ADENOVIRUS AND CELLULAR DNA

CELLULAR DNA METABOLISM AFTER INFECTION BY ADENOVIRUS TYPE 12.

By

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SCOPE AND CONTENTS:

The effect of infection by human adenovirus type 12 on the pre-existing cellular DNA was examined in an abortive system as well as a productive system. The results were discussed in relation to the known effects of infection on chromosomes and the mechanism by which such effects are induced.

The effects of viral infection on certain aspects of DNA replication in human KB cells were also studied. It was observed that the ligation of the "Okazaki fragments" was slower after infection by this virus. It was also observed that the inhibition of cell DNA synthesis by this virus is not a random process and that certain classes of DNA, as distinguished by their high buoyant density and by their high reiteration frequency, are selectively synthesized.

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ABBREVIATIONS

Ad 12	adenovirus type 12
Cot	the product of DNA concentration and the time of incubation
C/Co	the fraction of DNA reassociated
DNA	deoxyribonucleic acid
FUdR	5-flourodeoxyuridine
GC	guanine + cytidine
^{3}H-CdR	tritiated cytidine
HEK	human embryonic kidney
^{3}H-TdR	tritiated thymidine
mRNA	messenger RNA
p	density
p i	post infection
RNA	ribonucleic acid
S_1	single-stranded specific nuclease
SV40	simian virus 40
T-antigen	tumor antigen
UV	ultraviolet light

INTRODUCTION

I. The Genome of Higher Organisms

Genetic information in higher organisms is contained in DNA. The DNA is associated with proteins, forming the chromosomes. It is believed that each chromosome contains a single DNA duplex (Huberman, 1973; Kavenoff *et al.*, 1973; Petes *et al.*, 1973; Cryer *et al.*, 1973; Comings *et al.*, 1973; Callan, 1973; Taylor, 1974). The DNA duplex consists of two chains of polynucleotides which exist in a double helical structure. The main chain of each strand consists of deoxyribose residues joined by 3',5' phosphodiester bonds. The two strands are held together by hydrogen bonding between the amino and keto groups of the bases in each chain.

Distribution of the bases in the DNA of higher organisms is not random. An example is DNA blocks which are known as satellites because they can be separated from the bulk of DNA by centrifugation in $CsSO_4$ - heavy metal gradients or CsCl gradients as distinct peaks. Separation is essentially due to the enriched composition of the satellites in either G + C (guanine + cytosine) or A + T (adenine + thymine).

Satellite DNA has been observed in mouse (Waring and Britten, 1966; Yunis *et al.*, 1971), man (Corneo *et al.*,

1971; Saunders, 1974), guinea pig (Yunis and Yasmineh, 1970), *Drosophila* (Gall, 1973) and calf (Yasmineh and Yunis, 1971). They are located preferentially in the heterochromatic regions in the centromeres, telomeres or intercalated within other regions of the chromosomes. Their function is generally considered to be structural in nature.

Another characteristic feature of the genome of higher organisms is the presence of multiple copies of similar nucleotide sequences in the DNA (Britten and Cohen, 1968). Based on this property, the genome can be divided into three repetition categories (Walker, 1971; 1969): 1. The highly repetitive DNA which contains sequences repeated more than 10^5 times per haploid genome. The distinguishing features of this class of DNA is that it is generally the same DNA which is isolated as the satellite DNA and that it is not transcribed into RNA. 2. The intermediate repetitive DNA which contains sequences repeated between 10^2 and 10^5 times per genome. The characteristic feature of this DNA is that it shows a poor melting profile when isolated in its reassociated form. It is transcribed into RNA and has a broad distribution along the chromosomes. It consists of short sequences interspersed between nonrepetitive DNA at least in those organisms examined so far (Davidson *et al.*, 1973). 3. The unique or slow reassociating DNA which represents the bulk of the coding sequences.

The DNA of higher organisms replicates semiconservatively (Taylor *et al.*, 1957; Callan, 1973) in tandomly arranged units known as replicons (Huberman and Rigg, 1968; Taylor, 1974). Each replicon contains a centrally positioned initiation point. Replication proceeds bidirectionally from this point until meeting adjacent replicons (Hand, 1975). Replication of DNA among individual chromosomes is non-random (Taylor, 1974). The DNA replicated early in S-phase has a higher buoyant density, at least in all the heteroploid cells in culture examined so far (Tobia *et al.*, 1970; Bostoch and Prescott, 1971; Flamm *et al.*, 1971; Bostoch and Prescott, 1971). The DNA replicated in mid S-phase has an average density similar to that of bulk DNA, while that replicated late has a lower buoyant density. Generally, the DNA in the heterochromatic regions of the chromosomes are replicated late in the S-phase of the cell cycle. No information is available on the mechanism by which this process is regulated.

Synthesis of DNA is discontinuous. When cells are fed with a radioisotope precursor for very short periods the majority of label is incorporated into small pieces known as "Okazaki fragments" (Okazaki *et al.*, 1968). These fragments are then joined to form template-size molecules (Goulian, 1971; Huberman and Horwitz, 1973).

Three DNA polymerases are involved in the replication of eukaryotic genomes. These enzymes have been isolated

and characterized *in vitro* (Wiessbach, 1975; Bollum, 1975). DNA polymerase α is the major enzyme in rapidly growing cells. It is a high molecular weight protein (5-8S, 1.1 - 4.5 x 10^5 daltons) found predominantly in the cytoplasm and is sensitive to sulfhydryl group inhibitors. DNA polymerase β is a low molecular weight enzyme (3-4S, 4.5 x 10^4 daltons) and is restricted to the nucleus. It is a minor species in rapidly growing cells (5% of total polymerase activity) and is insensitive to sulfhydryl group inhibitors. DNA polymerase γ represents 1-2% of total cellular DNA polymerases. It is found in both nucleus and cytoplasm and is distinguished by its ability to copy ribohomopolymers at a higher rate than duplex DNA containing single-stranded gaps. No information on the *in vivo* properties of these enzymes is available.

II. Effect of Virus Infection on Host Genome

Viruses are a group of microorganisms which are dependent on the metabolic machinery of their host for their propagation. Their genetic information is contained in either DNA or RNA. They may have cubic or helical structure with or without a membranous envelope. Based on these and other physical and chemical properties, the animal DNA viruses have been classified into picodna-, papova-, adeno-, herpes-, and poxviruses while RNA viruses have been grouped into diplorna-, toga-, orthomyxo-, paramyxo-, rhabdo-, oncorna-, arena-, and coronavirus (Melnick, 1973).

Infection of mammalian cells by viruses may result in the inhibition of biosynthetic processes followed by cell death. In most cases this is correlated with the productive virus cycle. Infection may cause persistent changes of cellular growth and division as in transformation. Cellular transformation by viruses is characterized by the persistence of at least part of the viral genome in these cells. In the extreme cases, infection may have no detectable effect on the host cell while being propagated simultaneously (Luria and Darnell, 1968).

Virus infection has a variety of effects on the genome of the host. A well documented effect of infection is the induction of chromosome abnormalities such as chromosome pulverization, chromatid breaks, chromatid exchanges and chromosome rearrangements (Stich and Yohn, 1970; Nichols, 1970). Induction of such anamolies is dependent on virus dose, time after infection and the type of host. Polyoma, SV40, adeno, herpes, picorna, and Rous sarcoma viruses are the known examples which induce in their host one or more of the types of chromosome abnormalities listed above.

Infection by several viruses induces the fragmentation of host cell DNA. SV40 (Ritzi and Levine, 1973), polyoma (Ben-Porat and Kaplan, 1967), Epstein-Barr virus (Nonoyama and Pagano, 1972) and vaccinia (Parkhurst *et al.*, 1973) are examples. Fragmentation of cell DNA by SV40 and polyoma

has been observed late in infection and is shown to depend on the replication of these viruses. Early fragmentation is observed after infection of HeLa cells by vaccinia virus. Endonuclease activity associated with the virions has been found in SV40, polyoma and vaccinia. The role of these virion-associated nucleases in the fragmentation of cell DNA is, however, postulatory. The possibility that cellular nucleases may be involved in the fragmentation of cellular DNA by SV40 is supported by the observation that this effect is induced in productively infected AGMK cells but not in productively infected BSC1 and CV1 cells (Ritzi and Levine, 1970).

Another effect of infection by viruses on host genome is the induction of cell DNA synthesis. This effect is best observed in non-growing cells such as cells grown in the presence of low levels of serum or contact-inhibited cells. The level of DNA synthesis is minimal in these cells prior to infection. SV40 (Dulbecco *et al.*, 1965), polyoma (Dulbecco *et al.*, 1965; Branton and Sheinin, 1968), fowl pox (Gafford *et al.*, 1972), and cytomegalovirus (St Joer *et al.*, 1973) are some of the known examples of viruses to have such an effect. The induction of cell DNA synthesis after infection is due to the expression of functional gene(s) of the virus, since infection by UV- or heat-inactivated virus has no effect. Also, one temperature-sensitive mutant of polyoma, ts-3, does not induce cellular

DNA synthesis in 3T3 cells at the nonpermissive temperature (Dulbecco and Eckhart, 1970). The induction of cell DNA synthesis is observed only after infection with the low doses of the virus, in case of polyoma. Infection at high multiplicities has an inhibitory effect.

Inhibition of cell DNA synthesis is another effect which is observed after virus infection. This effect is observed after infection with either RNA viruses such as reo-, mengo-, Newcastle disease virus (Hand *et al.*, 1971), polio (Ackerman and Whal, 1966) and vesicular stomatitis virus (Yaoi and Amano, 1970) or with DNA viruses such as pox- (Moss, 1974), herpes-(Roizman and Furlong, 1974) and adenoviruses (Ginsberg *et al.*, 1967).

There is no general mechanism which explains the inhibition of cell DNA synthesis by viruses. Inhibition of cell DNA synthesis is observed after infection with UV-inactivated, VSV, reo- and poxvirus (Shaw and Cox, 1973; Guir *et al.*, 1971). The expression of all viral genes are therefore, not required for this effect. The inhibition of cell DNA synthesis after poliovirus infection is postulated to be due to the synthesis of arginine-rich proteins such as histones, since the omission of arginine from the medium prevents the inhibition of cell DNA synthesis without affecting virus production (Ackerman and Whal, 1966). This is further substantiated by the observation that the biosynthesis of histones is stimulated after infection by

this virus (Sokol *et al.*, 1965). A similar mechanism has been postulated for the inhibition of cell DNA synthesis after infection by herpesviruses. Two viral acid-soluble histone-like polypeptides have been reported to be associated with the isolated chromatin (Chantler and Stevely, 1973). However, there is no direct evidence that these proteins play a role in the alteration of cell DNA structure.

Another effect of virus infection is the covalent association (integration) of viral genome with the genome of the host. This effect is generally characteristic of those viruses which are able to induce benign or malignant tumors in some hosts and transform certain cells in culture. Viruses known to have this effect are: polyoma, SV40, adeno, herpes, and RNA tumor viruses (Tooze, 1973). Integration of RNA tumor viruses is assumed to proceed by way of a DNA intermediate which is synthesized by virion-associated reverse transcriptase after infection.

Integration of viral genome into the host cell DNA is observed in both lytically infected cells in which new viral particles are produced and in abortively infected cells in which the production of new viral progeny is blocked. Integration is considered to be a prerequisite for both tumor induction and cell transformation by these viruses.

III. Effect of Infection by Adenoviruses on Host Cell Genome

Adenoviruses are a group of DNA viruses which consist of a DNA-containing core surrounded by a protein capsid

composed of 252 capsomers arranged into an icosahedron (Philipson and Lindberg, 1974). Figure 1 shows a schematic drawing of an adenovirus with the major components of the capsid indicated. The DNA is a linear double-helix with the molecular weight of 20-25 x 10^6 daltons carrying information for 20-30 gene products. The proteins of the capsids have been highly purified and their function studied extensively.

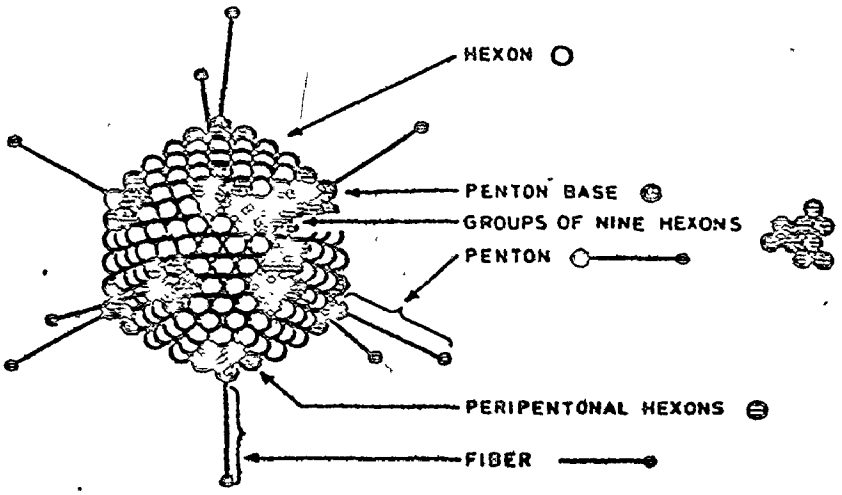


Fig. 1. Schematic drawing showing the icosahedral outline of the adenovirus capsid and the location of various components. Reprinted from Philipson and Pettersson (1973).

More than 80 different adenovirus types have been isolated from a variety of animals and all except avian adenoviruses contain one group-specific antigen determinant residing in the hexon.

The 30 human serotypes have been classified into

four groups on the basis of the GC-content of their DNA. This correlates with the extent of the oncogenecity of each group in hamsters (Green, 1970). The highly oncogenic group known as group A contains 48-49% G + C, the weakly oncogenic group known as group B contains 50-52% G + C, and the non-oncogenic group known as group C contains 57-59% G + C. Group D has the same G + C content as group C. This classification has been confirmed by heteroduplex mapping of the DNA of representative viruses within each group (Garon *et al.*, 1973).

The lytic cycle of human adenoviruses, studied mostly in cultures of human KB or HeLa cells, begins with the adsorption and the penetration of the virus into the host cell cytoplasm. This is followed by the removal of capsid proteins from the DNA protein core and the entry of the core into the nucleus. Uncoating is then completed in the nucleus. Transcription of early viral RNA (RNA synthesized prior to the onset of viral DNA synthesis) starts immediately. Viral DNA synthesis begins by 6-8 hr after infection by Ad 2 (Philipson and Petterson, 1973), a member of the group C. The onset of viral DNA synthesis for Ad 12, a member of group A, is at 12 hr post infection (Mak, 1969). Adenoviruses are assembled in the nucleus, but the viral proteins are synthesized in the cytoplasm and then transported to the nucleus for virion assembly.

Abortive infection by human adenoviruses has been observed in monkey cells and in hamster cells for Ad 12. In the monkey cells the virus particles enter the cell and the infection cycle proceeds up to DNA replication and production of late viral mRNA. The defective step seems to reside in the inability of ribosomes with late viral mRNA (RNA synthesized after the onset of viral DNA synthesis) for capsid proteins to form polysomes (Hashimoto *et al.*, 1973). With SV40 as a helper, the abortive cycle is converted to a lytic cycle. In the hamster cells, Ad 12 particles can penetrate and express early viral RNA (Mak, 1975) and T-antigen but the genome is not replicated (Ortin and Doerfler, 1975).

Adenoviruses interact with host cell genomes in a variety of ways. Induction of chromosome breaks has been observed in cells infected with highly oncogenic (types 12 and 18), weakly oncogenic (type 7), and non-oncogenic (type 2) adenoviruses (Stich, 1973). Chromosome breaks are best observed in abortively infected cells such as hamster cells (Cooper, 1968). Chromosome breaks are probably a reflection of fragmented DNA which has also been observed after infection of hamster cells (Doerfler, 1969; Strohl, 1973). Fragmentation of cell DNA is suggested to be due to the activity of an endonuclease associated with the pentons of the virion (Burlingham and Doerfler, 1972).

Studies on chromosomal abnormalities in adeno-infected permissive human cells is more difficult due to

the strong inhibition of mitosis. Chromosome breaks are observed in these cells when the virus is inactivated by UV. Mitosis is not inhibited after infection with UV irradiated virus (Stich *et al.*, 1968; Rainbow, 1970).

Another effect of infection by adenoviruses on host genome is the induction of cell DNA synthesis under certain conditions (Zur Hausen, 1973). Infection of resting hamster cells with adenovirus types 2,3,5,7,12 and 13 at high multiplicities induces cellular DNA synthesis. Similar results have been reported for the growth-arrested permissive human embryonic kidney (HEK) cells after infection with adenoviruses types 12 and 5 (Yamashita and Shimojo, 1969; Takahashi *et al.*, 1966).

The induction of hamster cell DNA synthesis by Ad 12 has been extensively studied by Strohl (1973). The cells are arrested in G_1-phase of the cell cycle by their growth in the presence of low serum and then infected with Ad 12. Stimulation of cell DNA synthesis is observed by 12 hr after infection reaching a peak by 21-24 hr at low multiplicities and by 16 hr at high multiplicities of infection. The difference between high and low multiplicities is interpreted as a result of high synchrony of infection obtained at high multiplicities. The stimulation of cell DNA synthesis is only for one round of replication and requires infectious virus and some transcription of viral genes.

Another effect of infection on the genome of the host is the early inhibition of DNA synthesis in lytically infected cells. At 6-8 hr after infection with adenoviruses the synthesis of cellular DNA begins to decline, and by 10-13 hr post infection about 90% of the newly synthesized DNA is viral (Ginsberg, 1969; Pina and Green, 1969). The synthesis of either host or viral DNA is not necessary for this effect. This has been shown by adding FUdR to uninfected and infected cells to stop DNA synthesis. The block is reversed with excess thymidine 10 hr later. Only viral DNA is made in the infected cells, whereas host DNA synthesis resumes in the uninfected controls (Ginsberg, 1969).

Inhibition of cell DNA synthesis seems to be at the level of initiation (Hodge and Scharff, 1969). This has been shown by using synchronized cultures. When viral DNA synthesis occurs during the G_1-phase of the cell cycle, cell DNA synthesis is not initiated. When viral DNA synthesis is "tuned" to begin during S-phase, the cellular DNA synthesis goes on but the round of cellular DNA replication fails to go to completion.

The mechanism of the induction of inhibition of cell DNA synthesis is not well understood. Levine and Ginsberg (1967) observed that the fiber antigen of type 5 inhibited the biosynthesis of cell DNA, RNA and proteins. No such effect was observed when fibers from Ad 2 or Ad 12 were used (Yamashita *et al.*, 1971). Levine and Ginsberg

(1968) also observed that the hexons as well as the fiber protein complexed with DNA *in vitro* and inhibited the activities of both DNA-dependent RNA polymerase and DNA polymerase activity. The authors postulated that the viral capsid proteins complex with the host DNA and block its synthesis *in vivo*. It is unlikely that the newly synthesized fiber has this effect since ts mutants negative for fiber and negative for viral DNA synthesis still inhibit host cell DNA synthesis (Wilkie *et al.*, 1973; Ledinko, 1974).

Some apparent contradictory effects of infection by adenoviruses on host cell genome (also observed with other viruses) should be emphasized at this point. Infection by either Ad 2 or Ad 12 results in the stimulation of host DNA synthesis in resting HEK cells (Yamashita and Shimijo, 1969). However, cell DNA synthesis is inhibited after infection of growing HEK cells by these same viruses (Ledinko *et al.*, 1969). It has been suggested that the induction of cell DNA synthesis is a temporary event and depends on the base level of DNA synthesizing enzymes which are used for the synthesis of viral DNA. Induction occurs only in those cells in which the base level of these enzymes are low (Pina and Green, 1969).

IV. Purpose of the Study

As reviewed in the previous section, chromosome breaks have been observed cytologically after infection of

of cells by adenoviruses (Cooper, 1968; Stich, 1973). The effects of infection at the level of cellular DNA were studied in the present work and the results are reported in the first part of the thesis. Adenovirus type 12 was used to infect both abortive and productive systems. It was hoped that this would give a better understanding of the mechanism.

Infection of human KB cells by adenovirus type 12 had been known to result in the early inhibition of host DNA synthesis (Pina and Green, 1969). The effect of such inhibition on certain aspects of cell DNA replication were examined in the second part of this work. It was felt that such studies might provide new information on virus-cell interactions at the same time and facilitate the understanding of the processes involved in the replication of the complex genome of eukaryotes..

MATERIALS AND METHODS

I. Maintenance and Propagation of Cell Cultures

A. Human KB cells

Monolayer cultures of human KB cells were grown in α MEM, a modified minimum essential medium (Flow Laboratories), supplemented with 10% fetal bovine serum (FCS), 1% fungizone (GIBCO), and antibiotics (100 μg/ml of streptomycin and 1.21 mg/ml of penicillin). Cells from confluent monolayers were subcultured by scraping them off of the glass with a sterile rubber policeman and seeding 10^6 of them in 32 oz screwcap glass bottles. They were maintained at 37°C in a humid incubator with 5% CO_2 and 95% air.

Suspension cultures were grown in MEM (Joklik, modified) suspension medium (GIBCO) supplemented with 5% horse serum. The cells were kept in suspension by constant agitation with a teflon magnetic bar over a magnetic stirrer. The number of cells per ml was maintained at 2-5 x 10^5 by regular dilution with medium prewarmed to 37°C.

B. Hamster embryo cells

Thirteen-day old hamster embryos were removed from the mother and washed with phosphate buffer saline (PBS) under sterile conditions. They were decapitated. The

internal organs and the four extremities were removed and the carcasses were washed with PBS. The carcasses were minced and transferred into a 250 ml flask and trypsinized with 0.25% trypsin in citrate saline (1% KCl and 0.44% sodium citrate) and constant agitation for 15 min at 37°C. To remove the large clumps, the suspension was filtered through several layers of cheese cloth into a 250 ml centrifuge bottle containing 10 ml of FCS. Growth medium was added to fill up the centrifuge bottle. The cells were pelleted by centrifugation for 5 min in an International centrifuge at 1000 rpm at room temperature. They were then resuspended in α MEM and about 2 x 10^7 of them were seeded in 32 oz glass bottles and incubated at 37°C. These were the primary cultures. Secondary cultures used in the experiments were obtained by trypsinization of the confluent primary cultures with 0.125% trypsin and seeding about 10^7 cells in 32 oz bottles.

II. Virus Preparation

KB cells grown in suspension were pelleted by low speed centrifugation and resuspended in infecting medium (Joklik, supplemented with 1% FCS) at 10^7 cells/ml. The virus (adenovirus type 12, Huie strain, 1000 particles/cell) was added and adsorbed for 90 min at 37°C. The culture was diluted to 2 x 10^5 cells/ ml by adding growth medium. The cells were harvested at 72 hr after infection. The

virus was isolated by a modification of the technique of Green and Pina (1963). Briefly, the pellet was resuspended in 0.01 M tris pH 8.1, sonicated and homogenized with equal volumes of Freon 113 at 4°C. The aqueous layer containing the virus was collected and the virus was sedimented onto a cushion of CsCl solution with a density of 1.44 g/cc. The virus was further purified by two cycles of equilibrium centrifugation in CsCl solution with a density of 1.34 g/cc at 33000 rpm at 5°C for more than 20 hr. Concentration of the virus was measured by its absorption at 260 nm. One absorbance unit (A_{260}) is about 4.5×10^{11} particles per ml. The virus was stored in tris buffer saline + 30% glycerol at -70°C.

III. UV Irradiation of the Virus

The virus was suspended in 1.5 ml of infecting medium in a 35 mm Falcon plastic petri dish and agitated by a micro-magnetic bar over a pre-cooled magnetic stirrer. The petri dish was kept at a distance of 10 cm from the ultra-violet light source (8 Watt General Electric Germicidal Tube) and was irradiated with an incident dose of 2.5×10^4 erg/mm^2. This dose reduces the surviving fraction of the virus to about 10^{-5} and its cell killing ability to 10^{-2} (Rainbow and Mak, 1973).

IV. Infection Procedures

Cells, grown to near confluency in 32 oz glass

bottles, were trypsinized and resuspended in 10 ml of infecting medium. About 3 x 10^6 cells (in 1.5 ml of infecting medium) were seeded in 25 cm^2 Falcon tissue culture flasks pre-incubated in the presence of 5% CO_2 + 95% air. Virus was added and adsorbed by slow shaking at 37°C for the indicated times (Figure 2). Close to maximum adsorption for both KB and hamster cells was achieved by 90 min of shaking. This time was chosen as the standard time of adsorption for all the subsequent experiments. Growth medium (3.5 ml) was added and infection was continued at 37°C for the times indicated in each experiment.

V. Isotopic-labelling Techniques

In order to examine the effects of virus infection on the cellular DNA synthesized prior to infection, the cells were pre-labelled with ^{3}H-TdR (0.5 µCi/ml , New England Nuclear, 5 ci/mmole) for 15-24 hr and then used for infection.

In order to study the cellular DNA synthesized after infection, the cells were pulse-labelled with ^{3}H-TdR (10 µCi/ml) for 30 min (or as indicated) at the indicated times after infection. They were then either used immediately or "chased" for the indicated times by replacing the medium containing the label with fresh medium after their washing with unlabelled medium. The cells had been labelled with ^{14}C-TdR (0.05 µCi/ml, New England Nuclear, 50 µCi/mmole) for 15-24 hr prior to infection in order to compare the properties of the newly synthesized DNA to the pre-existing bulk

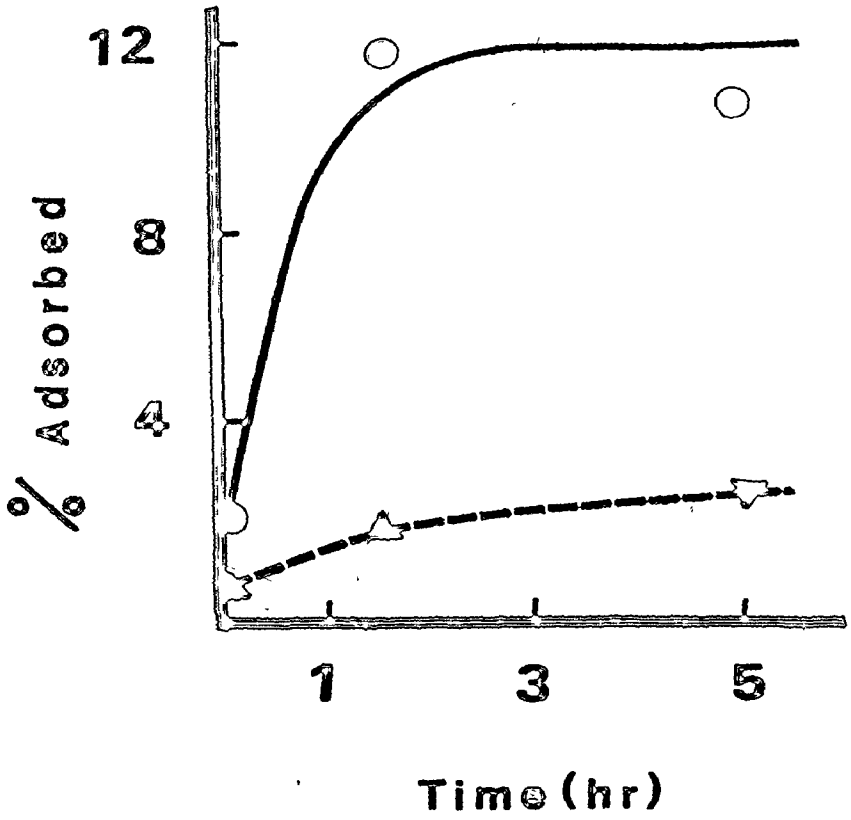


Figure 2: Efficiency of virus adsorption by human KB cells and by hamster embryo cells.

Cells were adsorbed with radioactive virus for the indicated times. They were then pelleted, washed with cold medium containing 10% serum and repelleted. After their suspension in 0.1 ml of PBS, they were collected onto nitrocellulose filters and the amount of radioactivity in each filter was determined. The amount of radioactivity in the input virus was 1.42×10^5 cpm. A------A, hamster embryo cells; o——o, KB cells.

Radioactivity was determined by precipitation of the labelled samples by 10% cold trichloroacetic acid (TCA) collecting onto 0.2 μ or 0.45 μ pore size cellulose nitrate filters and measuring the amount of radioactivity of the dried filter in 5 ml toluene containing omnifluor (4gm/litre, New England Nuclear).

In the experiments in which both ^{14}C and ^{3}H were used, the spill-over from the ^{14}C-channel to ^{3}H-channel was subtracted. There was no spill-over from ^{3}H-channel to ^{14}C-channel.

VI. Sucrose Gradient Sedimentation Techniques

A. Alkaline sucrose gradients

Centrifugation of single-stranded cellular DNA in alkaline sucrose gradients was carried out as described by Palcic (1972). The cells were trypsinized and resuspended in PBS. About 4-6 x 10^4 of them (in 10 μ l) were layered onto a lysing solution containing 0.5 M sodium hydroxide (NaOH),0.2% sodium dodecyl sulfate (SDS), 0.01 M ethylene-diamine-tetraacetic acid (EDTA) on top of a 5-20% linear sucrose gradient containing 0.3 M NaOH, 0.01% SDS, and 0.001 M EDTA. They were left to lyse at room temperature for 12-14 hr and then centrifuged using a SW27.1 rotor in a Beckman ultracentrifuge at 20000 rpm for 6-7 hr (or as indicated) at 20°C. The tubes were punctured at the bottom and one-hundred-drop fractions were collected. The DNA in

each fraction was precipitated by adding 10% cold TCA and then collected onto a 0.45 μ pore size nitrocellulose filter. Radioactivity of each filter was measured as described. The amount of radioactivity in each fraction was plotted as the percentage of total radioactivity in each gradient.

B. Neutral sucrose gradients

Neutral sucrose gradients were used to analyze double-stranded cellular DNA. A combination of the techniques of Okazaki *et al.* (1968) and Brewer (1972) was used. Briefly, 4-6 x 10^4 cells were layered onto a lysing solution containing 0.015 M sodium citrate (NaCit), 0.005 M EDTA, 0.15 M NaCl, and 2% sarkosyl, pH 7.5 (Bremer, 1972) on top of a 5-20% linear sucrose gradient containing 0.15 M NaCl, 0.015 M NaCit, 0.001 M EDTA and 0.1% SDS, pH 7.2 (Okazaki *et al.*, 1968). The remainder of the procedure was the same as described for alkaline sucrose gradients.

VII. Neutral CsCl Density Gradient Techniques

About 3 x 10^6 cells were lysed for 5 min in 4 ml of phosphate-EDTA-SDS buffer (0.1 M NaH_2PO_4, 0.001 M EDTA, 0.1% SDS and adjusted to pH 6 by adding 1 M Na_2HPO_4 solution). Pronase was added to a final concentration of 1 mg/ml and digestion was allowed for 30 min at 37°C. Tris-sarkosyl buffer (1 M tris, 10% sarkosyl, pH 9.2) was added to adjust the final molarity to 0.1 M tris - 1% sarkosyl, pH 7.3. CsCl powder was added to a density of 1.67. DNA was sheared into

pieces with an average molecular weight of 2×10^7 daltons (Figure 3) by passing the solution 5 times through a 21 gauge needle using maximum thumb pressure. Centrifugation was performed in a siliconized polyallomer tube for 65 hr at 40000 rpm in a titanium 50 rotor at 20°C after overlaying the solution with mineral oil.

VIII. Cell DNA Reassociation Techniques

A. Isolation of cellular DNA

About 10^8 cells were lysed in 50 ml of tris-SDS buffer (0.01 M tris, 0.1% SDS, pH 6.7) and treated with pronase (1 mg/ml) for one hr at 37°C. They were then extracted with buffer-saturated (tris-SDS buffer) phenol for 15 min at room temperature and precipitated (ppt) with two volumes of cold ethanol (EtOH) at 4°C for more than 4 hr. The precipitate was resuspended in double-distilled water. It was then digested with RNAse (50 μg/ml) in 0.1 x SSC (0.015 M NaCl, 0.0015 M sodium citrate) for 30 min at 37°C. After phenol extraction and EtOH precipitation, the DNA was dissolved in double-distilled water and dialyzed extensively against water and then against 0.01 M tris, pH 7.3.

B. Reassociation of cellular DNA

DNA, suspended in tris buffer (tris 0.01 M, pH 7.3), was sonicated for 5 min and broken into pieces about 900 base pairs in length and then chelex-treated to remove any traces of metal ions. It was then denatured by boiling

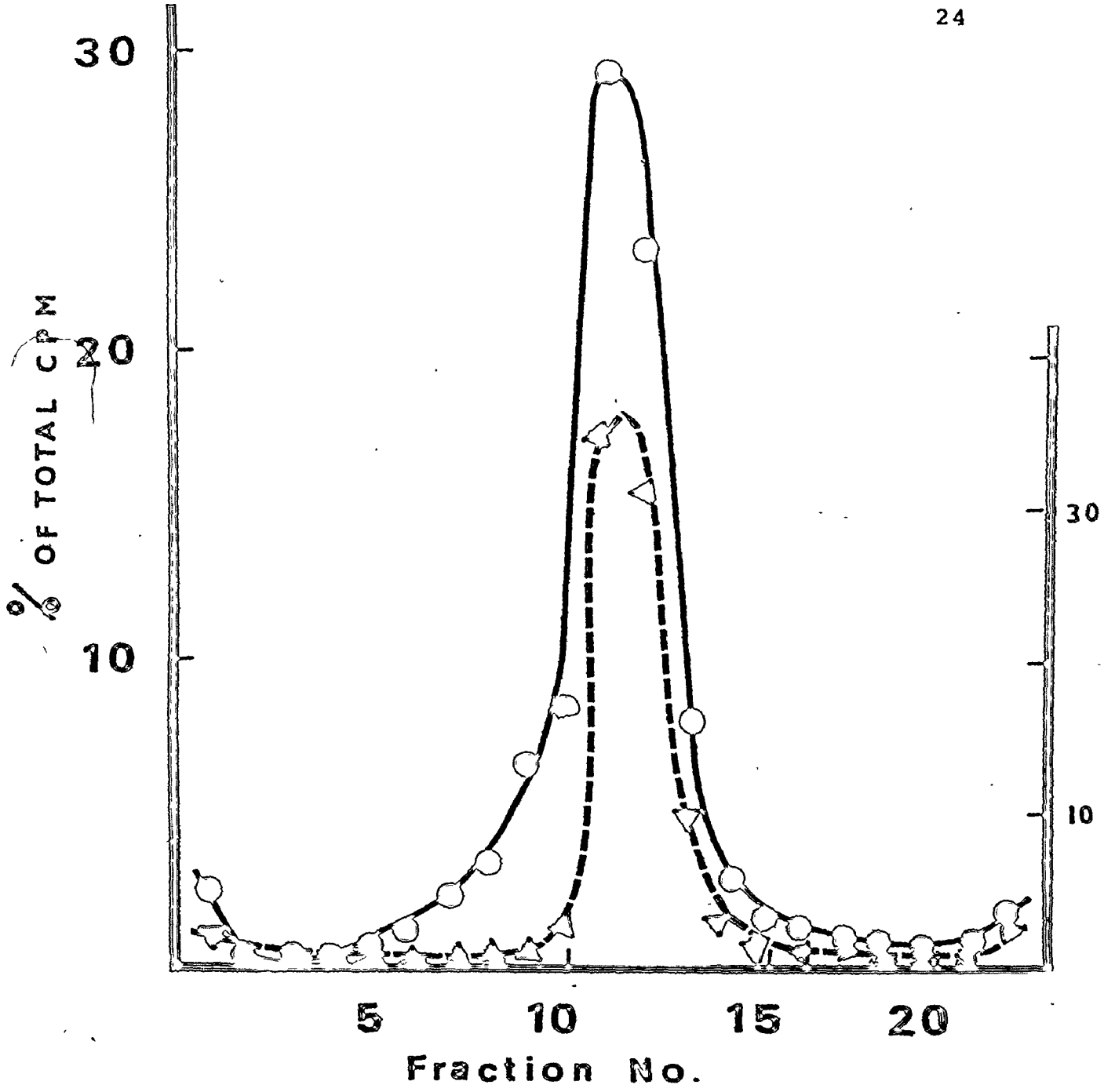


Figure 3: Centrifugation of sheared ^{3}H-labelled cellular DNA together with ^{14}C-labelled adenovirus type 2 in neutral sucrose gradients.

Sheared ^{3}H-labelled cellular DNA was mixed with ^{14}C-labelled adenovirus and then layered on top of 5-20% linear sucrose gradient and centrifuged as described earlier for 12 hr. o——o, ^{3}H; Δ_____Δ, ^{14}C.

for 5 min and cooled on ice rapidly. NaCl in tris buffer was added to bring the final salt concentration to 0.3 M. Reassociation was carried out in a siliconized 2 ml-volumetric flask at 37°C. Aliquots (50 µl) were taken at the indicated times and treated (untreated as input) with single-strand specific nuclease (S_1, from *Aspergillus oryzae*, Miles Lab.) as will be described in the following section.

C. S_1-nuclease digestion of single-stranded DNA

A modified technique of Sutton (1971) as suggested by Davidson *et al.* (1973) was used. A typical assay mixture consisted of: 1) 0.4 ml of tris-NaCl buffer (0.01 M tris, 0.3 M NaCl, pH 7.3); 2) 40 µl of denatured calf thymus DNA (1 mg/ml in double-distilled water); 3) 100 µl of zinc-acetate-NaCl buffer (0.01 M $ZnCl_2$, 0.3 M sodium acetate, 0.3 M NaCl, pH 4.5); 4) 50 µl of S_1-nuclease (1 mg/ml in 0.018 M $ZnCl_2$, 0.3 M sodium acetate, 3 M NaCl, pH 4.5, stored in 20% glycerol at -20°C).

The assay was at 37°C. Digestion of single-stranded DNA was complete by 30 min (Figure 4). Only 5% of double-stranded DNA was digested at this time. This time was chosen as the standard time of assay for all the subsequent experiments.

VIV. Filter Hybridization Techniques

A. Isolation of viral DNA

Purified virus was dialyzed against tris buffer M, pH [illegible] Phosphate-EDTA-SDS buffer (as

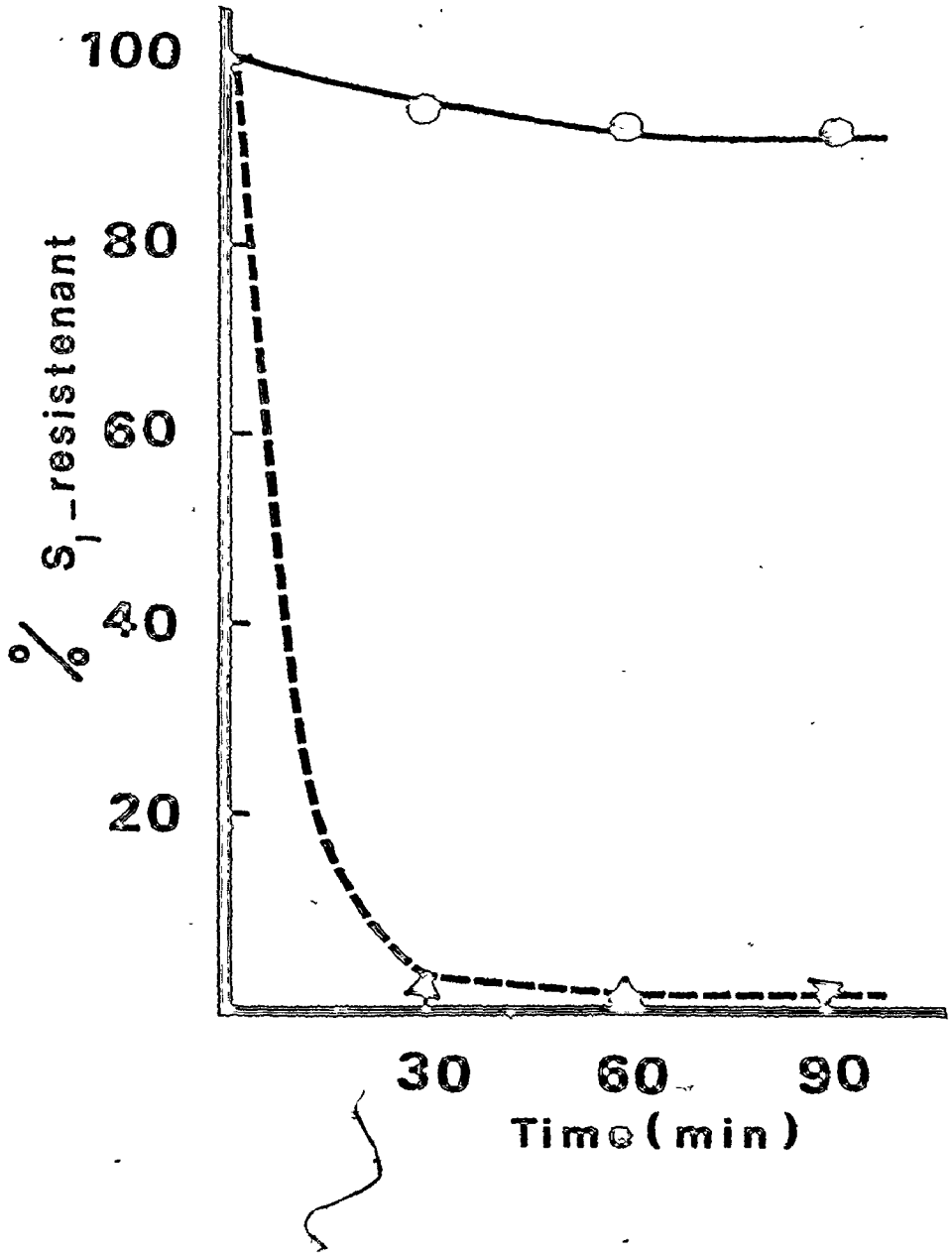


Figure 4: Digestion of denatured and native ^{3}H-labelled KB cell DNA by S_1-nuclease as a function of time.

Input 1.87 x 10^4 cpm. o——o, double-stranded native; Δ- - - -Δ, single-stranded.

described in section VII) was added to bring the pH down to 6.8 and then treated with pronase (1 mg/ml) at 37°C for 30 min. The DNA was extracted with buffer-saturated (phosphate buffer, 0.05 M, pH 6.8) phenol for 15 min at room temperature, three times. It was then dialyzed extensively against 0.1 x SSC.

B. Filter hybridization

Filter hybridization was essentially as described by Mak (1969). Briefly, unlabelled viral DNA in 0.1 x SSC was denatured by boiling and cooled rapidly on ice. SSC (20x) was added to give a final concentration of 2 x SSC. The solution was then filtered onto a nitrocellulose filter with 0.45 μ pore size which had been pre-soaked in 2 x SSC. The filter was dried at room temperature overnight and heated for 2 hr at 80°C.

Labelled DNA in 0.1 x SSC was sonicated for 1 min and denatured by boiling. The solution was adjusted to 2 x SSC containing 0.1% SDS and added onto the filters containing viral DNA. Hybridization was at 66°C for 24 hr. The filters were then rinsed extensively with tris-SSC buffer (0.003 M tris, 0.1 x SSC, pH 9.4) on each side to remove the unbound DNA. The amount of radioactivity in each filter was then determined.

RESULTS

I. Effect of Infection on Pre-existing Host Cell DNA

A. Effect of infection on pre-existing human KB cell DNA:

1. Fragmentation of cell DNA after infection: Human KB cells were pre-labelled with 0.5 μCi/ml of ^{3}H-TdR for 24 hr (about one cell generation) and then infected with 4.3×10^{5} virus particles per cell or mock infected for 10 hr. Their DNA was analyzed by centrifugation in alkaline sucrose gradients as described earlier. Recovery of input cpm was 90-95% in all gradients. Figure 5 shows that the majority of DNA in the mock-infected cells is in fractions 12-19. The molecular weight of this DNA has been estimated by Palcic (1972) and confirmed by the author to be 2.5×10^{8} daltons. No proteins are associated with this DNA. Note that a small percentage of DNA sediments very slowly. This could be degraded DNA which results in cell death (Williams *et al.*, 1974) during mock-infection. Note also that a small percentage of DNA sediments to the bottom of the gradient. This has been suggested by Palcic (1972) to be due to the attachment of some DNA molecules to nuclear membranes and lack of their dissociation during lysis.

Most of the DNA from the infected cells is in fractions 4-10 (Figure 5). The average S-value of this

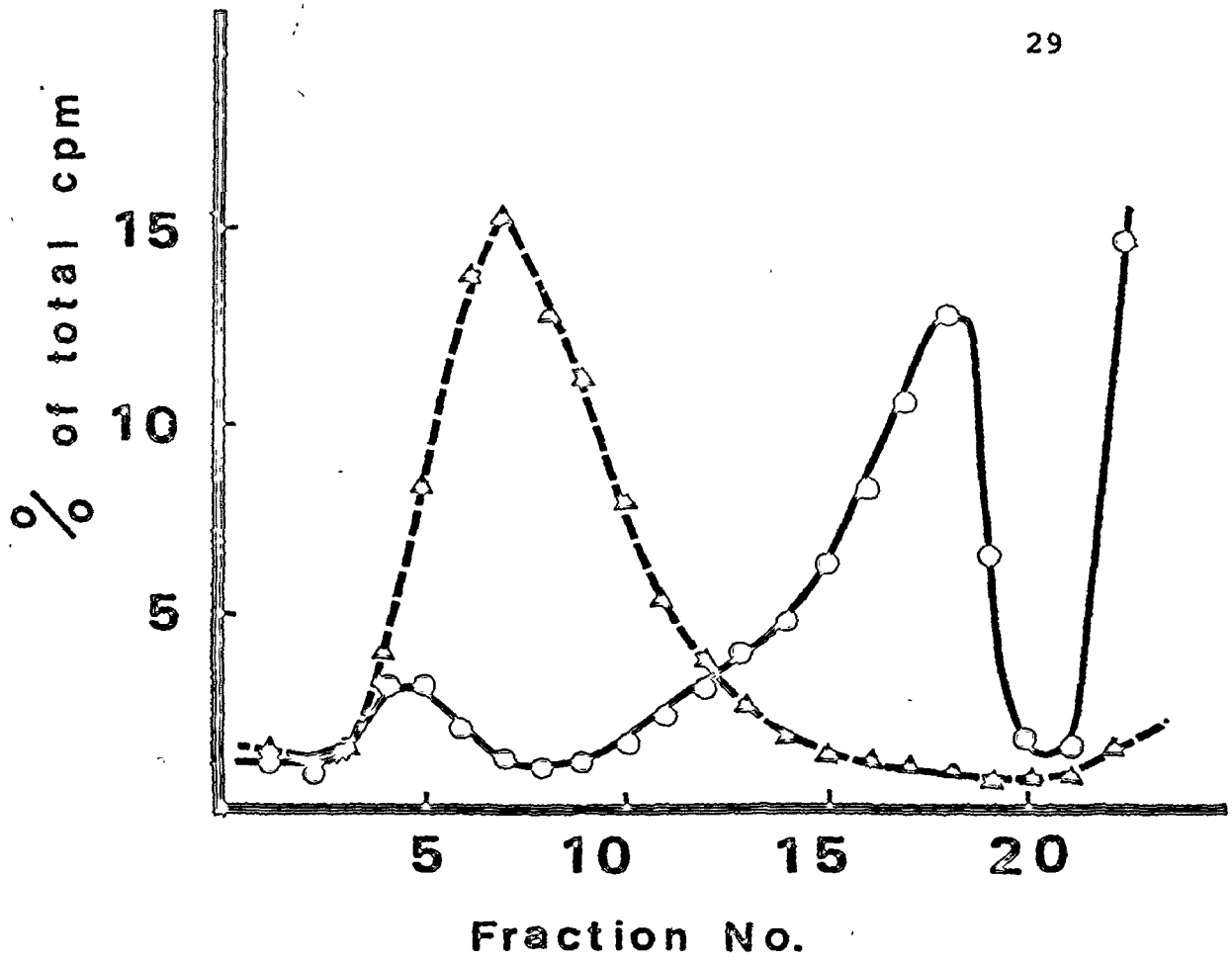


Figure 5: Fragmentation of cellular DNA after infection of human KB cells by Ad 12.

Human KB cells were pre-labelled with ^{3}H-TdR and then infected with 4.3 x 10^{5} virus particles per cell. Their DNA was analyzed by centrifugation in alkaline sucrose gradients. (Direction of sedimentation for all sucrose gradients is from left to right.) o———o, uninfected; ▲_____▲, infected.

fragmented DNA was estimated as 25 (McBurney *et al.*, 1972) and its molecular weight was calculated to be 4.7×10^6 daltons in alkaline sucrose gradients (Studier, 1965) using ^{14}C-labelled Ad 2 DNA as marker (Ad 2 DNA is 34S, Doerfler, 1969).

To determine whether or not the fragments were double-stranded, cells were pre-labelled with ^{3}H-TdR and then infected with 4.3×10^5 virus particles per cell or mock-infected. Their DNA was then analyzed by centrifugation in neutral sucrose gradients (Figure 6). Most of the DNA from uninfected cells is in fractions 20-23. The average S-value of this DNA from similar gradients was determined as 140 (McEwen, 1967) and its molecular weight was calculated to be 8×10^8 daltons (Eigner and Doty, 1965). The majority of DNA from the infected cells is in fractions 4-11. The average S-value of these fragments was estimated as 40 and their molecular weight as 3×10^7 daltons. The estimated size of both intact and fragmented double-stranded DNA is more than that of single-stranded DNA. This is probably due to differences in the methods of calculation of molecular weight.

2. Dependency of percentage of DNA fragmented on virus dose and time after infection: To study the kinetics of cell DNA fragmentation after infection, cells were pre-labelled with ^{3}H-TdR and then infected with the doses of virus ranging from 1.8×10^4 to 4.3×10^5 particles per cell for

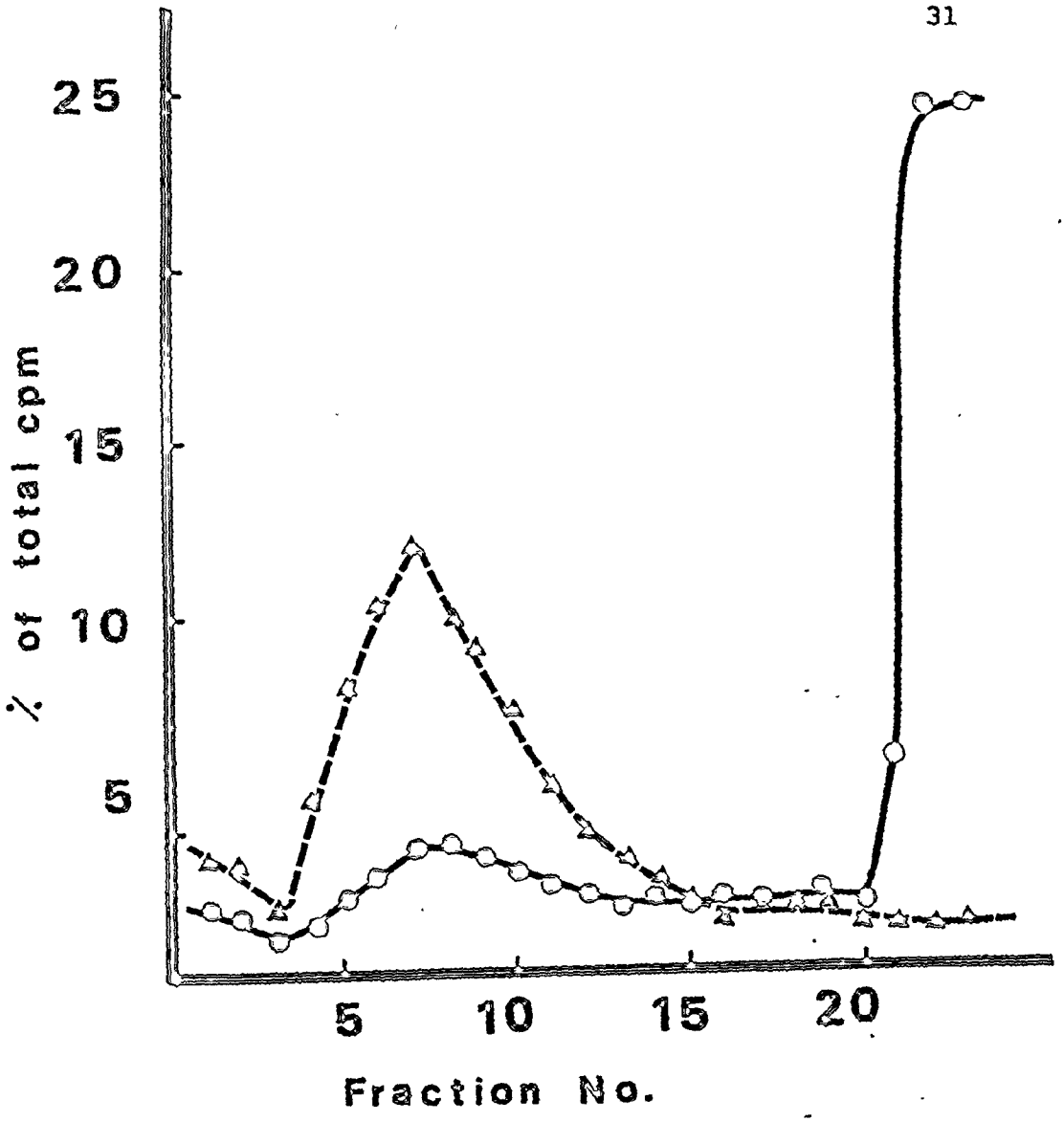


Figure 6: Double-strand breakage of human KB cell DNA after infection by Ad 12.

Cells were prelabelled with ^{3}H-TdR and infected with 4.3 x 10^{5} virus particles per cell or mock-infected. Their DNA was then analyzed by centrifugation in neutral sucrose gradients. o———o, uninfected; Δ- - -Δ, infected.

2 to 10 hr. Their DNA was then analyzed by centrifugation in alkaline sucrose gradients. The percentage of DNA fragmented by each dose of virus was determined by adding the amount of radioisotope in the peak containing the small molecular weight DNA and then dividing this by the amount of isotope in the total gradient.

Complete fragmentation of cell DNA occurs when the cells are infected with 4.3×10^5 virus particles per cell. Fragmentation is minimal at doses lower than 1.8×10^4 virus particles per cell (Figure 7A).

The percentage of breakage of cell DNA is dependent on time after infection (Figure 7B, a replot of Figure 7A). Fragmentation is observed when first examined after 2 hr post infection and increases further with time.

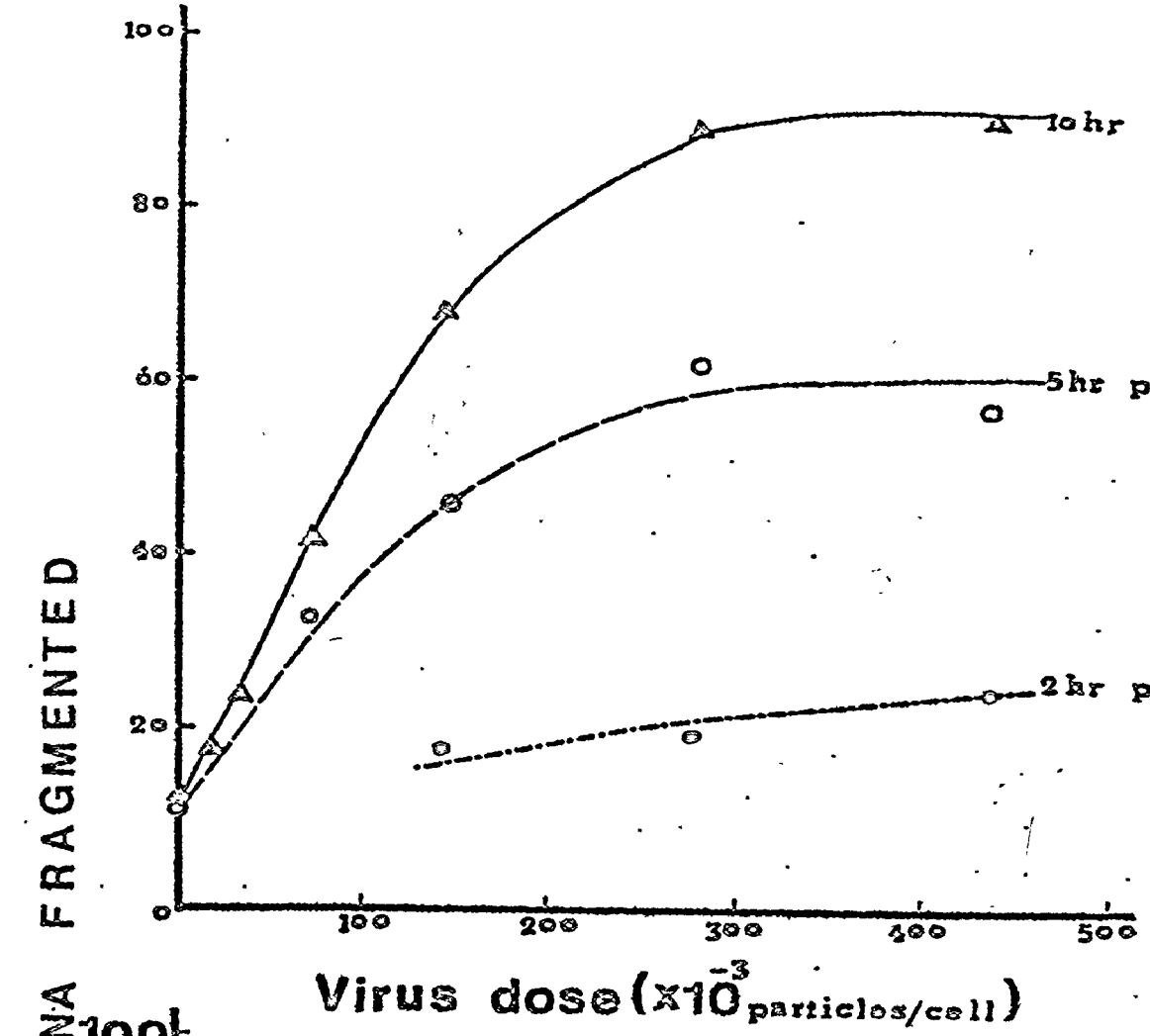
3. Fragmentation of cell DNA with low doses of virus: As indicated previously, fragmentation of cell DNA was minimal at doses of virus lower than 1.8×10^4 particles per cell by 10 hr post infection. To study the effect of infection by low doses late after infection, cells were pre-labelled with ^{3}H-TdR and then infected with 7×10^3 virus particles per cell. Their DNA was analyzed by alkaline sucrose gradient centrifugation at 10,31 and 46 hr post infection. The results show that the fragmentation of cellular DNA is considerably higher late after infection (Table 1). The significance of these results is, however,

Figure 7A: Dependency of the percentage of KB cell DNA fragmentation on virus dose.

Cells were pre-labelled with ^{3}H-TdR and infected with the indicated doses of virus. Their DNA was analyzed by centrifugation in alkaline sucrose gradients. Percentage of DNA fragmentation by each dose was determined and plotted against virus dose.

Figure 7B: Dependency of the percentage of cell DNA fragmentation on time after infection.

Replot of Figure 7A.


100
80
60
40
20
0
% OF DNA FRAGMENTED
10hr pi
5hr pi
2hr pi
100
200
300
400
500
Virus dose (x10⁻³ particles/cell)

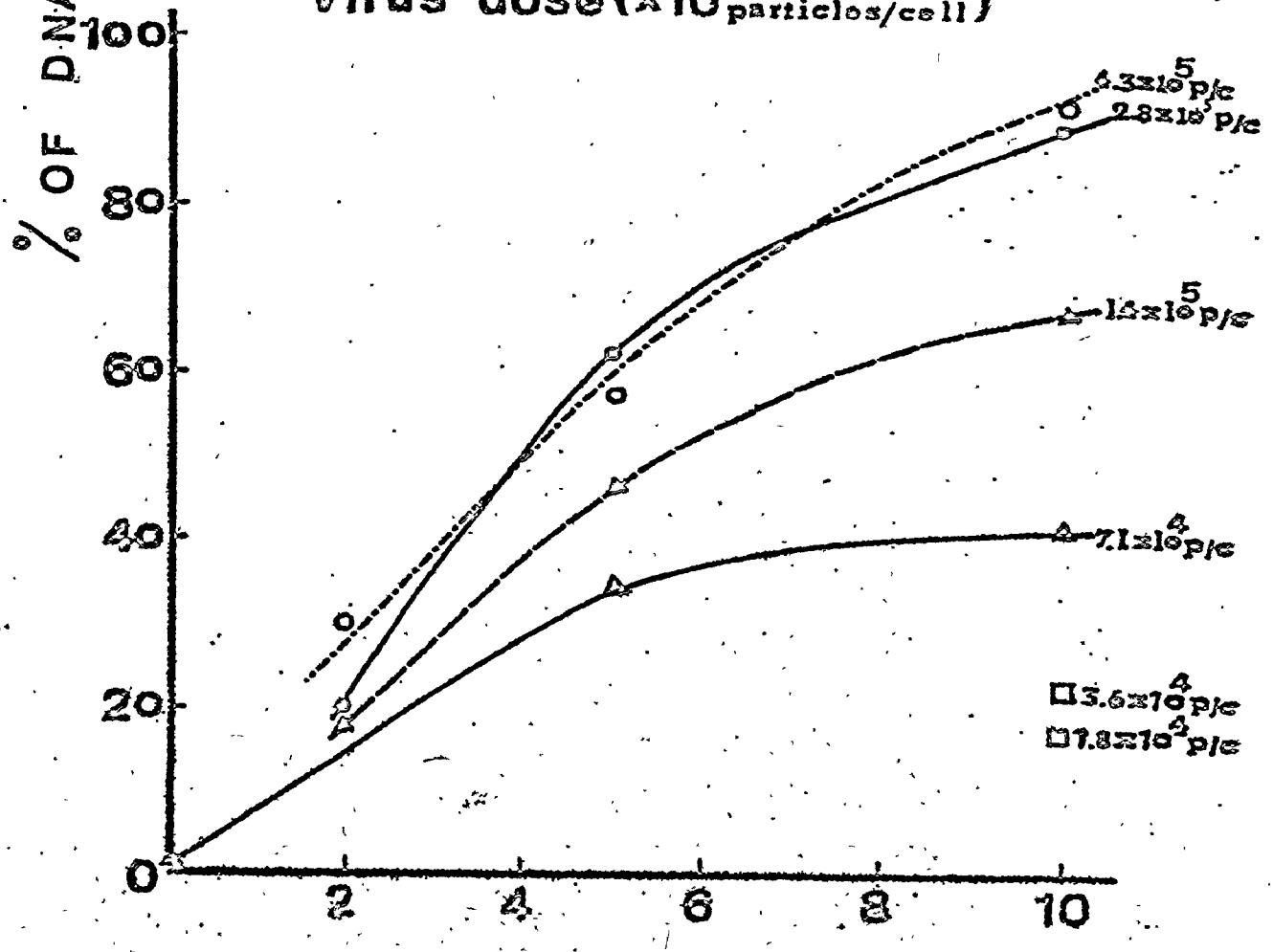

100
80
60
40
20
0
% OF DNA
4.3x10⁵ p/c
2.8x10⁵ p/c
1.4x10⁵ p/c
7.1x10⁴ p/c
3.6x10⁴ p/c
1.8x10⁴ p/c
2
4
6
8
10

Table 1

Fragmentation of Cellular DNA with Low Doses of Virus Late After Infection

Time (hr) p.i.	% DNA fragmented	
	mock-infected	infected
10	3	6.5
31	13.2	25.4
46	25.3	41.3

Cells were pre-labelled with ^{3}H-TdR and infected with 7×10^{3} virus particles per cell or mock-infected. Their DNA was analyzed by centrifugation in alkaline sucrose gradients. Percentage of cell DNA fragmented was determined at the indicated times.

obscured by the observation that considerable fragmentation of cellular DNA (due to radiolytic effects of isotopes over long durations) occurs also in the mock-infected cells.

4. Effect of infection by UV-irradiated virus on the fragmentation of cell DNA: To determine whether virus infectivity was required for the observed fragmentation of cellular DNA Ad 12 was UV-irradiated with 2.5×10^4 ergs/mm^2. This dose reduces virus survival to 10^{-5} without affecting its efficiency of adsorption (Rainbow and Mak, 1973). Cells pre-labelled with ^{3}H-TdR were infected with either UV-irradiated or non-treated virus for 10 hr. Their DNA was then analyzed by sedimentation in alkaline sucrose gradients. Infection by UV-irradiated virus induces the fragmentation of cell DNA to the same extent as infection by untreated virus (Table 2).

It should be mentioned that the dose of UV used in these experiments reduces the efficiency of the virus to inhibit colony formation down to 1% (Rainbow and Mak, 1973). The data (Table 2) seems to suggest that virus particles not capable of cell killing are still able to induce cell DNA fragmentation. (See Discussion.)

B. Effect of infection on pre-existing hamster embryo cell DNA

1. Fragmentation of cell DNA after infection: Infection of hamster cells by Ad 12 is non-productive (Doerfler, 1969).

Table 2

Effect of Infection by UV-irradiated Virus (1.35×10^5 particles/cell) on the Fragmentation of Cellular DNA

Type of virus	CPM in total gradient	CPM in small molecular weight DNA	% DNA fragmented
UV-irradiated	38511	26842	69.7
non-treated	44502	30973	69.6

Cells were pre-labelled with ^{3}H-TdR and infected with either UV-irradiated or non-treated virus. Their DNA was analyzed by alkaline sucrose centrifugation. Percentage of cell DNA fragmentation was determined from each gradient.

Neither viral DNA nor viral structural proteins are synthesized after the infection of these cells by Ad 12. It was, therefore, of interest to determine whether fragmentation of cell DNA was induced in these cells after infection by this virus.

Hamster embryo cells were pre-labelled with ^{3}H-TdR and then infected with 3 x 10^5 virus particles per cell. Their DNA was analyzed by alkaline sucrose gradient centrifugation at 20 hr post infection. The results (Figure 8) show that infection by Ad 12 induced the fragmentation of hamster embryo cell DNA. The size of the fragments were shown to be the same as those in KB cells.

2. Dependency of percentage of DNA fragmented on viral dose and time after infection: As stated in section A.2., the percentage of DNA fragmented after the infection of human KB cells was a function of viral dose. A similar study was made using hamster embryo cultures. Hamster cells were pre-labelled with ^{3}H-TdR and infected with doses of virus ranging from 1.8 x 10^4 to 3 x 10^5 particles per cell for 20 hr. Their DNA was then analyzed by centrifugation in alkaline sucrose gradients. As shown by Figure 9, the percentage of DNA fragmented after infection remains the same with increasing doses of virus ranging from 1.8 x 10^4 to 9 x 10^4 particles per cell. With doses higher than 9 x 10^4 the increase is linear.

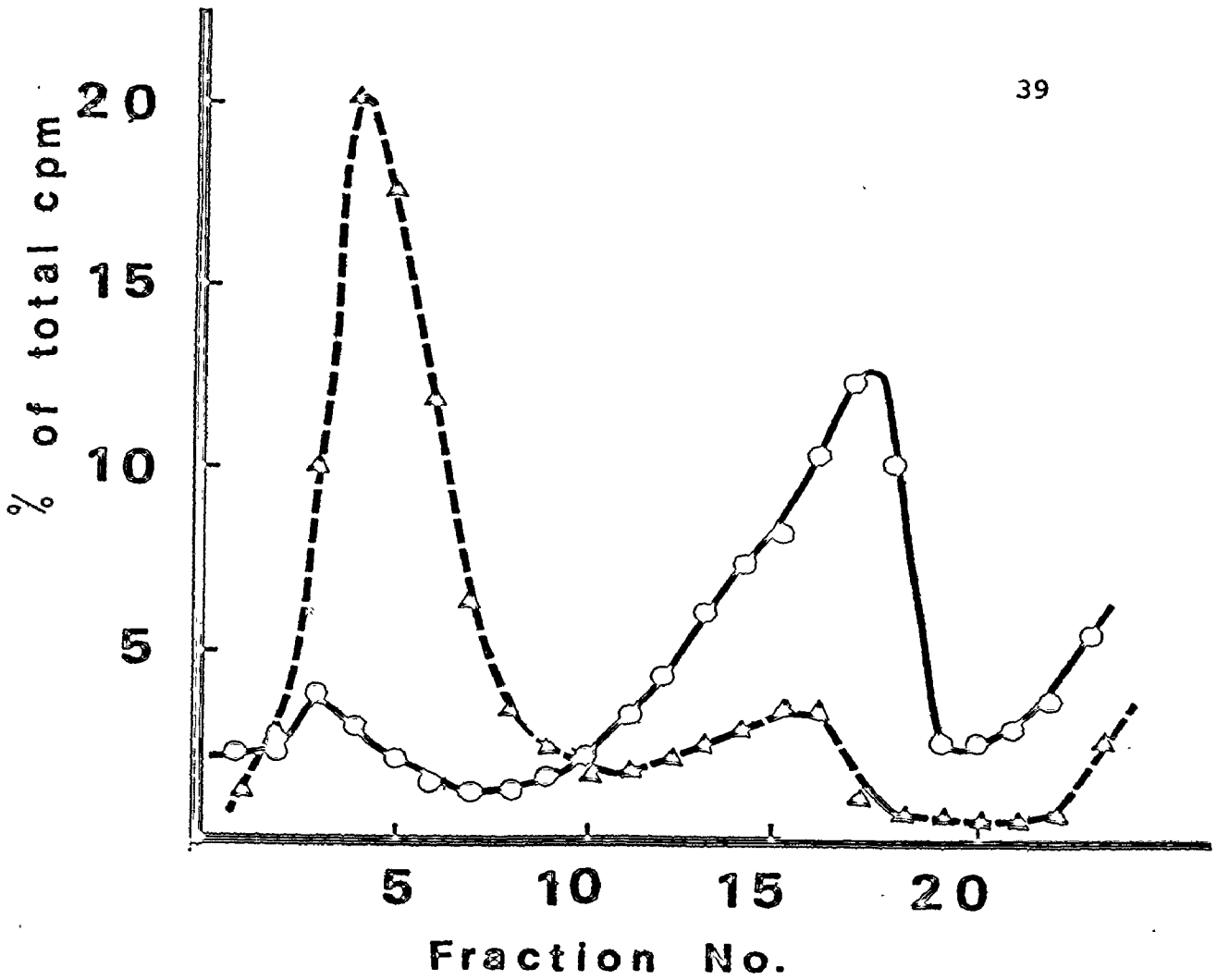


Figure 8: Fragmentation of hamster embryo cell DNA after infection by Ad 12.

Cells were pre-labelled with ^{3}H-TdR and infected with 3×10^{5} virus particles per cell. Their DNA was analyzed by centrifugation in alkaline sucrose gradients. ▲-----▲, infected; o———o, uninfected.

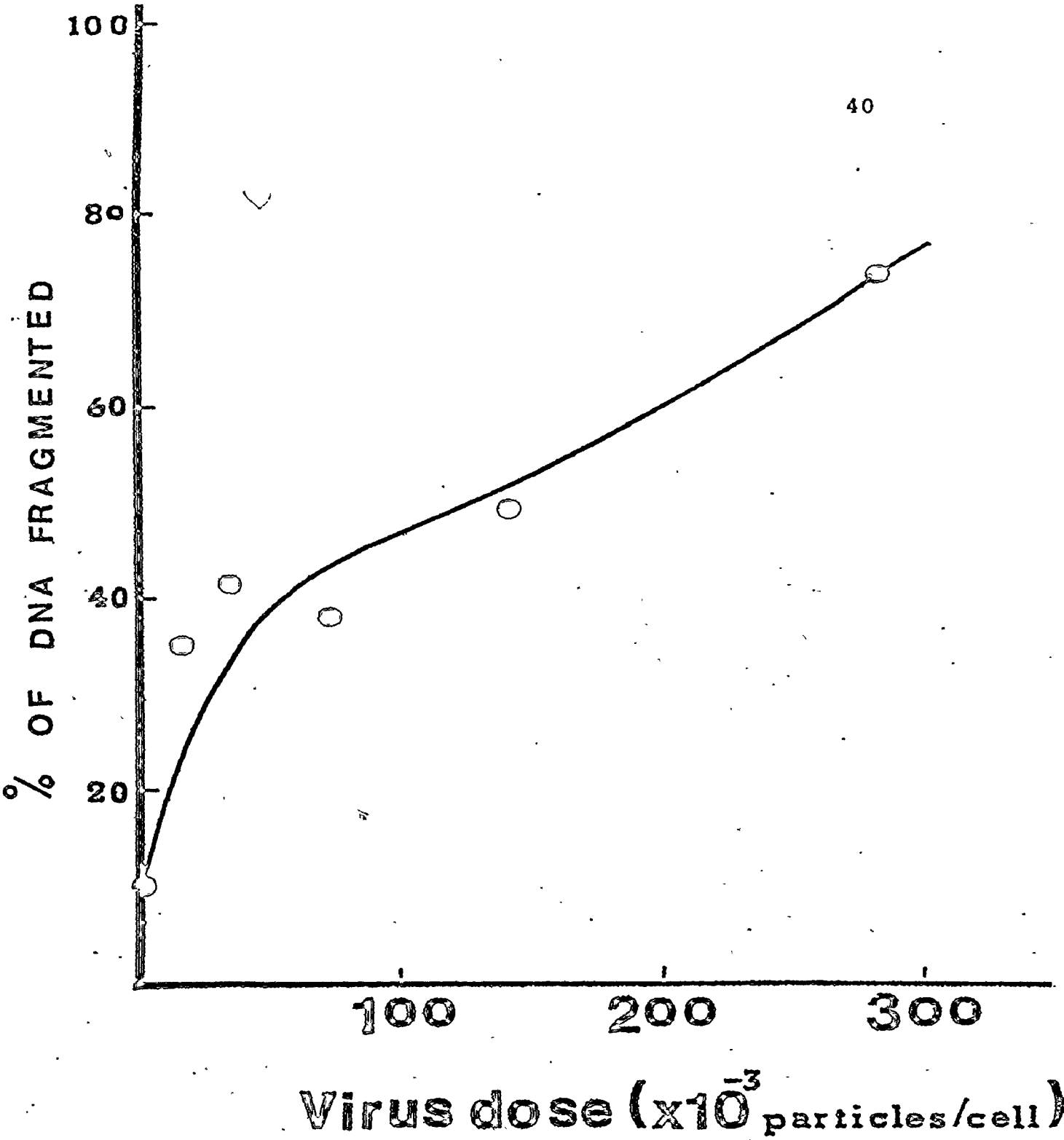


Figure 9: Dependency of the percentage of cell DNA fragmentation on virus dose.

Hamster embryo cells were pre-labelled with ^{3}H-TdR and infected with the indicated doses of virus. Their DNA was analyzed in alkaline sucrose gradients. Percentage of DNA fragmentation by each dose was determined.

The hamster embryo fibroblasts were derived from a variety of sources such as connective tissue, cartilage, muscle, linings of blood vessels, and nerve sheaths. The differential response of the cells, as noted above, to virus infection with respect to cell DNA fragmentation could be due to the differential sensitivity of cells with different origins. The results, reported in the following, support this possibility.

It was observed during the experiments that after infection with up to 9×10^4 virus particles per cell about half of the cells in the cultures were detached from the tissue culture flasks while the other half remained attached at 20 hr post infection. To determine whether there was differential cell DNA fragmentation in the two populations cells were pre-labelled with ^{3}H-TdR and then infected with 3.6×10^4 virus particles per cell for 20 hr. The detached cells were collected separately and the cells attached to the culture flasks were trypsinized. The DNA of each sample was analyzed by alkaline sucrose gradient centrifugation. As shown by Figure 10, 72% of DNA from detached cells sediments as small molecular weight DNA while only 25% of DNA from attached cells has small molecular weight.

It is unlikely that the infected cells detached from the tissue culture flasks are "dead" as they incorporate ^{3}H-uridine into RNA (Table 3). However, it is not clear from these results whether the low level of label incorporation

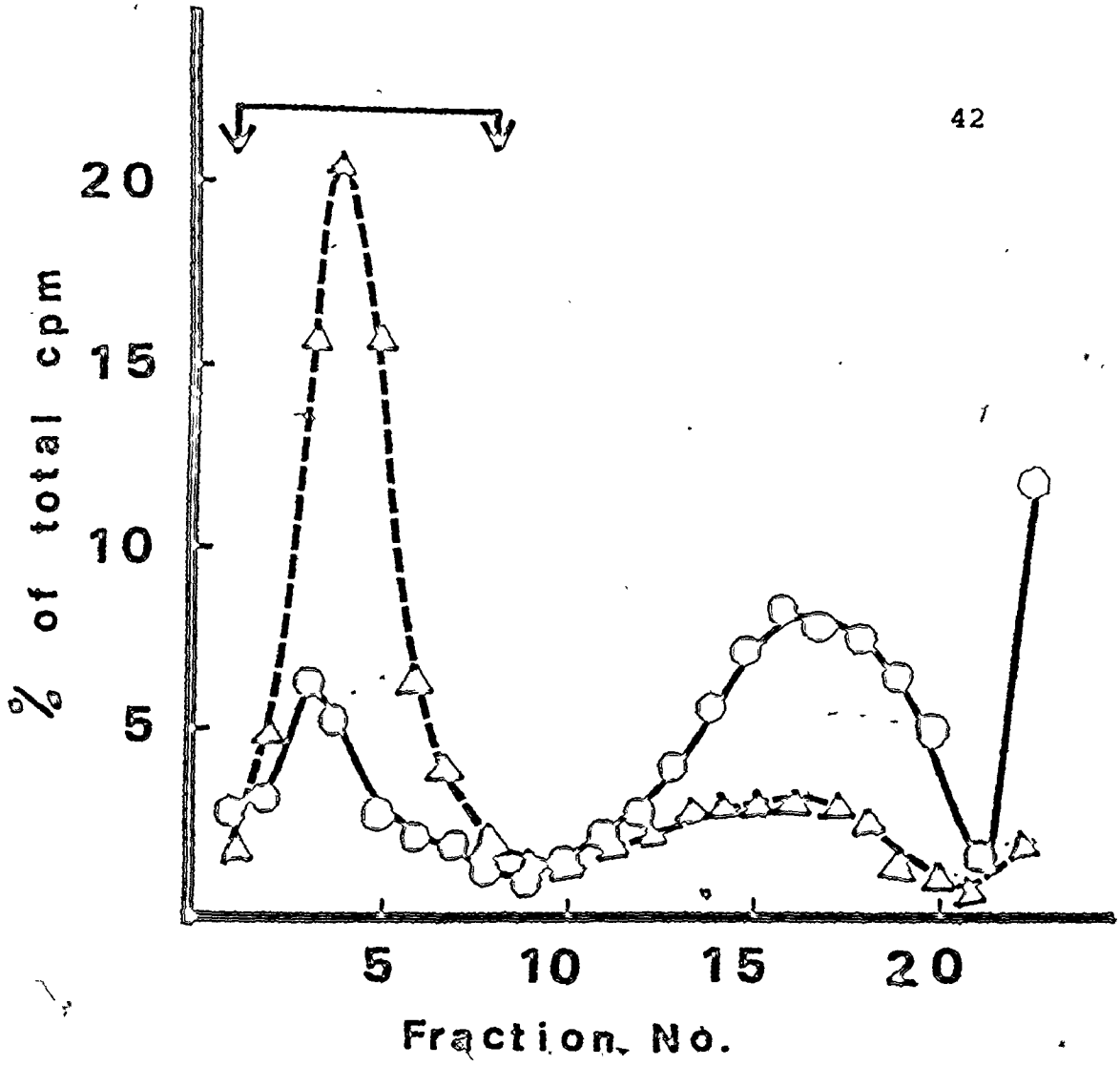


Figure 10: Fragmentation of cellular DNA in attached and detached infected hamster embryo cells.

Cells were pre-labelled with ^{3}H-TdR and infected with 3.6×10^{4} virus particles per cell. At 20 hr post infection, the detached cells were separated from the attached cells and their DNA was analyzed by alkaline sucrose gradient. A------A, detached cells; o———o, attached cells.

Table 3

Incorporation of ^{3}H-uridine into the RNA of Infected Detached and Attached Hamster Embryo Cells

Sample	CPM incorporated per 10^6 cells	% of control
Control cells	96345	-
Infected attached cells	60515	63
Infected detached cells	6839	7.1
Detached control	659	0.7

Hamster embryo cells were infected with 3.6×10^4 virus particles per cell or mock-infected. At 20 hr post infection, some cells became detached from the tissue culture flasks. Detached and attached cells were separated and pulse-labelled with ^{3}H-uridine for one hr. The amount of label incorporated into the RNA of each sample was measured by cold TCA precipitation.

is due to low level of RNA synthesis by all the cells or whether a small percentage of the cells are synthesizing RNA. Using autoradiography, Mak (personal communication) observed that there is incorporation of uridine into the RNA of all the floating cells.

To determine the relationship between increasing time after infection and percentage of DNA fragmented, cells were pre-labelled and infected with doses of virus ranging from 1.8×10^4 to 7.1×10^4 virus particles. Their DNA was analyzed at 10,20 and 26 hr post infection. Only 12% of cell DNA is fragmented by 10 hr with all the doses (Table 4). Extensive cell DNA fragmentation is observed after 20 hr of infection. Further breakage occurs with increasing time.

II. Effect of Infection on Host DNA Synthesis in Human KB Cells

A. Effect of infection on DNA chain growth

Infection of human KB cells by Ad 12 had been known to result in the early inhibition of cell DNA synthesis (Pina and Green, 1969). No studies had been made of the effect of infection by this virus on such aspects of the inhibition as changes in the rate of DNA chain elongation. Such studies have been difficult due to lack of sensitive techniques for examining unsheared large molecular weight DNA. In the technique of alkaline sucrose gradient, in which cells are directly lysed on top of the gradient, large

Table 4

Increasing Fragmentation of Hamster Embryo Cell DNA with Increasing Time After Infection

Virus dose (particles/cell)	% DNA fragmented		
	10 hr p i	20 hr p i	26 hr p i
1.8×10^4	11.3	35.3	41.1
3.2×10^4	12.6	41.2	48
7.1×10^4	12.7	38.4	53.7

Hamster embryo cells were pre-labelled with ^{3}H-TdR and infected with the indicated doses of virus for the indicated times. Their DNA was analyzed by alkaline sucrose gradient and the percentage of DNA fragmented was determined.

molecular weight DNA can be analyzed with minimal shearing (Palcic, 1972). This technique together with double-labelling techniques were used to study the effect of infection on DNA chain growth.

Cells were pre-labelled with ^{14}C-TdR and infected with 3 x 10^3 virus particles per cell or mock-infected for 8 hr. They were then pulse-labelled with ^{3}H-TdR for 15 min and either "chased" for 2.5, 4 and 6 hr or used immediately. Their DNA was analyzed in alkaline sucrose gradients.

The majority of label in a 15 min pulse is incorporated into lower molecular weight DNA in both infected and uninfected cells (Figure 11). This is consistent with the Okazaki model of DNA replication (Okazaki *et al.*, 1968) which is also observed in other cell types (Goulian, 1971).

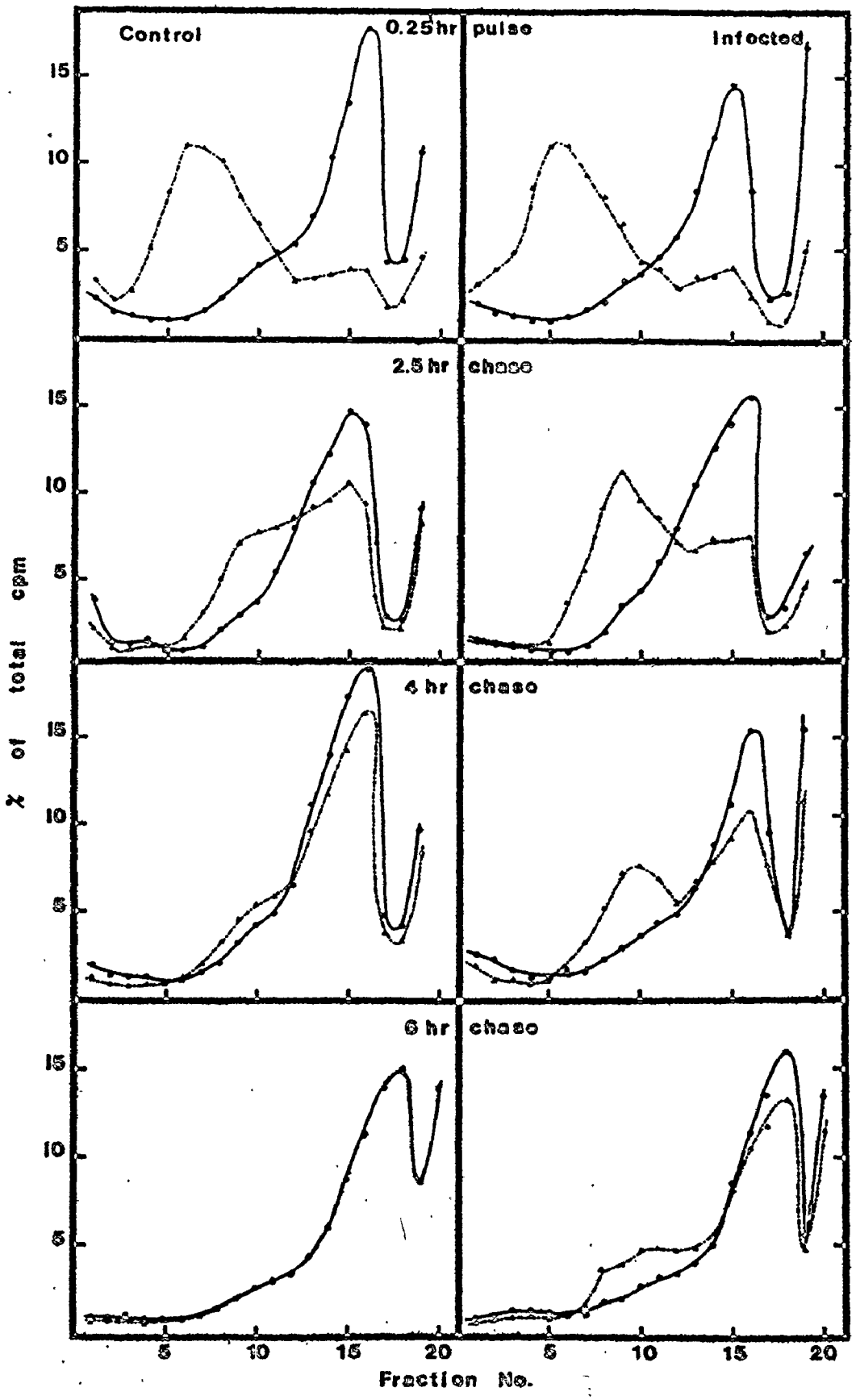
The low molecular weight fragments are converted to template size molecules. The process is complete by 6 hr in the uninfected cells (Figure 12). This is shown by the cosedimentation of ^{3}H-labelled DNA with pre-labelled ^{14}C-DNA (Figure 11). Chain elongation* is not complete in the infected cells even after 9 hr of chasing (Figure 12).

To study the effect of infection with higher doses of virus on DNA chain growth, cells were pre-labelled with ^{14}C-TdR and infected with 1.8 x 10^4 virus particles per cell and then pulsed with ^{3}H-TdR for 15 min at 8 hr post infection. Their DNA was then analyzed in alkaline sucrose gradients either immediately or after 6 hr of chasing.

* ligation of adjacent replicons has not been distinguished from the ligation of Okazaki fragments in this thesis.

Figure 11: DNA chain elongation in infected or mock-infected human KB cells.

Human KB cells were pre-labelled with ^{14}C-TdR and infected with 3×10^3 virus particles per cell or mock-infected. They were pulse-labelled with ^{3}H-TdR for 15 min at 8 hr post infection and chased for the indicated times. Their DNA was then analyzed in alkaline sucrose gradients. ▲_._._._▲, ^{3}H; o—o—o, ^{14}C-DNA.


Control
0.25hr pulse
Infected
2.5 hr chase
4 hr chase
6 hr chase
% of total cpm
Fraction No.
5
10
15
20

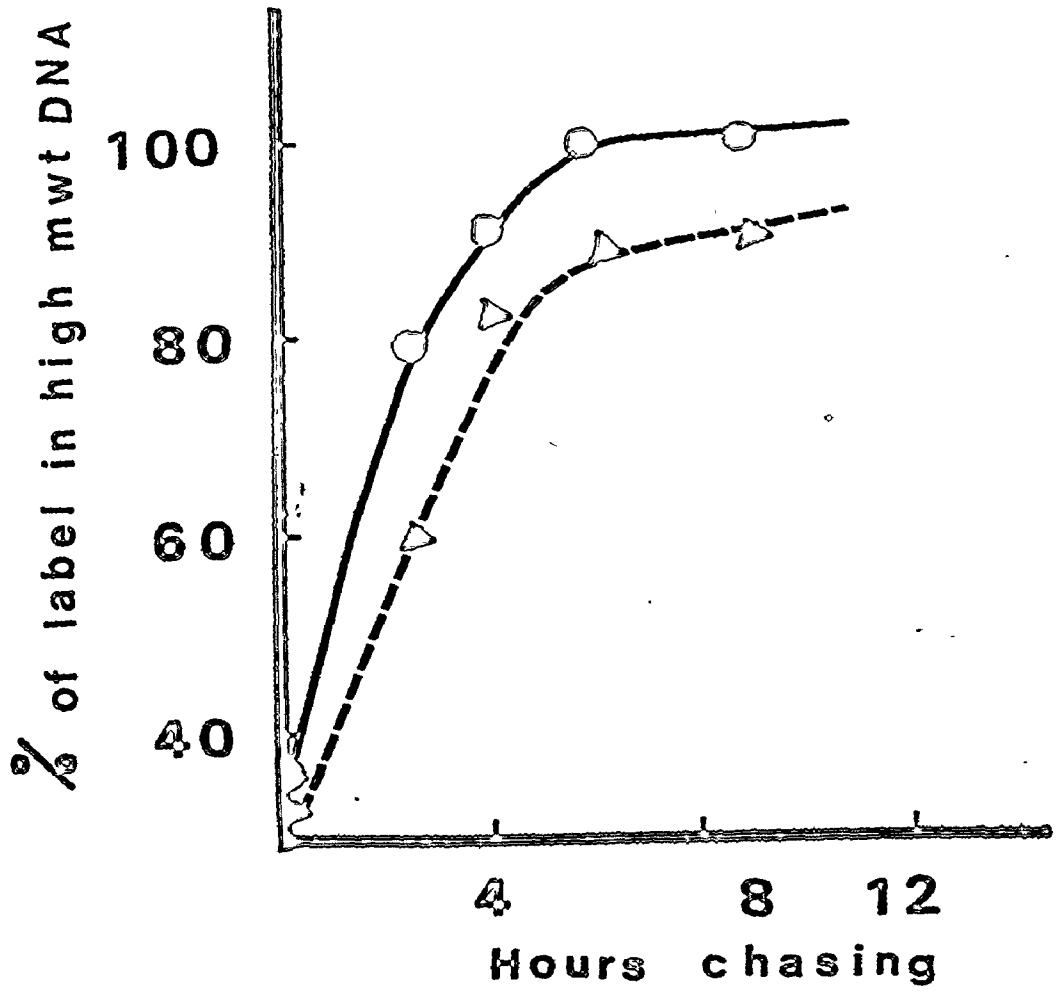


Figure 12: Conversion of pulse-labelled DNA into high molecular weight DNA from infected and uninfected KB cells as a fraction of time.

Percentage of pulse-labelled DNA converted into high molecular weight DNA was determined by measuring the percentage of pulse-labelled DNA under the ^{14}C-labelled high molecular weight DNA of Figure 11. o———o, control; A- - - - -A, infected.

Figure 13B shows that chain elongation is not complete by 6 hr of chasing. Note also that the size of DNA synthesized in a 15 min pulse is more heterogeneous and slightly larger than the size of DNA synthesized in both uninfected cells and cells infected with low doses of virus (compare Figure 13A to top panel of Figure 11). Also a higher percentage of ^{3}H-TdR is incorporated into DNA of high molecular weight.

B. The nature of newly synthesized DNA in the infected cells

1. Buoyant density of the newly synthesized DNA: a) Synthesis of DNA with high buoyant density in the infected cells: In a logarithmically growing culture of cells the buoyant density of DNA synthesized in a pulse is the same as that of pre-existing cell DNA (Figure 14A). Cells were pre-labelled with ^{14}C-TdR infected with 3 x 10^{3} virus particles/cell and pulse-labelled with ^{3}H-TdR for one hr at 8 hr post infection. Their DNA was then analyzed by neutral CsC. As shown in Figure 14B, the density of the newly synthesized ^{3}H-DNA is higher than the density of pre-existing ^{14}C-DNA in the infected cells. b) The high buoyant density of newly synthesized DNA is not due to its single-strandedness: The secondary structure of DNA synthesized in a short pulse is "destabilized". The DNA can be isolated as single or double-stranded according to extraction procedures used (Habener *et al.*, 1970). It was possible that the structure of DNA synthesized in a one hr pulse in the infected KB cells is destabilized,

Figure 13: Effect of infection with high doses of virus on cell DNA chain elongation.

Cells were pre-labelled with ^{14}C-TdR and infected with 1.8×10^4 virus particles per cell. They were pulse-labelled with ^{3}H-TdR for 15 min and/or chased for 6 hr at 8 hr post infection. Their DNA was then analyzed in alkaline sucrose gradients. Δ-----Δ, ^{3}H-DNA; o———o, ^{14}C-DNA. A, 15 min pulse; B, 4 hr chase.

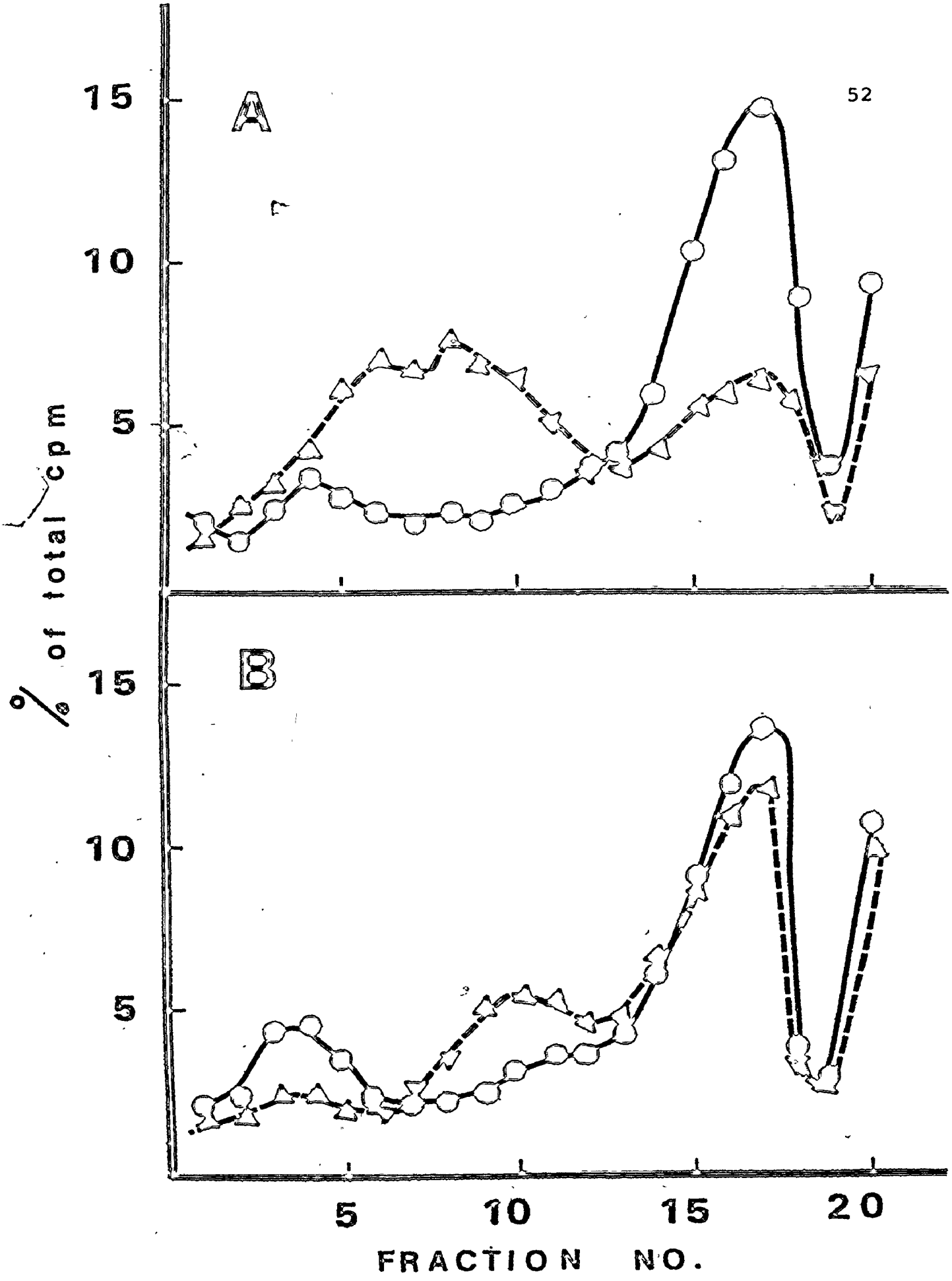
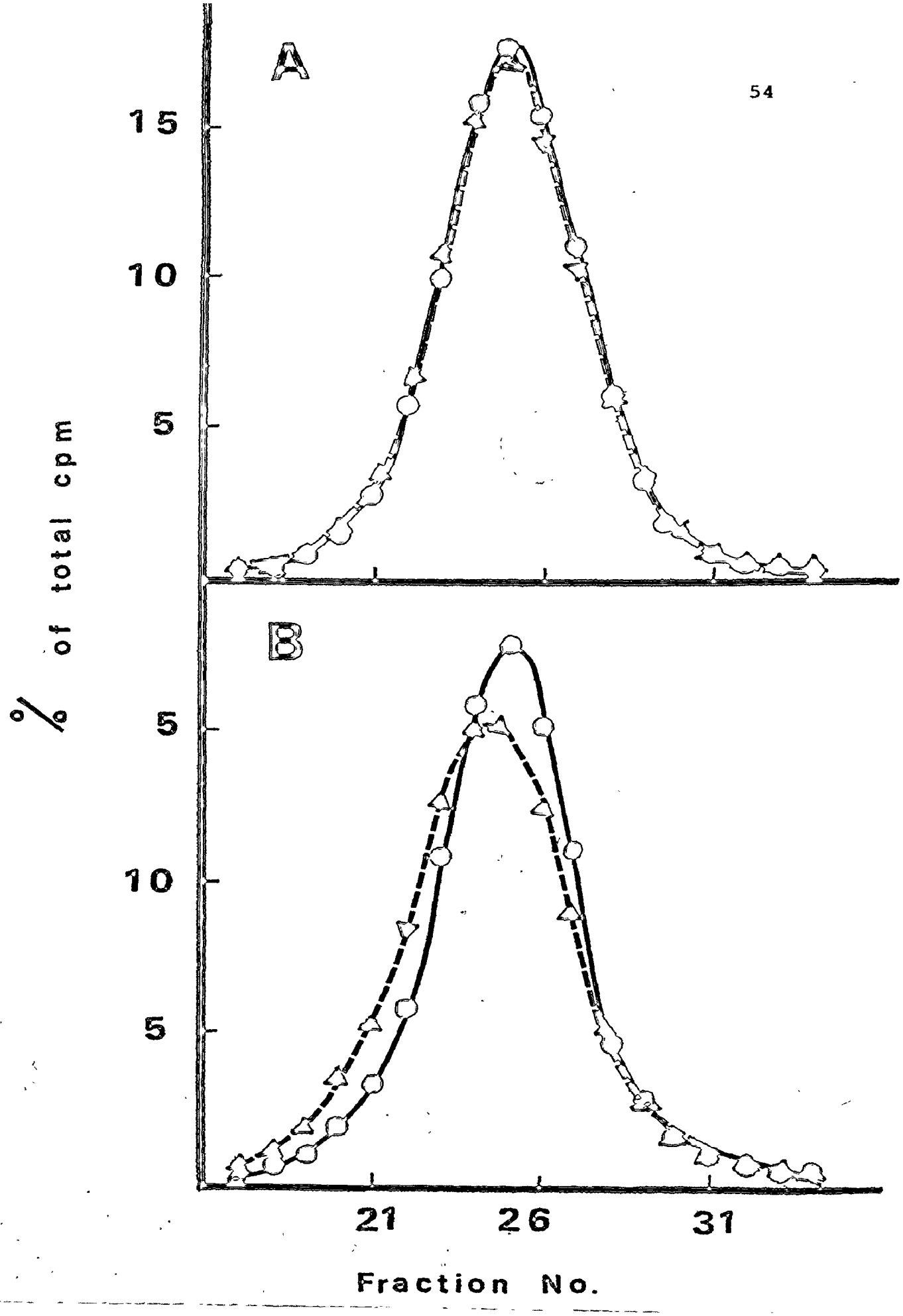

A
B
15
10
5
15
10
5
% of total cpm
5
10
15
20
FRACTION NO.

Figure 14: Synthesis of high buoyant density DNA in the infected human KB cells.

Cells were pre-labelled with ^{14}C-TdR and infected or mock-infected. They were pulse-labelled with ^{3}H-TdR for one hr at 8 hr after infection. Their DNA was then analyzed by centrifugation in neutral CsCl gradients. o———o, ^{14}C-DNA; ▲_____▲, ^{3}H-DNA. A, uninfected; B, infected. (Direction of sedimentation of all CsCl gradients is from right to left.)


A
B
15
10
5
10
5
% of total cpm
21
26
31
Fraction No.

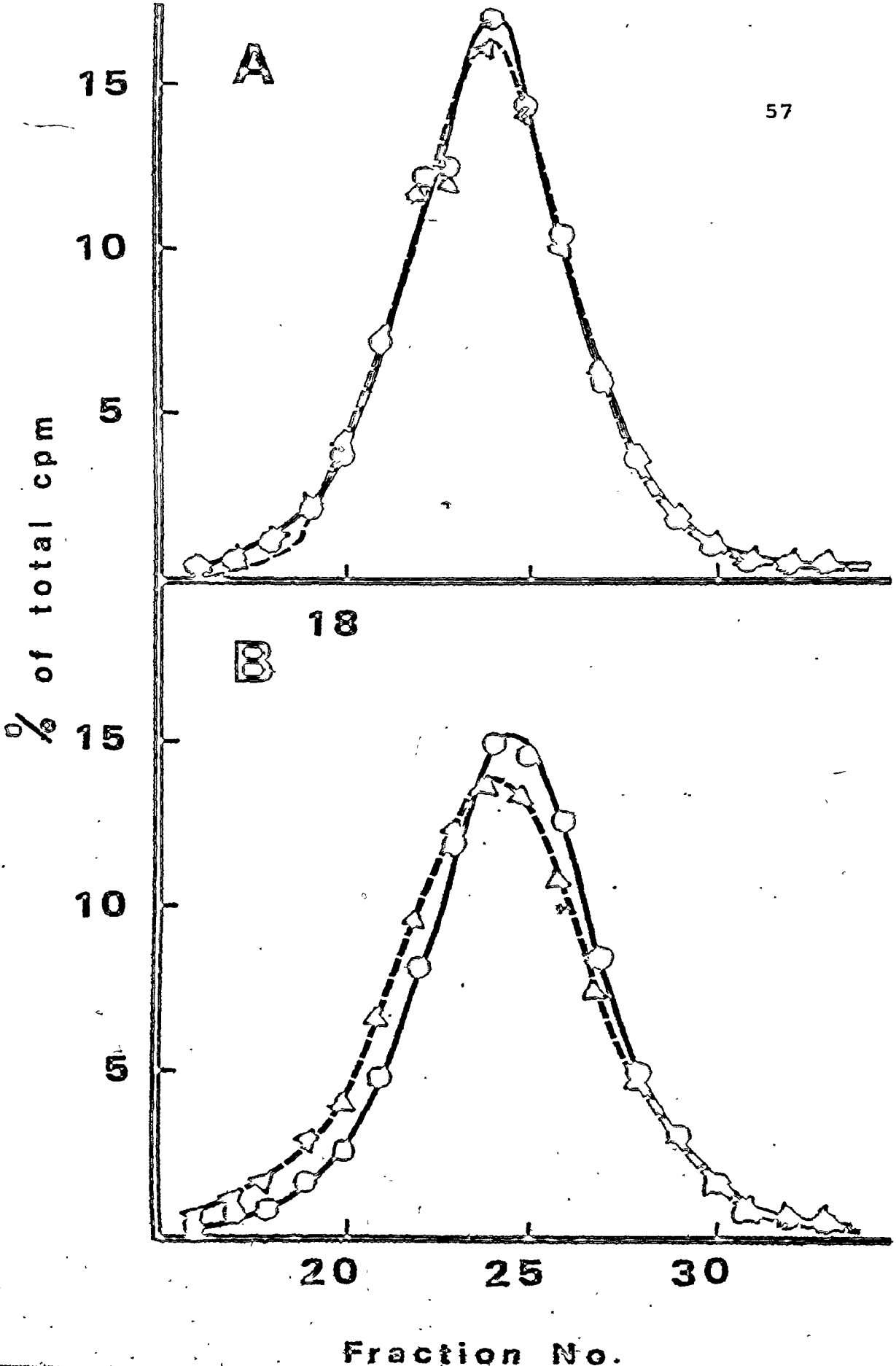
behaving as single-stranded DNA, and has,therefore, a higher buoyant density than the bulk DNA. The following experiments were done to examine this possibility.

Cells were pre-labelled with ^{14}C-TdR and infected or mock-infected. They were pulse-labelled with ^{3}H-TdR for one hr at 8 hr after infection and then chased for 6 hr. Their DNA was analyzed in CsCl. The density of the newly synthesized DNA in the control cells is the same as the density of the bulk DNA (Figure 15A). The density of the newly synthesized DNA in the infected cells is higher than the density of the bulk DNA even after 6 hr of chasing (Figure 15B). According to Painter *et al.* (1968) single-stranded DNA synthesized in a short-pulse (2 min or less) is converted to double-stranded DNA after one hr of chasing. It is, therefore, unlikely that the newly synthesized DNA from the infected cells remains single-stranded even after 6 hr of chasing.

To further rule out the possibility that the newly synthesized DNA in the infected cells was single-stranded, the cultures were infected or mock-infected as before. After 8 hr of infection, the infected cells were pulse-labelled with ^{3}H-TdR and the control cells were pulse-labelled with ^{14}C-TdR for one hr. The two were combined and treated with pronase and sheared as described before. Part of the sample was analyzed on CsCl gradients and the rest was analyzed on neutral sucrose gradients. As before, the newly synthesized DNA from the infected cells has a higher buoyant density

Figure 15: The buoyant density of newly synthesized DNA after 6 hr of chasing.

Cells were pre-labelled and infected or mock-infected as before. They were pulse-labelled with ^{3}H-TdR for one hr and chased for 6 hr. Their DNA was then analyzed in neutral CsCl gradients. ▲_____▲, ^{3}H-DNA; o——o, ^{14}C-DNA. A, control; B, infected.


A
B
15
10
5
15
10
5
% of total cpm
18
20
25
30
Fraction No.

than that of control cells (Figure 16). Figure 17 shows that the newly synthesized ^{3}H-DNA from infected cells cosediments with ^{14}C-DNA from control cells in neutral sucrose gradients and is, therefore, double-stranded and of the same size.

Further support for the double-stranded nature of newly synthesized DNA from the infected cells is the observation that more than 93% of the DNA from these cells is resistant to digestion by S_1-nuclease (Table 5).

In the Okazaki model of DNA replication, DNA synthesized in a short pulse is in the form of small fragments. The fragments are separated by small single-stranded gaps. These gaps cannot be detected by the techniques of isopicnic centrifugation in CsCl gradients (Probst *et al.*, 1974). It was, however, possible that in the infected KB cells inhibition of cell DNA synthesis resulted in creation of large single-stranded gaps and that the observed shift in density was due to the presence of large-single-stranded tails. To examine this possibility, cells were pre-labelled with ^{14}C-TdR and pulse-labelled with ^{3}H-TdR for one hr at 8 hr post infection. Their DNA was isolated by pronase-treatment followed by phenol-extraction as described before. The sample was divided into halves. One half was treated with S_1-nuclease for the digestion of single-stranded regions, and the other half was untreated. Each sample was analyzed separately by isopicnic centrifugation in neutral

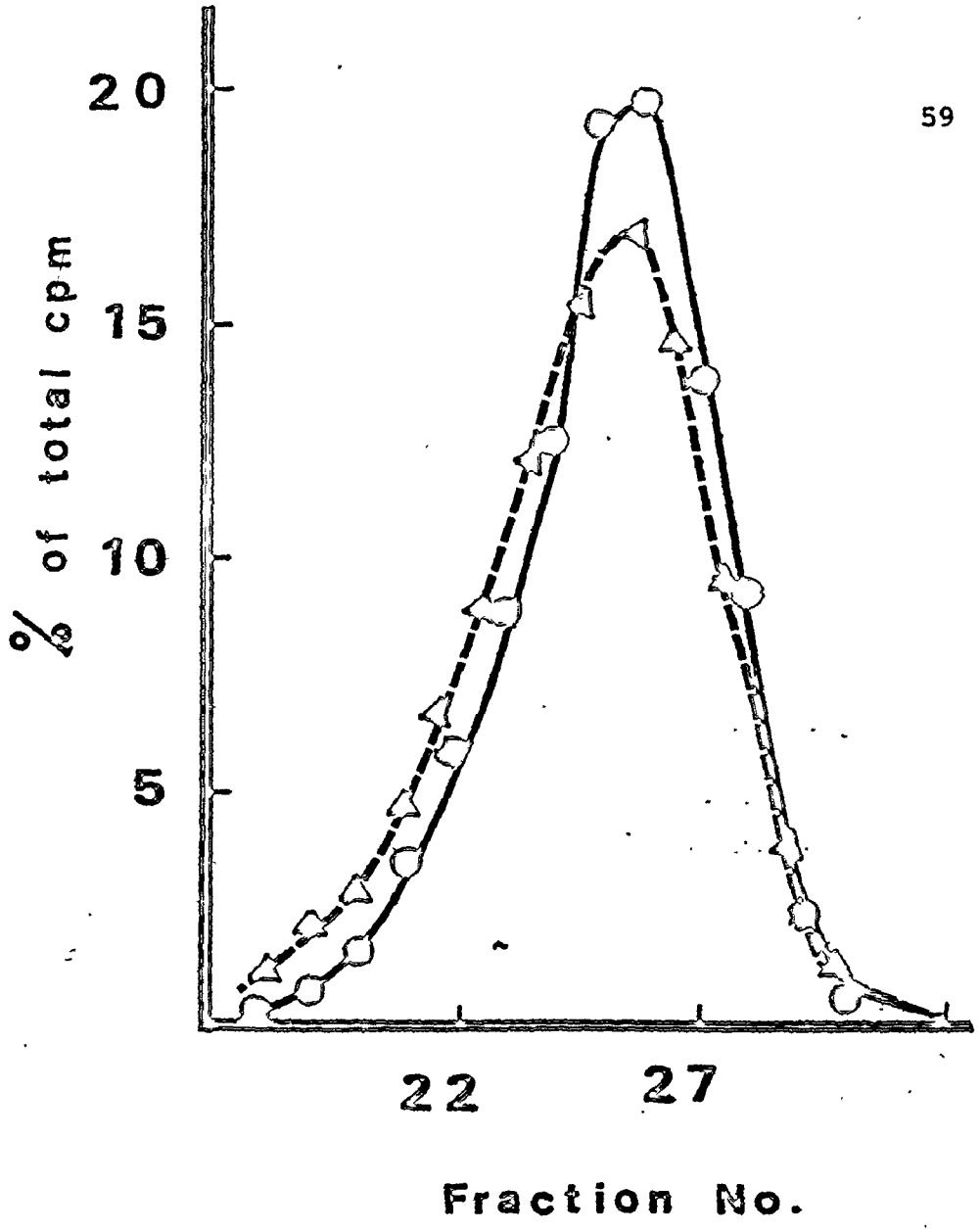


Figure 16: Simultaneous centrifugation of newly synthesized ^{3}H-DNA from infected cells with ^{14}C-DNA from control cells in neutral CsCl gradients.

▲——▲, ^{3}H-DNA; o——o, ^{14}C-DNA.

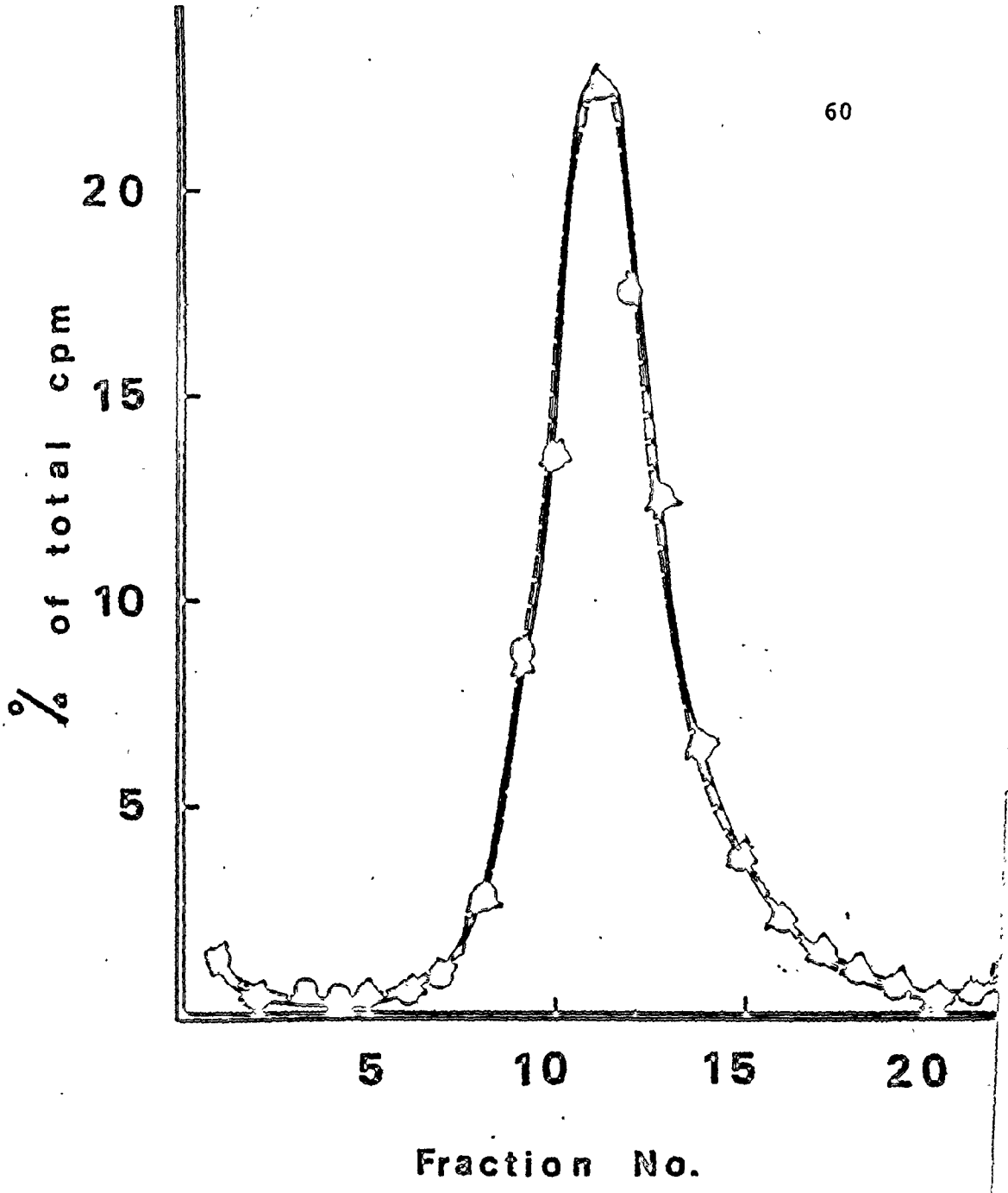


Figure 17: Cosedimentation of ^{3}H-DNA from infected cells with ^{14}C-DNA from control cells in neutral sucrose gradients.

▲——▲, ^{3}H-DNA; o----o, ^{14}C-DNA.

Table 5

Digestion of the Newly Synthesized DNA from the Infected Cells by S_1-nuclease

DNA sample	Input cpm	CPM resistant to S_1	% resistant to S_1
Native	4637	4325	93.3
Heat-denatured	4637	486	10.5

Cells were infected and pulse-labelled with ^{3}H-TdR at 8 hr after infection. Their DNA was isolated by pronase-treatment followed by phenol extraction and ethanol precipitation as described. Digestion with S_1-nuclease was at 37^{o}C, pH 4.5, for 30 min.

CsCl gradients. The buoyant density of newly synthesized DNA is higher than the density of pre-existing DNA in both nuclease-treated and untreated samples (Figure 18). c) The high buoyant density of newly synthesized cell DNA is not due to contamination with viral DNA: The buoyant density of Ad 12 DNA is slightly higher than that of host cell DNA (cellular DNA ρ = 1.701, Ad 12 DNA ρ = 1.709, Pina and Green, 1969). To examine whether the observed shift in density was due to contamination with newly synthesized viral DNA, cells were pre-labelled with ^{14}C-TdR and infected or mock-infected and pulse-labelled with ^{3}H-TdR at 9 hr post infection. Their DNA was analyzed in neutral CsCl gradients. The gradients were divided into heavy and light fractions as shown by Figure 19. Each fraction was hybridized to unlabelled viral DNA immobilized on filters. About 1% of the input label is bound to empty filters as background. The amount of label hybridized to viral DNA from either infected or mock-infected cells is not significantly higher than background whether from heavy or light fractions (Table 6 is a typical result). d) The higher buoyant density of newly synthesized DNA could be due to its higher G + C content: The buoyant density of DNA is related to its G + C content according to the relationship

$$\rho = 0.098\ (GC) + 1.66\ gm/cm^3$$

(Schildkraut *et al.*,1962). Therefore, the greater the G + C content, the higher the buoyant density. It was

Figure 18: The buoyant density of newly synthesized DNA from infected cells after their treatment with or without S_1-nuclease prior to their centrifugation in CsCl gradients.

A-----A, newly synthesized ^{3}H-DNA; o———o, pre-existing ^{14}C-DNA.

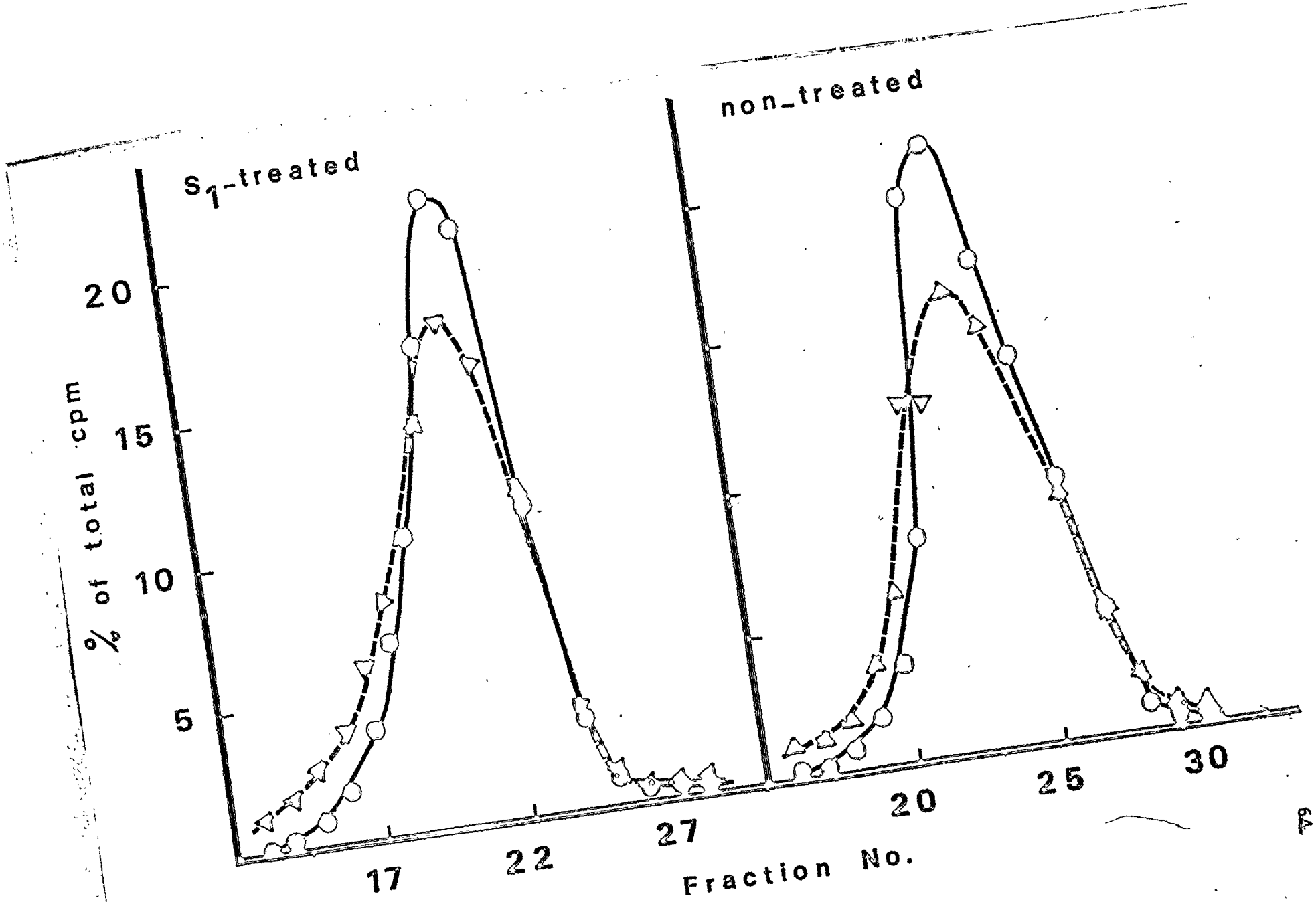

S1-treated
non-treated
% of total cpm
20
15
10
5
17
22
27
20
25
30
Fraction No.

Figure 19: CsCl gradient centrifugation profile of cell DNA used in hybridization experiments of Table 6.

A-----A, newly synthesized ^{3}H-DNA; o———o, pre-existing ^{14}C-DNA. A, control; B, infected.

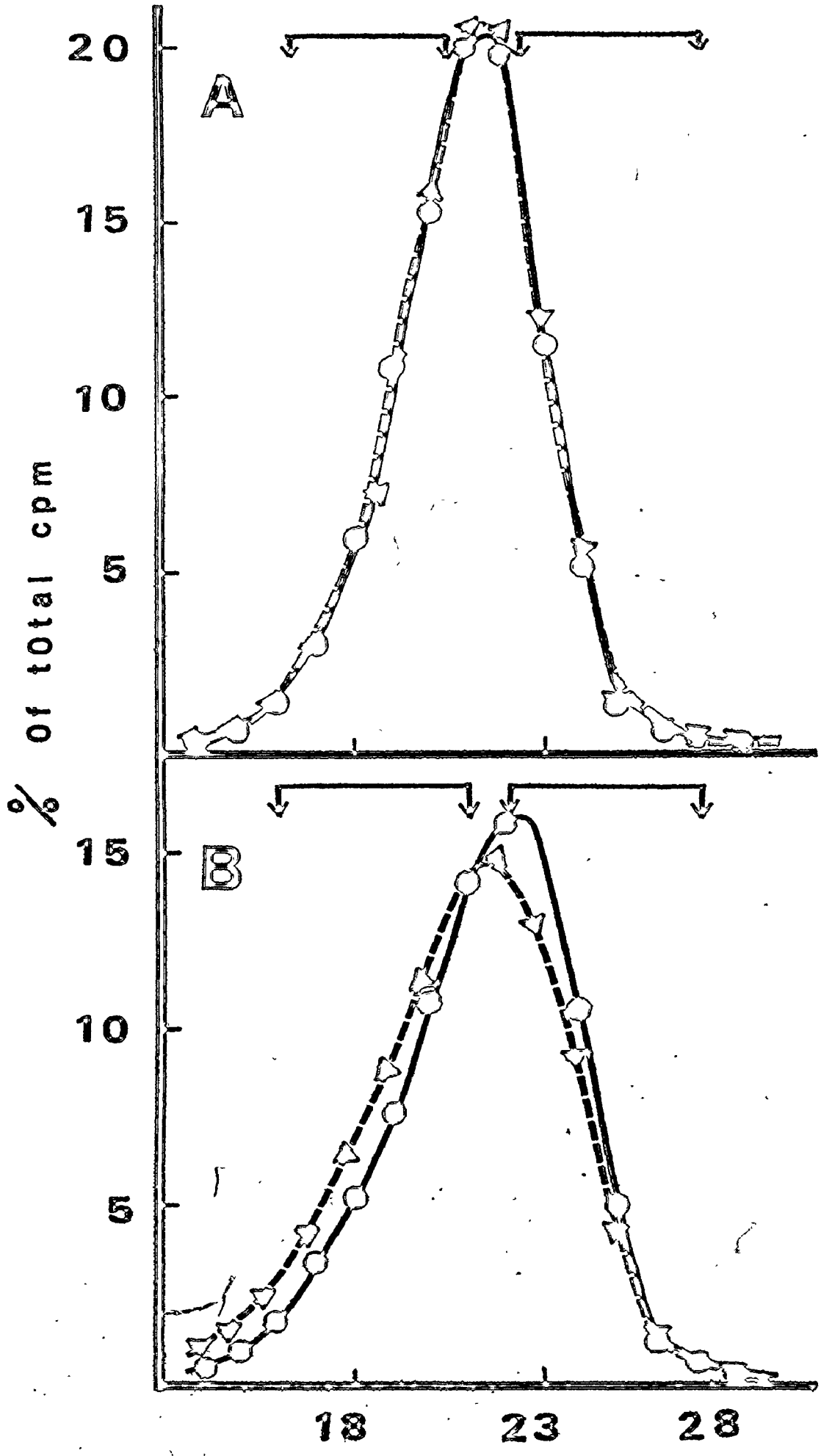

20
15
10
5
% of total cpm
A
15
10
5
B
18
23
28

Table 6

Hybridization of Newly Synthesized KB Cell DNA to Immobilized Viral DNA

Sample	Input cpm	Bound cpm	% bound
Empty filters	5305	59	1.1
Viral DNA	5305	3240	61.1
Control (heavy side)	85638	525	0.61
Control (light side)	50128	301	0.6
Infected (9 hr heavy side)	13217	216	1.6
Infected (9 hr light side)	11059	92	0.83

Cells pre-labelled with ^{14}C-TdR were infected or mock-infected and pulse-labelled with ^{3}H-TdR and centrifuged in CsCl gradients. Aliquots (20 µl) were taken to determine the ^{14}C-labelled radioactive peak. The gradient of DNA from infected cells was then divided so that 54% and 46% of the ^{14}C-label from the left and the right sides (referred to as heavy and light fractions, indicated by arrow in Figure 19) were pooled. The percentages for the control gradient were 63 and 37, respectively. The fractions were dialyzed against 0.1 x SSC and hybridized to cold viral DNA immobilized on nitrocellulose membrane filters as described.

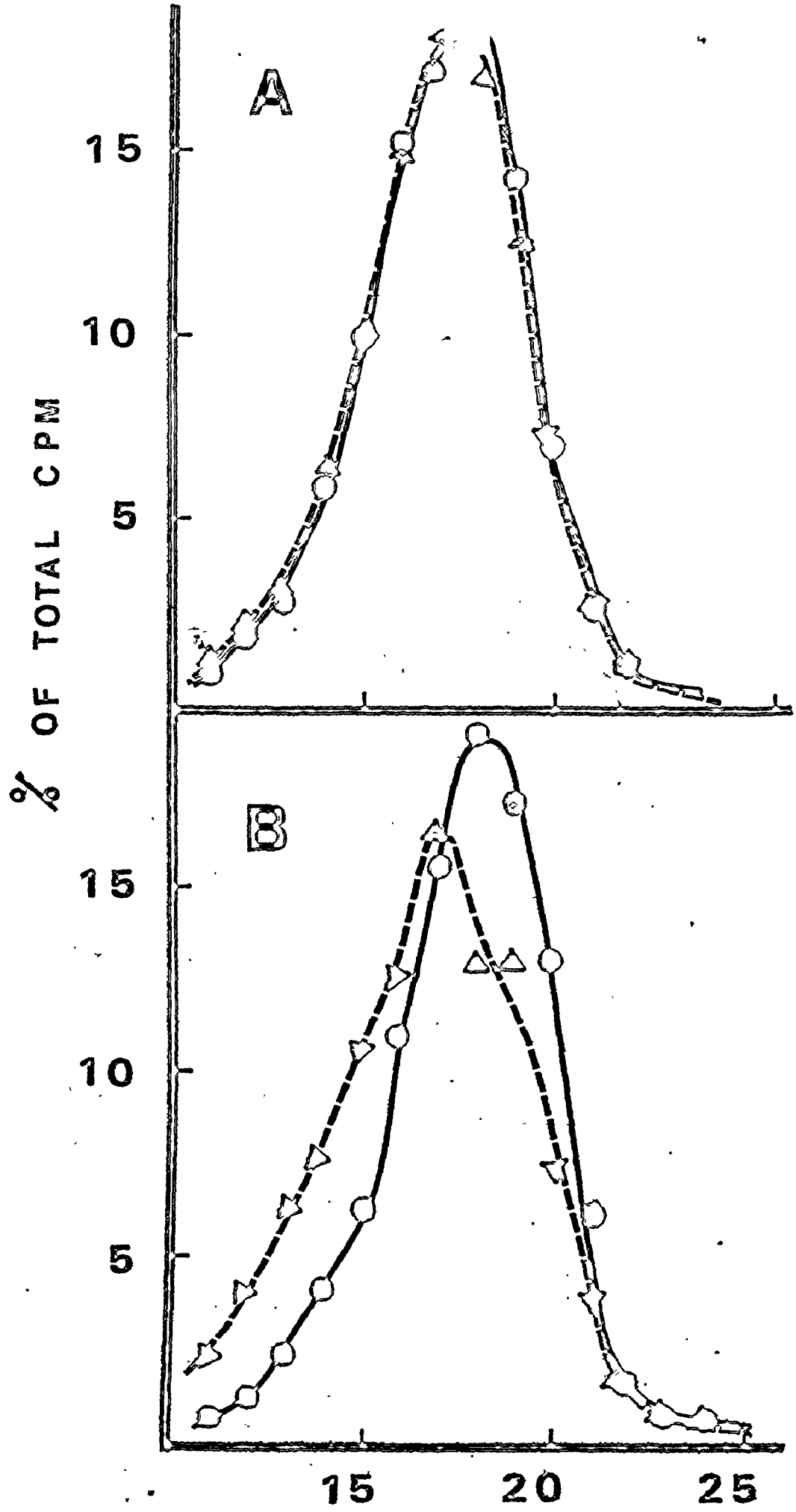
possible that the higher buoyant density of the newly synthesized DNA in the infected cells was due to the selective synthesis of GC-rich DNA. If this was the case, the shift should be more pronounced if deoxycytidine rather than deoxythymidine was used as a radioisotope precursor. To examine this possibility, cells were pre-labelled with ^{14}C-deoxycytidine (^{14}C-CdR) and then infected or mock-infected. After 8 hr of infection, the cells were pulse-labelled with ^{3}H-CdR for one hr and their DNA was analyzed by centrifugation in CsCl gradients.

As can be seen from Figure 20A, the density of newly synthesized DNA is the same as that of bulk DNA in the control cells. However, the density of newly synthesized DNA in the infected cells is significantly higher than that of bulk DNA (Figure 20B). Note also that the shift in density relative to bulk DNA is greater when deoxycytidine is used as label (compare Figure 20B to Figure 14B). This is consistent with the postulation that the higher buoyant density of the newly synthesized DNA in the infected cells is due to its higher G + C content.

The ratio of ^{3}H-DNA (newly synthesized DNA) to ^{14}C-DNA (DNA labelled for 24 hr prior to infection) for each point in the CsCl density gradient is a measure of the amount of DNA of different buoyant densities (Bostoch and Prescott, 1971). In the mock-infected human KB cells, there is uniform synthesis of DNA of all buoyant densities

Figure 20: High buoyant density of newly synthesized DNA from the infected cells after pulse-labelling with ^{3}H-CdR.

▲_____▲, ^{3}H-DNA; o———o, ^{14}C-DNA. A, control; b, infected.


A
B
15
10
5
15
10
5
% OF TOTAL CPM
15
20
25

(Figure 21). In the infected cells, however, there is preferential synthesis of high buoyant density DNA as indicated by the high $^{3}H/^{14}C$ ratio on the heavy side of DNA peak (Figure 21). If it is assumed that the buoyant density is related to CG-content in a straight forward manner (Schildraut *et al.*, 1962), then there is preferential synthesis of GC-rich DNA in the infected cells. This is consistent with the postulation that infection of human KB cells by Ad 12 induces the selective synthesis of those classes of DNA which are distinguished by their higher GC content. e) Dependency of the synthesis of high buoyant density DNA on time after infection and on viral dose: To study the kinetics of the synthesis of high buoyant density DNA cells were pre-labelled with ^{14}C-TdR and infected with 3 x 10^{3} virus particles per cell for 3 to 9 hr. The cells were pulse-labelled with ^{3}H-TdR for 30 min at each given time and their DNA was analyzed on neutral CsCl gradients. As shown in Figure 22, no shift in density is observed earlier than 7 hr post infection. The shift in density increases with time thereafter. It is interesting to note that the percentage of inhibition of cell DNA synthesis also increases with time (inset of Figure 22).

As stated above, the amount of shift in density increased as the percentage of inhibition of cell DNA synthesis increased. The percentage of inhibition of cell DNA synthesis was higher after infection with a higher dose of virus (Table 7).

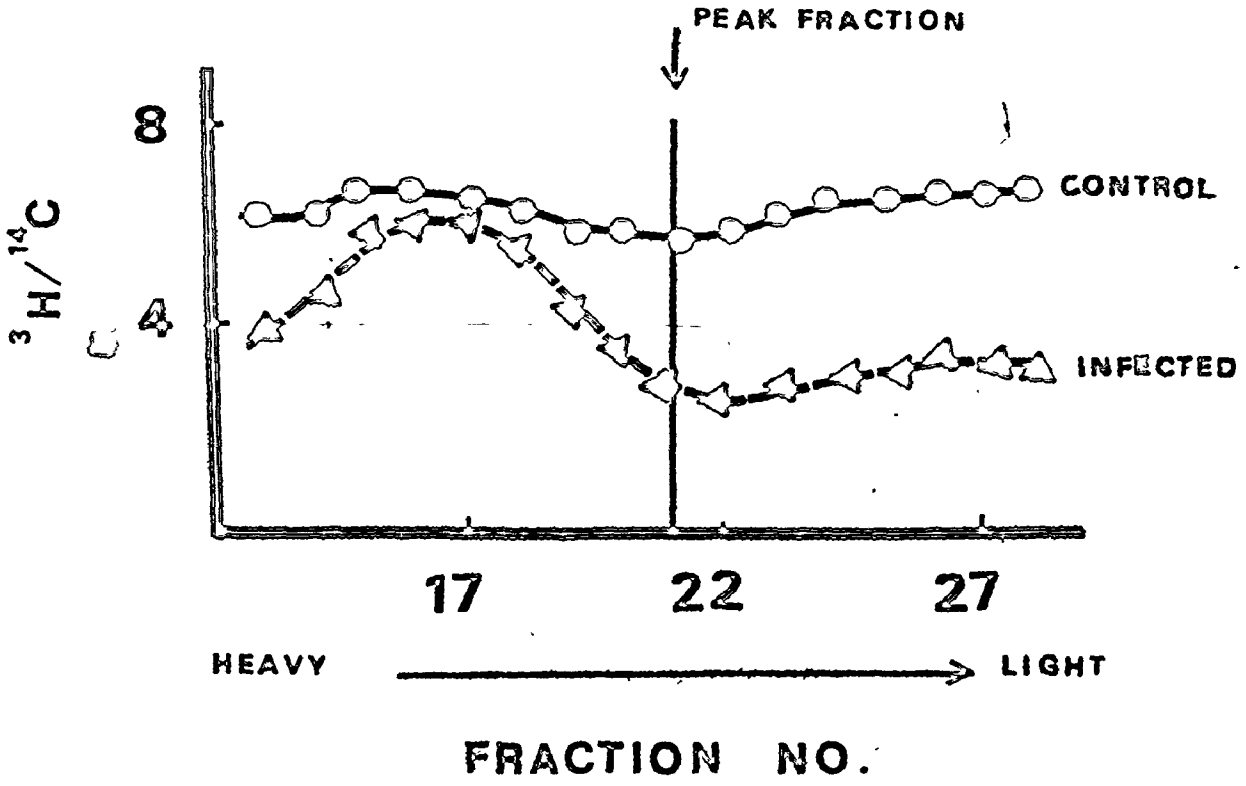


Figure 21: Differential synthesis of high buoyant density DNA in the infected KB cells.

Human KB cells were prelabelled with ^{14}C-TdR, infected or mock-infected, and pulse-labelled with ^3H-TdR for one hr. Their DNA was analyzed by centrifugation in CsCl gradients. The ratio of ^3H-DNA to ^{14}C-DNA in each fraction of the gradient was determined.

Figure 22: Synthesis of high buoyant density DNA in the infected cells as a function of time.

Percentage of inhibition of cell DNA synthesis (inset) was determined by comparing the ratio of ^{3}H-labelled DNA to ^{14}C-labelled DNA of the control cells to that of the infected cells. ▲_____▲, ^{3}H-DNA; o———o, ^{14}C-DNA.

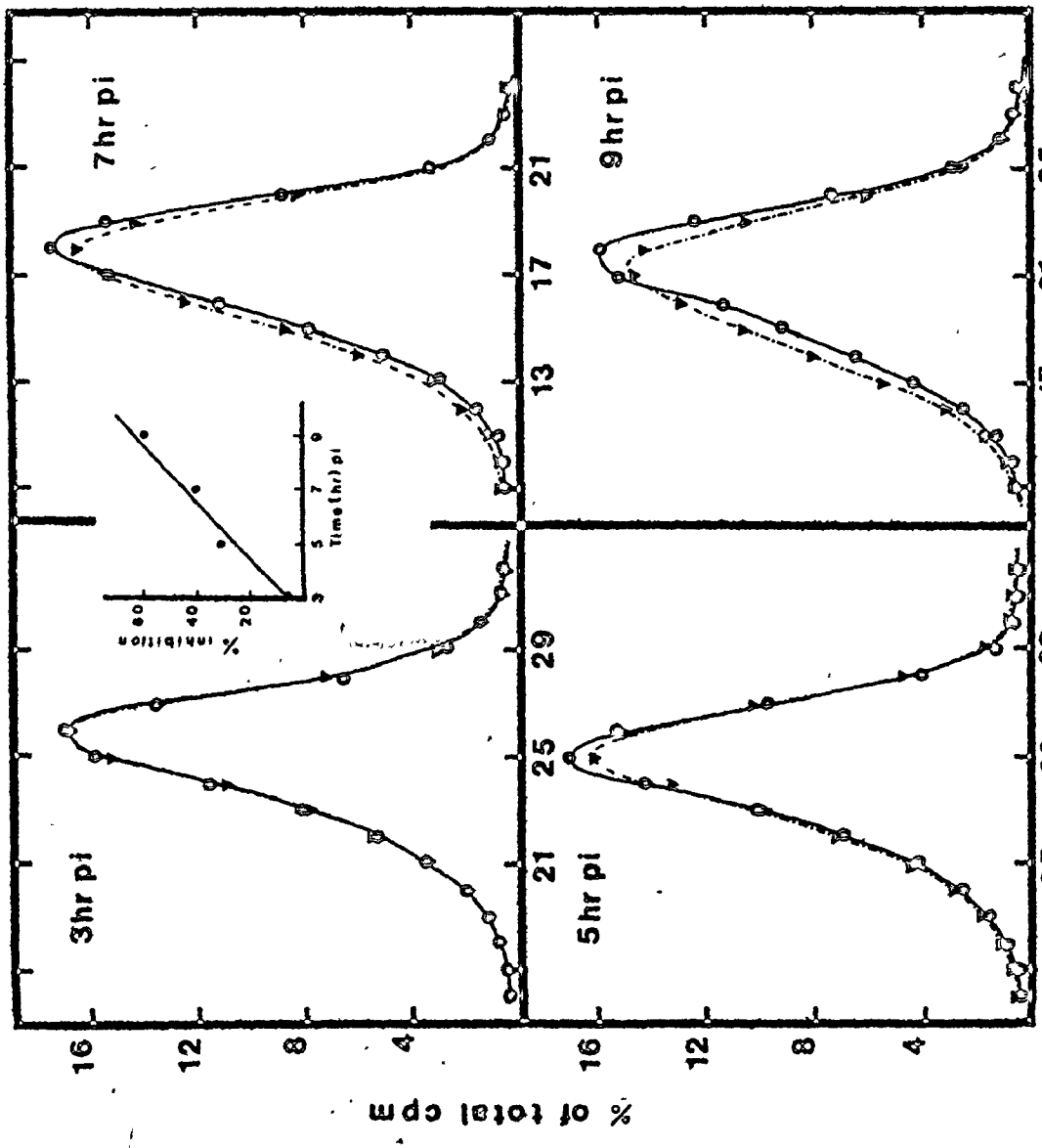

3hr pi
5hr pi
7hr pi
9hr pi
% of total cpm
Fraction No.
% inhibition
Time (hr) pi

Table 7

Dependency of Percentage of the Inhibition of Cell DNA Synthesis on Virus Dose

Sample	CPM in total gradient		^{3}H-TdR/ ^{14}C-TdR	% inhibition
	^{3}H-TdR	^{14}C-TdR		
Uninfected	139735	8433	16.6	-
Infected (3×10^{3} virus particles/cell)	87769	9205	9.5	43
Infected (3×10^{4} virus particles/cell)	21824	7631	2.86	82.8

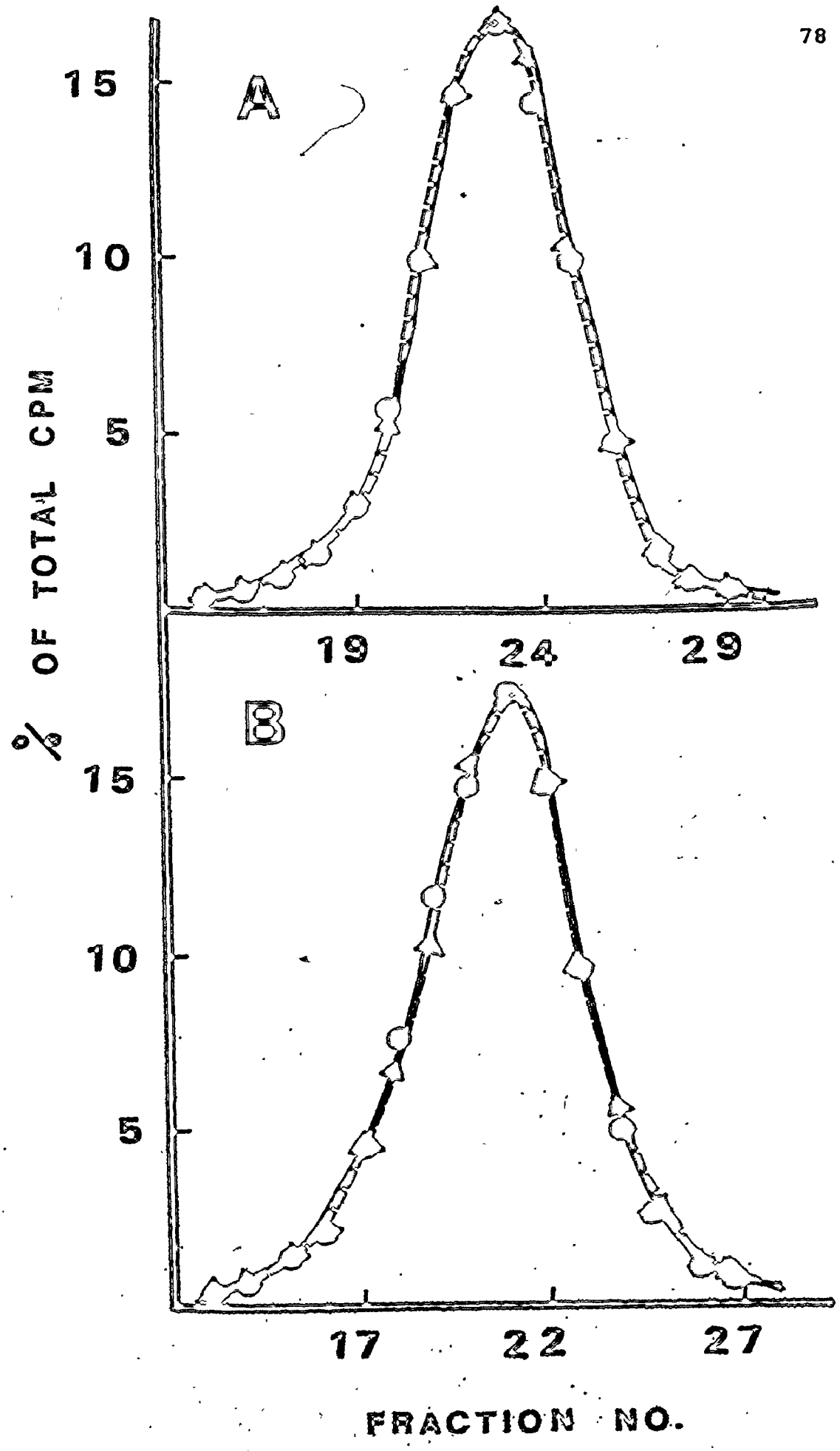
Cells were pre-labelled with ^{14}C-TdR and infected with the indicated doses of virus or mock-infected. They were pulse-labelled with ^{3}H-TdR for one hr at 8 hr post infection. Their DNA was analyzed in CsCl gradients.

It was, therefore, possible that infection by a higher dose of virus, which induces a greater inhibition of cell DNA synthesis induced also a greater shift in the buoyant density of the newly synthesized DNA. To examine this possibility cells were pre-labelled with ^{14}C-TdR and then infected with 3×10^4 virus particles per cell. After 8 hr of infection the cells were pulse-labelled with ^{3}H-TdR for one hr and their DNA was analyzed in neutral CsCl gradients. As shown in Figure 23, the density of the newly synthesized DNA is the same as the density of the bulk DNA for both infected and uninfected cells.

The normal cycle of virus propagation is inhibited after infection with high doses, probably due to the early fragmentation of cellular DNA followed by cell death. It therefore appears that the productive cycle of viral infection is required for the selective synthesis of high buoyant density DNA. f) Effect of infection by UV-irradiated virus on the synthesis of high buoyant density DNA: To determine whether viral infectivity was essential for the synthesis of high buoyant density DNA, the virus was UV-irradiated with 2.5×10^4 ergs/mm^2. The cells were pre-labelled with ^{14}C-TdR and infected with 3×10^3 particles of either UV-irradiated or non-treated virus and pulse-labelled with ^{3}H-TdR for one hr at 8 hr post infection. Their DNA was then analyzed by centrifugation in CsCl gradients.

Figure 23: The buoyant density of newly synthesized DNA after infection with high doses of virus.

▲-----▲, ^{3}H-DNA; o——o, ^{14}C-DNA. A, uninfected; B, infected with 3 x 10^{4} virus particles per cell.


A
B
15
10
5
19
24
29
15
10
5
17
22
27
% OF TOTAL CPM
FRACTION NO.

The buoyant density of the newly synthesized ^{3}H-DNA is the same as that of bulk DNA (Figure 24A) when cells are infected with UV-irradiated virus, while the density of newly synthesized ^{3}H-DNA is higher than that of bulk DNA when the cells are infected with untreated virus (Figure 24B). It should be pointed out that the amount of DNA synthesized in cells infected with untreated virus was only 30% of the amount synthesized in cells infected with UV-irradiated virus. This was determined by comparing the $^{3}H/^{14}C$ ratio from each gradient.

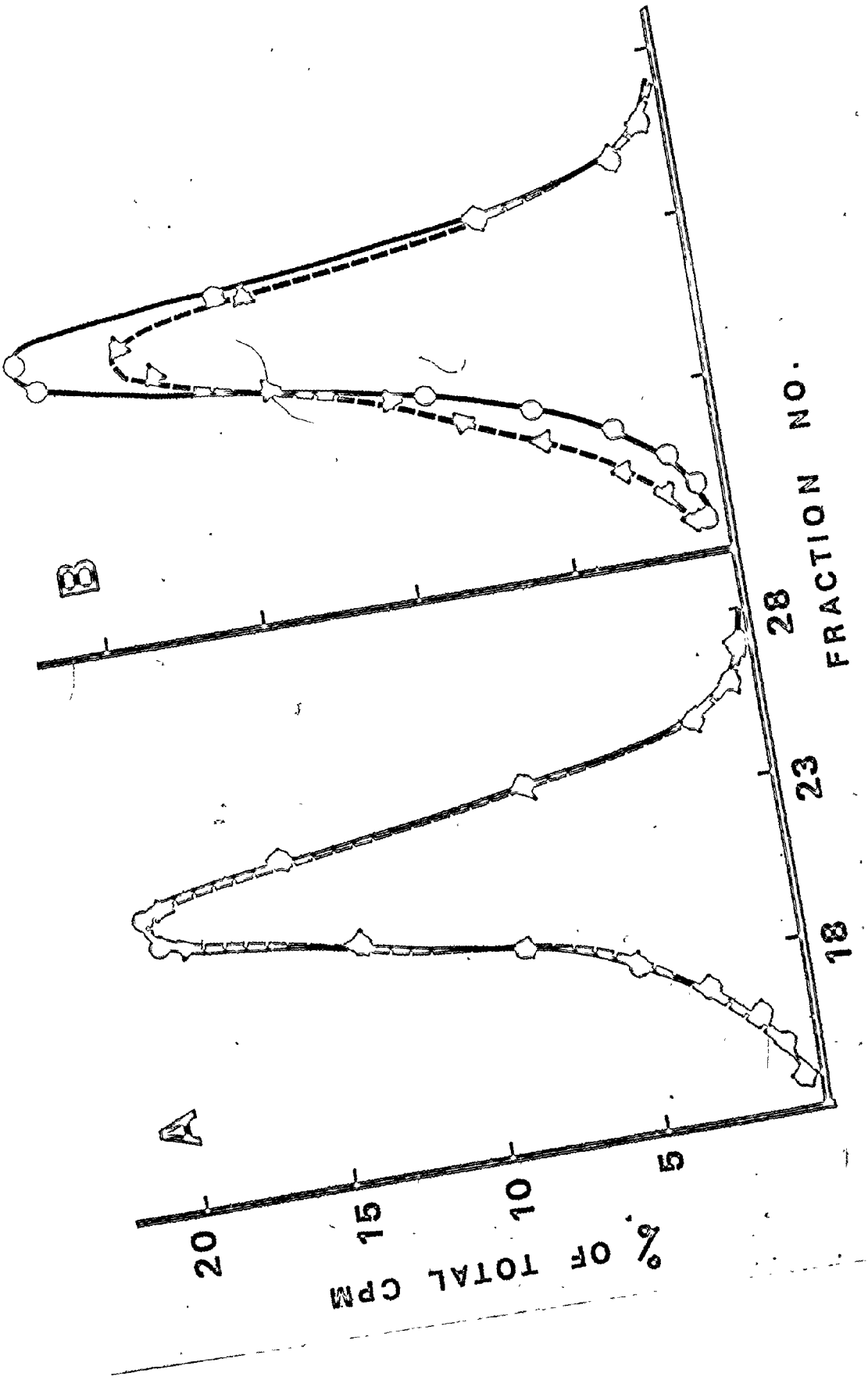
The shift in density is observed when the cells are infected with the virus that had been irradiated with a lower dose of UV (9×10^{3} erg/mm^{2}. This dose reduces the survival fraction of the virus to 10^{-2}.) However, the amount of shift in density is smaller when compared to the shift in density of the newly synthesized DNA from cells infected with unirradiated virus (compare Figure 25A and Figure 25B). Deoxycytidine was used as the radioisotope precursor in this experiment.

2. Reassociation kinetics of the newly synthesized DNA

When DNA is dissociated into two strands it can be reassociated into double-helix under appropriate conditions (Marmur *et al.*, 1963). Reassociation of complementary sequences results from their collision. The reaction is, therefore, second-order in nature and the rate of reaction (k) can be determined by the relationship (Britten and Cohen,

Figure 24: Synthesis of high buoyant density DNA in cells infected with UV-irradiated virus.

A_____A, ^{3}H-DNA; o———o, ^{14}C-DNA. A, DNA from cells infected with UV-irradiated (2.5×10^{4} erg/mm^{2}) virus; B, DNA from cells infected with non-treated virus.


B
A
20
15
10
5
% OF TOTAL CPM
18
23
28
FRACTION NO.

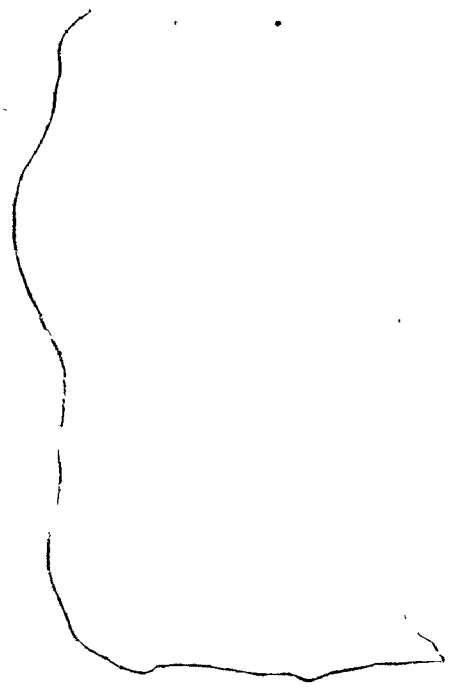
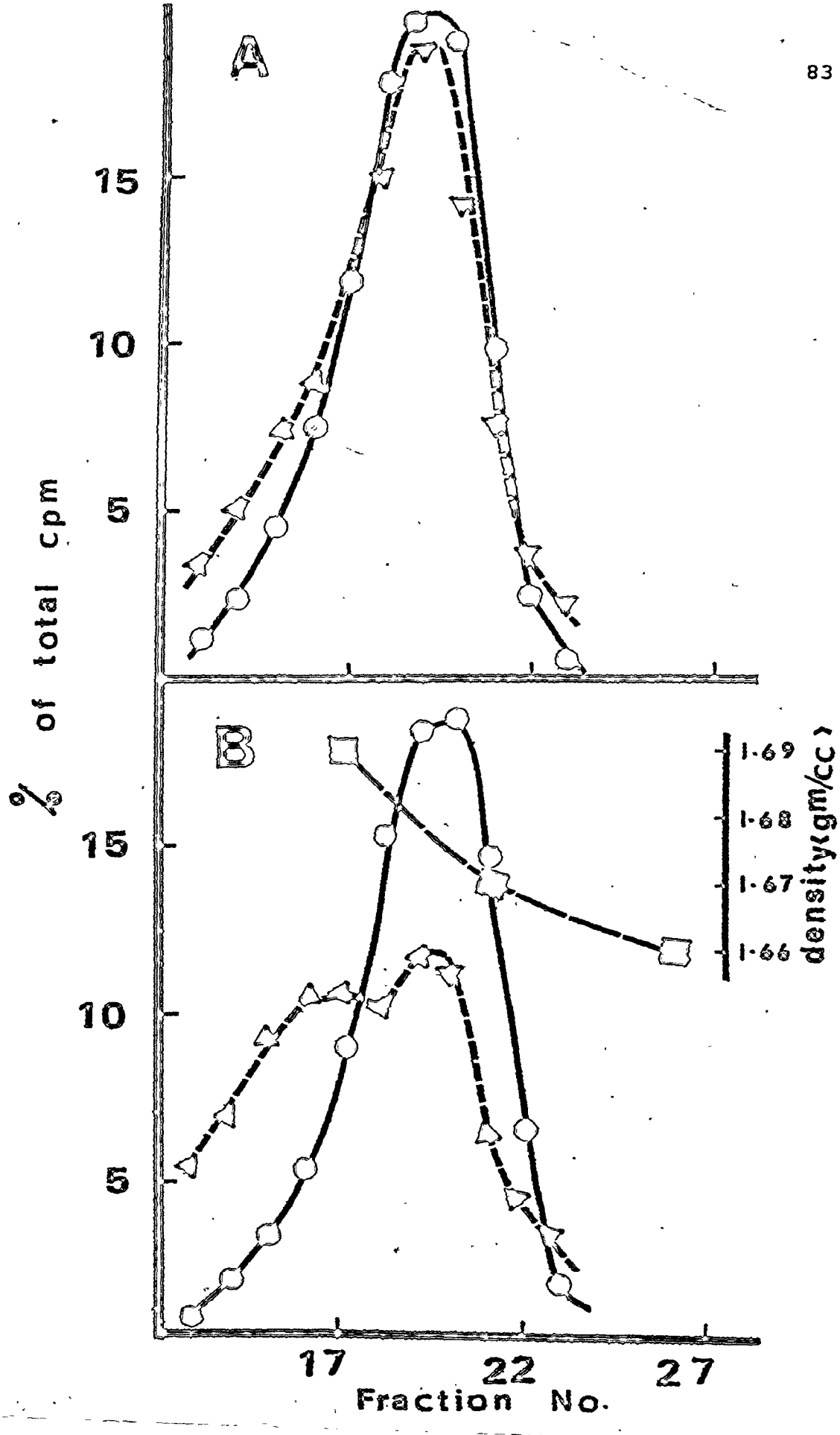


Figure 25: Synthesis of high buoyant density DNA after infection with virus which was irradiated with a low dose of UV (9×10^3 ergs/mm^2).

▲_____▲, ^{3}H-DNA; o———o, ^{14}C-DNA. A, DNA from cells infected with UV-irradiated virus; B, DNA from cells infected with untreated virus. Deoxycytidine was used as radioisotope precursor.


A
B
15
10
5
15
10
5
% of total cpm
1·69
1·68
1·67
1·66
density (gm/cc)
17
22
27
Fraction No.

$$C/Co = \frac{1}{1 + kCot}$$

The progress of reassociation can be plotted as a function of the logarithm of the product of DNA concentration (C , in moles of nucleotides) and the time of incubation (t).

When the fraction of DNA reassociated (C/Co) of higher organisms is plotted against "Cot", the resulting curve is complex and multi-component. A fraction of DNA reassociates slowly, as expected for the complex genome of eukaryotes. However, some classes of DNA reassociate with greater rates indicating the presence of multiple copies of these in the genome.

About 35% of the human genome has classes of DNA which are repeated more than once (Saunders, 1974). To determine whether infection by Ad 12 has any effect on the synthesis of these classes of DNA, KB cells were infected or mock-infected. The infected cells were pulse-labelled with ^{3}H-TdR and the control cells were pulse-labelled with ^{14}C-TdR for 2 hr at 7 hr post infection.

Infection could have induced the synthesis of multiple copies of certain classes of DNA to the extent that they would be reassociating with the same kinetics as repetitive DNA even if they were from the unique sequences. On the other hand, infection could have resulted in the selective synthesis of the repetitive sequences. To examine the latter possibility, the labelled infected and uninfected

cells were combined and mixed with 10^8 uninfected and unlabelled cells so that the contribution of the labelled DNA to the rate of reassociation would be essentially zero.

DNA was isolated as described earlier and sonicated. The size of the sonicated DNA was the same for DNA from infected and uninfected cells (Figure 26). This was important since the rate of reassociation is dependent on the size of denatured DNA (Britten *et al.*, 1975). Renaturation was at 67°C as described. Aliquots (50 µl) were taken at the indicated times and treated (untreated as input) with S_1-nuclease for the digestion of single-stranded DNA (Sutton, 1971; Davidson *et al.*, 1973) at 37°, pH 4.5 for 30 min. The percent of DNA digested by S_1-nuclease (C/Co) was plotted against "Cot" on a semilog scale. As shown by Figure 27, the reassociation of DNA follows a multi-component pattern with an apparent break at Cot of 10. About 20% of the newly synthesized ^{14}C-DNA from the mock-infected cells reassociates with a Cot of 10, while close to 30% of the newly synthesized ^{3}H-DNA from the infected cells reassociates with the same Cot value.

The higher percentage of the repetitive DNA synthesized in the infected cells is not an artifact of double-labelling techniques. The percentage of rapidly reassociating ^{3}H-DNA is the same as that of rapidly reassociating ^{14}C-labelled DNA when the two are isolated from uninfected cells (Figure 28.)

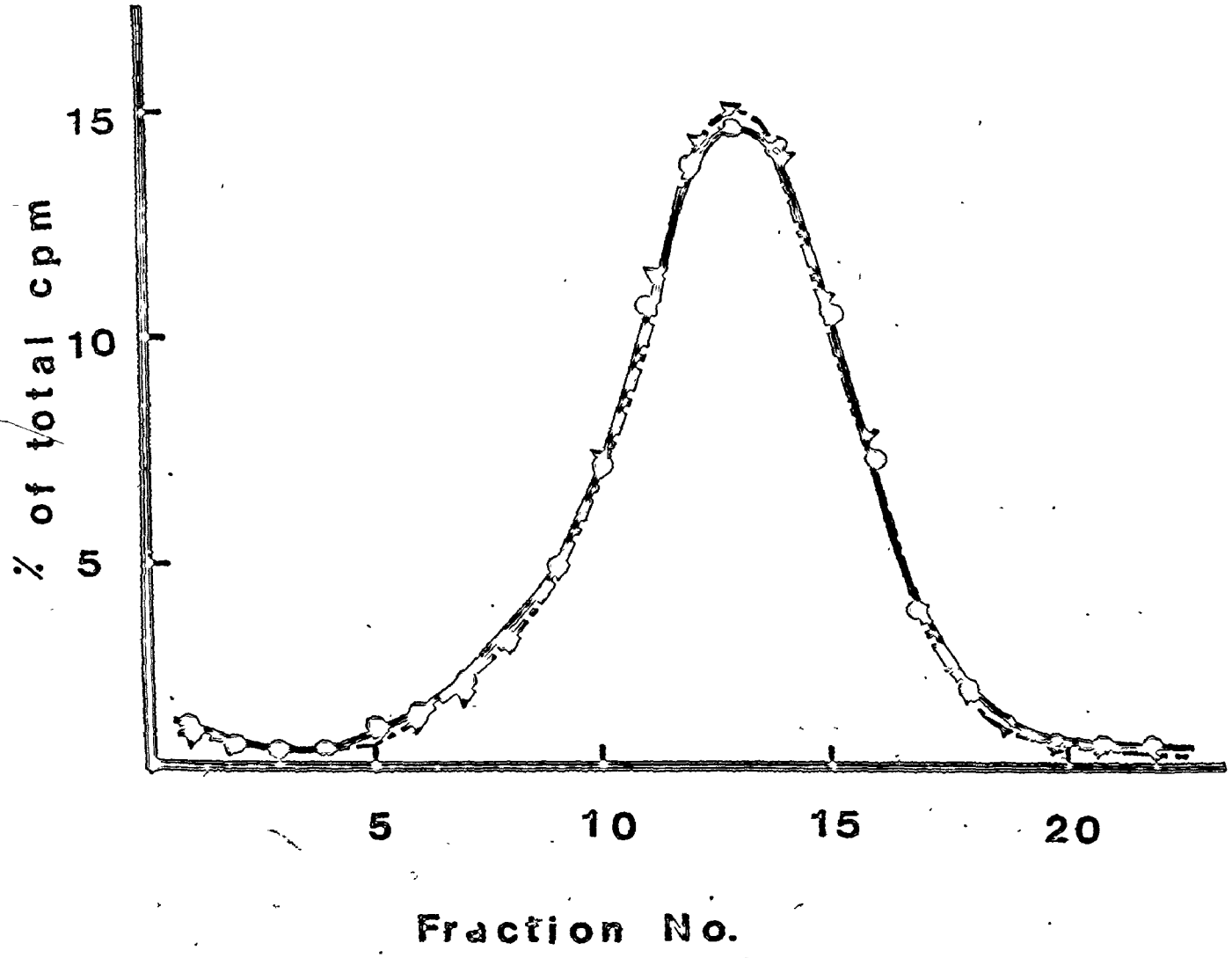
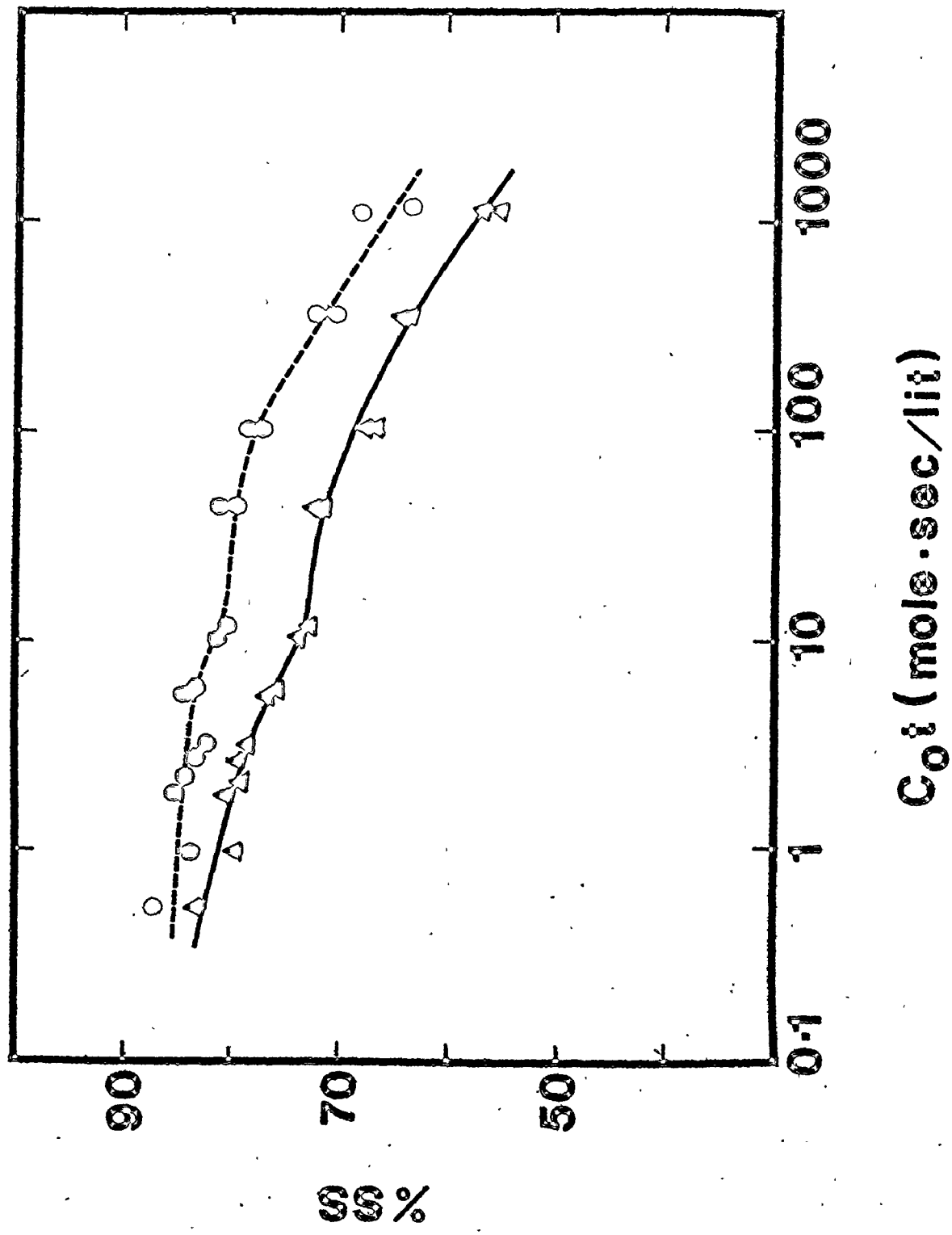


Figure 26: Cosedimentation of sonicated DNA from infected and uninfected cells in alkaline sucrose gradients.

^{3}H-DNA from infected cells mixed with ^{14}C-DNA from uninfected cells was sonicated and denatured in alkaline lysing solution on top of a 5-20% alkaline sucrose gradient for one hr. Centrifugation was in an SW40 rotor at 38000 rpm at 20°C for 23 hr. ▲_____▲, ^{3}H-DNA; o———o, ^{14}C-DNA.

Figure 27: Reassociation kinetics of newly synthesized DNA from infected and uninfected KB cells.

▲_____▲, ^{3}H-DNA from infected cells; o——o, ^{14}C-DNA from control cells. DNA concentration was 1.45 mg/ml. Specific activity of ^{3}H-DNA was 1.4×10^{4} cpm/mg and that of ^{14}C-DNA was 4.7×10^{3} cpm/mg.


90
70
50
SS%
0·1
1
10
100
1000
$C_o i$ (mole·sec/lit)

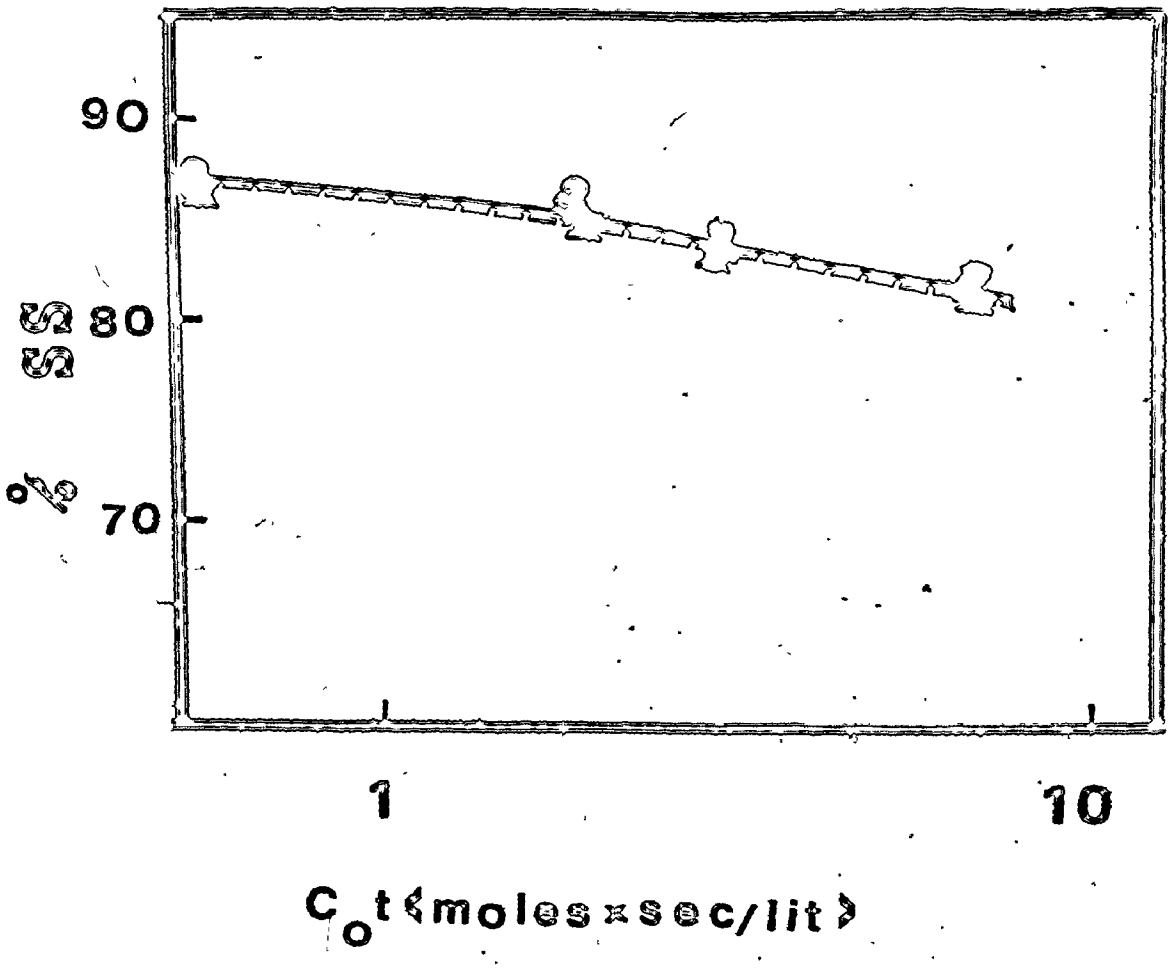


Figure 28: Reassociation kinetics of combined ^{14}C-labelled DNA and ^{3}H-labelled DNA isolated from uninfected KB cells.

DNA concentration was calculated from the control graph of Figure 27. o———o, ^{14}C-DNA; A_____A, ^{3}H-DNA.

It can, therefore, be concluded that a greater percentage of the newly synthesized DNA from the infected cells is from the repetitive sequences reassociating with a low Cot value.

DISCUSSION

The studies of the effect of infection by adenovirus type 12 on cell DNA metabolism will be discussed in two sections. In the first section the fragmentation of cellular DNA after infection and its relation to chromosome fragmentation will be discussed. In the second section, the discussion will be concerned with the effect of infection on some aspects of cell DNA replication. Some postulation on the mechanism by which these effects are induced will be made in each section.

I. Fragmentation of Cellular DNA After Infection by Ad 12

Human adenovirus type 12 induces chromosome fragmentation in abortively infected cells as shown by cytological techniques (Cooper, 1968). Only a small percentage of cells, i.e. those containing metaphase chromosomes at the time of observation, are studied by this technique. It, therefore, remained unclear whether chromosome fragmentation occurred generally in the whole population and was not detected due to the limit of resolution offered by such techniques. Another limitation of such techniques is that the molecular level at which this effect is exerted cannot be studied. Biochemical techniques were used in the present work to facilitate such studies.

Using the technique of alkaline sucrose gradients in which cell DNA is analyzed with minimum shearing (Palcic, 1972), it was shown that infection of hamster embryo cells by Ad 12 results in the fragmentation of the DNA of the majority of the cells (Figure 9). The percentage of DNA fragmented is dependent on both virus dose and time after infection as is the case with chromosome fragmentation (Cooper, 1968).

Fragmentation of hamster cell DNA after infection by Ad 12 has been reported by Doerfler (1969) and Strohl (1973). Both of these authors report only the fragmentation of the DNA which was synthesized after 25 hr of infection. The DNA fragmented, as reported in the present work, was labelled for about one generation prior to infection. It is not clear whether the fragmentation of the DNA synthesized after infection is the same phenomenon as the fragmentation of the pre-existing cell DNA.

Chromosome breaks are not observed in lytically infected cells (Cooper, 1968). It was therefore of interest to examine whether cell DNA fragmentation is induced in lytically infected cells.

Lytic infection of human KB cells results in the early fragmentation of cellular DNA (Figure 5). The percentage of DNA fragmented is dependent on both virus dose

and time after infection. Complete fragmentation of cell DNA is observed at 3-5 x 10^5 virus particles per cell. Cell DNA fragmentation is observed as early as 2 hr after infection. Fragmentation of cell DNA as early as 90 minutes after infection has been observed in HeLa cells infected by vaccinia virus (Parkhurst *et al.*, 1973). Fragmentation by this virus is also dependent on virus dose.

Fragmentation of cell DNA in KB and hamster embryo cells is the same at about 3 x 10^5 virus particles per cell even though the adsorption efficiency of the virus by the KB cells is four times higher than that by the hamster embryo cells. This is probably due to the fact that the receptors on the cell surface are saturated (about 10^4 receptors per cell, Philipson and Lindberg, 1974) in both cell types and, therefore, the same number of virus particles are entering in both.

A feature of infection of hamster embryo cells with respect to the fragmentation of cell DNA is that there appears to be two separable populations (Figure 10). One population requires low doses of virus for the complete DNA fragmentation. The other population requires high doses for the same effect. Heterogeneity in the populations can easily exist since whole embryos were used for the propagation of the cultures. It is possible that adsorption efficiency is different for certain cells derived from certain origins.

Significant fragmentation of cell DNA is not observed earlier than 20 hr after the infection of hamster cells (Table 4). This is in contrast to KB cells in which the complete fragmentation of cell DNA is observed by 10 hr of infection. The slow response of hamster cells to infection with respect to DNA fragmentation could be due to a difference in the rate of uncoating of the virus or transport of the virus or its components to the nucleus.

Chromosome fragmentation is observed in lytically infected cells when UV-inactivated virus is used (Stich, 1968; Rainbow, 1970). UV-irradiation of the virus allows the infected cells to enter mitosis and, therefore, makes it possible for chromosome fragmentation to be detected. Fragmentation of cellular DNA is induced equally by both UV-inactivated and untreated virus in the lytically infected cells (Table 2). Assuming that chromosome fragmentation is the same phenomenon as DNA fragmentation, it appears that two separable effects of infection are involved: 1) The inhibition of mitosis after infection; this is abolished by the UV-inactivation of the virus. 2) Chromosome (DNA) fragmentation which is observed even after infection with UV-inactivated virus.

Premature condensation of interphase chromosomes is induced when interphase cells are fused with mitotic cells (Johnson and Rao, 1970). The fusion of lytically infected cells with mitotic cells might induce premature

chromosome condensation in these cells. This would verify that chromosome fragmentation is a general effect of infection for all cells and that the molecular level at which this effect is exerted is DNA.

The mechanism by which cell DNA fragmentation is induced is unknown. It is possible that the fragmentation of cell DNA is a secondary result of cell "death" due to the high doses of virus. This, however, is unlikely since at the dose at which maximum percentage of DNA breakage was observed, the cells excluded trypan blue and incorporated labelled-uridine into their RNA.

Exposure of cells to external agents such as radio-isotopes, hypotonic shock, treatment with detergents and treatment with specific antibody induces the fragmentation of cell DNA to a homogeneous size of 5×10^6 daltons (Williams *et al.*, 1974). This fragmentation is thought to be due to the activity of an induced endonuclease of the cell. It is possible that the fragmentation of cell DNA by adeno infection is also due to the activity of such nucleases. It should, however, be noted that not all external agents induce the fragmentation of cellular DNA in the same manner. Treatment of KB cells with actinomycin-D results in the fragmentation of cellular DNA (Pater and Mak, 1974). There is a gradual reduction in the size of the fragments as the dose of the drug and the time of treatment increases. Moreover, in the adeno-infected cells lysosomes are intact (Aula and Nichols, 1968).

It is, therefore, unlikely that the fragmentation of cellular DNA is due to the breakage of lysosomes and the release of cellular nucleases.

Fragmentation of cellular DNA by vaccinia virus is postulated to be due to the activity of an endonuclease which is associated with the virion (Parkhurst *et al.*, 1973). An endonuclease activity has been found in association with the pentons of adenovirus (Burlingham and Doerfler, 1972). Viral DNA is hydrolyzed to fragments of 5×10^6 daltons in the presence of purified virions. The fragments are free of single-strand scissions.

The activity has also been detected in productively infected cells between 2-8 hr of infection. This early activity disappears and reappears later in infection and increases to 20 times the level of the early activity. These observations have led the authors to suggest that the endonuclease activity detected early in infection is due to the incoming virus and that the activity detected late in infection is due to the synthesis of the pentons. This suggestion is supported by the observation that in the abortively infected cells, in which the synthesis of structural proteins is blocked the endonuclease activity detected early in infection does not increase later in infection.

Several observations reported in the present work support indirectly the involvement of viral endonuclease in the fragmentation of cellular DNA.

Fragmentation of cellular DNA is induced in KB cells prior to the synthesis of viral DNA and late viral genes. The expression of the late viral genes are, therefore, not required for the fragmentation of cellular DNA. This is supported by the observation that cell DNA fragmentation is induced in the hamster embryo cells in which the expression of late viral genes are blocked.

The observation that cell DNA fragmentation is dependent on virus dose and that it is induced even after infection with UV-inactivated virus is suggestive of a role for the incoming virus in the fragmentation of cellular DNA. It should, however, be noted that since high doses of UV-irradiated virus were used, the possibility of multiplicity reactivation (a case in which cells are infected with more than one virus particle per cell; normal infectious cycle may be restored by the defective virus particles complementing each other's defect) cannot be ruled out.

Finally, the observations that the size of fragments is the same in both lytically and abortively infected cells and the fact that fragmentation is double-stranded in nature, are further support for the role of viral endonuclease in the fragmentation of cellular DNA.

A direct test for the role of penton-associated endonuclease would be the isolation of mutants which have temperature sensitive penton proteins. Infection at non-permissive temperatures should inhibit the fragmentation of cellular DNA.

Another test for the role of penton-associated endonuclease, is to infect cells with purified infectious viral DNA. Cell DNA fragmentation should not be observed at least early in infection if this effect is induced by the incoming viral proteins.

II. Replication of Human KB Cell DNA After Infection

A. Effect of infection on DNA chain growth

DNA replication in the majority of organisms is discontinuous (Okazaki, 1968; Goulian, 1971; Huberman and Horwitz, 1973). When cells are fed with a radioisotope precursor for very short periods (30 sec. - 2 min.) the majority of label is incorporated into small pieces known as "Okazaki fragments". These fragments are gradually joined together and molecules of the same size as the template eventually form.

In the human KB cells the majority of DNA synthesized in a 15 min pulse is also in the form of small molecular weight DNA (Figure 11). These fragments are joined to form template-size molecules. This process is complete by 6 hr. In the adeno-infected KB cells, however, the ligation of the newly synthesized DNA is slower. The slow rate of ligation seems to be independent of virus dose (compare Figure 13 to Figure 11), even though the synthesis of cell DNA is inhibited to a greater extent at a higher dose (Table 7).

It should, however, be noted that after infection with high doses of virus the DNA synthesized in a short

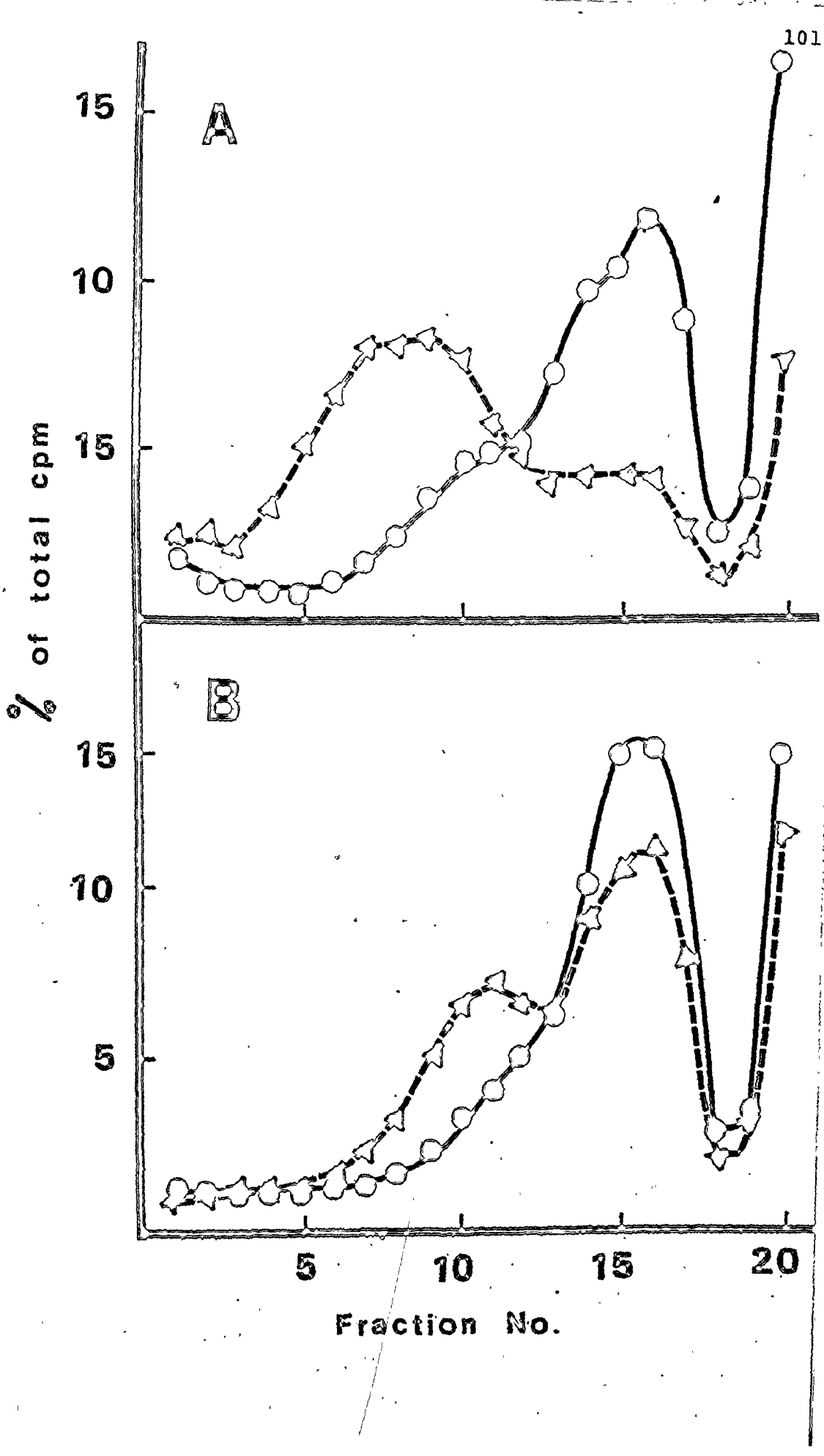
pulse is more heterogeneous in size and has a larger percentage of high molecular weight DNA. A similar effect is also observed when these cells are treated with actinomycin-D (Figure 29). The common feature is the presence of fragmented pre-existing cellular DNA in both infected and actinomycin-D-treated cells. The mechanism by which this effect is exerted is unknown. Mammalian cells can repair DNA damage induced by a wide variety of agents (Lieberman and Poirier, 1974). It is possible that the DNA fragmented in the KB cells due to infection with high doses of virus or treatment with actinomycin-D is being repaired simultaneously with normal replication.

The mechanism by which the normal rate of ligation is slowed down is unknown. No reduction in DNA ligase activity is observed earlier than 46 hr post infection (Ledinko, 1970). It is, therefore, unlikely that the slow rate of ligation is due to the inhibition of the activity of such enzymes.

Cellular DNA synthesis is induced in cells productively infected with polyoma virus (Cheevers *et al.*, 1972). The increased rate of DNA synthesis is thought to be due to increases both in the rate of initiation and in chain elongation early in infection. Two-thirds of the cellular DNA pulse-labelled late in infection accumulate as incomplete DNA strands. It is suggested that this is due to the faster rate of chain initiation as compared to the rate of chain elongation.

Figure 29: DNA chain elongation in actinomycin-D-treated KB cells

Human KB cells were pre-labelled with ^{14}C-TdR and then treated with 0.1 µg/ml of actinomycin-D for 3 hr. They were then pulse-labelled with ^{3}H-TdR for 15 min and/or chased for 4 hr. A, 15 min pulse; B, 4 hr chase. A-----A, ^{3}H-DNA; o———o, ^{14}C-DNA.


A
B
15
10
15
15
10
5
% of total cpm
5
10
15
20
Fraction No.

Whether a similar mechanism is operative in the case of adeno-infected KB cells has to be further investigated. It should, however, be remembered that infection of KB cells by Ad 12 has an inhibitory effect on cell DNA synthesis. Moreover, in the polyoma-infected cells the slow rate of ligation is not observed in the absence of viral DNA replication (Cheevers and Hiscock, 1973) while in the adeno-infected cells the slow rate of ligation is observed prior to the onset of viral DNA synthesis.

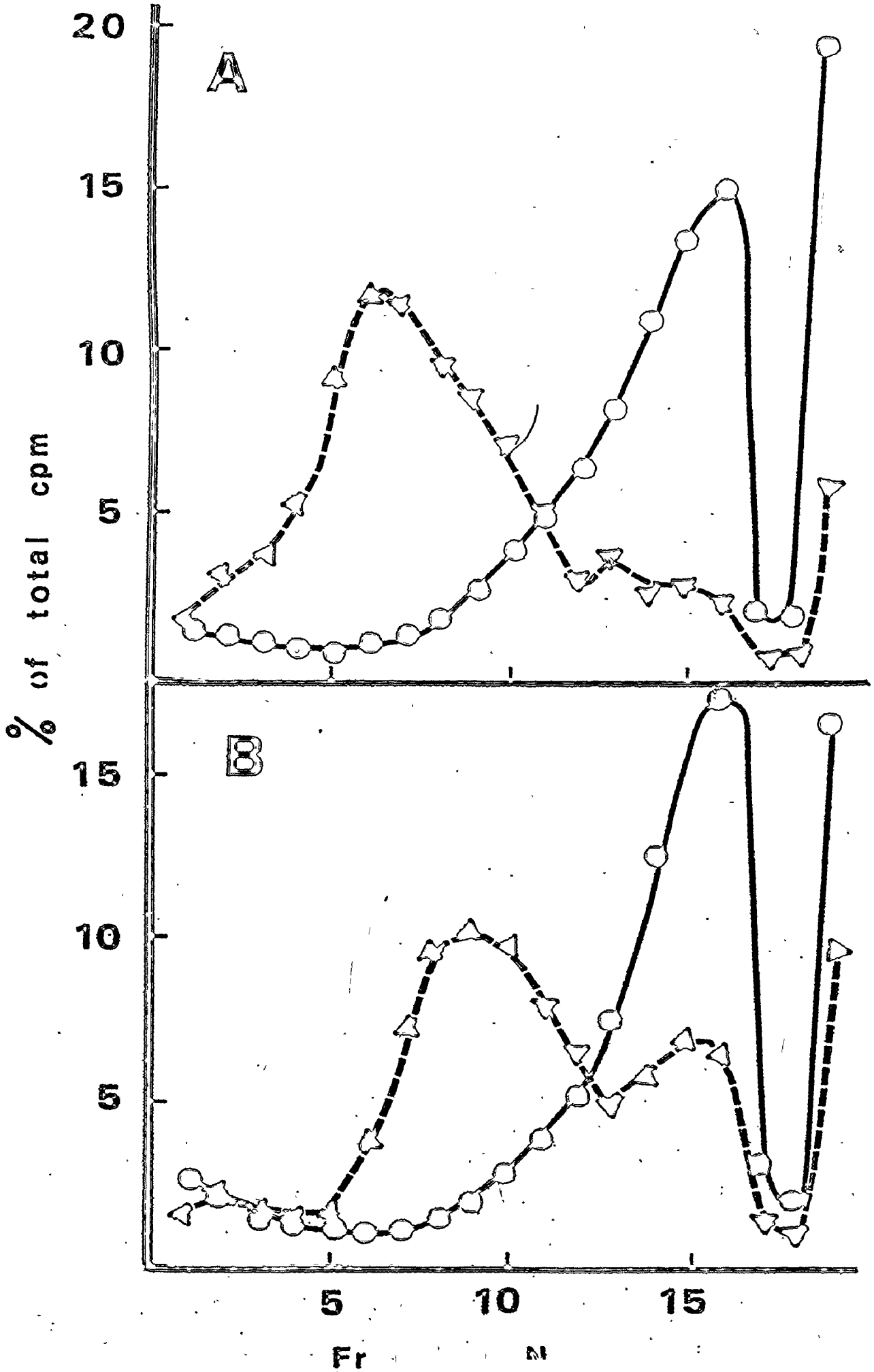
Finally, the ligation of cellular DNA synthesized in a short pulse is also slower when DNA synthesis is inhibited by treating KB cells with either actinomycin-D (Figure 29) or with excess thymidine (Figure 30). The slow rate of ligation in the adeno-infected cells could be a similar effect as in actinomycin-D treated cells and/or in cells treated with excess thymidine.

B. Characteristics of the newly synthesized KB cell DNA after infection

Newly synthesized DNA in Ad 12 infected KB cells has a higher buoyant density than the bulk DNA (Figure 14). This higher density is not due to single-strandedness of the newly synthesized DNA, to the presence of DNA with large single-stranded tails, or to contamination with newly synthesized viral DNA. The higher buoyant density is probably due to the selective synthesis of GC-rich classes of cellular DNA.

Figure 30: DNA chain elongation after the treatment of human KB cells with excess thymidine

KB cells were pre-labelled with ^{14}C-TdR and then pulse-labelled with ^{3}H-TdR for 15 min. They were either immediately used or chased for 4 hr in the presence of 100 µg/ml of unlabelled thymidine. A, 15 min pulse; B, 4 hr chase. ▲——▲, ^{3}H-DNA; ○——○, ^{14}C-DNA.


A
B
20
15
10
5
15
10
5
% of total cpm
5
10
15
Fr
N

The selective synthesis of high buoyant density DNA is dependent on time after infection. No shift in density is observed before 7 hr of infection (Figure 22). The shift in density increases with time thereafter. This is correlated with the increasing percentage of inhibition of cell DNA synthesis with time after infection.

There is also selective synthesis of repetitive DNA in the infected KB cells (Figure 27). Attempts were made during this work to determine whether this selectively synthesized reiterated DNA was included in the high buoyant density DNA. No success was achieved due to technical difficulties.

Selective synthesis of certain classes of cell DNA after infection by Ad 12 is the first report of such an effect by an animal virus. The mechanism by which this effect is induced is not known. DNA synthesized in the early S-phase of the cell cycle has a higher buoyant density than the bulk DNA in mouse L-cells, HeLa cells (Tobia *et al.*, 1970), and Chinese hamster cells (Bostoch and Prescott, 1971). In the chick fibroblast cells, some of the highly reiterated sequences which are included in the high buoyant density DNA fractions, replicate both early and late in S-phase, while repeated sequences of low buoyant density fractions replicate mainly late in the S-phase (Tapiero *et al.*, 1974). It is possible that after infection of KB cells by Ad 12 the synthesis of those classes of DNA which

replicate early in S-phase escape inhibition. Two possible ways by which this may happen are postulated in the following.

One possibility is that the cellular DNA polymerases have *in vivo* specificity with respect to the recognition of different classes of DNA as their template. Virus infection may either inhibit or modify the activity of some of these enzymes.

The major polymerase in a nuclear complex that is capable of synthesizing viral DNA sequences *in vitro* has been purified from Ad 2-infected KB cells and characterized as DNA polymerase γ (Ito *et al.*, 1975). This enzyme may also be involved in the synthesis of Ad 12 DNA. The *in vivo* template specificity of this enzyme is not known. It is tempting to postulate that this enzyme is specific for the synthesis of GC-rich classes of DNA. Selective synthesis of the GC-rich classes of host DNA would then be due to the activity of this enzyme as the active polymerase in the infected cells. Eventual switch-over to the synthesis of viral DNA may involve competition due to the excess of viral DNA as substrate.

The direct role of the functional genes of the virus in the selective synthesis of cell DNA cannot be excluded especially since no shift in density is observed when the cells are infected with UV-inactivated virus (Figure 24) or with high doses of virus in which early cell killing inhibits the production of viral progeny.

Although viral specific polymerases have not been so far isolated, genetic analysis has shown that two (Ledinko, 1974) or three (Shiroki and Shimojo, 1974) complementation groups are necessary for the replication of Ad 12 DNA. These could be genes coding for viral specific polymerases. Selective synthesis of certain classes of host DNA could be due to the recognition of these classes of DNA by such postulated viral enzymes. This is attractive to postulate since Ad 12 is also slightly richer in its GC-content than the bulk of cellular DNA (Green, 1970).

The biological significance of the selective synthesis of host DNA is not clear at this time. Integration of adenovirus DNA into the DNA of these cells during lytic infection has been observed (Doerfler *et al.*, 1974; Lee and Mak, personal communication). The site(s) at which integration occurs is not known. It is possible that those classes of DNA which are synthesized selectively are also the sites for the integration of the viral DNA.

SUMMARY

The effect of infection by adenovirus type 12 on pre-existing cellular DNA was studied in a productive system as well as in an abortive system. It was shown that infection by this virus induces the fragmentation of cellular DNA in both systems. The extent of fragmentation was dependent on both viral dose and time after infection. Fragmentation was not affected by infection with UV-irradiated virus. These results were discussed in relation to chromosome fragmentation and the mechanism by which these effects are induced.

The effect of infection by this virus on some aspects of DNA replication in human KB cells was also studied. It was shown that infection results in the early inhibition of cell DNA synthesis. This effect was accompanied by the slow ligation of newly synthesized DNA. The rate of ligation was not affected by a higher dose of virus.

The inhibition of cell DNA synthesis was shown not to be a random process. Certain classes of DNA which could be distinguished due to their high buoyant density were selectively synthesized. The selective synthesis of these classes of DNA was dependent on time after infection. This was correlated with the increased percentage of inhibition of cell DNA synthesis which is also observed with time. The

selective synthesis of high buoyant density DNA was not observed when the cells were infected with either high doses of virus or with UV-inactivated virus.

The reassociation kinetics of the newly synthesized DNA in both infected and uninfected KB cells was followed. It was observed that a greater percentage of the newly synthesized DNA from the infected cells was from the repetitive sequences.

Some postulation was made on the mechanism by which the above effects are induced in the infected cells.

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