STUDIES ON CYT MUTANT OF ADENOVIRUS TYPE 12.

BIOLOGICAL AND MOLECULAR STUDIES OF A LOW ONCOGENIC MUTANT OF HUMAN ADENOVIRUS TYPE 12

By

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SCOPE AND CONTENTS :

Physical and biological properties of a low oncogenic mutant (cyt mutant) of highly oncogenic human adenovirus type 12 were studied. The highly oncogenic parental strain contains much higher proportion of functionally defective particles than the cyt mutant. The role of defective virions was discussed in relation to oncogenicity of the viruses. Correlation of defectivity and viral DNA structure, as studied by electron microscopy, was made. Some defective virions have a specific deletion in their DNA. Also molecular mechanism for the reduction of virus yield by the cyt mutant was examined and it was found that the viral DNA synthesis was a limiting factor.

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INTRODUCTION

Viruses were defined by Lwoff and Luria, as submicroscopic entities whose genome is an element of nucleic acid, either DNA or RNA. Viruses reproduce within living cells (Luria 1967, Lwoff 1957). Viruses themselves are devoid of an energy producing system and their growth is strictly dependent on living cells. Different viruses have different host range, indicating that the virus-host relationship is specific. Viruses, consisting of genetic material enclosed in a protective coating, are the simplest entities of self-replicating units. The ability to maintain genetic continuity, with the possibility for mutation, is the only basis for considering viruses to be alive.

Up to the present time, about 600 animal viruses of various sizes and degrees of complexity are known, containing from five to several hundred genes and multiplying at different sites within the cell. The great variety in chemical composition, structure and site of viral replication, presumably reflecting differences in replicative patterns and induced cellular modifications, endows animal viruses with a unique role in dissecting cellular functions in molecular terms. In other words, these animal viruses provide important systems for elucidating the synthesis, function and regulation of macromolecules in mammalian cells. Since the mammalian cells contain as many as 10^7

genes, with thousands of different proteins being synthesized at any one time (Green 1966), it is difficult to analyze the individual biosynthetic process of these macromolecules, and their regulation at the molecular level. In contrast, viruses contain only a limited number of genes. Thus, it is technically possible to analyze in detail, the transcription and translation of virus specific macromolecules and the intracellular controls of these processes.

Virus-cell interaction

Infection of susceptible host cell by a virus results in two different types of response; productive infection and abortive infection. In productive infection which is referred to as permissive system, the virus can replicate and produce new infectious progeny. An invariant consequence of productive infection is structural and functional alterations in the host. Living cultures of infected cells often become rounded and develop an increased refractility. This cytopathic effect (CPE) is used as an index of the state of infection of a culture. One kind of morphological change occurring in infected cells is the formation of inclusion bodies. For example, poxviruses produce cytoplasmic inclusions, called factories, in infected cell. These are the site of viral DNA synthesis and of viral antigen accumulation (Cairns 1960).

The alterations in cellular metabolism are variable

depending on the virus-host cell system. For example, herpesvirus infection causes a complete cessation of host DNA synthesis, protein synthesis and a drastic modification of host RNA metabolism. On the other hand, the synthesis of cellular DNA is stimulated after infection by papovaviruses (reviewed by Tooze 1973). Induction of several enzymes involved in DNA synthesis (e.g., thymidine kinase, dCMP deaminase, DNA polymerase) is widely described for the detection of virus-induced biochemical modifications (Green 1966). In a total cell population, such events may end up with the death of the population or it may lead to an equilibrium of cell growth and cell death in the case of limited response (Belyavin 1963). However, there is an exception of this type of response. A typical example is the situation with RNA tumor viruses. The replication of RNA tumor viruses need not kill the host cell. The maturation of virus particles by budding from the plasma membrane does not usually cause CPE. Therefore cells infected with RNA tumor viruses can proliferate while producing viral progeny (Tooze 1973).

Productive infection

In general with DNA-containing viruses, replication proceeds in sequential fashion: adsorption to a specific receptor site on the plasma membrane, penetration, uncoating of the nucleic acid, synthesis of viral mRNA, translation of mRNA to early protein, replication of the viral DNA, synthesis of late protein, maturation of virus by self-assembly and release of infectious virus particles

(reviewed by Knight 1974). While the overall replication pattern may be similar, the individual biosynthetic steps may vary depending on the virus types, host cell types and their physiological state. As a consequence, yields of mature virus particles are variable.

Adenovirus multiplication

The replication of the adenoviruses has been extensively studied, mainly using adenovirus type 2 (Ad 2) in KB cells in culture by Green and his collaborators (Green 1962, 1966, 1970a). Upon entry into the nucleus, adenovirus DNA is transcribed into heterogeneous nuclear RNA of high molecular weight by host RNA polymerase (Chardonnet 1972, Ledinko 1971, Price 1972, Roeder 1970, Wallace 1972). The synthesis of viral specific mRNA can be detected by hybridization of pulse-labeled RNA with viral DNA (Bello 1969, Rose 1965, Thomas 1969). The poly-A sequence of 150 to 250 nucleotide long is post-transcriptionally added to high molecular weight RNA (Philipson 1971). Then the viral RNA is cleaved and transported to the cytoplasm and translated. During early infectious cycle (prior to the onset of viral DNA synthesis), only a portion of the adenovirus genome is transcribed (about 8 to 20 %) and translated (Fujinaga 1970). T-antigen is the most prominent early protein (Feldman 1966b, Gilead 1965).

Synthesis of viral DNA in KB cells infected with Ad 2 or Ad 5 begins in the nucleus at 6 to 7 hr after infection. The maximum rate of synthesis is reached about 13 hr post infection.

At this time the rate of host cell DNA synthesis is greatly inhibited so that more than 90 % of the newly synthesized DNA is viral (Ginsberg 1967, Pettersson 1973a). The replication of highly oncogenic group (e.g., Ad 12) is slower than that of other human adenoviruses (Mak 1968, Pina 1969).

After the onset of viral DNA replication, the transcriptional pattern is altered from that in early times. For example, (1) 80 to 100 % of the viral genome is transcribed (Fujinaga 1968), (2) most of the RNA associated with the polysome is virus specific (Raskas 1971). Late proteins including the viral structural proteins, the synthesis of which is strictly dependent on viral DNA synthesis, can be detected about 11 hr post infection with Ad 5. At about 13 to 14 hr after infection, maturation of progeny virus begins. This occurs in the nucleus where large quantities of viral protein accumulate late after infection. Arginine is an essential amino acid in this assembly process of mature virion (Rouse 1967, Russell 1968, Winters 1971).

Abortive infection

Abortive infection, in which the cell is nonpermissive for virus replication, is the other type of cellular response. Although virus can attach and penetrate into the host cell. Several virus-coded molecules are produced in varying degrees. Nonpermissive system has been known in

adenovirus infection; for example, Ad 2, Ad 7 and Ad 12 in African green monkey kidney (AGMK) cells. In AGMK cells, adenovirus can penetrate the cells and T-antigen, viral DNA and viral mRNA are synthesized(Baum 1968, Feldman 1966a, Friedman 1970, Rapp 1966, 1967). However, the synthesis of several viral proteins is not detected. Thus the translation of some late mRNA seems to be blocked in this system. Mature infectious virus particles are not produced unless they are co-infected with helper SV 40 (Feldman 1966a, Friedman 1970), with simian adenoviruses (Altstein 1968, Naegele 1967, Schlesinger1969), with Adeno-SV 40 hybrid viruses (Rowe 1965), or with the unidentified agent MAC (monkey cell-adapting component, Butel 1967). These studies are of special interest since they may furnish clues to the identification of genetic determinants which distinguish permissiveness from nonpermissiveness.

The Ad 12-hamster (BHK) cell system is a more restricted example of abortive infection. Although T-antigen is induced and at least part of the early genes are transcribed (Raska 1972, Strohl 1967), the viral DNA is unable to replicate (Doerfler 1969, 1970c). Consequently no viral structural proteins are detected and no virions are produced. Subsequently a small portion of BHK cells becomes transformed (Strohl 1970).

Cellular transformation by viruses

A special consequence of abortive infection is cell transformation. Transformation is defined as a heritable change in the properties of a cell, which is manifested by the loss of regulatory restraints of its growth potential (Black 1968). The cell transformation is most frequently recognized by changes in cell morphology, by a loss of contact inhibition, by agglutinability by plant lectins, by reduced requirement of serum for growth, and by ability to grow in agar suspension (reviewed by Tooze 1973). Thus a productive infection and cell transformation by DNA viruses are mutually exclusive.

Not only do tumor viruses affect host cell function and induce neoplastic transformation in vitro, but also they have been shown to produce tumors in vivo. The injection of the virus-transformed cells into the homologous animal also causes tumors.

The presence and continued expression of the complete viral genome or part of it in the virus-transformed cells are shown by; the appearance of new virus-induced antigens (for SV 40 (Black 1966, Diamandopouls 1968), for polyoma (Habel 1965), for adenovirus (Gilead 1965, Huebner 1964b)), the presence of virus-specific mRNA (for SV 40 (Aloni 1968, Martin 1969a, Oda 1968, Sauer 1968, Tonegawa 1970), for polyoma (Benjamin 1966, Martin 1969b), for adenovirus (Fujinaga 1966, 1967, 1969, 1970)) and some direct evidence for the presence of viral DNA (for SV 40 (Gelb 1971, Khoury 1974, Levine 1970, Ozanne 1973, Sambrook 1968, Westphal 1968), for polyome

(Westphal 1968), for adenovirus (Doerfler 1968, 1970a, Green 1970a, b, Pettersson 1973b, Sambrook 1974)). In the special case of SV 40 and polyoma transformed cells, infectious virus can be induced by fusion with permissive cells (Koprowski 1967, Watkins 1967) or by physical and chemical agents (Burns 1969, Fogel 1969, 1970, Gerber 1966), strongly indicating the persistence of an entire viral genome in transformed cells. In fact, using reassociation kinetics of defined segments of SV 40 DNA, Sambrook et al demonstrated that cells transformed by SV 40 contain complete sets of viral genes with different regions of the viral genome at different frequencies (Sambrook 1974).

Cell transformation with adenoviruses requires a large number of infectious units of virus and the frequency of transformation is low. Cells transformed by adenoviruses have a characteristic morphology (Strohl 1967). No infectious virus has been induced either by cell fusion technique with permissive cells or by treating the transformed cells with physical or chemical agents.

Cells transformed by Ad 2 contain viral DNA sequences integrated into the host chromosome (Pettersson 1973b). Pettersson and Sambrook, using DNA reassociation kinetics studies, showed that only a portion of the Ad 2 genome was present in transformed cells. Using specific Ad 2 DNA fragments generated by restriction endonucleases EcoRI and Hpa I, Sambrook et al showed that several Ad 2-transformed cell lines contain only 14 % of the total Ad 2 genome, which is mapped at the extreme left-hand end of the Ad 2 genome

(Sambrook 1974). Thus, it can be concluded that only a partial set of viral DNA sequences are required to maintain cellular transformation. Recently Graham et al have succeeded in transforming rat cells in vitro with DNA fragment, indicating that integrity of the whole viral genome is not required even for initiation of transformation (Graham 1974). They also mapped the isolated transforming segment of Ad 5 at the extreme left-hand end of the genome.

Viral transformation is governed to a great extent by two independent variables, heterogeneity in cells and viruses. The former is especially relevant to a population of primary cells. The latter is of increasing importance, since numerous recent studies have demonstrated the presence of defective particles in viral inocula. It has been shown that the defective hybrid virus SV 40-Ad 7 (PARA) can transform cells and induce tumors in vivo (Huebner 1964a, Rapp 1964, Rowe 1964). The technique of clonal analysis is one means by which the effects of cellular and viral heterogeneity can be analyzed. For example, the clonal analysis to detect heterogeneity of a transforming viral inoculum is based on the observation that hamster tumors induced by SV 40-Ad 7 hybrid virus may show either SV 40, adenovirus or mixed type morphology (Huebner 1964a).

Oncogenic human adenoviruses

It is not known at present time what properties oncogenic viruses must possess. Human adenoviruses consist of 31 serotypes,

some are oncogenic in rodents and others are not. Human adenoviruses are of particular interest since they are the first human viruses shown to possess oncogenic properties in newborn rats and hamsters (Huebner 1962, Trentin 1962), and adenovirus infections are very common in human populations as shown by the presence of antibodies directed against one or more of these viruses (Rowe 1953). Human adenoviruses have been divided into three groups on the basis of oncogenicity (Green 1970a). These are the highly oncogenic adenoviruses (Group A, types 12, 18 & 31), the weakly oncogenic adenoviruses (Group B, types 3, 7, 11, 14, 16 & 21) and the nononcogenic adenoviruses which transform rat embryo cells in vitro (Group C, types 1, 2, 5 & 6, Freeman 1967).

It has been noted that the DNA of each group of human adenoviruses has a characteristic GC content. There is a correlation between oncogenicity and the GC content of the DNA: 48-49 % GC for the highly oncogenic group A; 49-52 % for the weakly oncogenic group B; 57-59 % for the nononcogenic group C (Pina 1965). It is of interest to note that the GC content of highly oncogenic papovaviruses is 41-49 %. However, the correlation between low GC content of human adenovirus DNA and viral oncogenicity does not extend to simian adenoviruses; for example, the highly oncogenic simian adenovirus SA 7 has 58-60 % GC, while nononcogenic SV 15 has 56 % GC (Pina 1968).

Low oncogenic cyt mutant of adenovirus type 12

Viruses of the same serotype having different oncogenic potentials should be useful for understanding the mechanism of viral oncogenesis. However, the lack of substantial homology in the viral DNA sequences belonging to different groups makes it difficult to determine the viral gene functions essential for oncogenesis. By contrst, genetic aspects of the virus-cell interactions using viral mutants especially with respect to tumorigenicity should help to elucidate the viral gene functions involved in viral oncogenesis. By far the most popular class of viral mutants for the genetic analysis and physiological studies of the gene functions is the conditional lethal mutant, i.e., temperature-sensitive mutants. Ideally, genetic studies of these viruses can identify the product of each of the viral gene necessary for transformation.

A class of mutants, cytocidal (cyt) mutants have been isolated from highly oncogenic strain of Ad 12 (Takemori 1968). These mutants were spontaneously recovered from the parental virus stocks at a frequency of 0.001 %. Ultraviolet light irradiation of the parental stocks increased the proportion of the cyt mutant about five-fold. These mutants show a number of biological properties different from those of the parental strain: (1) They produce large clear plaques on primary human embryonic kidney cells, in contrast to the small fuzzy-edged plaques by the parental strain; (2) Cells infected with the mutants show a different CPE. Parental Ad 12 produces the "adenovirus-type CPE" characterized by Ginsberg as

marked rounding and aggregation of infected cells without cell lysis (Ginsberg 1962). However, the cells infected with these cyt mutants become neither rounded nor aggregated, but display early and extensive cellular destruction; (3) They are low tumorigenic in newborn hamsters, only 0-23 % of the animals injected with the cyt mutant produced tumors, while more than 80 % of the animals injected with the parental strain induced tumors; (4) They also lack the ability to transform newborn hamster kidney cells in vitro.

Similar class of mutants has been isolated by Yamamoto et al (Yamamoto 1972). Although some of the biological properties have been compared, no studies on the physical properties of the virions have been made.

This investigation was carried out to examine further the biological properties of the low oncogenic cyt mutant and to try to correlate them with the physical nature of the purified virion. It was hoped that these comparative studies of differences in viral functions between the cyt mutant and the parental Ad 12 would throw some light on the mechanisms involved in viral oncogenesis.

MATERIALS AND METHODS

I. Tissue Culture Techniques

A. Media

Media for cell cultures were purchased from Grand Island Biological Company, Grand Island, New York. Minimum Essential Medium (MEM) was used for monolayer cultures and MEM (Joklik modified) was used for suspension cultures. Routinely the media were prepared by dissolving the powdered media in glass-distilled water as directed and sterilized by filtration through a Millipore filter with a pore size of 0.22 microns.

B. Cell Lines

(1) KB cell

The cells were originally derived from human epithelial cheek carcinoma. The established line, obtained from Dr. Green, St. Louis, Missouri, was maintained either as monolayer cultures or as suspension cultures. Monolayer cultured were grown in MEM supplemented with 10 % fetal calf serum (FCS) plus 0.5 % Fungizone (GIBCO) and 0.5 % antibiotics mixture (40,000 units/ml of penicillin plus 7.5 mg ml of strepto

mycin sulphate), in 32 oz prescription screw-cap bottles. In some experiments, Falcon plastic bottles were used. The cells were kept in logarithmic growth phase by subculturing when they had become confluent. This was done by trypsinization (0.125 % trypsin in 1 % KCl and 0.44 % sodium citrate solution) and seeding about 2.5×10^6 cells in a fresh bottle. The monolayer cultures were incubated at 37°C in an atmosphere of 95 % air and 5 % carbon dioxide.

Suspension cultures were grown in MEM (Joklik modified) supplemented with 5 % horse serum plus 0.5 % Fungizone and 0.5 % antibiotics mixture in standard chemical reagent bottles with a pouring spout. The cells were kept at 37°C and constantly agitated by means of a teflon magnetic bar over a magnetic stirrer. They were kept in logarithmic growth phase at a cell density of about $2.5 - 4 \times 10^5$ cells/ml by daily dilutions using prewarmed fresh culture medium.

(2) HEp-2 cell

An established cell line derived from carcinoma of the larynx, human epidermoid cell (HEp-2 cell) was purchased from American Type Culture Collection, Rockville, Md. The cells were serially propagated as monolayer cultures as described for KB cell and maintained in MEM with 10 % FCS plus 0.5 % Fungizone and 0.5 % antibiotics mixture.

(3) HEK cell

Primary human embryonic kidney (HEK) cells were purchased from Biological Associates, Bethesda, Md., and kept as monolayer in

MEM supplemented with 10 % FCS plus 0.5 % Fungizone and 0.5 % antibiotics mixture as above.

II. Virological Techniques

A. Preparation of virus stocks

Purified viruses were used throughout this study.

(1) Human adenovirus type 12 parental strain

Human adenovirus type 12 (Ad 12, 1131 strain, Pereira 1964) was obtained from Dr. Stich, University of British Columbia, Canada. KB cells grown in suspension were infected with Ad 12 at an input multiplicity of 300 particles per cell at a concentration of 1×10^7 cells/ml. After 90 min adsorption at 37°C, the cells were diluted to a concentration of 3×10^5 cells/ml with prewarmed fresh MEM (Joklik modified) supplemented with 5 % horse serum plus 0.5 % Fugizone and 0.5 % antibiotics mixture. After 72 hr incubation, the cultures were harvested by low speed centrifugation (400 x g), and the cell pellets were resuspended in 0.01 M Tris buffer (Tris-Hydroxymethyl-Aminomethane, pH 8.1), and kept at - 70°C until purification.

Extraction and purification of the virus from the infected cells were carried out by the methods of Green and Pina (Green 1963) with slight modifications. Essentially, the virus was released from the infected cells by sonication at an ice-cold temperature. Cellular materials were further dissociated from the virus particles by three

successive homogenizations with ice-cold Freon 113 (Trifluoro-trichloro-ethane, Matheson Chemicals). Separation of the aqueous layer containing the virus from the Freon and the cellular debris was achieved by centrifugation at 1,200 x g for 3 min at 5°C. Then the virus was sedimented on a cesium chloride (CsCl) cushion having a density of 1.43 g/cc in 0.01 M Tris buffer (pH 8.1) by centrifugation using a Beckman SW 27 rotor at 20,000 rpm for 90 min at 10°C. Further purification of the virus was accomplished by two successive equilibrium centrifugations in CsCl. To do this, the virus solution was mixed with CsCl powder to give a density of 1.34 g/cc in 0.01 M Tris buffer (pH 8.1), and centrifuged at 33,000 rpm using a Beckman type 65 fixed angle rotor at 5°C. After 20 hr centrifugation, the virus was concentrated in a narrow band and was collected. The virus stock solution was diluted 7 to 8 fold with Tris-buffered saline (TBS, Winocour 1963) plus 20 % glycerol and stored at - 70°C until used. Storage under these conditions usually produced no significant loss in infectivity (Slonin 1969).

(2) Cyt mutant of Ad 12

Cytocidal mutants with low oncogenicity can be isolated from highly oncogenic Ad 12 (Takemori 1968). One of the clones isolated by Takemori et al was obtained from Dr. Stich (H 12 cyt 70). The propagation of the mutant was essentially the same as described for parental Ad 12 except for some modifications. KB cells were infected at an input multiplicity of 300 particles per cell. After 90 min adsorption at 37°C, the cells were incubated as monolayer cultures at a concentra-

tion of 2×10^7 cells per 32 oz prescription bottle in MEM plus 10 % FCS, 0.5 % Fungizone and 0.5 % antibiotics mixture. This monolayer culture minimized cellular destruction and was found to be essential for the propagation of this mutant virus. The infected cells were harvested at 40 hr after infection and the virus was purified in the same manner as described for Ad 12 parental virus.

(3) Defective virions of Ad 12

The defective lighter virions of Ad 12 (Huie strain) were separated by repeated equilibrium centrifugations in CsCl as described by Mak (Mak 1971).

(4) Radioactively labeled viruses

To obtain radioactively labeled viruses, ^{14}C-thymidine (TdR) was added to the culture at 0.05 µCi/ml (specific activity, 60 mCi/m M) at 10 hr after infection. To obtain ^{3}H-labeled virus having high specific activity, labeling schedule is as follows; 2.5 µCi/ml of ^{3}H-TdR (specific activity, 20 Ci/m M) was added to the culture at 13 hr, followed by the addition of 5 µCi/ml at 17.5 hr and another 2.5 µCi/ml of ^{3}H-TdR at 23 hr post infection. The culture conditions and purification procedures of the viruses were exactly the same as for unlabeled viruses.

(5) Bacteriophage Ø 29

Bacteriophage Ø 29 and its host, Bacillus subtilis strain 19 E were obtained from Dr. Takahashi. In order to obtain a pure clone of Ø 29, a single plaque formed on B. subtilis 19 E plate was picked. Phage from this plaque was used to infect cells grown on agar plates to produce phage Ø 29 in sufficient quantity for purification.

The purification of the phage was modified from that of Anderson et al (Anderson 1966). The crude lysates were clarified by centrifugation at 8,000 x g for 30 min at 5°C. Then the phage was sedimented from the cleared supernatant by centrifugation using a Beckman 19 rotor at 17,000 rpm for 150 min at 5°C. The phage pellets were resuspended in PBS-Mg solution (phosphate-buffered saline plus 5 mM $MgSO_4$, pH 7.5), and centrifuged again at 8,000 x g for 30 min to remove debris. Solid CsCl was added to the phage suspension to give a final density of 1.44 g/cc, and the mixture was centrifuged at 33,000 rpm for 18 hr at 5°C in a Beckman 65 rotor. The concentrated phage band was collected, and was dialyzed against 0.01 M Tris buffer (pH 8.1) and was immediately subjected to DNA extraction in a similar manner to that used for adenovirus DNA (see later section).

B. Analysis of viral functions

(1) Adsorption of virus to host cell

1×10^6 KB or HEp-2 cells were infected with purified ^3H-labeled viruses in 1 ml of infection medium (MEM (Joklik modified)

containing 1 % FCS plus 0.5 % antibiotics mixture) using a roller wheel (New Brunswick Scientific Instruments). After 90 min of incubation at 37°C, the cells and the supernatant medium were separated by low speed centrifugation (400 x g), and 0.2 ml samples of the supernatant were spotted onto nitrocellulose filters. For the cell associated fraction, the cell pellets were washed with PBS, resuspended into 0.5 ml fresh infection medium and 0.2 ml samples were added to the nitrocellulose filters. Each filter was dried at 80°C for 1 hr, and the radioactivity was counted by scintillation counter in 5 ml of toluene containing 0.02 g of Omniflour (New England Nuclear).

(2) Nuclear inclusion body formation

Standard cytological technique was used for examination of nuclear inclusion body formation in infected cell (Rainbow 1970). The cells were fixed with ethanol-acetic acid mixture (3 : 1) and stained with orcein.

(3) Cloning of cells

The technique used was modified after that of Puck and Marcus (Puck 1955). HEp-2 cells infected in suspension were seeded with appropriate dilutions on to 60 mm Falcon plastic petri dishes containing MEM supplemented with 15 % FCS, 2 % vitamin solution (GIBCO 100 x), 2 % amino acid mixture (GIBCO 100 x), 0.5 % Fungizone and 0.5 % antibiotics mixture. In addition to these, 0.3 % antiserum against Ad 12 virions from immunized rabbits (see later section) was added to prevent

reinfection by progeny viruses. The plates were incubated at 37°C in a moist chamber with 5 % CO_2 and 95 % air. On the 7th or 8th day the plates were fixed with methanol for 15 min at room temperature and rinsed with distilled water. The number of clones was scored after staining with Giemsa stain for 15 min. Under these conditions, the plating efficiency of noninfected cells varied from 90 to 100 %.

(4) Virus yield analysis

Amount of ^{3}H-thymidine incorporation into complete virions was used as a measure of virus yield of an infected culture. KB, HEp-2 or HEK cells were infected in suspension at an input multiplicity of 100 virus particles per cell, and incubated as monolayer cultures at 37°C. At 12 hr after infection, ^{3}H-TdR was added to the culture at a final concentration of 10 μCi/ml (specific activity, 4.7 μCi/μg), and labeling was allowed to proceed continuously. This large amount of ^{3}H-TdR used was to ensure the labeling of all virions and to minimize differences in pool size of different cultures. At 40 or 72 hr after infection, the cells were harvested, washed with PBS and lysed by 0.5 % sodium deoxycholate (DOC) for 30 min at room temperature. The resulting mixture was then extracted with an equal volume of ice-cold Freon 113. The extract was analyzed for virus content using rate zonal centrifugation together with ^{14}C-labeled Ad 2 virion as a marker. 0.5 ml samples were loaded on top of a 5 - 30 % linear sucrose gradient in PBS, pH 7.35 and centrifuged in a Beckman rotor SW 27.1 at 24,000 rpm for 35 min at 5°C. After centrifugation the fractions were collected from the bottom

of the tube. To each fraction an equal volume of ice-cold 10 % trichloroacetic acid (TCA) was added and the precipitate was filtered onto nitrocellulose filters, and washed with TCA. After drying the filters, the radioactivity was determined by liquid scintillation counting. Radioactivity sedimented in the position of marker (^{14}C-Ad 2 virion) was taken as an estimate of virus yield.

III. Tumor Induction

All operations were done as aseptically as possible. Purified virus preparations to be analyzed for tumorigenicity were dialyzed against PBS to remove CsCl. 1×10^9 virus particles in 0.1 ml PBS were injected subcutaneously into newborn hamsters using a 26-gauge needle. The animals were usually examined everyday up to seven months for tumors.

IV. Immunological Techniques

A. Source of antisera

(1) Anti T antiserum

Antiserum against T-antigen was obtained from tumor bearing hamsters. Tumors were induced by injection of purified virus preparations into newborn hamsters as described above. The tumor bearing hamsters were anesthesized with ether and the blood was collected by

Puncturing the heart with a 21-gauge needle. After storing overnight at 4°C, the serum was separated from the blood clot by centrifugation and stored at - 20°C until used.

(2) Anti V antiserum

Antiserum against adenovirus structural proteins (V-antigen) was obtained by immunizing rabbits with purified Ad 12. One part of 1.5×10^{11} virus particles of Ad 12 (in PBS) was mixed with one part of adjuvant (Bacto Adjuvant, Complete Freund, Difco Lab.), and 1 ml of this virus suspension was injected into a rabbit intraperitoneally and another 1 ml was injected intramuscularly at weekly intervals. On the 5th or 6th week, the animals were sacrificed and the serum was collected.

B. Indirect immunofluorescence techniques

2×10^5 infected HEp-2 cells were grown on cover slips in Leighton tubes as a monolayer. At different times after infection, the cells were washed three times with PBS, air-dried and fixed with carbon tetrachloride for 30 min at room temperature. Following three more rinses in PBS, the cells were incubated with anti T antiserum or anti V antiserum for 1 hr at 37°C in a moist chamber. Excess antisera were rinsed out with PBS. For the detection of T-antigen, the cells were incubated at 37°C with fluorescein-conjugated anti hamster globulin (rabbit origin, purchased from Roboz Surgical Instrument Co. Inc., Washington D.C.). For V-antigen, the cells were incubated with

fluorescein-conjugated anti rabbit globulin (sheep origin, purchased from Roboz Surgical Instrument Co. Inc.). Following a one hr incubation at 37°C, the cells were rinsed thoroughly with PBS and mounted with buffered-glycerin (9 parts glycerin and 1 part PBS). The slides were immediately examined for fluorescence under a fluorescent microscope.

V. Rate Zonal Sedimentation Techniques

Molecular size of viral DNA synthesized in infected cells was analyzed by rate zonal centrifugation. Virus-infected KB cells were labeled with ^{3}H-TdR at 20 μCi/ml (specific activity, 4.5 μCi/μg), either continuously from 13 to 40 hr after infection or with a short pulse for one hr at 23 hr post infection. After exposure to radioisotope, the cells were harvested by low speed centrifugation, followed by washing with PBS, resuspended in a small volume of PBS and were subjected to sedimentation analysis as described below.

A. Alkaline sucrose gradient sedimentation

The size of single-stranded DNA molecule was assayed by alkaline sucrose gradient sedimentation. To avoid shearing by pipetting the infected cell samples were lysed on top of a gradient (described below) with 0.5 % NaOH containing 10 mM EDTA, 0.1 M NaCl and 1 % SDS, together with purified ^{14}C-labeled Ad 2 virion as a marker, for 6 hr at room temperature. The lysate was centrifuged in a 5 to 20 % linear

alkaline sucrose gradient containing 0.3 M NaOH, 2 mM EDTA, 0.1 M NaCl and 0.1 % SDS, at 24,000 rpm for 13 hr at 20°C in a Beckman SW 27.1 rotor. About 40 fractions were collected from the bottom of the tube, and after neutralized with HCl, the radioactivity was determined in 10 ml of xylol-Triton X-114 mixture containing 0.05 g of Omniflour as described by Anderson et al (Anderson 1973). In some experiments the radioactivity in each fraction was determined by cold TCA precipitation and collection onto nitrocellulose filter.

B. Neutral sucrose gradient sedimentation

In a similar way, the size of double-stranded DNA molecule was analyzed in a 5 to 20 % linear sucrose gradient containing 0.01 M Tris buffer, pH 7.1, 0.15 M NaCl, 2 mM EDTA and 0.1 % SDS. In order to release the viral DNA from the infected cells, the cell suspension was treated for 2 hr at room temperature with lysing solution consisting of 1 x SSC (0.15 M NaCl plus 0.015 M sodium citrate), 5 mM EDTA, 1.5 % SDS and 200 µg of pronase (B grade, Calbiochem, Los Angeles, Calif., self predigested for 3 hr at 37°C). ^{14}C-Ad 2 marker was also added at the time of lysis. Centrifugation was at 23,000 rpm for 12.5 hr at 20°C in a Beckman SW 27.1 rotor. Fractionation and determination of radioactivity were carried out as described for single-stranded DNA.

VI. Molecular Hybridization Techniques

A. Preparation of DNA

(1) Viral DNA

Viral DNA was extracted from purified preparations of Ad 12 according to the procedure of Green and Pina (Green 1964). After the purified virus stock was dialyzed against 0.01 M Tris buffer (pH 8.1) to remove CsCl, the following were added to 2 ml of virus suspension; 0.15 ml of 0.05 M EDTA (pH 7.0), 0.15 ml of 1.0 M NaH_2PO_4 (pH 6.0), 0.1 ml of 10 % SDS and 40 µl of pronase (20 mg/ml). The sample was incubated at 37°C for 1 hr, and the viral DNA was extracted three times with distilled phenol at 4°C. The DNA solution was dialyzed thoroughly against several changes of 0.1 x SSC at 4°C.

(2) Cellular DNA

KB cells infected with either the parental strain or the cyt mutant of Ad 12 were labeled with 10 µCi/ml of ^{3}H-TdR (specific activity, 4.7 µCi/µg) for 2 hr at desired times after infection. After labeling, the cells were harvested and whole cell DNA was extracted by the method described by Mak (Mak 1969). The cell pellets were washed with PBS, resuspended in extraction buffer (1 x SSC, 0.1 M Tris buffer, pH 8.0, 0.3 M sodium trichloroacetate and 5 mM EDTA) and lysed by 0.5 % SDS for 10 min at 37°C. The lysate was then digested with 400 µg/ml of pronase at 37°C for 1 hr and extracted

three times with phenol. The aqueous layer was removed and precipitated by the addition of two volume of cold ethanol. The precipitate was collected by centrifugation at 8,000 x g for 15 min at 5°C. The sample was subsequently treated with pancreatic RNase (Worthington Biochem. Corp., 3 x cryst., preheated to 80°C for 10 min) at a concentration of 50 µg/ml for 30 min at 37°C, and was deproteinized by phenol extraction as before. The aqueous layer was further extracted with ether and the DNA solution thus obtained was dialyzed against 0.1 x SSC at 4°C with several changes.

B. DNA-DNA hybridization

The hybridization procedure was essentially based on the method described by Warnaar and Cohen (Warnaar 1966). Adenovirus DNA was denatured by heating at 100°C for 10 min in 0.1 x SSC and quickly chilled in an ice bath. The DNA solution was then adjusted to 2 x SSC and aliquots were filtered onto nitrocellulose filters (pore size of 0.45 µ). DNA was immobilized to the filters by baking at 80°C for 2 hr.

The isotopically labeled DNA samples to be analyzed for viral DNA were diluted in 0.1 x SSC and fragmented by sonication. The DNA was subsequently denatured by boiling, followed by a rapid cooling in an ice bath. The denatured DNA solution was immediately adjusted to 2 x SSC containing 0.1 % SDS, and a 1 ml of sample was placed in a vial containing DNA immobilized on a filter. The hybridization was

allowed for 20 hr by incubating at 65°C. The radioactivity bound to the filter was measured after exhaustive washing the filter with 0.1 x SSC containing 3 mM Tris buffer (pH 9.4) and drying at 80°C for 1 hr. The extent of DNA-DNA hybridization was determined by the radioactivity bound on the filter divided by the input radioactivity.

VII. Visualization of DNA Molecule by Electron Microscopy

Electron microscopy of DNA molecule was carried out according to the methods of Davis et al (Davis 1971b).

A. Mounting DNA by formamide technique

In the formamide technique, the DNA is mounted at a formamide and salt concentration such that double-stranded DNA is stable and single-stranded DNA appears as a stretched form which can be distinguished from double-stranded DNA. 40 µl of DNA sample was mixed with 10 µl of 0.05 % cytochrome c (Horse Heart, Type III, Sigma), 40 µl of formamide (Matheson, Coleman & Bell) and 10 µl of 10 x Tris EDTA (1 M Tris buffer plus 0.1 M EDTA, pH 8.5). 50 µl of this spreading solution was slowly applied across a slide glass ramp in a 90 mm square plastic petri dish containing 50 ml of hypophase solution consisting of 10 % formamide in 0.1 x Tris-EDTA. After the solution ran down the slide and the film spread on the hypophase solution, the film was allowed to stand for 30 to 60 seconds. The film was then picked up near the

slide-solution boundary by touching a copper grid (300 mesh) freshly coated with 0.5 % (w/v) Parlodion (Ladd Research Industry Inc., Vermont). The grid with the film was dipped into 1 x 10^{-4} M uranyl acetate (in 95 % ethanol) and stained for 30 seconds, then rinsed for 10 seconds in isopentane.

To increase the contrast of the final image, the rotary shadowing technique with heavy metal was applied. The specimen was rotated at a rate of 60 rpm with an angle of 5° at a distance of 4 cm from the heavy metal to be evaporated. 3 cm of Pt-Pd wire (Platinum(80 %)-Palladium(20 %), 0.2 mm diameter, Ernest F. Fullam Inc., N.Y.) was supported by precleaned tungsten wire (0.5 mm diameter, Electron Microscopy Science, Fort Washington, Pa.), and evaporation was achieved under vacuum of 10^{-5} torr in an Edwards vacuum evaporator.

B. DNA length measurement

The shadowed specimen was observed under the Philips EM 300 electron microscope using 20 μ objective aperture and 60 KV accelerating voltage, at step 4 magnification (calibrated as 5,372 times on the film). Micrographs were taken on Kodak EM films (# 4489). For measurement of the contour length of DNA molecule, the electron micrograph negatives were enlarged about 16 times by projecting on a screen using a Beseler slide projector, and the molecule was traced on tracing paper. Length was measured with a Keuffel and Esser 62 0300 map measurer

C. Heteroduplex formation

For the formation of heteroduplex molecules, phenol-extracted viral DNA was used. 14 µl each of two different adenovirus DNA (5 µg/ml) was mixed with 10 µl of 1 M Tris buffer (pH 8.0) and 4 µl of 0.25 M EDTA. To this DNA solution, 4 µl of 3 N NaOH was added and the denaturation proceeded for 10 min at room temperature. The resulting solution was neutralized with 4 µl of 3 N HCl. 50 µl of formamide was added to a final concentration of 50 %, and the DNA was renatured at 37°C for 15 hr. Examination of heteroduplex molecules was carried out according to the method described in the previous sections A and B.

RESULTS

I. Comparative Study of Cyt Mutant

A. Growth

It is generally necessary to use a purified virus preparation to study the properties and functions of the virus. Some biological functions have been studied by Takemori et al using crude virus stocks, i.e., lysates from cells infected with cyt mutants (Takemori 1968). However, such crude lysates contain components other than virus particles which may give nonspecific biological effects such as cytopathic effect (CPE). In this investigation, the physical and biological functions of the cyt mutant were studied using highly purified virus preparations.

Attempts to obtain highly purified virus stock of the cyt mutant using KB cells in suspension culture were not successful due to early cell lysis. Cell number in a culture infected with the cyt mutant began to decrease at about 24 hr after infection, leading to complete cell lysis by 72 hr. This early and remarkable cellular destruction confirms the cytocidal nature of this mutant as reported by Takemori et al (Takemori 1968).

It is possible that vigorous mechanical agitation in suspension culture coupled with fragility of the infected cells led to cell

lysis. But this could be avoided by infecting the cells in suspension and subsequently incubating them as monolayer cultures to minimize mechanical forces. The cyt mutant virus stock could then be obtained in highly purified form by repeated isopycnic gradient centrifugation in cesium chloride (CsCl) as described in Materials and Methods.

B. Yield of the virus

Amount of virus particles produced in KB cells infected with either adenovirus type 12 (Ad 12) parental strain or the cyt mutant was compared. The number of virus particles was determined by the absorption measurement at 260 nm. Table 1 summarizes the yields of several purified virus preparations. At a relatively low multiplicity of infection (m.o.i.), i.e., 200 or 300 virus particles per cell, KB cells infected with the cyt mutant produced only 10 to 18 % of that produced by parental virus infection. At m.o.i. of 3,000 particles per cell, however, cells infected with Ad 12 parental strain produced reduced virions; about 1/5 of that produced at low m.o.i.. This low yield at high m.o.i. is probably due to interference by defective particles present in the virus preparation. Nevertheless, the yield of the mutant does not seem to be significantly affected by input multiplicity, and it can be concluded that the yield of the cyt mutant virion is lower than that of the parental strain.

The low yield of the cyt mutant may be due to a poor recovery in the purification procedure, or due to the production of fewer virions

TABLE 1

Amount of Virions Produced by KB Cells after Infection with Ad 12 Parental Strain and Cyt Mutant

Virus	M.O.I. particles per cell	Virions produced per 10^7 cells [a]	
		Passage 1	Passage 2 [b]
Parental	200	-	8.0×10^{10}
	300	6.0×10^{10}	9.0×10^{10}
	3,000	-	1.8×10^{10}
Cyt mutant	200	-	1.2×10^{10}
	300	1.1×10^{10}	9.0×10^{9}
	1,500	-	8.4×10^{9}

a. Determined by optical density at 260 nm
1 OD = 3.5×10^{11} virions/ml (Mak 1971)

b. Passage 2 resulted from cells infected with purified virions from passage 1

by the cells infected with the cyt mutant. To distinguish these alternatives, the following method of estimating the total amount of virions from infected cells without purification was employed. The intracellular virus was labeled with ^{3}H-thymidine (TdR) and quantitated by rate zonal sedimentation after the virus was dissociated from the infected cells. Sufficient ^{3}H-TdR (2 μg/ml) was added to ensure its continued availability and to minimize the possible differences in precursor pools in different infected cultures. The amount of radioactivity sedimented in the position of complete virions in a sucrose gradient should give an estimate of the quantity of virions produced. Figure 1 shows typical profiles of ^{3}H-activity associated with virions from KB cells infected with the parental and the mutant viruses. ^{3}H-activity in parental virus infected cells gave a sharp band corresponding to complete marker virions (^{14}C-Ad 2); however, the radioactivity co-sedimenting with marker virus was much reduced after cyt mutant infections (Figure 1b). Thus these data are consistent with the results shown in Table 1 when total purified virions were determined by the absorption measurement.

C. Different methods of releasing virus from infected cells

It is still possible, however, that the mutant virions are more easily disrupted by sonication and fluorocarbon treatment during extraction procedure. Therefore, various methods of releasing the virus from infected cells were compared. Sodium deoxycholate has been

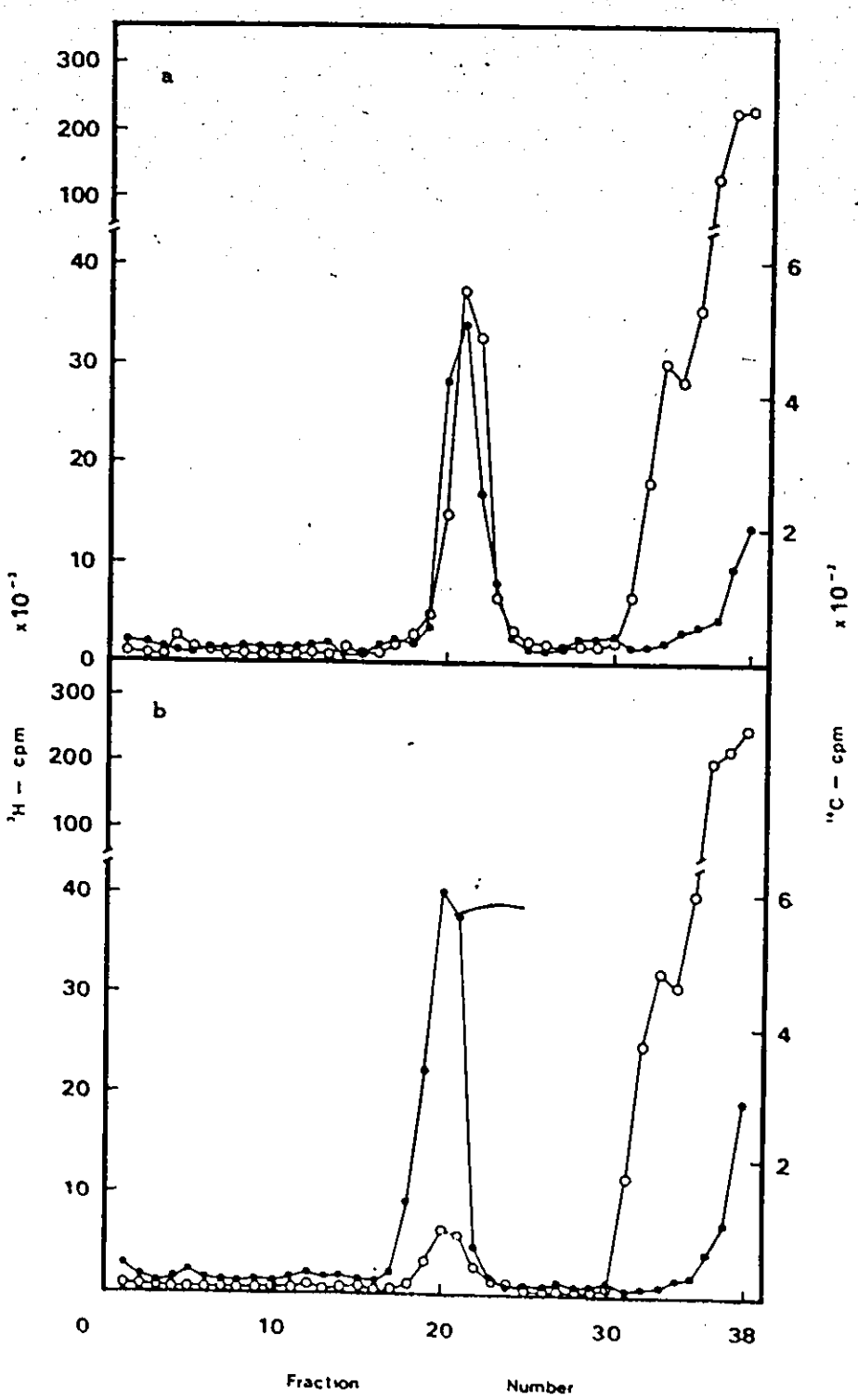
FIGURE 1

Sedimentation Profile of Lysates from KB Cells Infected with Ad 12 Parental Strain and Cyt Mutant in a 5 to 30 % Sucrose Gradient

Sedimentation is from right to left.

(a) KB cells infected with Ad 12 parental strain (○)
^{14}C-Ad 2 virions as marker (●)

(b) KB cells infected with cyt mutant (○)
^{14}C-Ad 2 virions as marker (●)


a
b
300
200
100
40
30
20
10
0
6
4
2
x10⁻³
³H – cpm
¹⁴C – cpm
0
10
20
30
38
Fraction Number

reported to be a good reagent for releasing the virus withour inactivating the infectivity of the virus and it has been used instead of sonication for purification of adenovirus type 5 by Lawrence and Ginsberg (Lawrence 1967). Infected cells were labeled with ^{3}H-TdR between 12 and 40 hr after infection. The amount of radioactivity from the cell lysates sedimenting as complete virions in sucrose gradients using different releasing methods is shown in Table 2. It can be seen that longer sonication time increased the radioactivity in the virion band and that a sonication time of greater than 90 seconds was sufficient to release maximum amount of virus from the cells. This table also shows that virus recovery from the infected cells was similar whether the cells were disrupted by sonication plus fluorocarbon treatment, deoxycholate alone, or deoxycholate plus fluorocarbon treatment. Therefore the reduced yield is not due to a selective damage of the cyt mutant virions by the method using sonication and extraction with fluorocarbon.

Due to the cytocidal nature of the mutant, virions may be released into the medium during prolonged incubation. In order to examine this possibility, the amount of extracellular virus was determined by ^{3}H-TdR labeling of the infected cells, sedimentation of the extracellular virions onto a CsCl cushion, and subsequent CsCl density equilibrium centrifugation of the virus fraction. The radioactivity in the virion band was taken as an estimation of the amount of virions present in the medium. Table 3 shows that only a small percentage of the total virions were found extracellularly both at

TABLE 2

Amount of Virus Recovered from KB Cells Infected with Cyt Mutant by Different Methods of Extraction

Time of sonication (seconds)	Methods of extraction	Total cpm in virion band
30	fluorocarbon	5,800
60	fluorocarbon	5,400
90	fluorocarbon	8,300
120	fluorocarbon	10,000
0	sodium-deoxycholate	9,000
0	sodium-deoxycholate plus fluorocarbon	10,200

a. Cell lysate prepared by indicated method was centrifuged in a 5 to 30 % linear sucrose gradient and the radioactivity sedimented in the position of complete virion was taken (see Materials and Methods).

TABLE 3

Amount of Virions Released into the Culture Medium from KB Cells Infected with Ad 12 Parental Strain and Cyt Mutant

Time after infection (hr)	Total cpm in virion band x 10^{-3} / 1 x 10^{7} cells				Total yield ratio $\frac{\text{cyt mutant}}{\text{parental}}$
	Parental strain		Cyt mutant		
	cell-associated[a]	in the medium[b]	cell-associated[a]	in the medium[b]	
40	760	1	155	8.5	0.24
72	1,100	2.5	195	13.5	0.19

a. Infected cells were lysed by DOC and after extraction with Freon, the lysate was analyzed by sucrose gradient sedimentation (see Materials and Methods).

b. The supernatant medium separated from cells was sedimented onto a CsCl cushion using a Beckman SW 27 rotor at 20,000 rpm for 90 min at 10°C. Then the virus containing fraction was analyzed by equilibrium centrifugation in CsCl (density of 1.34 g/cc), at 33,000 rpm for 20 hr at 5°C using a Type 65 rotor.

40 and 72 hr after infection under the experimental conditions. For the parental strain less than 0.2 % of the virus was released into the culture medium and for the cyt mutant infection the culture medium contained less than 7 %. Accordingly there is no serious loss of virions due to leakage from the cells into the medium. This experiment also shows that the total virus yield ratio of the cyt mutant to the parental strain does not change much from 40 to 72 hr post infection. It can be concluded that KB cells infected with the cyt mutant actually produce fewer virions than the cells infected with the parental strain.

D. Virus yield from different human cells

Since it has been reported that some cyt mutants can not grow in a subline of KB cell (KB-1, Takemori 1969), whether this reduced yield was due to the lower degree of permissiveness in our KB cell was examined. Three different human cells were chosen to compare the yield of the cyt mutant virion. KB, HEp-2 and HFK cells were infected with either the cyt mutant virus or the parental strain and labeled with ^{3}H-TdR, and the amount of virions produced was determined by sucrose gradient centrifugation as before. Figures 2 and 3 show sedimentation profiles of the extracts from infected HEp-2 and HFK cells respectively. As can be seen, sedimentation profiles are very similar to that of KB cells in both cases and the cyt mutant infected cultures gave lower radioactivity in the virion peak. The relative virus yields are shown in Table 4. Different cell lines

FIGURE 2

Sedimentation Profile of Lysates from HEp-2 Cells Infected with Ad 12 Parental Strain and Cyt Mutant in a 5 to 30 % Sucrose Gradient

Sedimentation is from right to left.

(a) HEp-2 cells infected with Ad 12 parental strain (○)
^{14}C-Ad 2 virions as marker (●)

(b) HEp-2 cells infected with cyt mutant (○)
^{14}C-Ad 2 virions as marker (●)

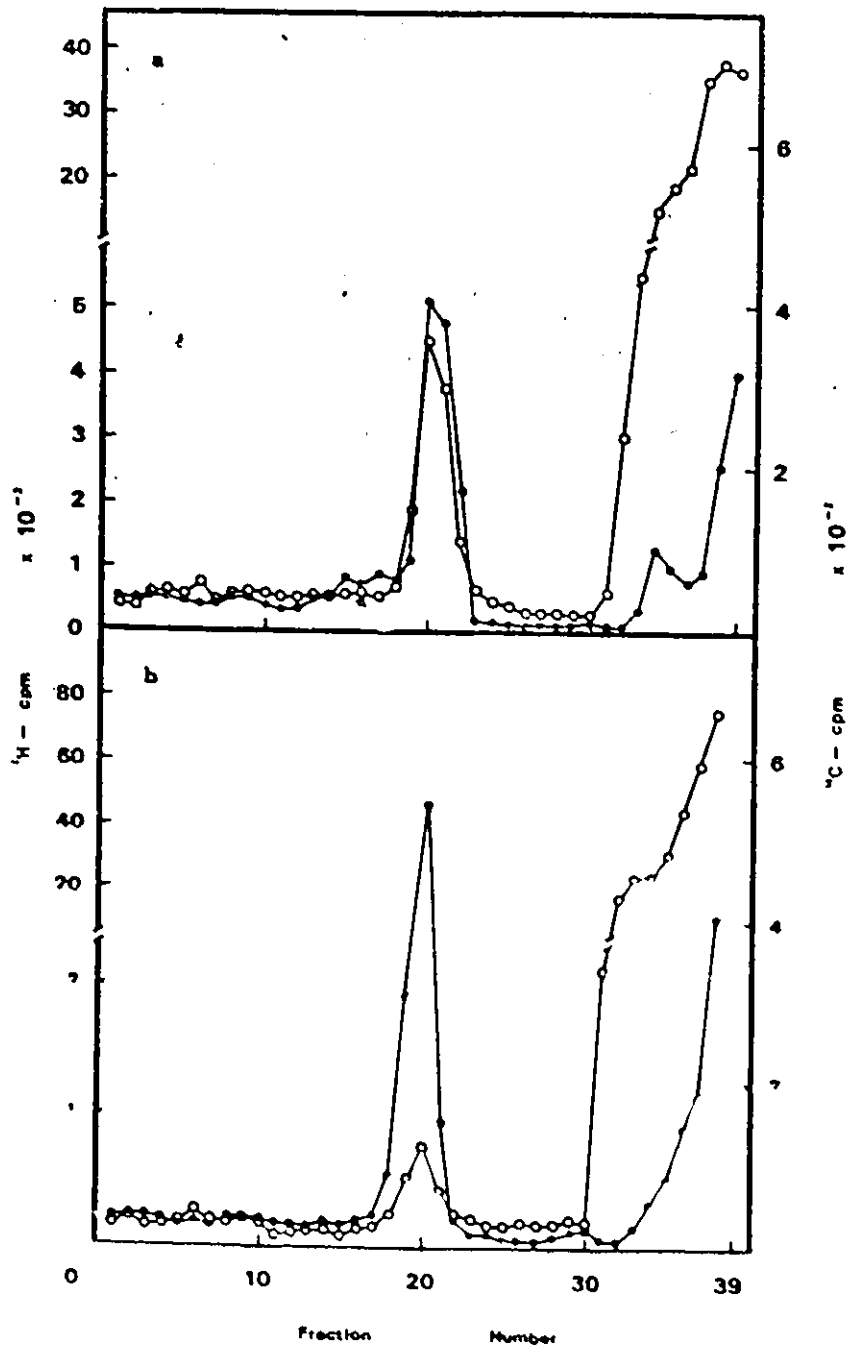

a
b
40
30
20
6
5
4
3
2
1
0
x 10⁻²
80
60
40
20
³H – cpm
¹⁴C – cpm
0
10
20
30
39
Fraction Number

FIGURE 3

Sedimentation Profile of Lysates from HEK Cells Infected with Ad 12 Parental Strain and Cyt Mutant in a 5 to 30 % Sucrose Gradient

Sedimentation is from right to left.

(a) HEK cells infected with Ad 12 parental strain (○)
^{14}C-Ad 2 virions as marker (●)

(b) HEK cells infected with cyt mutant (○)
^{14}C-Ad 2 virions as marker (●)

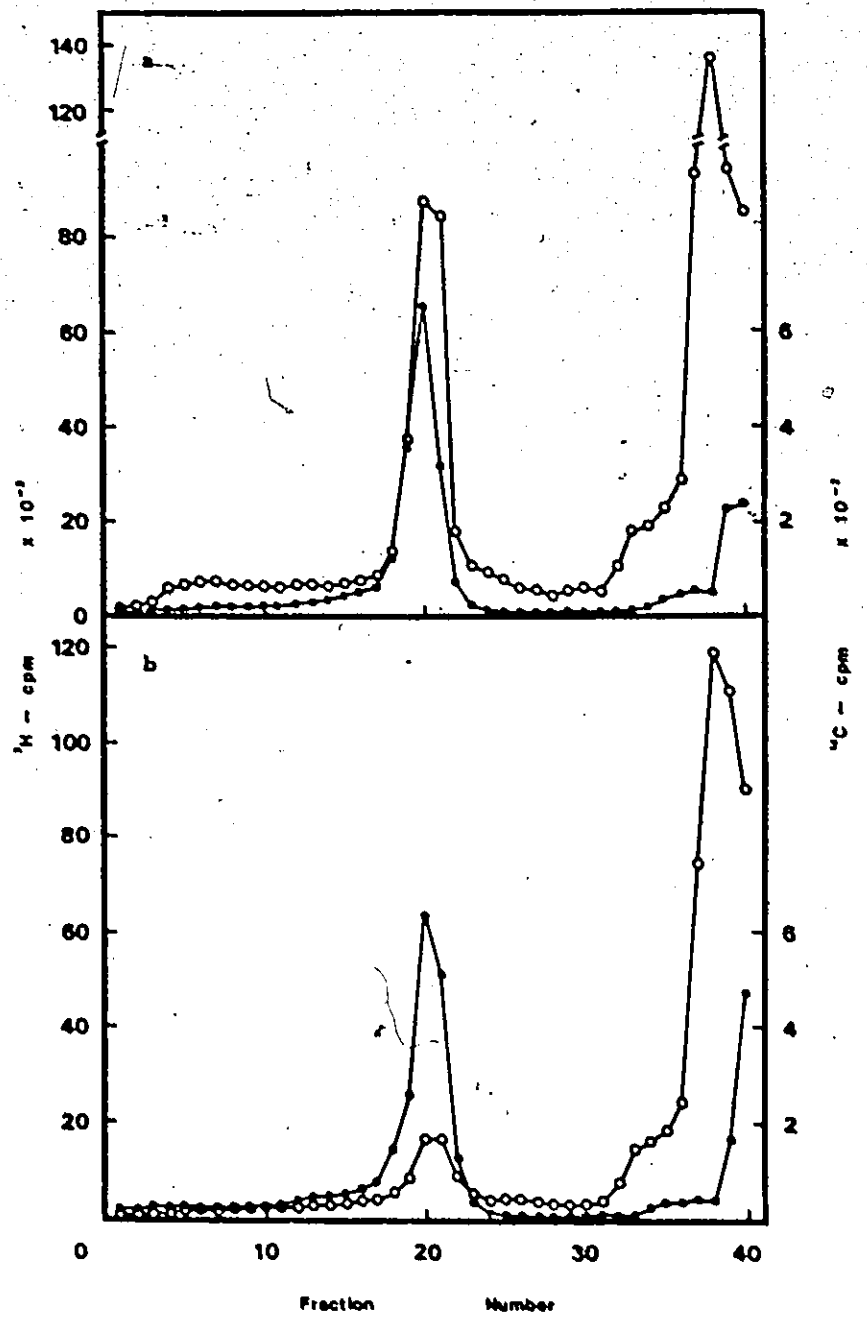

a
b
140
120
80
60
40
20
0
6
4
2
x 10⁻³
x 10⁻⁴
³H – cpm
¹⁴C – cpm
100
10
30
Fraction Number

TABLE 4

Amount of Virions Produced by Different Human Cell Lines and HEK Cells after Infection with Ad 12 Parental Strain and Cyt Mutant

Cell types	Total cpm in virion band x 10^{-3} per 1 x 10^{7} cells [a]		Yield ratio $\frac{\text{cyt mutant}}{\text{parental}}$
	Parental Ad 12	cyt mutant	
KB	340	70	0.21
HEp-2	50	13	0.26
HEK	897	203	0.23

a. Radioactivity in the virion band which cosedimented with ^{14}C-Ad 2 marker virion by sucrose gradient sedimentation (Figures 1, 2, & 3)

gave different quantities of radioactivity associated with complete virions, probably reflecting a difference in production of the virions, uptake of the radioisotope or a combination of the two among the cells. However, it can be seen that there is a similar reduction in virus yield in cyt mutant infected cultures independent of cell types used. Thus, it appears that the reduced production of complete virions in cyt mutant infection is probably a function of the virus itself and this is derived from "cyt mutation".

E. Properties of virions

(1) Physical properties of virions

Density of virus particles was compared in a CsCl density gradient (Figure 4). Under the experimental conditions used, a difference of 5 % in DNA to protein ratio could be detected; thus, the ratio of DNA to protein in the virion is probably the same for both parental strain and cyt mutant virions.

The sedimentation profile of cyt mutant viral DNA in an alkaline sucrose gradient as well as in a neutral sucrose gradient was identical to that of the parental viral DNA (Figures 5 & 6), indicating that the molecular size of viral DNA as both double-stranded and single-stranded form is identical in the two viruses. A difference of 10 % in the DNA size may be detected by this technique. However, a more sensitive technique of DNA length measurement using an electron microscope showed that there was no difference in the size

FIGURE 4

Distribution of Purified ^{3}H-Ad 12 Virions in a Cesium Chloride Density Gradient

Density of the gradient increases from right to left.

(a) Parental virions (○)

^{14}C-Ad 2 virions as marker (△)

(b) Cyt mutant virions (●)

^{14}C-Ad 2 virions as marker (△)

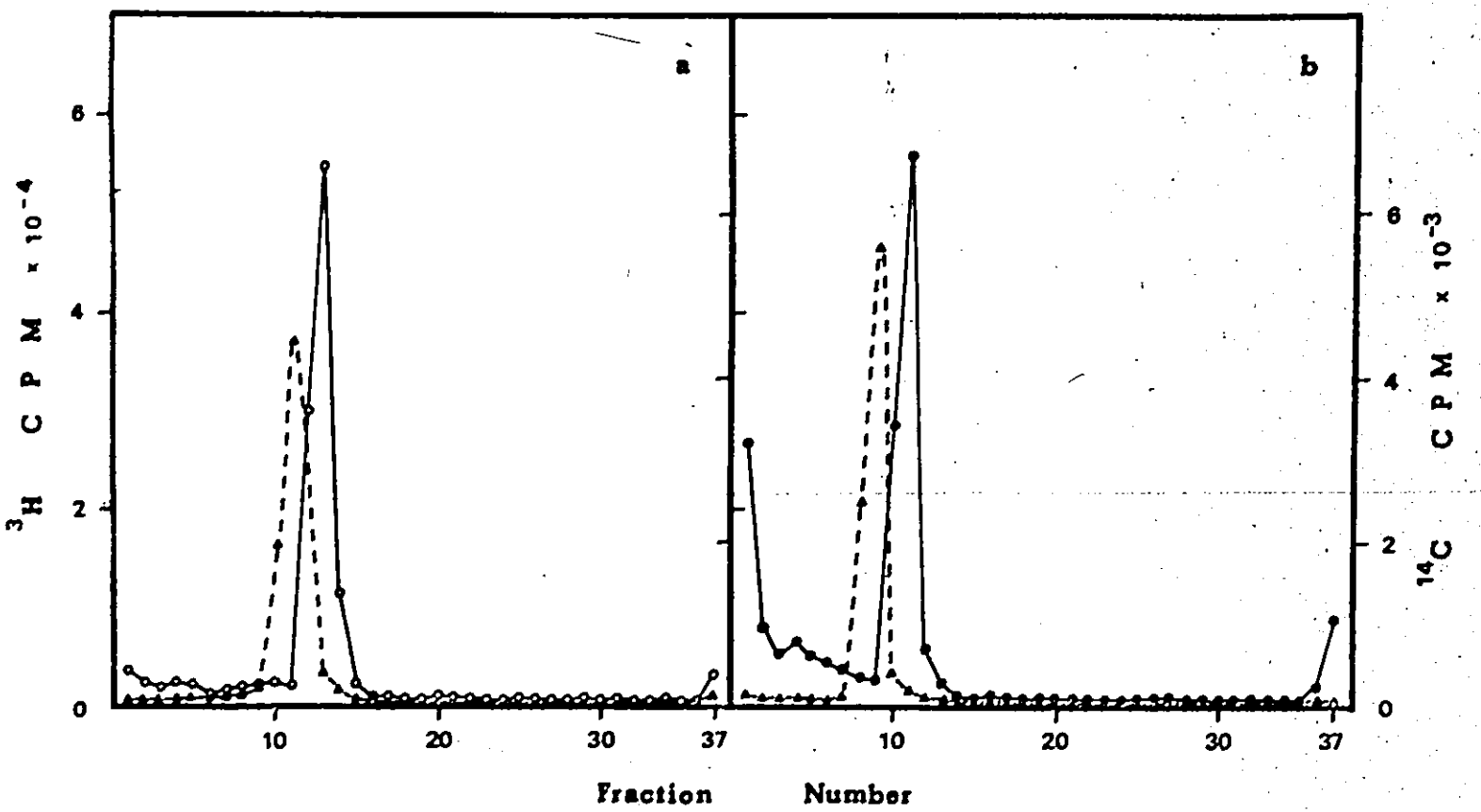

a
b
^3H CPM x 10^{-4}
^{14}C CPM x 10^{-3}
0
2
4
6
10
20
30
37
Fraction Number

FIGURE 5

Sedimentation Profile of Viral DNA in a 5 to 20 % Alkaline Sucrose Gradient

Sedimentation is from right to left.

(a) Ad 12 parental strain (○)

^{14}C-Ad 2 marker DNA (●)

(b) Cyt mutant (○)

^{14}C-Ad 2 marker DNA (●)

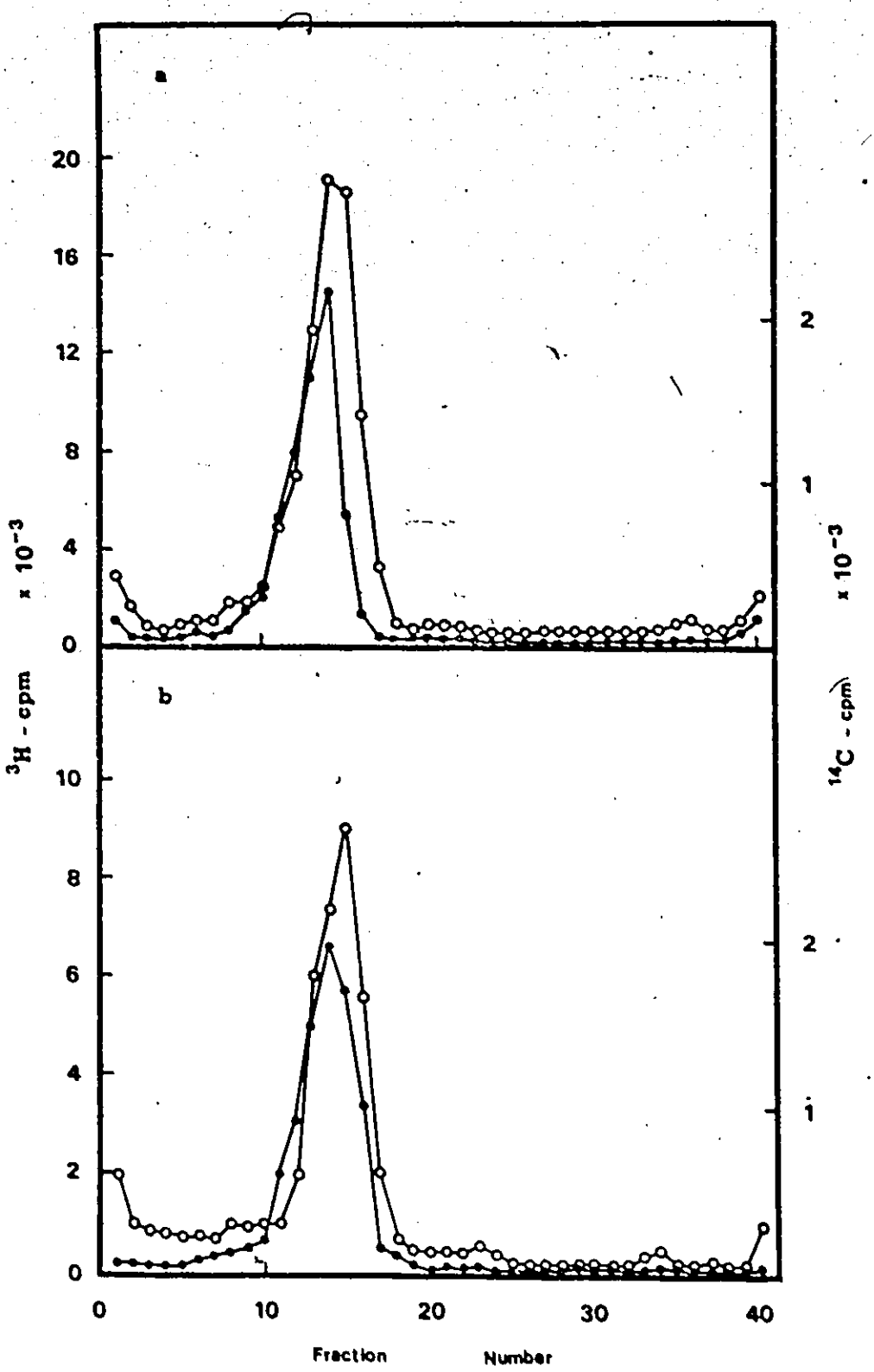

a
b
20
16
12
8
4
0
2
1
^{3}H - cpm x 10^{-3}
^{14}C - cpm x 10^{-3}
10
8
6
4
2
0
2
1
0
10
20
30
40
Fraction Number

FIGURE 6

Sedimentation Profile of Viral DNA in a 5 to 20 % Neutral Sucrose Gradient

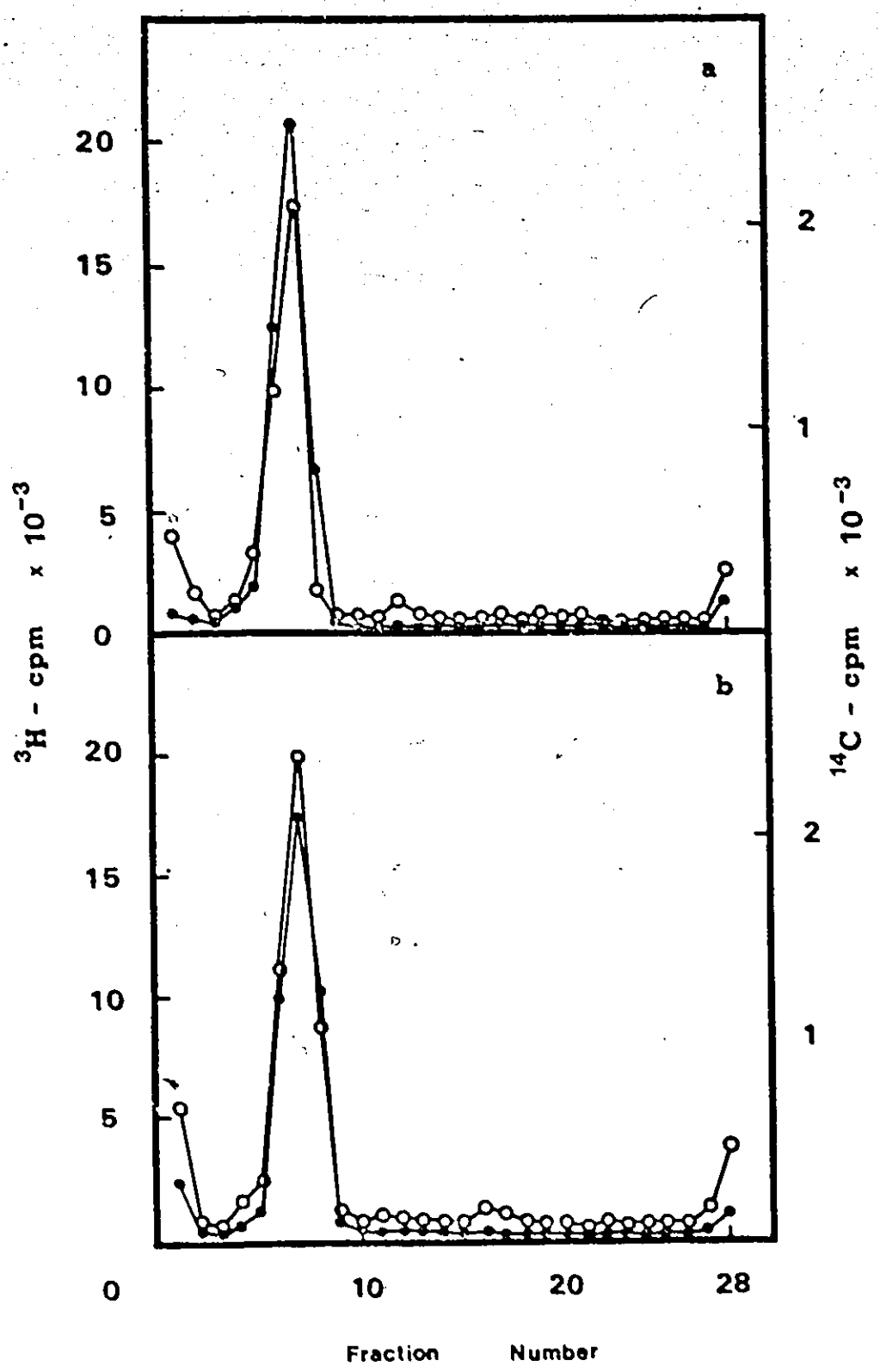
Sedimentation is from right to left.

(a) Ad 12 parental strain (○)

^{14}C-Ad 2 marker DNA (●)

(b) Cyt mutant (○)

^{14}C-Ad 2 marker DNA (●)


a
b
20
15
10
5
0
2
1
^{3}H - cpm x 10^{-3}
^{14}C - cpm x 10^{-3}
0
10
20
28
Fraction Number

of the DNA molecules (see later section).

(2) Biological properties of virions

(i) Cytological studies of infected cell

Cells infected with adenovirus display various degrees of nuclear change at various times after infection. For example, mid stage-infected cells are characterized by the appearance in the nuclei of electron dense inclusion bodies or condensation of chromatin materials. Later, the chromosomes are contracted and pulverized. Ginsberg described that characteristic intranuclear changes occur culminating in the formation of basophilic masses and development of crystalline, Feulgen-staining structures which are composed of virus particles (Ginsberg 1962).

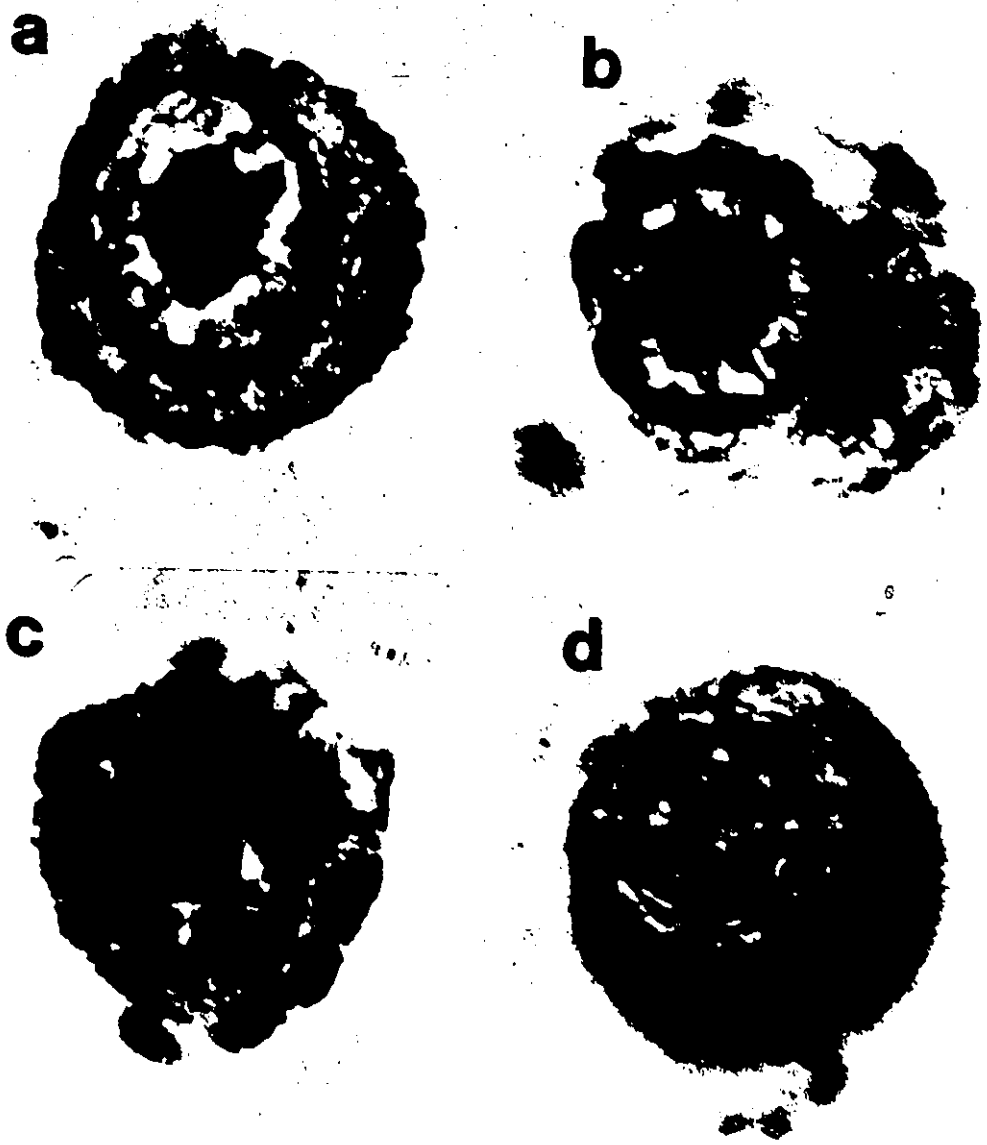
In this study, KB cells infected with Ad 12 parental strain and the cyt mutant were examined under a light microscope. Figure 7 demonstrates examples of orcein-stained cells. KB cells infected with the parental strain produced "typical" nuclear inclusion bodies characterized as dense condensation of chromosomal material centered in the nucleus of infected cells (Figure 7a,b). In contrast, cells infected with the cyt mutant showed morphologically different nuclear aggregation. Some of them were densely stained materials of which size was small and irregularly distributed in the nucleus (Figure 7c), or some cells had regions of nuclear condensation dispersed along the periphery of nuclear membrane (Figure 7d). This morphological

FIGURE 7

Photographs of Nuclear Inclusion Bodies in KB Cells Induced by Infection with Ad 12 Parental Strain and Cyt Mutant

(a),(b) KB cells at 40 hr after infection with parental strain

(c),(d) KB cells at 40 hr after infection with cyt. mutant


a
b
c
d

difference in nuclear change of the infected cells seen at the light microscopic level was more obvious in the fine details revealed in electron microscope observation of the ultrathin section of the cells (Figure 8). In the nucleus of parental virus infected cell, a big chromosomal condensation can be observed as an electron dense nuclear material (Figure 8a). An array of crystalline formation of matured virions and empty capsids are demonstrated in thin section specimen (Figure 8b). On the contrary, in cyt mutant infected KB cell, nuclear alteration is different from that made by parental virus infection (Figure 8c). So far, studies of ultrathin section specimens of the cells infected with the cyt mutant, have not revealed such a typical crystalline array as observed in parental virus infected cells at late infectious cycle.

(ii) Tumorigenicity

Tumorigenic potential of Ad 12 parental strain and the cyt mutant in purified preparation was tested in newborn hamsters. The large tumors induced by Ad 12 developed at the site of injection as described by Trentin et al (Trentin 1962). Table 5 summarizes results of tumor induction by Ad 12 parental strain and the cyt mutant. It can be seen that 100 % of the animals injected with the parental strain had tumor, while only 8 % of the animals injected with the cyt mutant produced tumor. This result confirms the high oncogenicity of parental Ad 12 (Trentin 1962, 1968). The number of animals injected with the parental strain was rather small in this experiment,

FIGURE 8

Ultrathin Section of KB Cells Infected with Ad 12 Parental Strain and Cyt Mutant

(a) KB cells at 40 hr after infection with parental strain

(b) Adenovirion crystals in nucleus of KB cells infected with parental strain

(c) KB cells at 40 hr after infection with cyt mutant

Arrows indicate virus particles

a
c

TABLE 5

Tumor Induction in Newborn Hamsters by Injection of Ad 12 Parental Strain and Cyt Mutant

Virus injected[a]	Dose (particles per animal)	Period of observation (months)	No. of tumors/ No. of hamsters injected	Positive (%)
Ad 12 parental	1×10^9	1.5	3/3	100
Cyt mutant	1×10^9	7	2/25	8

a. 0.1 ml of purified virus preparation previously dialyzed against PBS was injected subcutaneously.

however, throughout this work, tumors have been induced in many more hamsters by Ad 12 parental strain. On the contrary, the cyt mutant is much less tumorigenic in newborn hamsters and this is not due to delayed tumor development, confirming one of the characteristics of this mutant reported by Takemori et al (Takemori 1968).

(iii) Synthesis of T- and V-(structural) antigens

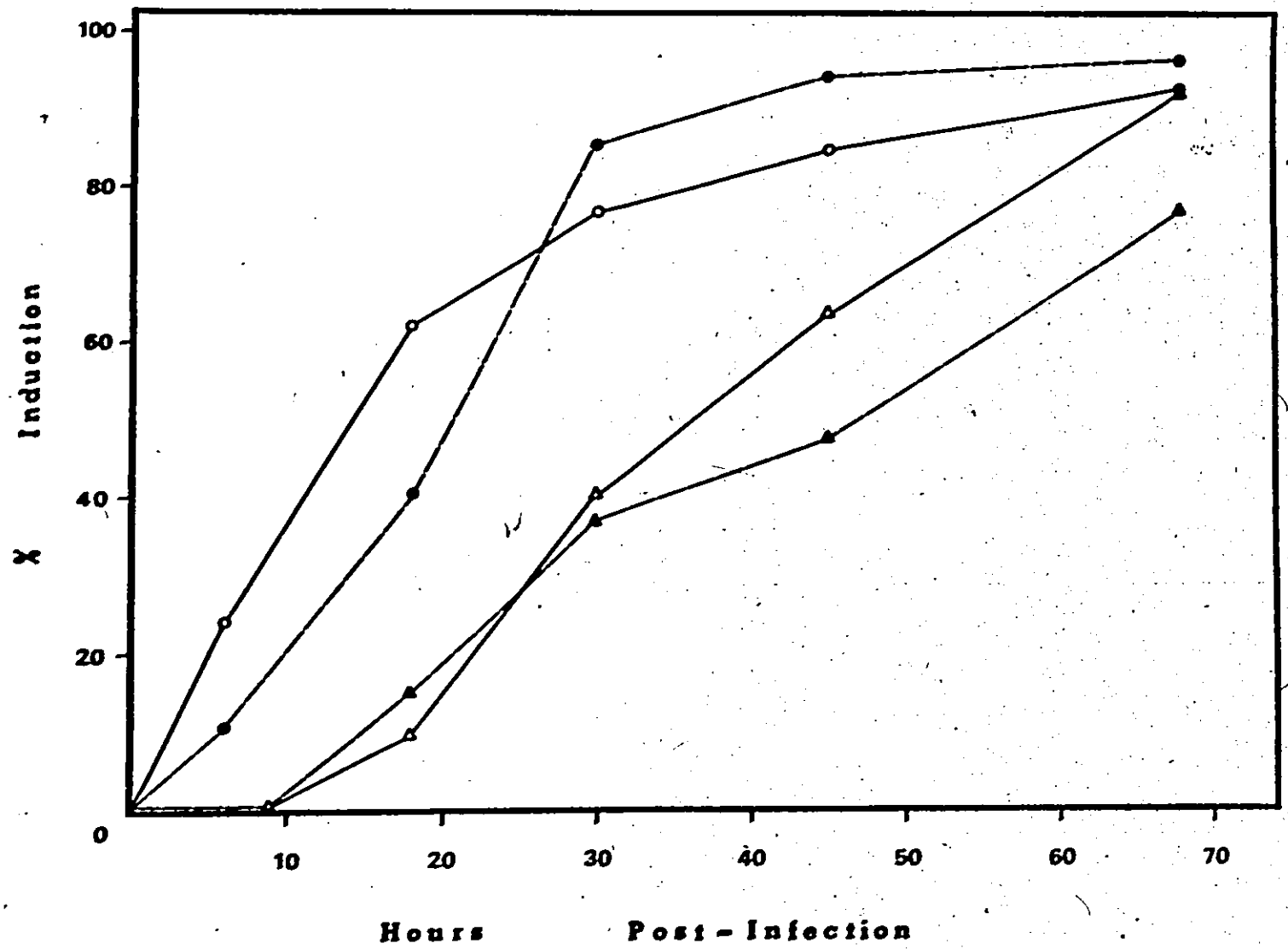
Indication of viral gene expression is the induction of new antigens in infected cells. A unique antigen, called tumor antigen (T-antigen), believed to be virus-coded is present in virus-induced tumors and virus-transformed cells. It is also induced in productively infected cells in tissue culture (Huebner 1962, 1963) and the induction is an early function of viral gene.

The time course of development of T-antigen as well as virus structural antigen (V-antigen) in infected HEp-2 cell was studied by indirect immunofluorescence technique. Figure 9 demonstrates that T-antigen was detected about 3 to 4 hr after infection, whereas V-antigen was not detected until about 15 hr after infection. These results confirm the kinetics of synthesis of intracellular viral antigens in KB cells infected with Ad 12 reported by Gilead and Ginsberg (Gilead 1965). It can also be seen that the kinetics of the production of the antigens was similar whether the cells were infected with either the parental strain or the cyt mutant. Thus the cytocidal effect on cells is probably not due to the accelerated development of the mutant compared with the parental virus.

FIGURE 9

Time Course of T- and V-antigen Synthesis
in Ad 12 Infected HEp-2 Cells

Symbols: T-antigen by parental strain (○)
T-antigen by cyt mutant (●)
V-antigen by parental strain (△)
V-antigen by cyt mutant (▲)


100
80
60
40
20
0
% Induction
10
20
30
40
50
60
70
Hours Post-Infection

(iv) Functional heterogeneity of the virions

The ratio of virus particle to its plaque forming ability for animal viruses is generally greater than unity; for example, the particle to infectivity ratio of Ad 12 has been reported to be 320 (Green 1967a). Furthermore, within a virus preparation, some virions are defective in that they are capable of limited viral functions (Blackstein 1969, Butel 1966 , Huang 1970, Uchida 1968a, b, 1969). By assaying several viral functions, such as cell killing ability, induction of early and late viral specific antigens; i.e., T- and V-antigens, respectively, in infected HEp-2 cells, the proportion of defectives in the population of parental virus preparation could be compared with that in cyt mutant preparation.

To ensure that cells in the same physiological state and under identical conditions of infection were used for different viral functions, all these viral functions were assayed with cells from the same infected cultures. After HEp-2 cells were infected with virus in suspension, a sample was taken and assayed for cell survival by cloning (see Materials and Methods). The remainder of the culture was seeded onto cover slips in Leighton tubes, incubated for 48 hr and the cells were prepared for immunofluorescence. The percentage of cells capable of forming clones and producing T- and V-antigens was determined for different input multiplicities (Table 6). As can be seen, for the same input multiplicity, the number of cells positive for T- and V-antigen formation was less than the number of cells killed. This effect was much more pronounced

TABLE 6

Effects of Input Multiplicity of Ad 12 Parental Strain and Cyt Mutant on the Induction of T- and V-antigens and Inhibition of Host Cell Cloning of HEp-2 Cells

Virus	Input multiplicity (virions/cell)	Percent T-antigen [a]	Percent V-antigen [a]	Percent surviving clones
Parental strain	0	0	0	100
	5	19.9	23.0	65.8
	25	30.4	56.1	1.98
	50	63.6	74.0	0.55
	100	92.7	89.7	0.086
	500	-	-	0.017
Cyt mutant	0	0	0	100
	5	6.4	19.8	90.8
	25	40.8	34.8	31.5
	50	70.7	66.1	8.93
	100	87.7	90.6	1.45
	500	-	-	0.24

a. Assayed at 48 hr after infection

in cultures infected with the parental strain than with the cyt mutant. To quantitate the number of input virus particles necessary for a particular function, the data were plotted into a graph, with the fraction of cells surviving that particular viral function against input multiplicity (Figure 10). Survival for various functions follows single-hit kinetics for relatively low m.o.i., as predicted by the Poisson distribution (Marcus 1958). Table 7 shows the input multiplicity of parental and mutant virus to give 63 % positive response for the various functions. It can be seen that in both cases there are more virions capable of killing cells than of inducing T- and V-antigens. However the ratio of antigen induction unit to cell killing unit is much higher in the parental strain, indicating that the parental virus contains a much higher proportion of defective virions capable of cell killing only.

(v) Adsorption of virus to host cell

It requires four to six times more cyt mutant virions than parental virions to give 63 % positive response for cell killing (Table 7). This difference may be a reflection of differential adsorption efficiency of the virus to host cell. Alternatively, the virus can adsorb but insufficient viral genes are expressed to cause cell killing. By using purified radioactively labeled viruses, adsorption efficiency of each virus to HEp-2 and KB cells was compared (Table 8A, B). About 16 to 22 % of the input virions adsorbed to HEp-2 cells while about 50 to 60 % of the input virions adsorbed to

FIGURE 10

Effects of Input Multiplicity (virions/cell) on Cell Killing and Induction of T- and V-antigens in HEp-2 Cells

The ordinate represents the percent of cells not showing that particular viral function.

(a) Clone formation by parental strain (□)

T-antigen induction by parental strain (○)

V-antigen induction by parental strain (△)

(b) Clone formation by cyt mutant (■)

T-antigen induction by cyt mutant (●)

V-antigen induction by cyt mutant (▲)

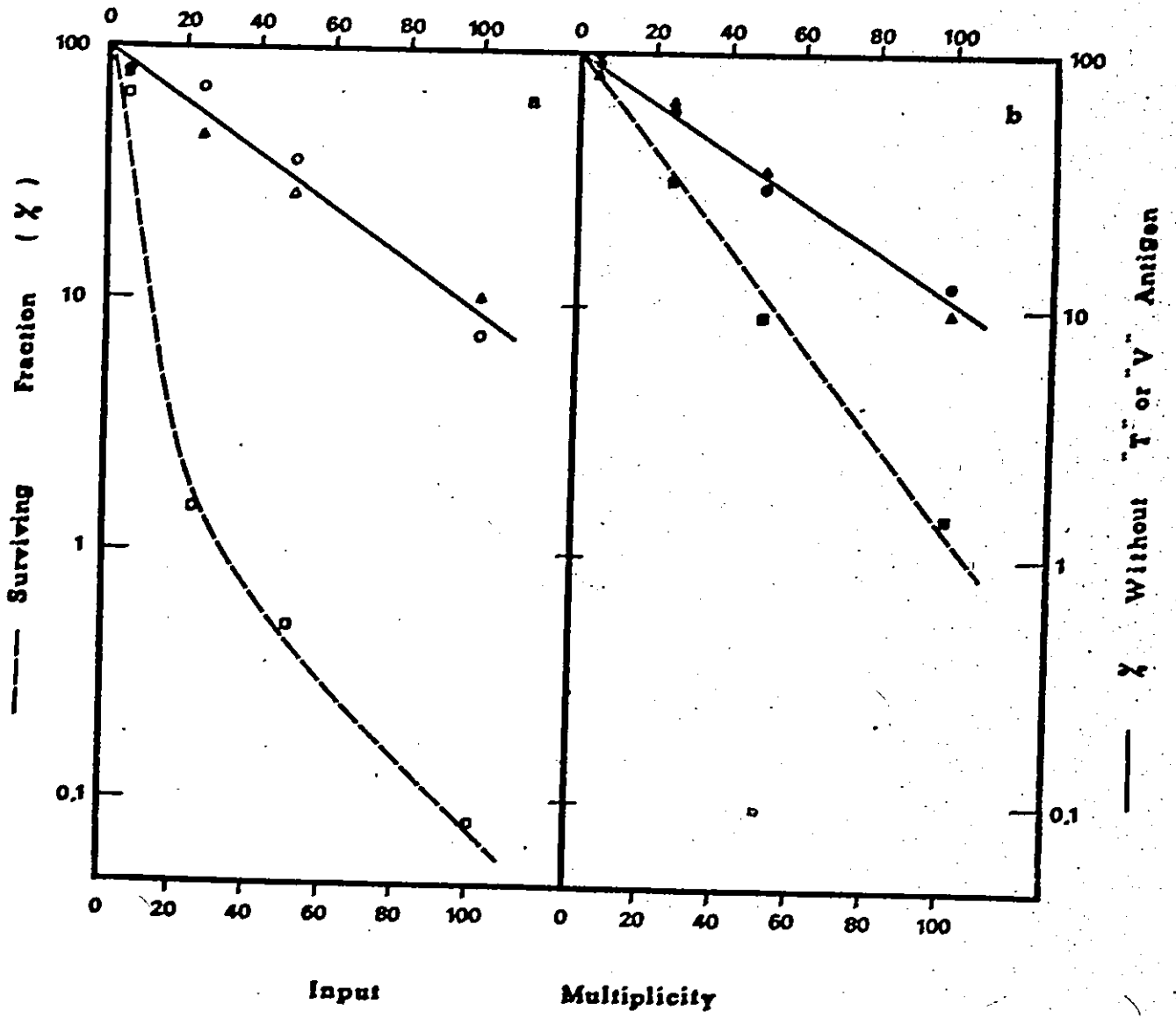

0
20
40
60
80
100
a
b
100
10
1
0,1
— Surviving Fraction (%)
— % Without "T" or "V" Antigen
Input
Multiplicity

TABLE 7

Number of Input Virions per Cell Necessary to Induce Positive Response in 63 % of the Cells in an Infected Culture of HEp-2 Cells

Virus	Cell killing	T-antigen induction	V-antigen induction	Ratio of V-antigen induction to cell killing
Expt. I				
Parental strain	6	41	40	6.7
Cyt mutant	23	44	45	1.9
Expt. II				
Parental strain	5	33	36	7.2
Cyt mutant	30	46	48	1.6

TABLE 8A

Adsorption of ^{3}H-Ad 12 Parental Virions and Cyt Mutant Virions to HEp-2 Cells [a]

Virus	Input multiplicity (virions/cell)	Cell associated cpm	Cpm remained in supernatant	% adsorbed
Parental strain	500	5,830	24,800	19.0
	100	1,305	6,610	16.5
Cyt mutant	500	1,275	6,540	16.3
	100	425	1,475	22.4

a. Adsorption period was 90 min

TABLE 8B

Adsorption of ^{3}H-Ad 12 Parental Virions and Cyt Mutant Virions to KB Cells [a]

Virus	Input multiplicity (virions/cell)	Cell associated cpm	Cpm remained in supernatant	% adsorbed
Parental strain	1,000	28,345	29,100	49.3
	500	16,515	14,080	54.0
	100	4,080	2,835	59.0
	50	1,630	1,595	50.5
Cyt mutant	1,000	8,225	7,080	53.7
	500	3,910	3,570	52.3
	100	1,045	1,005	50.7
	50	665	655	50.4

a. Adsorption period was 90 min

KB cells. Adsorption efficiency of the same virus varies with different host cells; yet, it can be seen that there is no difference between the cyt mutant and the parental strain. Thus, most of the virions from the parental virus population can adsorb and express the viral function of cell killing. On the other hand, high proportion of the cyt mutant virions can not express sufficient viral function to cause cell death, although they can adsorb to host cells.

II. Molecular Basis for Reduced Virus Yield in Cyt Mutant Infected Cells

It was found in section I that the production of the mature virion per infected cell is lower after cyt mutant infection than after infection with Ad 12 parental type strain, independent of the host cell used. The yield of virions depends on the synthesis of various viral components and the process of assembly. In this section, the amount of viral DNA synthesized in infected cells was studied to see if it is a limiting factor in virus production process.

A. Time course of viral DNA synthesis

The time course of viral DNA synthesis was determined by DNA-DNA hybridization. KB cells were infected with 200 virus particles per cell and pulse-labeled for 2 hr with ^{3}H-TdR at different times after infection. ^{3}H-DNA extracted from the infected cells was hybridized with two successive filter containing Ad 12 DNA. The results are shown in Table 9 and the time course of viral DNA synthesis is illustrated in Figure 11. As can be seen, a significant amount of viral DNA was detected at 14 hr after infection. The percentage of ^{3}H-DNA hybridized to viral DNA was almost 100 % at 24 hr post infection with the parental strain, suggesting that host DNA synthesis was completely

TABLE 9

Percent Hybridizable Viral DNA at Various Times after Infection of KB Cells with Ad 12 Parental Strain and Cyt Mutant [a]

Hours after infection	First hybridization		Second hybridization	Total cpm bound[d]	Total % bound[e]
	Input cpm[b] (A)	Bound cpm (B)	Bound cpm (C)	(D)	
Parental					
14	2,148	338	81	419	19.5
	2,148	322	139	461	21.5
24	2,278	2,140	67	2,207	96.9
	2,278	2,165	42	2,207	96.9
40	1,300	1,182	38	1,220	93.8
	1,300	1,244	27	1,271	97.8
Cyt mutant					
14	3,523	215	132	347	9.8
	3,523	212	136	348	9.9
24	2,195	757	131	888	40.5
	2,195	762	113	875	39.9
40	1,338	477	96	573	42.8
	1,338	472	94	566	42.3

a. ^{3}H-DNA extracted from infected cells was hybridized with 2µg of Ad 12 viral DNA.

b. Cpm of input ^{3}H-DNA was determined by cold 5 % TCA precipitation for each reaction.

c. After first hybridization, the unhybridized labeled DNA was removed, heat-denatured and rehybridized to a new filter containing 2µg of viral DNA as before.
Bound cpm = (column (A) - column (B)) x % hybridization in the 2nd.

d. Total cpm bound = column (B) + column (C)

e. Total % bound = column (D) / column (A)

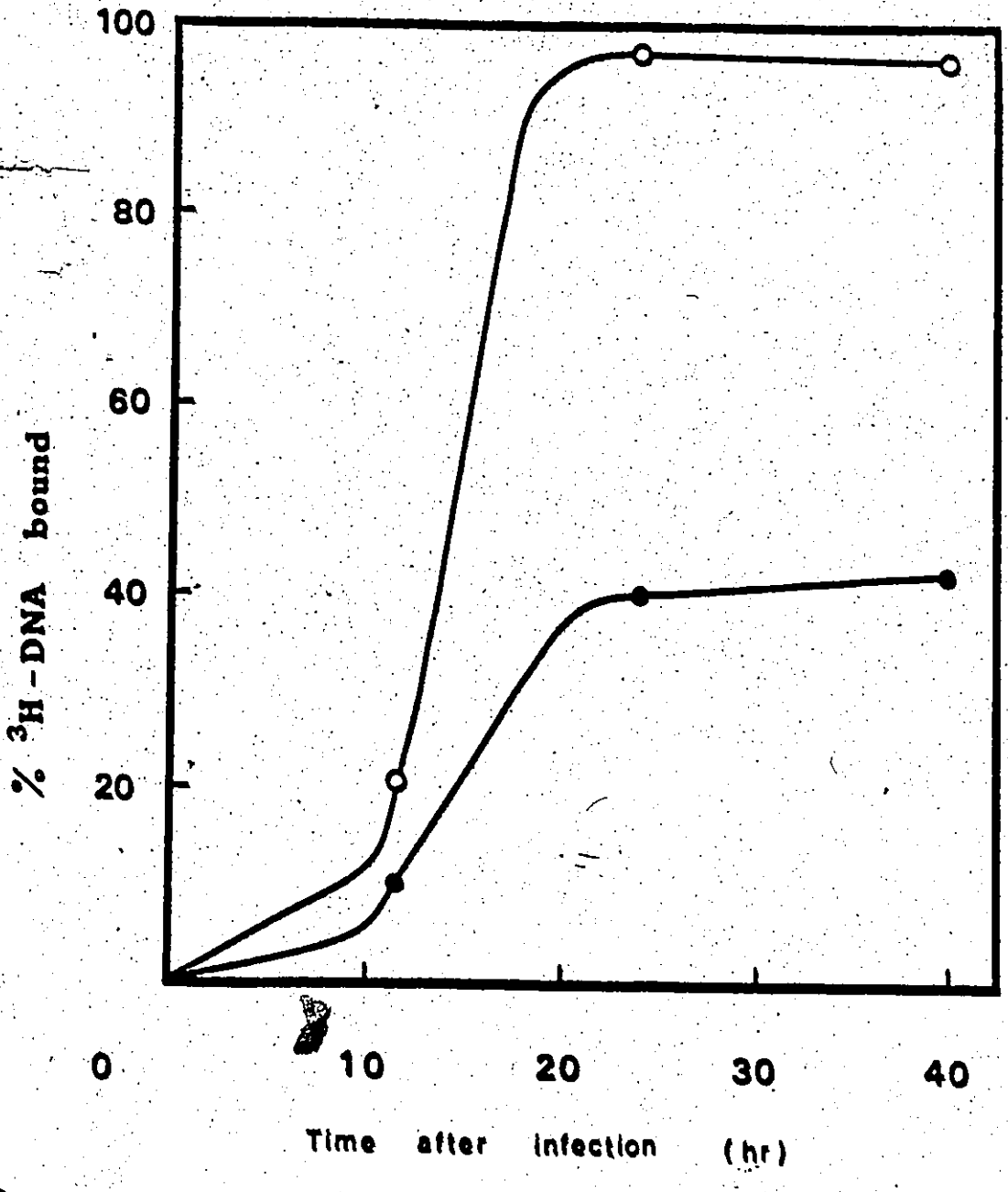
FIGURE 11

Synthesis of Viral DNA at Various Times after Infection of KB Cells with Ad 12 Parental Strain and Cyt Mutant

^{3}H-DNA extracted from infected KB cells was hybridized with 2 µg of Ad 12 viral DNA.

Symbols: DNA synthesis by parental strain (○)

DNA synthesis by cyt mutant (●)


100
80
60
40
20
0
10
20
30
40
% ^{3}H-DNA bound
Time after infection (hr)

shut off starting from 24 hr after infection. In cyt mutant infected cells, viral DNA synthesis began at about the same time and percentage of ^{3}H-DNA hybridized reached a plateau value of 40 at 24 hr. Thus, it appears that viral DNA synthesis was initiated at the same time whether the cells were infected with the parental strain or the cyt mutant. However, the cyt mutant did not shut off host DNA synthesis in contrast to parental virus infection under the identical experimental conditions.

B. Amount of viral DNA in infected cells

The total amount of viral DNA synthesized in KB cells at 40 hr after infection with the parental strain and the cyt mutant was compared. It was determined by the following equation:

$$\text{Total amount of viral DNA} = \frac{\text{Total count in viral DNA}}{\text{Specific activity of labeled viral DNA}}$$

Radioactivity incorporated into viral DNA was obtained by total cpm in infected cells multiplied by the fraction of ^{3}H-DNA hybridized to viral DNA. The specific activity of labeled viral DNA was determined by DNA-DNA saturation hybridization experiment. A small known amount of Ad 12 viral DNA (0.2 µg) immobilized on a nitrocellulose filter was hybridized with increasing amounts of ^{3}H-DNA extracted from the infected cells. The radioactivity at saturation should be equal to the amount of viral DNA immobilized on the filter. Therefore, the radioactivity at saturation divided by the amount of viral DNA on the filter will

give the specific activity of each preparation. Such a saturation hybridization curve is shown in Figure 12. The radioactivity bound on the filter increased with increasing amounts of ^{3}H-DNA input. Saturation was attained at 3,300 and 3,600 cpm hybridized, giving specific activity of 16,500 and 18,000 cpm/μg for the parental viral DNA and the cyt mutant DNA, respectively. Total amount of viral DNA at 40 hr post infection is given in Table 10. The parental strain produced 2.3 to 2.5 μg of viral DNA per 10^6 infected cells, whereas KB cells infected with the cyt mutant produced only 0.8 to 1.0 μg of viral DNA per 10^6 cells.

C. Molecular size of intracellular viral DNA

The reduced amount of viral DNA present in cyt mutant infected cells can account, in part, for the reduced yield of the cyt mutant virions. However, the study above does not show the nature of these DNA molecules, in particular, the size of the newly synthesized viral DNA. The next experiment was conducted to examine the size of viral DNA molecules made in infected cells. KB cells infected with the virus at m.o.i. of 200 particles per cell were labeled with ^{3}H-TdR from 13 to 40 hr after infection, and the lysate was analysed by rate zonal sedimentation in an alkaline sucrose gradient. Figure 13 shows sedimentation profiles of intracellular DNA in KB cells infected with the parental virus and the cyt mutant. In the parental virus infected cells, about 30 % of the total radioactivity was co-sedimented with

FIGURE 12

Saturation Hybridization Experiment of Immobilized Viral DNA with ^{3}H-DNA from KB Cells Infected with Ad 12 Parental Strain and Cyt Mutant

0.2 µg of immobilized Ad 12 DNA was hybridized with increasing amounts of ^{3}H-DNA extracted from KB cells at 42 hr after infection.

Symbols : Parental strain (○)

Cyt mutant (●)

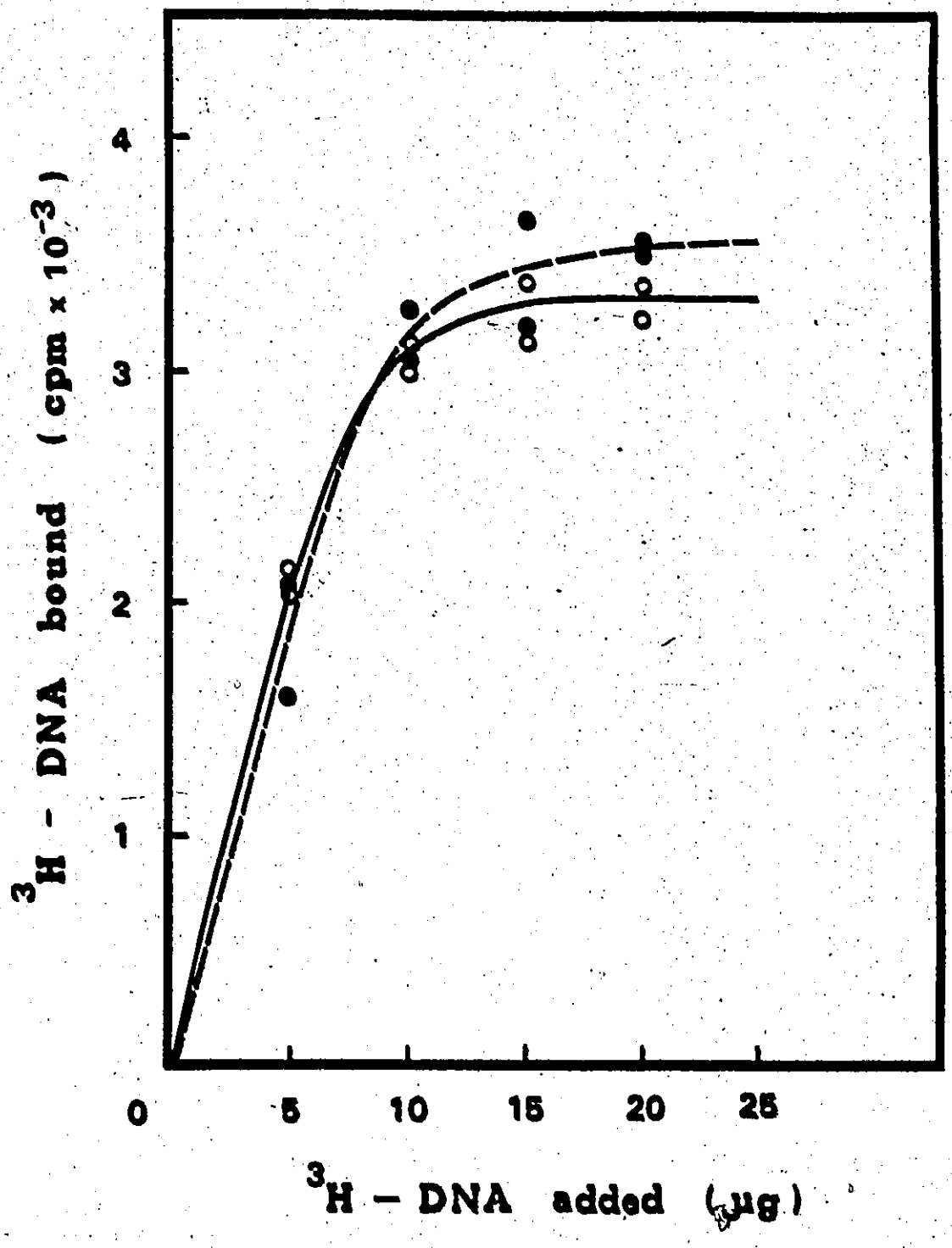

^{3}H - DNA bound (cpm x 10^{-3})
4
3
2
1
0
5
10
15
20
25
^{3}H – DNA added (µg)

TABLE 10

Total Amount of Viral DNA Synthesized at 40 hr after Infection of KB Cells by Ad 12 Parental Strain and Cyt Mutant

Infection	(A) Total radioactivity in 10^6 cells (cpm)	(B) Hybridizable DNA (%)	(C) Saturating amount in hybridization (cpm)	(D) Ad 12 DNA on filter (μg)	(E) Specific activity of viral DNA[a] (cpm/μg)	(F) Total amount of viral DNA[b] (μg/10^6 cells)
Expt. I						
Parental strain	37,620	100	3,300	0.2	16,510	2.28
Cyt mutant	34,930	43	3,600	0.2	18,000	0.83
Expt. II						
Parental strain	46,210	96	3,500	0.2	17,510	2.53
Cyt mutant	53,510	42	4,700	0.2	23,500	0.96

a. Specific activity = column (C)/column (D)

b. Total amount of viral DNA = column (A) x column (B)/column (E) x 100

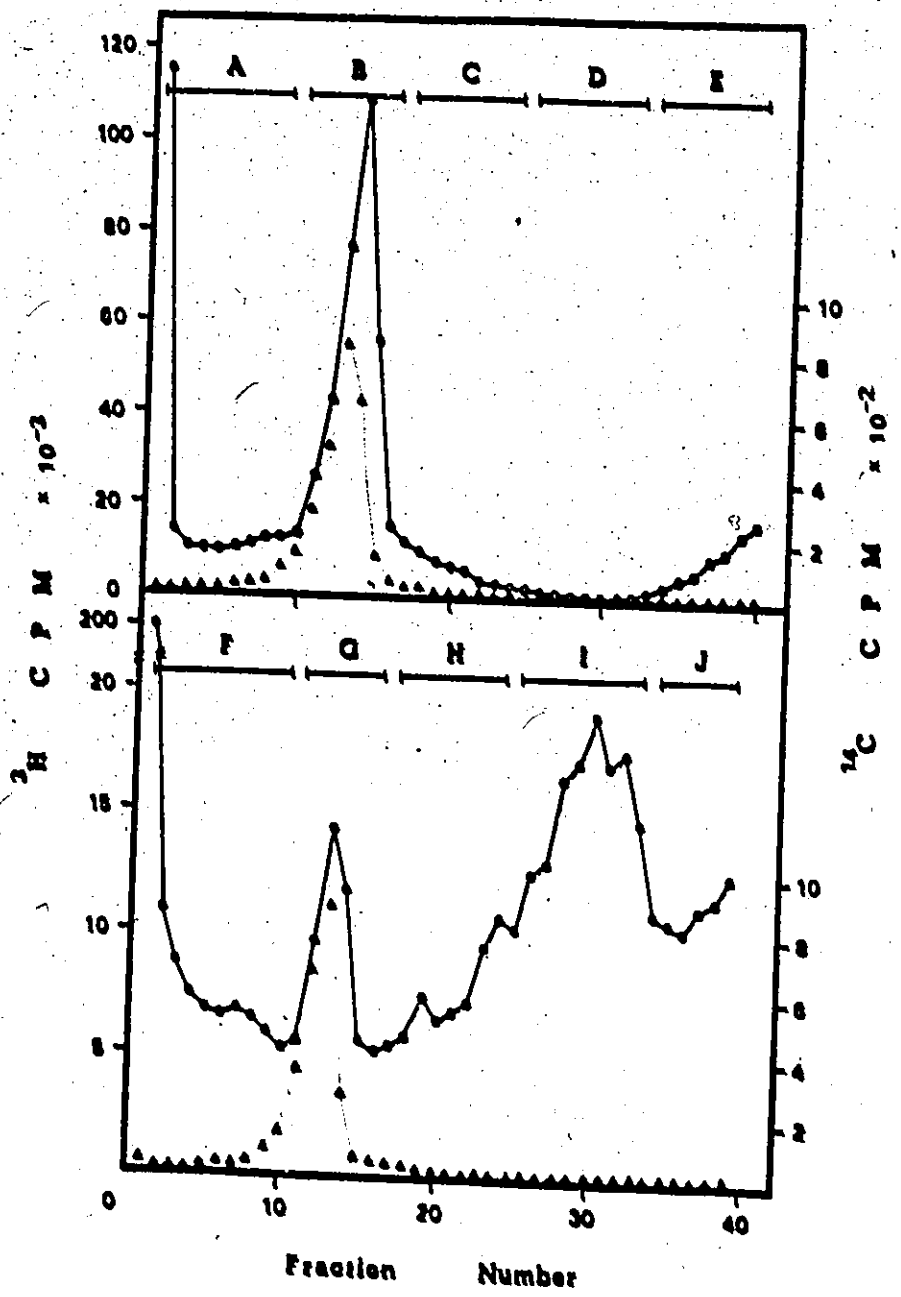
FIGURE 13

Sedimentation Profile of Intracellular DNA in KB Cells Infected with Ad 12 Parental Strain and Cyt Mutant in a 5 to 20 % Alkaline Sucrose Gradient

Sedimentation is from right to left.

upper panel : Parental virus infected cells (○)
^{14}C-Ad 2 marker DNA (△)

lower panel : Cyt mutant infected cells (●)
^{14}C-Ad 2 marker DNA (△)


A
B
C
D
E
F
G
H
I
J
120
100
80
60
40
20
0
200
20
15
10
5
0
10
20
30
40
Fraction Number
^3H C P M x 10^{-3}
^{14}C C P M x 10^{-2}
10
8
6
4
2

the marker viral DNA (^{14}C-Ad 2) showing production of DNA molecules of complete size. In contrast to this, the profile of the cyt mutant is very much different from that of the parental strain. The intracellular DNA was much more heterogeneous in size, ranging from 0.02 to 0.35 of the complete genome size of adenovirus DNA.

The ^{3}H-labeled intracellular DNA was also analyzed using neutral sucrose gradients. Figure 14 shows profiles of radioactivity. It can be seen that molecules smaller than a complete viral genome were also observed, indicating that small DNA fragments synthesized after cyt mutant infection were double-stranded.

To determine whether these small molecules are of viral origin, DNA-DNA hybridization experiment was performed. Fractions were pooled to five size classes as indicated in Figure 13(i.e., fractions A to E for the parental virus infected cells and F to J for the cyt mutant infected cells). After neutralization with HCl, the DNA was precipitated by ethanol, and hybridized to an excess amount of Ad 12 viral DNA immobilized on a nitrocellulose filter. The total amount of viral DNA in each size class was calculated. Table 11 shows the size distribution of intracellular viral DNA. As can be clearly seen, the size distribution of viral specific DNA made in infected cells is quite different between the two viruses. In the parental strain, about 70 % of the total viral DNA was found in the position of complete genome size class. On the other hand, the viral specific DNA of the cyt mutant produced in KB cells has a very heterogeneous size. It was found that the

FIGURE 14

Sedimentation Profile of Intracellular DNA in KB Cells Infected with Ad 12 Parental Strain and Cyt Mutant in a 5 to 20 % Neutral Sucrose Gradient

Sedimentation is from right to left.

(a) Parental virus infected cells (○)
^{14}C-Ad 2 marker DNA (●)

(b) Cyt mutant infected cells (○)
^{14}C-Ad 2 marker DNA (●)

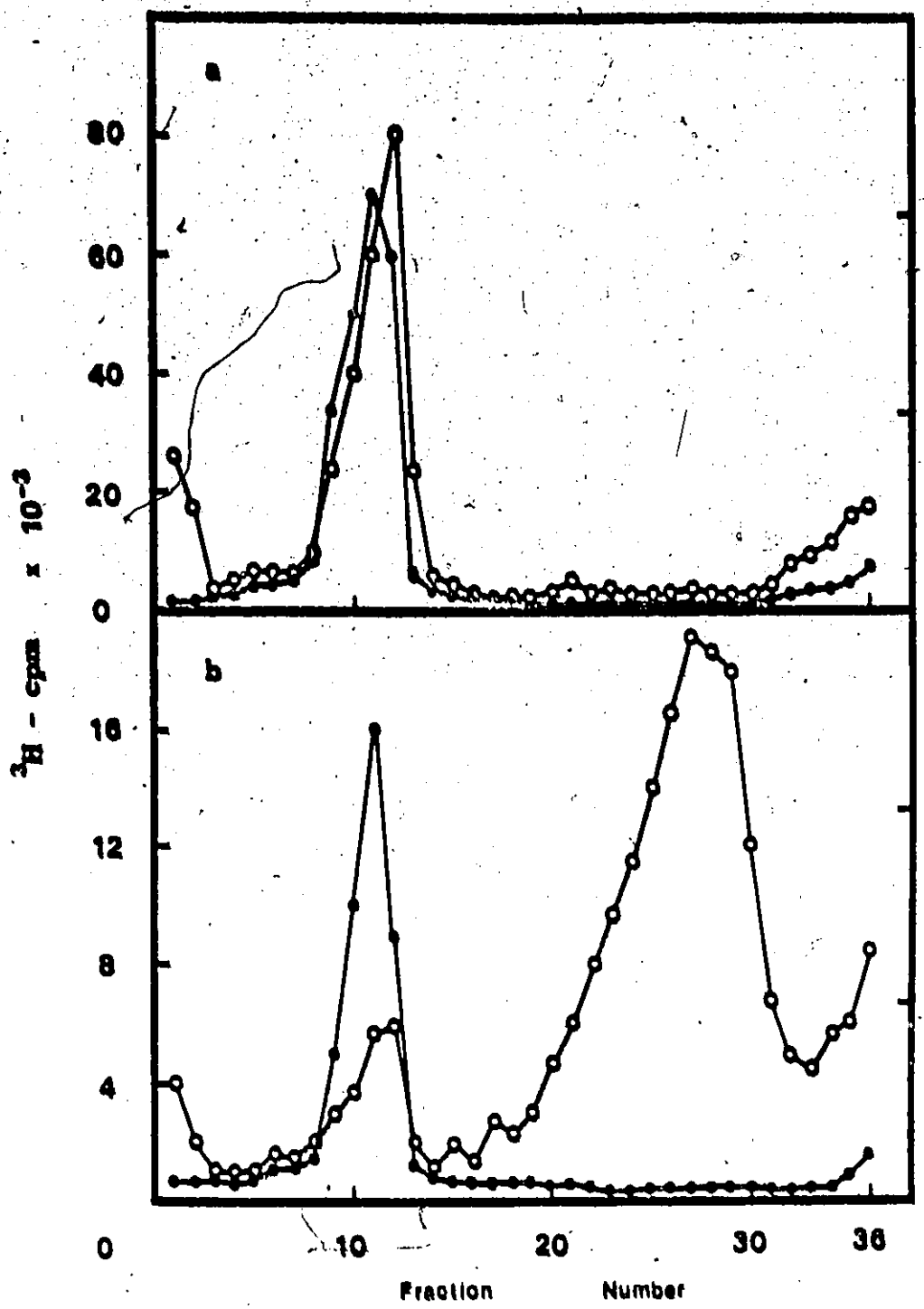

a
b
80
60
40
20
0
16
12
8
4
0
2
1
2
1
10
20
30
36
Fraction Number
^3H - cpm x 10^{-3}
^{14}C - cpm x 10^{-3}

TABLE 11

Size and Distribution of Intracellular Viral DNA after Infection of KB Cells with Ad 12. Parental Strain and Cyt Mutant

Fraction	(I) Molecular size[a]	(II) Total cpm	(III) % hybridizable[b]	(IV) Total viral DNA (cpm)[c]	(V) Amount of viral DNA[d]
Parental					
A	>1.37	223,000	14.6	32,700	7.4
B	1.26-0.73	355,000	88.9	316,000	71.4
C	0.66-0.26	66,000	81.8	53,600	12.1
D	0.22-0.04	25,000	46.3	11,800	2.7
E	<0.02	74,000	37.8	28,300	6.4
Cyt mutant					
F	>1.37	286,000	3.3	9,400	8.0
G	1.26-0.73	56,000	37.8	21,000	17.9
H	0.66-0.26	65,000	27.2	17,600	15.0
I	0.22-0.04	143,000	32.6	46,500	39.7
J	<0.02	72,000	31.7	22,700	19.4

a. As a fraction of complete viral genome.

b. ^{3}H-DNA recovered from each fraction in the gradient was hybridized with 1 µg of Ad 12 viral DNA.

c. Total viral DNA = column (II) x column (III)/ 100

d. Amount of viral DNA in each fraction is expressed as percent of total viral DNA in the gradient.

majority of the viral DNA is of less than half of the complete genome size and that only 18 % of the total newly made DNA is of intact size. Above all, about 40 % of the total viral DNA is in fraction I, which has average size of 0.07 of the whole viral genome. The total amount of viral DNA in cells infected with the parental strain was found to be 2.4 pg/cell (Table 10). Out of this, 71 % is of complete genome size (Table 11). Thus, the amount of viral DNA having complete genome size is 1.7 pg/cell. Similarly, the amount of the cyt mutant viral DNA in infected KB cell was estimated to be 0.16 pg/cell. Therefore, the reduction in virus yield may be accounted for by the reduced amount of viral DNA having complete genome size synthesized in cells infected with the cyt mutant.

D. Fragmentation of DNA

It was found that 75 % of the newly synthesized viral DNA in cyt mutant infected cells is smaller than the intact molecules present in the virion. To investigate further a molecular mechanism of the development of these smaller fragments in the mutant infected cells, two possibilities were considered. One possibility is that these fragments were made due to the synthesis of incomplete viral DNA molecules. The other possible explanation is that the cyt mutant could synthesize intact molecules, but subsequently a large proportion of them was degraded to smaller pieces. In order to distinguish these possibilities, pulse-chase experiment of viral

DNA was performed. At 23 hr after infection, at which time the viral DNA synthesis reached to maximum level (Figure 11), the cultures were exposed to ^{3}H-TdR for 1 hr and followed by a chase of 3 and 6 hr in the presence of excess nonradioactive TdR. The size of the DNA synthesized during 1 hr pulse-labeling and its fate after a chase was analyzed by rate zonal sedimentation in alkaline sucrose gradients. Figure 15 demonstrates the fate of the newly made DNA in both viruses. In 1 hr pulse-labeling of the parental virus infected cells (Figure 15a), 54 % of the total counts co-sedimented with the ^{14}C-Ad 2 marker DNA. After a 3 hr chase (Figure 15b), the radioactivity in major fraction increased to 74 % of the total counts, indicating a good production of mature molecules. After further chasing, 6hr (Figure 15c), the profile is similar to that of a 3 hr chase and there is neither an increase nor a decrease in the counts in the complete genome size fraction.

On the other hand, in KB cells infected with the cyt mutant, at 1 hr pulse-labeling, the sedimentation profile was exactly the same as that of parental virus infected cells (Figure 15d). The same proportion of the total radioactivity, i.e., 54 % of the total, was found in the ^{14}C-Ad 2 marker viral DNA region. This observation suggests that during 1 hr pulse-labeling, DNA made by the cyt mutant is the same as that made by the parental strain both qualitatively and quantitatively. However, when the infected cells were chased for different times after pulse labeling, the large molecules were eventually degraded into smaller fragments (Figure 15e,f). Quantitatively, after a 3 hr chase, 46 % of the total counts was found in the

FIGURE 15

Sedimentation Profile of Pulse-Labeled Intracellular DNA in KB Cells Infected with Ad 12 Parental Strain and Cyt Mutant in a 5 to 20 % Alkaline Sucrose Gradient

Sedimentation is from right to left.

(a) Pulse-labeled for 1 hr at 23 hr after infection with parental strain

(b) Followed by a 3 hr chase

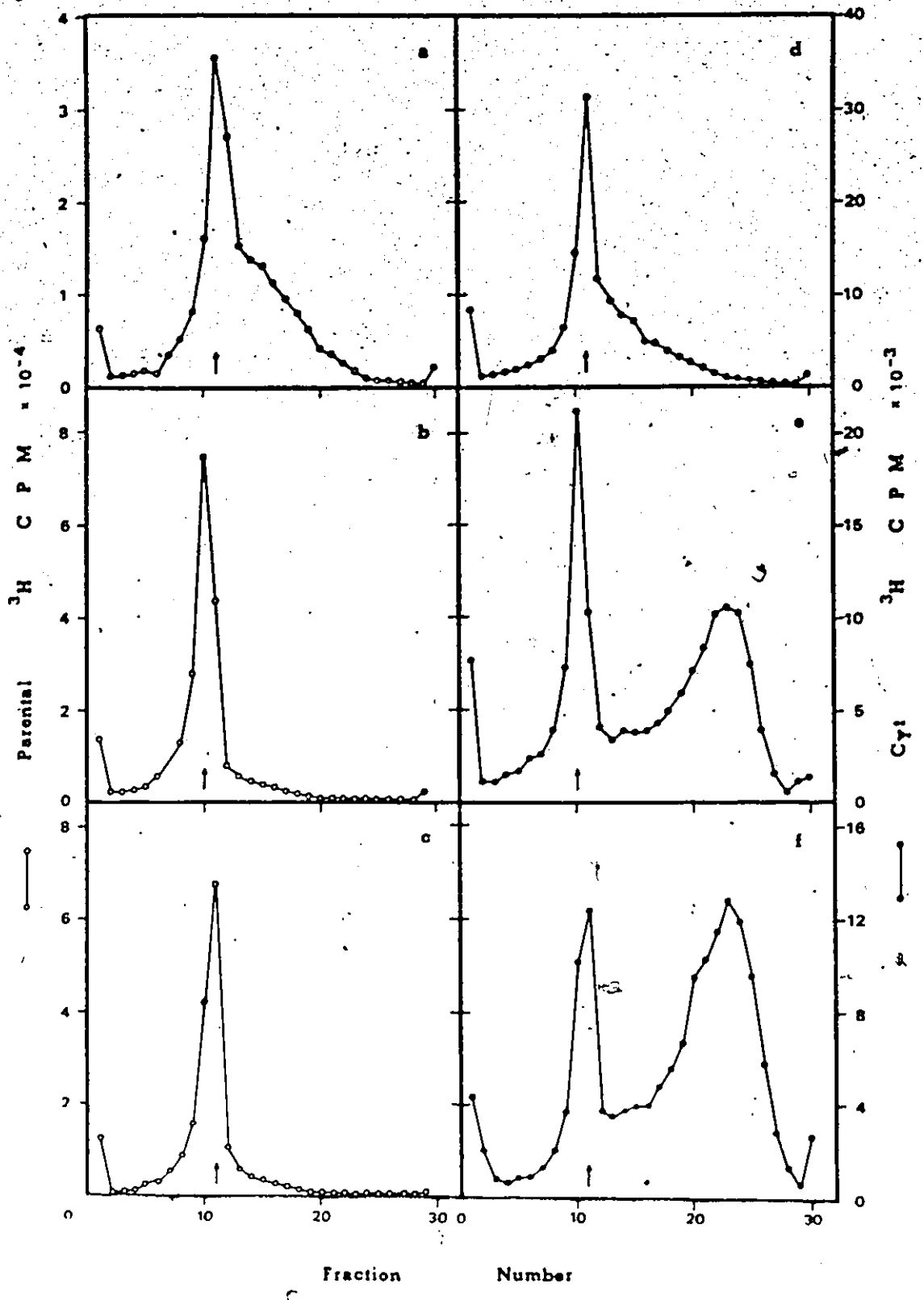
(c) Followed by a 6 hr chase

(d) Pulse-labeled for 1 hr at 23 hr after infection with cyt mutant

(e) Followed by a 3 hr chase

(f) Followed by a 6 hr chase

Arrows indicate the position of ^{14}C-Ad 2 marker DNA


a
b
c
d
e
f
Parental ^3H CPM × 10^{-4}
Cyt ^3H CPM × 10^{-3}
Fraction Number

heterogeneous small size class, leaving 30 % of the total radioactivity in the fraction of complete viral genome size. This degradation of DNA was much more enhanced after a 6 hr chase, at which time about 60 % of the total radioactivity was in the region of smaller molecules (less than 0.35 of the complete genome). Only 20 % of the molecules remained as a complete genome size. This profile of size distribution of the newly made DNA is quite similar to that of continuous labeling of the cyt mutant infected cells (Figures 13 & 14). Therefore these experiments clearly show that in cyt mutant infection, a viral DNA of complete viral genome size is normally synthesized at first, but subsequently a half to 2/3 of these molecules were degraded to smaller pieces.

E. Complementation by parental strain

It appears that functions associated with "cyt mutation" include the degradation of newly synthesized DNA, it is of interest to examine whether this function can be suppressed by the parental strain.

(1) Suppression of intracellular DNA degradation

Intracellular viral DNA in KB cells was continuously labeled with ^{3}H-TdR from 14 hr after mixed infection with the parental strain and the cyt mutant at an input multiplicity of 100 virus particles of each virus per cell. As a control, KB cells were also infected with either the parental strain or the cyt mutant alone at m.o.i. of 100

particles per cell. At 40 hr after infection, the cells were harvested and analyzed for DNA by alkaline sucrose gradient sedimentation as before. It can be seen from Figure 16 that most of the DNA synthesized intracellularly by a mixture of Ad 12 parental strain and the cyt mutant had complete viral genome size and that the proportion in this fraction was quite similar to that observed in cells infected with the parental strain alone. Thus an extensive degradation of DNA by the cyt mutant was almost completely suppressed by co-infection with the parental strain and the parental type character was dominant over the cyt character in this manner.

(2) Total yield of the virions

The same preparation of co-infection which was labeled with ^{3}H-TdR, was used for virus yield analysis by sucrose gradient as described before, and the amount of complete virions produced was compared with single infection. Table 12 shows a typical result. Total radioactivity recovered in the position of complete virions (^{14}C-Ad 2 marker) was much lower in cyt mutant single infection in agreement with the earlier result (Table 4). However, when the cells were simultaneously infected with both types of virus, the radioactivity sedimented as complete virions was almost equal to that of parental virus single infection. Thus the reduced yield of the virions by the cyt mutant was completely overcome by co-infection with the parental strain.

FIGURE 16

Sedimentation Profile of Intracellular DNA in KB Cells Infected with Ad 12 Parental Strain, Cyt Mutant, and a Mixture of Parental Strain and Cyt Mutant in a 5 to 20 % Alkaline Sucrose Gradient

Sedimentation is from right to left.

(a) Cells infected with parental strain (○)
^{14}C-Ad 2 marker DNA (●)

(b) Cells infected with cyt mutant (○)
^{14}C-Ad 2 marker DNA (●)

(c) Cells co-infected with parental strain and cyt mutant (○)
^{14}C-Ad 2 marker DNA (●)

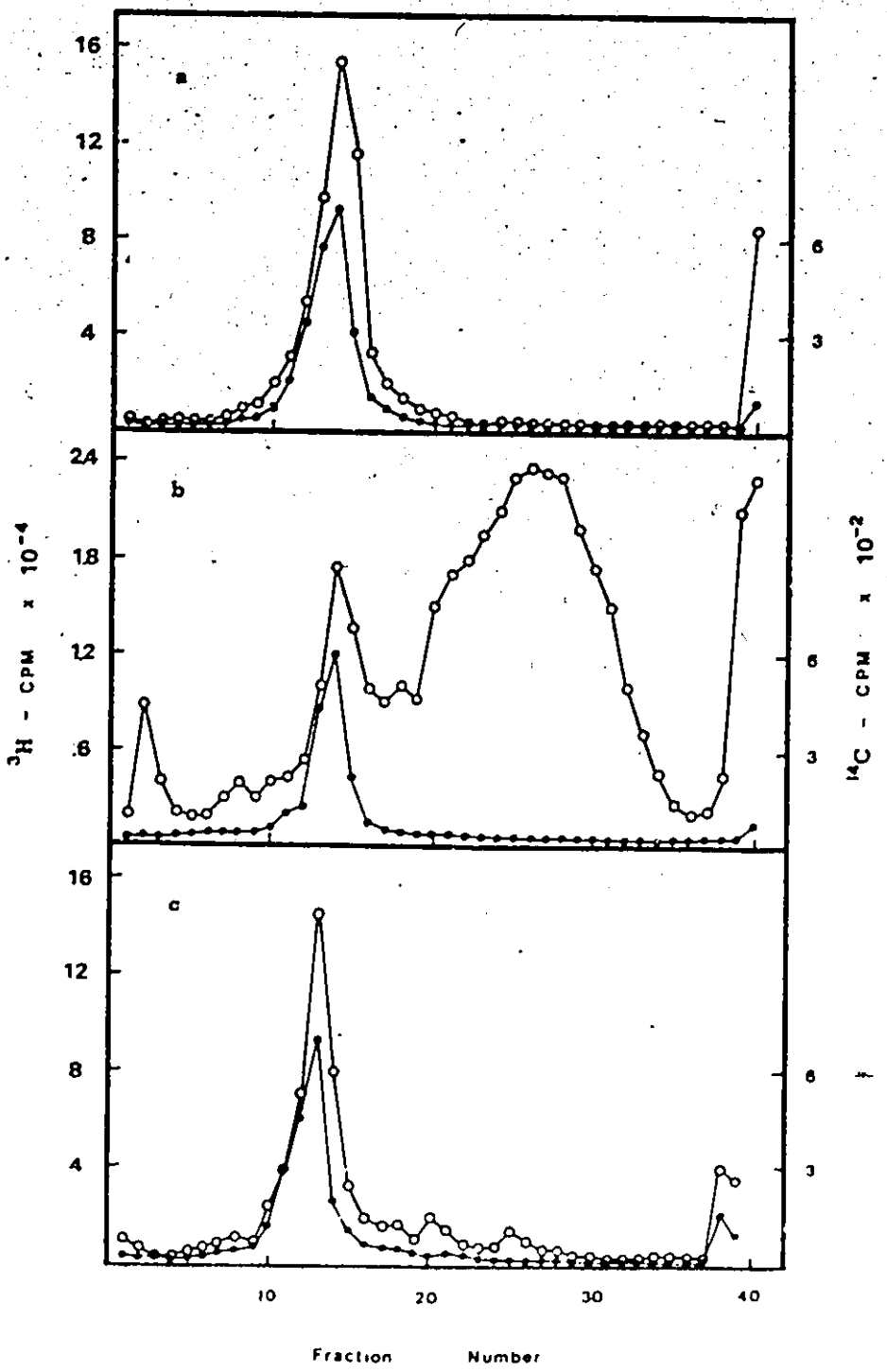

a
b
c
16
12
8
4
24
18
12
6
3
10
20
30
40
^3H - CPM x 10^{-4}
^{14}C - CPM x 10^{-2}
Fraction Number

TABLE 12

Amount of Virions Produced by KB Cells after Infection with Ad 12 Parental Strain, Cyt Mutant, and a Mixture of Ad 12 Parental Strain and Cyt Mutant

Infection	Total cpm in virion band[a] ($\times 10^{-3}$)
Parental strain	64.6
Cyt mutant	4.8
Parental plus cyt mutant	66.0

a. Radioactivity in the virion band which cosedimented with ^{14}C-Ad 2 marker virion by sucrose gradient sedimentation (see Materials and Methods)

(3) Super-infection by parental virus

It may be argued that this suppression of the cyt mutant properties in host cell by the parental strain is due to preferential exclusion of the cyt mutant virion by the parental virion at the time of adsorption or penetration of the virus to host cell. To test this possibility, a super-infection experiment by the parental strain was conducted.

KB cells were first infected with the cyt mutant virions and after 60 min adsorption, followed by a super-infection with Ad 12 parental virus at the same input multiplicity (100 particles per cell). At 14 hr post infection, ^{3}H-TdR was added to the culture and the viral DNA was continuously labeled up to 40 hr after infection. Intracellular DNA was analyzed by rate zonal sedimentation in an alkaline sucrose gradient. Figure 17 shows almost the same distribution of intracellular DNA molecules, whether the cells were infected with the cyt mutant and the parental strain simultaneously, or first infected with the cyt mutant, then super-infected with the parental strain. Therefore, the event of suppression by the parental strain must have occurred after the cyt mutant virions penetrated into KB cell rather than that the suppression was due to selective inhibition of adsorption and penetration.

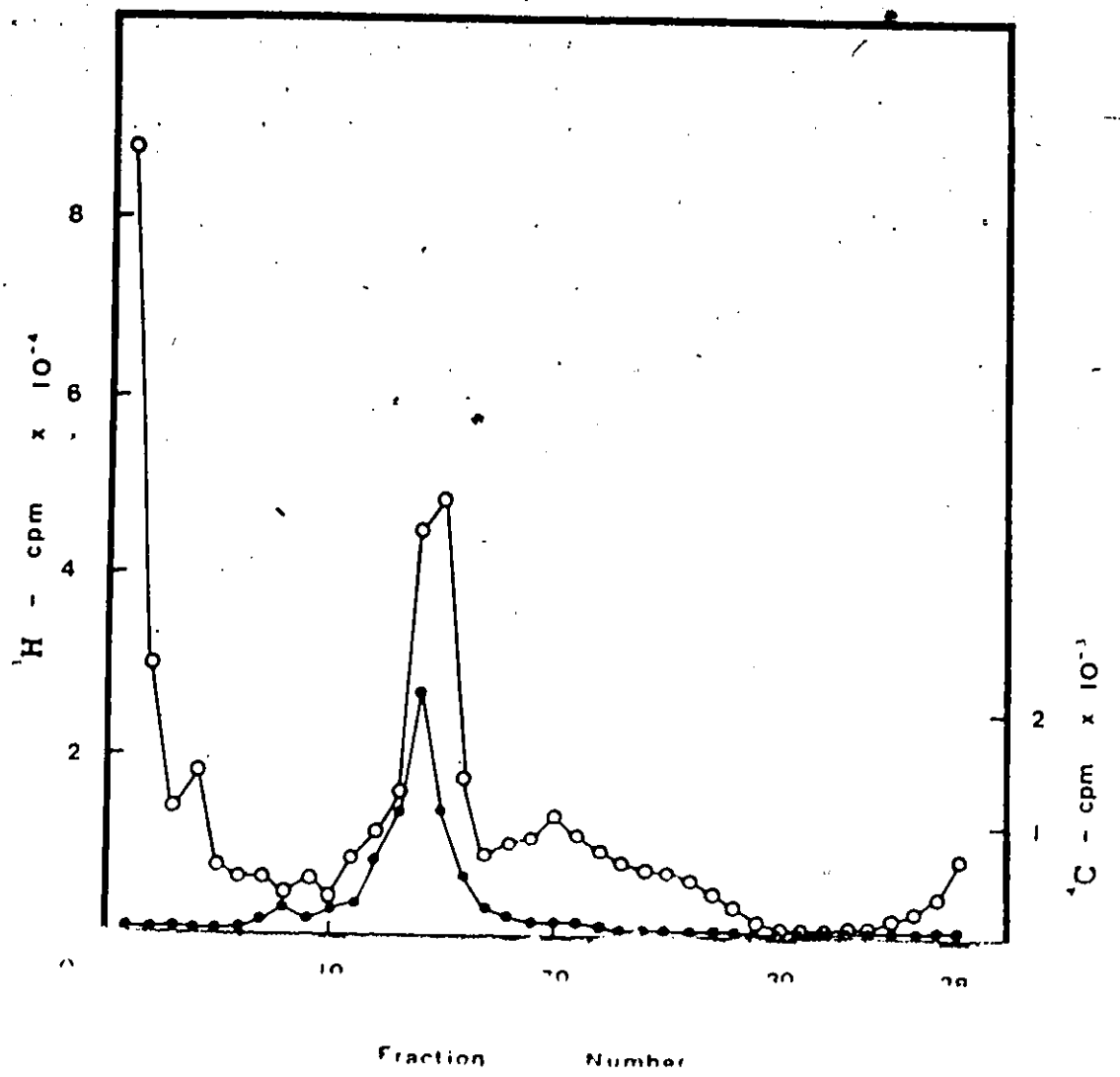
FIGURE 17

Sedimentation Profile of Intracellular DNA in KB Cells Infected with Cyt Mutant, then Super-infected with Parental Strain in a 5 to 20 % Alkaline Sucrose Gradient

Sedimentation is from right to left.

Symbols : Cells infected with cyt mutant, then super-infected with parental strain (○)

^{14}C-Ad 2 marker DNA (●)


8
6
4
2
^{3}H - cpm x 10^{-4}
2
1
^{14}C - cpm x 10^{-3}
Fraction Number

III. Electron Microscopic Studies on Adenovirus DNA

As shown in the previous section I, there were no detectable differences in physical properties of the cyt mutant virion from those of the parental virion. However, the difference in DNA size of the viruses may be below the level of detection by rate zonal sedimentation in sucrose gradients. In this section, differences in the adenovirus genome were examined in greater detail by an electron microscopic technique; heterogeneity in DNA sequence was also studied. Under the experimental conditions employed, the resolution could be obtained up to 35 to 100 base pairs long (Davis 1971a), which is about equal to 0.1 to 0.3 % of the adenovirus genome.

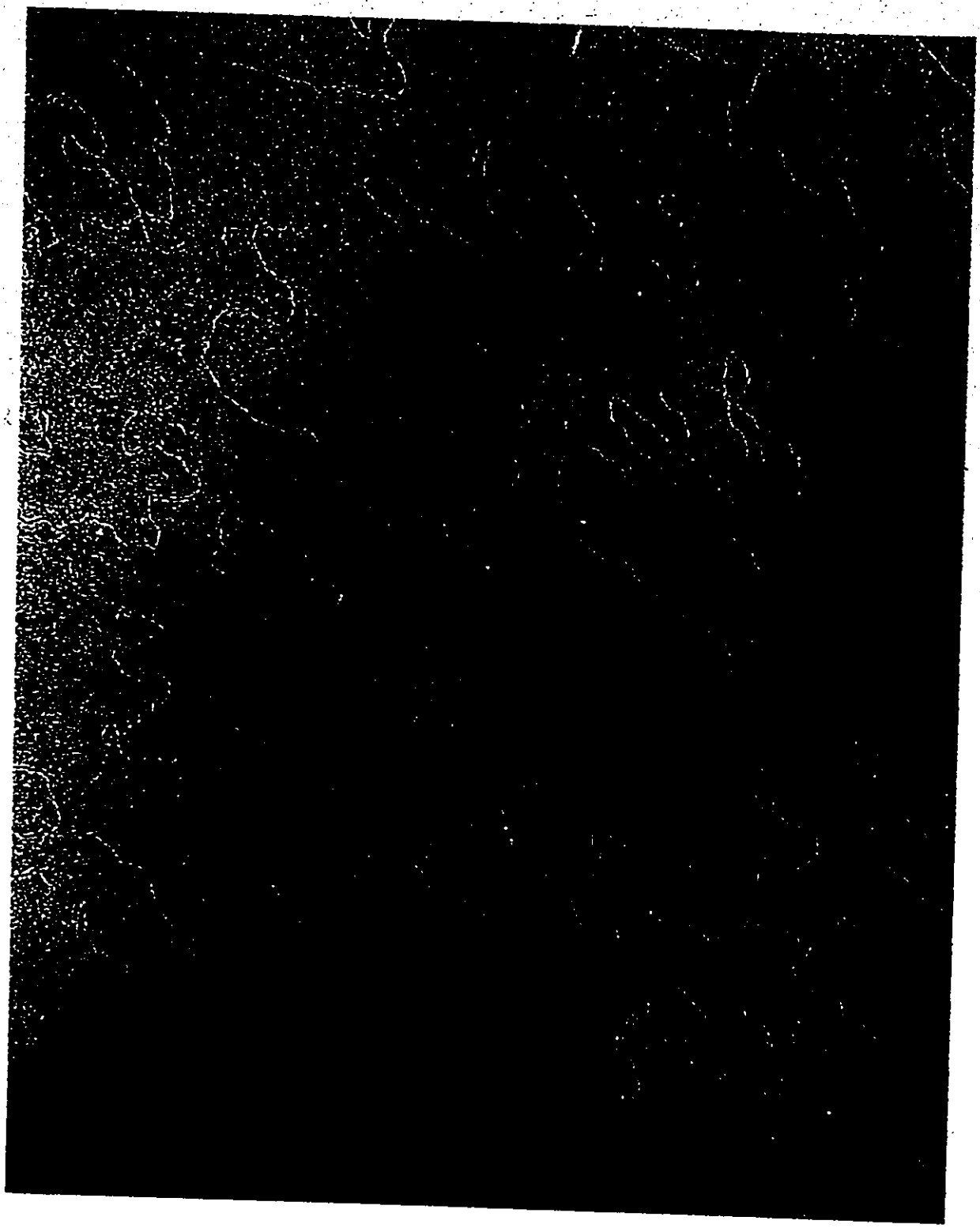
A. Measurement of the length of native DNA molecules

The native molecules of adenovirus DNA extracted from purified virions were examined under the electron microscope. The principle of the technique used was originally developed by Kleinschmidt and Zahn (Kleinschmidt 1959), and modified by Davis et al using a variation of the basic protein monolayer film-formamide technique (see Materials and Methods, Davis 1971b).

A representative picture of intact DNA molecules of Ad 12 is shown in Figure 18. In the formamide technique employed, the DNA

FIGURE 18

Electron Micrograph of Intact Ad 12 DNA Molecules



is mounted at a concentration of formamide and salt such that the double-stranded DNA is stable and if single-stranded DNA is present, it will appear as a curved filament which can be distinguished from the double-stranded DNA by its thickness (Davis 1971b). As can be seen, Ad 12 DNA is in a continuous linear double-stranded form; no circular forms were observed in the whole grids. It is also apparent that with careful extraction of the DNA from the purified virions, there is neither significant fragmentation of the molecule nor melting of the double-stranded DNA into the single-stranded form.

To determine the size of the native DNA molecules of Ad 12 parental strain and the cyt mutant preparation, the distribution of the total contour lengths of the DNA was examined. Since the absolute length of the molecules can vary from one preparation to another, depending on the conditions of the spreading and mounting the DNA for electron microscopy (Inman 1967), an internal calibration standard was thought to be necessary. Such a standard must be clearly and easily distinguishable from the DNA being studied and it would be preferable to use a linear double-stranded DNA.

DNA of bacteriophage Ø 29 of Bacillus subtilis is a linear double-stranded form and is about 6 μ in length (Figure 19, Anderson 1966, 1968, Ito 1973), which is about half the size of adenovirus DNA. Therefore Ø 29 DNA is suitable for use as a standard. Figure 20 shows an electron micrograph of a mixture of Ad 12 parental type DNA and Ø 29 DNA spread together. As can be seen in the picture, the molecules sometimes tended to appear as highly looped configura-

FIGURE 19

Electron Micrograph of Bacteriophage Ø 29 DNA Molecules

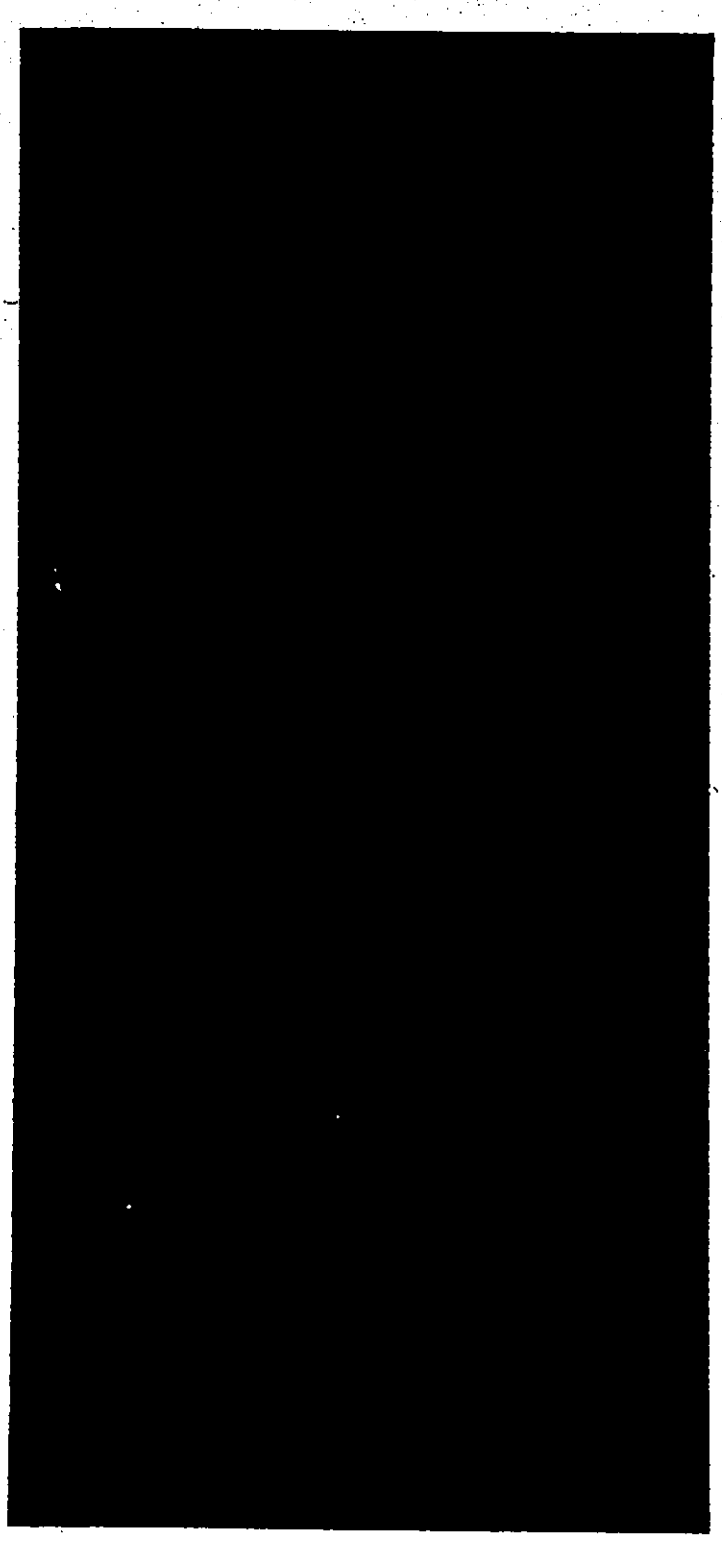


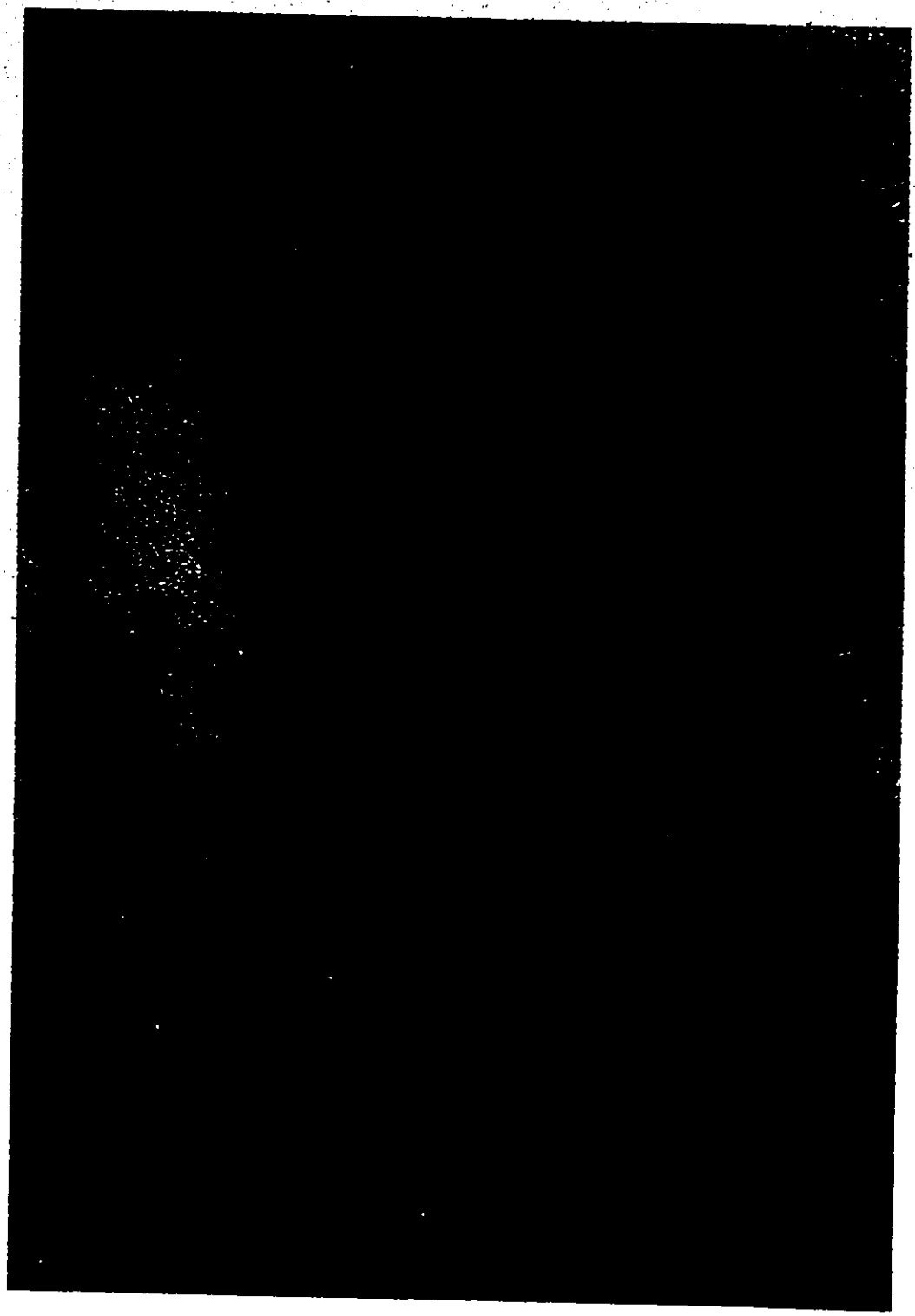
FIGURE 20

Electron Micrograph of Intact DNA Molecules of

Ad 12 and Bacteriophage Ø 29

Symbols : Ad 12 DNA (Ad)

Ø 29 DNA as an internal standard (Ø)



tions or so-called "flower-like structure", which make measurement of the length impossible. In order to get accurate measurement of the contour length, only molecules that were fairly well stretched and with no or a few infolding were selected for measurement. Calculation of the actual length of the molecule was given by the following formula:

$$\text{Contour length } (\mu) = \frac{\text{Measured length after tracing (cm)} \times 10^4}{5{,}372 \times 15.85}$$

where, 5,372 is a magnification factor under the electron microscope and 15.85 is an enlargement factor of projection of the electron micrograph negatives onto a screen.

The length distribution of each preparation was plotted in a histogram (Figure 21), and summarized in Table 13. As shown in Table 13, the mean length of Ad 12 DNA molecule was found to be 11.07 μ. This value is not widely different from those have been reported by Green et al (11.0 μ, 12.8 μ; Green 1967b) and by Doerfler and Kleinschmidt (10.7 μ; Doerfler 1970b). The absolute lengths are slightly different between the two preparations probably due to variation in the spreading conditions. The ratios of the length of adenovirus DNA to that of the standard Ø 29 DNA are 1.777 and 1.781 for the parental viral DNA and the cyt mutant DNA, respectively. T-test for difference in means between these preparations revealed that there was no difference in viral DNA size at 5 % level of signifi cance. Therefore, it can be concluded that the mean length of the DNA molecule in the cyt mutant virion is exactly the same as that of the

FIGURE 21

Histogram of Contour Lengths of DNA Molecules of Adenovirus and ϕ 29

(a) Distribution of Ad 12 parental virus DNA moelcules

(b) Distribution of cyt mutant virus DNA molecules

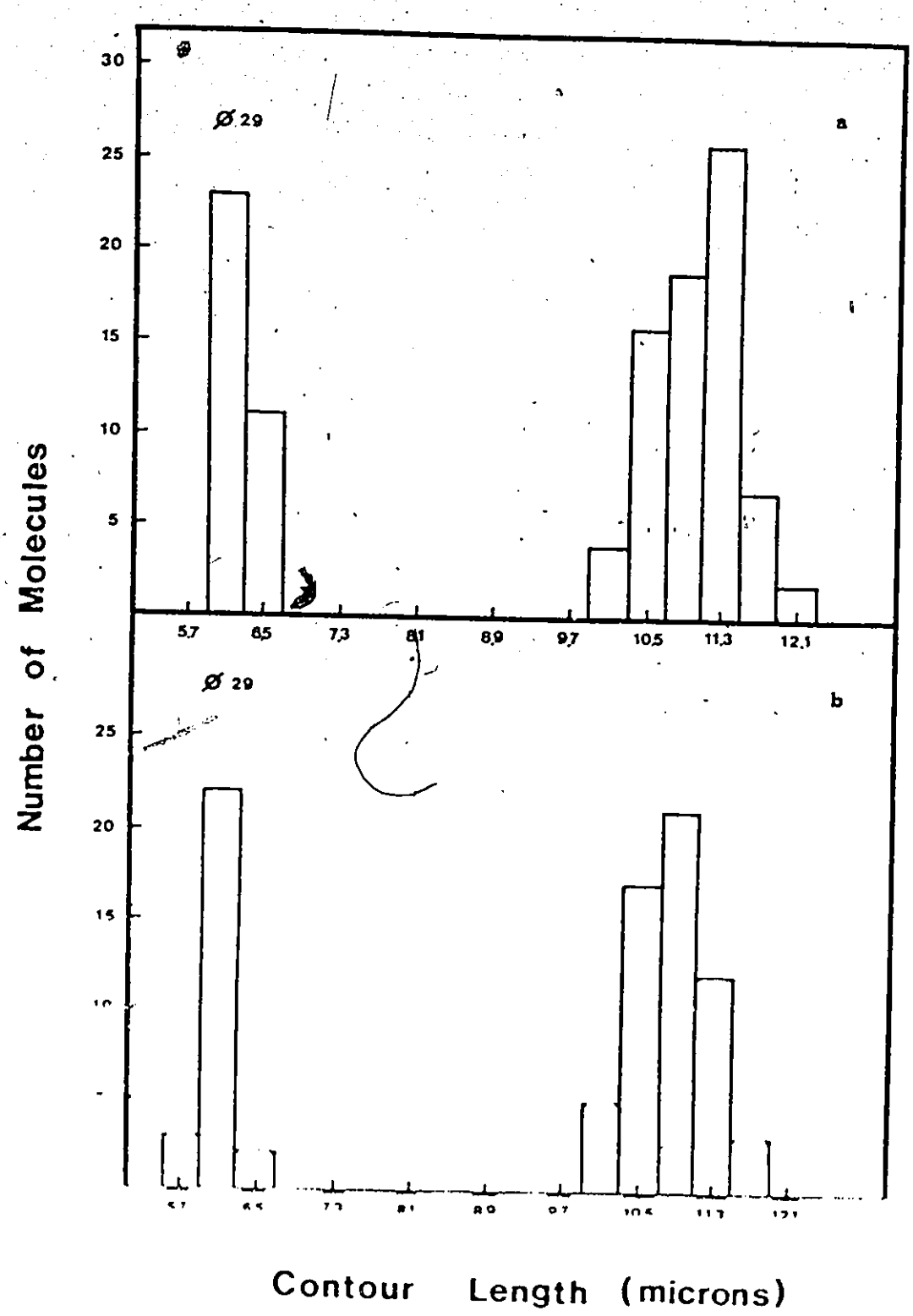

Number of Molecules
Ø 29
a
Ø 29
b
30
25
20
15
10
5
5,7
6,5
7,3
8,1
8,9
9,7
10,5
11,3
12,1
Contour Length (microns)

TABLE 13

Contour Length of Native DNA Molecules of Ad 12 Parental Virion, Cyt Mutant Virion and Bacteriophage Ø 29

Viral DNA	No. of molecules measured	Mean length (μ)	Standard deviation (μ)	Ratio of Ad / Ø 29
Prep. I				
Parental strain	74	11.07 (0.05)[a]	0.42	1.777
Ø 29	34	6.23 (0.02)[a]	0.13	
Prep. II				
Cyt mutant	58	10.83 (0.05)[a]	0.30	1.781
Ø 29	27	6.08 (0.03)[a]	0.13	

a. Standard error

parental strain, confirming the result on size estimation by rate zonal sedimentation (Figures 5 & 6).

Analysis of variance (F-test) shows that there is no difference between Ad 12 parental viral DNA and the cyt mutant DNA populations, while there is a significant difference in variance between adenovirus DNA and bacteriophage Ø 29 DNA populations. Adenovirus DNA appears to be more heterogeneous in size distribution than Ø 29 DNA, suggesting that this heterogeneity may probably contribute to a higher proportion of heterogeneity in biological functions of Ad 12 as described in the previous section.

B. Visualization of reassociated DNA

The advent of visualization of DNA molecules under an electron microscope allows one to study the sequence homology of two related DNA preparations (Garon 1973). Under suitable conditions, homologous and heterologous regions of DNA molecules can be physically mapped. In principle, if a mixture of two identical double-stranded DNA, AA' is denatured and renatured, the self-annealed product will entirely consist of the homoduplex AA' which is recognized as a complete double stranded form by electron microscopy. However, if a mixture of two different DNAs having partially homologous sequences, AA' and BB', is allowed to anneal, the hybrids will consist of each type of homoduplex, AA' and BB', and in addition to these, the heteroduplexes, AB' and A'B will be expected. If there is a partial

sequence heterogeneity between the two molecules, such as deletion or substitution, such heteroduplexes can be recognized as double-stranded molecules having partial single-stranded loops.

Purified preparations of Ad 12 parental virions appear to be more heterogeneous than those of the cyt mutant virions in biological functions and to contain a higher proportion of defective virions capable of cell killing only (section I). It is of interest to study whether the viral DNA molecules are heterogeneous with respect to their sequence. If small deletions and/or substitutions are present in some of viral DNA, it should be possible to detect them using a heteroduplex analysis. The same technique can be used to analyze any difference in viral DNA sequence between the parental strain and the cyt mutant.

(1) Self-annealing of Ad 12 parental DNA and cyt mutant DNA

Renaturation of the DNA was performed in the presence of 50 % formamide to avoid the damage of DNA due to prolonged incubation at relatively high temperature. In order to check the intactness of the DNA molecules during annealing under this condition, the size distribution of Ad 12 DNA after incubation for 8 hr at 37°C was examined by an alkaline sucrose gradient sedimentation. As can be seen in Figure 22, no fragmentation of the DNA molecules could be observed. This observation was further confirmed by measurement of the reassociated molecules under the electron microscope (see later section). About 90 % of the DNA molecules have been reassociated in

FIGURE 22

Sedimentation Profile of Reassociated Ad 12 DNA in a 5 to 20 % Alkaline Sucrose Gradient

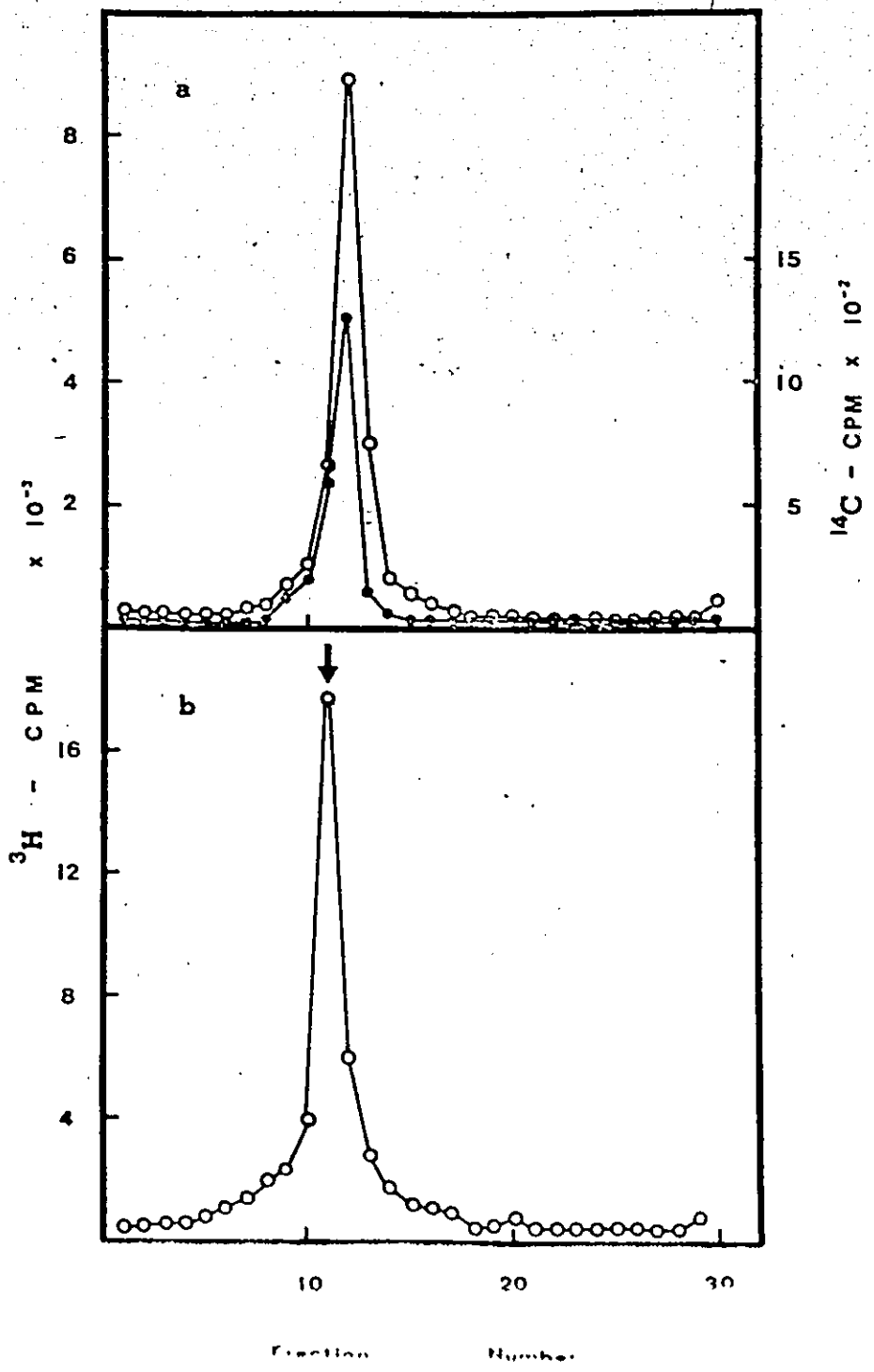
Sedimentation is from right to left.

(a) ^{3}H-Ad 12 native DNA (○)

^{14}C-Ad 2 marker DNA (●)

(b) ^{3}H-Ad 12 reassociated DNA (○)

Arrow indicates the position of ^{14}C-Ad 2 marker DNA


a
b
8
6
4
2
15
10
5
16
12
8
4
10
20
30
^{3}H - CPM x 10^{-3}
^{14}C - CPM x 10^{-3}
Fraction Number

heteroduplex formation. The extent of DNA reassociation was determined by the percentage of the fraction which was resistant to digestion by the single-stranded specific nuclease S 1 (see Appendix for detail of reassociation versus time).

Electron micrographs of each self-annealed DNA of the parental strain and the cyt mutant are shown in Figures 23 and 24. It can be seen that there are several configurations of reannealed molecules; (1) completely reannealed double-stranded forms, (2) aggregated configurations of both double-stranded and single-stranded DNA, and (3) double strands having a partial single strand tail. All these configurations except the first type make an interpretation difficult. However, in both preparations, there were essentially no double-stranded molecules having single-stranded loops. Thus, these results suggest that sequence heterogeneity in the viral DNA was not detectable.

(2) Hybrids between parental viral DNA and cyt mutant DNA

Absence of gross heterogeneity in DNA sequence of the parental strain and the cyt mutant was shown by the examination of self-annealed DNA molecules. However, it may be possible that substitutions may be present in either one of these two viral DNA molecules. This possibility can be tested by heteroduplex analysis. Figure 25 shows an electron micrograph of hybrid DNA molecules between the parental DNA and the cyt mutant DNA. No heteroduplex molecules with single-stranded loops were observed, indicating that neither

FIGURE 23

Electron Micrographs of Self-Annealed DNA Molecules of Parental Strain

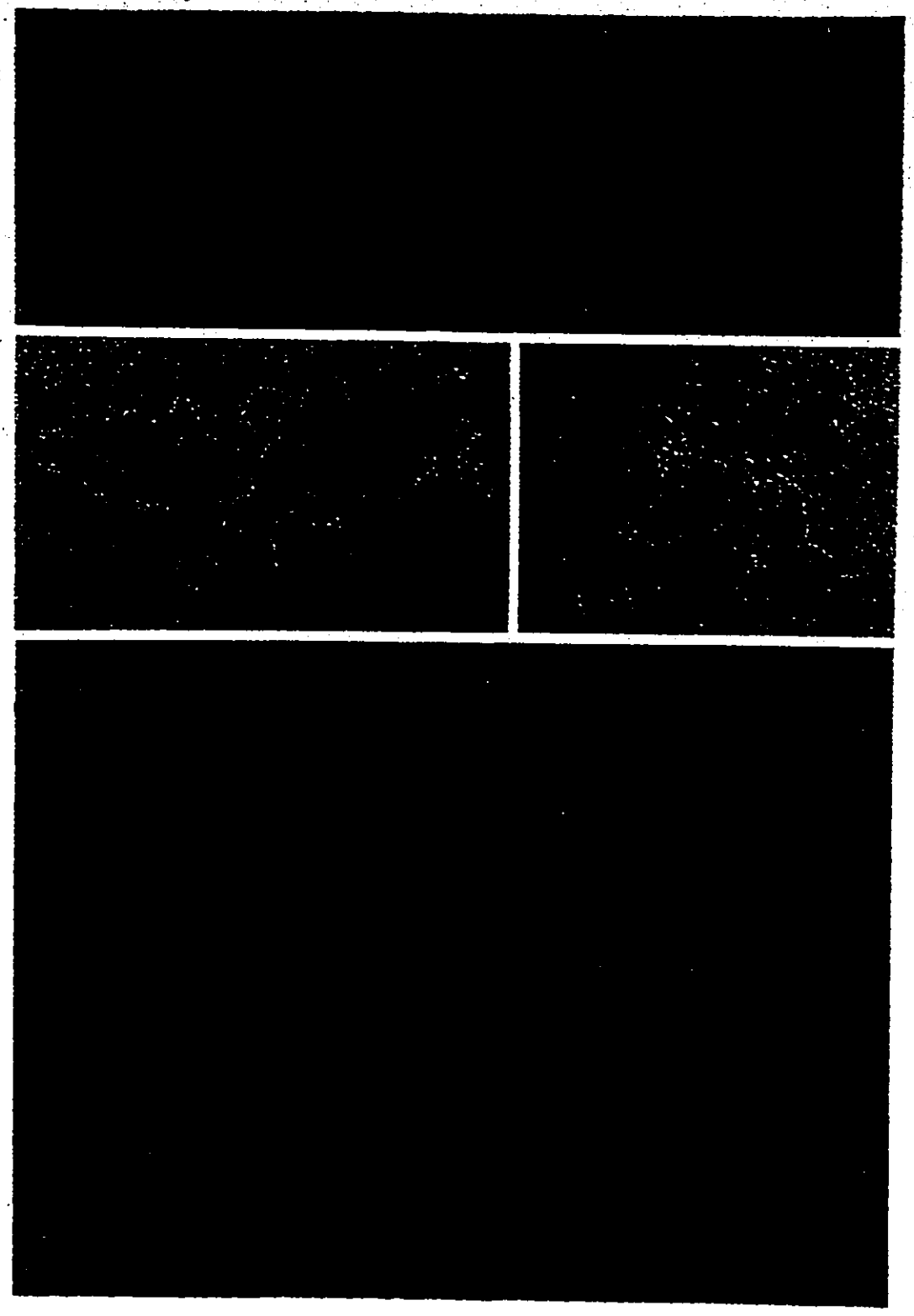


FIGURE 24

Electron Micrographs of Self-Annealed

DNA Molecules of Cyt Mutant

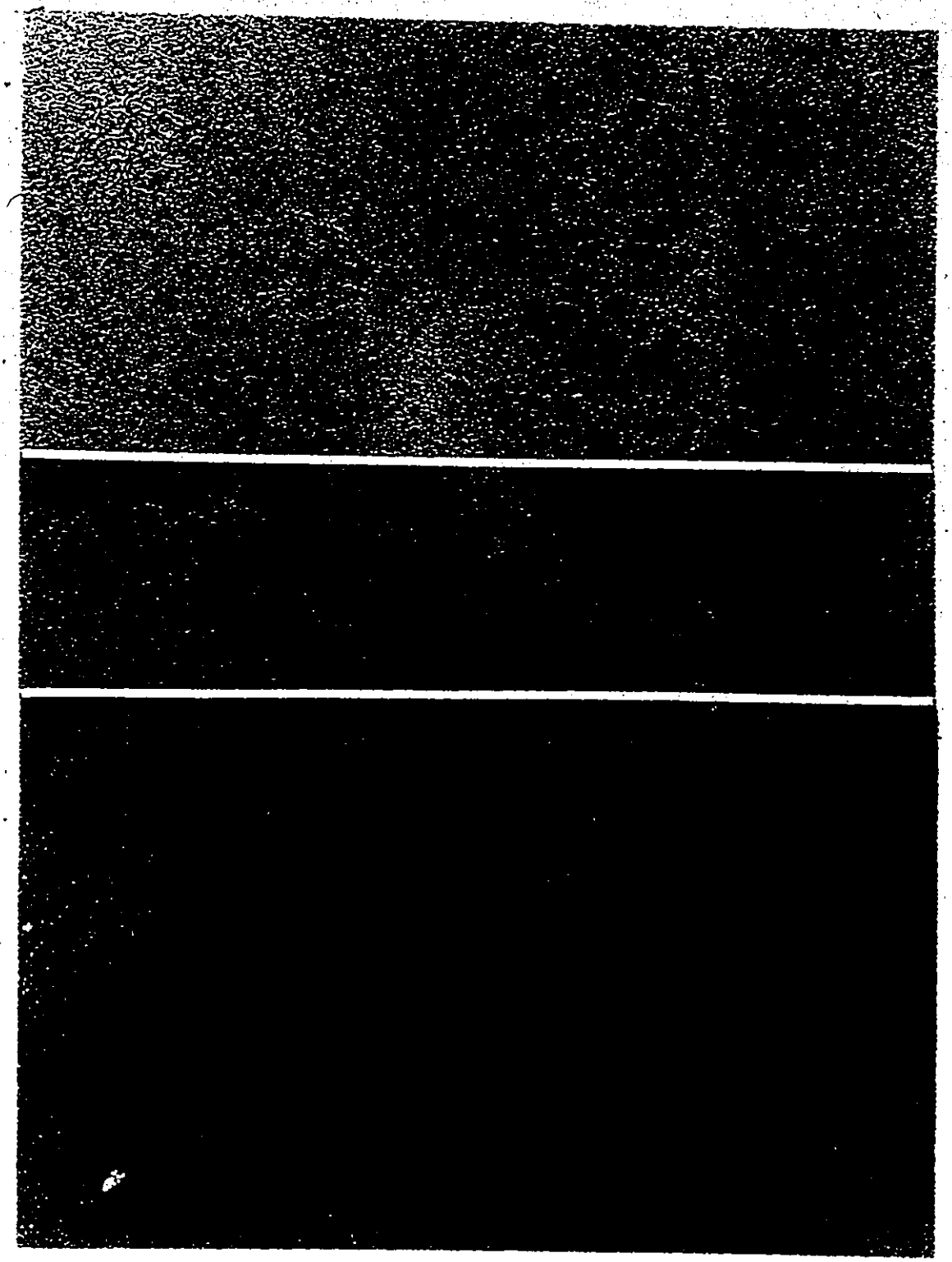
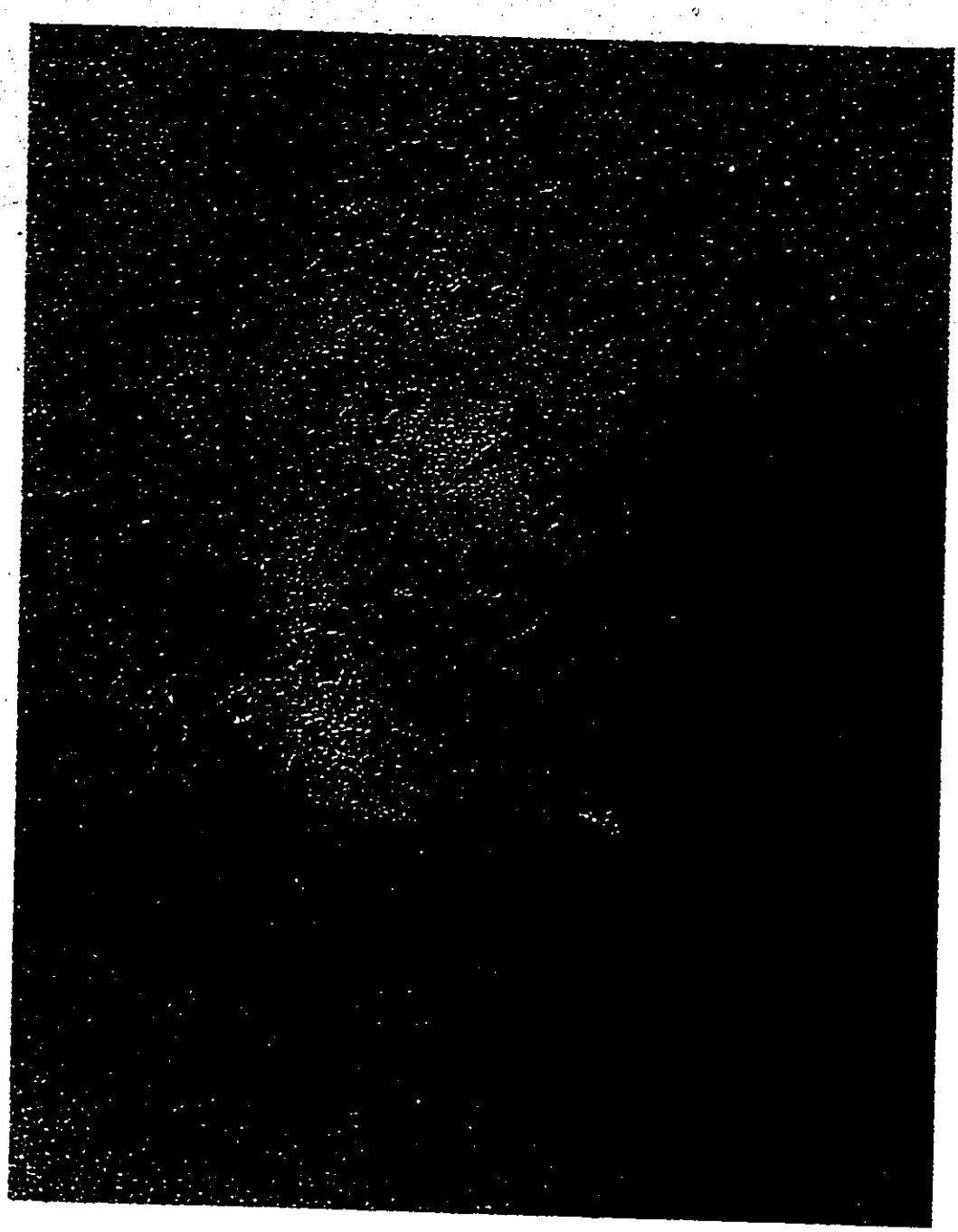


FIGURE 25

Electron Micrograph of Hybrid DNA Molecules of Parental Strain and Cyt Mutant



substitutions nor deletions could be detected.

C. Heteroduplex of defective Ad 12 DNA

A different strain of Ad 12 (Huie) has been shown to have functional heterogeneity (Rainbow 1970). In this strain, defective virions with lighter density than infectious one were detected subsequently by Mak (Mak 1971). These defective particles can carry out limited viral functions, such as inhibition of cell cloning and T-antigen induction as efficiently as the nondefective virions. Some of their properties, however, differ from those of the nondefective virions; they show a reduced plaque forming ability or a reduced formation of nuclear inclusion bodies in KB cells. Thus, these defective particles could probably account for the functional heterogeneity of the virions in Ad 12. It is of interest, therefore, to study the sequence alteration in the genome of these defective virions by an electron microscope heteroduplex technique.

In the first place, the size of the native DNA molecule of the defective virion was measured and the contour length of intact DNA was calibrated using Ø 29 DNA as an internal standard. Figure 26 shows a distribution of DNA lengths from defective virion together with that of Ø 29 DNA. Again the absolute lengths of the molecules are somewhat different between the preparations, however, the ratio of the length of the defective Ad 12 DNA to that of Ø 29 DNA is 1.658, which is significantly smaller than that of the

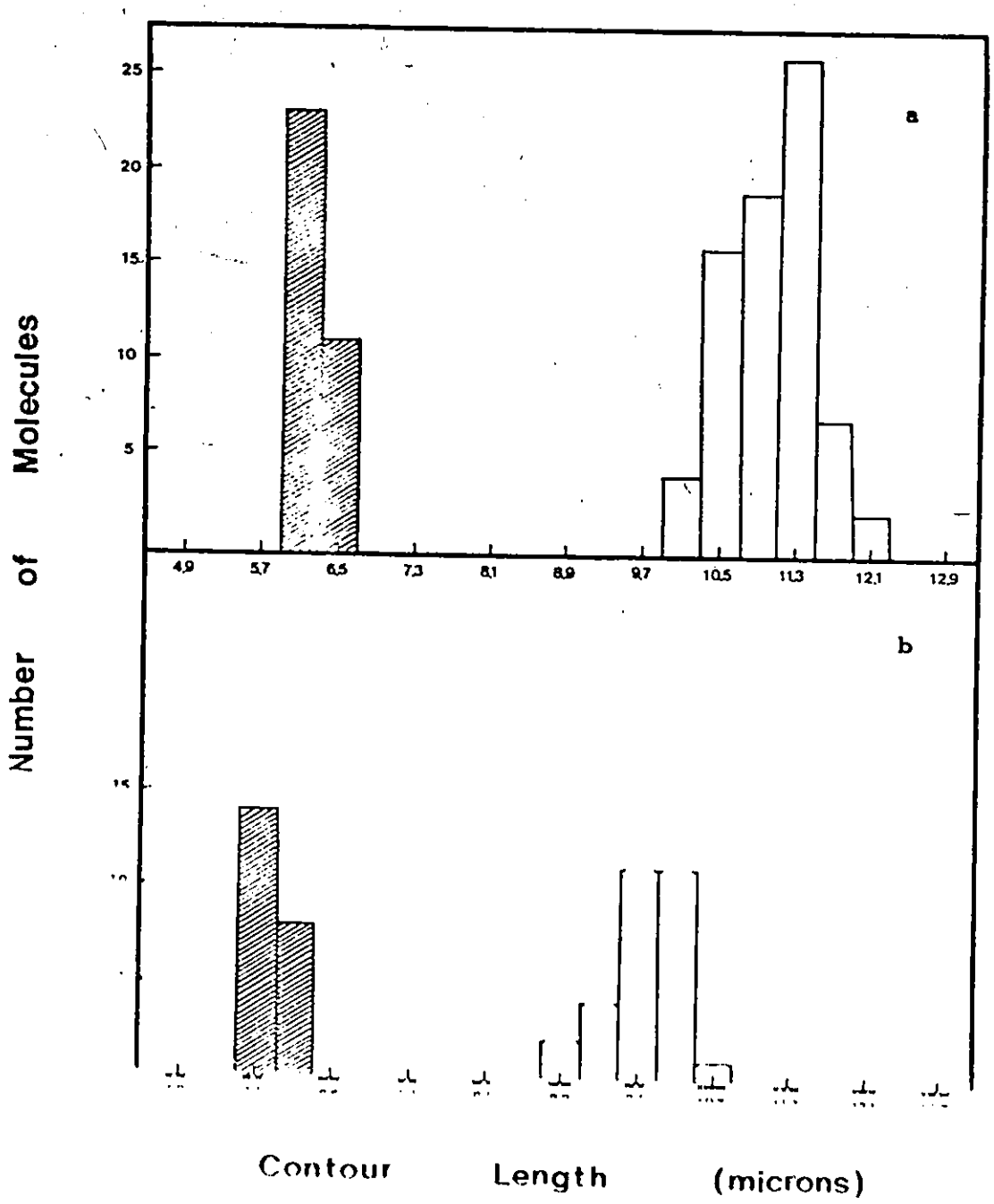
FIGURE 26

Histogram of Contour Lengths of DNA Molecules of Ad 12 Defective Virion and Ø 29

(a) Distribution of nondefective Ad 12 DNA molecules

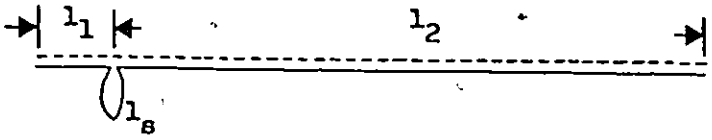
(b) Distribution of defective Ad 12 DNA molecules

Histograms with oblique lines are Ø 29 DNA as calibration standards.


Number of Molecules
25
20
15
10
5
a
4.9
5.7
6.5
7.3
8.1
8.9
9.7
10.5
11.3
12.1
12.9
b
Contour Length (microns)

nondefective DNA to Ø 29 DNA (1.777). Statistical analysis for difference in mean lengths of the DNA molecules revealed that there was a significant difference between these two viral DNA sizes with a 5 % level of significance. Therefore the defective adenovirus appears to have undergone a deletion of approximately 7 to 8 % of its genome.

The location of the deleted region can be mapped by the heteroduplex technique as illustrated in the following diagram:



$l_1 / (l_1 + l_s + l_2)$ gives the position of the deletion with respect to one end of the genome and l_s is the deleted region. Also, $l_1 + l_2$ should be equal to the length of the defective viral DNA, while $l_1 + l_s + l_2$ should be equal to the length of the nondefective viral DNA.

Equal amounts of DNA from defective virions and nondefective virions were denatured and reassociated. The reassociated DNA molecules were examined by electron microscopy. A typical heteroduplex molecule is shown in Figure 27. It can be seen that a single stranded loop is located near one end of the molecule. A total of 54 such heteroduplex molecules with a single stranded deletion loop was analyzed to determine the extent and location of the deletion. The results are shown in Figure 28 with a thicker line representing the extent and location of the deletion. All deletions map at almost

FIGURE 27

Electron Micrograph of Heteroduplex Molecule Produced by Nondefective and Defective Viral DNA of Ad 12

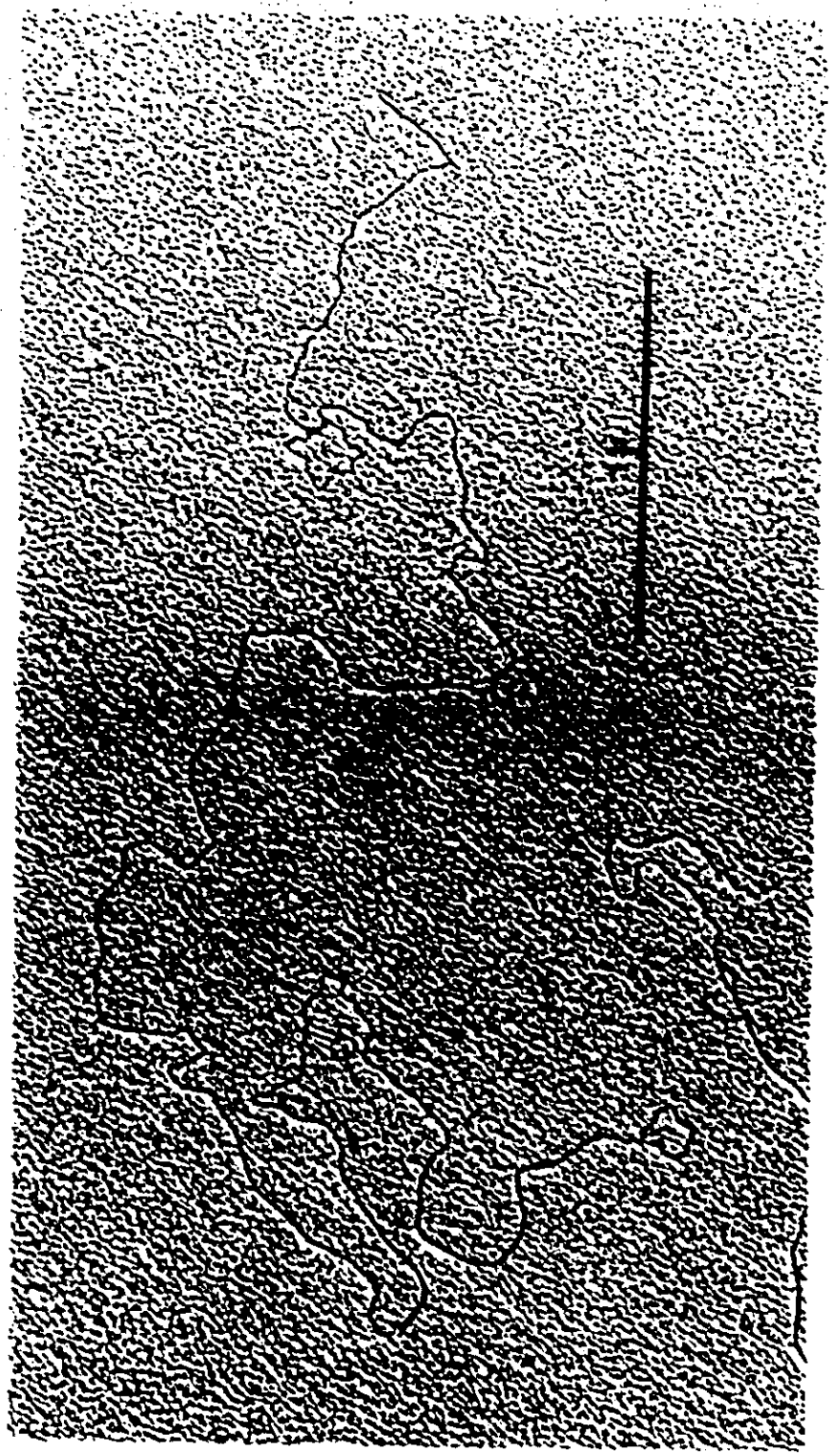
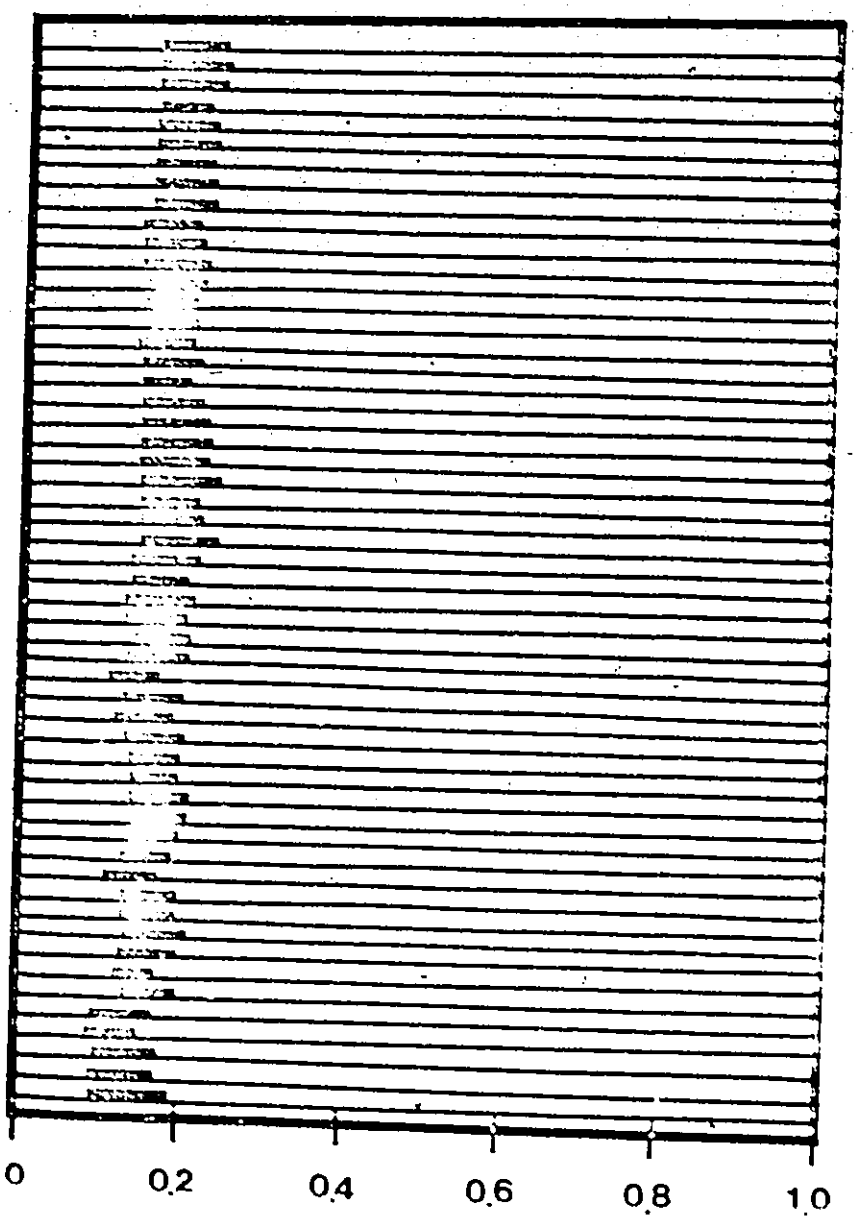


FIGURE 28

Mapping of the Deletion of Ad 12 Defective Viral DNA

Thicker lines indicate the region deleted on the adenoviral genome expressed as a fractional contour length.


0
0.2
0.4
0.6
0.8
1.0
Fractional Contour Length

the same region, ranging from 0.128 to 0.204 of the total length of the genome on the average. The fraction of the deletion (0.076) coincided well with independent measurement of the native defective DNA molecules as shown above. Also the average length of $l_1 + l_2$ was found to be 9.71 μ, compared to 9.75 μ for the native defective DNA molecules. Total length of $l_1 + l_s + l_2$ was calculated to be 10.50 μ on the average, compared to 10.45 μ for the nondefective DNA molecules. Thus, these heteroduplex molecules are really composing of strands; one from the nondefective DNA molecule and the other from the defective DNA molecule. The unique position of this deletion was also confirmed by the absence of such a heteroduplex molecule in a self-annealed preparation of defective viral DNA.

DISCUSSION

Regulation and control of biosynthetic process in mammalian cells are very complicated. A given specialized cell does not grow at any site. Each has only certain cell types for its neighbors and each cell type has its own unique pattern of differentiation. However, any of change in this regulated proliferation and differentiation can lead to abnormal growth which is collectively called cancer. This uncontrolled growth leads to localized masses of single cell types often referred to as tumors, and the resulting tumor often quickly leads to disease symptoms and culminates in the eventual death of the afflicted organism. At the present time, the nature of the change or loss of regulatory control of normal cell growth at the molecular level remains a total mystery. But there is general agreement that cancer is a disease at the cellular level, that occurs when a hereditary change somehow transforms a single normal cell into a cancer cell.

For the initiation of neoplastic proliferation, some event must trigger nonlethal changes in the cell that enable the expression of new functions--- enzymes, antigens and growth control. Many external agents called carcinogens, such as tumor viruses, ionizing radiation, ultraviolet light and various chemical compounds have been shown to increase the frequency of the cancerous transformation.

However, the mechanism of tumor induction by many of these agents is not known. The trigger for this cellular transformation may sometimes be a spontaneous somatic mutation or an environmental irritant. Whatever the stimulus, the mutation must be stable and when the newly induced cancer cell divides, both of its progeny carry the cancer property. Thus most carcinogens seem to act directly or indirectly by altering the genetic makeup of one or more host chromosomes.

There is evidence that the interaction between carcinogen and host cell may be more specific for tumor viruses than for chemical or physical agents. This specificity is documented by such features as common tumor-specific antigens and the persistence of functional viral genetic material in virus-transformed cells (reviewed by Black 1968, Green 1970a). The advent of animal cell culture allows one to study the tumor virus-cell interaction more precisely and quantitatively. The information thus obtained can be utilized to understand the process of viral oncogenesis.

As discussed in the Introduction, the virus-cell system is a useful tool for the study of cellular regulation. In general, to effect these complex changes in a mammalian cell a tumor virus should be capable of stable interactions with susceptible cells resulting most notably in latency and/or transformation. The virus must initiate cell transformation and stimulate cellular DNA synthesis but cannot impair cell functions requisite for mitosis. If virus genes are required to maintain transformation, as shown

with mutants of such oncogenic viruses as polyoma (Benjamin 1970, Eckhart 1971) and Rous Sarcoma Virus (Martin 1970), the virus genome must be associated with cellular DNA and replicated. Accordingly, limited virus transcription and protein synthesis might occur, and cellular transformation may partly be under the control of viral genes. Thus, study of virus-cell interaction is an ideal system for understanding of the molecular mechanism of regulation.

Human adenoviruses are the first human viruses shown to possess oncogenic potentials in newborn rodents. There is a wide spectrum in oncogenicity among different serotypes and their oncogenicity has been correlated to some of physical properties, such as GC content of the viral DNA and its genome size (Green 1970a). However, there is a limitation in the correlation between low GC content of human adenovirus DNA and its oncogenic potential. This cannot be extended to simian adenoviruses. To voercome this problem, other experimental approach such as genetic studies are necessary.

Genetic studies using viral mutants would be a powerful approach to elucidate the role of specific viral genes in a productive infection as well as in a nonproductive infection. In cells productively infected with a mutant, the process of viral replication is interrupted at a specific point. This allows examination of the events that have preceded the blocked step and determination of what is required to continue the process of viral

replication. The same approach using viral mutants with respect to transforming ability can be used to study what viral function is necessary in the course of cell transformation (for polyoma and SV 40 reviewed by Eckhart 1974). It is now recognized that such transformation does not require expression of the full complement of the viral genome. In fact, viruses unable to replicate are often capable of cell transformation. Graham and van der Eb have shown that not only intact Ad 5 DNA but also fragments of viral DNA can transform rat cells in vitro (Graham 1973). These results, therefore, indicate that integrity of the whole viral genome is not necessary even for the initiation of transformation.

The comparative study of highly oncogenic human adenovirus type 12 and its low oncogenic mutants is an ideal system for the elucidation of viral functions and tumorigenicity. Viruses of the same serotype having different oncogenic potentials are more advantageous than those belonging to different groups for the study of physical properties of the virions which have been studied by Green and his coworkers (Green 1970a). In this investigation, physical and biological properties of a low oncogenic mutant (cyt mutant) of Ad 12 were examined and biological functions were correlated with the physical properties of the purified virions.

There were no detectable differences in the density of virion or in viral DNA size as shown by sedimentation studies. Also using the more sensitive technique of electron microscopy, no difference in molecular size of viral DNA could be observed. An

electron microscopic study of viral DNA showed that no heteroduplex molecules having single strand loops were present in the preparation of either self-annealed DNA of the cyt mutant or hybrid DNA with the parental viral DNA. Therefore, the cyt mutation probably does not involve a gross change in the virus structure, deletion or substitution. It must be a very small alteration which is below detection by the techniques used. This point is also supported by the genetic data by Takemori et al showing that some cyt mutants can spontaneously revert to wild type (Takemori 1969).

The cyt mutant can induce T-(tumor) antigen in permissive cells with an equal efficiency as the parental strain (Figure 9). It is known that Ad 12-induced tumors or cells transformed by Ad 12 express T-antigen, which may be required for the maintenance of tumors or transformed state (Huebner 1963, 1964b). Therefore the low oncogenicity of the cyt mutant is not due to the lack of ability to induce T-antigen in infected cells. However under the condition used, one can not rule out the possibility of a minor alteration in T-antigen.

Other biological activities of the purified viruses, such as inhibition of cell cloning and induction of viral structural (V)-antigen in HEp-2 cells were determined. It was found that there are more virions capable of inhibiting cell cloning than of inducing the antigen. However, the ratio of particles required for the viral specific antigen induction to that for cell killing is higher in the parental strain than in the cyt mutant (3.5 to 4.5 times, Table 7), indicating that there is a greater degree of heterogeneity in the parental virus population than in the cyt mutant population. This

functional difference of the virus may presumably reflect the degree of viral gene expression in the infected cells. Similar results were presented by Strohl in Ad 12-BHK cell, nonpermissive system (Strohl 1969). He concluded that an infected cell synthesizing T-antigen is likely to be killed. The data presented in this thesis indicate that T-antigen synthesis is not necessary to inhibit a cell from forming a clone. Thus the viral genes responsible for cell killing are not identical to those for T-antigen induction. In conclusion, there is a greater proportion of defective particles capable of only cell cloning inhibition in highly oncogenic parental Ad 12 preparations than in low oncogenic cyt mutant preparations. The basis for the formation of these defectives is not known. But it is possible that defective particles may play an important role in tumorigenesis. The lower proportion of defective virions in cyt mutant preparation may account for its low oncogenicity. It is of interest to note that nononcogenic Ad 2 also shows a low degree of heterogeneity compared to Ad 12 highly oncogenic strain (Rainbow 1970).

A different strain of Ad 12 (Huie) was also shown to produce defective particles by high multiplicity of infection in KB cells (Mak 1971). In this investigation, an electron microscopic visualization technique was applied to quantitate the size of DNA molecule of this defective virion, and it was shown that the defective adenovirus appears to have undergone a deletion of approximately 7 to 8 % of its genome. Heteroduplex formation by reassociation of a mixture of the defective and nondefective viral DNA clearly indicated the

single stranded loop due to a deletion in one strand (Figure 27). Physical mapping of the position of this deletion showed that all deletions map at almost the same region, ranging from 0.128 to 0.204 of the total fractional length of the genome. The unique position of this deletion was further confirmed by the absence of such a heteroduplex molecule in self-annealed preparations of the defective viral DNA. Thus, in the lytic system, development of this type of defective virion during high input multiplicity of infection seems to be quite a selective process so that only one particular type of deletion mutant is accumulated. This is in contrast to the situation in SV 40, as shown by Brockman et al (1973), Mertz et al (1974) and Yoshiike et al (1974), where the heteroduplex deletion map is random on the SV 40 genome. At the present time, we do not know precisely the gene product(s) of this deleted region. However, the defective virus can induce T-antigen and inhibit cell cloning as efficiently as the infectious virus. It may be concluded that the deleted region is not necessary for the expression of these viral functions, but it is absolutely required for plaque formation (Mak 1971). It is not known at which end of the adenoviral DNA molecule this unique deletion is. Further precise physical mapping of the genes can be achieved by using specific DNA fragments generated by restriction endonucleases and heteroduplex studies. Preliminary results of such an experiment showed that there were no heteroduplex molecules having single strand loops in hybrid molecule between defective viral DNA and fragment "A" of Eco RI digests of Ad 12 DNA (35.6 % of the genome from right hand end, Mulder 1974). Therefore, these data suggest that the

deletion may be located between 12.8 % and 20.4 % of the genome from the left hand end.

Some functions of the genome of tumor viruses are more resistant to physical or chemical inactivation than the function of replication. Defendi and Jensen demonstrated that SV 40 and polyoma virus irradiated with ultraviolet light or by gamma rays, not only retained their oncogenicity for newborn hamsters, but actually exhibited an enhanced tumorigenicity when compared to that of the nonirradiated viruses. However, the infectivity of the irradiated samples had been inactivated (Defendi 1967). The authors postulated that oncogenicity would be a property of defective virus particles. In irradiated samples, the function of viral replication is inactivated, thus increasing the proportion of these hypothetical defective particles. These defective particles, in turn, would be responsible for the observed enhancement in oncogenicity. They speculated the molecular mechanism is that the fragments of viral DNA responsible for oncogenesis may be more easily incorporated into the cell genome if the integrity of the whole viral DNA is altered by radiation.

UV irradiation of oncogenic viruses usually results in the more rapid inactivation of the ability of a virus to replicate than to transform cells in cultures (for polyoma (Basilico 1965, Benjamin 1965, Interjet 1967), for SV 40 (Aaronson 1970), for adenovirus (Casto 1968, Finklestein 1969, Yamamoto 1971)). In other words, the cellular cytopathic effect of these UV-irradiated viruses is

generally reduced or removed long before the oncogenic potential is eliminated. Consequently, an increase in the transforming frequency of the virus was observed after low dose of UV irradiation of the virus. Similar result was obtained in herpes simplex virus (HSV) type 2 by UV-irradiation of the virus to demonstrate the possible oncogenic activity of HSV (Duff 1971a, b, Kutinova 1973).
The resultant hamster embryo fibroblast cell populations transformed by UV-irradiated HSV contain HSV-specific antigens.

A defective SV 40 genome integrated into Ad 7 DNA (PARA-Ad 7) has been described (Huebner 1964a, Rapp 1964, Rowe 1964). PARA-Ad 7 is defective in its biological functions, that is, it cannot code for SV 40 coat proteins, but it produces SV 40 type tumors in newborn hamsters, induces SV 40 T-antigen and tumor specific transplantation antigen (TSTA). The discovery and subsequent characterization of PARA-Ad 7 provided convincing evidence that late functions associated with the synthesis of capsid proteins and maturation of progeny virions are not required for the induction of SV 40 T-antigen, TSTA or oncogenicity.

The PARA Ad 7 particle is also naturally defective. Although it can initiate events of its replicative cycle, but it is unable to complete it without a helper adenovirus (Butel 1966, Rapp 1967). It has been reported that the inability of PARA to complete its replicative cycle may be responsible for the relatively high transforming frequency. If a critical step is blocked, either by genome inactivation or inactivation of a cellular function, the

PARA-Ad 7 may exhibit increased potential to transform the host cell (Duff 1972).

Isolation of defective particles has been achieved by physical methods. Uchida et al demonstrated that the defective particle of SV 40 was separated as virion with lighter density than the normal nondefective standard virion using CsCl density gradient centrifugation (Uchida 1968a, b, 1969). Defective virions are usually produced by serial undiluted passages of the virus or by high multiplicity of infection of permissive host cell. These virions were proved to be deletion mutants which contain shorter circular DNA molecules of various lengths depending on their density (Yoshiike 1968). The antigen formers (T particles) which produce at least T-antigen but not capsid (V)-antigen have much reduced infectivity. These exist abundantly in the defective virion population (Uchida 1968a), and they can transform mouse 3T3 cells with the same efficiency as plaque formers (Uchida 1969).

As reviewed above, functional heterogeneity in a given virus population may have an important role in viral oncogenesis. My experimental data are consistent with the idea that [illegible] may be significant in viral oncogenesis.

The cyt mutant shows a number of biological properties different from those of the parental strain: different type of CPE in permissive cells, low oncogenicity in newborn hamsters, inability to transform hamster embryo cells in vitro, and induction of surface changes of infected cells. These characters of the cyt mutant are

lost upon reversion. In addition to these cyt characters, cytotoxicity of the cyt mutant is prominent. Cells infected with the cyt mutant become very fragile. Consequently, extensive cellular destruction occurs at late times after infection. At the molecular level, it was found that intracellular DNA was extensively degraded (section II). This phenomenon may account for the destruction of infected cells. The degradation of DNA by the cyt mutant could be due to (1) adenovirus penton associated endonuclease, (2) virus-induced nuclease which is probably cyt mutant specific, or (3) participation of preexisting host cell nuclease which is activated upon virus infection. However, the first alternative, i.e., penton associated endonuclease of Ad 12, seems to be an unlikely explanation. Burlingham and Doerfler reported that the degradation product by this enzyme is rather homogeneous, having about 1/4 of the intact adenovirus DNA size. Thus, it suggests that the endonuclease may have some site specificity on adenovirus DNA (Burlingham 1971,1972). In contrast to this, the situation in cyt mutant infection is rather different: (1) The fragment size is rather heterogeneous, some of the molecules remain intact; (2) Degradation is not specific to adenovirus DNA but cellular DNA is also degraded; (3) Co-infection with the parental virus suppresses the fragmentation of DNA by the cyt mutant. Therefore, it is unlikely that the degradation of DNA is due to the endonuclease associated with incoming adenovirions. Rather, a different type of nuclease may be involved in DNA degradation by the cyt mutant infection. At present, whether this is due to newly induced enzyme by the cyt mutant infection or activation

of a preexisting cellular enzyme, cannot be distinguished. But the nuclease activity must be cyt mutant specific in either case.

It was observed that viral DNA was also drastically degraded by the cyt mutant. This leads to the observed low yield of the virus (section I). The reduction in virus yield of the cyt mutant can be entirely accounted for by the following observations: (1) the average cell infected with the cyt mutant produces about 1/3 the amount of the viral DNA produced by a cell infected with the parental strain; (2) only 18 % of the viral DNA is of the whole genome size in the cyt mutant, while 71 % of the DNA is of viral genome size in the parental strain.

It is of interest to note that co-infection with the parental strain can prevent the degradation of DNA by the cyt mutant (Figure 16). It can also complement the virus yield (Table 12). It has been reported that co-infection prevents the cellular destruction (Takemori 1968). Thus, it is possible that DNA degradation and cellular destruction by the cyt mutant may be linked. The result of the co-infection experiment indicates that the cyt character of the mutant is recessive. However, the mechanism of suppression of the cytotoxicity accompanying the cyt mutant infection by the parental virus is not known.

Whatever the mechanism, the cellular destruction representing the cytotoxicity of the cyt mutant may also play a role in tumorigenesis. It is possible to speculate that the low oncogenic cyt mutant can initiate transformation as efficiently as the parental

strain, but a certain cytotoxic effect may subsequently prevent the establishment of neoplastic transformation. Although the cellular destruction by the cyt mutant was tested in a permissive system, it is possible that in a nonpermissive system (Ad 12-hamster cell), the same cytotoxic effect may occur (Takemori 1968). Therefore, the cellular destruction rather than the inability to transform cells in vitro may account for the low oncogenicity of the cyt mutant.

Co-operation in tumor induction of the cyt mutant by a mixed injection with low tumorigenic field strain of Ad 12, or weakly oncogenic Ad 7 or Ad 3 (Takemori 1968) may be explained by this hypothesis as follows: The early event(s) of transformation may be initiated by the cyt mutant and the subsequent destructive effect may be prevented by the co-operating adenoviruses. Consequently, tumorigenic potential should be restored to the similar level to that of highly oncogenic adenovirus when both viruses are used. The hypothesis is compatible with other observation that the sera from hamsters bearing tumors induced by a mixture of the cyt mutant and Ad 7 contain antibodies against Ad 12 as well as Ad 7 (Takemori 1968, unreported observation). Furthermore, cells from two tumors thus induced contained Ad 12 specific antigen (Takemori 1968).

SUMMARY

Physical and biological properties of highly oncogenic human adenovirus type 12 and its low oncogenic mutant (cyt mutant) were compared. As a gross character of the purified virion, there was no difference in density of the virus particles and in DNA size, as determined by sedimentation studies and electron microscopy. However, the cyt mutant showed a number of biological properties different from those of the parental strain; (1) early cell lysis in KB cell suspension culture, (2) low tumor incidence in newborn hamsters, and (3) reduced yield of the virions independent of host cells.

The molecular mechanism of this reduced yield of the complete virions by the cyt mutant was investigated to determine if viral DNA synthesis was the limiting factor. Extensive degradation of intracellular viral DNA accounted for the observed low yield of the cyt mutant virions. The total amount of DNA synthesized by the cyt mutant having the viral genome size was only some 1/10 to 1/12 of that synthesized by the parental strain in KB cell. This DNA degradation by the cyt mutant, which could be due to a virus-induced endonuclease, was completely suppressed by co-infection with the parental virus and the amount of total virus produced was almost equal to that produced by the parental strain alone.

Defective virus particles present in purified virus stocks were demonstrated by assaying several viral functions; i.e., T- and V-antigen induction and inhibition of cell cloning. The results suggested that there was a functional heterogeneity in virus population. There was a higher proportion of defective particles, capable of only cell cloning inhibition, in the parental virus preparation compared to that in the cyt mutant preparation. This observation is compatible with the idea that the defective particles may play an important role in viral oncogenesis.

Visualization of viral DNA molecules was achieved by electron microscopy. No sequence alteration in the viral genome of the cyt mutant could be detected. However, the defective virus of a different strain of Ad 12 could be separated from the nondefective particles. The DNA molecule of this defective virus was examined by electron microscopy and it was shown that it had undergone a deletion of approximately 7 to 8 % of its genome. The deleted region of the viral genome was physically mapped by a heteroduplex technique at a unique position, ranging from 0.128 to 0.204 of the fractional length of the viral genome.

APPENDIX

Assay for DNA-DNA Reassociation in Formamide

The specificity of DNA-DNA reassociation and DNA-RNA hybridization depends strongly on the difference between melting temperature (Tm) and temperature of reassociation reaction. According to Marmur and Doty, the maximum rate of renaturation reaction of nucleic acid occurs some 25°C below its Tm (Marmur 1961). However, the exposure of nucleic acid to such high temperature with prolonged incubation presents serious disadvantages such as strand scission and depurination, and this is particularly unfavorable for heteroduplex study. To overcome this problem, formamide has been introduced by Bonner et al to allow the use of lower temperatures for the reaction (Bonner 1967). The correlation of reaction of thermal stability of the hybrid to formamide concentration was determined by two groups; (1) Tm is reduced by 0.72°C per each 1 % formamide (McConaughy 1969), or (2) 0.60°C per each 1 % formamide (Bluthmann 1973). Although there is a slight discrepancy between these two measurements, advantage of using formamide at low temperature was confirmed. Therefore, in the course of this investigation, formamide was used in the experiment of heteroduplex formation at low temperature. The degree of reassociation was determined by the fraction of

double-stranded form which is resistant to single strand specific nuclease S 1 digestion.

Following is a typical example of DNA-DNA reassociation mixture:

100 µl of 1 M Tris (pH 8.0)
40 µl of 0.25 M EDTA
280 µl of ^{3}H-DNA (23 µg/ml)
40 µl of 3 N NaOH ..... denatured for 10 min at room temperature

40 µl of 3 N HCl ..... neutralized
500 µl of formamide

Total 1.0 ml

Reassociation was carried out at 37°C. At various times after incubation, 0.15 ml of sample was taken, 1.35 ml of 0.01 M Tris (pH 8.0) was added to this, and the sample was dialyzed against 0.01 M Tris (pH 8.0) for 6 hr at an ice-cold temperature to remove formamide. The mixture was then subjected to S 1 nuclease digestion. Following is a reaction mixture:

700 µl of ^{3}H-DNA reassociated mixture
100 µl of 0.3 M NaCl
150 µl of 0.2 M acetate buffer (pH 4.5)
20 µl of 1 mg/ml heat-denatured calf thymus DNA
20 µl of 10 mM $ZnCl_2$
10 µl of 1 mg/ml nuclease S 1

Total 1.0 ml

Following incubation at 37°C for 1 hr, the aliquots were precipitated by the addition of cold TCA and filtered through

membrane filter (pore size 0.20 μ). After drying the filter, the radioactivity remained on the filter was counted by liquid scintillation system. Table 14 shows the result. The extent of DNA reassociation increased with increasing incubation times. 90 % of the DNA molecules was reassociated after 3 hr incubation under these experimental conditions.

The Cot curve was drawn through these points using the theoretical curve of second-order reaction determined by the equation:

$$\frac{C}{Co} = \frac{1}{1 + K\,Co\,t}$$ (Britten 1968)

where, Co : initial concentration of DNA in reaction mixture

C : concentration of DNA remained in single strand

t : reassociation time

K : reaction constant

This formula can also be expressed as:

$$\frac{Co}{C} = K\,Co\,t + 1$$

in which Co/C is a function of t, and Figure 29 shows such a Cot curve. As can be seen, reassociation of Ad 12 DNA under the experimental conditions used, followed second-order kinetics. Cot value at half-reassociation ($Cot_{1/2}$) is 2.24×10^{-2} OD·hr. This is similar to the value obtained under different conditions of reassociation (Lee, personal communication).

TABLE 14

DNA-DNA Reassociation in 50 % Formamide

Time of reassociation (min)	Input cpm[a] (A)	Cpm remained after S1 digestion[b] (B)	% reassociated[c]
5	5,443	1,002	18.4
30	8,389	4,949	59.0
60	7,583	5,759	75.9
90	8,320	6,500	78.1
120	8,140	6,784	83.3
180	8,273	7,452	90.1

a. Cpm of input ^{3}H-DNA was determined by cold 5 % TCA precipitation.

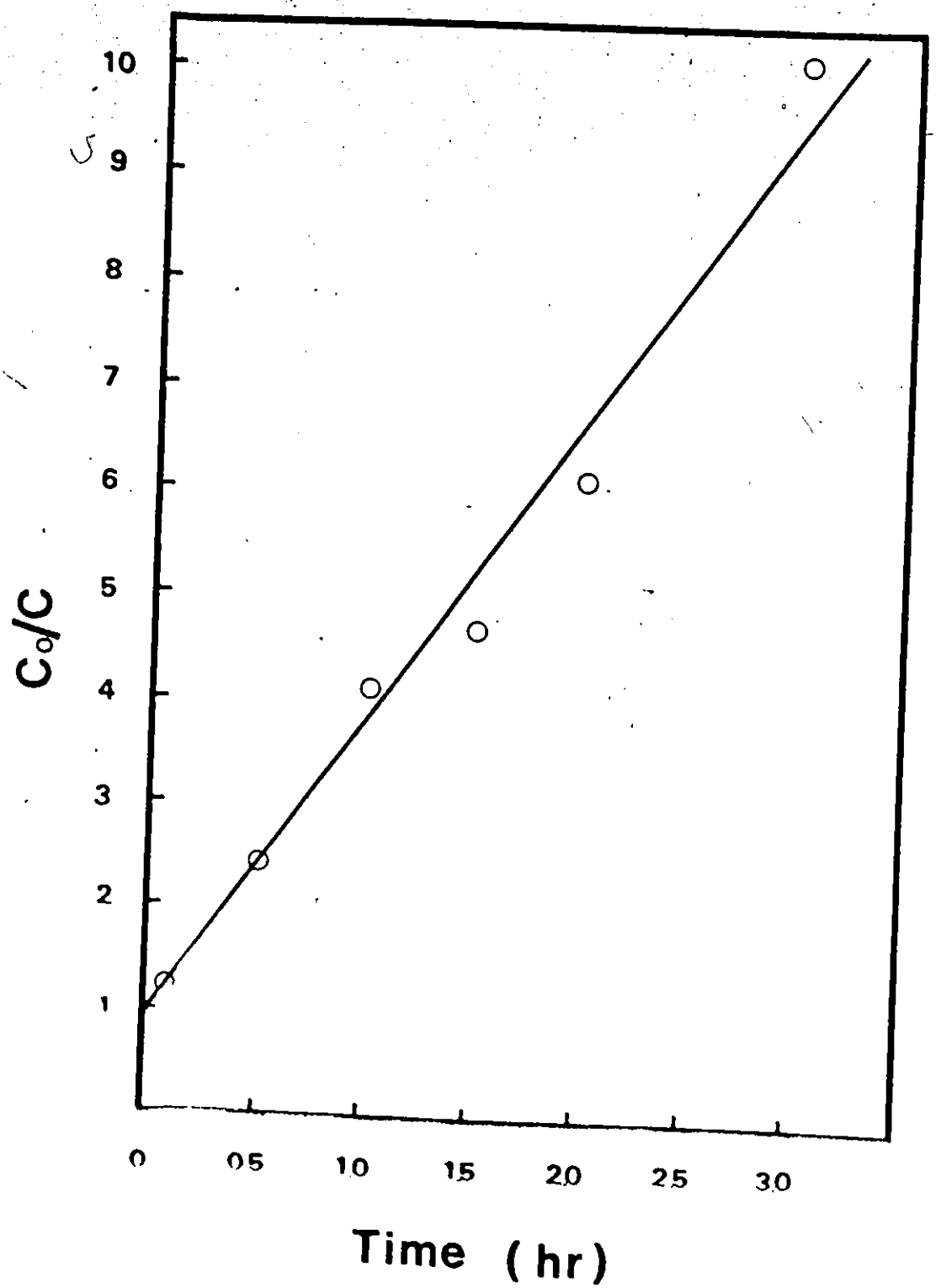
b. After reassociation, the sample was dialyzed against 0.01 M Tris (pH 8.0) and was digested with S 1 nuclease. Cpm resistant to S 1 nuclease was determined by cold 5 % TCA precipitation.

c. The extent of reassociation = column (B) / column (A)

FIGURE 29

Reassociation Kinetics of Ad 12 DNA

in 50 % Formamide


10
9
8
7
6
5
4
3
2
1
C_o/C
0
05
10
15
20
25
30
Time (hr)

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