REPLICATION AND TRANSCRIPTION OF ADENOVIRUS TYPE 2 AFTER ULTRAVIOLET IRRADIATION

REPLICATION AND TRANSCRIPTION OF ADENOVIRUS TYPE 2 AFTER ULTRAVIOLET IRRADIATION

by

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SCOPE AND CONTENTS:

When adenovirus type 2 is irradiated with ultraviolet light before infection of KB cells, a significant reduction of virus yields is observed. The ability of the irradiated viral DNA as template for DNA and RNA synthesis was examined. Special emphasis was placed on quantitative determinations of viral DNA and both quantitative and qualitative analysis of viral RNA in infected cells.

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INTRODUCTION

Regulation and control of biosynthetic processes at the molecular level is a fundamental biological problem which must be studied to reach an understanding of differentiation and development. In the past thirty years various systems have emerged to provide useful probes to the understanding of molecular events. The discovery of phage by d'Herelle as replicating units dependent on bacterial host machinery started a new generation of fruitful discoveries. The elucidation of DNA in 1953 by Watson, Crick, and Wilkins opened up a new horizon on the study of genetics and replication. Since DNA (with the exception of the RNA viruses) was recognized as the universal hereditary material consisting of genes transmissable predictably from one generation to the next, it has intrigued scientists how this mechanism is organized and regulated; why some genes are "on" and others "off", i.e., the mechanisms of expression and repression.

Escherichia coli became the popular system of the '60's since it was easily maintained, and it replicated itself quickly. Exhaustive studies were endeavoured by geneticists, enzymologists, biochemists

and molecular biologists alike. Invaluable information has been obtained concerning the mechanisms of DNA replication, RNA transcription and translation into proteins. E. coli has been used to study the intricacies of various bacterial components, their composition and function(s).

Though the study of prokaryotes is both interesting and important, it has nevertheless some limitations. Many feel that a direct extrapolation of processes in bacterial cells to higher organisms is far too simple-minded. The eukaryotic cell contains many times more DNA with greater complexity than the bacterial cell and carries vast amounts of information. So many complex processes are going on simultaneously and thousands of proteins are being synthesized at any one time, that it seemed virtually impossible to segregate individual processes for detailed analysis. However attempts to unravel the eucharyotic system have improved considerably during the past few years due to technical progress of isolation and examination of various sub-cellular components and the ability to reproduce, in vitro, conditions similar to the natural environment of these components.

A. The Study of Animal Viruses

The use of animal viruses offers an important tool for the eventual understanding of

regulation and control of the expression of genetic information. Viruses are completely dependent on their host's machinery and vary in their effects upon the host. Over 500 animal viruses have been described (Green, 1966), varying in complexity and mechanism of replication. They have been classified into two main groups according to their nucleic acid composition: the RNA-viruses including the arboviruses, myxoviruses, picornaviruses and reoviruses, and the DNA-viruses including adenoviruses, herpes-viruses, papovaviruses and poxviruses.

The response of the host cell to infection with virus is of two main types. One is a lytic infection when the virus literally takes over the cell's machinery to produce more infectious particles and subsequently causes cell death or lysis. The other type of infection is the abortive infection where the maturation process appears incomplete and the host cell may become transformed. In either case metabolic alterations can be observed. There may be a stimulation (Melnick et al, 1964; Brenswick and Rapp, 1968; Raškas et al, 1971) or inhibition (Green, 1966; Levine and Ginsberg, 1968; Ho and Washington, 1971) of macromolecular synthesis, the formation of inclusion bodies as seen in Adenovirus infection (Ginsberg, 1962), and the appearance of virus -

specific antigens. If the infection is abortive and leads to transformation, the cells lose contact inhibition and control over cellular division. The DNA - viruses have been studied extensively for their capacities to induce tumor - formation when injected into animals. For instance Adenoviruses types 12, 18 and 31 are highly oncogenic in hamsters (Huebner et al, 1962) and papovaviruses SV40 and polyoma also cause tumors in new-born rodents (Eddy, 1963). The persistence of viral genes in transformed cells has been demonstrated by the detection of neoantigens (Huebner et al, 1964), the continued synthesis of virus - specific mRNA (Wall and Darnell, 1971; Axel et al, 1972), enzymic alterations (Kit et al, 1970; Gallagher et al, 1971), or the detection of viral DNA by rescue of the virus from the transformed cell by cell fusion with a permissive host (Gerber, 1966) or with the aid of a helper virus (Sarma et al, 1970).

The virus - infected cell system is ideal for studies of both virus and cellular molecular events. One can introduce an extremely small amount of genetic information leading to profound reproducible changes in the host cell metabolism. Due to a virus' ability to shut off or stimulate various cellular processes, these can be selectively analysed. The smallest DNA - viruses, the papovavirus, contain a

double-helical DNA with a molecular weight of 3×10^6 (Melnick, 1962) equivalent to approximately 5,000 nucleotides and capable of synthesizing about 8 proteins. Since viruses utilize the host's machinery for replication, transcription and translation of their limited information, it is thus possible to gain insight on regulation at the cellular level.

B. The Adenoviruses

The biosynthetic events leading to production of infectious viral particles follows an orderly sequence which has been extensively studied by Green and his co-workers (Green, 1966; Green et al, 1967 and 1968; Lawrence and Ginsberg, 1967; Green et al, 1970). Adenovirus type 2 has a double-helical DNA of molecular weight 23×10^6, synthesizing at least 8-10 proteins. Replication of the virus is intranuclear with translation occurring in the cytoplasm of infected cells. Viral DNA and RNA are synthesized at the time of host macromolecular synthesis inhibition. Ginsberg (Ginsberg et al, 1967) has postulated several control steps which might cause this observed host inhibition. He believes that the viral DNA protein core and capsid protein inhibit host replication, the viral structural proteins complexing with DNA, as the histones do. Furthermore, since the rate of viral RNA synthesis

increases 5 X the rate of host RNA synthesis, these RNA species may compete for host ribosomal subunits. Finally the viral components are assembled into mature infectious progeny virions.

More recent studies further indicate why Adenovirus can be used as a model study for eukaryotic cells. Ad 2 DNA replication has been shown to be semi-conservative. Furthermore, viral RNA is believed to be processed similarly to eukaryotes. Georgiev (Lukanidin et al, 1972) has proposed a general scheme for the transport of mRNA via globular protein particles which he calls informofers. These informofers are believed to detach RNA from the DNA template, giving rise to ribonucleoprotein complexes. Virus - specific RNA has been detected within these complexes, extracted from Ad 2 infected cells, by hybridization to viral DNA. Processing nucleases then degrade non-informative RNA sequences; in contrast to this, Green (Parsons et al, 1971) has found all the nucleotide sequences of Ad 2 mRNA to be transported from the nucleus into the cytoplasm, where it is found associated with the polysomes. Green has suggested that late virus - specific RNA sequences are transcribed as large precursor RNA molecules within the cell nucleus which are subsequently cleaved to smaller RNA pieces prior to translation in the cytoplasm, a

mechanism also proposed in the eucharyotic cell. A further line of evidence is the fact that polyadenylic acid (poly (A)) has been found in the large heterogeneous nuclear RNA and in the smaller mRNA of mammalian cells, as well as in vaccinia virus mRNA and adenovirus specific nuclear and polyribosomal RNA (Philipson et al, 1971; Edmonds et al, 1971; Darnell et al, 1971). Darnell and Philipson have indicated that poly (A) is added posttranscriptionally to the nuclear RNA, suggesting that the poly (A) is located near a terminus of nuclear RNA molecules. Indeed Perry (Molloy et al, 1972) has shown these sequences to be located at the 3'-OH termini of HeLa and L-cell mRNA.

Another parallel between viral and eucharyotic systems is the fact that translation occurs in the cytoplasm. It has been shown in poliovirus (Baltimore, 1969) and in vaccinia virus (Katz and Moss, 1970) that large polypeptides may be synthesized and specifically cleaved to smaller peptides, another example of post-transcriptional control. Thus, it can be seen that the DNA - viruses, including Adenoviruses, are ideal subjects for studies of control and regulation in eukaryotes.

C. Radiation Effects on Viral Functions

Various agents have been used to produce lesions in the DNA of biological systems. Irradiation

of genetic material has been found a convenient method since the incident dose can be carefully measured and the doses varied. Ultraviolet irradiation is known to produce mainly thymidine dimers (Howard-Flanders, 1968; Smith and Hanawalt, 1969) whereas X - rays and γ - rays give rise to other photoproducts as well such as single - or double-stranded breaks (Lytle and Ginoza, 1969; Bohne et al, 1970). The structural defects induced inhibit replication of DNA (Yamashita et al, 1971) until they are corrected by a repair mechanism (Setlow and Carrier, 1964; Wilkin, 1969; Kelly et al, 1969; Driedger and Grayston, 1971). In some cases such as patients with the clinical condition Xeroderma pigmentosum, this mechanism is lacking due to a deficiency in repair enzyme. When these individuals are exposed to U.V.-irradiation from sunlight, they develop from severe skin burns to fatal skin cancers. The study of radiation and repair is important also since it is known that actively dividing cells, such as bone marrow and reproductive gonads, are most susceptible to radiation, and since radiation is used as a medical treatment for various diseases.

Viruses offer an excellent approach for the study of radiobiological problems. Viruses can be conveniently kept at low temperatures during the

irradiation procedure, and after infecting a suitable host, the expression of the "defective" genome and subsequent repair can be followed. It has been suggested that repair occurs via a host mediated repair mechanism (Sauerbier, 1964; Zavadova and Zavada, 1968) which may be similar to that observed in animal or bacterial cells. Since this mechanism may be similar to the control and regulation mechanisms of normal cells (Haynes, 1966; Cleaver, 1969), it is worthwhile to examine it in detail.

Another important use of U.V.-irradiation is that it can alter the final outcome of infection. For example tumor induction may be enhanced, or a transformation may replace a normally lytic infection. U.V.-irradiated herpes simplex virus type 2 has been shown to transform hamster embryo fibroblast cells in vitro (Duff and Rapp, 1971), suggesting that U.V.-irradiated non-plaque-forming virus particles still retain sufficient information to transform mammalian cells. It has also been reported that U.V.-irradiated herpes simplex virus type 1 can transform the deoxythymidine kinaseless mouse L cells to a TdR kinase positive phenotype (Munyon et al, 1971).

In this investigation, Adenovirus type 2 was irradiated with U.V. and subsequent replication

was followed. Main emphasis was placed on the quantitative and qualitative analysis of viral nucleic acids following infection of human KB cells with irradiated virus as compared to infection with non-irradiated Ad 2.

MATERIALS AND METHODS

I. TISSUE CULTURE TECHNIQUES

The human cell line KB (Eagle, 1965) was used throughout the course of this investigation. The cells were maintained either as monolayer cultures or as suspension cultures. Monolayer cultures were grown in sterile minimum essential medium (GIBCO), supplemented with 10% Fetal Calf Serum (FCS) (GIBCO) and 1% Fungizone (GIBCO), on 32 oz. screwcap prescription bottles. The cells were kept in logarithmic phase by subculturing when they had formed a confluent sheet. This was done either by scraping the cells with a sterile rubber policeman or by trypsinization (0.125% trypsin in 1% K Cl and 0.44% Sodium citrate) at 37°C and seeding about 3×10^6 cells per 32 oz. bottle. The monolayers were kept at 37°C in a humid incubator with an atmosphere of 95% air and 5% CO_2. Suspension cultures were grown in standard chemical reagent bottles in sterile culture medium (Joklik, modified for suspension cultures, GIBCO) supplemented with 5% Horse Serum (HS) and 1% Fungizone. The cells were kept at 37°C and constantly agitated by means of a teflon magnetic bar over a magnetic stirrer.

They were kept in logarithmic phase at a density of about 2-4 x 10^5 cells/ml by daily dilutions using culture medium pre-warmed to 37°C.

II. VIROLOGICAL TECHNIQUES

1. Purification of the Virus: Only purified human adenovirus type 2 (Ad 2) was used throughout this study. KB cells in suspension were used for cultivation of the virus. They were infected with Ad 2 at a multiplicity of infection (MOI) of about 100 plaque-forming-units (PFU) per cell. The infected cells were harvested at about 48 hours post-infection by low speed centrifugation. The virus was isolated and purified by the method of Green and Piña (Green and Piña, 1963) modified by Rainbow (Rainbow, 1971). Briefly, the cell pellet was resuspended in 0.01 M Tris pH 8.1 and disrupted by sonication. The sonicate was homogenized with equal volumes of Freon 113 at 4°C. The virus containing aqueous solution was separated from the Freon and cell debris by centrifugation. The virus present in the supernatant was sedimented onto a cesium chloride cushion of density 1.44 by high speed centrifugation. Further purification was achieved by two cycles of equilibrium centrifugation in cesium chloride. The titer of the purified virus was determined by plaque-assay and the virus

was stored in Tris Buffer Saline (TBS) + 20% glycerol at -45°C.

2. Viral Assay: The plaque-assay (Green et al, 1967) was used to determine virus titers. KB cells obtained from monolayer cultures were seeded onto plastic dishes (Falcon Plastics, Los Angeles, California) at a concentration of 10^6 cells/60 mm dish in 5 ml of minimum essential medium supplemented with 2% Amino Acids (GIBCO), 2% Vitamins (GIBCO), 10% FCS, 1% Fungizone, and 2% Antibiotics (6×10^6 units/ml Penicillin and 7.4×10^3 µg/ml Streptomycin). The plates were incubated for 2 days in a CO_2 incubator until the cells reached confluency. Then the medium was removed and the cell sheet washed with 5 ml of Phosphate Buffer Saline (PBS) (Rainbow, 1971). Virus suspended in PBS + 1% Antibiotics + 1% FCS was adsorbed to the cells at 37°C for 120 min. in a CO_2 incubator with occasional shaking. The cells were overlayed with 5 ml of overlaying agar, consisting of 2.5 ml of 1.8% Noble Agar + 2.5 ml of plaquing medium (Rainbow, 1971), allowed to gel at room temperature, and incubated at 37°C. On the fifth day post-infection, 5 ml of overlaying agar were again added. On the tenth day the plates were overlayed with 5 ml of overlaying agar containing neutral red to stain the living non-infected cells. The total number of plaques per plate was determined.

3. Inclusion Body Formation: Inclusion body formation was routinely examined as a quick check for infected cells. Infected cells kept in suspension cultures were sedimented by low speed centrifugation, swelled in 1% Na citrate and fixed in Carnoy's fixative (3:1: EtOH : Glacial Acetic Acid). The cells were placed on a slide, stained with orcein, washed in absolute alcohol and scored for the presence of nuclear inclusion bodies.

III. IRRADIATION TECHNIQUE

The virus used for the irradiation experiments was suspended in 1.5 ml of culture medium in a 35 mm plastic petri dish (Falcon Plastics) and agitated by means of a micro-magnetic bar over a pre-cooled magnetic stirrer. The petri dish was kept at a distance of 4 inches (10 cm.) from the Ultraviolet light source (8 Watt General Electric Germicidal Tube, Number G875), and the virus was irradiated for varying periods of time in seconds. The incident dose was determined from a Blak-Ray short wave UV-meter, model J-225 (Ultraviolet Products, Inc., San Gabriel, California).

IV PREPARATION OF LABELLED NUCLEIC ACIDS

1. Labelling and Extraction of RNA: 300 ml of infected cells were centrifuged for 15 min. at 1000 RPM in an IEC Model RP-2 centrifuge and resuspended in

60 ml of pre-warmed suspension medium + 5% HS + 1% Fungizone. The cells were concentrated in this way to reduce the total amount of radioactive precursors used per experiment. They were labeled for two hours at 37°C with H^3 - Uridine (labeled in the 5-position) at a concentration of 10 μ Ci/ml (specific activity of 25 - 30 Ci/m mole; The Radiochemical Center, Amersham). The cells were harvested by low speed centrifugation, washed three times in Earles Saline Buffer, resuspended in 0.5 ml of sucrose buffer (0.25 M sucrose, 0.01 M Tris pH 7.4, 0.01 M KCl, 0.001 M Mn^{++}, 0.001 Mg^{++}), and stored at -45°C until extraction.

From this step on, all glassware used in handling RNA was pre-heated at 180°C for 4 hours to eliminate RNase. The procedure for RNA extraction was a modified method of Sherrer (Sherrer et al, 1966; Sherrer, 1970). The frozen pellet was thawed slowly at 4°C to avoid breakdown of the cells and subsequent release of degradative enzymes such as RNase. The cells were then centrifuged for 10 min. at 1000 RPM in a clinical centrifuge at room temperature, and resuspended to 30 x the pellet volume in Extraction Buffer (0.01 M Na acetate pH 5.2, 0.05 M Na Cl, 0.001 M Mn^{++}, 0.001 M Mg^{++}) in glass-stoppered Erlenmeyer flasks. Sodium lauryl sulphate (SDS) was added to give a final concentration of 1% to lyse the cells. Immediately after the addition of SDS, an equal volume

of redistilled phenol at 65°C was added. The flask content was shaken manually for 3 min. in a 65°C water bath, and cooled for 5 min. in a mixture of ice: methanol (2:1), giving a temperature of -15°C, until phenol crystals appeared on the sides of the flask. The mixture was centrifuged for 1 min. at 12,000 RPM at 4°C in a Sorvall Superspeed Model RC2-B centrifuge. The bottom phenol layer was removed with a 50 ml plastic syringe and the aqueous phase was re-extracted twice at 65°C, the first time with phenol 20 x the volume of the original cell pellet for 3 min., the second time with phenol 10 x the pellet volume for 2 min. The final aqueous layer was measured and re-extracted with 1/2 its volume of phenol saturated with extraction buffer for 5 min. at room temperature. The aqueous layer was adjusted to 0.1 M Na Cl and precipitated overnight with ethanol at -20°C. The RNA was collected by centrifugation at 15,000 RPM at 4°C in a Sorvall centrifuge. The RNA was precipitated once more with ethanol and finally dissolved in 0.01 M Tris pH 7.0 + 0.01 M Na Cl to give a final concentration of about 1 mg/ml. The purity of the preparation was checked by determining the optical density (O.D.) at 260 nm and 280 nm. A $\frac{260\text{ nm}}{280\text{ nm}}$ O.D. ratio of greater than 2 was considered

acceptable. The RNA concentration was determined from O.D. readings at 260 nm, where 1 mg/ml RNA gives an O.D. reading of 24.

2. Labelling and Extraction of DNA: 100 ml of infected cells were labeled with Methyl - H^3 - Thymidine of specific activity of 15-20 Ci/m mole (The Radiochemical Center, Amersham) at a concentration of 2 μ Ci/ml. The cells were incubated for 1 hour at 37°C, washed three times with cold Earles saline, resuspended in 0.5 ml of sucrose buffer, and stored at -45°C until extraction.

The method of DNA extraction was essentially that used by Mak (Mak, 1969). The cell pellet was thawed gently at 4°C, centrifuged for 10 min. at 1000 RPM in a clinical centrifuge, and resuspended in 10 ml of Extraction Buffer (0.15 M Na Cl, 0.015 M Na citrate, 0.1 M Tris pH 8.0, 0.005 M EDTA, 0.3 M Na tricloroacetate). SDS was added to a final concentration of 1% and incubated for 15 min. at 37°C to lyse the cells. To digest proteins, pronase (B-grade, Calbiochem., Los Angeles, California; predigested for 2 hours at 37°C) was added at a concentration of 400 μg/ml and incubated at 37°C for 4 hours. Equal volumes of phenol, saturated with Extraction Buffer was added and the sample was shaken for 10 min. at room temperature, cooled for 5 min. in ice, and centrifuged 10 min. at 13,000 RPM

in a Sorvall centrifuge Model RC2-B. The phenol layer was removed and the aqueous layer was re-extracted twice in a similar manner. The aqueous layer was removed and precipitated overnight with 2 volumes of ethanol at -20°C. The precipitate was collected by centrifugation for 15 min. at 15,000 RPM in the Sorvall centrifuge, and resuspended in 3 ml 0.1 x SSC (1 x SSC = 0.15 M Na Cl + 0.015 M Na citrate). The suspension was treated with RNase (3 x cryst., Worthington Biochem. Corp.; preheated at 90°C for 10 min. to destroy DNase) at a concentration of 50 µg/ml for 30 min. at 37°C. SDS was then added to a final concentration of 0.5% and the phenol extraction procedure described above was repeated three times. The final aqueous layer was dialysed for 2 days against 0.1 x SSC. The purity of the preparation was determined as for RNA preparations, and the concentration by reading the optical density at 260 nm where 1 O.D. unit equals 50 µg of DNA per ml.

3. Preparation of Viral DNA: Viral DNA was extracted from purified Ad 2. After the virus suspension was dialysed against 0.01 M Tris pH 8.1 to remove cesium chloride, to each ml of virus suspension, the following was added: 0.15 ml 0.05 M EDTA pH 7.0, 0.15 ml 1 M Na $H_2PO_4 \cdot H_2O$ pH 6.0, 0.1 ml 10% SDS, and 100 µg pre-digested pronase. Incubation was carried out for 1 hour at 37°C to disrupt the virus. The suspension

was extracted 3 x at room temperature with phenol saturated with 0.1 M Na Cl and 0.05 M Na PO_4 pH 6.7. The viral DNA was then dialysed extensively with 0.1 x SSC and stored at -45°C.

V QUANTITATIVE AND QUALITATIVE ANALYSIS OF VIRAL NUCLEIC ACIDS

1. Quantitative Determination of Radioactivity in Labeled Nucleic Acids: After infected cells were labeled with either H^3 - Uridine or H^3 - Thymidine, the amount of radioactivity associated with the nucleic acids was determined by TCA precipitation. Briefly, 2 ml of cells were sedimented by low speed centrifugation and the supernatant discarded, the pellet was washed 3 x with Phosphate Saline Buffer (0.14 M Na Cl, 0.003 M K Cl, 0.008 M Na_2 HPO_4, 0.0015 M KH_2PO_4) and resuspended in Na acetate pH 5.1. An aliquot of cells was treated for 20 min. with 4°C 10% TCA in the presence of 100 µg of salmon sperm DNA (Mann Research Laboratories) added as carrier. The precipitate was filtered onto cellulose nitrate filters, and the filters dried and counted in 5 ml toluene containing 4 gm/ml Omnifluor (New England Nuclear).

Occasionally, the amount of radioactivity incorporated into DNA was determined by hot TCA extraction (Burton, 1956). Briefly, cells labeled

with H^3 - Thymidine were treated with cold 10% TCA for 20 min. The precipitate was collected by centrifugation and washed once with cold TCA. 10% TCA was added to the precipitate and heated at 90°C for 20 min. The supernatant, containing the radioactivity originally associated with H^3 - DNA, was collected. The pellet was washed once with 10% TCA and the wash pooled with the previous supernatant. The radioactivity in a 0.2 ml aliquot of this solution was determined by adding 15 ml of Aquasol (New England Nuclear) and counting in a Beckman scintillation counter. Control experiments showed that this procedure recovered all the radioactivity associated with DNA (Mak, personal communication).

2. Hybridization Procedure: The hybridization procedure is based on the method originally described by Gillespie and Spiegelman (Gillespie and Spiegelman, 1965). DNA, denatured by alkaline treatment (pH 12.8) and subsequent neutralization, was filtered onto nitrocellulose filters and irreversibly bound to the filters by heating at 80°C for 2 1/2 hours. H^3 - RNA dissolved in 2 x SSC containing 0.1% SDS (1 ml) was added to the filters in a capped scintillation vial. Hybridization was carried out at 66°C for 20 hours. The filters were washed exhaustively with 2 x SSC, treated with 5 ml of RNase in 2 x SSC (20 μg/ml) and washed again with 2 x SSC. The radioactivity bound to the filters was determined by

counting in toluene containing omnifluor as described. For DNA - DNA hybridizations, the procedure was essentially that of Gillespie (Gillespie, 1968). H^3 - DNA, fragmented by sonication for 1.5 min. was dissolved in 2 x SSC, 0.01 M Tris pH 7.4, and 0.1% SDS and hybridized at 66°C for 20 hours to viral DNA bound to nitrocellulose filters. The filters were washed with 0.003 M Tris pH 9.4 + 0.1 x SSC, dried, and the radioactivity determined by counting in a liquid scintillation system.

3. Melting Profiles of H^3 - RNA - DNA Hybrids: After hybridization, RNase treatment, and washing, filters containing H^3 RNA - DNA hybrids were placed in glass Sorvall tubes + 2.5 ml 0.1 x SSC containing 0.1% SDS and incubated for 10 min. at 5°C intervals from 25°C to 95°C. After 10 min. incubation at each temperature, 0.5 ml of solution was removed and the radioactivity determined by cold TCA precipitation as described in Section V - 1. The volume in the Sorvall tubes was maintained at 2.5 ml by addition of fresh 0.1 x SSC + 0.1% SDS.

4. Sedimentation Studies of Viral H^3 - RNA: Sedimentation patterns of viral H^3 - RNA species were obtained by sucrose density centrifugation. 5 to 20% sucrose gradients over a 50% sucrose cushion were prepared in cellulose nitrate tubes of 1" diam. by 3 1/2" (Spinco Division of Beckman Instruments, Inc.) using

a gradient maker (Buchler Instruments). The sucrose used was Crystalline Grade RNase - free (purchased from Schwarz-Mann) dissolved in 0.1 M Na Cl, 0.01 M Tris pH 7.5, 0.001 M EDTA and 1% SDS. 1.5 ml of H^3 RNA containing 800 µg was gently overlayed onto the gradients and centrifuged for 14 hours at 18°C at 23,000 RPM in an SW27 rotor in a Beckman L2-65B Ultracentrifuge. The gradients were then collected in 1 ml fractions using an ISCO Model D Density Gradient Fractionator monitored at 254 nm by an ISCO UV Analyser. 0.02 ml aliquots of each fraction were removed and radioactivity determined. Fractions were pooled and ethanol - precipitated twice at -20°C with 100 µg phenol - extracted yeast RNA as carrier (Na salt, British Drug House Ltd.). The RNA was resuspended in 0.1 x SSC and hybridized to viral DNA as described in Section V - 2.

RESULTS

SECTION I

1. Ad 2 Survival After U.V. Irradiation.

To determine the effects of U.V. on the survival of Ad 2, virus suspensions, irradiated and non-irradiated, were diluted ten-fold in sterile Tris Buffer Saline + 20% Glycerol and frozen. After thawing, they were diluted in PBS + 1% FCS and the titer was measured by plaque-assay. The results of typical experiments are shown in Table I. It can be seen that irradiating Ad 2 with U.V. for 40 sec, 100 sec and 125 sec reduced the titer to 10-15%, 1% and 0.5% of that of the non-irradiated virus respectively.

2. Virus Yield After Infection with U.V.-Irradiated Virus.

KB cells were infected with non-irradiated Ad 2 and Ad 2 irradiated with graded doses of U.V., at a MOI of 25 PFU/cell, assayed before irradiation. Aliquots of cells were removed at various times post-infection and assayed for virus yields. The cells were sedimented by low speed centrifugation; the pellet was resuspended with sterile TBS + 20%

UV DOSE (secs)	VIRUS SURVIVAL			
	EXPT. 1		EXPT. 2	
	PFU/ml	% SURVIVORS	PFU/ml	%SURVIVORS
0	6×10^8	100.0	3×10^8	100·0
40	6×10^7	10.0	5×10^7	16.7
100	8×10^6	1.3	3×10^6	1.0
125	4×10^6	0.67	1×10^6	0·33

TABLE I Survival of Ad 2 After U.V.-Irradiation

glycerol and sonicated for 1.5 min to release the virus. Appropriate dilutions were made in PBS + 1% FCS and the virus titer was measured by plaque-assay. As shown in Table II, infecting KB cells with non-irradiated virus gave a final virus yield of $\sim 10^4$ PFU per cell, in agreement with that reported by Green (Green and Daesch, 1961). However infection with irradiated virus led to a drastic reduction in final virus yield. The results shown in Table II, Expt. 1, would seem to indicate a higher virus yield after 125 sec UV than after 100 sec UV. However variation between adsorption efficiency and in the plaque-assay as well as the condition of the infected cells in the suspension cultures could account for this result. It is difficult to distinguish the effects upon virus yield between 100 sec and 125 sec when survival is down only by a factor of 2 (Table I). However it is still valid to compare the relationship between molecular and biological events of these infected cultures. The average burst-size, defined as PFU per virus-producing cell, was found to be lower for cells infected with U.V.-irradiated virus (Mak, personal communication). Figure 1 illustrates the results obtained in Expt. 2 using non-irradiated virus and virus irradiated for 100 sec U.V. The graph shows that, even after 75 hours post-infection, the theoretical maximum virus yield was not recovered.

UV DOSE (secs)	TIME P.I (hrs.)	VIRUS YIELD (PFU/cell)	
		EXPT. 1	EXPT. 2
0	20	—	4.6×10^3
	30	—	7.3×10^3
	48	3.3×10^4	9.9×10^3
	60	—	8.5×10^3
40	48	4.7×10^3	—
	60	1.5×10^3	—
100	20	—	0
	30	—	3.3
	48	8.3×10	2.8×10^2
	60	2.0×10^2	4.2×10^2
	75	—	1.3×10^3
125	48	3.8×10^2	—
	60	1.8×10^3	—

TABLE II Virus Yield After Infection With U.V.-Irradiated Virus

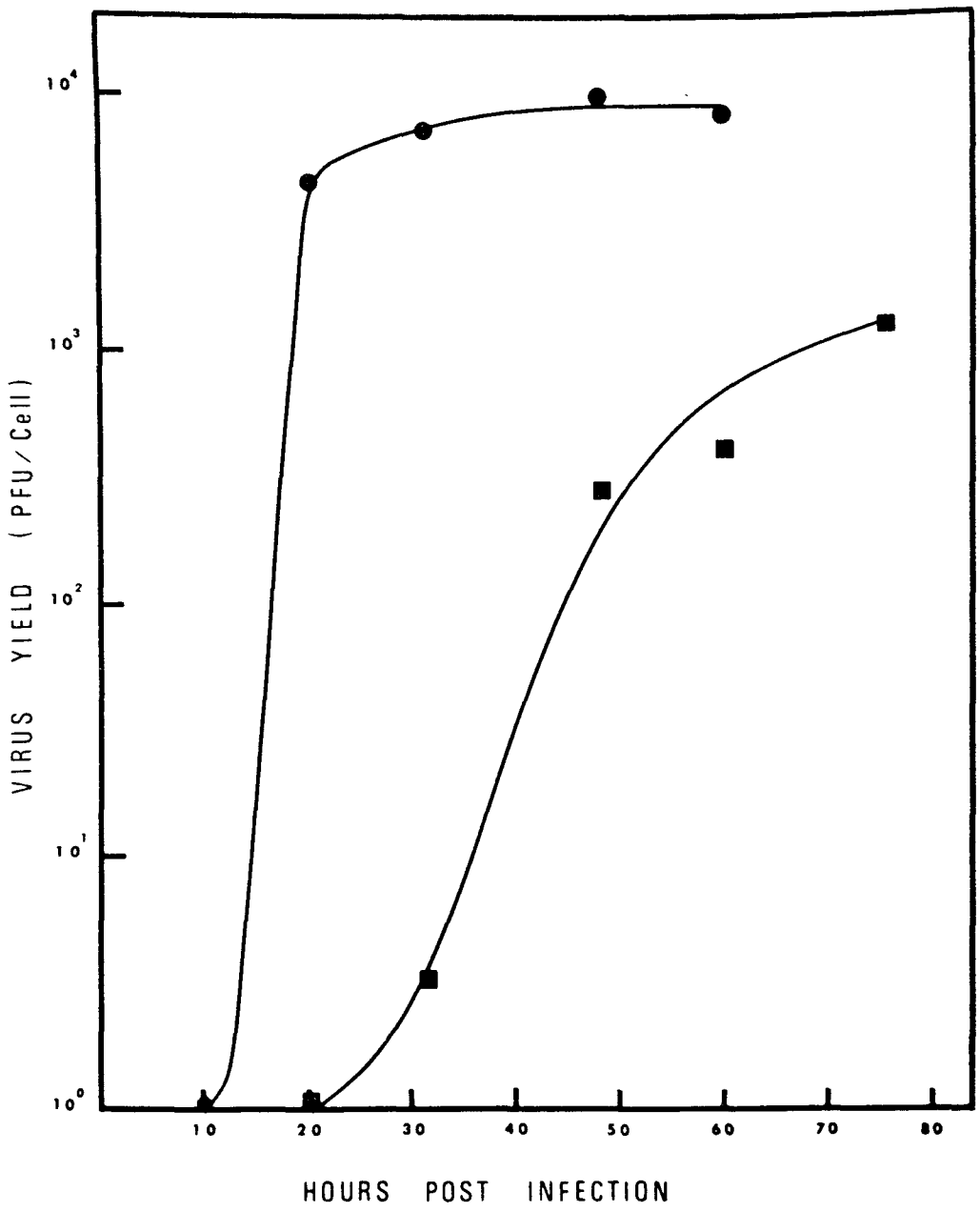

Figure 1. Virus Yield Expressed as PFU/Cell. Symbols: Virus Yields From Cells Infected with Non-Irradiated Ad 2, ●; and with 100 sec U.V.-Irradiated Ad 2, ■.

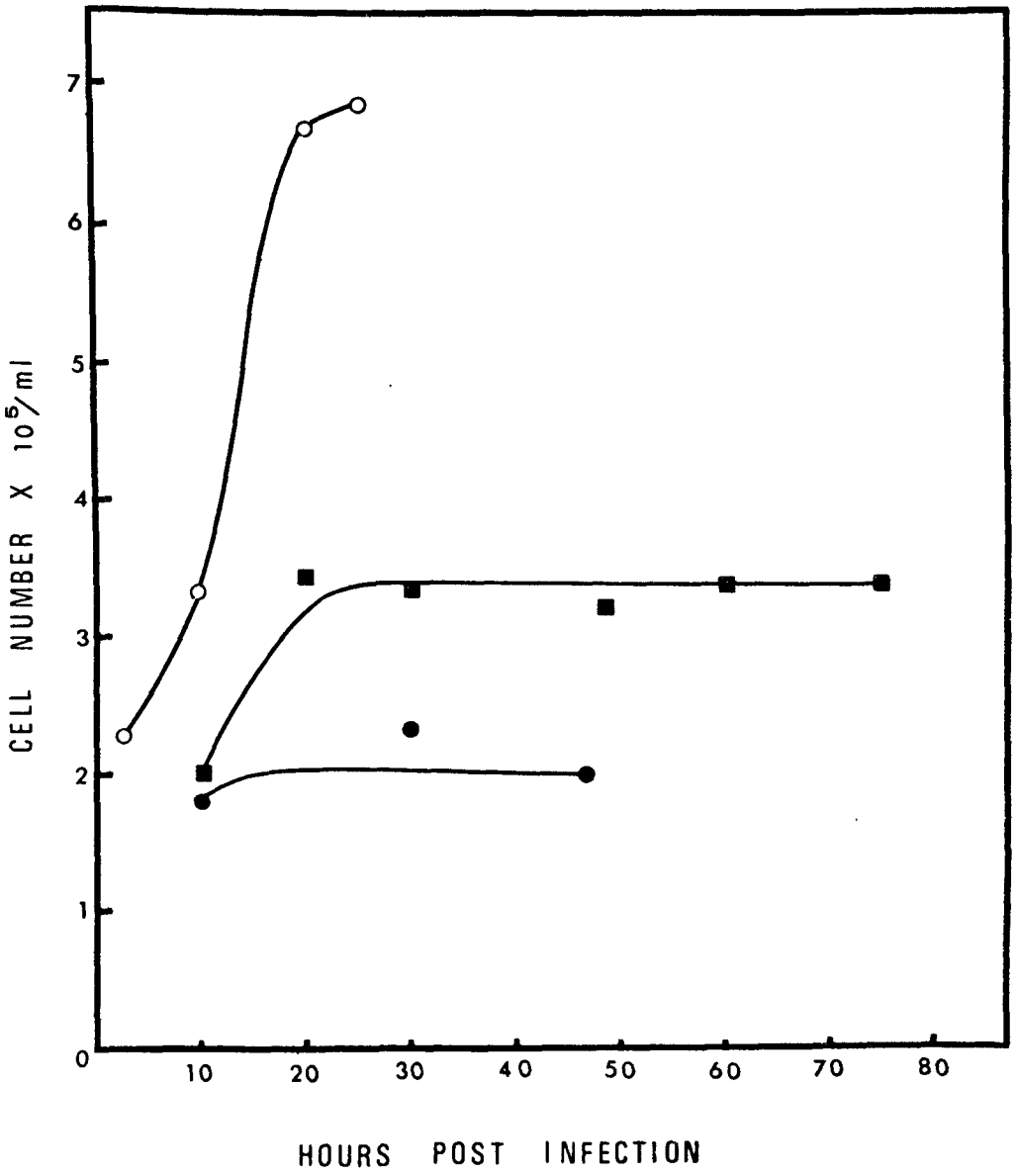

Figure 2. Growth of KB Cells, Non-infected, (o); Infected with Ad 2, (●); and Infected with U.V.-Irradiated Ad 2 for 100 sec, (■).

3. Growth of Non-Infected and Virus-Infected KB Cells.

KB cells were infected with Ad 2 at a MOI of 100 PFU/cell, or with the same amount of Ad 2 but U.V.-irradiated for 100 sec. Figure 2 illustrates cell concentration as a function of time post-infection for these cultures and for a non-infected culture. It can be seen that the non-infected cells had a doubling time of about 24 hours while cells infected with non-irradiated virus ceased dividing shortly after infection. Cells infected with the irradiated virus increased slightly in numbers but also reached a plateau level. Therefore cessation of cell growth was due to virus infection. The delay in this cessation after infection with U.V.-irradiated virus may be due to a delay in the initiation of viral replication processes.

SECTION II

VIRAL DNA ANALYSIS AFTER INFECTION OF KB CELLS WITH U.V. IRRADIATED VIRUS

In this and in the following sections, viral replication in the irradiated system was analysed at the molecular level. Quantitative studies on viral DNA are presented in this section, while quantitative and qualitive analysis of viral RNA will be described in Section III. In all experiments Anti-Ad-2 serum, obtained from immunized rabbits, was added after infection to neutralize all unadsorbed virus and to reduce re-infection by the progeny virus.

1. Rate of Incorporation of H^3 T into Total DNA

KB cells were infected with non-irradiated and U.V.-irradiated Ad 2. At various times post-infection, aliquots of cells were labeled with 5 μ Ci/ml H^3 Thymidine for 30' at 37°C and the cells were precipitated with cold 5% TCA and the radio-activity determined by liquid scintillation counting. The amount of H^3 Thymidine incorporated per cell is shown in Figure 3. It can be seen that there was an inhibition of incorporation of H^3 Thymidine in all cultures whether infected with irradiated or non-irradiated virus. The values shown represent an average estimate since not all cells were

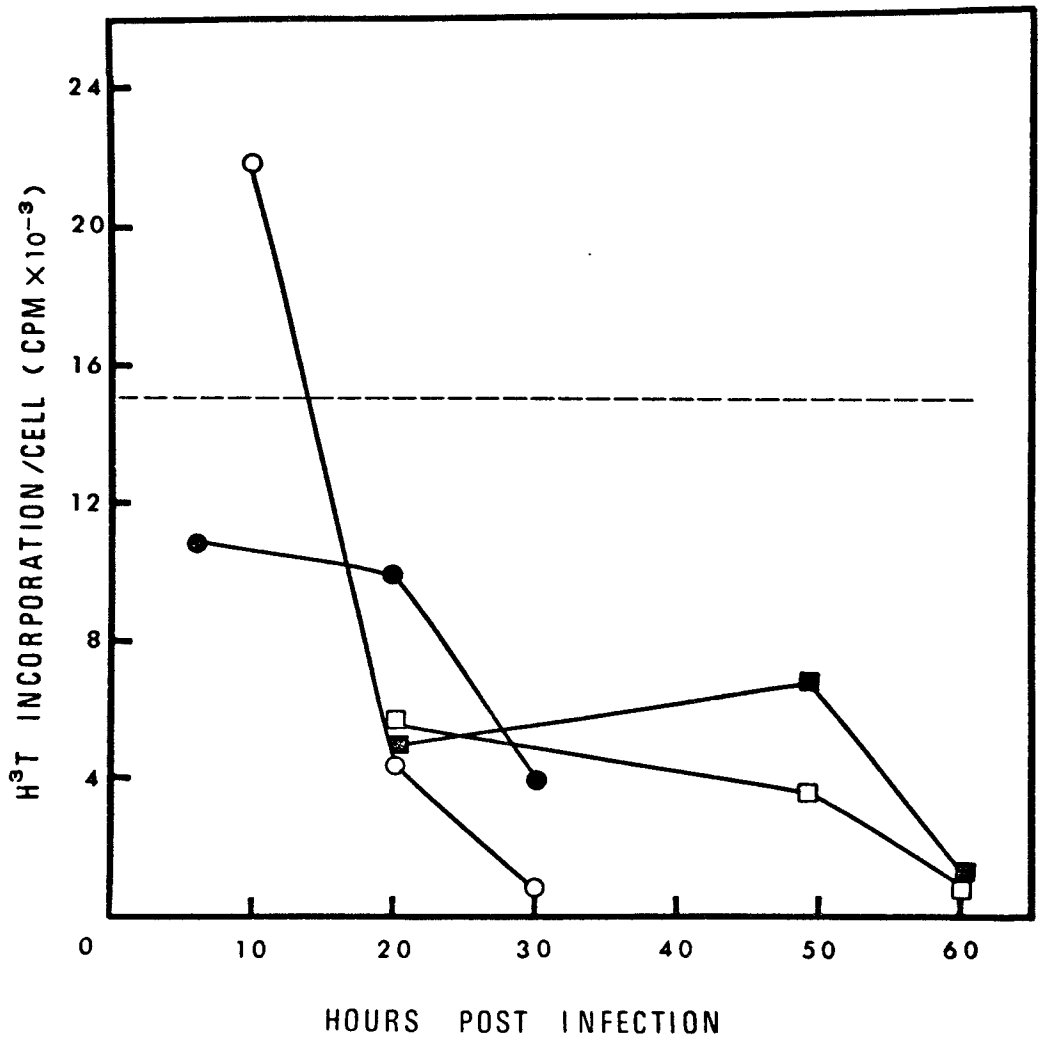

Figure 3. Average Amount of H^3-Thymidine Incorporated into DNA per Cell during a 30 min Incubation with the Labelled Precursor. Symbols: Dotted line, non-infected cells; cells infected with Ad 2 irradiated with U.V.:0 sec, ●; 40 sec, ○; 100 sec, ■; 125 sec, □,

synthesizing DNA at the labeling time.

2. Viral DNA Synthesis

Cells were infected with virus, irradiated with graded doses of U.V., at a MOI of ~ 25 PFU/cell, assayed before irradiation. At various times post-infection, aliquots of cells were labeled with H^3 Thymidine at 2μ Ci/ml for 1 hour at 37°C. DNA was extracted with phenol and viral DNA was detected by DNA - DNA hybridization. The hybridization reaction was found to be specific for viral DNA as KB cell DNA did not hybridize to viral DNA bound to nitrocellulose filters (McPherson, 1969).

The results obtained are shown in Figure 4. In the non-irradiated control, viral DNA synthesis began at 6 hours post infection and reached a maximum of 60% total DNA synthesis by 20 hours post-infection. However viruses irradiated for 40 sec, 100 sec and 125 sec U.V. began their DNA replication around 10 hours, 18 hours, and 20 hours post infection respectively, and subsequently increased to values similar to the control. Thus U.V. irradiation caused a delay of viral DNA replication which appeared to be dose-dependent, followed by a recovery process which could be due to repair of the damaged DNA. The downward trend of the percentage

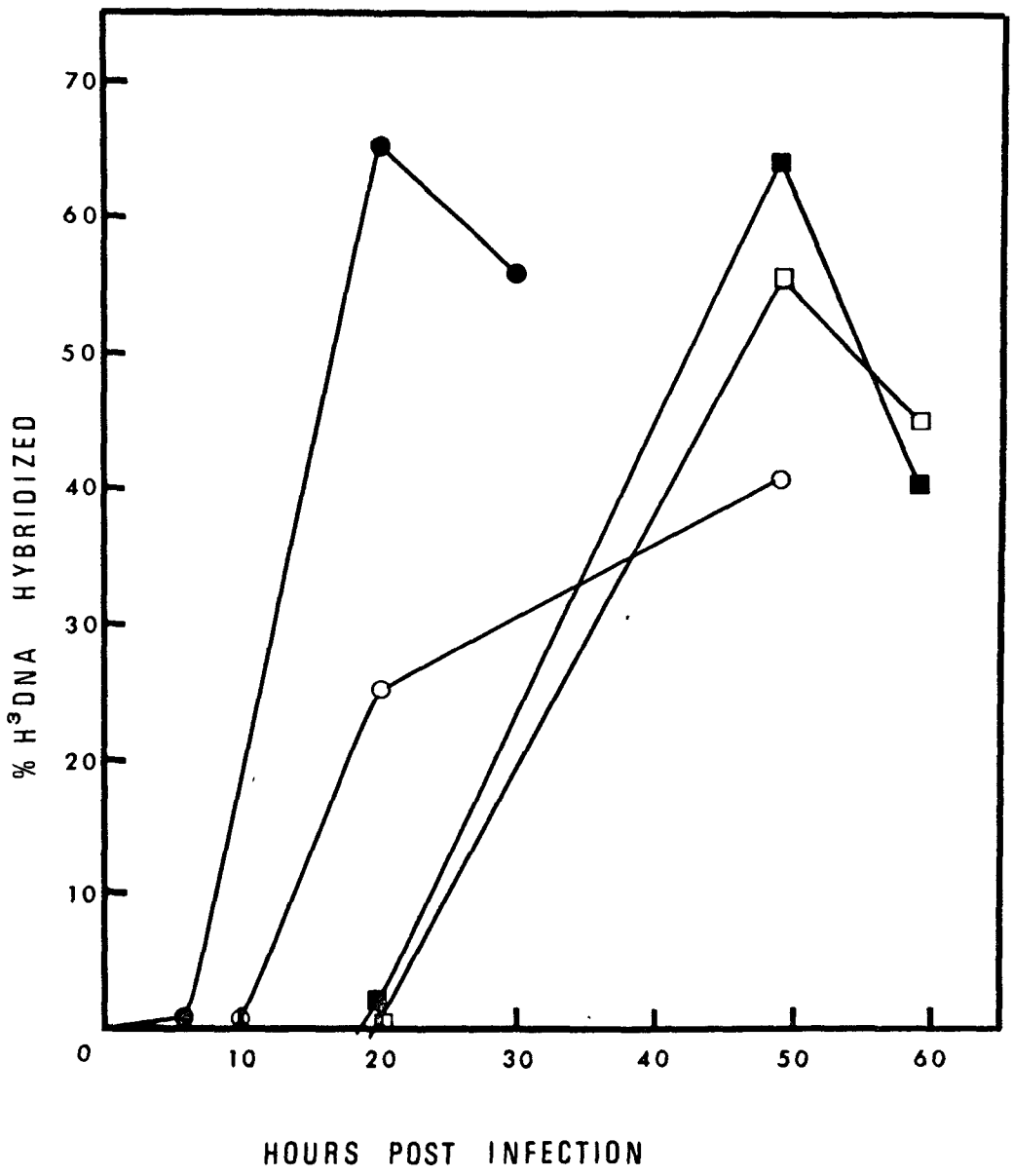

Figure 4. Viral DNA Synthesized at Different Times Post-Infection. The % H^3-DNA hybridized was measured as CPM bound to viral DNA on filters.
Symbols: Cells infected with virus U.V.-irradiated for o sec, ●; 40 sec, o; 100 sec, ■ , 125 sec, □ .

hybridizable DNA at late times was probably due to renaturation of viral H^3 DNA during the hybridization incubation. McPherson has shown that if the H^3 DNA was denatured and rehybridized, the percentage H^3 DNA is increased to maximum values (McPherson, 1969).

3. Calculating Amount Viral H^3 DNA Per Infected Cell

Since the preceding experiments confirmed the synthesis of viral DNA, using irradiated virus, it was of interest to determine the amount of viral DNA made by infected cells. To estimate the amount of viral DNA in μg per cell, the following equation was utilized (Mak, 1969):

$$\frac{\text{Total } H^3 \text{ T Incorporated in DNA} \times \%\ H^3 \text{ DNA Hybridizable to Viral DNA}}{\text{Specific Activity of Viral DNA (CPM/μg)}}$$

or simply: $\frac{\text{CPM } H^3\text{T in tDNA} \times \%\ \text{vDNA H°}}{\text{vDNA Sp. Act.}}$

To obtain the total H^3 T in total DNA, the cells were heated with 10% TCA at 90°C to render the DNA acid soluble, after the precursor pools had been extracted by cold TCA. The radioactivity in the 90°C TCA hydrolysate was determined

by scintillation counting using Aquasol.

The percentage of viral DNA in total H^3 DNA was determined by DNA - DNA hybridization. The specific activity of viral H^3 DNA was obtained from saturating a small amount of viral DNA immobilized on filters. Increasing amounts of total H^3 DNA were hybridized to 0.1 μg of cold viral DNA bound to nitrocellulose filters. The maximum CPM bound should give the specific activity of the viral DNA since the amount bound was the same as that on the filters, i.e. 0.1 μg. Saturation curves are illustrated in Figure 5. It can be seen that no saturation was obtained with DNA obtained at 6 hours post-infection with non-irradiated virus and from 10 hour post-infection with 100 sec U.V.-irradiated virus. This was expected since at early times post-infection there were few copies of viral DNA made. However, samples taken later times post-infection gave saturation values. The specific activites of viral DNA from cells infected with non-irradiated virus and virus irradiated for 100 sec U.V. are shown in Table III. They decreased with time after infection. These results indicate that either viral DNA was accumulating in the cells, or that rate of viral DNA synthesis decreased, or a combination of both.

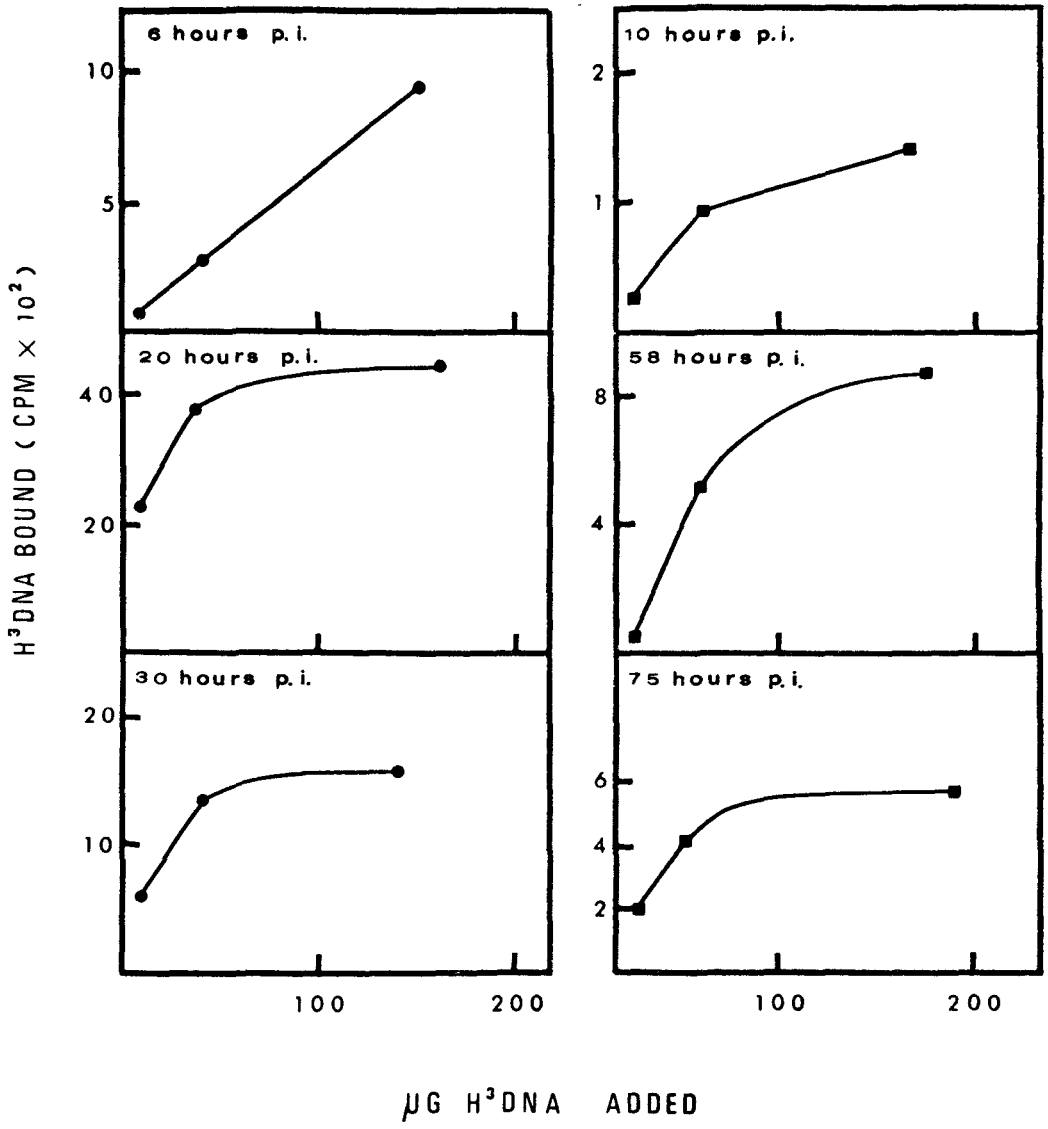

Figure 5. Saturation of Immobilized Viral DNA with H^3 DNA, non-irradiated, ●; 100 sec U.V. ■.

UV DOSE (secs)	TIME P.I. (hrs)	VIRAL DNA SPEC. ACTIVITY (CPM/μg)
0	20	84×10^3
	30	21×10^3
40	20	115×10^3
	48	6.6×10^3
100	20	17×10^3
	58	4.7×10^3
125	20	19×10^3
	58	7×10^3

TABLE III Specific Activity of Viral DNA in CPM/μg.

Returning to the original equation for calculating amount of viral DNA per infected cell and substituting the values obtained above, we can observe the amount of viral DNA in pg/cell in Table IV. It must be noted that these values are not absolute but relative since counting efficiency may vary; however, the effects of U.V. on the amount of viral DNA synthesized is valid since within an experiment, all the samples would have the same counting efficiency. The table indicates that in all cases, significant amounts of viral DNA per cell was made.

4. Correlating Infectious Virus Yield to Amount of Viral DNA per Infected Cell

It was of interest to determine the relationship between the amount of viral DNA present in an infected cell and the virus yield of this cell. Table V illustrates the PFU/viral DNA ratio of infected cells at various times post-infection. It can be seen that though significant amounts of viral DNA were synthesized by the cells infected with U.V.-irradiated virus, fewer infectious virus were produced. This suggests that the amount of viral DNA present in the cell was probably not the limiting factor leading to a reduced virus

UV DOSE (secs)	TIME P.I. (hrs)	VIRAL DNA (pg/cell)
0	20 30	2.7 4.7
40	20 48	2.1 2.0
100	48 58	2.4 4.1
125	48 58	2.6 2.1

TABLE IV Amount of Viral DNA in Picograms/Cell

UV DOSE (secs)	TIME P.I. (hrs)	VIRUS YIELD (PFU/cell)	VIRAL DNA (pg/cell)	$\frac{\text{PFU}}{\text{VIRAL DNA}}$
0	20	4.6×10^3	2.5	1.8×10^3
	30	7.3×10^3	4.2	1.7×10^3
100	48	1.8×10^2	1.9	0.9×10^2
	58	3.0×10^2	3.2	0.9×10^2
125	58	3.8×10^2	2.6	1.5×10^2

TABLE V Relationship Between Virus Yield in PFU to the Amount of Viral DNA per Cell

yield but rather a defective or deficient transcription or translation mechanism; or alternately virus assembly may be deficient.

SECTION III

VIRAL RNA ANALYSIS AFTER INFECTION OF KB CELLS WITH U.V.-IRRADIATED VIRUS

1. Rate of Incorporation of H^3 Uridine into Total RNA

KB cells were infected with non-irradiated or U.V.-irradiated Ad 2. At various times post-infection, cells were labeled with H^3 Uridine at 10 μ Ci/ml for 30' at 37°C and the cells were TCA precipitated and the radioactivity determined. The rate of incorporation of labeled precursor into the cells is shown in Figure 6. It can be seen that there was an initial rise in the amount of H^3 Uridine incorporation followed by rapid inhibition at later times post-infection in both non-irradiated and irradiated systems.

2. Viral RNA Synthesis

At various times post-infection with non-irradiated and irradiated virus, cells were labeled with H^3 Uridine at 10 μ Ci/ml for 2 hours at 37°C, and the RNA was extracted with phenol and hybridized to viral DNA. As seen in Figure 7, viral RNA synthesis in the non-irradiated control began as early as 2 hours post infection and reached

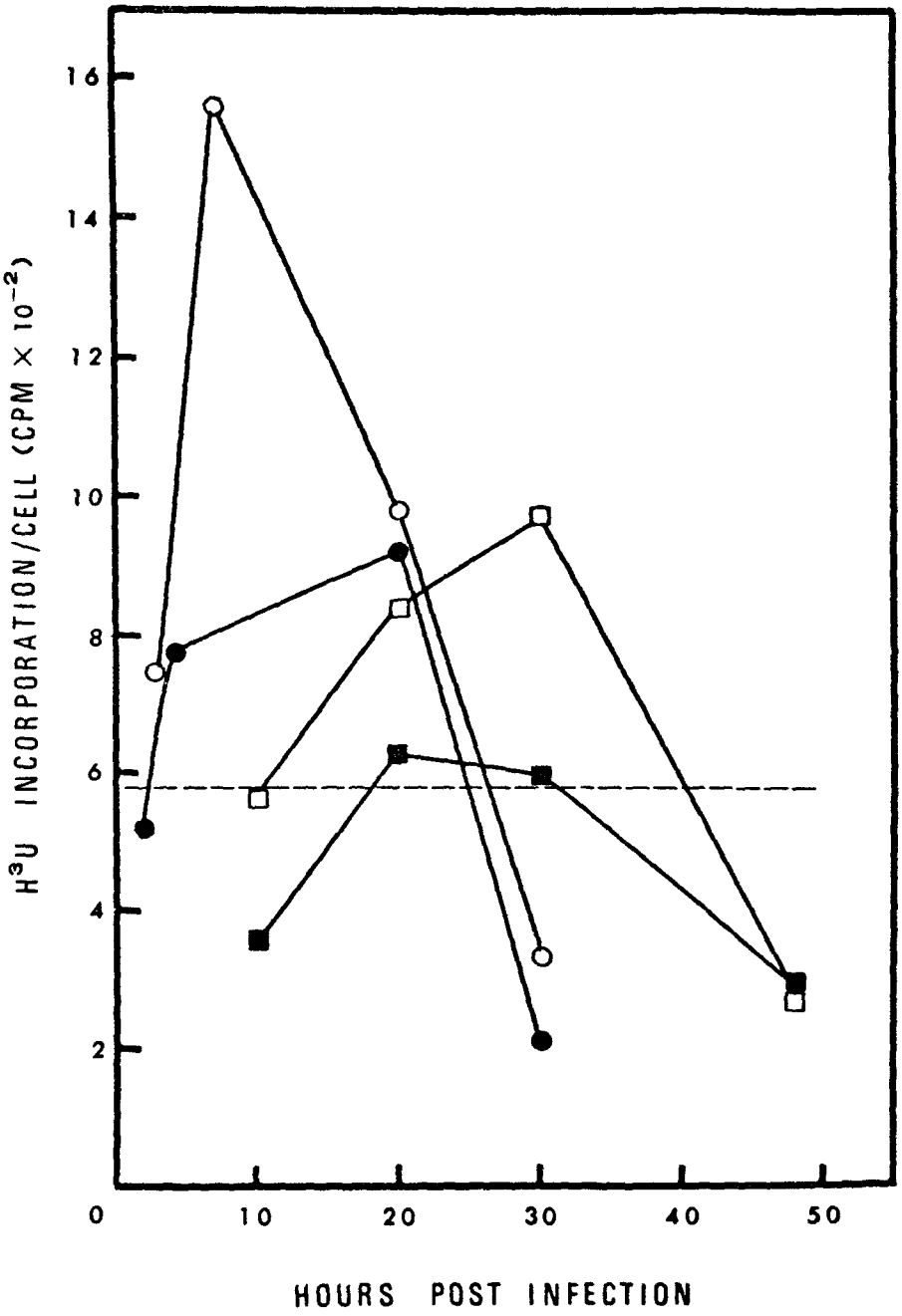

Figure 6. Average Amount of H^3-Uridine Incorporated into RNA per Cell during a 30 min. Incubation with the Labelled Precursor. Symbols: Dotted line, non-infected cells; cells infected with virus U.V.-irradiated for 0 sec, ●; 40 sec, o; 100 sec, ■; 125 sec, □.

a maximum of 44% total H^3 RNA by 25 hours post-infection. Viral RNA synthesis in cells infected with virus irradiated for 40 sec, 100 sec and 125 sec was detected from 10 hour, 20 hours and 20 hours respectively with subsequent rise at later times post-infection. These results indicated that U.V. irradiation of the virus caused a dose-dependent delay of the synthesis of viral specific RNA, as was shown for viral DNA synthesis. The kinetics of recovery appeared to be similar for both DNA and RNA, suggesting that RNA synthesis is coupled to DNA synthesis.

3. Correlation Between Amount of Viral DNA and Viral RNA Synthesized in Infected Cells

Both virus-specific DNA and RNA were synthesized in cells infected with U.V.-irradiated virus. It was of importance to determine the capacity of the viral DNA to be transcribed into messenger RNA, since the latter plays an important role in the production of virus. This capacity for transcription could be estimated by determining the rate of virus-specific RNA synthesis per viral DNA molecule. The rate of viral RNA synthesis could be estimated by multiplying the rate of H^3-Uridine incorporation and the percentage total RNA hybridizable to viral DNA. These calculations are shown in Table VI. Assuming the RNA precursor pools in all cultures to be

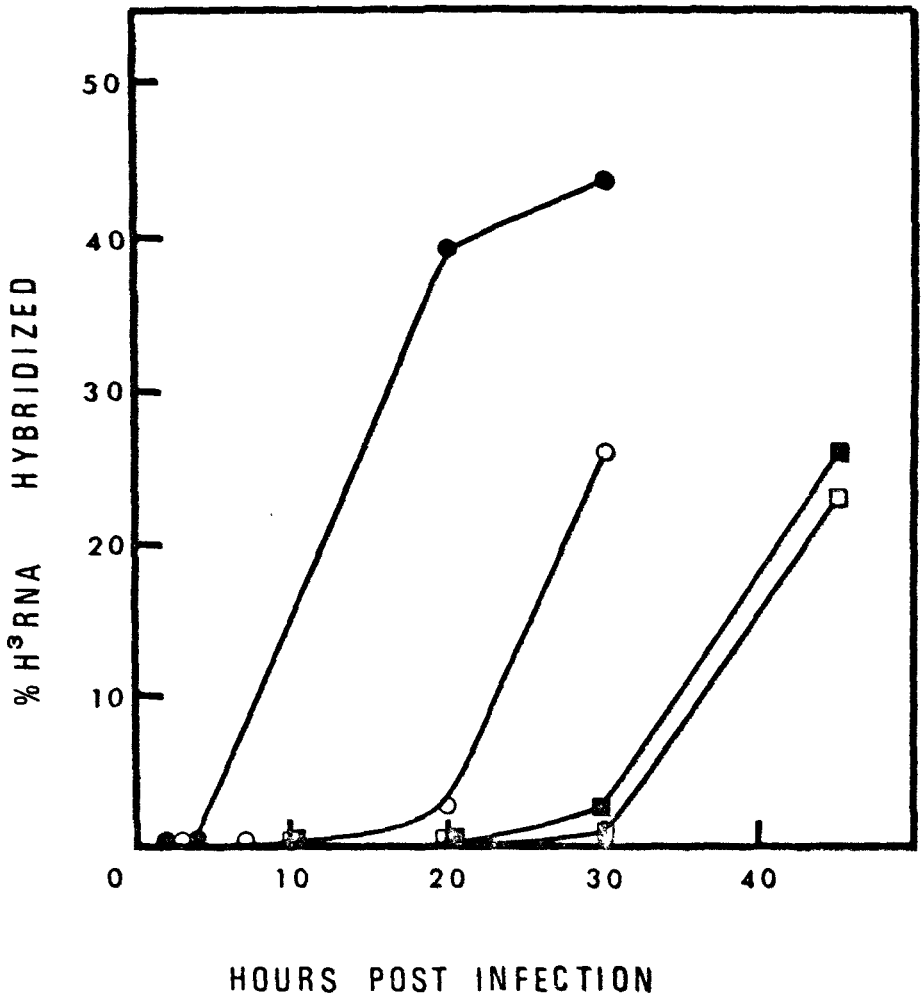

Figure 7. Viral RNA Synthesized at Different Times Post-Infection. The % H^3-RNA hybridized was measured as CPM bound to viral DNA on filters. Symbols: 0 sec, ●; 40 sec, o; 100 sec, ■; 125 sec, □.

the same, it can be seen that viral DNA synthesized by the non-irradiated and irradiated virus had similar capacities for transcription.

4. Elution of Viral H^3 RNA From H^3 RNA - DNA Hybrids

From the previous experiments, it was found that viral RNA was made in KB cells after infection with U.V.-irradiated virus, after an initial delay. Some qualitative studies on viral RNA were performed to examine whether the RNA made in the irradiated system was similar to that observed in the non-irradiated control. One criterion is the determination of the Tm value of RNA - DNA hybrids, that is, the temperature at which 50% of the RNA is released from the hybrid.

Cells were infected as usual with non-irradiated and U.V.-irradiated virus. At various times post-infection, samples were labeled with H^3 Uridine for 2 hours at 37°C, the RNA was extracted with phenol, and viral H^3 RNA - viral DNA hybrids were prepared by hybridization. The viral H^3 RNA was released from the hybrids by incubation for 10 min at various temperatures with 5°C increments and the percentage of H^3 RNA released measured. Figure 8

UV DOSE (secs)	TIME P.I. (hrs)	VIRAL DNA (pg/cell)	H^3 VIRAL RNA (CPM/cell)	$\frac{H^3 \text{ VIRAL RNA}}{\text{VIRAL DNA}}$
0	20	2.5	.12	.048
	30	4.2	.05	.012
100	48	1.9	08	.042
	58	3.2	.07	.022
125	48	2.6	.03	.012

TABLE VI Relationship Between Amount Viral H^3 - RNA to the Amount of Viral DNA per Cell

illustrates typical melting profiles obtained.

The Tm values obtained from Figure 8 are shown in Table VII along with their corresponding average G + C content using a calibration curve by Bolten and McCarthy (Bolten and McCarthy, 1963). It can be seen from Table VII and Figure 8 that early viral RNA in all cases had a lower G + C content as well as a broader melting profile. However there seemed to be a recovery phenomenon in all cases, as late viral RNA showed sharper melting profiles with Tm values of 73°C corresponding to an average G + C content of 54%. These observations suggest that early RNA had a more heterogenous and perhaps smaller size distribution than late RNA.

5. Molecular Size Analysis of Viral RNA

The size distribution of viral RNA synthesized early and late after infection in both irradiated and non-irradiated systems was examined by sucrose density centrifugation. KB cells were infected with non-irradiated Ad 2 and Ad 2 U.V.-irradiated for 100 sec. At various times post-infection, RNA was labeled with H^3-Uridine and extracted with phenol. A maximum of 800 µg of H^3 RNA was layered onto 5-20% sucrose gradients, centrifuged, and collected in 1 ml fractions. The

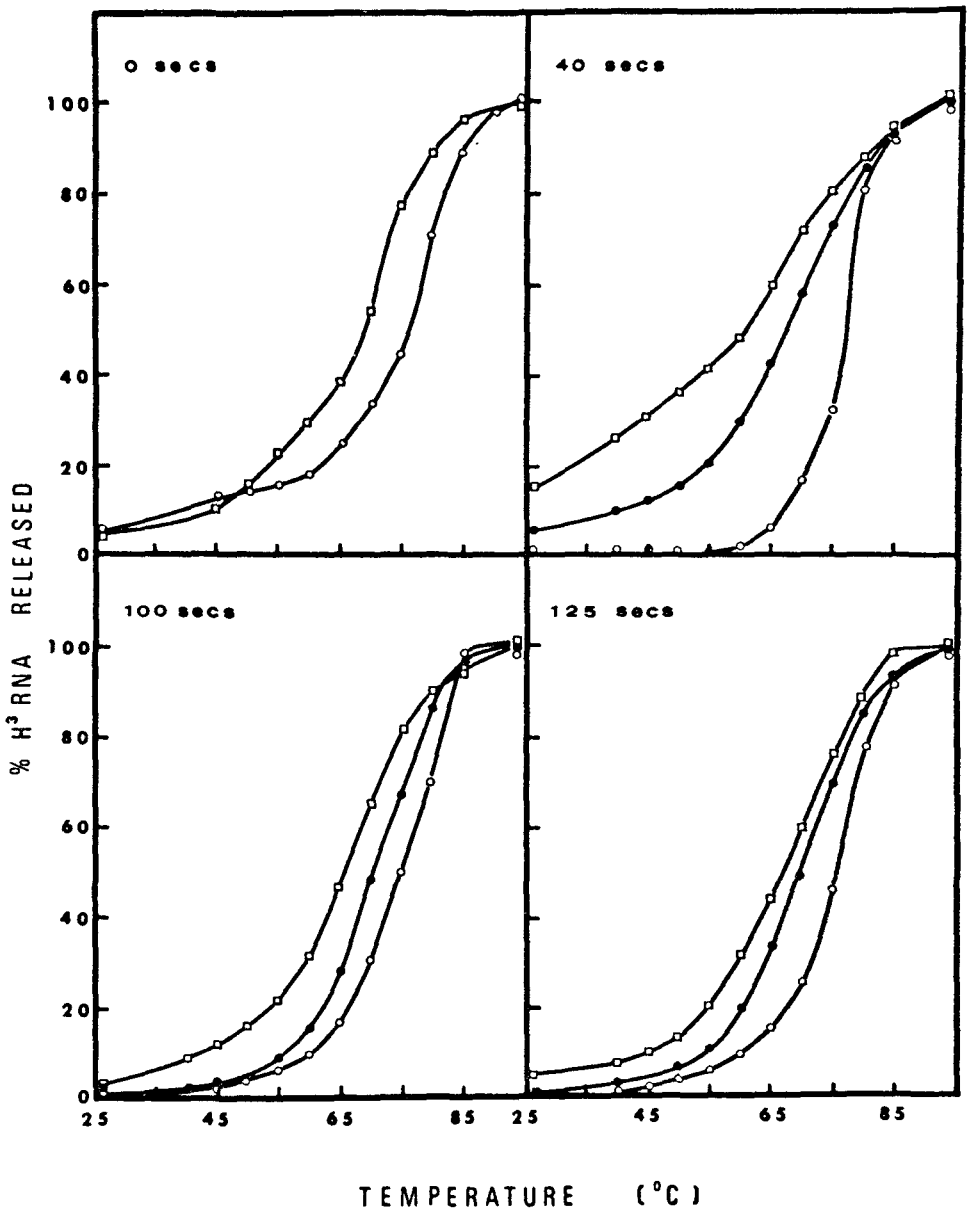

Figure 8. Melting Profiles of H^3 - Viral RNA - DNA Hybrids, Irradiated with Different UV Doses (sec). Symbols: early viral RNA, □; RNA synthesized at the onset of DNA synthesis, ●; late RNA, o.

UV DOSE (secs.)	TIME P.I. (hrs.)	Tm VALUE (°C)	AVERAGE G + C CONTENT (%)
0	2	68	46
	4	67	45
	20	76	54
	30	76	54
40	3	62	40
	7	67	45
	20	76	54
	30	76	54
100	10	66	44
	20	70	48·5
	30	76	54
	45	76	54
125	10	66	44
	20	69	47
	30	76	54
	45	76	54

TABLE VII Tm Values of H^3 RNA and Their Corresponding G + C Content

radioactivity in an aliquot from each fraction was determined by TCA precipitation; the fractions were then pooled into threes, twice ethanol precipitated, hybridized to viral DNA, and the amount of virus specific RNA (in CPM) of each fraction was determined. The distribution of virus-specific H^3 RNA is shown in Figures 9 and 10, expressed as % of total virus-specific H^3 RNA in the whole gradient. Figure 9 shows results from the cells infected with non-irradiated virus. It can be observed that 4 hours post-infection H^3 viral RNA consisted essentially of RNA species of molecular weight less than 29S. However, the 20 hours sample showed a greater proportion of viral H^3 RNA having sedimentation values greater than 33S. At 30 hours post-infection, a higher proportion of the viral H^3 RNA appeared at about 10S. It should be noted that by 30 hours post-infection, the cycle of infection was almost completed (see Figure 3). Figure 10 shows sedimentation profiles of viral H^3 RNA extracted from cells infected with 100 secs U.V.-irradiated virus. A shift from lower molecular weight viral RNA species to higher molecular weight species during the time course of infection was also observed. The approximate S-values obtained from Figure 9 and 10 are listed in Table VIII. Green has also observed

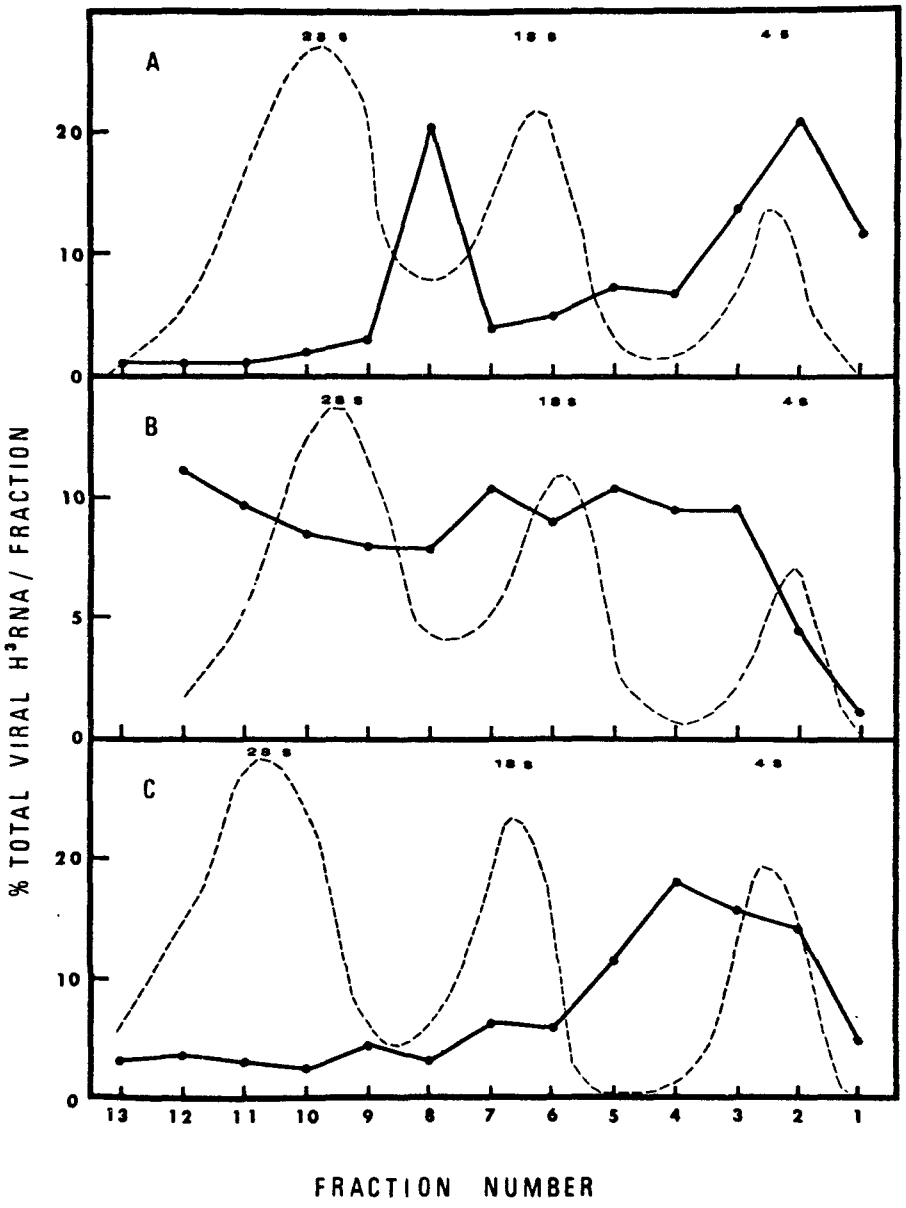

Figure 9. Sucrose Gradient Profile of H^3 Viral RNA From Non-Irradiated Virus Infected Cells.
Graph A = 4 hr p.i. RNA ---- OD Profile
Graph B = 20 hr p.i. RNA
Graph C = 30 hr p.i. RNA

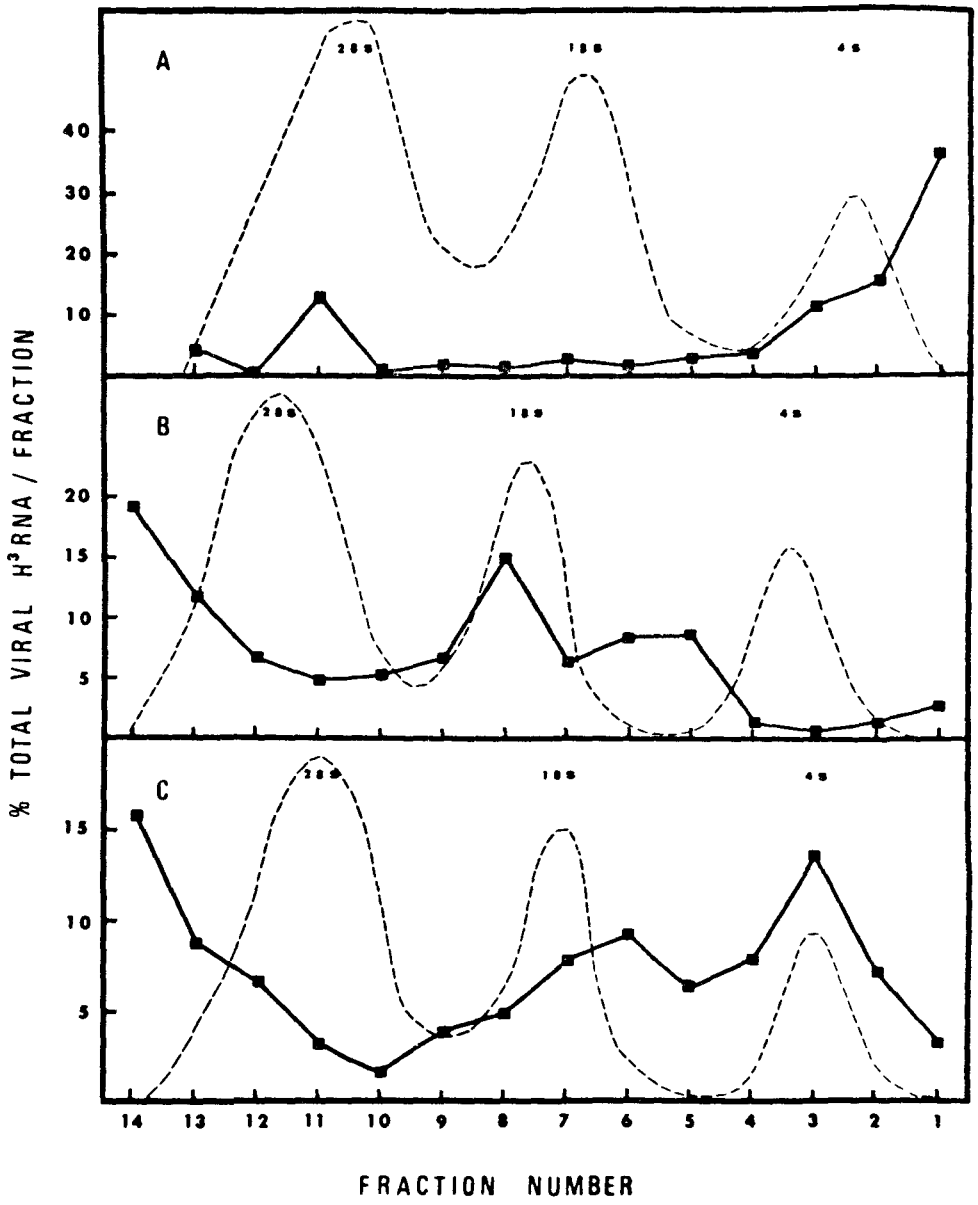

Figure 10. Sucrose Gradient Profile of H^3 Viral RNA From 100 sec. U.V.-Irradiated Virus Infected Cells.
Graph A = 10 hr p.i. RNA --- OD profile
Graph B = 30 hr p.i. RNA
Graph C = 48 hr p.i. RNA

UV DOSE (secs.)	TIME P.I. (hrs.)	APPROXIMATE S-VALUES
0	4	4, 23,
	20	8, 14, 20, >32
	30	9, 18, >33
100	10	8, 28
	30	14, 20, >34
	48	7, 16, >33

TABLE VIII Sedimentation Estimates of H^3 - Viral RNA Synthesized After Infecting KB Cells with Irradiated and Non-Irradiated Virus.

an increase in size of cytoplasmic viral m RNA from early to late times post-infection (Green et al, 1970). He has reported early cytoplasmic m RNA species of S-values of 16S, 22S, and possibly 24S, and late cytoplasmic m RNA species of S-values of 12S, 15S, 17S, 23S, 26S, 30S, 36S. It is somewhat difficult to conclude exact sedimentation values of viral RNA species described here due to the much poorer resolution of sucrose gradient centrifugation compared to gel electrophoresis technique used by Green. The essential point remains, however, that there was a shift to higher molecular weight species in both control and U.V.-irradiated systems. However, this shift was again delayed by U.V.

According to the procedure of Sherrer (Sherrer, 1970), the RNA must be treated with DNase for hybridization experiments. It should be pointed out that DNase treatment was omitted since commercially available DNase degraded H^3 RNA as illustrated in Table IX. It can be seen that the enzyme digested ~97% of H^3 DNA, however only 29-48% of H^3 RNA remained TCA precipitable. Sucrose gradient centrifugation of H^3 RNA incubated with and without DNase also revealed the presence of contaminating RNase causing breakdown of H^3 RNA. The omission of DNase treatment did not interfere with DNA - RNA hybridization reactions.

NUCLEIC ACID	CPM[a] −DNase	CPM[a] +DNase*	% CPM[a] RECOVERED
H^3-DNA	11,933	285	2·4
H^3-DNA	8636	248	2·8
H^3-RNA	4309	1291	29.0
H^3-RNA	1490	727	48.0

* Incubation was 1 hour at 37°C
a, TCA precipitable CPM

TABLE IX RNase and DNase Activities of "RNase-free" DNase

DISCUSSION

The effects of ultraviolet irradiation upon various biological systems has been extensively studied both in vivo and in vitro. The most stable photoproducts resulting from irradiation of phage, bacterial or mammalian DNA are pyrimidine dimers, especially those between adjacent thymine bases. Other minor photoproducts include base alterations or substitutions, as well as single-strand or double-strand breaks with high doses of U.V. These lesions may eventually lead to the death of the organism or be repaired and allowing the organism to survive.

Repair replication has been studied in detail in prokaryotes and is thought to proceed via one of two mechanism, excision-repair, or post-replicative recombination (Howard-Flanders, 1968). Excision-repair has been described in mammalian cells following ultraviolet irradiation (Painter *et al*, 1970), X-rays (Painter and Cleaver, 1970), or alkylating agents (Roberts *et al*, 1968). Painter has also shown that irradiated HeLa cells resume normal replication after the damaged DNA has undergone replication. However, in contrast to bacterial cells

and protozoans such as Tetrahymena pyriformis where a large numer of bases are excised during repair, it would appear that repair patches in U.V.-irradiated human cells are relatively small, ~30 nucleotides (Edenberg and Hanawalt, 1972). Still other mechanisms of repair have been described in rodent cells (Chiu and Rauth, 1972) where thymine dimers are not excised, but probably by-passed. Although some workers favor a passive, non-enzymatic mode of repair replication (Elkind et al, 1972), more recently a U.V.-specific endonucleolytic activity was found in human cell extracts (Bacchetti et al, 1972), further strengthening the possibility that some repair replication in mammalian cells may be enzymatically similar to the prokaryotic cell.

Rainbow (Rainbow, 1970) has examined the effects of ultraviolet irradiation upon survival of adenovirus type 2 and the efficiency of production of thymine dimers and single-stranded breaks in the viral DNA. He found that about one single-strand break and approximately 30 dimers were necessary to produce one lethal hit, suggesting that some of the photolesions were repaired or could be tolerated by the host cell during viral multiplication.

Four major steps are required for the production of infectious virions, a) replication

of viral DNA, b) transcription of viral RNA, c) translation of viral RNA into proteins, and d) assembly of the viral components. In this investigation the effects of U.V. on viral DNA replication and its transcription, and the correlation of these functions with virus survival were examined.

It has been shown that U.V.-irradiated viruses adsorb as efficiently to host cells as non-irradiated virus (Rainbow, 1970) and that the virus uncoats normally (Gilead and Ginsberg, 1966). The rates of incorporation of labeled precursor into the DNA and RNA of cells infected with U.V.-irradiated virus was examined. It was shown in Figure 3 that there was an inhibition of incorporation of H^3 - Thymidine which was independent of U.V. dose and preceded viral DNA synthesis. This observation suggests that inhibition of host DNA synthesis represented the expression of early viral function insensitive to U.V. This inhibition may be due to some viral components such as the fiber antigen (Levine and Ginsberg, 1965; Ginsberg et al, 1967), or some function of the viral genome which is expressed before viral DNA replication. It has been shown (Gilead and Ginsberg, 1966; Casto, 1968) that these functions such as "T" - antigen induction are much less sensitive to U.V. The relative importance of these alternatives cannot be determined

at the present. Incorporation of H^3 - Uridine into total RNA (Figure 6) also revealed a dose independent inhibition; however, this was following an initial apparent stimulation of incorporation in cells infected with both irradiated and non-irradiated virus. The reason for this stimulation is not known at present.

U.V.-irradiation caused an overall delay in the lytic cycle of Ad 2, i.e. a delay in cessation of cellular division (Figure 2), and a delay in the synthesis of viral DNA (Figure 4) and viral RNA (Figure 7). These delays were dose-dependent and followed by a recovery phenomenon which may be due to repair of the damaged viral template. Aaronson (Aaronson, 1970) has suggested host cell reactivation of U.V.-damaged SV40. These results also suggest that viral RNA synthesis is coupled to viral DNA synthesis after irradiation as without, unlike the decoupling of these two processes by ^{32}P decay in radiosensitive strains of E. coli (Davern, 1968), and low-dose irradiation of E. coli (Hanawalt and Setlow, 1961).

The amount of viral DNA synthesized after infecting cells with U.V.-irradiated Ad 2 was calculated and correlated with the yield of infectious virus. It was found that at 30 hours post-infection with non-irradiated Ad 2, almost

all the viral DNA present was incorporated into infectious virions. This is in contrast with the 5-10% efficiency of utilization of viral DNA reported by Ginsberg (Ginsberg and Dixon, 1961). However, the utilization of viral DNA in cells infected with U.V.-irradiated virus was much less efficient, i.e. approximately 6% of the viral DNA was detected in infectious virions. It was concluded that U.V.-irradiation of Ad 2 decreased the assembly of viral DNA into infectious virions. It is unlikely that viral DNA was assembled into non-infectious or defective virions which would be undetected in the plaque-assay, and also would increase the particle/PFU ratio, since the actual number of virions produced was drastically reduced (Mak, personal communication).

Since the amount of viral DNA was not the limiting factor leading to reduced virus yields, the transcription ability of this DNA as well as the viral RNA synthesized post-irradiation was examined. Table V indicated the ratio of viral RNA/viral DNA as a rough measure of transcription activity, and similar values were found in all cases. The characteristics of viral RNA were further analysed by elution from H^3 RNA-viral DNA hybrids and sucrose gradient sedimentation.

Elution profiles of H^3 RNA - DNA hybrids demonstrated broader profiles with low Tm values for early viral RNA with a change over to sharper profiles and high Tm values for late viral RNA indicating that these RNA represent different species. The pattern of the RNA from cells infected with irradiated virus showed a real recovery phenomenon, indicating recovery of amount as well as quality of viral mRNA.

Sucrose gradient analysis also indicated a recovery phenomenon as the sizes of viral RNA species increased during the course of infection with both non-irradiated and irradiated Ad 2. The transcription of RNA on an irradiated template has been examined both in vivo and in vitro by other workers. At sufficiently low doses of U.V., DNA synthesis of *E. coli* is blocked without affecting overall RNA or protein synthesis (Sibatani and Mizuno, 1963). However increasing U.V. dose leads to a corresponding decrease of macromolecular synthesis. Rörsch and co-workers (Kroes *et al*, 1963; Rörsch *et al*, 1963) found that the synthesis of larger species of RNA was preferentially inhibited while low molecular weight RNA accumulated. Sauerbier has noted that U.V.-irradiation of T_4 phage leads to transcription of shorter RNA chains, both in vivo

and in vitro, which progressively become longer as the damaged DNA is repaired (Sauerbier et al, 1970). Hagen (Hagen et al, 1965) has also observed that U.V.-light reduces rapidly the ability of isolated calf-thymus DNA to "prime" the synthesis of RNA.

Several workers have attempted to elucidate the reason(s) for transcription of shorter RNA species. It has been suggested (Michalke and Bremer, 1969; Sauerbier et al, 1970) that RNA polymerase molecules are liberated at the site of U.V. lesions on the DNA. Furthermore the rate of RNA chain initiation is reduced. Hagen (Hagen et al, 1970) has suggested the formation of new binding sites for RNA polymerase which, however, are no longer capable of giving rise to RNA chains but compete with normal initiation sites. Evidence for this arose from the fact that U.V.-irradiated DNA slowed RNA synthesis on untreated DNA template in vitro (Zimmerman et al, 1965). Recent evidence in yeast (Koch and Kiefer, 1972) would indicate an increased RNA synthesis per cell after irradiation especially of RNA of less than 5S. This would suggest that RNA synthesis may play a part in repair and recovery phenomena, however these workers used relatively low doses of U.V. in their experiments.

From the experiments done in this investigation, it appears that neither viral RNA nor DNA were the limiting factors leading to reduced virus yields after infection with U.V.-irradiated virus. Although transcription seems to recover, perhaps the translation products of these molecules are abnormal leading to shorter or defective polypeptides. There is also the possibility of the absence of a maturation or assembly protein. Further work in this system, such as the analysis of labeled viral proteins, made post infection, using polyacrylamide gel electrophoresis, is necessary to elucidate this. Another likely possibility is that the viral functions are completely recoverable after a delay during which repair goes on, however by this time, the cell machinery, the milieu upon which the virus depends, has ceased functioning and thus cannot translate the viral messages or assemble the viral components.

APPENDIX

The usual method for determination of molecular size of viral specific RNA was by sucrose gradient density centrifugation of purified RNA followed by hybridization as presented in the previous section. It would be less time consuming to hybridize first, elute the viral specific RNA from the RNA - DNA hybrids, and then analyse the RNA by sucrose gradient centrifugation to determine molecular size. In order to examine the molecular size of viral specific RNA by hybridization, subsequent elution, and analysis on sucrose gradients, it is also necessary to minimize the degradation of RNA. The following methods were originally attempted to this effect.

1. Low Temperature Hybridization

The use of lower temperatures in the presence of formamide for the formation of RNA - DNA hybrids was first exploited by Bonner et al (Bonner, Kung, Bekhor, 1967). The advantages of using low temperature hybridization included increase retention of DNA bound to nitrocellulose filters, decreased background binding of input RNA, as well as prevention of RNA degradation and

depurination seen with prolonged incubation at elevated temperatures. Furthermore, McCarthy (McConaughy, Laird, and McCarthy, 1969) has correlated formamide concentration to thermal stability of the hybrid. He found using RNA - DNA hybrids from varied sources that 1% formamide reduced the Tm by 0.72°C. Gros (Kourilsky, Manteuil, Zamansky, Gros, 1970) has also studied RNA - DNA hybridization at low temperatures in the presence of urea and found that though the reaction rates were somewhat slower than at elevated temperatures, the hybrids formed were more specific in terms of hybrid structure. Considering the described advantages of this method, low-temperature hybridization in the presence of formamide was attempted to detect Ad 2 viral-specific RNA.

Cells were infected with virus and RNA was labeled with H^3 Uridine at 18 hours and 24 hours post-infection. The RNA was extracted with phenol and subjected to low temperature hybridization with viral DNA at various temperatures and salt concentrations.

a) Effects of Temperature: Table X shows that no significant differences in percentage H^3 RNA hybridized to viral DNA was found between 66°C

HYBRIDIZATION CONDITIONS	% HYBRIDIZED 18 hr p.i. RNA	% HYBRIDIZED 24 hr p.i. RNA
25°C 50% Formamide	8	8
37°C 50% Formamide	20	20
66°C NO Formamide	21	22

TABLE X Hybridization of H^3 - Viral RNA to Viral DNA at Various Temperatures in the Presence of Formamide.

hybridization and 37°C hybridization using 50% formamide. The salt concentration used was 2 x SSC in all cases and the time of incubation was 20 hours.

b) Effects of Ionic Strength: Hybridization of H^3 RNA to viral DNA was performed at 37°C with 50% Formamide in the presence of varying ionic strength ranging from 0.5 x SSC to 8 x SSC. The percentage of RNA hybridized increased from 0.5 x SSC to 2 x SSC, after which increasing ionic strength had no significant effect. Therefore the conditions chosen for hybridization were 37°C with 50% formamide in 2 x SSC.

c) Treating RNA - DNA Hybrids with RNase: When RNA - DNA hybrids are prepared at elevated temperatures, it is customary to treat the filters with RNase to eliminate all non-specific binding of RNA to the filters and unstable hybrids. When filters containing H^3 RNA - DNA hybrids formed at 37°C (50% formamide) were treated with RNase, there was a 50% loss of radioactivity compared to hybrids formed at 66°C. This suggested that the RNA in the hybrid was less tightly bound to DNA.

d) Melting Profiles of Hybrids Formed at 37°C and at 66°C: H^3 RNA - DNA hybrids were prepared by hybridization at 37°C in the presence of 50% formamide, omitting RNase treatment, and by hybridization

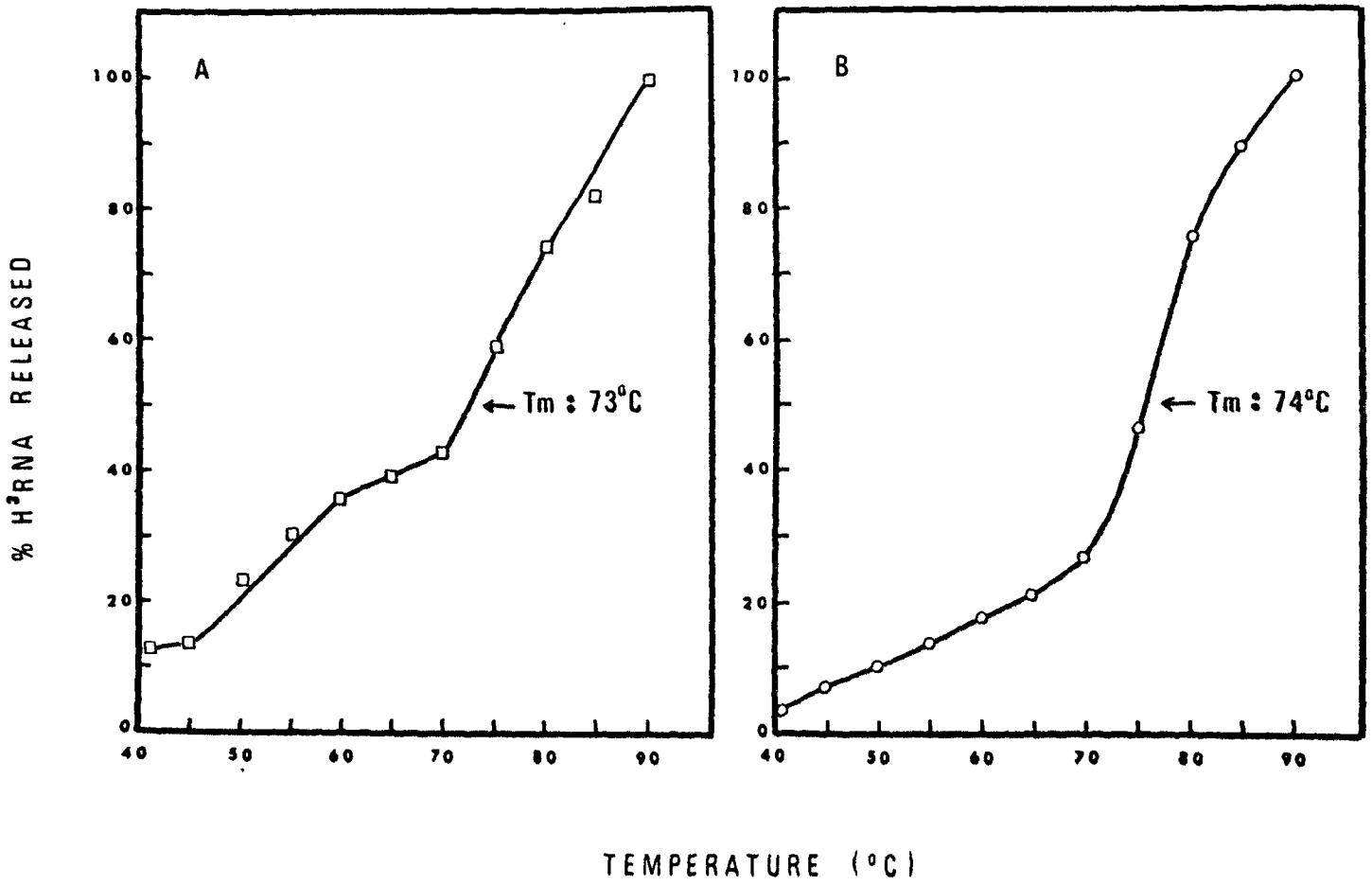

Figure 11. Melting Profiles of Hybrids Formed at 66°C, Graph A; and at 37°C + 50% Formamide, Graph B.

at 66°C, and subsequently eluted. As seen in Figure 11, both hybrids had relatively similar melting profiles and Tm values.

2. Molecular Size of Virus-Specific RNA After Hybridization and Elution.

In order to ascertain the degree of degradation of virus-specific RNA during hybridization and elution, the following experiment was carried out. Cytoplasmic extract of Ad 2 infected KB cells treated with 0.02 µg/ml Actinomycin D (Perry, 1963; Roberts and Newman, 1966; Kay et al, 1969) and labeled with H^3 Uridine, was prepared by a method similar to that of Penman (Penman, 1966). The cells were disrupted by 4°C 0.5% Triton x 100 and 0.5% Na deoxycholate for 1 hour. After the nuclei were pelleted by centrifugation, ethanol was added to the supernatant and the precipitate was resuspended in 0.1 M Na Cl, 0.01 M Tris pH 7.5, 0.001 M EDTA, 0.5% SDS. C^{14} r RNA markers were added to the extract and layered on 5-20% sucrose gradients, centrifuged for 14 hours at 21,000 RPM in an SW27 rotor at 20°C. The gradients were fractionated and the radioactivity in each fraction determined. Figure 12 indicates that H^3 RNA is distributed throughout the gradient indicating heterogeneity of size distribution.

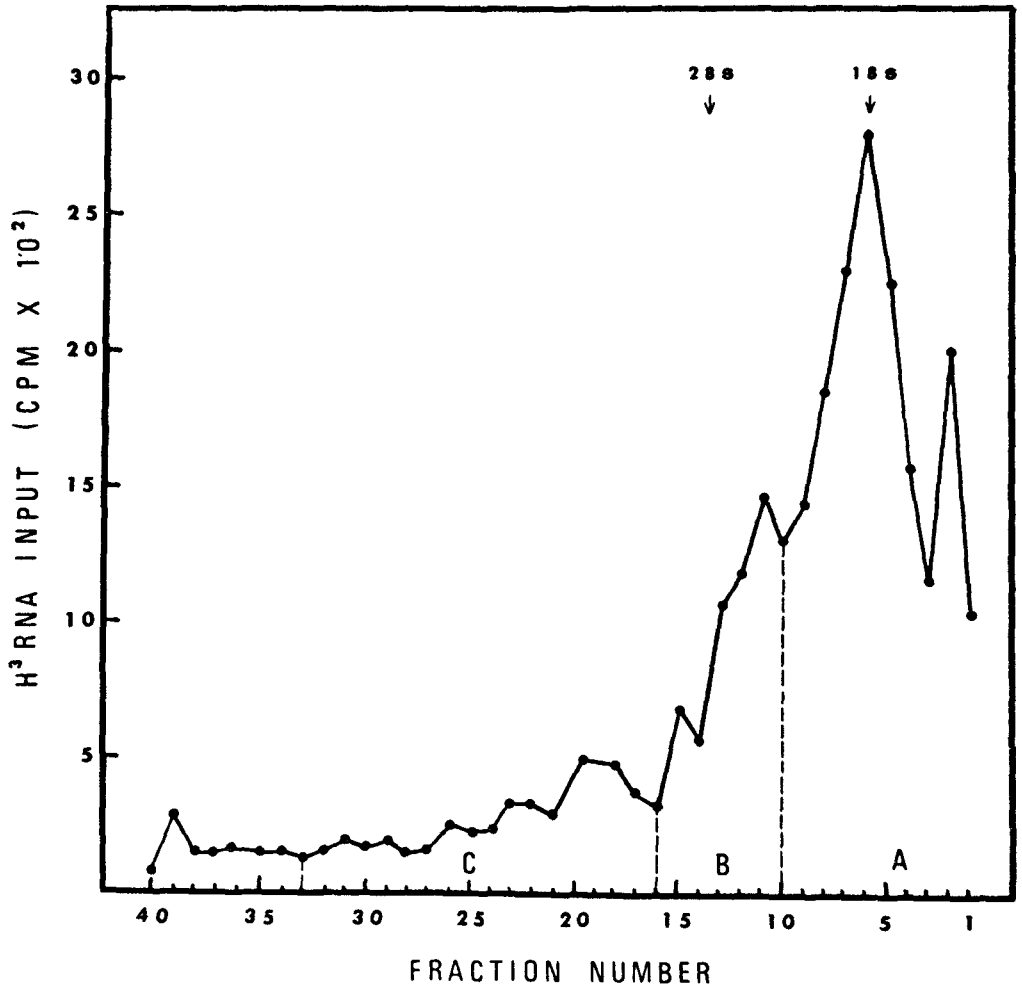

Figure 12. Sucrose Gradient Profile of Cytoplasmic H^3 RNA From Ad 2 Infected Cells. The dotted lines indicate gradient cuts.

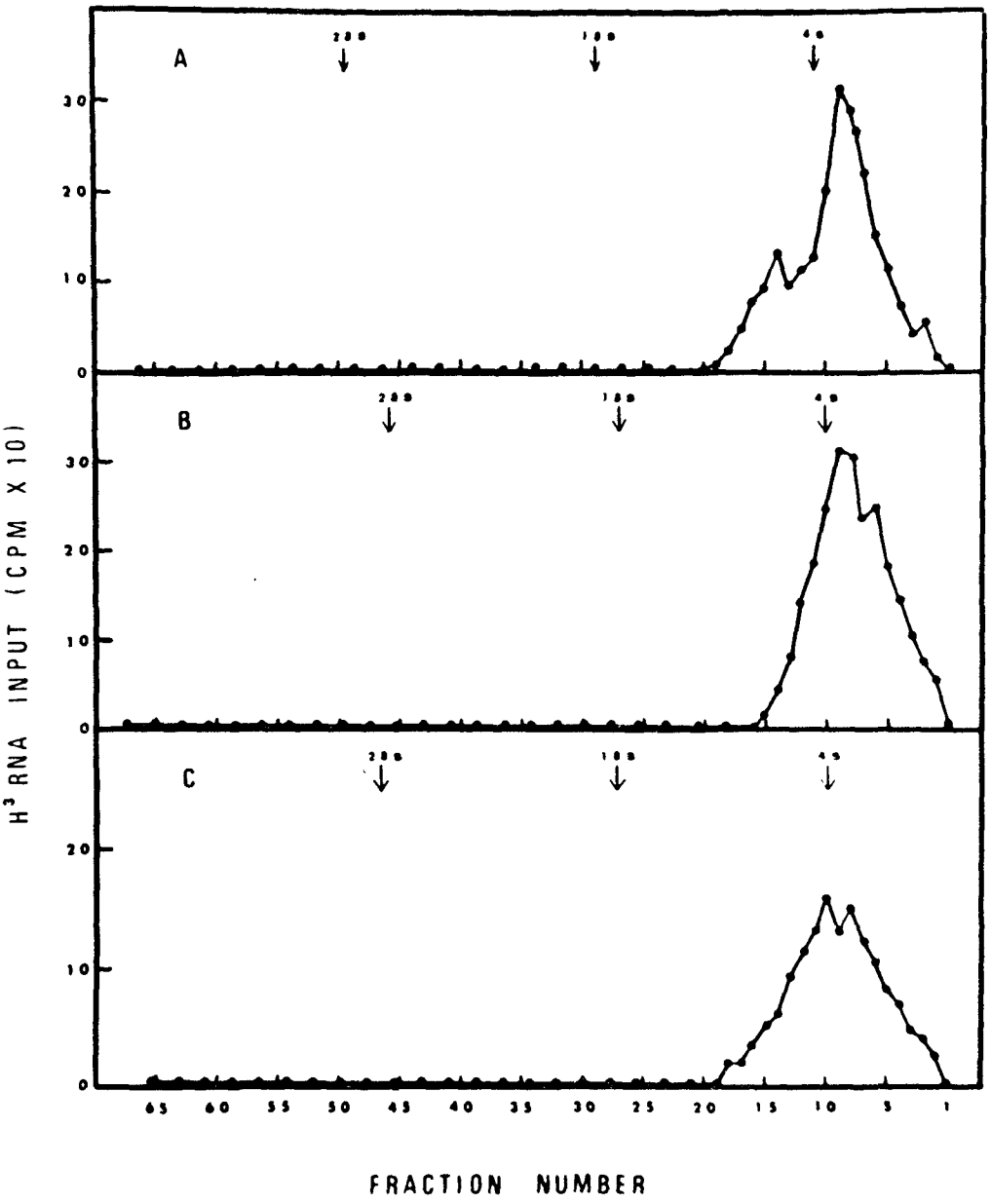

Figure 13. Sucrose Gradient Profile of Viral H^3 RNA Extracted From Cuts of the Previous Gradient (Figure 12).
Graph A = Group A
Graph B = Group B
Graph C = Group C

Fractions were pooled into groups A, B, and C, treated with pronase for 1 hour at 37°C, the RNA extracted with phenol and dialyzed for 48 hours at 4°C against NTE buffer (0.1 M Na Cl, 0.01 M Tris pH 7.4, 0.001 M EDTA). The purified RNA was hybridized to viral DNA at 37°C in 50% formamide and the viral RNA eluted from the hybrid and re-centrifuged on sucrose gradients. It can be seen from Figure 13 that the extraction and/or hybridization and elution techniques degraded H^3 RNA from groups A, B, and C to sizes of less than 5S.

The problems arising from the inability of treating the H^3 RNA - DNA hybrids formed at 37°C in the presence of formamide with RNase as well as failure of a proper elution technique led to the discontinuation of this method. Furthermore, Smith (Schmeckpeper and Smith, 1972) pointed out further disadvantages. They found reaction rates of hybridization to be much slower at low temperatures and that the hybrids were much less specific than those formed at 66°C. They also pointed out that in their system the value obtained by McCarthy of 1% formamide reducing the Tm value of hybrids 0.72°C did not apply below 45°C.

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