. Virus samples were mixed with equal volumes of anti-VSV antiserum and anti-FLV antiserum of different dilutions. The mixtures were then incubated at 37°C for 30 minutes. The surviving virus was titrated by plaque assay. A control VSV(VSV) preparation grown on NIH-3T3 cells was incubated in the same manner only with anti-FLV antiserum of similar dilutions and the residual virus was then titrated. The result shown in Fig. 19 shows that the anti-FLV antiserum did not have any VSV-neutralizing activity but, on the other hand, there was a reduction in the VSV(FLV) titer when serially diluted anti-FLV antiserum was used.

SECTION II. Physical Studies of FLV, VSV and VSV(FLV) Pseudotype

Characterization of FLV by Isopycnic Gradient Centrifugation

As isopycnic and rate zonal gradient procedures are to be subsequently employed to purify and characterize virus particles, preliminary experiments were carried out to determine the stability of FLV in different potential gradient solutions. To do this, 0.2 ml of FLV stock (spleen extract) was incubated with 1.0 ml of different test solutions (as shown in Table 20) for 50 hours at 4°C. The residual plaque forming activity and the spleen focus forming activity were then determined. The result as presented in Table 20 shows that the virus responsible for producing XC plaques was not greatly inactivated in sucrose, glycerol and potassium tartrate solutions under these conditions. The spleen focus forming activity also showed good stability in sucrose and potassium tartrate but appeared to be inactivated by glycerol. Whether this latter observation is due to the actual direct inactivation of the SFFV component of FLV by glycerol or is due to some other reason has not been determined. Cesium chloride (30%) appeared to inactivate both the XC and the spleen focus forming activities. From these results it would appear that sucrose, potassium tartrate or glycerol would all be suitable gradient forming materials for the purification of the XC plaque forming component of FLV.

TABLE 20

Effect of Different Test Solutions on the Plaque Forming Activity and the Spleen Focus Forming Activity of FLV

Test Solution	XC-Plaque Forming Activity in PFU/ml	Spleen Focus Forming Activity in PFU/ml
0 (original titer)	1.0×10^7	3.9×10^4
PBS	5.0×10^6	1.4×10^4
40% Sucrose	3.7×10^6	1.9×10^4
40% Glycerol	3.8×10^6	6.0×10^2
30% Cesium Chloride	4.8×10^5	6.6×10^2
30% Potassium Tartrate	3.3×10^6	1.8×10^4

0.2 ml of *in vivo* grown FLV (spleen extract) was incubated with 1 ml of the test solutions for 50 hours at $4^{\circ}C$. The plaque forming activity of the mixtures was determined by XC-assay and the focus forming activity by spleen focus assay.

Density Gradient Centrifugation of FLV in Sucrose

Concentrated FLV stock grown in tissue culture was used for this study. Virus stock (0.2 ml) was layered on 5 ml of 15 - 50% sucrose gradients containing PBS - 0.002 M EDTA. Centrifugation was carried out in an SW 50 rotor at 100,600 g for 3 hours at 4°C. For comparison, a parallel run was performed with concentrated VSV stock. Figure 20 and 21 show the distribution of densities and the plaque forming activities along the gradients. The result shows that the FLV particles have a density of 1.14 - 1.17 gm/ml as compared to 1.17 - 1.18 gm/ml of VSV.

Density Gradient Centrifugation of FLV in Potassium Tartrate Gradients

Potassium tartrate had been used successfully by McCrea et al. (1961) for density studies of influenza A, influenza B and New Castle Disease virus, by Brown et al., for VSV (1967) and by O'Connor et al. (1964) for the density study of Rauscher leukemia virus. In this experiment, 0.2 ml of virus was mixed directly with 5.0 ml of 30% or 35% potassium tartrate solution made in PBS - 0.002 M EDTA and put into 5 ml tubes of the SW 50 rotor. Centrifugation was carried out at 100,600 g at 4°C for 48 hours. A parallel run was performed with VSV. As seen in Fig. 22 the maxima of both the visible band and the infectivity of FLV occur at a density of about 1.22 - 1.23 gm/ml . The VSV infectivity and visible band occur at a density of 1.17 gm/ml.

FIGURE 20

Buoyant Density of FLV in Sucrose Gradient

Concentrated virus stock (0.2 ml) was layered on 5 ml of 15 - 50% sucrose gradient in PBS - 0.002 M EDTA. Centrifugation was carried out in SW 50 rotor at 100,600 g for 3 hours at 4°C. Fractions (15 drops) were collected by puncturing the bottom of the tube and tested for XC-plaque forming activity.

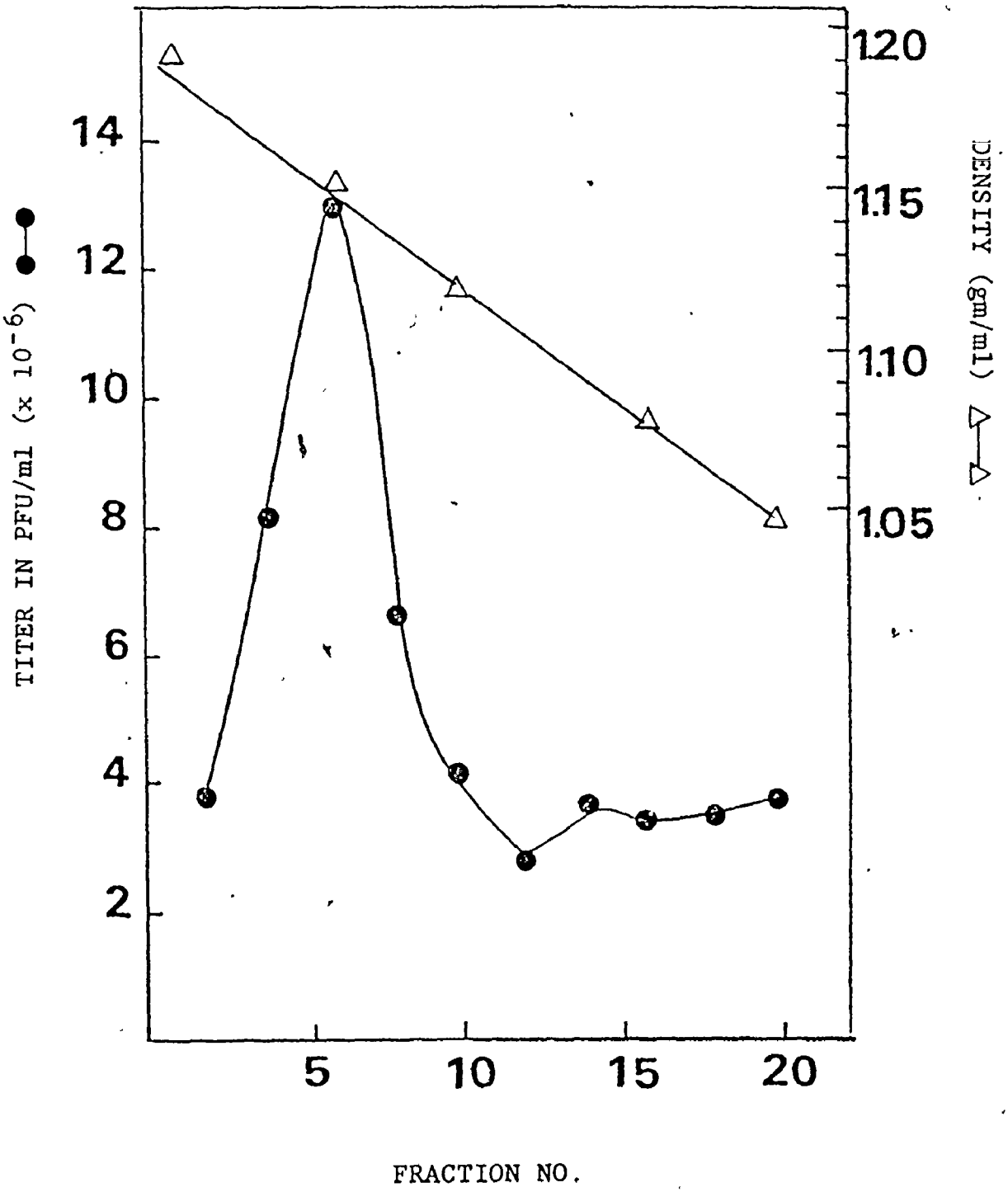

TITER IN PFU/ml (x 10⁻⁶)
14
12
10
8
6
4
2
1.20
1.15
1.10
1.05
DENSITY (gm/ml)
5
10
15
20
FRACTION NO.

FIGURE 21

Buoyant Density of VSV in Sucrose Gradient

Concentrated virus stock (0.2 ml) was layered on 5 ml of 15 - 50% sucrose gradient in PBS - 0.002 M EDTA. Centrifugation was carried out at 100,600 g for 3 hours at 4°C. Fractions (15 drops) were collected by puncturing the bottom of the tube and tested for plaque forming activity.

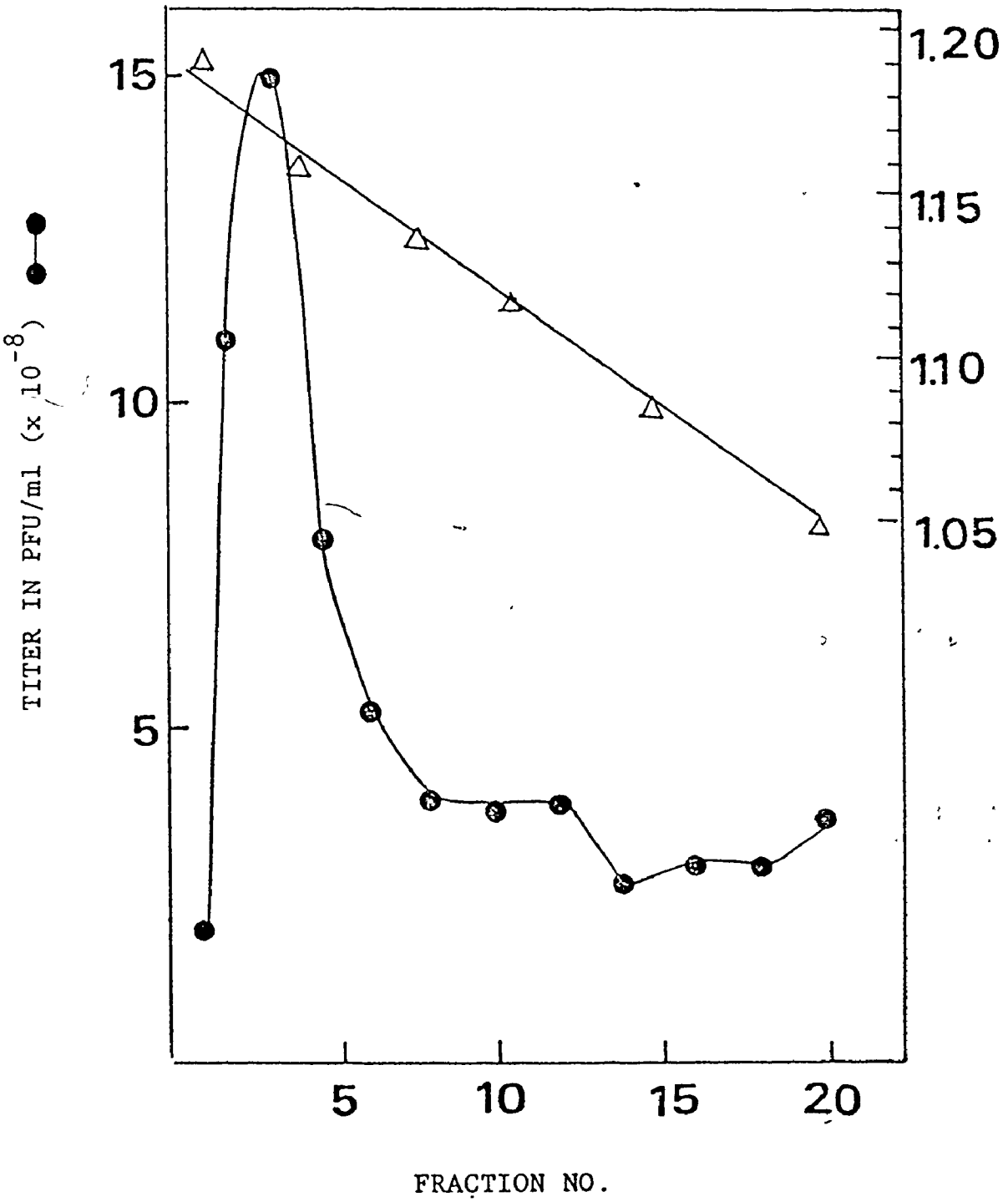
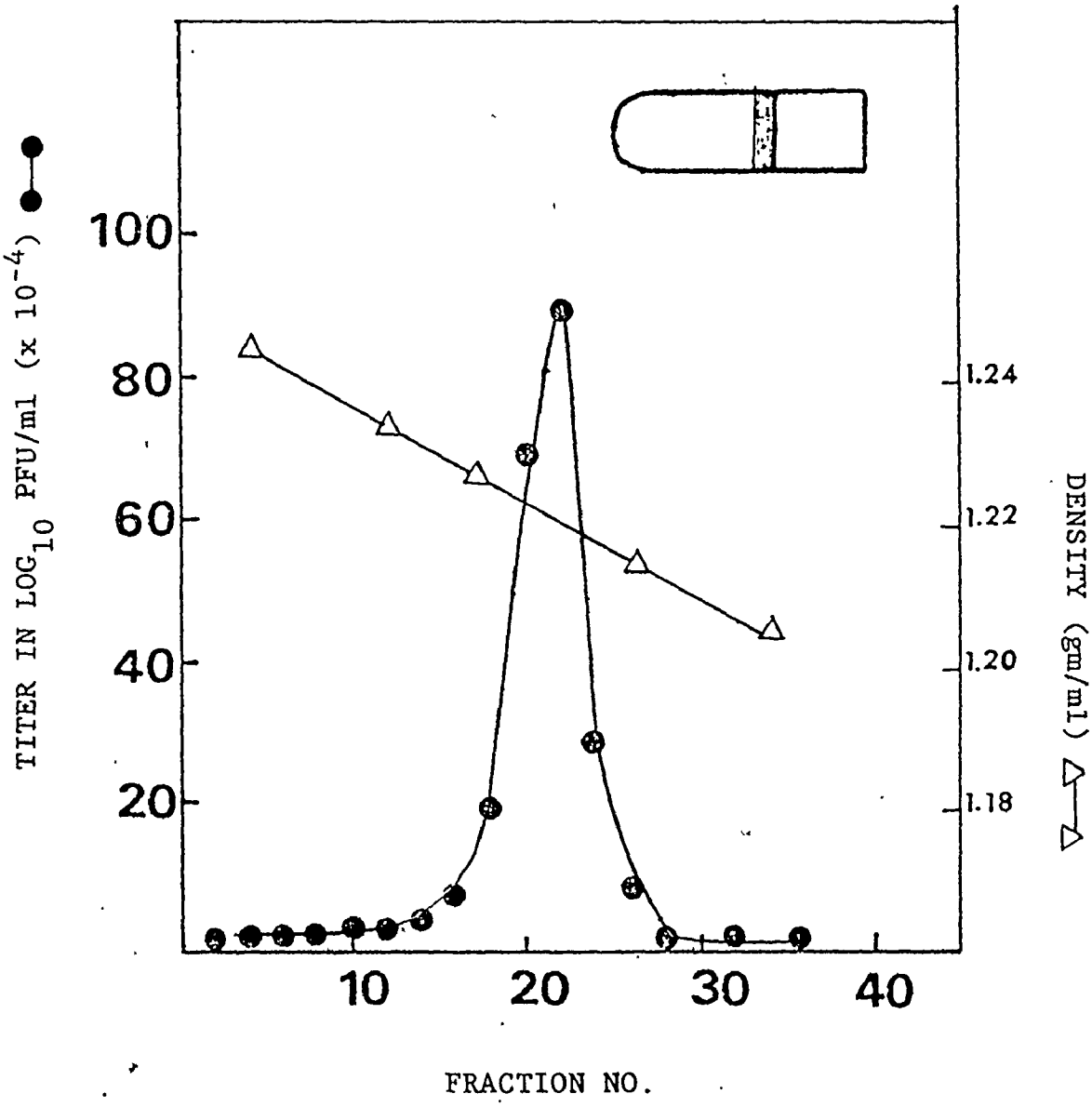

15
10
5
1.20
1.15
1.10
1.05
5
10
15
20
TITER IN PFU/ml (x 10^{-8})
DENSITY (gm/ml)
FRACTION NO.

FIGURE 22

Buoyant Density of FLV Plaque Forming Activity

in Potassium Tartrate Gradient

Some 0.2 ml of concentrated FLV was mixed with 5 ml of 35% potassium tartrate solution and centrifuged at 100,600 g for 48 hours at 4°C. Eight drop fractions were collected by puncturing the bottom of the tube and tested for XC-plaque forming activity. The inset of the figure shows the visible light scattering band.


TITER IN LOG10 PFU/ml (x 10-4)
100
80
60
40
20
10
20
30
40
FRACTION NO.
1.24
1.22
1.20
1.18
DENSITY (gm/ml)

as seen in Fig. 23.

The distribution of reverse transcriptase activity following gradient analysis of Friend virus was determined in a similar but separate experiment. The samples collected from the gradient were first dialyzed overnight against PBS at 4°C to remove the potassium tartrate as it has been observed (Table 21) that potassium tartrate was inhibitory to the reverse transcriptase assay. After dialysis the reverse transcriptase activity of the samples was determined. Figure 24 indicated that the reverse transcriptase activity was maximum at a density of approximately 1.22 - 1.23 gm/ml. The density of the maxima of the infectivity and the reverse transcriptase activity of FLV in potassium tartrate gradient appears to be significantly different from the maxima of infectivity in sucrose gradient. On the other hand, the apparent density of VSV is the same in both gradient solutions.

Density Gradient Centrifugation of VSV(VSV) and VSV(FLV) in Sucrose

To compare the buoyant densities of VSV(VSV) and VSV (FLV) on sucrose gradient, this experiment was carried out. The densities were determined on 15 - 60% sucrose gradients in PBS - 0.002 M EDTA. Figure 25 indicates that there is no essential difference between the buoyant densities of VSV(VSV) and VSV(FLV). Both of them have a density of about 1.18 - 1.19 gm/ml.

FIGURE 23

Buoyant Density of VSV Plaque Forming Activity

in Potassium Tartrate Gradient

Some 0.2 ml of concentrated VSV stock was mixed with 30% potassium tartrate solution and centrifuged at 100,600 g for 48 hours at 4°C. Eight drop fractions were collected by puncturing the bottom of the tube and tested for the presence of plaque forming activity. The inset of the figure shows the visible light scattering band.

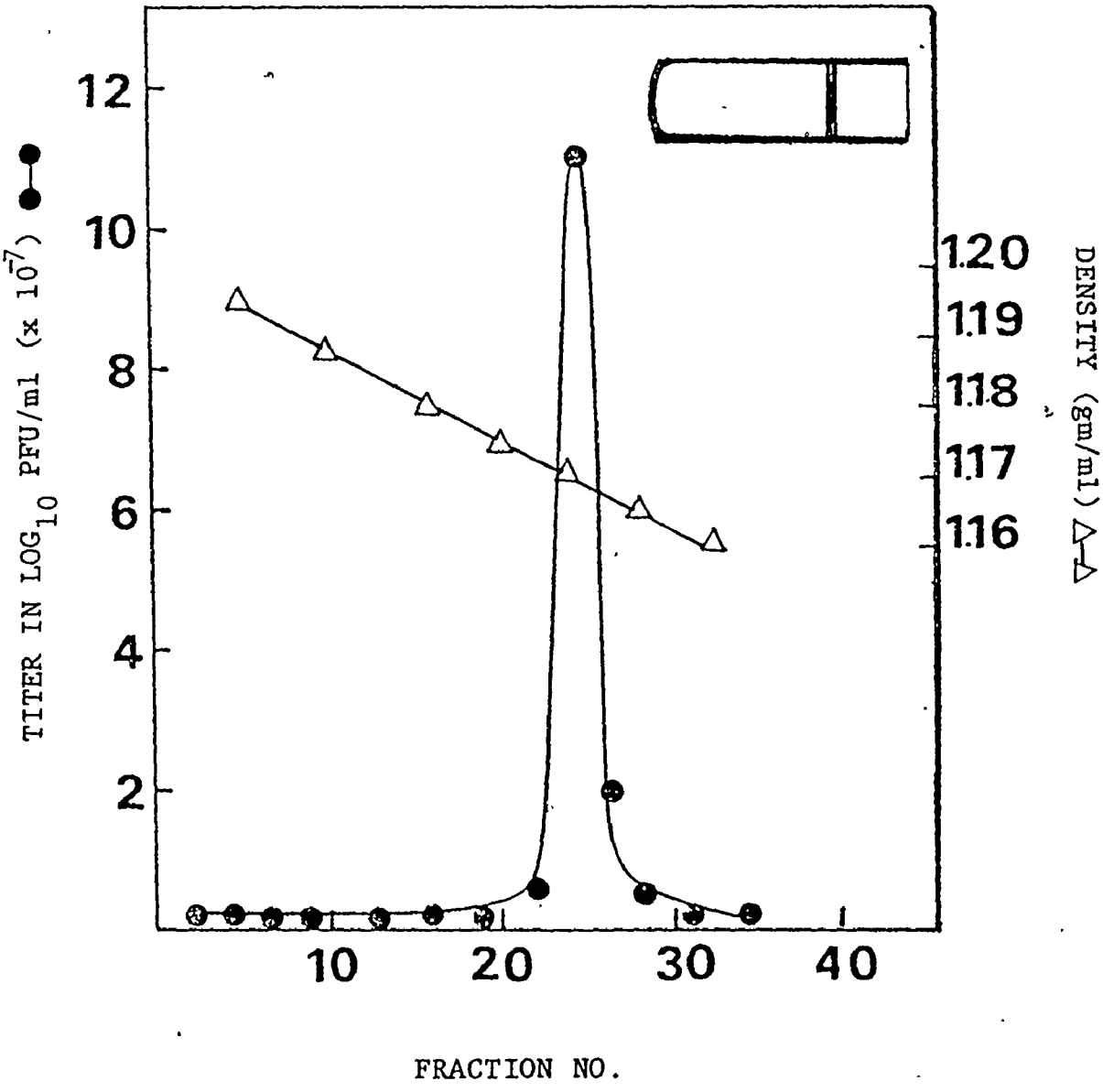

12
10
8
6
4
2
TITER IN LOG10 PFU/ml (x 10-7)
10
20
30
40
FRACTION NO.
1.20
1.19
1.18
1.17
1.16
DENSITY (gm/ml)

TABLE 21

Incorporation of ^{3}H-TTP by RNA-Dependent DNA Polymerase of FLV in Presence of Sucrose and Potassium Tartrate

Samples	cpm Incorporated
FLV in PBS	24,941
FLV in 25% Sucrose	16,691
FLV in 35% Potassium Tartrate	608

0.1 ml of FLV (about 50 µg of protein) was used for the assay. The final concentration of the reactants was the same as described in Table 2. The reaction was performed in 0.2 ml volume. The mixtures were incubated for 45 mins at 37°C.

FIGURE 24

Buoyant Density of FLV Reverse Transcriptase Activity

Some 0.2 ml of concentrated FLV stock was mixed with 35% potassium tartrate solution and centrifuged at 100,600 g for 48 hours at 4°C. Eight drop fractions were collected by puncturing the bottom of the tube. The fractions were dialysed overnight against PBS and then tested for reverse transcriptase activity following the method described in 'Materials and Methods'.

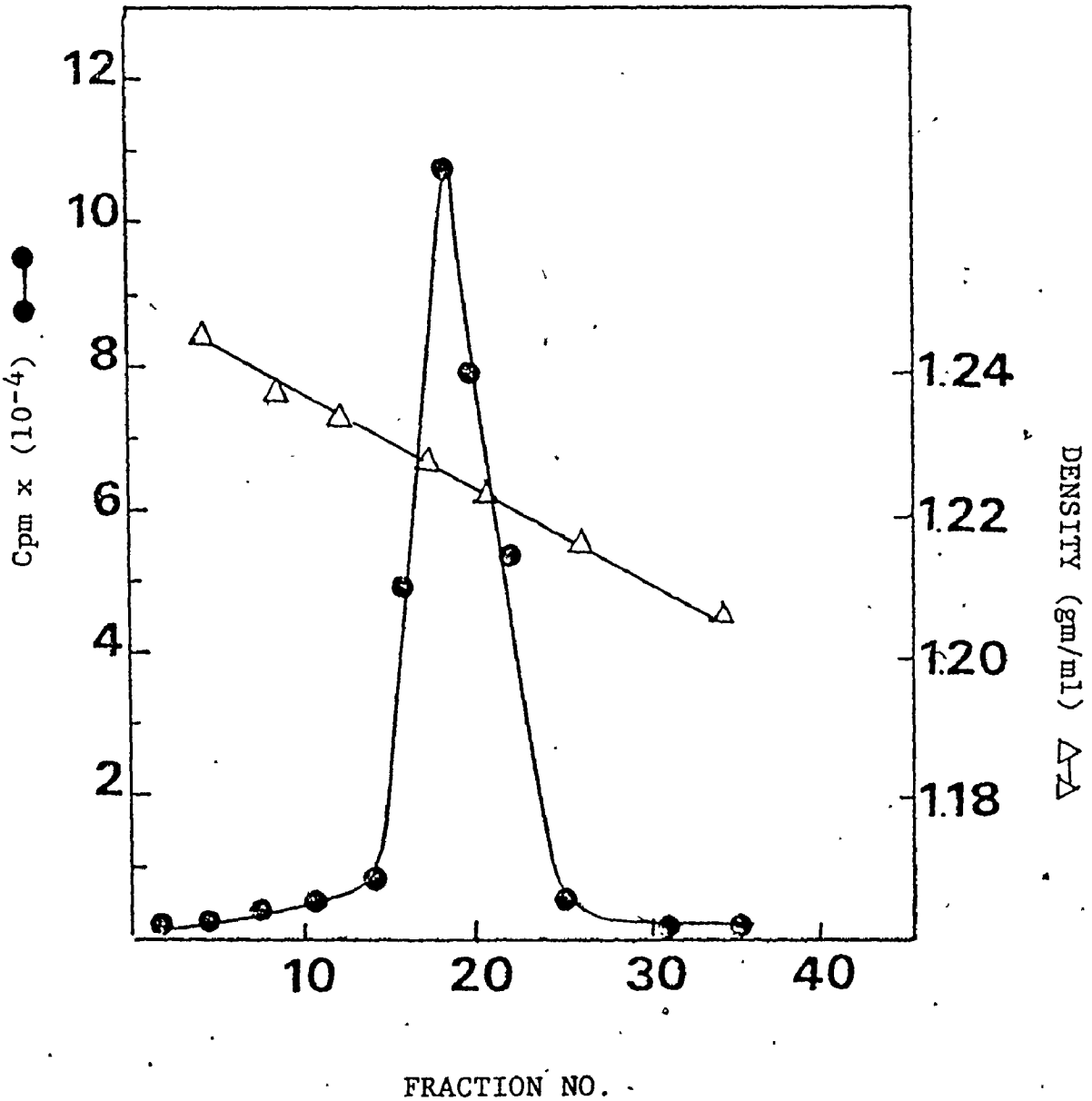
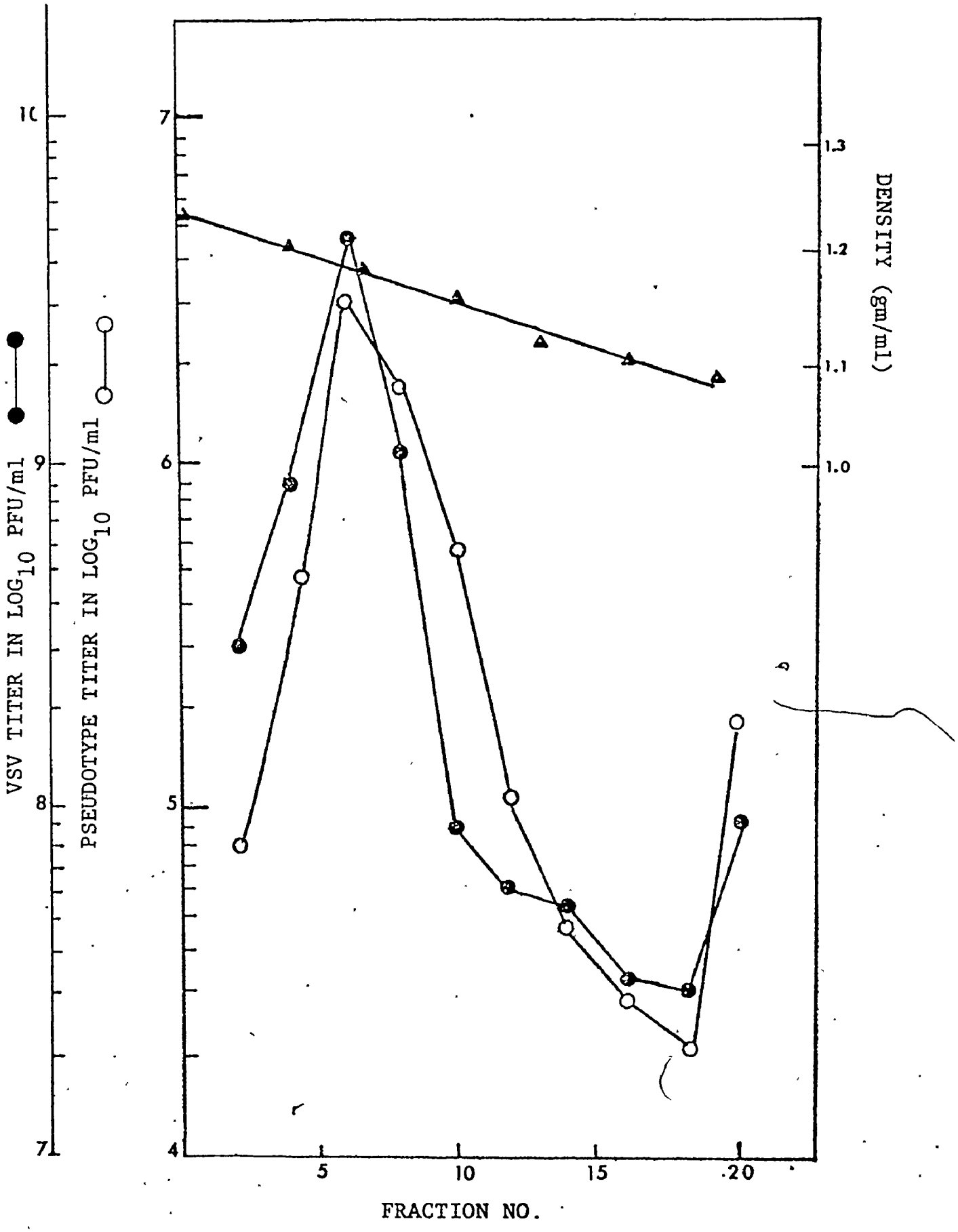

12
10
8
6
4
2
Cpm x (10^{-4})
1.24
1.22
1.20
1.18
DENSITY (gm/ml)
10
20
30
40
FRACTION NO.

FIGURE 25

Buoyant Densities of VSV(VSV) and VSV(FLV) Particles in Sucrose Gradient

Some 0.2 ml of concentrated virus stock containing pseudotype was layered on 5 ml of 15 - 60% sucrose gradient in PBS - 0.002 M EDTA and centrifugaed at 100,600 g for 3 hours at 4°C. Fractions (15 drops) were collected by puncturing the bottom of the tube and tested for VSV(VSV) and VSV(FLV) plaque forming activities.


VSV TITER IN LOG10 PFU/ml
PSEUDOTYPE TITER IN LOG10 PFU/ml
DENSITY (gm/ml)
FRACTION NO.
10
9
8
7
7
6
5
4
1.3
1.2
1.1
1.0
5
10
15
20

Rate Zonal Centrifugation of VSV(VSV) and VSV(FLV) in Sucrose Gradients

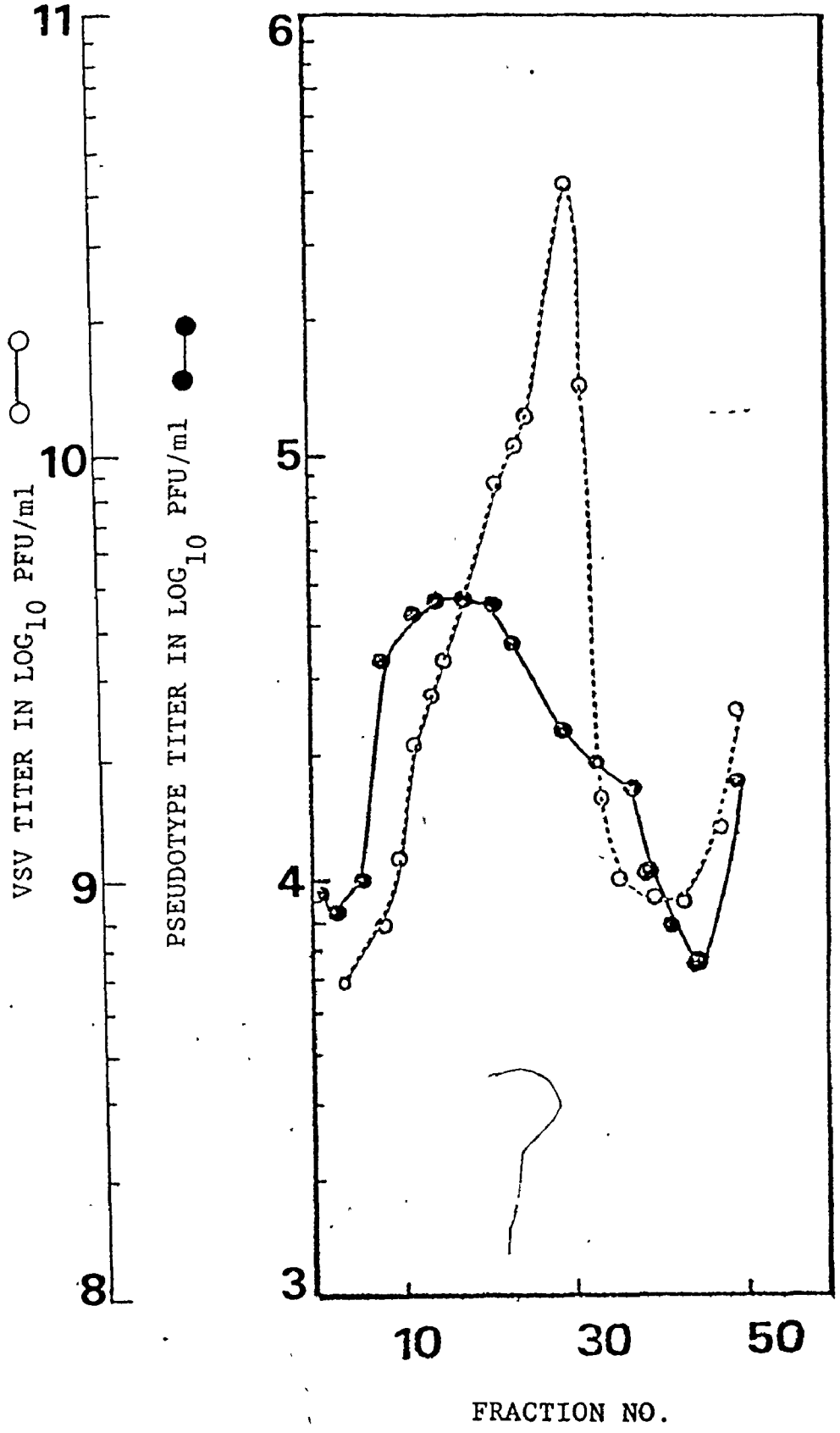
To analyse the sedimentation velocities of VSV(VSV) and VSV(FLV) particles, the concentrated virus stocks containing 5 x 10^{10} PFU/ml of VSV and 1 x 10^{7} PFU/ml of pseudotypes were layered on 5 - 40% preformed sucrose gradient in PBS - 0.002 M EDTA. Centrifugation was carried out in SW 27.1 rotor at 81,000 g for 35 minutes at 4°C. Figure 26 shows the distribution of VSV(VSV) and VSV(FLV) plaque forming activities along the gradient. This result suggests that the VSV(FLV) particles had higher sedimentation coefficient value than that of VSV(VSV) particles.

To find out whether this apparent sedimentation of VSV (FLV) pseudotypes is due to aggregation of the particles, the following two experiments were performed. The rationale of the first experiment was as follows. If the pseudotype particles are self-aggregated, it would be expected that sonication would increase the pseudotype titer assuming that the pseudotype particles were no more sensitive to inactivation by sonication than wild-type VSV(VSV). If the pseudotype particles were aggregated to viruses other than VSV(FLV), either to VSV(VSV) or to FLV(FLV) or to cellular material, then sonication would not greatly affect the pseudotype titer. On the other hand, if some of the apparent pseudotype plaques are actually the result, say, of normal VSV(VSV) protected in clumps of FLV(FLV), then sonication in the presence of anti-VSV antiserum may be expected to expose and neutralize this virus and reduce the apparent pseudotype titer. Fractions from the previous

FIGURE 26

Sucrose Rate Zonal Centrifugation of VSV(VSV) and VSV(FLV) Virus Stock

Some 0.2 ml of virus stock containing VSV(VSV) and VSV(FLV) was layered on 18 ml of 5 - 40% sucrose gradient in PBS - 0.002 M EDTA and centrifuged at 81,000 g for 35 minutes at 4°C. Fractions (20 drops) were collected by puncturing the bottom of the tube and tested for the presence of VSV(VSV) and VSV(FLV) plaque forming activities.


11
10
9
8
VSV TITER IN LOG10 PFU/ml
6
5
4
3
PSEUDOTYPE TITER IN LOG10 PFU/ml
10
30
50
FRACTION NO.

experiment containing the highest titer of pseudotype particles were pooled. After incubation with anti-VSV antiserum at 37°C for 30 mins, portions of the sample were sonically treated for different periods of time to disaggregate the virus, and then were incubated again at 37°C for 30 mins. The result presented in Fig. 27 shows that sonication for 3 mins under the conditions employed had no effect on VSV(VSV) and only a small effect on the total pseudotype titer. It would appear then that the pseudotype particles sedimenting more rapidly than VSV(VSV) on sucrose gradients are the result of aggregation or combination with either VSV(VSV) or FLV(FLV) particles or with cellular material.

In the second experiment, concentrated virus stocks containing pseudotypes were sonically treated for 15 seconds and then examined by sedimentation analysis on 5 - 40% sucrose gradients. As seen in Fig. 28, both VSV(VSV) and VSV(FLV) pseudotype particles now banded at the same position in the gradient showing essentially the same sedimentation coefficients. A comparison of Figs. 26 and 28 suggests that significantly more of the VSV(FLV) pseudotype population was in aggregate form prior to sonication than was the case for the VSV(VSV) population. This result suggests that either the pseudotype particles aggregate more readily to other viruses or cellular debris or that the mechanism of pseudotype formation increases the probability that larger particles are produced. This point shall be further considered in the 'Discussion'.

FIGURE 27

Effect of Sonication on VSV(FLV) Pseudotypes Collected from the Sucrose Gradient

Fractions containing the highest amount of pseudotypes from the previous experiment were pooled and incubated with anti-VSV antiserum for 30 minutes at 37°C. After incubation, the mixture was sonicated for different lengths of time and incubated again at 37°C for 30 minutes and then tested for plaque forming activity.

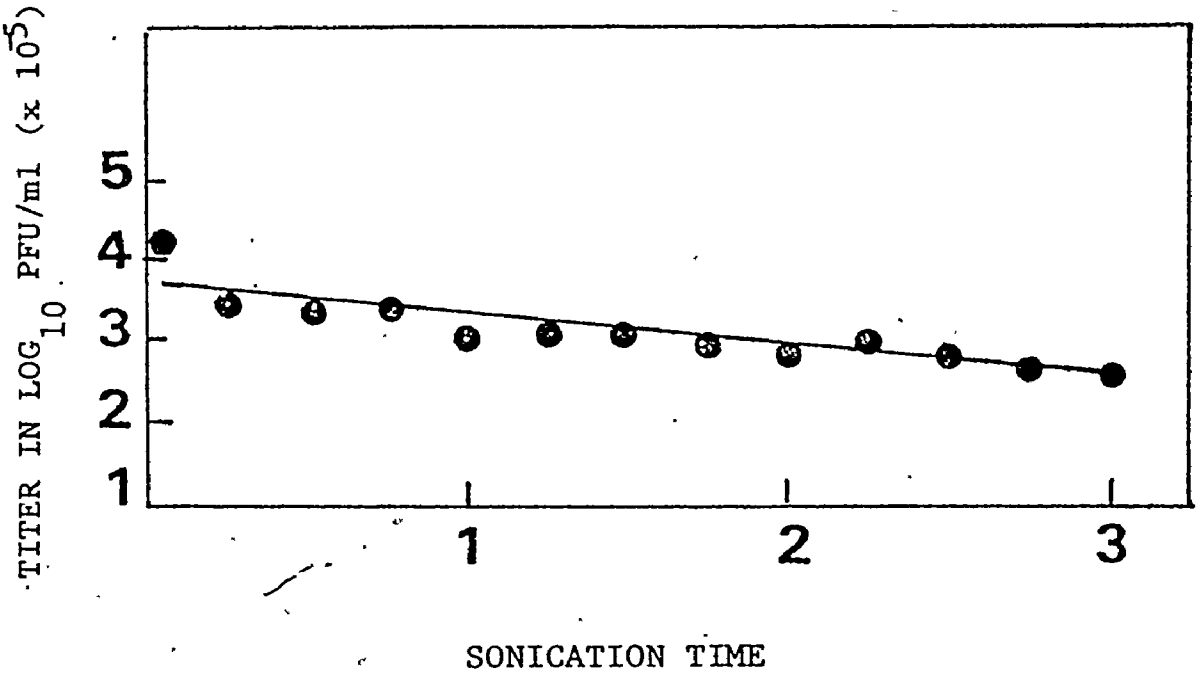
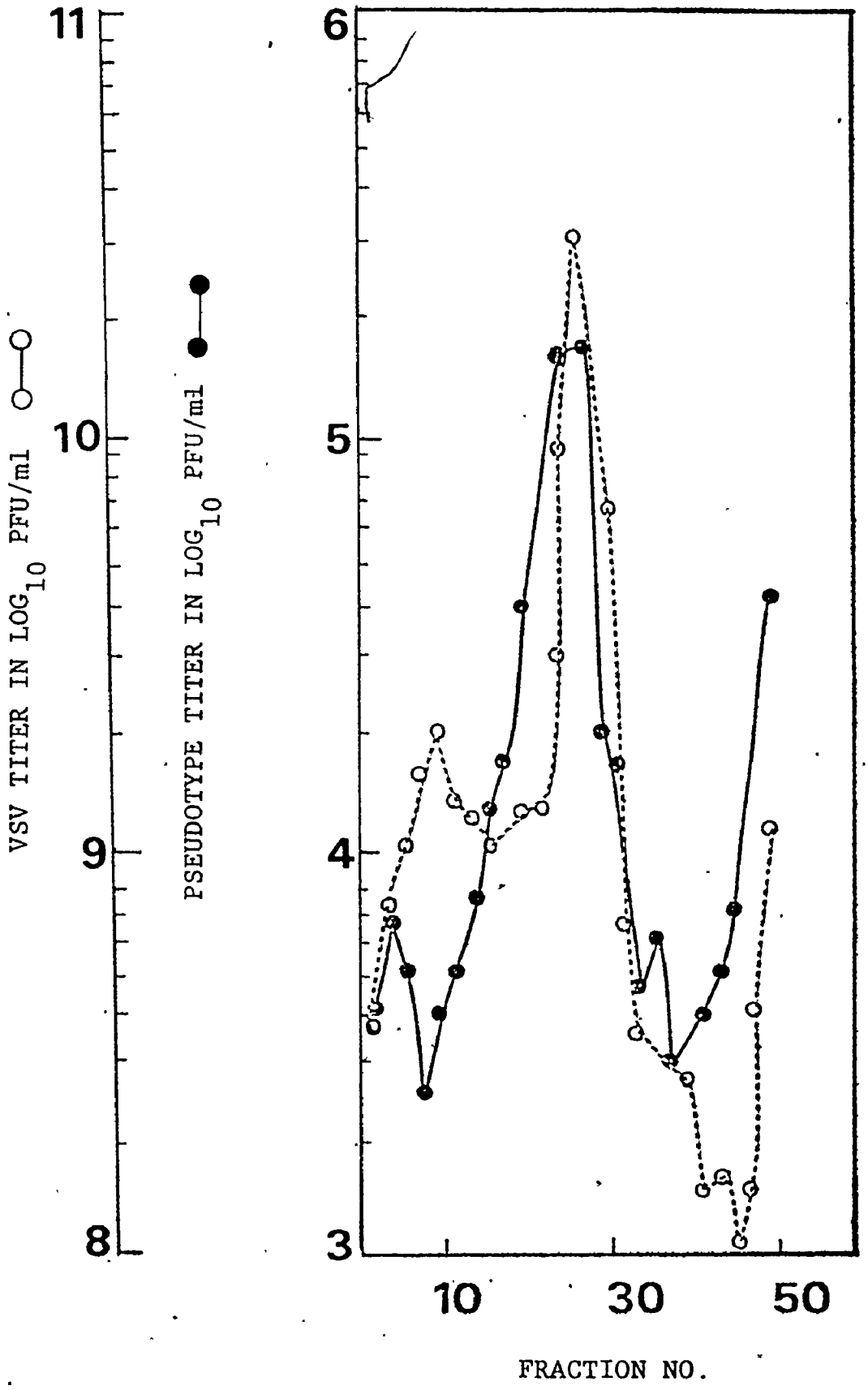

TITER IN LOG10 PFU/ml (x 10-5)
5
4
3
2
1
1
2
3
SONICATION TIME

FIGURE 28

Sucrose Rate Zonal Centrifugation of VSV(VSV) and VSV(FLV) Virus After Sonication

Concentrated virus stocks containing VSV(VSV) and VSV(FLV) particles was sonicated for 15 seconds and then layered on 18 ml of 5 - 40% sucrose gradient and centrifuged for 35 minutes at $4^{o}C$. Fractions were collected by puncturing the bottom of the tube and tested for the presence of VSV(VSV) and VSV(FLV) plaque forming activities.


11
10
9
8
6
5
4
3
10
30
50
VSV TITER IN LOG10 PFU/ml
PSEUDOTYPE TITER IN LOG10 PFU/ml
FRACTION NO.

SECTION III. Intracellular Events in the Synthesis of Murine Leukemia Viral Glycoprotein

In the experiments of this section, the effect of several inhibitors of macromolecular synthesis on the production of VSV(FLV) pseudotypes was studied and an attempt was made to relate these observations to the synthesis of FLV specific glycoproteins in the infected cells.

Effect of Nucleic Acid Inhibitors on Pseudotype Formation

Actinomycin D inhibits the production of RNA tumour viruses but does not inhibit the formation of VSV. To study its effect on VSV(FLV) synthesis, the following series of experiments were performed.

(i) In the first experiment, 1×10^6 NIH-3T3 cells were seeded in 60 mm plastic tissue culture dishes and infected with FLV at an MOI of 100 PFU/cell one day later. Actinomycin D (2.5 µg/ml) was added to the cells at 3, 6, 9, 12 hours after infection with FLV. The FLV infected cells were in turn superinfected with VSV 12 hours after FLV infection. Actinomycin D was again added to the cultures and the virus was harvested 12 hours after VSV infection. The total yield of VSV and the titer of VSV(FLV) pseudotypes were determined by plaque titration. Table 22 shows that no significant VSV(FLV) pseudotypes were produced in cultures which had been infected with FLV for less

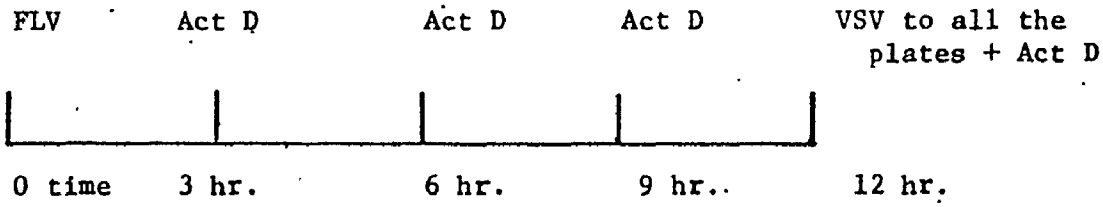
TABLE 22

Application of Actinomycin D at Different Times after FLV Infection and its Effect on VSV(FLV) Pseudotype Synthesis

Application of Act D, Hours After FLV Inf.	Total VSV Titer In PFU/ml	Pseudotype Titer in PFU/ml
3	2.6×10^8	3.5×10^2
6	4.1×10^8	4.0×10^2
9	6.7×10^8	2.5×10^3
12	8.0×10^8	4.6×10^4
12 (No Act)	1.0×10^9	8.5×10^4

Approximately 1×10^6 NIH-3T3 cells seeded in 60 mm plastic petri dishes were infected with FLV at an MOI of 100 PFU/cell. Cells were preincubated with 10.0 µg/ml of polybrene prior to FLV infection. At different times after FLV infection, the cells were incubated with actinomycin D (2.5 µg/ml). Twelve hours after infection with FLV, all the cells were infected with VSV at an MOI of 5 PFU/cell and actinomycin D was added again after infection with VSV. Finally, the virus was harvested after 12 hours of VSV infection, and the plaque forming assay was performed.

Scheme of the Experiment:



than 9 hours prior to actinomycin D addition. This experiment suggests that there is an early actinomycin D sensitive stage in murine virus replication which is essential for the formation of VSV (FLV) pseudotypes. It would seem reasonable to expect that this sensitive stage is the DNA-dependent RNA synthesis which is required for murine leukemia virus replication.

As shall be considered more fully in the 'Discussion', the half-life of cellular messenger RNA may be of 2-3 hours in the presence of actinomycin D (Penman et al., 1963; Craig et al., 1971). It is possible then that the failure to observe VSV pseudotypes when actinomycin D was present in the culture for 6 to 9 hours prior to VSV infection might have been due to the loss of newly synthesized Friend viral mRNA over this time period. In order to examine this possibility, NIH-3T3 cells infected with FLV at an MOI of 100 PFU/cell were superinfected with VSV in the presence of actinomycin D (2.5 μg/ml) at 3, 6, 9, 12 and 24 hours after FLV infection. Each culture was harvested 12 hours after VSV superinfection and the plaque assay was performed to determine the total and the pseudotype titer. As seen in Table 23 no significant pseudotype level was observed at times earlier than 9 hours post infection of FLV. It would, therefore, seem safe to conclude that the experiment measures the kinetics of synthesis of FLV component necessary for pseudotype production. It would further seem reasonable to expect that the required FLV component is a Friend viral glycoprotein.

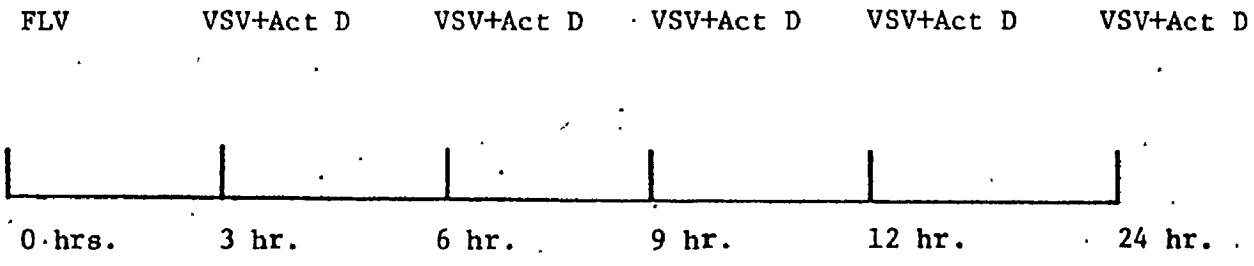
TABLE 23

Infection of FLV-Infected Cells with VSV at Different Times After FLV Infection and the Production of VSV(FLV) Pseudotype

Hour After FLV Infection	Total VSV Titer in PFU/ml	Pseudotype Titer in PFU/ml
0	7.5×10^8	1.0×10^2
3	3.0×10^8	1.5×10^2
6	5.7×10^8	4.0×10^2
9	4.3×10^8	5.3×10^3
12	6.1×10^8	3.3×10^4
24	6.7×10^8	1.2×10^5

Approximately 1×10^6 NIH-3T3 cells seeded in 60 mm petri dishes were infected with FLV at an MOI of 100 PFU/cell after 24 hours of seeding. Cells were incubated with 10 µg/ml of polybrene for 1 hour prior to FLV infection. At different times after FLV infection, the cells were superinfected with VSV at an MOI of 5 PFU/cell and actinomycin D (2.5 µg/ml) was added post infection of VSV. Viruses were harvested after 12 hours of VSV infection and the plaque assay was done on NIH-3T3 cells.

Scheme of the Experiment



(ii) While the above experiments strongly suggest the requirement for de novo RNA synthesis prior to pseudotype formation, they do not exclude the possibility that some FLV glycoprotein synthesis may occur from the parental input RNA genomes. To examine this possibility, NIH-3T3 cells were infected with FLV at an MOI of 100 PFU/cell and actinomycin D (2.5 µg/ml) was added immediately after infection. The FLV infected cells were then superinfected with VSV at an MOI of 5 PFU/cell at 6, 12 and 24 hours after FLV infection and after an additional 12 hours incubation the virus was harvested. The plaque assay was performed to determine the total VSV yield and the pseudotype titer. The result in Table 24 shows that no significant yield of VSV (FLV) pseudotype could be detected in any case while the total yield of VSV remained unaffected.

Since it is possible that actinomycin D may have secondary effects on the ability of input Friend viral RNA to act as a messenger, as a further refinement of the above experiment an inhibitor of DNA synthesis, cytosine arabinoside (ara C) was added at a concentration of 50 µg/ml immediately after infection of NIH-3T3 cells with FLV at an MOI of 100 PFU/cell. The FLV infected cells were superinfected with VSV after 6, 12 and 24 hours of FLV infection and the virus was harvested after 12 hours of VSV infection. The result presented in Table 25 shows that there was no significant synthesis of pseudotypes even after 24 hours of FLV infection when Ara C was present. The total yield of VSV was inhibited slightly under the influence of Ara C. This inhibition more apparent in 24 hours sample was probably due to some cytotoxic effect of Ara C resulted

TABLE 24

Effect of Actinomycin D on the Production of VSV(FLV) Pseudotypes when the Drug Added Immediately After FLV Infection

Time of VSV Superinfection after FLV infection in hours	VSV Titer in PFU/ml	Pseudotype Titer in PFU/ml
6	6.6×10^8	$<1.0 \times 10^2$
9	2.5×10^8	$<1.0 \times 10^2$
12	2.4×10^8	$<1.0 \times 10^2$

Approximately 1×10^6 NIH-3T3 cells were seeded in 60 mm plastic tissue culture dishes. Twenty-four hours after seeding, the cells were incubated with 10 µg/ml of polybrene solution for 60 mins. At the end of the incubation period, the cells were infected with FLV at an MOI of 100 PFU/cell. Medium containing 2.5 µg/ml of actinomycin D was added immediately after infection with FLV. These FLV infected cells were superinfected with VSV at an MOI of 5 PFU/cell at 6, 9, or 12 hours after FLV infection. Virus was harvested after 12 hours of VSV infection and plaque-forming assay was performed to determine the total VSV and the pseudotype titers.

TABLE 25

Effect of Cytosine Arabinoside on the Production of VSV(FLV) Pseudotypes

Time after FLV Infection	Total VSV Titer in PFU/ml	Pseudotype Titer in PFU/ml
0	1.7×10^8	$<10^2$
6	5.3×10^7	$<10^2$
12	3.5×10^7	$<10^2$
24	5.5×10^6	3.5×10^2

Approximately 1.0×10^6 NIH-3T3 cells were seeded in 60 mm plastic tissue culture dishes. Twenty-four hours after seeding the cells were incubated with 2 ml of 2.5 µg/ml polybrene solution for 1 hour. After the incubation, the cells were infected with FLV at an MOI of 100 PFU/cell. Ara C (50 µg/ml) was added immediately after the infection. These FLV infected cells were superinfected with VSV at an MOI of 5 PFU/cell at different times after FLV infection as mentioned in the Table. Plaque forming assay was performed to determine the total VSV and the pseudotype titer.

from prolonged incubation.

These two experiments suggest that no significant FLV glycoprotein synthesis results from the direct translation of the input parental genome RNA prior to DNA transcription.

(iii) As seen above, some nine hours of FLV infection and RNA synthesis are required prior to VSV infection in order to allow pseudotype formation. In order to determine the temporal requirement of murine leukemia virus RNA synthesis in the formation of pseudotype particles, 3T3 cells chronically infected with Friend leukemia virus (FN-3T3) were pre-treated with 2.5 μg/ml of Actinomycin D for 3, 6, 9 and 12 hours prior to superinfection with VSV. VSV infection was done at an MOI of 5 PFU/cell and actinomycin D was added again post infection of VSV. Viruses were harvested after 12 hours of infection with VSV. The result in Table 26 shows that inhibition of transcription by actinomycin D for 12 hours before VSV infection greatly reduced the pseudotype yield, while pretreatments of less than 6 hours had virtually no effect on pseudotype production. This result can be interpreted to mean that either the m-RNA of the FLV specific glycoprotein required for pseudotype formation or the FLV glycoprotein itself was stable during the 6 hour time period prior to VSV infection.

Effect of Protein Synthesis Inhibition on Pseudotype Formation

The effect of cycloheximide, an inhibitor of both VSV and cellular protein synthesis, on pseudotype production was studied in

TABLE 26

Effect of Actinomycin D on the Production of VSV(FLV) Pseudotypes by Cells Chronically Infected with FLV

Time of Application of Act D prior to VSV Infection	Total VSV Titer in PFU/ml	Pseudotype Titer in PFU/ml
-12 hours	3.0×10^7	5.0×10^2
-9 hours	9.5×10^7	4.1×10^3
-6 hours	1.1×10^8	1.1×10^4
-3 hours	1.2×10^8	1.0×10^4
-0 hours	1.2×10^8	1.2×10^4

Approximately 1×10^6 FN-3T3 cells were seeded in 60 mm plastic petri dishes and incubated with actinomycin D (2.5 µg/ml) at different times prior to VSV infection. MOI of VSV infection was 5 PFU/cell and actinomycin D was added again post-infection of VSV. Virus was harvested after 12 hours of infection.

the following experiment. Friend virus infected FN-3T3 cells were seeded in two series of plastic petri dishes. Both series of cells were preincubated with 20 µg/ml of cycloheximide for different lengths of time prior to VSV superinfection. The cells in one series of plates were washed with medium to reverse the effect of cycloheximide before infection with VSV. The cells in the second series of plates were not washed and cycloheximide was also added post-infection with VSV. In one plate cycloheximide was present only after infection with VSV. Viruses were harvested after 12 hours of VSV infection and the titers of VSV(VSV) and VSV(FLV) were determined.

As seen from the results of Table 27, preincubation with cycloheximide of chronically infected FN-3T3 cells for 3 to 12 hours prior to VSV infection had no significant effect on level of pseudotype production as long as the drug was removed at the time of superinfection with VSV. The continual presence of cycloheximide after VSV infection effectively inhibited both wild type VSV and hence pseudotype production. Had protracted incubation periods in cycloheximide resulted in proportional decrease in pseudotype levels it may have been possible to conclude that depletion of existing FLV glycoprotein occurred during the period of inhibition. In the absence of any significant effect, it is not possible to discriminate between a number of possible temporal relationships between the FLV glycoprotein and pseudotype production.

TABLE 27

Effect of Cycloheximide on the Production of VSV(FLV) Pseudotypes

Series	Preincubation Time in hours	Total VSV Titer in PFU/ml	Pseudotype Titer in PFU/ml
Drug	12	5.9×10^7	4.2×10^4
Washed	9	6.3×10^7	3.9×10^4
	6	5.8×10^7	4.7×10^4
	3	5.2×10^7	5.3×10^4
No Drug	0	4.2×10^7	4.9×10^4
Drug	12	1.6×10^4	$<1.0 \times 10^2$
Not washed	9	1.3×10^4	$<1.0 \times 10^2$
	6	2.0×10^4	$<1.0 \times 10^2$
	3	1.7×10^4	$<1.0 \times 10^2$
Drug	Added post-inf. of VSV	1.1×10^4	$<1.0 \times 10^2$

Approximately 1×10^6 FN-3T3 cells were seeded in 60 mm plastic petri dishes. The cells were preincubated with cycloheximide (20 µg/ml) for different lengths of time as stated in the Table. In one series of plates the drug was washed out before infection with VSV and no drug was added post infection. In the second series the drug was not removed and added after VSV infection also. VSV infection was done at an MOI 5 PFU/cell and the viruses were harvested after 12 hours of infection. Plaque forming assay was performed to determine the total yield and the pseudotype titer.

Effect of Glucosamine

Glucosamine inhibits avian sarcoma virus replication by blocking the synthesis of viral glycoprotein (Hunter et al., 1974; Lewandowski et al., 1975). The production of VSV in the presence of glucose is unaffected by glucosamine but is inhibited by glucosamine in the presence of fructose. This inhibition appears to be mediated through the reduction of viral RNA synthesis caused by UTP depletion (Scholtissek et al., 1975).

The effect of glucosamine on the synthesis of VSV(FLV) pseudotypes was studied. FN-3T3 cells which are chronically infected with FLV were superinfected with VSV after 24 hours of seeding at an MOI of 5 PFU/cell. Glucosamine was added post infection of VSV at a concentration of 20 μmoles/ml in Eagle's MEM containing either glucose or fructose or tryptose phosphate broth. Viruses were harvested after 12 hours of VSV infection. The result in Table 28 shows that there was inhibition of the synthesis of both VSV and the pseudotypes by almost 100-fold when glucosamine was applied in the presence of fructose or tryptose phosphate broth. In the presence of glucose, glucosamine inhibits the yield of VSV by 10-fold while corresponding pseudotype yield was reduced by 100-fold. This result suggest that glucosamine in the presence of glucose had a greater inhibitory effect on FLV specific glycoprotein synthesis than it had on VSV glycoprotein and RNA synthesis. The relative decrease in VSV pseudotype production under these conditions of infection also suggests that majority of the VSV(FLV) pseudotypes are assembled using FLV glycoprotein synthesized after VSV infection.

TABLE 28

Effect of Glucosamine on the Production of VSV(FLV) Pseudotypes

Medium	Glucosamine	Total VSV Titer in PFU/ml	Pseudotype Titer in PFU/ml
Glucose (10 mM)	0	6.1×10^7	2.4×10^4
	20 µmoles/ml	6.7×10^6	1.5×10^2
Fructose (10 mM)	0	6.5×10^7	5.2×10^3
	20 µmoles/ml	2.0×10^5	$<10^2$
Tryptose Phosphate Broth (10%)	0	3.6×10^7	4.5×10^3
	20 µmoles/ml	4.0×10^5	$<10^2$

Some 1.0×10^6 FN-3T3 cells seeded day before were infected with VSV at an MOI of 5 PFU/cell. MEM with or without glucosamine was added immediately after the infection in presence of either glucose of fructose or tryptose phosphate broth in the concentrations mentioned in the Table. The medium containing tryptose phosphate broth was 90% deficient in amino acids and 75% deficient in glucose. Virus was harvested 12 hours after infection and the plaque forming assay was performed to determine the VSV and the pseudotype titer.

Effect of Ethidium Bromide on the Production of Pseudotypes

Ethidium bromide, an inhibitor of mitochondrial transcription and translation, has been shown to inhibit RNA tumour virus production and transformation in de novo infected cells (Roa and Bose, 1974, 1975; Guntaka et al., 1975). This inhibition is effective at a concentration as low as 0.5 - 0.7 µg/ml but prolonged incubation of cells with the drug is necessary to get the maximum effect. In the presence of ethidium bromide, normal quantitites of viral DNA are synthesized but integration of this DNA into cellular DNA is blocked. This in turn inhibits viral replication. Inhibition of integration by ethidium bromide is thought to be due to its interference with the formation of the closed circular superhelical DNA which may be necessary for successful integration (Guntaka et al., 1975). The drug does not have any effect on viral production in chronically infected cells (Bader, 1973).

The effect of ethidium bromide on the production of pseudotype particles from cells newly infected with FLV was examined. Some 7 x 10^5 NIH-3T3 cells were incubated for 20 hours in medium containing 1.0 µg/ml of ethidium bromide. At the end of the incubation period, the cells were infected with FLV at an MOI of 100 PFU/cell and ethidium bromide was again added in the medium. After 24 hours of FLV infection, the cells were superinfected with VSV and ethidium bromide continued to be present in the medium. Viruses were harvested after 12 hours of VSV infection and the total VSV and the pseudotype titers were determined.

The result in Table 29 shows that the presence of ethidium bromide did not reduce the pseudotype yield to any degree. In this experiment, the FLV production after ethidium bromide treatment was not monitored. Roa and Bose (1974) using Balb-3T3 cells found inhibition of murine sarcoma virus production at ethidium bromide concentrations of 0.7 µg/ml when incubated 15 hours prior to infection with the virus. Guntaka et al., (1975) found 90% inhibition of integration of viral DNA into cellular DNA at a drug concentration of 0.5 µg/ml when the duck cells were incubated with the drug 8 hours prior to infection with avian sarcoma virus. Based on these results the conditions employed in the experiment of this thesis should be sufficient to greatly inhibit FLV production. The fact that no apparent reduction of pseudotype yield occurred raises two interesting possibilities which cannot unfortunately be resolved with the available data but suggest further work in this area may be important to our understanding of viral gene expression. The first possible explanation is simply that even though the bulk of the virus DNA may be prevented from integrating, sufficient viral DNA is integrated and utilized to produce FLV glycoprotein to yield normal levels of pseudotype particles. If this occurs in the absence of significant FLV production, then it suggests a dissociation of FLV virion production and glycoprotein synthesis. The second possibility, that the unintegrated linear duplex DNA copy of the viral genome serve as a template for the transcription of virus-specific messenger RNA for glycoprotein synthesis also suggests a dissociation between the two viral functions.

TABLE 29

Effect of Ethidium Bromide on the Production of VSV(FLV) Pseudotypes

Time after FLV Infection	Presence of Drug	Total VSV Titer in PFU/ml	Pseudotype Titer PFU/ml
0 hr	0	1.1×10^8	$<10^2$
	1 µg/ml	1.5×10^8	$<10^2$
24 hr	0	1.0×10^8	2.0×10^4
	1 µg/ml	1.1×10^8	2.2×10^4

Some 7×10^5 NIH-3T3 cells were incubated with medium containing 1 µg/ml ethidium bromide. After 20 hours of incubation, the cells were infected with FLV at an MOI of 100 PFU/cell and ethidium bromide was again added in the medium. Superinfection with VSV was done at an MOI of 5 PFU/cell after 24 hours of FLV infection and ethidium bromide continued to be present in the medium. Viruses were harvested after 12 hours of VSV infection and plaque forming assay was performed to determine the total VSV and the pseudotype titer.

DISCUSSION

In the work described in this thesis, an attempt has been made to detect and characterize the pseudotypes of vesicular stomatitis virus and murine leukemia virus. Some intracellular events involved with the synthesis of murine leukemia viral glycoprotein have been studied using pseudotype production as a tool.

Detection and Characterization of Pseudotype Particles

A number of cell lines chronically infected with murine leukemia viruses have been used as hosts for superinfection with VSV to produce pseudotype particles. The level of pseudotypes obtained in all the cases examined has been the order of 0.1% of of the total wild type VSV yield. This relatively low level of pseudotype necessitates the use of various controls to ensure that it is indeed pseudotype particles which are being investigated. For example, it is possible that VSV ribonucleoprotein (RNP) which can infect cells (Szilagyi and Uryvayev, 1973) would remain unneutralized by anti-VSV antiserum and appear as apparent pseudotypes in the assay used.

That this is unlikely is suggested for a number of reasons. The antiserum employed to neutralize the VSV particles is raised in rabbits against whole virus and is known from complement

fixation studies (Kang and Prevec, 1970) to contain antibodies to ribonucleoprotein components. While no direct evidence that these RNP particles may be inactivated is presented, the recent observation of the neutralization of transcriptase activity by anti-NS antibody (Imblum and Wagner, 1975) would suggest that this may occur. Secondly, whereas the infectivity of RNPs is highly dependent on the presence of polycation (Szilagyi and Uryvayev, 1973), no such dependence was observed in the pseudotype assay. Infectivity of "pseudotype" particles was greatly reduced in murine leukemia virus infected cells, a fact which is consistent with exclusion of a particle with MuLV envelope properties but is not expected in the use of infectious VSV-RNP. The fact that apparent pseudotypes are observed only from MuLV infected cells is also consistent with the particles assayed being true pseudotypes. Finally, the dose-dependent inactivation of VSV(FLV) pseudotype particles by anti-FLV antiserum is proof that these particles possess the neutralizing antigen of the Friend leukemia virus. In all of the observed properties, therefore, the infectivity seen in the pseudotype assay appears to be due to the genuine VSV(MuLV) pseudotype particles, that is, virions having the genetic constitution of VSV and the neutralizing antigens of MuLV.

Thus, the VSV(MuLV) particles have all or part of the VSV-specific component giving rise to neutralizing antibody substituted by the corresponding antigens of murine leukemia viruses (Zavada, 1972). In the VSV system, it has been clearly demonstrated by Kelley et al., (1972) that the VSV antigen against which neutralizing antibody is directed is the virus-specified glycoprotein G of the virion. Whether

VSV proteins other than G protein may be substituted for by MuLV proteins has not been firmly established, however, it was shown by Zavada (1972) that of the three (I, III and V) complementation groups of the Indiana serotype of VSV, only mutants of group V (the defect is thought to be in the gene coding for glycoprotein G) were complemented through pseudotype production in MuLV infected cells at the non-permissive temperature.

In the MuLV system, the glycoprotein 69/71 has been shown to constitute the surface knobs of the virion (Strand and August, 1973; Witte et al., 1973). Monospecific antiserum raised to purified glycoprotein 69/71 has been shown to neutralize the infectivity of MuLV (Steeves et al., 1974). It would seem probable on the basis of these observations that the VSV(MuLV) pseudotypes contain at least the MuLV glycoproteins 69/71. Whether they also contain other MuLV proteins has not yet been determined.

In this regard the fact that pseudotype particles produced from any of Friend-, Rauscher-, Moloney- or Gross virus infected cells are excluded from all of these infected cell lines shows a feature common to all of these MuLV virus types. If, as seems highly likely from the considerations presented above, only the envelope antigens are exchanged in the VSV(MuLV) pseudotypes, then the interference observed within the MuLV system must be due to MuLV virion glycoproteins. The fact that the MuLV strains used differ in some cases in both the type-specific and sub-group specific determinants present in the glycoprotein 69/71 suggests that it is the common group-specific determinant of glycoprotein 69/71 which plays some role in the interference

process.

In order to begin to understand the possible mechanisms through which pseudotype particles may arise, it is necessary to briefly consider some of the events which may be occurring in coinfected cells. Cells preinfected with MuLV will have some MuLV glycoproteins on their plasma membrane prior to VSV superinfection. As VSV synthesis proceeds, VSV specific glycoproteins are also inserted into the cellular plasma membrane. When VSV nucleocapsid structure begins to assemble, they may bud through part of the membranes modified by VSV glycoprotein to give VSV(VSV) particles or by MuLV glycoprotein to give VSV(MuLV) pseudotype particles. Conversely, as has been shown by Weiss et al., (1975), the RNA tumour virus genome may acquire a VSV envelope. Pseudotypes have been produced between different kinds of enveloped viruses, for example, VSV and paramyxo SV 5 (Choppin and Compans, 1970) or VSV and fowl plague virus (Zavada and Rosenbergova, 1972). The phenomenon of pseudotype formation leads to the concept that the recognition of membrane-associated viral glycoprotein by the viral ribonucleoprotein may not be highly specific. It has been shown in case of VSV and sindbis virus (Brown et al., 1974) and in case of semiliki forest virus (Garoff and Simons, 1974) that the surface projections extend through the viral membrane into close proximity with the viral nucleocapsid which probably suggests that associations may be involved between the two. The fact that pseudotype particles may be formed between diverse antigenically distinct virus types indicates that if a protein-protein interaction of this type occurs, it is not highly specific.

In spite of this apparent lack of specificity it is nonetheless possible that the relatively low yield of pseudotype particles is, at least in part, due to selection by the ribonucleoprotein of appropriate cell membrane areas for budding. It is conceivable, for example, that relatively few glycoproteins homologous to the ribonucleoprotein in a raft of glycoprotein specified by the coinfecting virus may be sufficient to affect a recognition by the RNP and a subsequent budding of virus through this region. In this case it would be expected that the majority of all pseudotype particles would in fact contain mixtures of both viral glycoproteins. Mixed particles of this type have indeed been observed in coinfections in which pseudotypes are produced (Zavada et al., 1975). Since it is not known how many VSV specific glycoproteins must be present on a virus envelope in order to render it neutralizable by anti-VSV antiserum, it is not possible to state that pseudotype particles totally lacking VSV G protein are indeed produced.

Alternatively, it is possible that pseudotype production is totally non-specific, the RNP budding through the membrane at any site modified by a viral glycoprotein. In this case, the relative amount of any pseudotype particle might be proportional to the relative amount of cell surface occupied by the particular glycoprotein. That this factor is operative in at least some cases of pseudotype production is suggested by the fact that infections yielding high levels of avian tumour viruses also produce a high level of VSV pseudotypes.

In most instances of pseudotype production, it is possible that both features, glycoprotein availability and some degree of selectivity, are involved. A particular mechanism of pseudotype production which is suggested from some of the data in this thesis is the possibility that pseudotype particles actually result from the "hitchhiking" of ribonucleoproteins through an area of membrane which is already involved in budding the coinfecting virus. It is not uncommon in the maturation of enveloped virus to observe relatively long strings of particles which appear to have been produced by budding continuously from one particular spot on the membrane. The pseudotype particles resulting from this mechanism of formation could consist of one or more RNP particles of one virus together with at least one RNP of the other virus type enveloped in one virus envelope. This model, at least in part, obviates the need for RNP-glycoprotein recognition, since the second glycoprotein need not specifically initiate the budding but needs only be in the right place as the first RNP buds out of the cell.

The presence of multi-genome or heteropolyploid particles of VSV have been shown by Baltimore (1975), Howatson (1970), and Prevec (personal communication). Weiss et al. (1975) have mentioned that preliminary electron microscopic studies by Pegrum have indicated that stocks of virus containing pseudotype of VSV and RNA tumour viruses contain bizarre forms of VSV often possessing more than one nucleocapsid. While direct electron microscopic examination of the pseudotype-containing virus stocks used in this thesis was carried

out, it was not possible with the low level of pseudotype particles in the preparation to determine if such heteropolyploid particles were indeed present. The results of the sedimentation experiments present in the 'Results' is however consistent with this particular model. Considering the relative shape and size of the VSV and MuLV ribonucleoproteins, it is possible to suggest that sonication of the ribonucleoproteins and associated envelope fragment at significantly lower energies than would be required to begin to disrupt the individual virion. Sonication could thus result in infectious VSV(MuLV) pseudotype particles having the sedimentation coefficient of the normal VSV(VSV) particle as was observed.

Intracellular Events regarding the Synthesis of FLV Glycoproteins

It has been shown in this thesis that pseudotype production can be used as a tool to study certain intracellular events concerning the metabolism of murine leukemia viral glycoprotein.

VSV inhibits host cell protein synthesis in several cell lines (Yamazaki and Wagner, 1970; Wertz and Younger, 1970). At an MOI of 100 PFU/cell, protein synthesis was inhibited to 7% of the control rate in infected L cells and at 10 PFU/cell it was inhibited to 24% of the control rate. The relative decrease of VSV(FLV) pseudotype production at high MOI of VSV infection (100 PFU/cell) may be direct consequence of this effect. FLV-specific protein synthesis may be inhibited by VSV infection in parallel with the effect on host cell protein synthesis. The concurrent reduction in FLV glycoproteins on the cell surface thus leads to decreased

synthesis of pseudotypes.

Experiments with glucosamine has shown that majority of glycoproteins incorporated in VSV(FLV) pseudotypes are synthesized after infection with VSV. Since, in this experiment the MOI of VSV infection was only 5 PFU/cell, a significant amount of FLV-specific glycoprotein synthesis probably went on even after VSV infection. Moroni (1971) has shown that in Rauscher leukemia virus infected cells there exists either a small intracellular pool and/or high turnover rate of the viral glycoproteins. Also, Baluda and Nayak (1969) have shown that in AMV-infected cells 10% of ^{14}C-glucosamine-labelled glycoprotein is incorporated into virions within an hour after synthesis. If, as is likely, a similar situation exists in FLV infected NIH-3T3 cells then it is reasonable to expect that the glycoproteins being utilized for pseudotype formation are synthesized at relatively short times (1 to 2 hours) prior to release from the cell. Since VSV infection at an MOI of 5 PFU/cell would allow some cellular and hence probably FLV protein synthesis to continue, FLV glycoprotein would continue to be made after VSV infection at times when VSV ribonucleoproteins have been assembled and begin to mature by budding.

It is interesting that the studies with actinomycin D presented in this thesis show that DNA-dependent RNA transcription may be inhibited for up to six hours prior to VSV superinfection with no significant reduction in the pseudotype yield. At a simple level this fact may be reconciled with the results of the glucosamine

experiment to suggest that m-RNA for MuLV glycoproteins has a long half-life under these conditions. This would allow continual synthesis of FLV glycoprotein and hence no reduction in pseudotype level. It has been shown by several groups of workers (Perry and Kelley, 1973; Singer and Penman, 1973; and Greenberg, 1972) that the m-RNA in mammalian cells is indeed long-lived under normal conditions. In the presence of actinomycin D however the half-life of the mjaority species of m-RNA is of the order of 2 to 4 hours (Penman et al., 1963; Craig et al., 1971). The reason for the shorter messenger life span in the presence of the antibiotic has not been resolved. Since actinomycin D was employed in the pseudotype experiments, it becomes more difficult to determine whether the lack of effect on pseudotype yield is due to the stability of the m-RNA or instead to the stability of FLV glycoproteins synthesized at relatively longer times prior to pseudotype formation. While the glycoproteins in RNA tumour virus infected cells may have a high turnover rate under normal conditions as described above, the presence of actinomycin D and consequently the absence of newly synthesized tumour virus RNA and hence turmour virus RNP may result in the net accumulation of leukemia viral glycoprotein on the surface of the cells to be used for the production of pseudotypes. In this case, therefore, it is stability of the FLV glycoprotein which is responsible for the production of normal yields of pseudotypes even after prolonged treatment with actinomycin D.

This model and the models considered in the 'Results' section in an effort to explain the production of pseudotype particles in the presence of ethidium bromide, both bring forward an interesting question not yet answered in the RNA tumour virus systems: does the same RNA molecule function both as a m-RNA in the cell and as the genome in the virion? The RNA extracted from the virion has some of the characteristics of other known m-RNA species. There is one poly A sequence of 200 nucleotides long at the 3'-OH end of 30 - 40S RNA (Wang and Duesberg, 1975; and Lai and Duesberg, 1972), either 60 - 70S or 35 - 40S RNA extracted from virus can be translated *in vitro* to yield virus-specific proteins (Helm and Duesberg, 1975; Twardzik *et al.*, 1973; and Gielken *et al.*, 1972), and apparently, RNA possessing the same sequence as virion RNA functions as m-RNA in the infected cells (Fan and Baltimore, 1973; and Leong *et al.*, 1972). Despite the obvious potential of the virion RNA for m-RNA function there is no evidence that the input viral genome is used for any function other than to serve as template for the DNA synthesized by the virion associated reverse transcriptase enzyme. The demonstration by Takano and Hatanaka (1975a and b) that viral RNA is not degraded *in vivo* after being used as template leaves open the possibility that it may subsequently function as m-RNA. In an experiment presented in this thesis, cells infected with FLV at an MOI of 100 PFU/cell in the presence of either actinomycin D or cytosine arabinoside failed to yield any pseudotype particles when superinfected with VSV. While a negative experiment of this type is not particularly helpful, it does say that to the level of sensitivity afforded by the pseudotype

assay, no FLV glycoprotein was detected in the absence of DNA synthesis or DNA-dependent RNA synthesis.

By direct visualization of infected cells using the scanning electron microscopy, Wong and MacLeod (1975) have detected murine leukemia virions on the infected cell membrane as early as 8 hours postinfection. This particular result is obtained using the temperature-sensitive Moloney leukemia virus ts-3 isolated by Wong and McCarter (1974) at the non-permissive temperature of 39°C under which the virus undergoes all stages of replication upto budding but do not release from the infected cells. This report is probably the minimum reported interval between the initial infection and the expression of progeny virion in murine leukemia virus infected cells. Assay of the release of the MuLV plaque forming units or labelled virions or reverse transcriptase of which the reverse transcriptase assay is the most sensitive technique, could not detect any progeny virus in de novo infected cells earlier than 14 - 16 hours (Salzberg et al., 1973; Fleissner et al., 1975). Fleissner et al. (1975) have found that p30, p15, p12 and p10 proteins are detectable in Rauscher and Gross murine leukemia virus infected cells after 16 hours of infection. Using the cytotoxic inhibition test the G-CSA antigen on Gross leukemia virus infected cells was undetected at 8 hours postinfection but was detected by 16 hours. Using an immunofluorescence technique Chuat et al., (1971) have detected the expression of group-specific antigen on murine tumour virus infected cells 15 hours postinfection. In the work of this thesis, the VSV(FLV) pseudotypes were first detected where cells were VSV 9 hours FLV Sin

the VSV growth curve and maximum pseudotype level reaches a plateau by 6 ~ 7 hours after VSV infection, it is therefore possible to use the pseudotype procedure to detect the presence of functional MuLV glycoprotein on the infected cell membrane within 15 - 16 hours following MuLV infection. The pseudotype assay is, in these circumstances, at least as sensitive as most other available procedures and more sensitive than some. The general applicability and relative simplicity of the procedure make the pseudotype approach deserving of further investigation.

BIBLIOGRAPHY

Aaronson, S.A. and Todaro, G.J. (1968). Development of 3T3-like lines from Balb/C mouse embryo cultures: transformation susceptibility to SV 40. J. Cell. Physiol. 72:141.

Ali, M. and Baluda, M.A. (1974). Synthesis of avian oncornavirus infected chicken cells. J. Virol. 13:1005.

Aoki, T., Old, L.J. and Boyse, E.A. (1966). Serological analysis of leukemia antigens of the mouse. Natl. Cancer. Inst. Mon. No. 22, Pg. 449.

Aoki, T., Boyse, E.A. and Old, L.J. (1968). Wild-type Gross leukemia virus. I. Soluble antigen (GSA) in the plasma and tissues of infected mice. J. Natl. Cancer Inst. 41:87.

Aoki, T., Boyse, E.A., Old, L.J., de Haven, E., Hammerling, U., and Wood, H.A. (1970). G (Gross) and H-2 cell-surface antigens: Location on Gross leukemia cells by electron microscopy with visually labeled antibody. Proc. Natl. Acad. Sci., USA 65:569.

Axelrad, A. and Steeves, R.A. (1964). Assay for Friend leukemia virus in mice: studies with the spleen forus assay method. Virology 24:513.

Bablanian, R. (1975). Structural and functional alterations in cultured cells infected with cytocoidal viruses. Adv. Virus Res., Vol. 20.

Bader, A.V. (1973). Role of mitochondria in the production of RNA-containing tumour viruses. J. Virol. 11:314.

Bader, J.P. (1964). The role of deoxyribonucleic acid in the synthesis of Rous sarcoma virus. Virology 22:462.

Bader, J.P. (1966). Metabolic requirements for infection by Rous sarcoma virus. I. The transient requirement for DNA synthesis. Virol. 29:444.

Baltimore, D. (1970). Viral RNA-dependent DNA Polymerase. Nature 226:1209.

Baltimore, D., Huang, A.S. and Stampfer, M. (1970). RNA synthesis of Vesicular Stomatitis virus. II. An RNA polymerase in the virion. Proc. Natl. Acad. Sci., USA 66:572.

Baltimore, D. and Smoler, D. (1971). Primer requirement and template specificity of the DNA polymerase of RNA tumour viruses. Proc. Natl. Acad. Sci., USA 68:1507.

Baltimore, D., Verma, I.M., Drost, S. and Mason, W.S. (1974). Temperature sensitive DNA polymerase from Rous sarcoma virus mutants. Cancer 34:1395.

Baluda, M.A. (1972). Widespread presence, in chickens, of DNA complementary to the RNA genome of avian leukosis viruses. Proc. Natl. Acad. Sci., USA 69:576.

Baluda, M.A. and Nayak, D.P. (1969). Incorporation of precursors into ribonucleic acid, protein, glycoprotein and lipoprotein of avian myeloblastosis viru. J. Virol. 4:554.

Beard, J.W. (1963). Avian virus growths and their etiologic agents. Adv. Cancer Res. 7:1.

Bernhard, W. (1958). Electron microscopy of tumour cells and tumour viruses. Cancer Res. 18:491.

Bernhard, W. (1960). The detection and study of tumour viruses with the electron microscope. Cancer Res. 20:712.

Bishop, J.M., Levinson, W.E., Quintrell, N., Sullivan, D., Fanshier, L. and Jackson, J. (1970a). The low molecular weight RNAs of Rous sarcoma virus. I. The 4S RNA. Virol. 42:182.

Bishop, J.M., Levinson, W.E., Quintrell, N., Sullivan, D., Fanshier, L. and Jackson, J. (1970b). The low molecular weight RNAs of Rous sarcoma virus. II. The 7S RNA. Virol. 42:927.

Biswal, N., McCain, B. and Benyesh-Melnick, M. (1971). The DNA of murine sarcoma-leukemia virus. Virol. 45:706.

Boettiger, D., Love, D.N. and Weiss, R.A. (1975). Virus envelope markers in mammalian tropism of avian RNA tumour viruses. J. Virol. 15:108.

Bolognesi, D.P., Luftig, R. and Shaper, J. (1973). Location of RNA tumour virus polypeptides. I. Isolation of further virus substructure. Virol. 56:549.

Bolognesi, D.P., Huper, G., Green, R.W. and Graf, T. (1974). Biochemical properties of oncornaviruses. Biochim. Biophys. Acta. 355:220.

Bonar, R.A. and Beard, J.W. (1959). Virus of avian myeloblastosis. XII. Chemical constitution. J. Natl. Cancer Inst., 23:183.

Both, G.W., Moyer, S.A. and Banerjee, A.K. (1975). Translation and identification of the m-RNA species synthesized in vitro by the virion associated RNA polymerase. Proc. Natl. Acad. Sci., USA 72:274.

Brown, F., Martin, S.J., Cartwright, B. and Crick, J. (1967). The RNAs of the infective and interfering components of Vesicular Stomatitis virus. J. Gen. Virol. 1:479.

Brown, F., Smale, C.J. and Horzinek, M.C. (1974). Lipid and protein organization in Vesicular Stomatitis and Sindbis viruses. J. Gen. Virol. 23:455.

Cannani, E. and Duesberg, P.H. (1972). Role of subunits of 60-70S avian tumour virus RNA in its template activity for the viral DNA synthesis. J. Virol. 10:23.

Cannani, E., Helm, K.V.D. and Duesberg, P.H. (1973). Evidence for 30-40S RNA as precursors of the 60-70S RNA of Rous sarcoma virus. Proc. Natl. Acad. Sci., USA 70:401.

Cartwright, B., Smale, C.J. and Brown, F. (1970a). Dissection of Vesicular Stomatitis virus into the infective ribonucleoprotein and immunising components. J. Gen. Virol. 7:19.

Cartwright, B., Talbot, P. and Brown, F. (1970b). The proteins of biologically active subunits of Vesicular Stomatitis virus. J. Gen. Virol. 7:267.

Choppin, P.W. and Compans, R.W. (1970). Phenotypic mixing of envelope proteins of the parainfluenza virus SV 5 and Vesicular Stomatitis virus. J. Virol. 5:609.

Chuat, J., Lasquellee, F., L'Hirondel, A. and Boiron, M. (1971). Studies on murine sarcoma virus. II. Detection of group specific antigens by immunofluorescence. Int. J. Cancer 7:101.

Coffin, J. and Temin, H.M. (1971). Ribonuclease sensitive DNA polymerase activity in uninfected and rat cells infected with Rous sarcoma virus. J. Virol. 8:630.

Craig, N., Perry, R.P. and Kelley, D.E. (1971). Life-time of the messenger RNAs which code for ribosomal proteins in L cells. Biochim. Biophys. Acta 246:493.

Dahlberg, J.E., Sawyer, R.C., Harada, F., Taylor, J.M., Faras, A.J., Levinson, W.E., Goodman, H.M. and Bishop, J.M. (1974). Transcription of DNA from the 70S RNA of Rous sarcoma virus. I. Identification of a specific 4S RNA which serves as a primer. J. Virol. 13:1126.

Dales, S. and Hanafusa, H. (1972). Penetration and intracellular release of the genomes of avian RNA tumour viruses. Virol. 50:440.

Deutsch, V. and Berkaloff, A. (1971). Ann. de l'Inst. Pasteur. 121:101.

Duesberg, P.H. (1968). Physical properties of Rous sarcoma virus DNA. Proc. Natl. Acad. Sci., USA 60:1511.

Duesberg, P.H. (1970). On the structure of RNA tumour viruses. Curr. Topic. Microbiol. Immunol. 51:79.

Duesberg, P.H. and Robinson, W.S. (1966). Nucleis acid and proteins isolated from the Rauscher mouse leukemia virus (MLV). Proc. Natl. Acad. Sc. U.S. 55:219.

Dulbecco, R. and Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 99:167.

Earle, W.R. (1943). Production of malignancy in vitro. J. Natl. Cancer Inst. 4:165.

Elder, K.T. and Smith, A.E. (1974). Methionine transfer RNAs associated with avian oncornavirus 70S RNA. Nature 247:435.

Emerson, S.V. and Wagner, R.R. (1972). Dissociation and Reconstitution of the transcriptase and template activities of Vesicular Stomatitis B and T virions. J. Virol. 10:297.

Emerson, S.V. and Wagner, R.R. (1973). L protein requirement for an in vitro RNA synthesis by Vesicular Stomatitis virus. J. Virol. 12:1325.

Erikson, E. and Erikson, R.L. (1972). Transfer ribonucleic acid synthetase activity associated with avian myeloblastosis virus. J. Virol. 9:231.

Fan, H. and Baltimore, D. (1973). RNA metabolism of murine leukemia virus: Detection of virus specific RNA sequences in infected and uninfected cells and identification of virus-specific messenger RNA. J. Molec. Biol. 80:93.

Faras, A.J., Garapin, A.C., Bishop, J.M. and Goodman, H. (1973). The characterization of low-molecular weight RNAs associated with 70S RNA of Rous sarcoma virus. J. Virol. 12:334.

Faras, A.J., Dahlberg, J.E., Sawyer, R.C., Harada, F., Taylor, J.M., Levinson, W.E., Bishop, J.M. and Goodman, H.M. (1974). Transcription of DNA from the 70S RNA of Rous sarcoma virus. II. Structure of a 4S RNA primer. J. Virol. 13:1134.

Farmilo, A.J. and Stanners, C.P. (1972). Mutant of Vesicular Stomatitis virus which allows DNA synthesis and division in cells synthesizing viral RNA. J. Virol. 10:605.

Fischinger, P.J., Tuttle-Fuller, N., Huper, G. and Bolognesi, D.P. (1975). Mitosis is required for production of murine leukemia virus and structural protein during de novo infection. J. Virol. 16:267.

Fleissner, E. and Tress, E. (1973). Isolation of a ribonucleoprotein structure from oncornaviruses. J. Virol. 12:1612.

Fleissner, E., Ikeda, H., Vitetta, E.S., Tress, R., Hardy, Jr., W., Stockert, E., Boyse, E.A., Pincus, T. and O'Donnell, P. (1975). Characterization of murine leukemia virus-specific proteins. Cold Spring Harbor Symp. Quant. Biol. 39:1057.

Follett, E.A.C., Pringe, C.R., Wunner, W.H., and Skehel, J.J. (1974). Virus replication in enucleate cells: Vesicular Stomatitis virus and influenza virus. J. Virol. 13:394.

Galasso, G.J. (1967). Enumeration of VSV particles and a demonstration of the growth kinetics by electron microscopy. Proc. Soc. Exp. Biol. Med. 124:43.

Gantt, R.R., Stromberg, K.J. and de Oca, F.M. (1971). Specific RNA methylase associated with avian myeloblastosis virus. Nature 234:35.

Garof, H. and Simons, K. (1974). Location of the spike glycoproteins in the Semliki Forest virus membrane. Proc. Natl. Acad. Sci., USA 71:3988.

Geering, G., Old, L.J. and Boyse, E.A. (1966). Antigens of leukemias induced by naturally occurring murine leukemai virus: Their relation to the antigens of Gross virus and other murine leukemia viruses. J. Exp. Med. 124:753.

Gielken, A.L.J., Salden, M.H.L., Bloemendal, H. and Konings, R.N.H. (1972). Translation of oncogenic viral RNA and eukaryotic messenger RNA in the E. coli cell-free system. FEBS Lett. 28:348.

Gilden, R.V., Oroszlan, S. and Huebner, R.J. (1971). Coexistence of intraspecies and interspecies specific antigenic determinants on the major structural polypeptide of mammalian C-type viruses. Nature New Biol. 231:107.

Green, R.W., Bolognesi, D.P., Schafer, W., Pister, L., Hunsmann, G., and de Noronha, F. (1973). Polypeptides of mammalian oncornaviruses. I. Isolation and serological analysis of polypeptides from murine and feline C-type viruses. Virol. 56:565.

Green, M., Grandgenett, D., Gerard, G., Rho, H.M., Loni, M.C., Robins, M., Salzberg, S., Shanmugam, G., Bhaduri, S. and Vecchio, G. (1975). Properties of oncornavirus RNA-directed DNA plymerase, the RNA template and the intracellular products formed early during infection and cell transformation. Cold Spring Harbor Symp. Quant. Biol. 39:975.

Greenberg, J.R. (1972). High stability of m-RNA in growing cultured cells. Nature 240:102.

Grubman, M.J., Moyer, S.A., Banerjee, A.K. and Ehrenfeld, E. (1975). Sub-cellular localization of Vesicular Stomatitis virus messenger RNAs. Biochem., Biophys. Res. Comm. 62:531.

Grubman, M.J., Ehrenfeld, E. and Summers, D.F. (1974). In vitro synthesis of proteins by membrane bound polysomes from Vesicular Stomatitis virus infected HeLa cells. J. Virol. 14:560.

Guntaka, R.V., Mahy, B.V.J., Bishop, J.M. and Varmus, H.E. (1975). Ethidium bromide inhibits appearence of closed circular viral DNA and integration of virus-specific DNA in duck cells infected by avian sarcoma virus. Nature 253:507.

Hackett, A.J., Zee, Y.C., Schaffer, F.L. and Talens, L. (1968). Electron microscopic study of the morphogenesis of Vesicular Stomatitis virus. J. Virol. 2:1154.

Hartley, J.W., Rowe, W.P., Capps, W.I. and Huebner, R.J. (1969). Isolation of naturally occurring viruses of the murine leukemia virus group in tissue culture. J. Virol. 3:126.

Hatanaka, M., Kakefuda, T., Gilden, R.V. and Callan, E.A.O. (1971). Cytoplasmic DNA synthesis induced by RNA tumour viruses. Proc. Natl. Acad. Sci., USA 68:1844.

Helm, K.V.D. and Duesberg, P.H. (1975). Translation of Rous sarcoma virus RNA in a cell-free system from ascites Krebs II cells. Proc. Natl. Acad. Sci., USA 72:614.

Howatson, A.F. (1970). Vesicular Stomatitis and related viruses. Adv. Virus Res. 16:195.

Huang, A.S. and Wagner, R.R. (1965). Inhibition of cellular RNA synthesis by non-replicating Vesicular Stomatitis virus. Proc. Natl. Acad. Sci., USA 54:1579.

Huang, A.S. and Wagner, R.R. (1966). Comparative sedimentation coefficient of RNA extracted from plaque-forming and defective particles of VSV. J. Molec. Biol. 22:381.

Huang, A.S., Baltimore, D. and Stampfer, M. (1970). RNA synthesis of Vesicular Stomatitis virus. III. Multiple complementary messenger RNA molecules. Virol. 42:946.

Huang, A.S., Besmer, P., Chu, L. and Baltimore, D. (1973). Growth of pseudotypes of Vesicular Stomatitis virus with N-tropic murine leukemia virus coats in cells resistant to N-tropic viruses. J. Virol. 12:659.

Hunsmann, G., Moenning, V., Pister, L., Seifert, E. and Schafer, W. (1974). Properties of mouse leukemia viruses. VIII. The major viral glycoprotein of Friend leukemia virus. Serological, interfering and hemagglutinating capacities. Virol. 62:307.

Hunter, E., Friis, R.R. and Vogt, P.K. (1974). Inhibition of avian sarcoma virus replication by glucosamine. Virol. 58:449.

Ihle, J.N., Hanna, Jr., M.G., Schafer, W., Hunsmann, G. and Bolognesi, D.P. (1975). Polypeptides of mammalian oncornaviruses. III. Localization of p15 and reactivity with natural antibody. Virol. 63:60.

Ikeda, H., Hardy, Jr., W., Tress, E. and Fleissner, E. (1975). Chromatographic separation and antigenic analysis of proteins of oncornaviruses. V. Identification of a new murine viral protein, p15(E). J. Virol. 16:53.

Kacian, D.L., Watson, K.F., Burny, A. and Spiegelman, S. (1971). Purification of the DNA polymerase of avian myeloblastosis virus. Biochim. Biophys. Acta 246:365.

Kang, C.Y. and Prevec, L. (1969). Proteins of Vesicular Stomatitis virus. I. Polyacrylamide gel analysis of viral antigens. J. Virol. 3:404.

Kang, C.Y. and Prevec, L. (1971). Proteins of Vesicular Stomatitis virus. III. Intracellular and extracellular appearance of virus-specific proteins. Virology 46:678.

Kelley, M.J., Emerson, S. and Wagner, T. (1972). The glycoproteins of Vesicular Stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody. J. Virol. 10:1231.

Kennel, S.J., Del Villano, B.C., Levy, R.L. and Lerner, R.A. (1973). Properties of an oncornavirus glycoprotein: Evidence for its presence on the surface of virions and infected cells. Virol. 55:464.

Klein, E. and Klein, G. (1964). Antigenic properties of lymphomas induced by the Moloney agent. J. Natl. Cancer Inst. 32: 547.

Klement, V. Rowe, W.P., Hartley, J.N. and Pugh, W.E. (1969). Mixed culture cytopathogenicity: a new test for growth of murine leukemia viruses in tissue culture. Proc. Natl.

Acad. Sci., USA 63:753.

Knipe, D., Rose, J.K. and Lodish, H.F. (1975). Translation of individual species of Vesicular Stomatitis viral m-RNA.

Koentris, T.G., Soeiro, R. and Fields, B. (1973). Host restriction of Friend leukemia virus. Role of the viral outer coat. Proc. Natl. Acad. Sci., USA 70:2549.

Lai, M.M.C. and Duesberg, P.H. (1972). Adenylic acid-rich sequence in RNAs of Rous sarcoma virus and Rauscher mouse leukemia virus. Nature 235:383.

Leong, J.A., Garapin, A.C., Jackson, N., Fanshier, L., Levinson, W.E., and Bishop, J.M. (1972). Virus-specific ribonucleic acid in cells producing Rous sarcoma virus. Detection and characterization. J. Virol. 9:891.

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

Lewandowski, L.J., Smith, R.E., Bolognesi, D.P. and Halpern, M.S. (1975). Viral glycoprotein synthesis under conditions of glucosamine block in cells transformed by avian sarcoma viruses. Virol. 66:347.

Lilly, F. and Nathenson, S.G. (1969). Studies on the FMR antigens. Transplant. Proc. 1:85.

Lilly, F. and Steeves, R. (1974). Antigens of murine leukemia viruses. Biochim. Biophys. Acta 355:105.

Linial, M. and Mason, W.S. (1973). Characterization of two conditional early mutants of Rous sarcoma virus. Virol. 53:258.

Loni, M.C. and Green, M. (1975). Virus-specific DNA sequences in mouse and rat cells transformed by the Harvey and Moloney murine sarcoma viruses detected by in situ hybridization. Virol. 63:40.

Love, D.N. and Weiss, R.A. (1974). Rseudotypes of Vesicular Stomatitis virus determined by exogeneous and endogeneous avian tumour viruses. Virol. 57:271.

McCrea, J.F., Epstein, R.S. and Barry, W.H. (1961). Use of potassium tartrate for equilibrium density-gradient centrifugation of animal viruses. Nature 189:220.

McDonnell, J.P., Garapin, A., Levinson, W.E., Quintrell, N., Fanshier, N. and Bishop, J.M. (1970). DNA polymerases of Rous sarcoma virus: Dilineation of two reactions with actinomycin. Nature 228:433.

McSharry, J.J., Compans, R.W. and Choppin, P.W. (1971). Proteins of Vesicular Stomatitis virus and of phenotypically mixed Vesicular Stomatitis virus-simian virus 5 virions. J. Virol. 8:722.

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

Mizutani, S., Temin, H., Kodama, M. and Wells, R.T. (1971). DNA ligase and exonuclease activitities in virions of Rous sarcoma virus. Nature 230:232.

Mizutani, S. and Temin, H.M. (1971). Enzymes and nucleotides in virions of Rous sarcoma virus. J. Virol. 8:409.

Molling, K., Bolognesi, D.P., Bauer, H., Busen, N., Plassman, H.W. and Hausen, P. (1971). Association of the viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA-DNA hybrids. Nature New Biol. 234:240.

Moroni, C. (1972). Structural proteins of Rauscher leukemia virus and Harvey sarcoma virus. Virol. 47:1.

Morrison, T., Stampfer, M., Baltimore, D. and Lodish, H.F. (1974). Translation of Vesicular Stomatitis virus m-RNA by extracts from mammalian and plant cells. J. Virol. 13:62.

Moyer, S.A. and Banerjee, A.K. (1975). Messenger RNA species synthesized in vitro by the virion-associated RNA polymerase of Vesicular Stomatitis virus. Cell 4:37.

Mudd, J.A. and Summers, D.F. (1970). Protein synthesis in Vesicular Stomatitis virus infected HeLa cells. Virol. 42:328.

Nakai, T. and Howatson, A.F. (1963). The fine structure of Vesicular Stomatitis virus. Virol. 35:268.

Nakata, Y. and Sakamoto, Y. (1971). Abstr. Jap. Cancer Conf., 30th, pp. 76.

Nermut, M.V., Frank, H. and Schafer, W. (1972). Properties of mouse leukemia viruses. III. Electron microscopic appearance as revealed after conventional preparation techniques as well as freeze-drying and freeze-etching. Virol. 49:345.

O'Connor, T.E., Rauscher, F.J. and Zeigel, R.F. (1964). Density gradient centrifugation of a murine leukemia virus. Science 144:1144.

Old, L.J., Boyse, E.A. and Stockert, E. (1964). Typing of mouse leukemias by serological methods. Nature 201:777.

Old, L.J., Boyse, E.A. and Stockert, E. (1965). The G (Gross) leukemia antigen. Cancer Res. 25:813.

Parks, W.P., Noon, M.C. Gilden, R. and Scolnick, E.M. (1975). Serological studies with low-molecular weight polypeptides from the Moloney strain of murine leukemia virus. J. Virol. 15:1385.

Parsons, J., Coffin, J., Haroz, R., Bromley, P. and Weissman, C. (1973). Quantitative determination and location of newly synthesized virus-specific ribonucleic acid in chicken cells infected with Rous sarcoma virus. J. Virol. 11:761.

Pasternak, G. (1967). Differentiation between viral and new cellular antigens in Graffi leukemia of mice. Nature 214: 1364.

Penman, S., Scherrer, K., Becker, Y. and Darnell, J. (1963). Polyribosomes in normal and poliovirus infected HeLa cells and their relationship to m-RNA. Proc. Natl. Acad. Sci., USA 49:654.

Perry, R.P. and Kelley, D.E. (1973). Messenger RNA turnover in mouse L cells. J. Molec. Biol. 79:681.

Pincus, T., Rowe, W.P. and Lilly, F. (1971). A major genetic locus affecting resistance to infection with murine leukemia viruses. II. Apparent identity to a major locus described for resistance to Friend murine leukemia virus. J. Exp. Med. 133:1234.

Rettenmier, C.W., Dumont, R. and Baltimore, D. (1975). Screening procedure for complementation-dependent mutants of Vesicular Stomatitis virus. J. Virol. 15:41.

Roa, R.C. and Bose, S.K. (1974). Inhibition by ethidium bromide of the establishment of infection by murine sarcoma virus. J. Gen. Virol. 25:197.

Roa, R.C. and Bose, S.K. (1975). Requirement for cellular protein synthesis in reversal of ethidium bromide-induced inhibition of cell transformation by murine sarcoma virus. Proc. Natl. Acad. Sci., USA 72:4337.

Robinson, W.S. and Baluda, M.A. (1965). The nucleic acid from avian myeloblastosis virus compared with the RNA from the Bryan strain of Rous sarcoma virus. Proc. Natl. Acad. Sci., USA 54:1686.

Rosenthal, L.J. and Zamecnik, P.C. (1973). Amino-acid acceptor activity of the "70S-associated" 4s RNA from avian myeloblastosis virus. Proc. Natl. Acad. Sci., USA 70:1184.

Ross, J., Scolnick, E.M., Todaro, G.J. and Aaronson, S.A. (1971). Separation of murine cellular and murine leukemia virus DNA polymerases. Nature New Biol. 231:163.

Ross, J., Tronick, S.R. and Scolnick, E.M. (1972). Polyadenylate rich RNA in the 70S RNA of murine leukemia-sarcoma virus. Virol. 49:230.

Rowe, W.P., Pugh, W.E. and Hartley, J.W. (1970). Plaque-assay techniques for murine leukemia viruses. Virol. 42:1136.

Salzberg, S., Robin, M.S. and Green, M. (1973). Appearance of virus-specific RNA, virus aprticles and cell surface changes in cells rapidly transformed by the murine sarcoma virus. Virol. 53:186.

Sarkar, N.H. and Moore, D.H. (1968). Internal structure of mouse mammary tumour virus as revealed after Tween-ether treatment. J. de Microscopie 7:539.

Sarkar, N.H., Nowinski, R.C. and Moore, D.H. (1971). Helical nulceocapsid structure of the oncogenic ribonculeic acid viruses (Oncornaviruses). J. Virol. 8:564.

Sarkar, N.H., Moore, D.H., and Nowinski, R.C. (1972). Symmetry of the nucleocapsid of the oncornaviruses. In 'RNA viruses and host genome in Oncogensis'. Ed. P. Emmelot and P. Bentvelzen, North Holland, Amsterdam, pp. 71.

Sarma, P.S., Turner, H.C. and Huebner, R.J. (1967). A viral interference test for mouse leukemia viruses.. Virol. 33:180.

Sawyer, R.C., Harada, F. and Dahlberg, J.E. (1974). Virion-associated RNA primer for Rous sarcoma virus DNA synthesis: Isolation from uninfected host cells. J. Virol. 13:1302.

Schafer, W., Fischinger, P.J., Bolognesi, E.H. and Pister, L. (1972). Properties of mouse leukemia viruses. II. Isolation of viral components. Virol. 47:210.

Schincariol, A.L. and Howatson, A.F. (1972). Replication of Vesicular Stomatitis virus. II. Separation and characterization of virus-specific RNA species. Virol. 49:766.

Scholtissek, C., Rott, R. and Klenk, H.D. (1975). Two different mechanisms of the inhibition of enveloped viruses by glucosamine. Virol. 63:191.

Shanmugam, G., Vecchio, G., Attardi, D. and Green, M. (1972). Immunological studies on viral polypeptide synthesis in cells replicating murine sarcoma-leukemia virus. J. Virol. 10:447.

Shoyab, M., Evans, R.M. and Baluda, M.A. (1974). Presence in leukemic cells of avian myeloblastosis virus-specific DNA sequences absent in normal chicken cells. J. Virol. 14: 47.

Shoyab, M., Baluda, M.A. and Evans, R. (1974). Acquisition of new DNA sequences after infection of chicken cells with avian myeloblastsis virus. J. Virol. 13:331.

Singer, R.H. and Penman, S. (1973). Messenger RNA in HeLa cells: Kinetics of formation and decay. J. Molec. Biol. 78:321.

Steeves, R.A. (1968). Cellular antigen of Friend virus-induced leukemias. Cancer Res. 28:338.

Steeves, R.A., Strand, M. and August, J.T. (1974). Structural proteins of mammalian oncogenic RNA viruses: Murine leukemia virus neutralization by antisera prepared against purified envelope glycoprotein. J. Virol. 14:187.

Stephenson, J.R., Reynolds, R.K. and Aaronson, S.A. (1972). Isolation of temperature-sensitive mutants of murine leukemia virus. Virol. 48:749.

Stephenson, J.R., Tronick, S.R. and Aaronson, S.A. (1974). Analysis of type specific antigenic dterminants of two structural polypeptides of mouse RNA C-type viruses. Virol. 58:1.

Strand, M. and August, J.T. (1971). Protein kinase and phosphate acceptor proteins in Rauscher murine leukemia virus. Nature New Biol. 233:137.

Strand, M. and August, J.T. (1973). Structural proteins of oncogenic RNA viruses. Interspec II, a new interspecies antigen. J. Biol. Chem. 248:5627.

Strand, M. and August, J.T. (1974). Structural proteins of mammalian oncogenic RNA viruses: Multiple antigenic determinants of the major internal protein and envelope glycoprotein. J. Virol. 13:171.

Strand, M. and August, J.T. (1975). Structural proteins of RNA tumour viruses as probes for viral gene expression. Cold Spring Harbor Symp. Quant. Biol. 39:1109.

Szilagyi, J.F. and Uryvayev, L. (1973). Isolation of an infectious ribonucleoprotein from Vesicular Stomatitis virus containing an active transcriptase. J. Virol. 11:279.

Takano, T. and Hatanaka, M. (1975a). Fate of viral RNA of murine leukemia virus after infection. Proc. Natl. Acad. Sci., USA 72:343.

Takano, T. and Hatanaka, M. (1975b). DNA:RNA hybrid in cell infected by murine leukemia virus. Cold Spring Harbor Symp. Quant. Biol. 39:1009.

Temin, H. (1963). The effects of actinomycin D on growth of Rous sarcoma virus in vitro. Virol. 20:577.

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

Temin, H.M. (1971). Mechanism of cell transformation by RNA tumour viruses. Ann. Rev. Microbiol. 25:609.

Temin, H.M. and Baltimore, D. (1972). RNA directed DNA synthesis and RNA tumour viruses. Adv. Virus. Res. 17:129.

Todaro, G.J. and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17:299.

Tooze, J. (1973). The molecular Biology of tumour viruses. Cold Spring Laboratory.

Toyoshima, K. and Vogt, P.K. (1969). Enhancement and inhibition of avian sarcoma viruses by poycations and polyanions. Virol. 38:414.

Tronick, S.R., Stephenson, J.R. and Aaronson, S.A. (1973). Immunological characterization of a low molecular weight polypeptides of murine leukemia virus. Virol. 54:199.

Tsuchida, N., Robin, M.S. and Green, M. (1972). Viral subunits in cells transformed by RNA tumour viruses. Science 176:1418.

Tsuchida, N. and Green, M. (1974). Intracellular and virion 35S RNA species of murine sarcoma and leukemia viruses. Virol. 59:258.

Twardzik, D., Simonds, J., Oskarson, M. and Portugal, F. (1973). Translation of AKR-murine leukemia viral RNA in an E. coli cell-free system. Biochem. Biophys. Res. Comm. 52:1108.

Varmus, H.E., Levinson, W.E. and Bishop, J.M. (1971). Extent of transcription by the RNA-dependent DNA polymerase of Rous sarcoma virus. Nature New Biol. 233:19.

Varmus, H.E., Weiss, R.A., Friis, R.R., Levinson, W. and Bishop, J.M. (1972). Detection of avian tumour virus-specific nucleotide sequences in avian cell DNAs. Proc. Natl. Acad. Sci., USA 69:20.

Varmus, H.E., Vogt, P.K. and Bishop, J.M. (1973a). Integration of DNA specific for Rous sarcoma virus after infection of permissive and non-permissive hosts. Proc. Natl. Acad. Sci., USA 70:3067.

Varmus, H.E., Bishop, J.M. and Vogt, P.K. (1973b). Appearance of virus-specific DNA in mammalian cells following transformation by Rous sarcoma virus. J. Molec. Biol. 74:613.

Varmus, H.E., Guntaka, R.V., Deng, C.T. and Bishop, J.M. (1975). Synthesis, structure and function of avian sarcoma virus-specific DNA in permissive and non-permissive cells. Cold Spring Harbor Symp. Quant. Biol. 39:987.

Vecchio, G., Tsuchida, N., Shanmugam, G. and Green, M. (1973). Virus-specific messenger RNA and nascent polypeptide in polyribosomes of cells replicating murine sarcoma-leukemia viruses. Proc. Natl. Acad. Sci., USA 70:2064.

Vigier, P. and Golde, A. (1964). Effects of actinomycin D and of mitomycin C on the development of Rous sarcoma virus. Virol. 23:511.

Wagner, R.R. and Huang, A.S. (1966). Inhibition of RNA and interferon synthesis in Krebs-2 cells infected with Vesicular Stomatitis virus. Virol. 28:1.

Wagner, R.R., Snyder, R.M. and Yamazaki, S. (1970). Proteins of Vesicular Stomatitis virus. Kinetics and cellular sites of synthesis. J. Virol. 5:548.

Wagner, R.R., Prevec, L., Brown, F., Summers, D.F., Sokol, F. and MacLeod, R. (1972). Classification of Rhabdovirus proteins: a proposal. J. Virol. 10:1228.

Warburg, O. and Christian, W. (1941). Biochem. Z. 310:384.

Ware, M. and Axelrad, A. (1972). Inherited resistance to N- and B-tropic murine leukemia viruses in vitro: Evidence that congenic mouse strains SIM and SIM.R differ at the FV-1 locus. Virol. 50:339.

Weiss, R.A., Boettiger, D. and Love, D.N. (1975). Phenotypic mixing between Vesicular Stomatitis virus and avain tumour viruses. Cold Spring Harbor Symp. Quant. Biol. 39:913.

Wertz, G.W. and Younger, J.S. (1970). Interferon production and inhibition of host synthesis in cells infected with Vesicular Stomatitis virus. J. Virol. 6:476.

Witte, O.N., Weissman, I.L. and Kaplan, H.S. (1973). Structural characteristics of some murine RNA tumour viruses by lactoperoxide iodination. Proc. Natl. Acad. Sci., USA 70:36.

Witte, O.N. and Weissman, I.L. (1974). Polypeptides of Moloney sarcoma-leukemia virions: their resolution and incorporation into extracellular virions. Virol. 61:575.

Witter, R., Frank, H., Moennig, V., Hunsmann, G., Lange, J. and Schafer, W. (1973a). Properties of mouse leukemia viruses. IV. Hemagglutination assay and characterization of hemagglutinating surface components. Virol. 54:330.

Witter, R., Hunsmann, G., Lange, J. and Schafer, W. (1973b). Properties of mouse leukemia viruses. V. Hemagllutination-inhibition and direct hemagglutination tests. Virol. 54:346.

Wong, P.K.Y., Holloway, A.F. and Comrack, D.V. (1971). A search for recombination between temperature sensitive mutants of Vesicular Stomatitis virus. J. Gen. Virol. 13:477.

Wong, P.K.Y. and MacLeod, R. (1975). Studies on the budding process of a temperature sensitive mutant of murine leukemia virus with a scanning electron microscopy. J. Virol. 16:434.

Yamazaki, S. and Wagner, R.R. (1970). Action of interferon. Kinetics and differential effects on viral function. J. Virol. 6:421.

Yaoi, Y. and Amano, M. (1970). Inhibitory effect of UV inactivated Vesicular Stomatitis virus on initiation of DNA synthesis in cultured chick embryo cells. J. Gen. Virol. 9:69.

Yaoi, Y., Mitsui, H. and Amano, M. (1970). Effect of UV irradiated Vesicular Stomatitis virus on nucleic acid synthesis in chick embryo cells. J. Gen. Virol. 8:165.

Yoshikura, H. (1970). Dependence of murine sarcoma virus infection on the cell cycle. J. Gen. Virol. 6:183.

Zavada, J. (1972). Pseudotypes of Vesicular Stomatitis virus, with coat of murine leukemia and avian myeloblastosis virus. J. Gen. Virol. 15:183.

Zavada, J., Zavadova, Z., Malir, A. and Kocent, A. (1972). VSV pseudotype produced in cell line derived from human mammary carcinoma. Nature New Biol. 240:124.

Zavada, J. and Rosenbergova, M. (1972). Phenotypic mixing of Vesicular Stomatitis virus with fowl plaque virus. Acta Virologica 16:103.

Zavada, J., Bubenik, J., Widmaier, R. and Zavadova, Z. (1975). Phenotypically mixed Vesicular Stomatitis virus particles produced in human tumour cell lines. Cold Spring Harbor Symp. Quant. Biol. 39:907.

Zee, Y.C., Hackett, A.J. and Talens, L. (1970). Vesicular Stromatitis virus maturation sites in six different host cells. J. Gen. Virol. 7:95.

ADDITIONAL REFERENCES

Imblum, R.L. and Wagner, R.R. (1975). Inhibition of viral transcriptase by immunoglobulin directed against the nucleocapdis NS protein of VSV. J. Virol. 15:1357.

Kang, C.Y. and Prevec, L. (1970). Proteins of Vesicular Stomatitis Virus. II. Immunological Comparisons of Viral Antigens. J. Virol. 6:20.

Wang, L.H. and Duesberg, P.H. (1974). Properties and location of poly (A) in Rous sarcoma virus RNA. J. Virol. 14:1515.

Wong, P,K.Y. and McCarter, J.A. (1974). Studies of two temperature-sensitive mutants of Moloney leukemia virus. Virol. 51: 424.