DNA SYNTHESIS IN HUMAN KB CELLS INFECTED WITH ADENOVIRUS TYPE 2

STUDIES ON DEOXYRIBONUCLEIC ACID SYNTHESIS IN HUMAN KB CELLS INFECTED WITH HUMAN ADENOVIRUS TYPE 2

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SCOPE AND CONTENTS:

Human adenovirus, type 2, was utilized to investigate its effects on the host cell population. The progression of KB cells through the various phases of the cell cycle after infection was studied, with special emphasis of DNA metabolism. At different times after infection, the rate and the amount of viral DNA synthesized was determined and their efficiency of incorporation into virious was investigated.

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INTRODUCTION

Deoxyribonucleic Acid Synthesis in Human KB Cells Infected with Adenovirus, Type 2

Viruses, in general, have long been recognized as genetic agents which upon infection function to alter the metabolism of the host. By exposing cells cultivated *in vitro* to the action of viruses, it is possible to control the amount and the quality of the exogenous genetic material, the physiological and nutritional conditions of the host cells at the time of the introduction of the material, genetic and environmental influences which, acting at various times after infection, may affect the destiny of the virus-infected cell. It should be possible, moreover, to identify some specific biochemical alterations that follow viral infection and to find out what role they play in the viral maturation cycle and/or subsequent changes in cellular structure and function.

The introduction into living cells of viral nucleic acids quickly results in new functions appearing. The major system to study these viral induced modifications has been the bacteriophage-bacterium system. Cohen (14, 13) recognized the production of the T-even bacteriophages in

E. coli occurs by a sequence of very ordered processes. An example of such an ordered and controlled process is evident from the studies conducted by Pizer (78) concerning the phage induced new enzymes required for the synthesis of unique T-even phage DNA pyrimidine nucleotide, 5-hydroxymethyl deoxycytidylic acid. It has been through elaborate explorations of the mechanism(s) operative in the virus-infected cell that has founded today's modern molecular theories of biochemistry and genetics. It was primarily through investigations conducted on the multiplication of the T-even phages in E. coli that have substantiated the "central dogma" (103) of molecular biology, that is the relationship between DNA-RNA and protein.

Not unlike the bacteriophage system, animal viruses under proper conditions will elict a cellular response upon infection of a host cell. Animal virus infection of a host cell can be of a productive nature, characterized by a cycle of viral replication that eventually kills the cell, or of an abortive nature whereby the viral maturation process appears incomplete. In either form of infection, however, alterations in the normal cell metabolism and structure can be found, such as the induced synthesis of adenovirus-specific (T) antigen and viral DNA synthesis (8,70,89).

Animal viruses show a distinct advantage besides a lytic or non-lytic infection process above the bacterial

systems in that their studies can be performed at the whole animal, tissue or cellular level of anatomical organization. The DNA containing viruses, that is herpesviruses, papovaviruses, poxviruses and adenoviruses, have been intensively studied as to their tumorgenicity in various host animals. For example, Adenovirus, particularly types 12, 18 and 31 have been shown to be highly oncogenic in hamsters (19,53,76) while papovaviruses such as polyoma and SV40 have also been demonstrated oncogenic in newborn hamsters (30,31). It was soon established that the DNA containing animal viruses were also capable of transforming suitable host cells in tissue culture, Adenovirus (36,68,84,102), papovaviruses (94,100), poxviruses (18,32), and herpesviruses (19). Transformation involves a change in the growth properties of the target cell normally including the acquisition of the ability to grow under conditions where untransformed cells remain static. Cells explanted from tumors in animals into tissue culture are usually very similar in properties, such as the presence of neoantigens and the ability to proliferate under suboptimal conditions to cells initially transformed in tissue culture. Cells transformed in vitro also proved oncogenic upon introduction into appropriate animals even when virus free (1,70).

The basic problem of virology is the understanding of the process by which a virus multiplies after entering the cell. The advent of mammalian cell tissue culture

allows one to study virus cell interaction in a more precise manner because the following features have been made possible:

(1) a host cell population under defined and reproducible culture condition,

(2) uniform, synchronous and dramatic response of the cell population upon infection,

(3) production of a large amount of progeny virus by each cell,

(4) reliable and reproducible methods for infectivity assays for the viral population,

(5) large quantities of virus allowing effective purification.

Adenoviruses belong to a group of DNA-containing viruses which include herpesviruses, papovaviruses and poxviruses, by the nature of their DNA core material (17). Of the DNA viruses only the poxviruses (vaccinia) is a cytoplasmic virus; the others, adenovirus (31 human types), herpesviruses (herpes simplex) and the papovaviruses (SV40, polyoma) are all assembled in the nucleus of the infected cell.

The major difference distinguishing these four groups of viruses resides in the size of their DNA genomes. The DNA of the papovaviruses has a molecular weight that averages about 3×10^6 daltons (19) and can code for about 5 to 10 proteins, that of the herpesviruses ranges

from 60 - 70 X 10^6 dalton (87) while poxviruses are the largest genome of 167 X 10^6 daltons (54). The DNA of the adenoviruses has an average molecular weight of 23 X 10^6 daltons and occupies an intermediate position (48) in size and capable to code for at least 9 proteins (63,64,105).

Infection of a normal cell population with a new piece of genetic apparatus through viral infection of a cell is manifest in changes in the cell's architecture, biochemistry and dynamics as illustrated by the formation of inclusion bodies and cell size, enzyme activities, and transformation or cell death. In particular, interest has been focused on the possible mechanism(s) for the synthesis of virus specific genetic material. One may expect that the mechanisms of viral DNA synthesis to be different depending upon the specific requirements of different DNA viruses for biosynthesis. These requirements may be determined by such factors as the size of the viral genome, rate of multiplication or host cell conditions, etc. Although cytological methods such as radioautography may provide some information, the nature of viral DNA replication requires studies on the macromolecular pathways at the molecular level. Besides looking at the final product, viral DNA, enzymes involved in its synthesis have also been investigated intensively. Green (45) found that the DNA-synthesizing system showed very modest increases in DNA polymerase and various kinase activities between the Ad 2 infected and uninfected KB cells. Vaccinia virus

infected KB cells, however, showed several enzymes of the DNA synthesizing system to be increased in activity (45,55). The finding that polyoma and SV40 initiate DNA synthesis after infection of murine cell cultures (51,90) has also lead to a biochemical analysis of the DNA-biosynthesis pathway. Kit (57,58) among others have contributed information on increased activity of six major DNA pathway enzymes in the case of productive infection with papovaviruses; while four DNA synthesis enzymes are shown to have increased activity under abortive SV40 infection (59). Of the DNA containing viruses, adenoviruses show a DNA synthesis system most compatible with the normal cell functions.

Thus from investigations at the cellular and biochemical level, viral agents definitely alter the normal cell machinery and regulatory processes after infection in a very efficient and sometimes dramatic fashion.

The nature of the animal virus infection to facilitate the study of the regulation and control of cellular processes is necessarily supplemented with the study of the sequence of macromolecular events which are required for viral synthesis. The viral nucleic acid once it has entered the host cell must perform two types of functions. On one hand, it causes the synthesis of specific products, such as coat protein and enzymes; on the other hand, it replicates itself, culminating in the production of infectious progeny virions in the case of a lytic type of infection.

Certain key features found during the time course study of the productive infectious cycle of Adenoviruses are reiterated here in order to provide a basis for discussion of the thesis topic. The events involve in Adenovirus infection of a cell progress through a number of ordered steps:

(a) penetration and transport of the infectious virion(s) to the synthesis sites (20),

(b) transcription of specific regions of the parental genome (38,50),

(c) viral RNA-ribosome complex (37),

(d) synthesis of viral specific enzymes (45),

(e) replication of viral DNA (40,42),

(f) translation of viral specific RNA to viral substructures (105),

(g) and, finally, the assembly of components into mature infectious progeny virions.

These are the events which, one may assume, are maximally under the control of the viral genome. To carry them out to a successful conclusion, that is the production of infectious progeny virions, the virus needs the milieu of a permissive cell system.

Exploitation of the science of "cell biology" has and continues to be a very complex process since the mammalian cell has more than a million genes and thousands of different peptides being synthesized at any given time. Under these conditions cell biology has turned to animal viruses to

probe and analyze the mechanisms and regulation of such processes as replication, transcription and translation within the mammalian cell.

The data to be presented demonstrate that adenovirus, type 2 infection of human KB cells do indeed distrupt normal cell dynamics and morphology along with drastic alterations in DNA biosynthesis during the viral maturation process. Finally, some of the features of viral specific DNA biosynthesis were examined, permitting a comparison between the DNA content of the infected cell to the amounts of infectious virus isolated from an infected cell.

MATERIALS AND METHODS

A. TISSUE CULTURE TECHNIQUES

1. Glassware

(a) Monolayer Culture Vessels: "Saniglass" screwcap prescription bottles (Brown Glass and Supplies, Montreal, Quebec) in the 32 oz. size were used for growing cells on a glass surface. The bottles were used as received without additional cleaning; subsequent usages were preceded by standard cleaning and sterilization procedures for tissue culture glassware.

(b) Suspension Culture Vessels: Standard chemical reagent bottles were obtained in sizes ranging from 500 ml. to 4 litre capacities. The bottles were modified by the Glassblowing Department of the University to provide a pouring spout. The addition of a teflon magnetic stirring bar provided a suitable suspension culture vessel for large quantities of cells. Repeated utilization of the suspension vessel was feasible following standard tissue culture washing and sterilization.

2. Media

(a) Minimum Essential Medium, Eagle's salts (EMEM) (28): This medium was prepared from powdered constituents. The purchase of "Powder Media" provided a rapid, efficient and uniform method of preparation of EMEM. This medium was used mainly for growing cells on glass or plastic surfaces. The medium was purchased from Grand Island Biological Co., Grand Island, New York (Gibco, Catalogue Number F-12).

(b) Minimum Essential Medium, Joklik modified: As with EMEM, Joklik modified MEM was purchased from "Gibco" (Catalogue Number F-13). This medium has been specially modified by Gibco for use in suspension cultures.

(c) Preparation of Media: Routinely, 10-30 litres of media were prepared by dissolving the powdered medium in glass-distilled water as directed. The liquid tissue culture media were then sterilized by filtration through a glass fiber pre-filter and a Millipore membrane, GS type, with a pore size of 0.22 microns. Filtered media were dispensed into sterile 32 oz. Brockway glass (Brown Bottle and Supplies, Montreal, Quebec) and stored at 4°C.

3. Cell Line

Human epithelial cheek carcinoma cells, KB, (29) were originally supplied by Dr. M. Green, St. Louis, Missouri. The established cell line has since been

continued with propogation either on glass surfaces or in suspension culture. The KB cell line was used throughout this investigation for experiments, preparation of purified virus, and for titration of virus.

(a) Maintenance of Cell Cultures: Propogation of KB cells was accomplished by scrapping a monolayer culture growing on a glass surface with a soft rubber-policeman until the entire cell sheet was disrupted. The cell clumps were then dispersed by vigorous tituration with a 5 ml. pipette before transferring a small portion to another culture bottle to which 50 ml. of pre-warmed fresh EMEM containing 10% fetal calf serum was added. Maintenance monolayer cultures were maintained in EMEM containing 2.5% fetal calf serum without any antibiotic supplement.

In order to obtain large numbers of cells for experimental purposes, suspension cultures were used. A suspension culture may be started by scrapping several monolayer cell cultures, as above, and resuspending the cells in MEM (Joklik's modified) containing 5% horse serum. Sufficient suspension growth medium was added in order to give a cell density of 200,000 cells/ml. Subsequent subculturing was performed by volume dilution with prewarmed (37°C) suspension growth medium containing 5% horse serum to maintain the cell density between 200,000 to 400,000 cells/ml.

(b) Cell Stocks: To ensure a supply of cells in case of contamination, KB cells may be frozen and stored for an indefinite period of time. A sample of exponentially growing KB cells were concentrated from a culture vessel to a density of 10^7 cells/ml. by centrifugation, then resuspended in EMEM plus 10% fetal calf serum plus 10% dimethyl sulphoxide. For freezing, 1 ml. sample of cells were placed in glass ampoules (Johns Glass Co., Toronto) and sealed by fusing the ampoules' glass neck in a flame before being slowly frozen and stored in liquid nitrogen cryostat (85,93). Periodically a cell ampoule was thawed quickly and the cells were recovered by standard monolayer culturing conditions with frequent changes of growth medium.

B. VIROLOGICAL TECHNIQUES

1. Reagents

(a) Tris Buffer: Tris buffer was prepared from commercial THAM or TRISMA (hydroxymethyl aminomethane - Fisher Scientific Co., Catalogue Number T395 or Sigma Chemical Company, St. Louis, Missouri, Catalogue Number T-1503) in a 1 M solution then diluted to 0.01 M solution with double distilled water and adjusted to pH 8.1 with concentrated HCl.

(b) Tris Buffer Saline, TBS: TBS was prepared in a manner similar to that of Winocour (106) in a 5 fold concentrated form:

Sodium Chloride	80.0 gms.
Potassium Chloride	3.8 gms.
Disodium Hydrogen Phosphate	1.0 gms.
Tris Buffer, 1 M (pH 7.4)	300.0 mls.
Double Distilled Water to	2000 mls.

This 5x TBS stock solution was sterilized by filtration and stored at 4°C until use. The virus preservative fluid was prepared by diluting the 5x stock solution with sterile distilled water to 1x TBS and adding 20% sterile reagent grade glycerol.

(c) Cesium Chloride: The cushion CsCl solution used in virus purification was prepared by dissolving 41 gms. of pure CsCl powder (British Drug Houses, Toronto) and 59 ml. of 0.01 M Tris buffer, pH 8.1, (density 1.44 gms/cc)

while a CsCl solution of density of 1.339 gm/cc was prepared in a similar manner using 34 gms. of CsCl and 66 ml. of 0.01 M Tris buffer, pH 8.1.

2. Preparation of Adenovirus, Type 2

Logarithmically growing KB cells in suspension culture, at a concentration of about 200,000 cells/ml., were sedimented by centrifugation in an International Equipment Centrifuge, (Model RP-2) at 350 x g for 15 min. at room temperature. The cell pellet (3×10^8 cells) was resuspended in 31 ml. of fresh prewarmed to 37°C, MEM (Joklik's modified) containing 1% fetal calf serum. A sufficient sample of Ad-2 stock (usually 1ml.), previously titred to determine its infectivity (49) was added to the concentrated cell suspension so as to give a infectious input of 100 PFU/cell. The virus inoculum was allowed to absorb to the cells for 60 min. with the suspension continuously agitated at 37°C. Following absorption, the infected cells were diluted with 1500 ml. of fresh, prewarmed to 37°C, MEM (Joklik's modified) containing 5% horse serum giving a cell concentration of 200,000 cells/ml. The infected suspension culture was harvested after 48 hours (43) by centrifugation at 350 x g for 15 mins. at 4°C using a IEC Model PR-2 centrifuge. Following removal of all the supernatant culture medium, the cell pellet was resuspended in 10 ml. of 0.01 M Tris buffer, pH 8.1, and

frozen at -45°C until purification.

The methods of Green and Pina (44) were used with some modifications to purify preparations of Ad-2. Distruption of the infected cells was accomplished by three rapid cycles of freezing and thawing, or by ultrasonic vibrations generated from a Biosonik III system's needle-probe, Model BP III 40 T (Bronwill Scientific, Rochester, New York). To minimize heating the sample was held in an ice-bath during sonication. It was found that most efficient for cell disruption and virus release if sonication was performed for exactly 2 mins. at 30% power output (300 watts acoustical energy at full power).

Cellular material was further dissociated from the virus by two successive homogenization with cold Freon 113 (triflorotrichloroethane-Matheson chemicals) using a Sorvall Omni-mixer, Model OM-1150 (I. Sorvall Inc., Norwalk, Conn.) operating at a speed setting of 7.5 for 1 min. Centrifugation at 1200 x g for 5 min. at 4°C in IEC, Model RP-2 centrifuge was sufficient to separate the Freon 113 and the cellular debris from the aqueous layer which contains the virus. The aqueous layer was added to a graduated buriet and layered slowly on top of a CsCl cushion, density = 1.44 gms/cc in a nitrocellulose tube. The virus was sedimented on top of the CsCl cushion following centrifugation in a SW 27 rotor at 20,000 rpm for 120 min. at 4°C in a Beckman L2-65B preparative

ultracentrifuge (Beckman Instruments Ltd., Palo Alto, California).

The upper portion of the supernatant was aspirated and the virus containing opalescent band at the interphase was removed using a pipette. This virus solution was then mixed with CsCl powder and the density was adjusted to 1.34 gms/cc and centrifuged at 35,000 rpm for 20 hours at 4°C using a 65 rotor in a Beckman L2-65B centrifuge. This duration and speed allows a density gradient to form in the tube and the virus was concentrated in a narrow band.

Collection of the virus band was accomplished by piercing the centrifuge tubes from the bottom and collecting the drops that showed the opaque virus band. If the virus preparation was going to be used as stock seeds or experimental infection procedures it was subsequently diluted aseptically with TBS plus 20% glycerol (92) and stored frozen at -45°C in a Revco Freezer (Revco Inc., West Columbia, South Carolina).

3. Titration of Ad-2 - Plaque Assay

Infectivity of Ad-2 was measured using the plaque assay method (49). The unit of infectivity will be expressed as plaque forming units (PFU).

(a) Overlay Medium: Complete overlay medium was prepared by adding aseptically the following ingredients:

EMEM (2X)	82	ml.
Amino Acid Mixture (100X) . . . . BME, MA #13602 (Gibco)	2	ml.
Vitamin Solution (100X) . . . . . BME, Cat. #104 (Gibco)	2	ml.
L-glutamine (2.0 mM)	2	ml.
Arginine (2.1%) (86,88)	0.7	ml.
NaOH (1.0 N).	0.4	ml.
Horse Serum	12	ml.
Fetal Calf Serum	12	ml.
Sodium Bicarbonate (1 M).	6.0	ml.
Antibiotic Solution	2.0	ml.
Fungizone (100X) . . . . . Amphotericin B 250 mcg/ml Cat. #529 (Gibco)	0.2	ml.

(b) Antibiotics: The antibiotic solution used in the overlay medium consisted to penicillin and streptomycin. Penicillin G, sodium salt, U.S.P., 1.650×10^3 units/mg., and streptomycin sulphate, 740 µg/ml., were purchased from General Biochemicals (Chagrin Falls, Ohio). Penicillin and streptomycin were prepared as a single stock solution by dissolving aseptically each component in sterile double distilled water, penicillin at 6×10^6 µ/ml., streptomycin at 7.4×10^3 µg/ml.

(c) Phosphate Buffered Saline (PBS): This reagent was prepared as a 10 x concentrated stock solution in the following manner:

NaCl	80.0 gms.	
KCl	2.0 gms.	- A - Dissolved in 800 ml. of distilled water
Na_2HPO_4 . . .	11.5 gms.	
KH_2PO_4	2.0 gms.	
$CaCl_2$	1.0 gms.	- B - Dissolved in 100 ml. of distilled water
$MgCl_2.6H_2O$ . .	1.0 gms.	- C - Dissolved in 100 ml. of distilled water

Units A, B, C, were sterilized separately by filtration through a millipore filter, pore size 0.22 microns before being mixed.

The stock solution was diluted with sterile glass distilled water as needed.

(d) Assay: For the plaque assay, 10^6 exponentially growing KB cells suspended in 10 ml. of EMEM supplemented with 10% fetal calf serum were placed in a 60 mm. Falcon plastic petri dishes (Falcon Plastics, Los Angeles, California) and incubated at 37°C with 5% CO_2 and maintained at 90-100% humidity. Twenty-four hours later, a complete cell sheet would be formed. The medium was removed and cells were washed once with warmed EMEM, and inoculated with 0.2 ml. of the virus, appropriately diluted in PBS containing 0.1% bovine serum albumin. After absorption for 90 min. at 37°C with intermittent rocking of the dishes to distribute the viral inoculum evenly over the cells, the plates were overlaid with 5 ml. of nutrient agar. Nutrient agar was constituted immediately before use from completed overlay

medium diluted 1:1 (v/v) with 1.8% (w/v) noble agar, warmed to 45°C. After 5 days of incubation, the plates were overlaid with an additional 5 ml. of white nutrient agar. At the 9th day, the plates were again overlaid but the nutrient agar contained 0.0043% of neutral red. Plaques were observed after the eleventh day of incubation and the plaque number was recorded daily until constant.

C. CYTOLOGICAL TECHNIQUES

1. Reagents

(a) Colchicine: Colchicine powder (Alkaloid U. S. P., Fisher Scientific Co., New York) was used at a concentration of 10^{-6}M in order to provide an efficient mitotic inhibitor (11).

(b) Orcein Stain: As a general cytological stain, a 2% solution of orcein (Fisher Scientific Co.) was prepared in 45% galcial acetic acid as described by Fogh (34).

(c) Hypotonic Solution: A 1% sodium citrate (w/v) solution was prepared in distilled water.

(d) Hypertonic Solution: This solution was as a 2 x concentration of PBS.

(e) Hydrolysis Solution: various concentrations of HCl were used for hydrolysis, prepared from reagent grade HCl (Allied Chemicals, Morristown, New Jersey) and distilled water.

(f) Feulgen Stain: The Schiff reagent was prepared in a manner similar to that of Swift (96). The method consisted of dissolving 0.5% (w/v) of basic fuschin (Fisher Scientific Co., Catalogue Number F-98) in boiling distilled water, following the solution was cooled to 50°C and filtered to remove residual stain crystals. Formation of active Schiff reagents was accomplished by adding 10 ml. of 1 N HCl and 1 gm. of powdered potassium metabisulphite

for every 100 ml. of the filtrate. This solution was stored for twenty-four hours in a dark solution bottle at 4°C. Decolouration of the solution was accomplished by activated charcoal (Norite) treatment and filtration until a clear solution was obtained. The stain was prepared fresh before staining of experimental slides.

(g) Sulphite Wash Solution: Excess Schiff reagent was washed from the stained material by passing the slides through the following solution:

Potassium metabisulphite 10% aqueous solution	5 ml.
HCl, 1 N	5 ml.
Distilled Water	90 ml.

2. Preparation of Slides

(a) For Microspectrophotometry Measurements: The method used for microspectrophotometric analysis necessitated the shrinkage of the tissue culture cells in a uniform manner to a relatively spherical form in which the chromophore groups were quite condensed. This requirement was fulfilled by resuspending the cells, which were sedimented from the culture medium by centrifugation, with 2 ml. of hypertonic PBS for 10 mins. at room temperature. Two ml. of acid alcohol fixative (3 parts of absolute ethanol to 1 part glacial acetic acid) was added to the cell suspension and let stand for 5 min. Then the cells were sedimented by

centrifugation and resuspended in a similar manner in 2 ml. of fresh fixative and let stand for 5 min. The fixed cells were again sedimented and all residue supernatant fluid was carefully drained from off the cell pellet. The cells were resuspended in a few drops of fresh fixative and applied drop-wise to a clean microscope slide (Clay Adams Inc., New York, Catalogue Number A1450). The cells were air-dried overnight before being stained by the Feulgen method.

(b) For Cytological Examinations: Slides were treated essentially the same as in (a) but pre-treatment with 1% sodium citrate, 10 mins. at 37°C was used instead of the hypertonic treatment. The cells were stained immediately after drying with orcein instead of Feulgen.

3. Feulgen Staining

In several qualitative studies (3 , 23) of the Feulgen reaction it has been noted that the critical transition of deoxyribonucleic acids to apurinic acid was very sensitive to the duration and temperature at which the acid hydrolysis was conducted. In this investigation the hydrolysis reaction was performed on hydrated specimens either in 1 N HCl, 60°C for exactly 12 min. (96) or in 5 N HCl at room temperature for 1 hr. (22 , 23 , 35) with comparable results. Following hydrolysis the slides were washed in distilled water then placed in

fresh Schiff reagent, which was contained in black taped staining bath at room temperature. After 60 mins. staining the material was passed through 3 rinses of the sulphite washing solution followed by one wash in distilled water. The stained cell preparations were then processed for radioautograph as outlined later. In order to achieve a uniformity in the staining reaction, the usual case was to arrange to stain one slide per experimental time in conjunction with other sampling times.

4. Radioautographic Techniques

Microscope slides containing Feulgen stained cells were coated with photographic emulsion in the following manner:

Ilford Nuclear Research Emulsion, K-5, in gel form was purchased from Ilford Ltd., Essex, London, England. The technique for coating slides is that of Kopriwa and Leblond (61). Operating under a Kodak safelight filter, Wratten Series No. 2, the gel emulsion was melted in a waterbath at 55°C for 30 min., then diluted with distilled water, also at 55°C, in a ratio of 1:1. The solution was mixed slowly with a glass rod, and residual air bubbles were carefully scooped from the surface with a clean slide; the emulsion was then ready for coating the slides. Two slides, back to back, were dipped vertically into the emulsion, for

1-2 seconds, removed from the emulsion and excess fluid was allowed to drain onto filter paper. The backs of the slides were wiped free of melted emulsion, and the slides placed in a rack for drying; a slide box with large openings cut into its base and top, so that only the frame structure remained. The drying rack was placed in a light-proof drying box equipped with a variable speed electric air-blower. Emulsion coated slides were dried in this apparatus in a gentle air stream at room temperature for 30 min. The experimental slides were then transferred to light-proof slide box, containing a dehydrating agent (Drierite) and the edges of the box were wrapped with light-tight electrical tape. The slides were stored at 4°C. Following 1-2 weeks exposure the radioautograms were developed in D-19 developer (Kodak Ltd., Rochester, New York) for 2 min., rinsed in water, fixed in acid-rapid fixer (Kodak Ltd., Rochester, New York) for 5 min. and washed for 20 min. in gently running water, all operations were performed at 19°C.

All photographic procedures were conducted under a Kodak safelight filter, Wratten Series No. 2. In a similar manner as outlined above, the level of background fog on a test slide was assessed for each new batch of emulsion.

5. Microspectrophotometry

Many chemical compounds of cells are detectable by means of induced colour reaction with specific stains (24). Colour induction is possible by a specific chemical reaction such as in the case of the Feulgen reaction in which a colour complex is generated in the presence of DNA. The stoichiometric nature of the reaction allows a quantitative determination of the amount of reactive groups present in the sample, i.e., arbitrary units of DNA per cell nucleus.

The two wavelength method of microspectrophotometry was employed in this study (71 , 74 , 80) using a Leitz microspectrophotometer, Model MPV (W. A. Carveth Ltd., Toronto) equipped with an adjustable iris diaphragm to facilitate measurements of the photometric field over a cell nucleus.

(a) Calculations and Operation: In spite of the fact that Mendelsohn (72) has greatly simplified the calculation for the two-wavelength method, they are still very tedious and specific values might be acceptable but fall outside the range of published values. Thus it was decided to compute the relative chromophore content per cell from the general formula of Patua (74) with the aid of the I.B.M. 7040 computer at McMaster University.

General Formula: $$m_t = \frac{B\ L_a\ C}{k_a \ln 10}$$

$$C = \frac{1}{2 - Q} \ln \frac{1}{Q - 1}$$

$$Q = \frac{L_b}{L_a} = \frac{1 - Tb}{1 - Ta}$$

Parameters: m_t = total amount of chromophore

Ta, Tb = transmission at wavelength a and b respectively

k_a, k_b = absorptivity at wavelength a and b respectively

a and b refer to two wavelengths such that

$$k_b = 2k_a$$

B = the area of the photometric field

In practice, one selects from a radioautographic slide, a single non-labelled Feuglen stained, cell nucleus and adjust the microscope stage in such a manner so as to position the cell nucleus in the optical path incident upon the photometer attachment. By means of a variable measuring iris diaphragm in the monocular tube it was possible to restrict the image area to that of cell nucleus. The exact coordination of the measuring field with the object to be measured can thus be achieved, i.e., the value B in the general formula. Maintaining a constant measuring field the transmission values were obtained for the two specific wavelengths (Ta, Tb) by measuring the absorptivity of the cell nucleus relative to the absorptivity of a clear

area adjacent to the nucleus by means of a light-measuring photometer. The transmission values for each wavelength were then substituted into the general formula in order to calculate the total chromphore content (m_t) for one specific nucleus. The process of randomly selecting another nucleus adjusting the measuring field and determining the transmission values for each wavelength was then repeated.

6. Rate of DNA Synthesis

The rate of incorporation of ^{3}H-TdR into the acid insoluble fraction of a cell sample was used to measure the rate of DNA synthesis. Uninfected and Ad-2 infected cells were incubated in the usual manner and at various times after infection a cell sample was removed from each culture and pulse labelled with ^{3}H-TdR (1 μCi/ml., Sp. Act. 16.1 Ci/mM) added to the culture medium at 37°C. The duration of the radioactive pulse ranged from 1 hour at early times after infection, to 3 hours at later stages of viral maturation. Following incubation with the isotopic DNA precursor a known cell concentration, determined by triplicate hemocytometer determinations, was rapidly chilled by the addition of ice-cold PBS to stop further isotope incorporation. The sample was washed with cold PBS before a 1 ml. aliquot containing at least 10^5 cells was added to 2 ml. of ice-cold 10% (w/v) trichloroacetic acid. The sample was incubated for 20 min in a ice bath before the

acid insoluble cell fraction was washed unto a membrane filter by suction. The filter was dried in a scintillation vial and radioactivity was counted in 5 ml. of scintillation flor (Omniflor - New England Nuclear Ltd.) using a liquid scintillation spectrometer. The rate of isotope incorporation was expressed as cpm of ^{3}H-TdR in the acid insoluble cell fraction per cell per hour of pulse labelling.

Radioactivity bound to filters was determined in 5 ml. of toluene containing 0.02 gms. Omniflor (New England Nuclear, Catalogue Number NEF-906) when liquid scintillation counting was employed.

D. BIOCHEMICAL TECHNIQUES

1. Preparation of Adenovirus Type 2 DNA

Viral DNA was extracted from purified preparations of Ad-2 (see Section B-2). After collection of the virus band from the CsCl equilibrium centrifugation the virus was dialysed against 0.01 M Tris buffer, pH 8.1 for 48 hrs. with several changes of buffer. Viral DNA was extracted by a method similar to Green and Pina (46, 77) from 1 ml. of purified virus by incubating the virus in a buffer containing: 150 lambda of 0.05 M EDTA, pH 7.0; 150 lambda of 1 M $NaH_2PO_4.H_2O$, pH 6.0; 15 lambda of 1 M cysteine HCl, and 20 lambda of papain (27.5 mg/ml) for 1 hour at 37°C. To the mixture was added 150 lambda of 5% sodium dodecyl sulfate in 45% ethanol and further incubated in room temperature for 30 min. The solution was deproteinized using redistilled phenol saturated with 0.1 M NaCl and 0.05 M NaH_2PO_4 and gentle agitation at 4°C for 15 min. Centrifugation at 10,000 rpm for 10 min. in a Model RC2-B Sorvall centrifuge was sufficient to separate the aqueous supernatant layer containing the viral DNA from the phenol layer. The aqueous layer was removed and subjected to 2 additional deproteinizationsas above. The aqueous layer containing viral DNA was then dialysed for at least 48 hrs. against several changes of 0.1 x SSC at 4°C before being used for biochemical studies.

2. Preparation of KB cellular DNA

Cellular DNA was extracted from whole cells in a manner similar to Marmur (66) and Thomas (99). The procedure called for resuspending approximately 0.5 ml. of cell pellet in 9.5 ml. of a buffer consisting of 1 x SSC (0.15 M NaCl + 0.015 M sodium citrate), 0.1 M Tris buffer, pH 8.0, 0.005 M EDTA, and 0.3 M sodium-trichloroacetate; subsequently referred to as the "DNA buffer". To the cell suspension in the "DNA buffer" was added 400 micro-liters of pronase (Calbiochemical, predigested at a concentration of 20 mg/ml for 120 min. at 37°C to destroy DNA'se activity) and sodium dodecyl sulfate to a final concentration of 0.5%. The cells were lysed in this buffer and incubated at 37°C for 3 hours. The viscous solution was deproteinized by three phenol extractions (phenol was distilled and saturated with the "DNA buffer" prior to use). The aqueous supernatant was removed by centrifugation at 10,000 rpm for 10 mins. at 5°C in a Sorvall centrifuge, Model RC2-B (I. Sorval Inc., Norwalk, Conn.). The DNA was precipitated out of the aqueous phase by the addition of 2 1/2 volumes of cold absolute ethanol for 24 hrs. at 4°C. The precipitate was collected by centrifugation at 15,000 rpm for 20 min. at 5°C then dissolved in a small volume of 0.1 x SSC. The solution of nucleic acid was adjusted to an ionic strength of 1 x SSC by the addition of 20 x SSC before being treated with RNA'se (50 µgm/ml - heat activated

at 90°C for 10 mins. to destroy DNA'se activity) for 30 mins. at 37°C. The DNA solution was deproteinized by three separations using redistilled phenol saturated with 1 x SSC as before. Residual phenol was removed from the aqueous phase containing the DNA by three extractions with water saturated ether. The aqueous phase was then dialysed for 48 hrs. against several changes of 0.1 x SSC at 4°C.

3. Quantitation of DNA

The concentration of DNA was determined from ultra violet absorption measurements made using a Beckman DU spectrometer (Beckman Instruments, Fullerton, California) or an Unicam SP 500 spectrophotometer (Canlab., Toronto, Canada). The conversion factor of Sueoka (95) that a concentration of 1 µg/ml of DNA is equivalent to 0.021 OD 260 µm units was used throughout this investigation.

4. DNA-DNA Hybridization

(a) Immobilization of DNA on Nitrocellulose Filters: The DNA was dissolved in 0.1 x SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate; n x SSC represents SSC concentration by a factor n times) and alkaline denatured (pH 12.8 for 10 mins. using 5 N NaOH in 2 x SSC). Following neutralization with 1 N HCl in 2 x SSC the ionic strength of the solution was adjusted to 2 x SSC using a 20 x SSC solution and the volume adjusted with 2 x SSC in order to

give the desired concentration of DNA in a 5ml. aliquots. Nitrocellulose membrane filter (0.45 μ, Sartorius, Catalogue Number SM-11305) presoaked in 2 x SSC, were placed on a suction-filtering apparatus and washed with 2 x SSC (33) 50 ml. through each side, before 5 ml. samples of denatured DNA were suction-filtered through the washed filters. The filters were rewashed with 2 x SSC, placed in scintillation vials and dried at room temperature (overnight) and heated at 80°C for 2 hrs. before use (12,104).

(b) Hybridization Reaction: The DNA sample, isotopically labelled, to be analyzed was diluted in 1.5 ml. of 0.1 x SSC and fragmented by ultrasonic sonication. The sonication was performed using a Biosonik III ultrasonicator (Bronwill Inst., Rochester, New York) fitted with a needle-probe, and operating at 30% power for 30 seconds (21).

The sample was adjusted to the desired concentration of DNA/ml. with 0.1 x SSC, before denaturing the sample in a boiling water bath, for 10 mins., followed by rapid cooling in ice. Immediately, the denatured DNA solution was adjusted to 2 x SSC, with 20 x SSC, 10^{-2} M Tris buffer, and 0.1% SDS, and a 1.0 ml. sample, pH 7.0, was placed in a scintillation vial containing an immobilized DNA filter. The vial was placed in a 65-67°C water bath and stoppered. The reaction mixture was incubated for at least 22 hrs. Following incubation the membrane filter was removed from the reaction mixture and exhaustively suction washed with

0.1 x SSC + 3 x 10^{-3} M Tris buffer solution, pH 9.4 (60). The extent of DNA-DNA hybridization was determined by drying and counting bound labelled DNA in a liquid scintillation counter.

(c) Determination of Percent Hybridizable DNA: To determine what fraction of the input DNA is hybridizable to the immobilized DNA on the filter, a known volume of the input DNA solution was precipitated in the presence of carrier protein by the addition of ice-cold 0.3 M trichloroacetic acid. The precipitate was retained on a membrane filter after passing the material through a suction-filtration apparatus. The filters were washed with cold 5% trichloroacetic acid, dried at 80°C before the radioactivity was counted in a liquid scintillation spectrophotometer. The extent of hybridization of the input DNA was expressed as the percentage of counts of radioactivity bound to the DNA immobilized on the filters with respect to the total counts of radioactivity in the input solution as determined by the above method, throughout this study.

RESULTS

I. VIROLOGICAL SYSTEM

1. Morphology and Purity

Adenovirus type 2 infected KB cells (m.o.i. of 100 PFU/cell) were harvested from a suspension culture and the virus was extracted and purified according to the methods described in the MATERIALS AND METHODS. Crude viral material was taken from the initial pelleting step, shown in Fig. 1-1 and centrifuged under equilibrium centrifugation conditions. Normally after the first equilibrium centrifugation several bands of varying density were observed, however, only the heavy major band was collected and re-centrifuged under isopynic equilibrium centrifugation conditions. The virus band after the second equilibrium centrifugation is shown in Fig. 1-2. It can be seen that only a sharp, single, translucent, white band is present in the centrifuge tube. Examination by negative staining of a small portion of this band with ammonium molybdenate (69) showed many virus particles (Fig. 2). The virus band was free of debris and consisted of mainly full, intact virions, however, some virions were broken probably during the

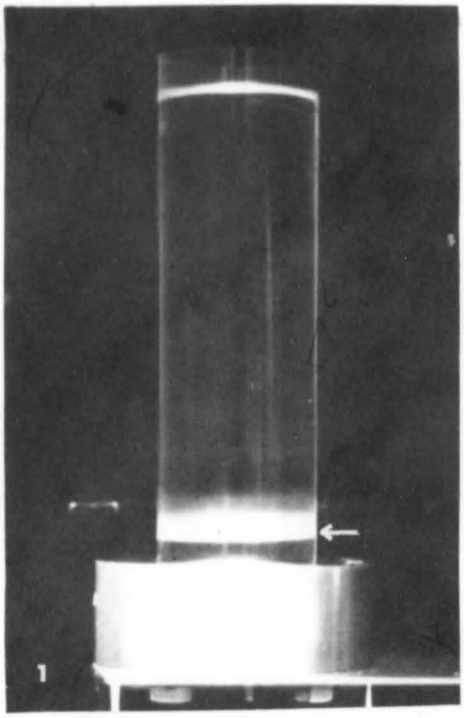
FIGURE 1

Adenovirus, type 2 purification and titration

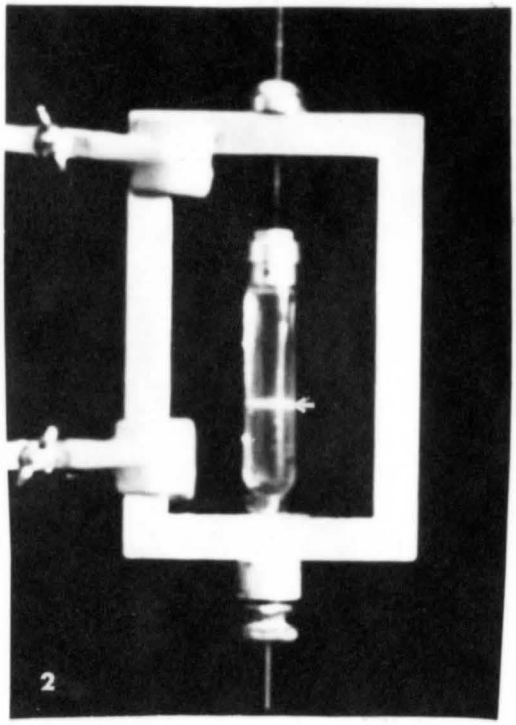
1-1. Viral material sedimented on top of a CsCl cushion (density 1.44 gms/cc) following centrifugation in a SW 27 rotor at 20,000 rpm for 120 min.

1-2. Ad 2 band following two successive isopynic equilibrium centrifugation in CsCl (density = 1.34 gms/cc) using a 65 rotor at 35,000 rpm for 20 hours.

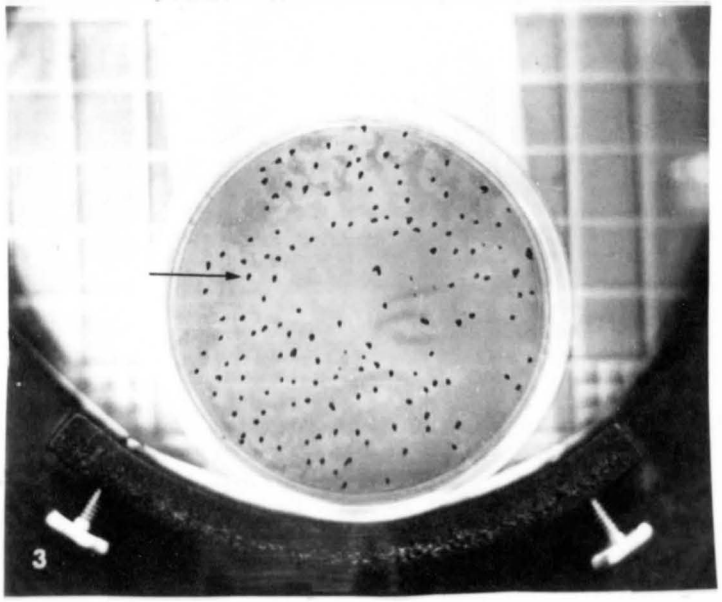
1-3. Ad 2 plaques formed on a KB cell monolayer stained with neutral red after 10 days incubation.



1



2

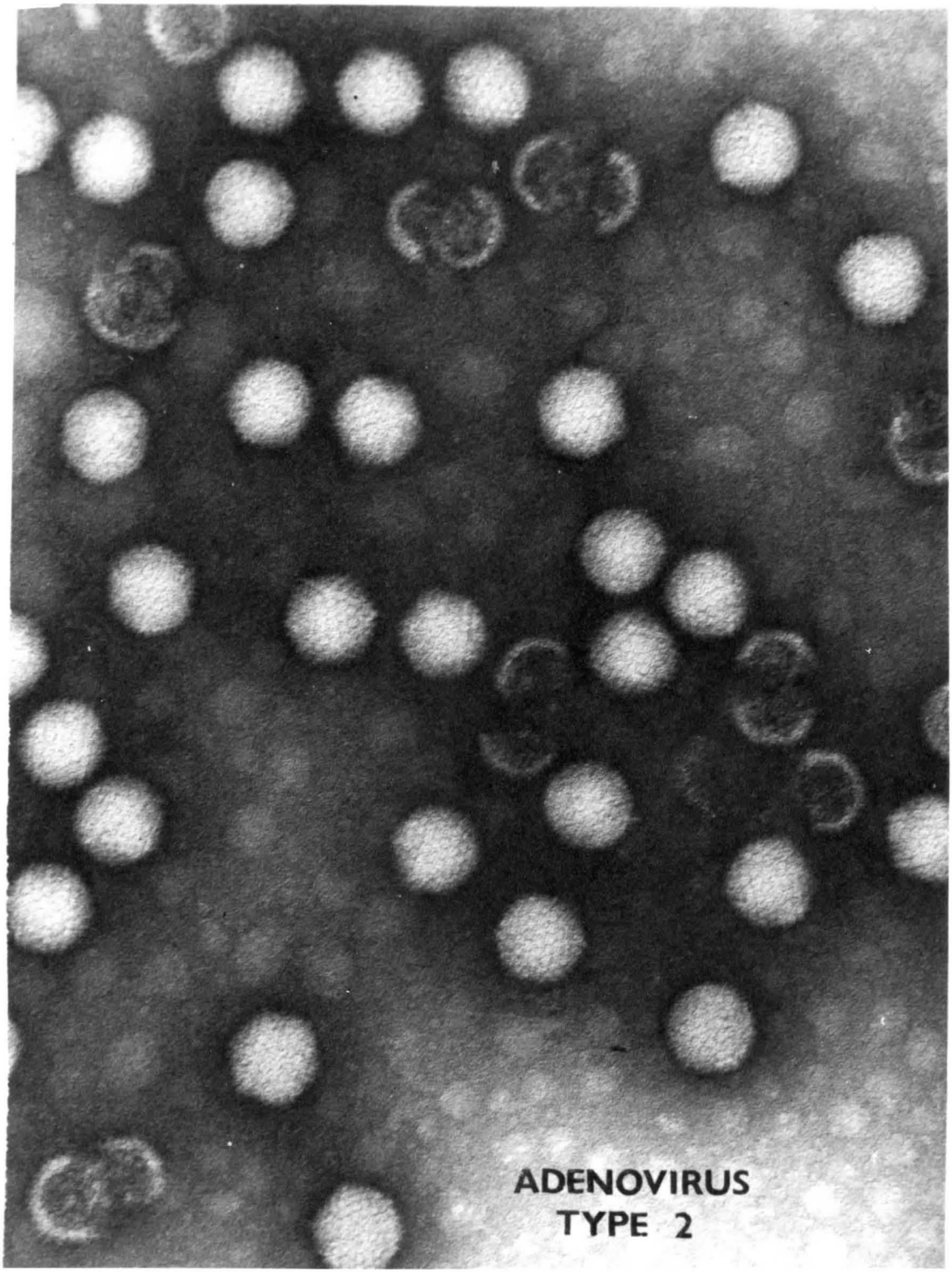


3

FIGURE 2

Adenovirus type 2

Electron micrograph of a portion of a purified preparation of Ad 2 negatively stained with ammonium molybdenate and examined in a Philips EM-300 electron microscope. Mag. X 100,000. Micrograph prepared by Mr. D. Stoltz.


ADENOVIRUS
TYPE 2

negative staining procedures. The virion's capsid was naked and conformed to a icosahedron besides consisting of many polygonal-like capsomers, all of which are characteristic of Adenovirus . The virus preparations examined with the electron microscope were free of any detectable adeno-associated virus (2,9,73).

2. Criteria for Complete Infection

To study the effects of Ad 2 infection on a cell population it is necessary to establish that all the cells in the culture were infected under the experimental conditions, that is an m.o.i. of 100 PFU/cell. This problem was studied by preparing a sample of Ad 2 infected KB cells, 48 hours after infection, for the electron microscope. Thin sections were cut from the sample fixed in 3.5% glutaraldehyde and 2% osmium tetroxide and embedded in Epon. The sections were stained in uranyl acetate and lead citrate and examined in the electron microscope. Observation of 86 cells on the grid showed 92% of the cell nuclei contained discernable viral aggregates similar to that shown in Fig. 3.

Additional evidence was obtained as to total viral infection from an infective center assay. A sample of cells from an Ad 2 infected culture, 2 hrs. after infection, were counted and diluted in EMEM containing 5% human serum (41) and 5% fetal calf serum to a definite cell

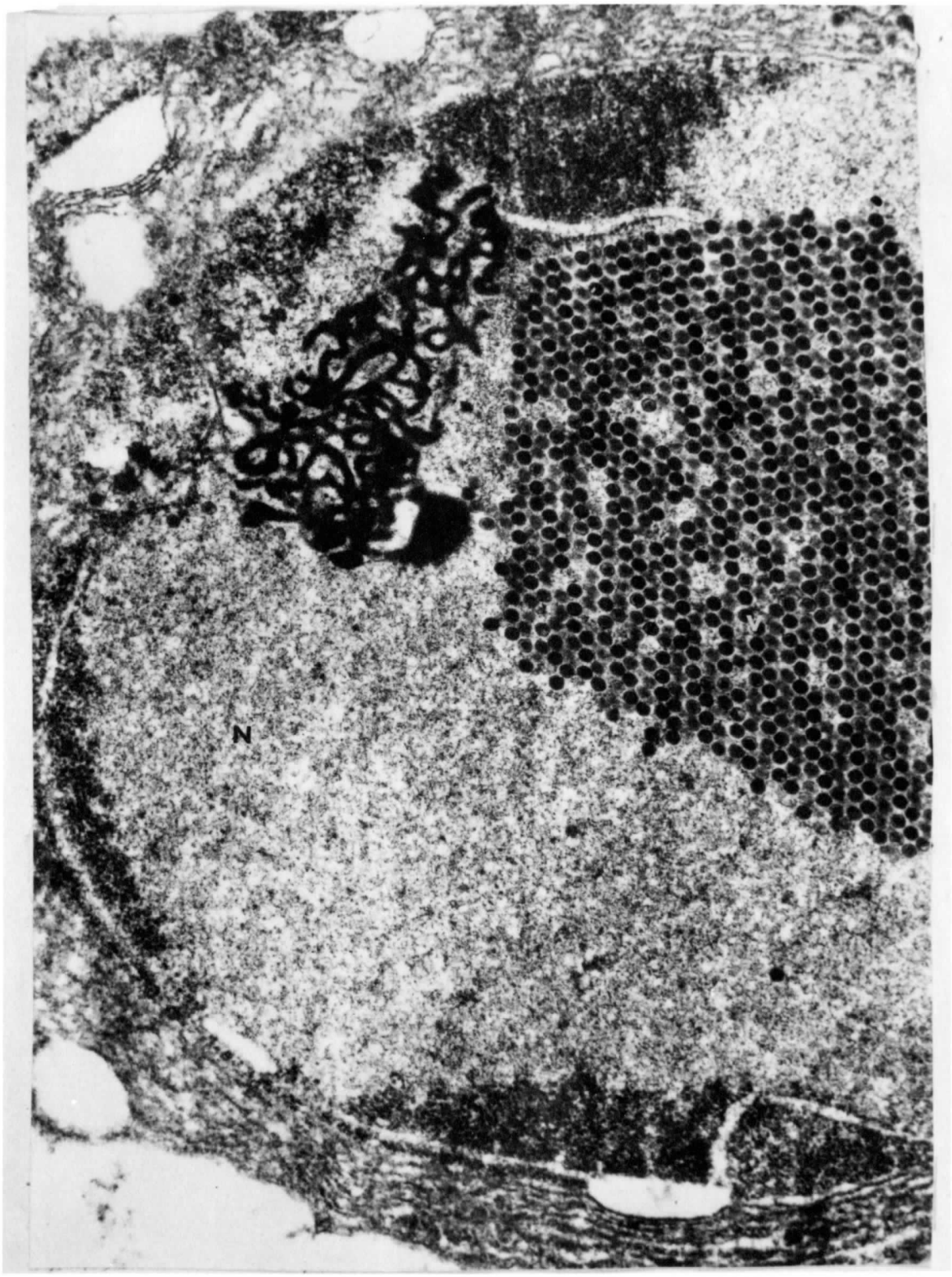
FIGURE 3

Electron micrograph of Ad type 2 infected KB cell, 48 hours after infection.

The sample was fixed in gluteraldehyde and osmium tetroxide and stained with uranyl acetate and lead citrate before being examined with a RCA EM-3H electron microscope operating at 50 Kilovolts.

Mag. X 64,000.

N = nucleus, V = Ad 2 aggregate.


N

concentration before being plated onto a KB cell monolayer grown in a 60 mm plastic dish (Falcon Plastics). The monolayers were overlaid with 1 ml. of growth medium supplemented with molten agar just before use to a concentration of 0.9% (v/v), without removing the infected cell inoculum. The plates were processed as outlined for the plaque assay described in the MATERIALS AND METHODS. After 10 days incubation the results of the assay were scored. The results are shown in Table 1.

Table 1

Infective Center Assay

Time after Infection (hrs.)	Percentage of Cells Plated that formed Plaques
2	67, 72

A plaquing efficiency of 69% should be considered as a maximal value for an infective center assay of Ad 2 infection of KB since the efficiency of 46-67% has been reported (41,83).

The failure to obtain a plaque yield of 100% may be due to cell damage during transfer operations, failure of some cells to adhere to the cell sheet, or insufficient infectious virus released from the infected cell to cause detectable re-infection.

However, from the observation of infected cells under

the electron microscope and the data obtained from the infectious center assay, it would appear that Ad 2 infection of KB cell under the experimental conditions was rapid and complete.

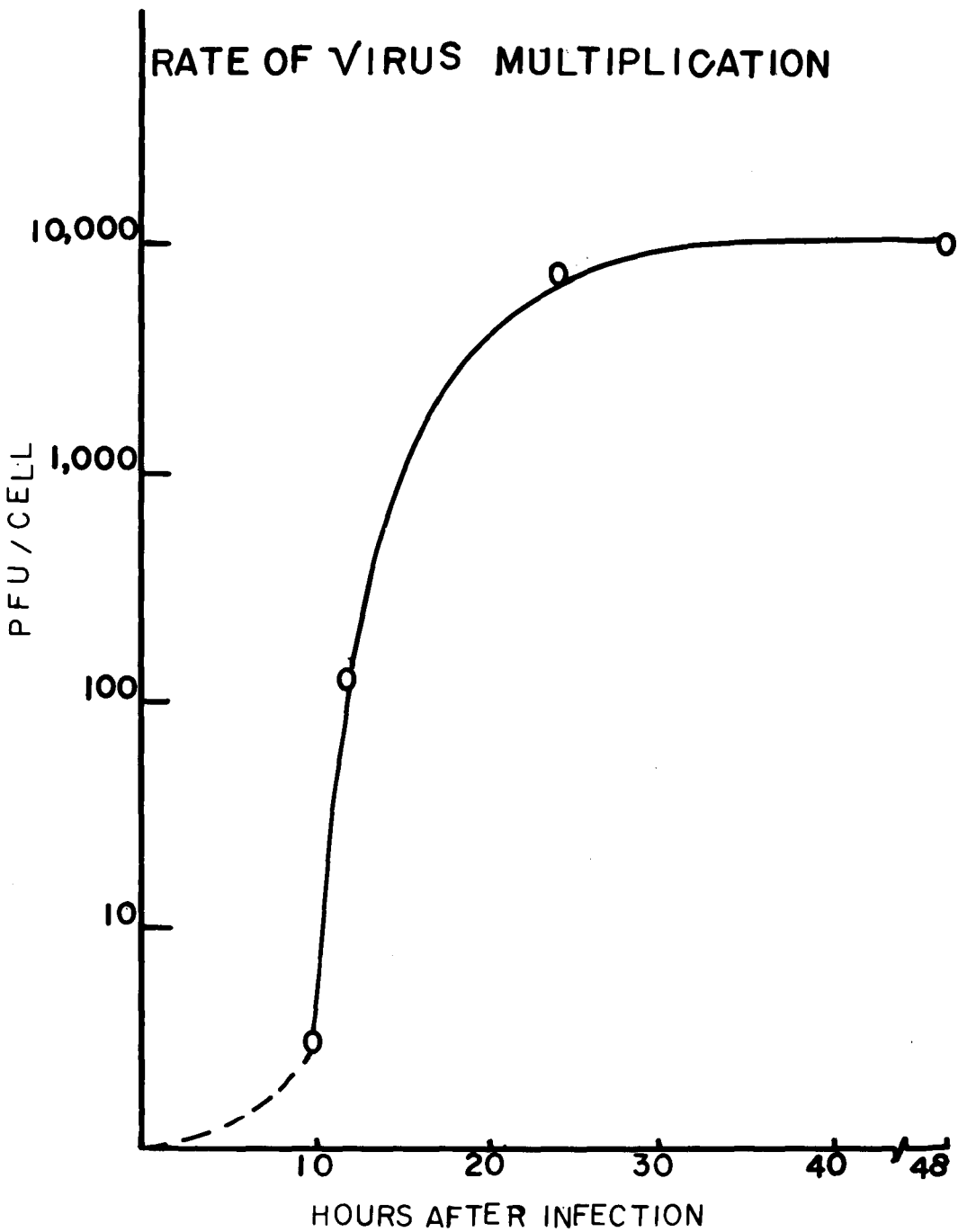
3. Virus Multiplication in KB Cells

KB cells infected with Ad 2 at a m.o.i. of 100 PFU/cell were incubated at 37°C in a suspension culture. At various times after infection, a 5 ml. amount of the infected culture was centrifuged, and the cells resuspended in 5 ml. of PBS + 0.1% serum albumin. The cell concentration was determined, and the sample was stored at -20°C. After thawing, the cells were distrupted at 0°C by a 2 min. pulse of sonication from a Biosonik III needle-probe operation at 30% power. The infectious virus titre was determined in terms of PFU/cell by means of the plaque assay method. After 10 days incubation sufficient viral reinfection of adjacent cells had occurred to produce discernable large clear plaques approximately 3-4 mm. in diameter in the pink stained cell monolayer within the assay plates. Ad 2 plaques formed on a KB cell monolayer are shown in Fig. 1-3. It can be seen from the Ad 2 growth assay shown in Table 2 and Fig. 4 that the intracellular virus titre was very low up to the 10th hours after infection, then rose sharply to reach a maximum titre of 9,000 PFU/cell at

FIGURE 4

Ad 2 Multiplication in KB Cells

Growth of intracellular infectious Ad 2 in Human KB cells. Titration by means of the plaque assay at various times after infection. The cells were infected with 100 PFU/cell of Ad 2.


RATE OF VIRUS MULTIPLICATION
10,000
1,000
100
10
PFU/CELL
10
20
30
40
48
HOURS AFTER INFECTION

24 hours after infection. Cytological examination of the infected cell culture at 48 hrs., a time when the maximum virus titre was reached, revealed only 63% of the cells showed distinct intranuclear inclusion body formation although the entire culture showed cells with abnormal nuclear morphology. Results from other experiments indicate that a final titre of intercellular Ad 2 could range from 6,000 to 20,000 PFU/cell, thus possibly reflecting an altered physiological condition of the infected KB cell.

Table 2

The Rate of Viral Multiplication

Hours after infection	Total intracellular infectious virus produced per cell (assayed in terms of PFU/cell)
10	3
12	120
24	6,800
48	9,000

II. CYTOLOGICAL SYSTEM

1. General Features of an Adenovirus, type 2 Infected Cell Population

(a) Colony Inhibition: In order to examine the fate of the Ad 2 infected cells, a colony inhibition assay was conducted. The exact mechanism of viral interference, with the process of cytokinesis is unknown, however, cell death may be due to disruption of the integrity of the host DNA since virus can induce chromosome breakages very efficiently (16). The biological phenomena of cell growth can be detected in vitro by seeding a definite number of single cells, incubated under optimal growth conditions, and examining the macroscopic colonies originated from these single cells. Such an assay was conducted for cells taken from and infected and uninfected cell culture. Residual virus infection was hindered by supplementing the growth medium with 5% human serum (41). Also optimal growth conditions were achieved by conditioning the growth medium with 60,000 KB cells (irradiated with 5000 rads of gamma rays from a ^{137}Cs source, Department of Health Physics, McMaster University). Data obtained after a 10 day growth period, as shown in Table 3, indicate 78% of the uninfected cells plated formed colonies while no colonies arose from the infected cells.

Although no colony formation was observed from the infected cell culture, it is still possible that the infected cells can go through the division cycle a number of times before finally arrested - see section (b).

Table 3

Colony Formation of Ad 2-Infected and Uninfected Human KB Cells

Cells	Number of cells plated per dish	Total number of cells plated	Number of colonies found per dish	Percent of cells plated which formed colonies
Uninfected	200	800	149, 160 146, 170	78
Infected	500	1000	0, 0	0
Uninfected + Infected	200 500	1400	160, 151	78

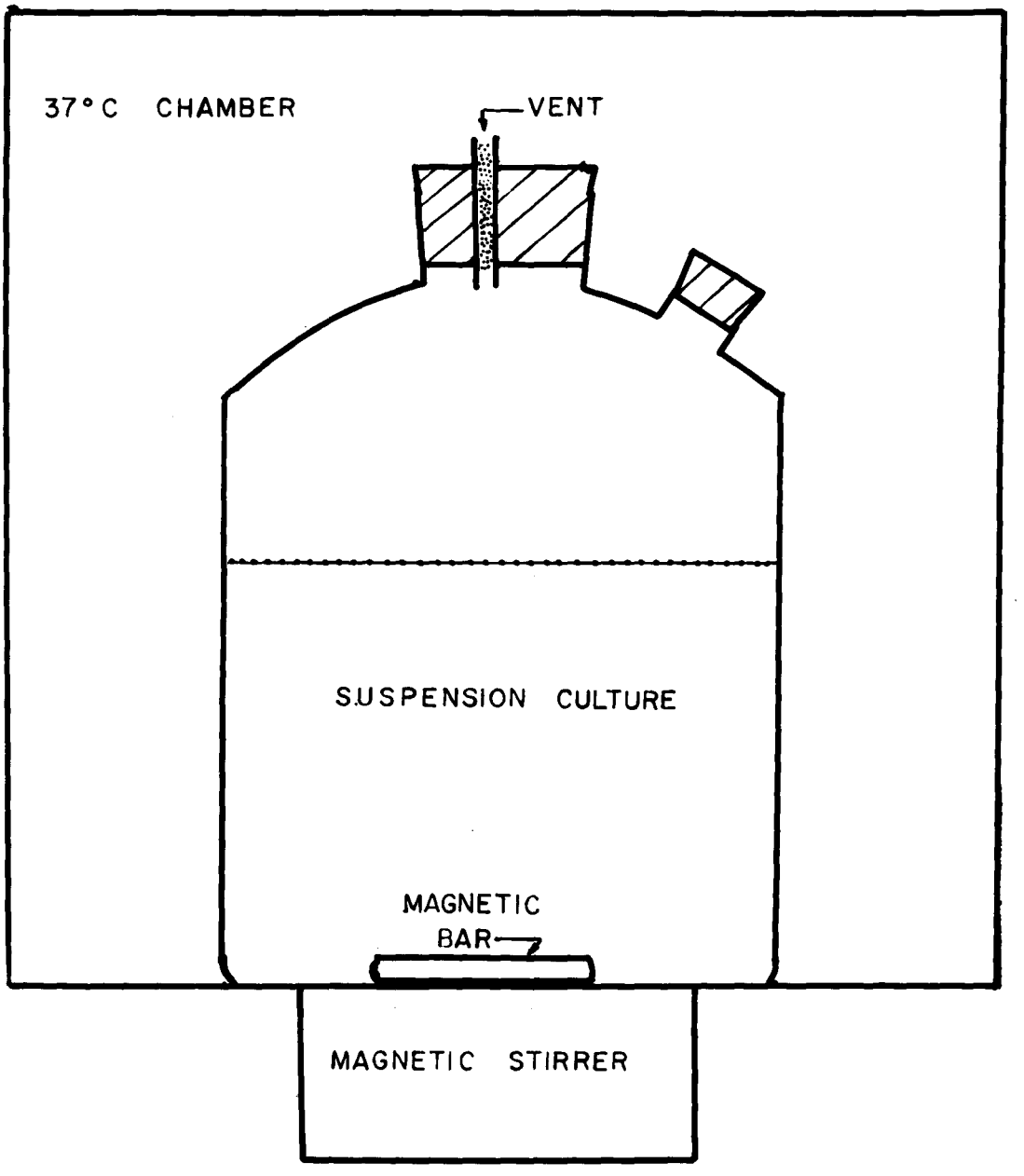
(b) Cell Growth: Logarithimically growing KB cells were maintained in suspension culture, at a cell concentration of $2X10^5$ cells/ml. using a culture vessel shown in Fig. 5. Under normal growth conditions the cell number would double every 22 to 24 hours as determined by counting a sample from the suspension culture with the aid of a hemocytometer. Actively growing KB cells in suspension culture conditions usually would form small clumps consisting of 2 to 6 cells together. After infection with Ad 2 (m.o.i. 100 PFU/cell), the cell concentration would increase slightly in the infected culture but quickly would level off to a constant

FIGURE 5

Suspension Culture Vessel

Suspension cultures of KB cells were maintained in large quantities, 1-4 liters, in such vessels for experimental purposes.

MODIFIED VESSEL FOR SUSPENSION CULTURES



value for the duration of the viral maturation cycle. The uninfected cell culture treated in an identical manner, however, would show a normal pattern of cell growth (Fig. 6-1). Without interference, the suspension culture could increase to a concentration of about 10^6 cells/ml. before leveling off. Besides an alteration in the normal population growth features of a cell culture infected with Ad 2, the infected cell population was comprised of single KB cells instead of the normal small cell clumps, suggesting that infection has altered the surface of the cells.

(c) Mitotic Index: Microscopic studies on the cells from an infected cell population revealed a sharp reduction in the frequency of mitotic figures as compared to the uninfected cell population at late times after infection. The normally growing cell population showed about 2.7% of the cells were in some stage of mitotic division. The mitotic activity of an Ad 2 infected cell population declined rapidly after an initial normal level during the first 7 hours after infection, but by 24 hours after infection was undetectable. These data are given in Table 4 and shown in Fig. 6-2. These data are consistent with the cell growth data showing a slight increase in the cell number of the infected cell population after infection, but remaining constant at later times.

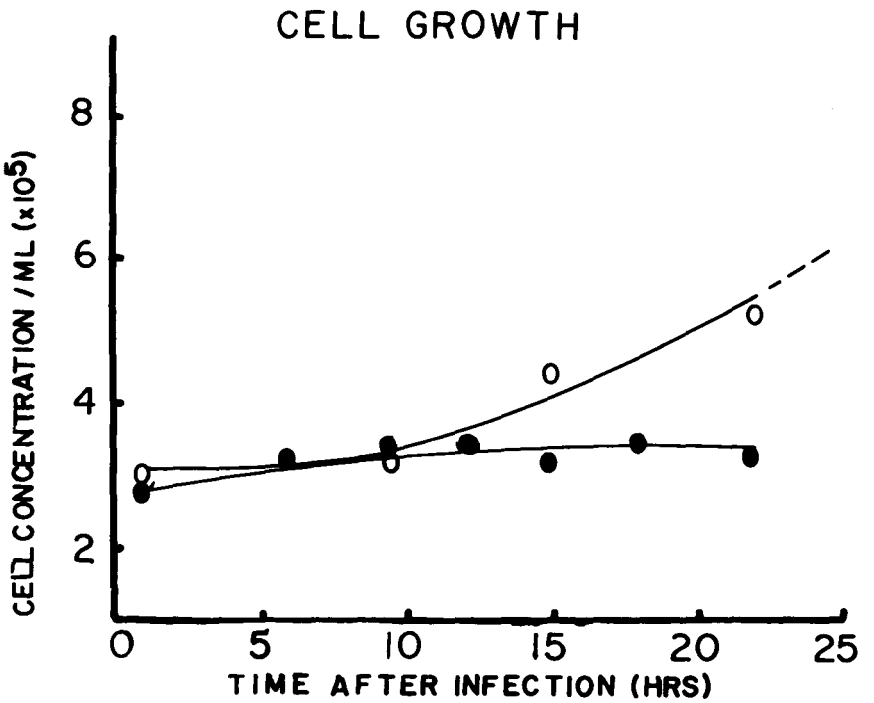
FIGURE 6

6-1. Cell growth of suspension cultures of KB cell at various times after infection.

6-2. Mitotic index of KB cells at various times after infection.

Symbols: 0 = uninfected

● = infected with Ad 2


CELL GROWTH
CELL CONCENTRATION / ML (x10⁵)
8
6
4
2
0
5
10
15
20
25
TIME AFTER INFECTION (HRS)

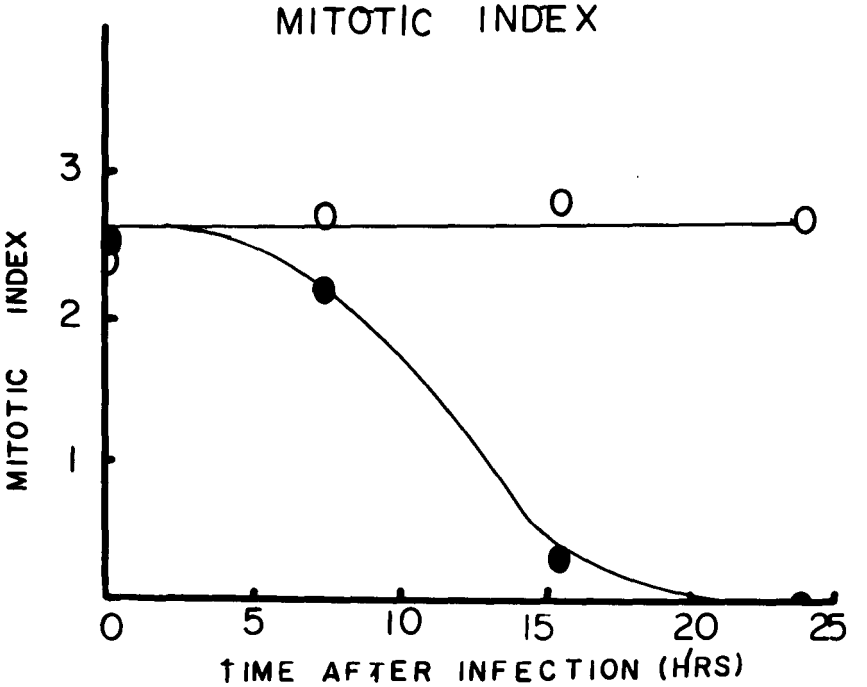

MITOTIC INDEX
MITOTIC INDEX
3
2
1
0
5
10
15
20
25
TIME AFTER INFECTION (HRS)

Table 4

Mitotic Index

Time after Infection (hours)	Mitotic Index* uninfected	infected
0	2.4	2.5
7½	2.7	2.2
15½	2.8	0.3
24	2.6	0.0

* The mitotic index was obtained from microscopic examination of at least 500 cells stained with orcein.

(d) Inclusion Body Formation: Characteristic of Adenovirus infected cells is the formation of large, Feulgen staining intranuclear corpuscles (7). The mature inclusion bodies shown in Fig. 7-3 were first detectable 24 hours after infection. Microscopic studies of stained KB cells infected with Ad 2, showed all the cells from the infected culture to have a drastic nuclear alterations, 48 hours after infection as compared to cells taken from a uninfected culture (Fig. 7-1). Ad 2 infection of human KB cells at a m.o.i. of 100 PFU/cell resulted in 63% of the infected cells having dense nuclear inclusion bodies while the remaining infected cells showed various degrees of basophilic nuclear condensations, however, the same infected cell sample revealed 92% of the cells contained viral aggregates examined in the electron microscope (see Section I-2). These results showed that not only the function of the cells has been changed, the morphology is also drastically altered.

FIGURE 7

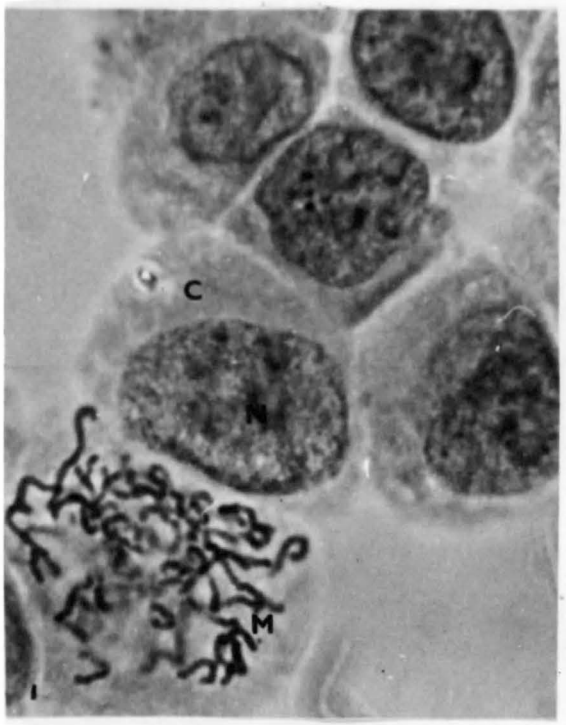
Photomicrographs of KB cells

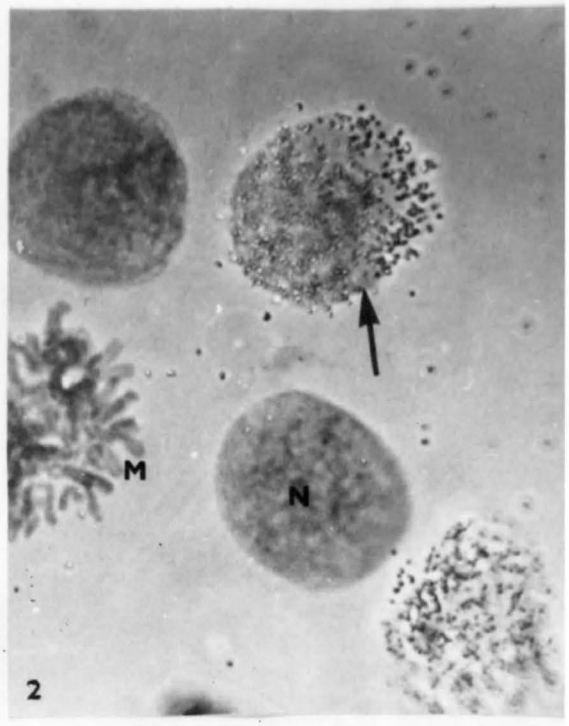
7-1. Human KB cells uninfected, orcein stained, Mag. 1100 X. M = Mitotic figure, N = Nucleus, C = Cytoplasm.

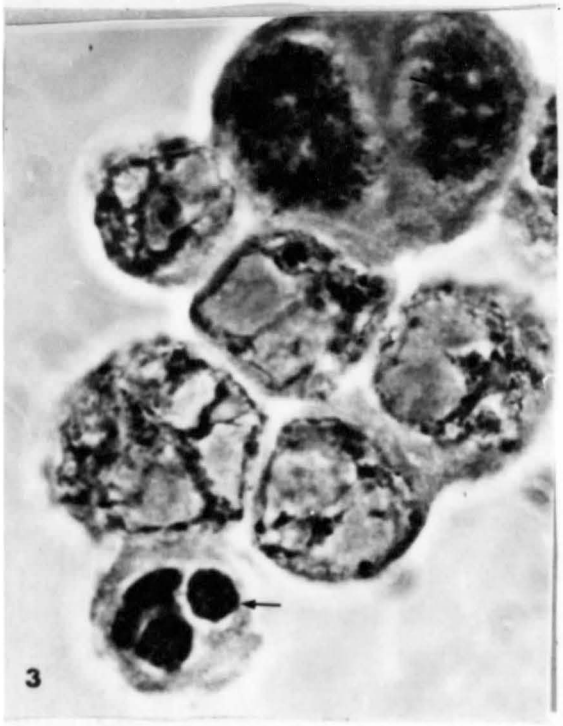
7-2. Radioautograph of uninfected KB cells, Feulgen stained. Mag. 1100 X. Arrow denotes silver grains from ^{3}H-TdR incorporation. N = Nucleus, M = Mitotic figure.

7-3. Adenovirus type 2 infected KB cells, 48 hours after infection. Abnormal nuclear morphology is present in all cells with internuclear inclusion bodies (arrow). Mag. 1000 X.

7-4. Radioautograph of Ad 2 infected KB cell, 48 hours after infection. Silver grains were present over every cell and its inclusion body. Mag. 1100 X.


C
N
M
1


M
N
2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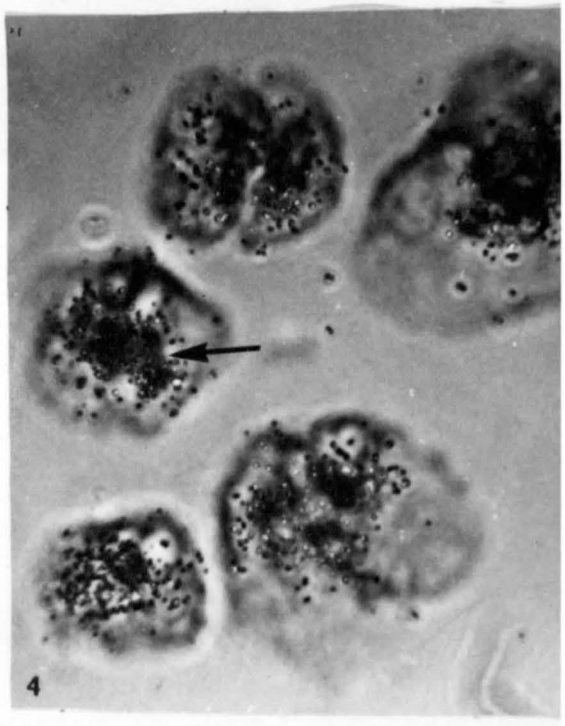

4

2. Cell Cycle Analysis

Mammalian cells pass through two main phases during their growth, interphase and mitosis. With the introduction of quantitative cytological techniques and radioactive precursors, it became possible to subdivide the interphase period into S, DNA synthesis, G_1, pre-DNA synthesis and G_2, post DNA synthesis, phases.

(a) Percent "S" Phase Cells: A study of the DNA synthesis period, S phase, of mammalian tissue culture cells is facilitated by radioautographic detection of the incorporation of a radioactive DNA precursor molecules, such as ^{3}H-TdR into the cells. Uninfected and Ad 2-infected KB cells by pulse labelling a portion of these cultures at various times after infection with ^{3}H-TdR and subsequently the portion of cells synthesizing DNA was determined using radioautography. Figure 7-2, 7-4 gives a photomicrograph of cells labelled with ^{3}H-thymidine. It can be seen that the ^{3}H-TdR was incorporated into the nuclear area for both the infected and non-infected control cells. The percentage of labelled cells detected by this manner remained relatively constant in the uninfected cell population (Table 5) (Fig. 8). The infected cell population showed a rapid increase in the percentage of cells actively synthesizing DNA from the 6-7 hour after infection, by 24 hours after infection

92% of the infected cell population were engaged in DNA synthesis, at that time.

Table 5

Incorporation of ^{3}H-TdR into DNA of Uninfected and Ad 2 Infected, KB Cell Cultures*

Time after Infection (hours)	Radioautography (% cells labelled)	
	Uninfected	Ad 2-infected
0	34.3	-
2	32.5	33.6
4	32.3	35.4
6	32.6	36.9
8	33.8	46.5
12	-	60.0
16	34.5	65.1
18	-	81.0
20	32.4	-
24	34.3	92.7
48	33.9	98.0

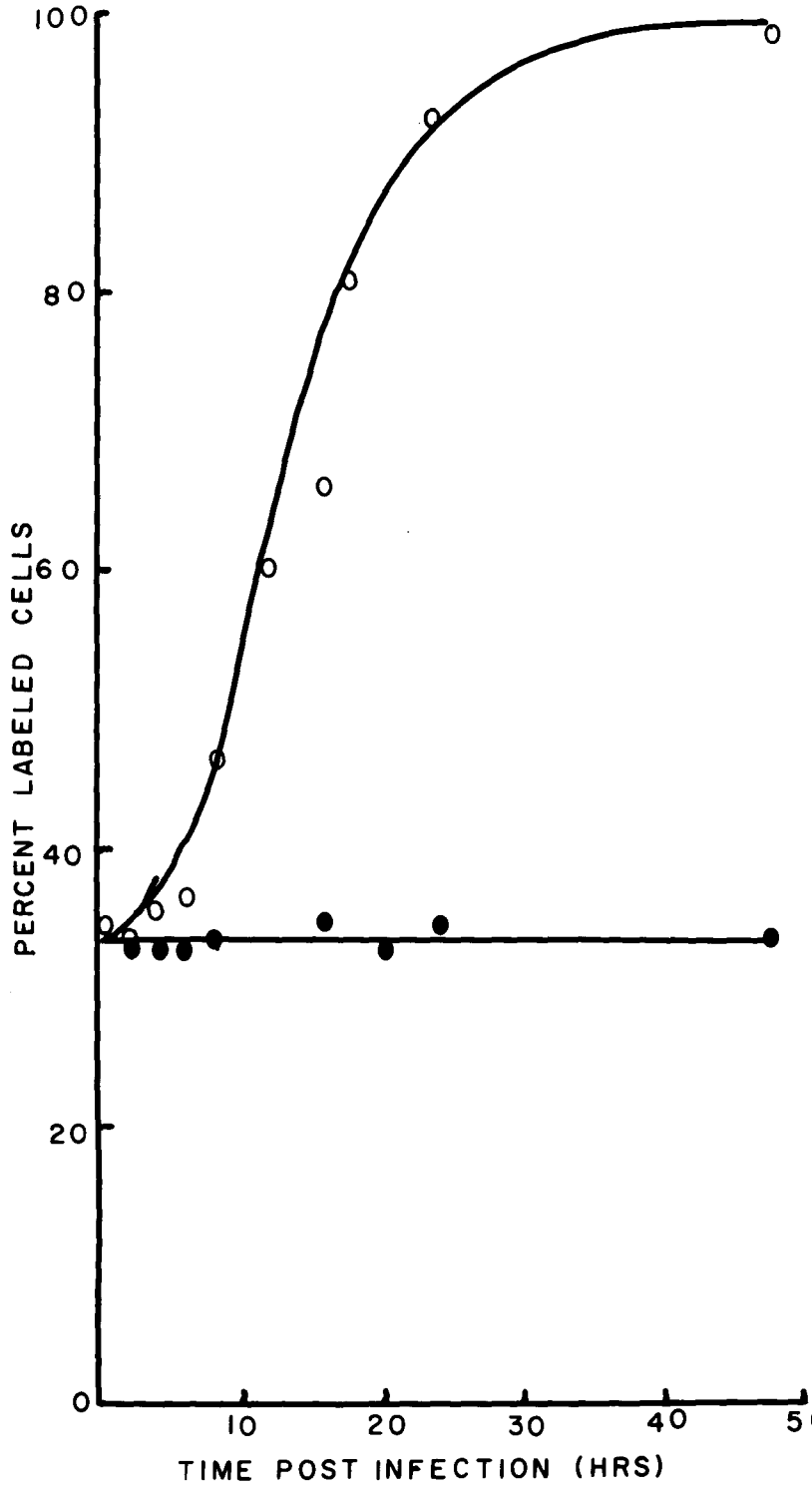
* KB Cells were infected (m.o.i. 100 PFU/cell) with Ad 2 and at various times an aliquot was pulse labelled (60 min.) with ^{3}H-TdR (1 µCi/ml., Sp. Act. 16.1 mCi/mM). Microscopic analysis of at least 1000 cells was conducted to determine the percentage of labelled nuclei. "Sham" infected control cultures were processed in a similar manner.

It was observed that at 48 hours, after infection the labelled nuclei showed fewer silver grains than a uninfected cell nuclei and a somewhat localized pattern over the dense internuclear inclusion bodies. This localization and distribution of silver grains is shown in Fig. 7-2 and 7-4.

FIGURE 8

Percentage of DNA Synthesizing Cells at Different Times After Infection with Ad 2 at a m.o.i. of 100 PFU/cell

Open circles represent infected cell population while the closed circles correspond to values obtained from labelling uninfected cell populations. At least 1000 cells were examined radioautographically for each time.


100
80
60
40
20
0
PERCENT LABELED CELLS
10
20
30
40
50
TIME POST INFECTION (HRS)

These results further showed that the pattern of DNA synthesis in the infected population was altered beginning 6-7 hours after infection.

(b) DNA Content of Uninfected and Ad 2-Infected KB Cells During the Viral Eclipse Period: Studies of the interphase growth characteristics were initiated in light of the unusual growth and DNA metabolism data obtained for the Ad 2-infected cell population. It is of interest to study if the normal progression of cells from G_1 to S and S to G_2 were altered by Ad 2 infection. This can be done by checking the proportion of cells in the various phases (G_1, S and G_2) at different times after infection. Since G_2 cells contain twice the amount of DNA as G_1 cells and they are not labelled with a pulse of tritiated thymidine, it is possible to determine the fraction in G_1 and G_2 compartments by measuring the DNA content (26) of individual non-labelled cells. This problem was investigated at various times after infection by pulse labelling a sample of cells from an infected and uninfected cell population with ^{3}H-TdR then preparing Feulgen stained radioautographs for microspectrophotometry analysis of the individual cells. The portion of the cell sample synthesizing DNA (S period) were detected as labelled cells while the balance of the cell population was ascertained from the relative dye content bound using the two wavelength method of

microspectrophotometry.

In order to obtain the correct wavelengths used in the determination of the amount of DNA by this method, the absorption spectrum of the dye was determined as shown in Fig. 9. It can be seen that the maximum absorption occurs at 540 mμ. In order to satisfy the conditions for the two wavelength method, the wavelengths selected were 540 mμ and 483 mμ.

Determination of the distribution of cells containing different amounts of DNA (arbitrary units) are shown in Fig. 10. It can be seen in first panel the DNA content of metaphase plates show relatively uniform values with a mean of 121 units, and a standard deviation of 17 units. It can be seen also that in the uninfected culture, there consists a population of cells with DNA values clustering about 60 units, about 1/2 of the metaphase mean DNA content. These would be the G_1 cells. The uninfected culture also shows a small population of cells with DNA values clustering around 120 units. These would be the G_2 cells. The uninfected cell population showed very few cells in the G_2 phase because of errors in sufficiently condensing the DNA material or more likely a very short duration of the normal G_2 period for KB cells. Examination of cells from the Ad 2 infected culture, at 1 and 3 hours after infection reveal a cell distribution similar to the uninfected cells. At 5 hours after infection, the G_1 cells

FIGURE 9

Absorption spectrum for Feulgen stained Human KB Cell.

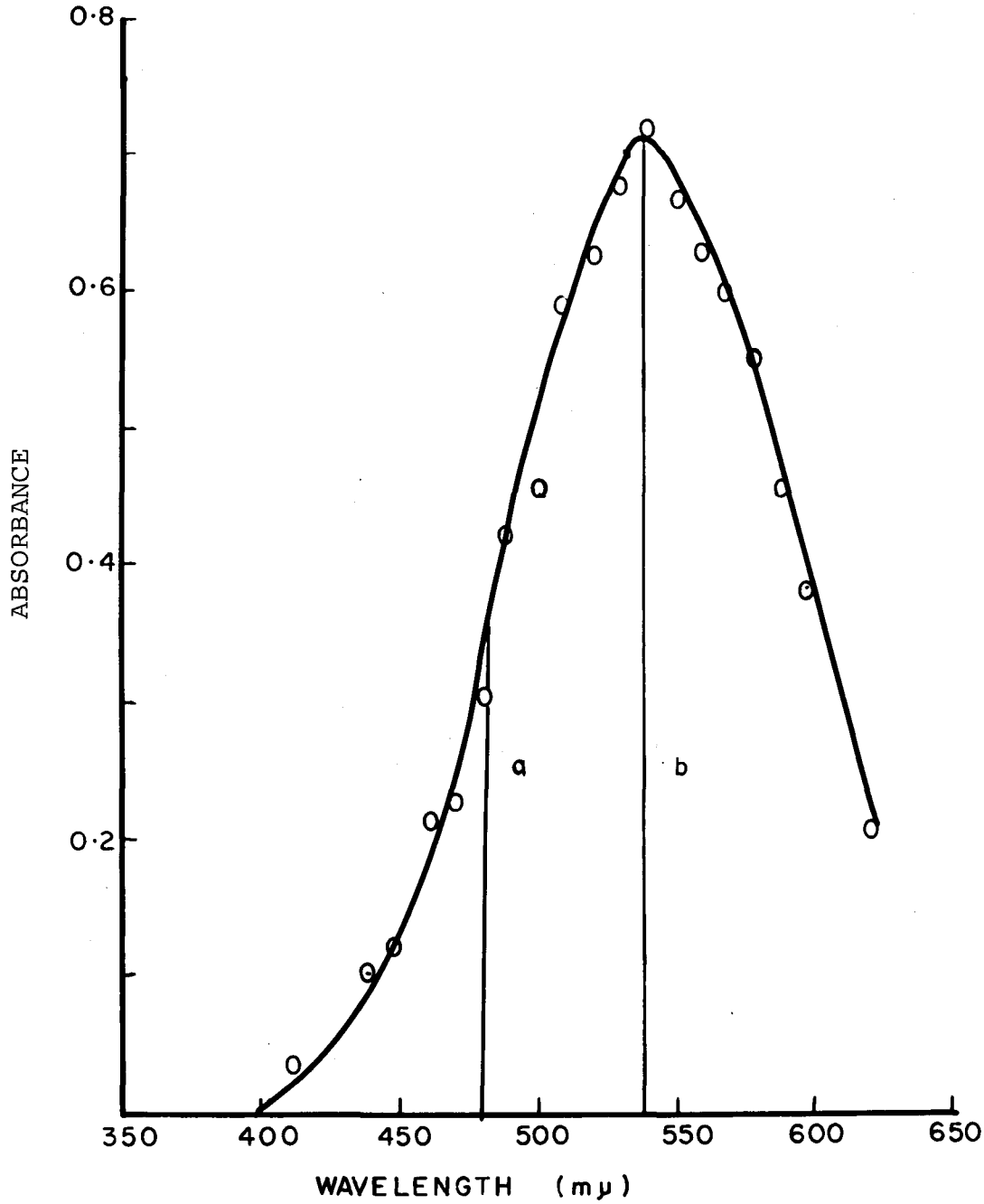
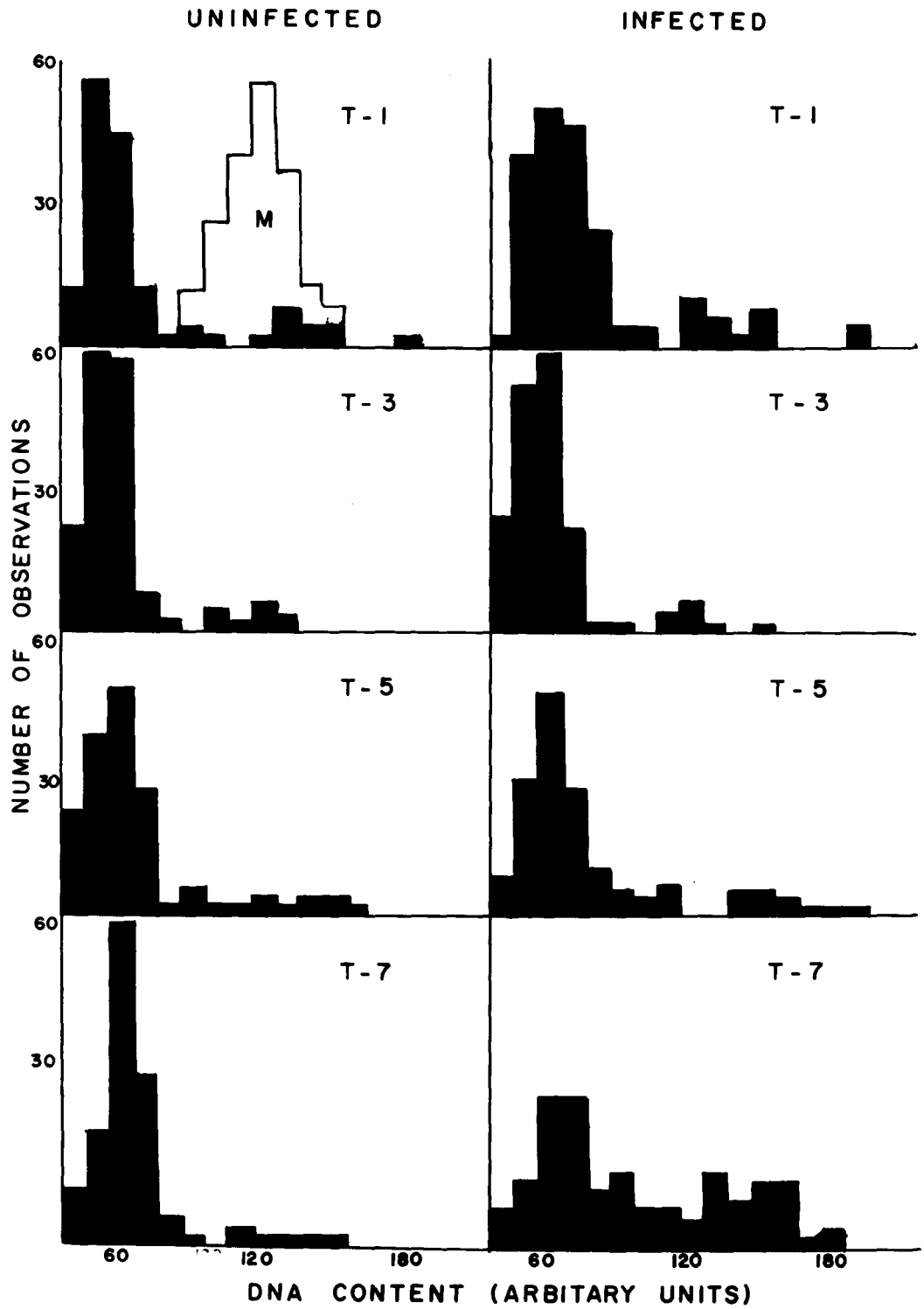

0·8
0·6
0·4
0·2
ABSORBANCE
a
b
350
400
450
500
550
600
650
WAVELENGTH (mμ)

FIGURE 10

DNA Content of Human KB Cells at Various Times After Infection with Ad 2

Histograms determined from microspectrophotometry data on dye content of individual Feulgen stained interphase cells. (M) denotes the dye content of colchocine accumulated metaphase plates. (T) denotes the time after infection at which the radioautographs were prepared.


UNINFECTED
INFECTED
T-1
T-1
M
T-3
T-3
T-5
T-5
T-7
T-7
60
30
60
30
60
30
60
30
NUMBER OF OBSERVATIONS
60
120
180
60
120
180
DNA CONTENT (ARBITARY UNITS)

showed a slight skewing towards higher DNA values. By 7 hours after infection, the infected cell population showed a substantial increase in a population of G_2 cells and an even more pronounced shift in G_1 cells to higher DNA values, with a reduction in the frequency of G_1 cells.

These results suggest that some of the infected cells possess a DNA content between G_1 and G_2 values but are not synthesizing DNA. It is possible that these cells were originally in "S" phase and then its DNA biosynthesis was shut off at 5-7 hours after infection. At the same time some cells in S phase go into G_2 phase to increase the G_2 population, along with a compensating flow of G_1 cells into S phase.

3. Rate of DNA Synthesis

As was indicated from Section II-2, it was observed that the amount of ^{3}H-TdR incorporated was reduced in the infected cells, 48 hours after infection, i.e., autoradiographs of infected cells, showed the number of grains in the ^{3}H-TdR labelled infected cells were fewer than the control.

The next series of experiments show a quantitative measurement for this reduction.

The rate of DNA synthesis was determined by the extent that ^{3}H-TdR was incorporated into the acid-insoluble cell fraction. Preliminary studies indicated that the

amount of radioactivity associated with the acid-insoluble cell material was proportional to the number of cells precipitated (Table 6-1), and to the duration of the pulse labelling (Table 6-2) (shown in Fig. 11-1 and 11-2 respectively). At various times after infection a defined number of cells was labelled with ^{3}H-TdR and the radioactivity determined by cold trichloroacetic acid precipitation. Radioautographs of the same cells were prepared and subsequently the percent of labelled cells was determined. The rate of DNA synthesis per labelled cell $\left(\frac{\text{cpm of } ^{3}\text{H-TdR incorporated}}{\text{(cell number)(percent labelled cells)(duration of labelling)}}\right)$ infected with Ad 2 (m.o.i. 100 PFU/cell) differs markedly from that of the uninfected cells by a very rapid decline during the initial 24 hours after infection. Table 6-3, Fig. 11-3. The rate of DNA synthesis continued to decline on a per labelled cell basis throughout the course of viral maturation within the infected cell. The uninfected cells also showed a reduced rate of DNA synthesis at later times after infection due to possible crowded culture conditions.

Table 6

Rate of DNA Synthesis°

(^{3}H-TdR incorporation into acid insoluble cell material)

6-1. ^{3}H-TdR incorporation versus number of cells*

Cell Concentration (X 10^5 cells/ml)	^{3}H-TdR incorporated (cpm X 10^3)
2	1.75
4	2.7
6	3.8
8	4.9
10	6.0

6-2. ^{3}H-TdR incorporation versus time of incubation**

Labelling Duration (hours)	^{3}H-TdR incorporated (cpm)
½	213, 228
1	654, 702
1½	1100, 986
2	1280, 1200
2½	1460, 1340
3	1873, 1924

° Radioactivity was determined by precipitating the labelled whole cells with cold 10% TCA and assaying the incorporation as described in MATERIALS AND METHODS.

* KB cells were labelled with ^{3}H-TdR (1 µCi/ml) (Sp. Act. 16.1 mC/mM) for 1 hour at 37°C.

** Ad 2 infected KB cells were labelled at 37°C with ^{3}H-TdR (1 µCi/ml) (Sp. Act. 16.1 mCi/mM) before 200,000 cells were precipitated.

Table 6 (continued)

6-3. ^{3}H-TdR incorporation into labelled cells at various times after infection

Time after Infection (hours)	Rate of DNA Synthesis* (cpm/cell/hr) X 10^3		Duration of Labelling (Hours)
	Uninfected	Infected	
0	104.2	-	1
6	-	52.0	1
9	75.0	31.2	1
11	-	23.4	1
18	85.3	23.0	2
20	-	21.4	2
24	81.0	19.7	3
36	72.2	10.5	3
48	87.8	1.4	3

* Rate of DNA synthesis per labelled cell =

$$\frac{\text{cpm of } ^{3}\text{H-TdR incorporated}}{(\text{cell number})(\text{percent labelled cells})(\text{duration of labelling})}$$

FIGURE 11

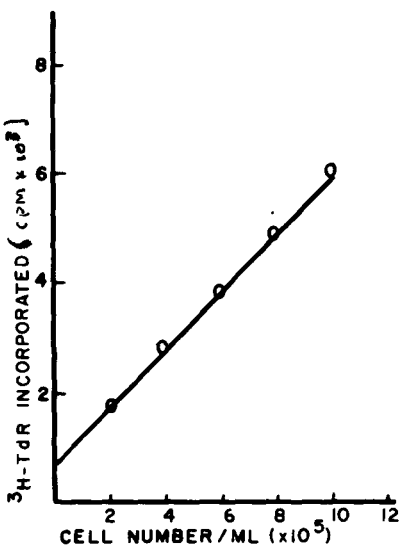
Rate of DNA Synthesis in Suspension Cultures of KB Cells After Infection with Ad 2

11-1. Amounts of ^{3}H-TdR incorporated into acid insoluble cell material for various cell concentrations. See text for details of pulse labelling.

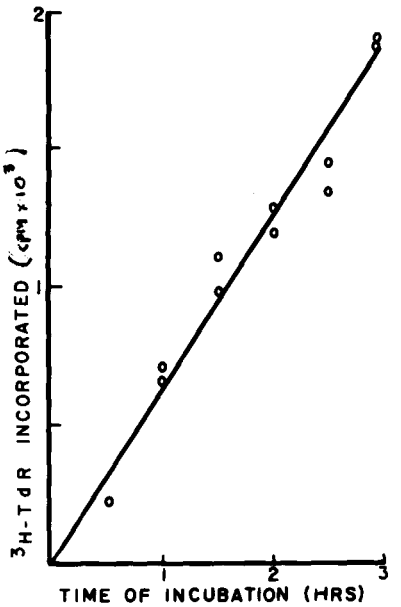
11-2. Amounts of ^{3}H-TdR incorporated into acid insoluble cell material for various pulse labelling periods.

11-3. ^{3}H-TdR incorporation into acid insoluble cell material at various times after infection with Ad 2. 0 = uninfected cells ● = Ad 2 infected cells.

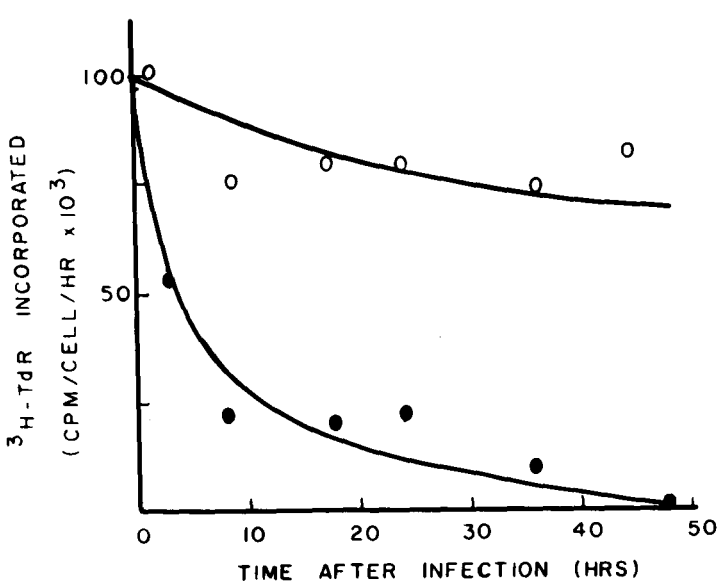
1


^{3}H-TdR INCORPORATED (CPM x 10^3)
2
4
6
8
10
12
CELL NUMBER/ML (x10^5)

2


^{3}H-TdR INCORPORATED (CPM x 10^3)
1
2
3
TIME OF INCUBATION (HRS)

3


^{3}H-TdR INCORPORATED
(CPM/CELL/HR x 10^3)
100
50
0
10
20
30
40
50
TIME AFTER INFECTION (HRS)

III. BIOCHEMICAL STUDIES ON VIRAL DNA SYNTHESIS

1. Hybridization Experiments

(a) Purity of Extracted DNA: The absorption spectrum of purified Ad 2 DNA shown in Fig. 12, is typical of a DNA solution and allows its concentration to be determined from the OD_{260} value (0.0211 OD_{260} units = 1 µg/ml). All DNA preparations, viral or infected cell, showed a sharp UV absorbance peak in the range 256 - 260 mµ and an OD_{260}/OD_{280} ratio of about 2, thus indicating a relatively pure DNA sample (26).

The radioactivity of the ^{3}H-DNA preparation was measured by precipitating a small defined volume of the ^{3}H-DNA sample with cold 10% TCA in the presence of a carrier protein onto a membrane filter and counting the filter in a liquid scintillation spectrometer. Alternatively, the ^{3}H-DNA sample could be spotted onto a glass fiber filter and the radioactivity determined in the usual manner. Comparison of the two methods to determine the radioactivity of a specific concentration of ^{3}H-DNA which was free of contaminating protein revealed the former method to underestimate the cpm by approximately 30%. The method of choice however was that of cold TCA precipitation since the spotting method does not remove the small acid soluble material. Specific activity of all ^{3}H-DNA samples was

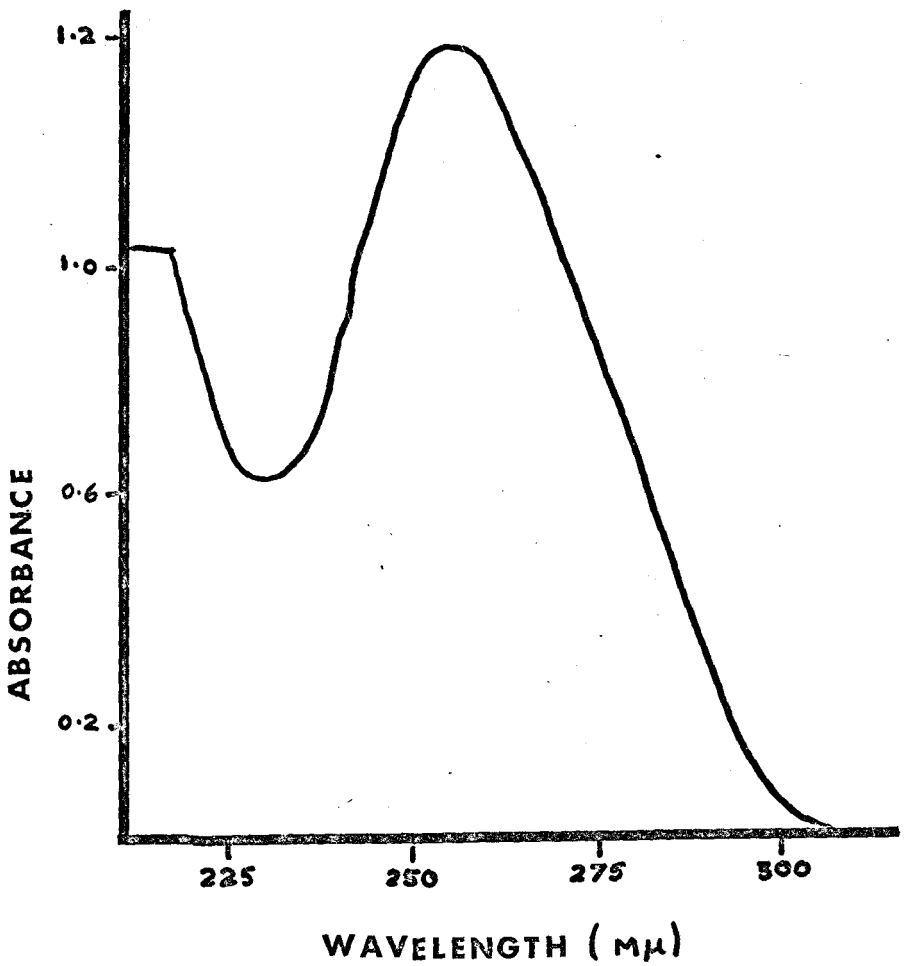
FIGURE 12

Specific Activity Determination of Ad 2 ^{3}H-DNA
A sample of ^{3}H-DNA extracted from purified Ad 2 was resuspended in 0.1 X SSC before the ultraviolet absorbance spectrum was obtained using a scanning spectrophotometer. The radioactivity associated with an known concentration of ^{3}H-DNA was determined by two methods, cold 10% TCA, and direct spotting, as described in the text before Specific Activity (cpm/μg) was determined.

FIGURE 12

Specific Activity Determination of Ad 2 (^{3}H-DNA)

Ultraviolet Absorbance



$\frac{OD_{260}}{OD_{280}} = \frac{1.2}{0.61} = 1.97$

Concentration = 57.1 μg/ml.
(0.021 OD_{260} units = 1 μgm/ml)

Radioactivity

^{3}H-DNA Concentration μg/ml	Cold TCA cpm/ml	Spotting cpm/ml
5.7	111,179	145965
Sp. Act. cpm/μg	19,628	25,620

thus determined as cpm/μg of DNA.

(b) Efficiency of Retention of DNA on Nitrocellulose Filters: The nature of DNA-DNA hybridization studies resides in the annealing of complementary strands, one of which is immobilized on membrane filters and the other, usually radioactively labelled is in solution. It is necessary to demonstrate that the denatured DNA was quantitatively retained by the filters throughout the hybridization procedure. ^{3}H-DNA from Ad 2 was denatured by alkaline, diluted in 2 X SSC and filtered onto nitrocellulose membrane filters, dried at room temperature and heated at 80°C for 2 hours. The amount of ^{3}H-DNA retained was found by determining the radioactivity associated with the filters. As can be seen from Fig. 13-1, the nitrocellulose filter could retain quantitatively an amount of DNA proportional to the concentration added, under the immobilization procedures. The efficiency of retention was about 96%.

It was also necessary to show that the amount of DNA immobilized on the filters remained bound at the end of the hybridization procedure. This was investigated by preparing membrane filters containing immobilized ^{3}H-DNA, carrying them through the annealing reaction and determining the degree of retention of ^{3}H-DNA at each step.

Table 7

Retention of Immobilized DNA During the Hybridization Reaction[1]

Treatment[2]	cpm on Filter[3]	Percent of ^{3}H-DNA filtered[4]
1. Alkaline denatured (2 X SSC)	2527 2698	∿100
2. Alkaline denatured (6 X SSC)	2530 2784	∿100
3. Heat denatured (100°C, 10 min.)	2755 2452	∿100
4. Alkaline denatured Wash with Tris buffer (pH 9.4) before drying filters.	575 623	2.3
5. Procedure 1, incubated at 66°C for 24 hrs. and washed with Tris buffer (pH 9.4)	2585 2540	99

[1] Denatured Ad 2 (^{3}H-DNA), 0.21 µg, 2677 cpm, was immobilized on each replicate filter. After various treatments, cpm bound were measured in a liquid scintillation counter.

[2] Procedure 1 and 2 are alkaline denatured ^{3}H-DNA applied to nitrocellulose filters under ionic conditions of 2 X SSC or 6 X SSC respectively, dried, and counted as outlined in the MATERIALS AND METHODS.
Procedure 5 is the DNA-DNA hybridization reaction.

[3] Each value represents a separate filter.

[4] cpm bound to a membrane filter before treatment normalized to 100%. Average of duplicate filters.

In this experiment, the efficiency of retention under different denaturation and immobilization conditions were also studied. As can be seen from Table 7, 2 x SSC and 6 x SSC gave similar results; denaturation by heat or alkaline made no difference. However, the filters must be heated to 80°C to stabilize the immobilized DNA on the filter before it is suitable for DNA-DNA hybridization studies.

The results of the experiments shown in Table 7 indicate that nitrocellulose filters will retain quantitative amounts of denatured DNA and once the DNA has been stabilized by heating the filter to 80°C it is firmly affixed to the membrane even under conditions of exhaustive washing with a high pH buffer.

(c) Fragmentation of DNA: DNA-DNA hybridization occurs when complementary strands of DNA bind to form a stable duplex molecule. Involved in the DNA-DNA hybridization, two competing reactions are operating,

(1) the hybridization between the immobilized DNA and the ^{3}H-DNA in solution, and,

(2) the hybridization between ^{3}H-DNA-^{3}H-DNA in solution.

The latter process reduces the annealing efficiency. The rate of formation of the hybrid molecules may depend on the relative size of the reacting molecules. Fragmentation of the DNA molecule was accomplished by sonication before

being used in the annealing reaction. A sample of ^{3}H-DNA was sonicated for various periods of time before the efficiency of hybridizing with homologous DNA on nitrocellulose filters was determined. As can be seen from Table 8, and Fig. 13-2, maximal annealing efficiency was achieved with a 30 sec. sonication pulse.

An interesting feature of this experiment was the fact that cell DNA mixed with viral ^{3}H-DNA prior to sonication, as outlined previously did not noticeably interferewith the efficiency of viral DNA annealing efficiency.

The other conditions necessary for reduced solution hybridization of input ^{3}H-DNA is the utilization of a small quantity of input ^{3}H-DNA relative to the amount of DNA immobilized on the filter. In the subsequent hybridization studies the ratio of input ^{3}H-DNA to immobilized DNA was maintained at a value less than unity except were saturation conditions were necessary.

Table 8

Fragmentation of DNA[a]

Duration of sonication (min)	cpm Bound to Ad 2 DNA			
	Ad 2 ^{3}H-DNA only[b]	% Maximal Binding	Ad 2 ^{3}H-DNA + Cell DNA	% Maximal Binding
0	4093	.43	3526	38
½	9520	100	9285	100
1	9139	96	8725	94
2½	8853	93	8820	95
5	7901	83	6868	74

[a] Ad 2 (^{3}H-DNA) 20µg was sonicated in 1.5 ml of 0.1 x SSC then diluted to 0.9 µg/ml (11,900 cpm) and 1 ml was hybridized with 2 µg of Ad 2 DNA immobilized on nitrocellulose filter for 22 hrs at 66°C. Likewise 20 µg of Ad 2 (^{3}H-DNA) was mixed with 20 µg of non-labelled KB cell DNA and treated as above.

[b] Each value represents the cpm bound to a separate filter after the background non-specific binding was subtracted.

FIGURE 13

DNA-DNA Hybridization Studies

13-1. Efficiency of Retention of DNA on Nitrocellulose Filters.

Ad 2 (^{3}H-DNA), Sp. Act. 16,500 cpm/µg, was alkaline denatured and applied to 2 X SSC washed filters at various concentrations in a 5 ml. volume. The filters were washed in 2 X SSC, dried, and radioactivity counted in the usual manner.

13-2. Efficiency of Hybridization as a Function of Sonication Time.

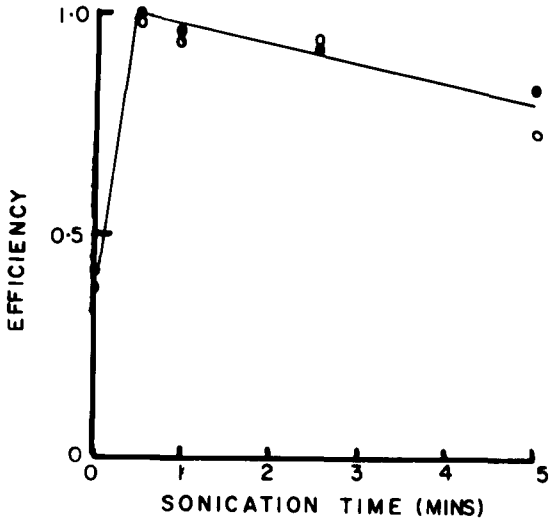
Ad 2 (^{3}H-DNA) or Ad 2 (^{3}H-DNA) + KB cell DNA were sonicated for various periods before an aliquot was removed and adjusted to hybridize to Ad 2 DNA on a filter.

● close circles represent viral to viral DNA annealing,

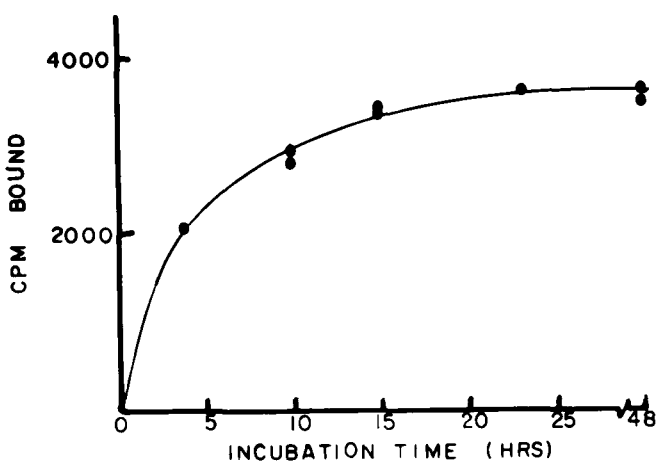
O open circles represent viral + cell DNA to viral annealing.

13-3. Kinetics of Hybrid Formation.

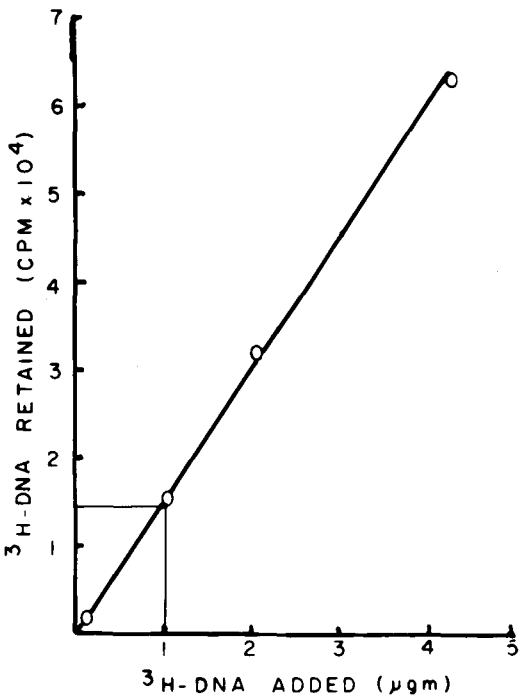
Rate of formation of ^{3}H-DNA-DNA complex when 0.16 µg of Ad 2 (^{3}H-DNA) was hybridized to 3 µg of Ad 2 DNA on a filter and incubated for different durations at 66°C.


1·0
0·5
0
EFFICIENCY
0
1
2
3
4
5
SONICATION TIME (MINS)

2


4000
2000
CPM BOUND
0
5
10
15
20
25
48
INCUBATION TIME (HRS)

3


7
6
5
4
3
2
1
^{3}H-DNA RETAINED (CPM x 10^{4})
1
2
3
4
5
^{3}H-DNA ADDED (μgm)

1

(d) Specificity of the Hybridization Reaction: To insure that the hybrid molecule retained by the filter was formed by hydrogen bonding between homologous DNA molecules, various combinations of DNAs were matched. As shown in Table 9 , hybridization was achieved only with homologous DNAs with 95% viral annealing efficiency and about 20% annealing efficiency for cell DNA. The 20% hybridizable cell DNA could be accounted for by the great redundancy in cell DNA replication(101). Of particular interest in light of the studies to be presented later was the fact that with a combination of KB cell and Ad 2 DNAs analogous to some degree to the case of the macromolecules extracted from an infected cell, strict homologous annealing remained.

Table 9

Specificity of the Hybridization Reaction[a]

Expt.	Input DNA[b] (cpm/ml)		cpm bound to filter[c] with				B. subtilis[d]
	KB cell	Ad 2 ^{14}C-DNA	KB cell DNA		Ad 2 DNA		
			^{3}H	^{14}C	^{3}H	^{14}C	^{14}C
1	640	-	134(21%)	-	0	-	N.D.
2	639	528	125(20%)	0	0	500(95%)	N.D.
3.	-	695	-	0	0	666(96%)	N.D.
4	-	4200	N.D.	N.D.	N.D.	N.D.	7(0%)

[a] Hybridization was carried out by using various combinations of labelled DNAs against homologous and heterologous DNAs immobilized on filters.

[b] Input concentrations were KB cell ^{3}H-DNA (0.1 µg/ml, Sp. Act. 6800 cpm/µg) or Ad 2 ^{14}C-DNA (0.12 µg/ml, Sp. Act. 4800 cpm/µg).

[c] Radioactivity bound to either 10 µg of KB cell DNA, or 3.6 µg of Ad 2 DNA or 10 µg of B. subtilis DNA. Background counts were subtracted. Brackets denote percentage of input cpm bound.

[d] B. subtilis DNA filters were a gift from Mr. C. Chow

N.D. = not done

(e) Kinetics of Hybrid Formation: The rate of annealing of complementary subunits of DNA can be observed experimentally by following the appearance of radioactive binding of input DNA to homologous DNA on nitrocellulose filters. The results presented in Table 12 and in Fig. 13-3 show that maximal binding between viral DNAs occurred after 15 hours of incubation at 66°C.

Table 10

Kinetics of Hybrid Formation

Incubation time (hrs.)	Input ^{3}H-DNA (cpm/ml)	cpm Bound	Percent Hybridized
4	3620	2078	57
10	3620	2853, 2948	80
15	3620	3373, 3416	93
23	3620	3687, 3605	100
48	3620	3502, 3663	99

[a] Denatured Ad 2 ^{3}H-DNA (0.16 µg, 3620 cpm) was annealed with 3.0 µg of Ad 2 DNA immobilized on nitrocellulose filters. After various periods of incubation the extent of radioactivity bound was determined in a liquid scintillation spectrometer.

[b] Cpm bound to separate filters after blank filter, non-specific binding, cpm were subtracted.

[c] Percent of input radioactivity bound.

2. Characterization of Intercellular DNA at Various Times after Infection of KB Cells with Ad 2

(a) Percent Hybridizable Viral and Cell DNA: Having the methodology of formation of stable DNA-DNA complexes on nitrocellulose filters firmly established, it was now possible to study DNA biosynthesis in Ad 2 infected KB cells throughout the replicative cycle of the virus. Ad 2 infection of KB cells, m.o.i. 100 PFU/cell as shown in the previous section causes:

(1) a rapid decline in the rate of DNA synthesis in affected cells, and,

(2) a rapid increase in the percentage of cells from an infected population that are engaged in DNA synthesis from the 6 to 48 hour period after infection as shown by radioautography.

The nature of the newly synthesized DNA molecules whether it is viral or cell, within the infected cell could now be examined by conducting hybridization experiments. At various times after infection a sample of the infected cell culture was pulse labelled with ^{3}H-TdR and a portion of the extracted ^{3}H-DNA was hybridized with either viral or cell DNA immobilized on filters as described in the MATERIALS AND METHODS.

The results presented in Table 11 and shown in Fig. 14 indicate a major alteration in the nature of the

DNA synthesis after Ad 2 infection.

A sample of infected cell ^{3}H-DNA prior to 6 hours after infection was found only to anneal with KB cell DNA. The maximum percent hybridizable cell DNA found at early times after infection, after viral absorption, was only 20%. Coincident with the increase in the percentage of labelled cells as found by radioautography, hybridization experiments revealed an increasing percentage of hybridizable viral DNA within the infected cells from the 6-7 hour after infection. By about 15 hours after infection some 92% of the input radioactive DNA became bound to viral filters while the percentage hybridizable cell DNA gradually reduced to undectable amounts by 24 hours after infection.

Hybridization experiments revealed a very unusual feature of ^{3}H-DNA extracted from infected cells late in the maturation period of the virus, that is from 20 to 48 hours after infection. Although no hybridizable cell DNA was present in the sample of extracted ^{3}H-DNA, the amount of hybridizable viral ^{3}H-DNA showed an abrupt decrease from nearly 100% to only about 42% by 48 hours after infection. This finding was very unusual in light of continued DNA synthesis activity within the infected cells at late times after infection.

Table 11

Percent Hybridizable Viral and Cellular DNA During the Viral Maturation Cycle

Time After Infection (hours)	Input ^{3}H-DNA cpm/ml	Cell DNA cpm bound	Cell DNA % bound	Viral DNA cpm bound	Viral DNA % bound
0	8360	1670	19	0	0
6	2180	349	16	74	3
9	700	101	14	154	22
11	1860	148	8	1113	60
15	8420	420	5	7784	92
20	6640	90	1.4	5636	85
24	4660	22	0.5	3538	76
36	1300	0	0	911	70
48	240	0	0	100	42

[a] ^{3}H-DNA was extracted from Ad 2 infected KB cells at various times after infection and annealed to either cell or Ad 2 DNA immobilized on nitrocellulose filters.

[b] Input ^{3}H-DNA was determined in the usual manner as cpm associated with cold TCA insoluble precipitate. Input ^{3}H-DNA was less than 1 µg/ml.

[c] Input ^{3}H-DNA was hybridized with 3 µg of Ad 2 or 25 µg of KB cell DNA immobilized on separate nitrocellulose filters for 24 hours at 66°C. The respective filters were washed and counted in the usual manner. The values represent the average of duplicate filters with background, non specific ^{3}H-DNA binding, cpm subtracted, usually <1% of input.

[d] The percent of input ^{3}H-DNA bound to either cell or viral filter; data shown in Fig. 14.

FIGURE 14

Percent Hybridizable Viral and Cell ^{3}H-DNA at Various Times After Infection

^{3}H-DNA, isolated at different times after infection was annealed with 3 µg of Ad 2 DNA or 25 µg of KB cell DNA on nitrocellulose membranes. The percentage of input ^{3}H-DNA bound to respective filters is plotted as time after infection

▼ percent of ^{3}H-DNA input binding to KB cell DNA filters

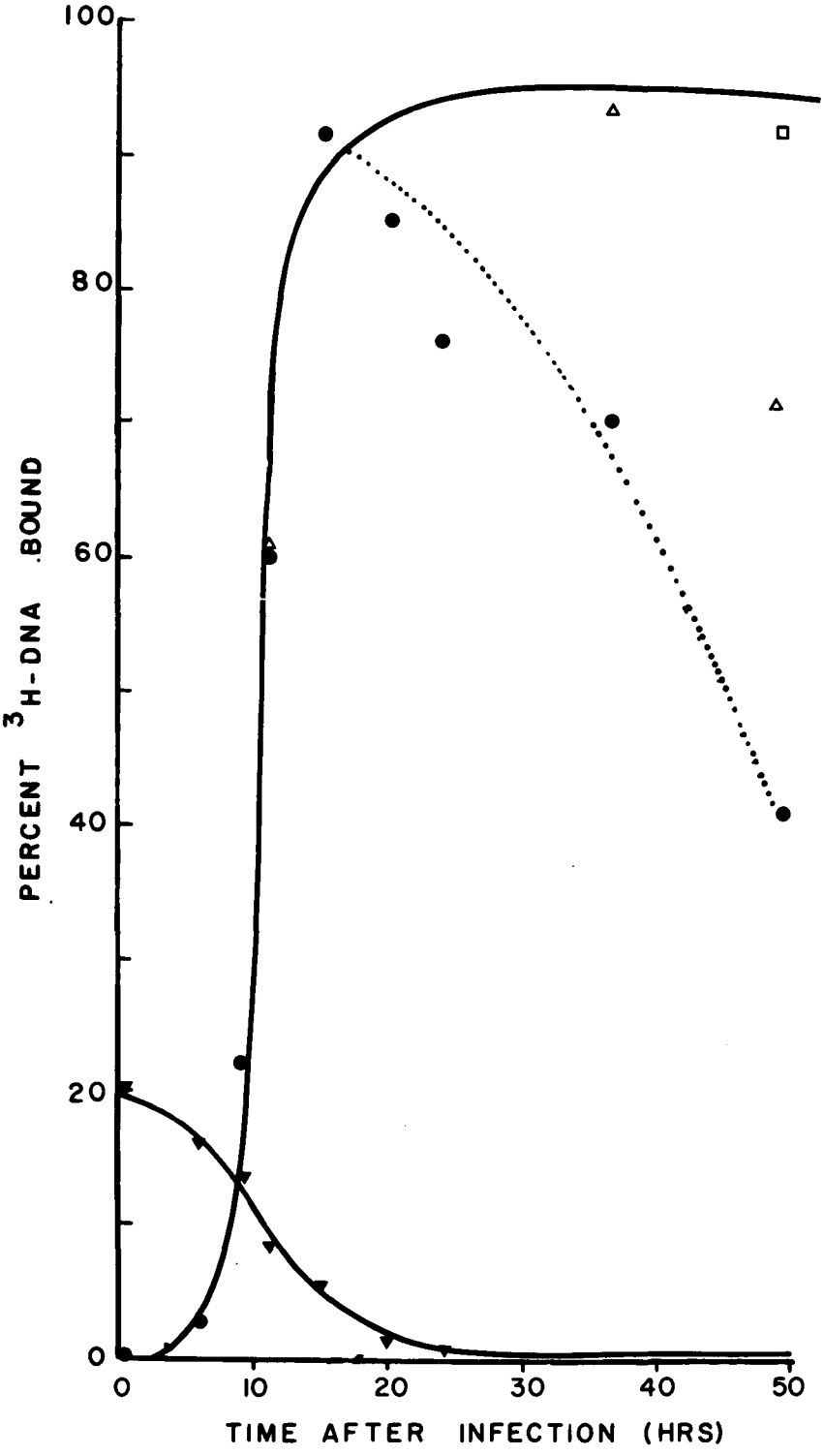
● percent of ^{3}H-DNA input binding to Ad 2 DNA filters

••• dotted line represents the first annealing reaction

△ percent of ^{3}H-DNA input recovered from the first hybridization vial that binds to viral DNA filters, i.e., percentage of ^{3}H-DNA input bound on two viral filters

two viral filters

□ percentage of ^{3}H-DNA input bound to 3 viral DNA filters


100
80
60
40
20
0
PERCENT ^{3}H-DNA BOUND
0
10
20
30
40
50
TIME AFTER INFECTION (HRS)

(b) Unusual Feature of ^{3}H-DNA extracted from Ad 2 infected KB Cells at late times after infection: The results of the preceding section have shown that the species of DNA synthesized during a 3 hour pulse labelling period, 48 hours after infection, to take the extreme case, show no hybridizable cell DNA and very low amounts of hybridizable viral DNA present in the sample of extracted ^{3}H-DNA. Because of this finding and that from infected cell incorporation of ^{3}H-TdR it was decided to expand the hybridization reaction in order to clarify the nature of late synthesized DNA within an Ad 2 infected cell. Samples of ^{3}H-DNA extracted from labelled cells at 11, 36 and 48 hours after infection were annealed to viral DNA immobilized on nitrocellulose filters. Following incubation for 24 hours at 66°C the balance of the input ^{3}H-DNA was removed, redenatured by heating to 100°C for 10 mins. and annealed to a second filter containing viral DNA as before. Following each cycle of hybridization the extent of the original input ^{3}H-DNA bound to the respective filters was determined. The procedure was continued until all of the input ^{3}H-DNA could be accounted for. The results of a series of repeated hybridization on early and late samples of infected cell ^{3}H-DNA are shown in Table 12.

At early times after infection, 11 hours, rehybridization of the input ^{3}H-DNA resulted in very little increase in the amount of ^{3}H-DNA annealing. The residual radioactivity could

be accounted for as cell DNA, and also indicated that the annealing reaction was very efficient. A quite different case was observed for ^{3}H-DNA extracted at 48 hours after infection when rehybridized. As shown in Table 11, the first annealing reaction removed only some 42% of the Ad 2 ^{3}H-DNA from the input solution, but after 3 cycles of annealing nearly the total (92%) radioactivity of the input ^{3}H-DNA was found to Ad 2 DNA. The results of cyclic rehybridization studies thus indicated that:

(1) nearly all species of labelled viral DNA present in early samples were annealed to the viral DNA containing filters under the experimental conditions, and

(2) at later times after infection, 36 and 48 hours, the viral DNA synthesized during the labelling period was less efficiently annealed under normal one step hybridization procedures. These results confirm that ^{3}H-TdR incorporated only into viral DNA.

Table 12

Exhaustive Hybridization[a]

Hours After Infection	1st Hybridization			2nd Hybridization			3rd Hybridization			Total[d]
	Input[b] cpm	Bound[c] cpm	% Bound	Input cpm	Bound cpm	% Bound	Input cpm	Bound cpm	% Bound	% Bound
11	1860	1133	60.8	1113	15	1.5	N.D.	-	-	61
36	1300	911	70	461	305	66	N.D.	-	-	93
48	240	100	42	170	70	41	57	50	87	92

a ^{3}H-DNA extracted from infected cells was hybridized with viral DNA, 1st hybridization before the input was redenatured and hybridized to a new filter containing viral DNA.

b Input ^{3}H-DNA was determined by cold TCA precipitation for each reaction

c cpm bound to filter containing 3 µg of Ad 2 DNA, values represent separate filters after non-specific binding has been subtracted.

d The percentage of 1st input ^{3}H-DNA bound on all filters.

N.D. = not done.

3. Intracellular Ad 2 DNA

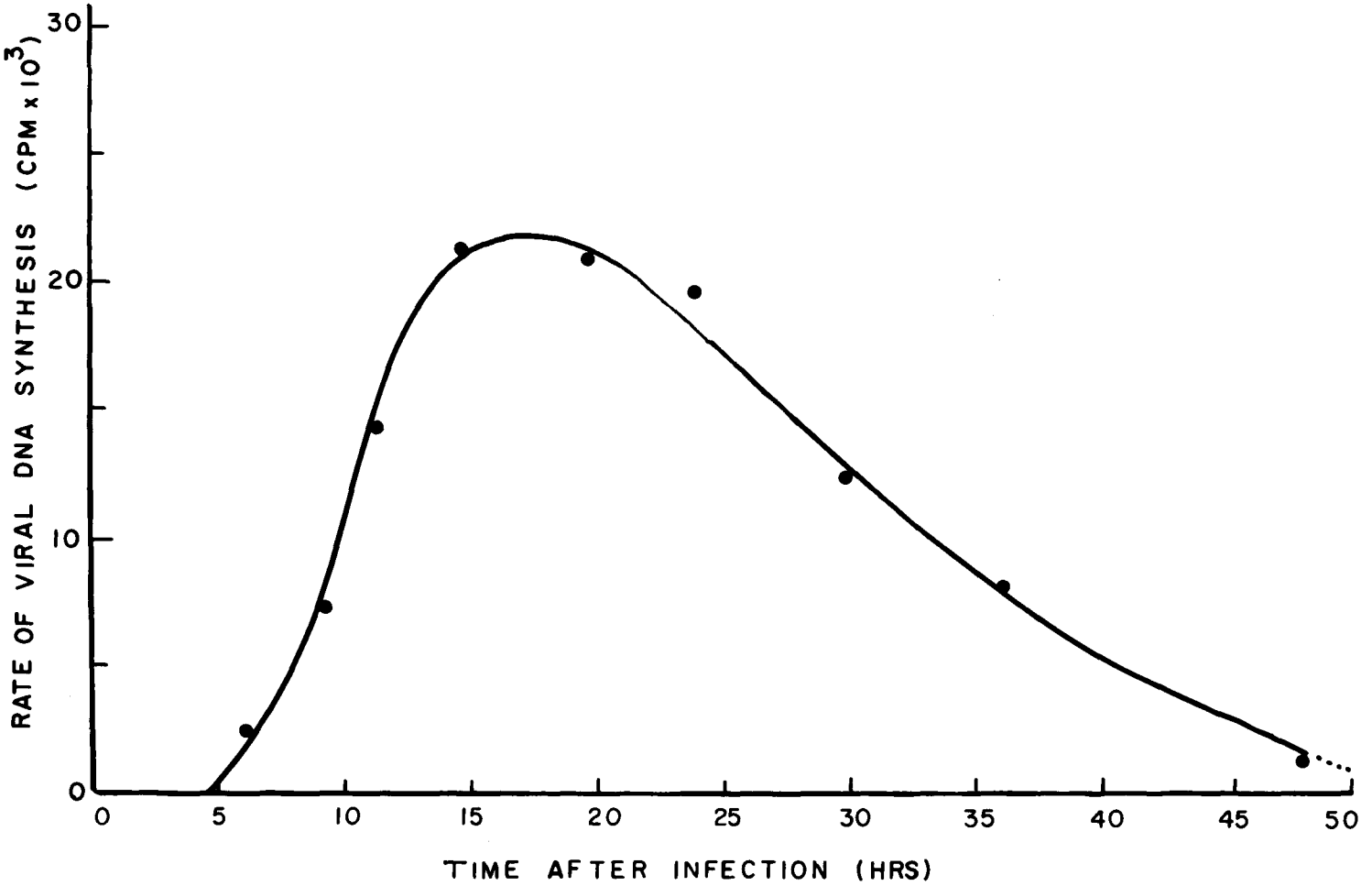
(a) The Relative Rate of Viral DNA Synthesis: From the product of the rate of total DNA synthesis per cell and the percentage hybridizable viral ^{3}H-DNA present at various times after infection, (see section II-3 and III-2) it was possible to arrive at the relative rate of intracellular viral DNA synthesis. As shown in Fig. 15, the rate of viral DNA synthesis increases rapidly from 6-7 hours after infection until a maximum rate about 18 hours after infection, then declines gradually for the balance of the viral maturation cycle. It was interesting to note the maximal relative rate of viral DNA synthesis coincides with a time when the viral hybridization studies showed nearly 100% hybridizable viral DNA made during a ^{3}H-TdR pulse labelling period, that is about 15-18 hours after infection.

(b) The Relative Amount of Viral DNA at Various Times After Infection: Having the relative rate of viral DNA synthesis, it was possible to quantitate the relative amount of viral specific DNA synthesized up to certain times in the maturation cycle of the virus. The relative amount of viral DNA present was calculated by integrating the curve shown in Fig. 15 as follows: $\int_{o}^{t} f(DNA)\,dt$.
Where f(DNA) is the rate of viral DNA synthesis. Integration

FIGURE 15

Relative Rate of Ad 2 DNA Synthesis

The product of the rate of ^{3}H-TdR incorporation and the percentage hybridizable viral DNA per cell was plotted as time after infection.


RATE OF VIRAL DNA SYNTHESIS (CPM x 10^3)
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
TIME AFTER INFECTION (HRS)

of the rate of viral DNA synthesis thus provided the necessary data to examine the relative amount of viral DNA made within an infected cell at various times after infection.

(c) The Absolute Amount of Viral DNA at Various Times After Infection: Although the results of the preceeding sections revealed the relative amount of viral DNA present in cpm at various times after infection, it was necessary to convert the data into meaningful absolute terms of micrograms of Ad 2 DNA per cell at a definite time by means of a conversion step. The key to the conversion of relative amounts in cpm to absolute amounts in µg of viral DNA present intracellularly during the viral maturation process lay with an accurate determination of the specific activity of only viral ^{3}H-DNA extracted from the infected cell. A determination of viral ^{3}H-DNA specific activity could be attempted from either CsCl centrifugation or saturation hybridization studies. Although the densities of KB cell DNA and Ad 2 DNA are sufficiently different (48) (cell DNA = 1,700 gm/cc; Ad 2 DNA = 1.716 gms/cc), pilot experiments revealed many technical difficulties in quantitatively separating the DNA species by CsCl equilibrium centrifugation. The method of choice thus reverted to specific activity determination of extracted viral ^{3}H-DNA by means of DNA-DNA saturation hybridization experiments.

A DNA-DNA saturation hybridization study was conducted using purified viral DNA. A series of filters containing 0.2 µg of immobilized viral DNA were hybridized with increasing amounts of Ad 2 ^{3}H-DNA. As can be seen in Fig. 16-1, it was possible to bind only 7600 cpm to the filters even when the µg of input ^{3}H-DNA (Sp. Act. 38,270 cpm/µg) was greater than 10 fold the amount of DNA immobilized on the filter. Assuming the binding efficiency of the homologous DNA strands is 100% then at the saturation level the counts per minute bound should be equivalent to the amount of DNA contained on the filter. This was indeed the case when the specific activity of the ^{3}H-DNA was determined, that is 0.2 µg of viral DNA on the filter will bind the equivalent counts per min of 0.2 µg of ^{3}H-DNA. It has also been demonstrated by Mak (65) that filters containing various amounts of immobilized viral DNA will plateau out, i.e. cpm bound, with increasing amounts of labelled DNA added at a value equivalent to the amount of DNA on the filter. Once all the sites of the immobilized DNA are occupied by homologous labelled input DNA, it was impossible to bind more labelled DNA even when the saturated DNA filter was washed with high pH buffer, dried and rehybridized with excess labelled DNA.

In light of this, it was now possible to examine the specific activity of <u>only</u> viral ^{3}H-DNA extracted from infected cells since cell DNA also extracted in the preparation

does not interferewith annealing efficiency of homologous DNA species. As shown in Fig. 16-2 and 16-3, it was possible to add increasing amounts of labelled infected cell DNA, 37 or 48 hours after infection, to filters containing 0.2 µg of Ad 2 DNA to a saturation cpm plateau level. In this way a value for the specific activity (cpm/µg) of only intracellular viral ^{3}H-DNA was obtained. The new specific activity determinations for extracted viral ^{3}H-DNA from saturation experiments showed the Sp. Act. of the 48 hour sample of ^{3}H-DNA to increase from 370 cpm/µg to 685 cpm/µg , likewise, the 36 hour ^{3}H-DNA Sp. Act. rose from 1400 cpm/µg to 6750 cpm/µg after eliminating the dilution effect of unlabelled cell DNA. This now allowed the conversion of the integral of the relative rate of viral DNA synthesis in cpm to be plotted in terms of total viral DNA (µgm) present within the infected cell to various times after infection.

We can consider the sample calculations for the µgm of viral DNA present in the infected cell, 48 hours after infection, in the following manner. The rate of DNA synthesis per labelled cell at this time is 1.4×10^{-3} cpm/cell/hour (see Section II-3) or the total radioactivity incorporated into the viral DNA during a 3 hour labelling period is 4.2×10^{-3} cpm/cell since no hybridizable cell DNA was detected. From the DNA-DNA saturation experiment, it was shown that the specific activity of viral ^{3}H-DNA

FIGURE 16

Saturation Hybridization Studies

Saturation of immobilized DNA with ^{3}H-DNA isolated from purified Ad 2 or with ^{3}H-DNA extracted from infected KB cells.

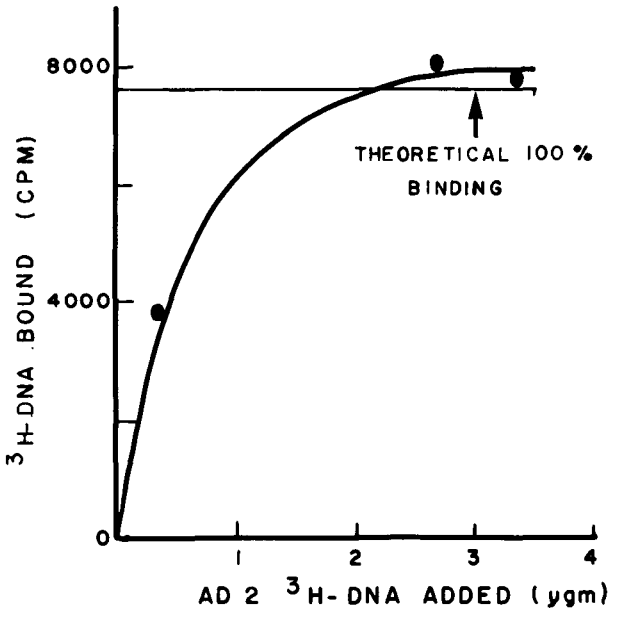
16-1. Membrane filters containing 0.2 µg of denatured Ad 2 DNA were annealed with increasing amounts of sonicated, denatured Ad 2 (^{3}H-DNA), Sp. Act. for Ad 2 ^{3}H-DNA is 38,270 cpm/µg.

16-2 and 16-3. Membrane filters containing 0.2 µg of denatured Ad 2 DNA were annealed with increasing amounts of sonicated, denatured, infected cell extracted ^{3}H-DNA.

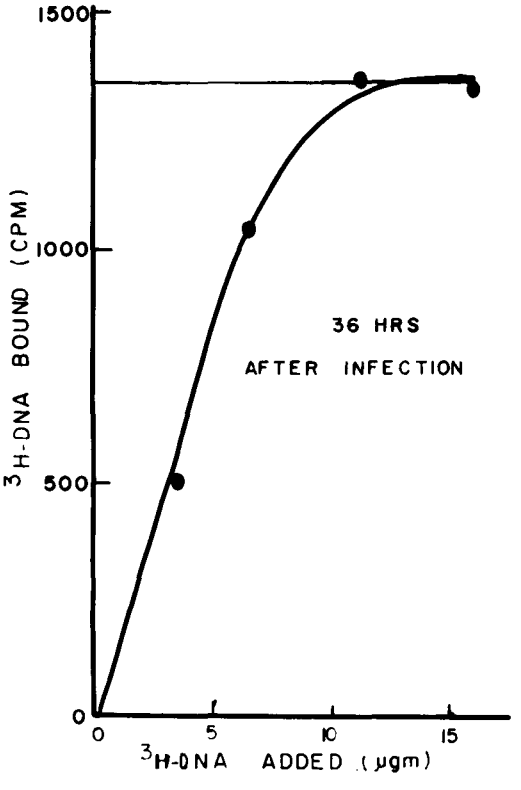
16-2. ^{3}H-DNA extracted 36 hours after infection, Sp. Activity 1400 cpm/µg.

16-3. ^{3}H-DNA extracted 48 hours after infection, Sp. Activity 370 cpm/µg.

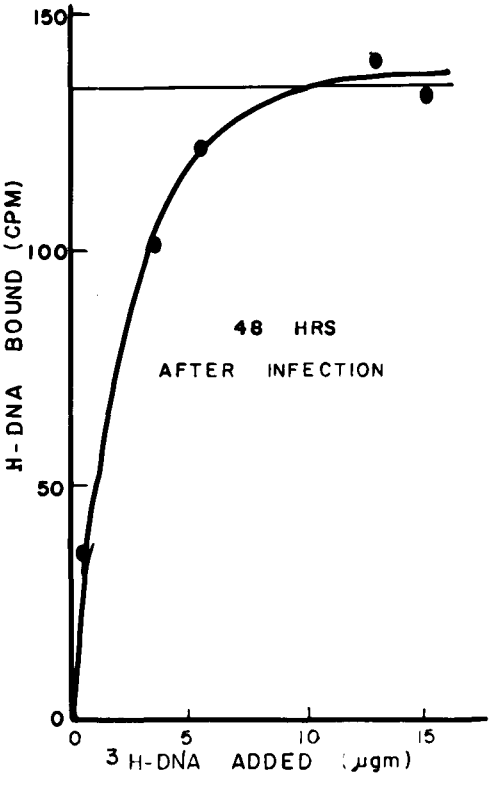
Calculations from the saturation curves showed the 36 hour sample contained Ad 2 ^{3}H-DNA of Sp. Act. equal to 6750 cpm/µg and the 48 hour sample to contain Ad 2 ^{3}H-DNA of Sp. Act. equal to 685 cpm/µg.


8000
4000
0
THEORETICAL 100 %
BINDING
^{3}H-DNA BOUND (CPM)
1
2
3
4
AD 2 ^{3}H-DNA ADDED (μgm)

1


1500
1000
500
0
^{3}H-DNA BOUND (CPM)
36 HRS
AFTER INFECTION
0
5
10
15
^{3}H-DNA ADDED (μgm)

2


150
100
50
0
H-DNA BOUND (CPM)
48 HRS
AFTER INFECTION
0
5
10
15
^{3}H-DNA ADDED (μgm)

3

made during this interval around 48 hours after infection was 680 cpm/μg. By dividing the total cpm incorporated by the viral ^{3}H-DNA specific activity (cpm/μg), it is possible to show that 0.618×10^{-5} μg of viral DNA were present during the labelling period, 48 hours after infection.

Integration of the relative rate of viral DNA synthesis curve shown in Fig. 15, that is the function $\int_{o}^{48} f(DNA)dt$ represents an area of 8.870×10^{3} units^{2}. Thus, 1 square unit is equivalent to 0.6967×10^{-9} μg of viral DNA.

From this value we expect 0.5547×10^{-5} μg viral DNA to be present at 36 hours after infection based on the area of integral $\int_{o}^{36} f(DNA)dt$. This value corresponds fairly well with the data achieved from specific activity determinations for the ^{3}H-DNA sample 36 hours after infection which indicated some 0.466×10^{-5} μg of viral DNA was present. The balance of the rate of viral DNA synthesis curve was thus calculated from an average value from 36 hours and 48 hour samples of ^{3}H-DNA for the equivalent amount of viral DNA (μg) to unit area of the integration curve. This value was 0.6381×10^{-9} μg of viral DNA is equivalent to one square unit of the integration curve. As can be seen from Table 13 and shown in Fig. 17, the total amount of intracellular viral DNA obtained from the conversion outlined above rose from about 10 hours after infection to

a maximum value of 0.618×10^{-5} µg of viral DNA present at 48 hours after infection. The amount of intracellular viral DNA 48 hours after infection thus is nearly identical to the diploid cells DNA complement of 6 picograms.

(d) Efficiency of Virus Formation: It was interesting, having obtained the total amount of viral DNA within an infected cell ar various times after infection to study what proportion of the viral DNA molecules are incorporated within a protein coat to form mature infectious virions also at various times after infection. By extracting viral DNA from a defined infectivity sample of purified Ad 2 it was possible to arrive at a value of infectivity, PFU, to DNA content (µgm). The experiment was conducted by harvesting and purifying Ad 2, assaying its infectious titre (PFU/ml), then determining the total amount of viral DNA extractable from 1 ml. of the same virus stock as described in the MATERIALS AND METHODS. Having determined the PFU and amount of extractable viral DNA per ml. it was calculated that one PFU was equivalent to 4×10^{-10} µg of viral DNA. This value is a minimal estimate due to errors in the plaque assay and extraction yields of DNA from the virus. If this value is compared to a theoretical DNA content per virion from the molecular weight of Ad 2 DNA of 23 million (48); one achieves a value of 3.83×10^{-11} µg of viral DNA per virion. From the "burst size" of Ad 2

infected cells, as shown previously, each cell could produce 10,000 PFU, or using the DNA/PFU conversion state above, this is equivalent to 4×10^{-6} µg of viral DNA/cell. But if we consider the theoretical DNA content per virion and a burst size of 10^4 PFU/cell we achieve a equivalent of 3.83×10^{-7} µg of viral DNA. In light of these observations, it appears that at least 10 virions are required for one PFU.

With the conversion of PFU of Ad 2 particles to Ad 2 DNA it was possible to take the data obtained from the rate of viral multiplication (see Section I-3) and converted it into micrograms of viral DNA encapsulated per cell. As shown in Table 13 and Fig. 17, it was now possible to correlate in absolute terms the µgms of viral DNA which are removed from the intercellular viral DNA pool to form infectious virions on a per cell basis. As indicated by the last column of Table 13, the efficiency of encapsulation of viral DNA reaches a maximum value of about 80% around 20 hours after infection. However, at later times in the viral maturation cycle, with continued viral DNA synthesis only 63% of the viral DNA is encapsulated into infectious virions.

FIGURE 17

Intracellular Ad 2 DNA

The total amount of Ad 2 intercellular DNA per cell represented as full circles - ●, and the total amount of Ad 2 encapsulated into infectious virion per cell represented as open circles - O, are plotted as a function of time after infection.

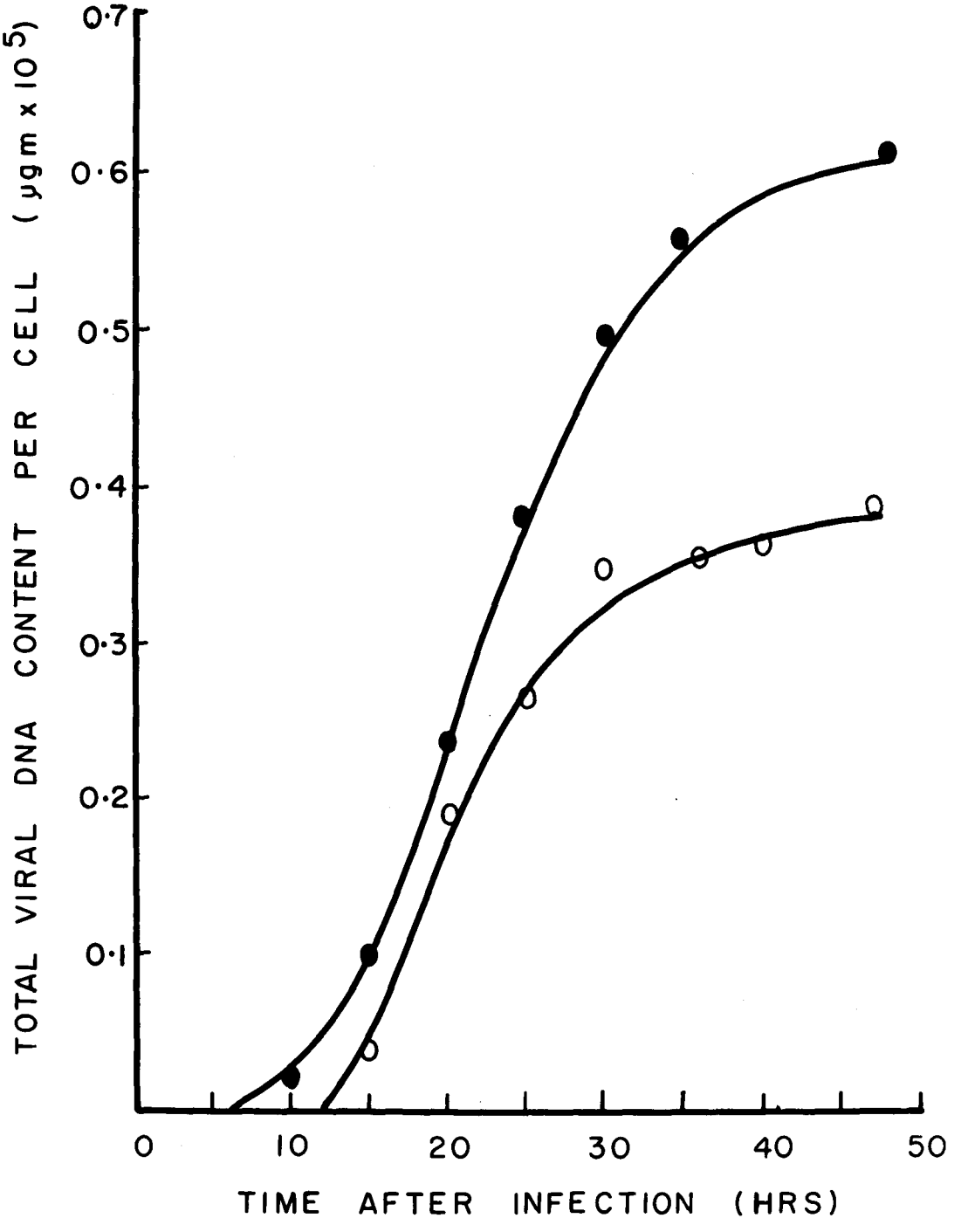

TOTAL VIRAL DNA CONTENT PER CELL (μgm x 10^5)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
10
20
30
40
50
TIME AFTER INFECTION (HRS)

Table 1[3]

Efficiency of Viral DNA Encapsulation into Infectious Virions

Time After Infection (hours)	Total Viral[a] DNA made/cell	Virus[b] Growth PFU/cell	Viral DNA[c] Encapsulated μg X 10^{-5}	Efficiency of[d] Encapsulation
0	0.0	-	-	-
6	0.0	-	-	-
9	0.0268	3	0.001	4
11	N.D.	N.D.	N.D.	N.D.
15	0.1038	720	0.037	36
20	0.2365	4000	0.190	80
24	0.3783	6600	0.266	71
36	0.5573	9000	0.358	62
48	0.6026	10,000	0.380	63

[a] Total cpm of ^{3}H-TdR incorporated during pulse labelling period into viral DNA divided by specific activity of viral ^{3}H-DNA determined from saturation hybridization for 36, and 48 hour samples. Balance extrapolated from $\int_{o}^{t} f(DNA)\,dt$ function (cpm)

[b] See Section I-3

[c] Conversion of 1 PFU = 10 particles = 3.8 X 10^{-10} μg of viral DNA.

[d] Percentage of column c/column a

DISCUSSION

In a lytic infection of human KB cells with adenovirus, type 2, there is a drastic alteration in the normal cell functions as evident from a series of cytological and biochemical experiments conducted in course of this thesis. The discussion is divided into two sections, with first an examination of the effects of Ad 2 infection on the whole cell population and the individual infected cells; followed by an examination of biochemical events, specifically DNA biosynthesis, occurring after infection.

On the cellular level, it can be seen that none of the cells taken from an infected culture could give rise to macroscopic colonies. In other words, all the cells were killed after infection with Ad 2, m.o.i. 100PFU/cell. However, there is still a slight increase in the cell number in the infected culture suggesting that some of the cells could divide at least once after Ad 2 infection. The observation of a gradual decrease in the mitotic index of the infected cell culture after infection is consistent with this interpretation.

With the drastic changes in the infected cell population where is also a drastic alteration in the morphology of the individual infected cells after infection. The major

cellular alteration was the appearance of large intranuclear inclusions. The development of intranuclear inclusion is characteristic of an adenovirus infected cell (7). Cytochemical analysis of these inclusion bodies based on the positive Feulgen staining feature have been used to suggest that they contain a large amount of DNA (6,7,27,81). Microscopic examination of cells from an infected culture show cytological alterations beginning about 16 hours after infection. However, at a time when maximum virus yields are achieved approximately 36 hours after infection, all the infected cells were morphologically altered. The appearance of viral particles within all the infected cells at this time after infection was also revealed by electron microscopic studies. Interpretation of the cytological alterations can thus be held as an actual reflection of the development of virus particles. The chemical nature of these characteristic nuclear structures provides additional support for the presence of intranuclear virus, and probably reflect the viral synthesis and aggregation of DNA.

Even though the morphological changes of the infected cells were not apparent until quite late times after infection, the metabolic functions of the cells have been drastically altered. Radioautographic analysis of the infected cells pulse labelled with tritiated thymidine revealed a sharp rise in the percentage of labelled cells from 6-7 hours after infection. At a time when all cells

show mature virus production the radioautograms of these cells showed all the infected cultures were labelled as opposed to about 30% of the uninfected cells. Therefore, the evidence indicates that the normal control mechanism of DNA synthesis has been upset. As a result of Ad 2 infection of KB cells some of the cells normally not in DNA synthesis are now induced into DNA synthesis. Under normal conditions of cell growth, a cell would control synthesis of DNA only within the interphase period, denoted "S phase". The typical cell cycle is controlled into four distinct periods G_1 phase (pre DNA synthesis), S phase (DNA synthesis), G_2 phase (post DNA synthesis and M phase (mitosis), however after viral infection studies showed only S phase cell activity.

A number of investigators have made observations on the virus-induced changes in the DNA synthesizing enzymes which mediate DNA replication before virus maturation begins.

Although, Green et al. (45) have shown the replication of type 2 and type 4 adenoviruses to require the functioning of the DNA synthesizing enzymes, there was little alteration in the activities of these enzymes. In this respect, the basic pattern in adenovirus multiplication in KB cells would appear to be different from that of the T-even bacteriophages (15), vaccinia virus (56), polyomavirus (25), and SV40 (39) stories where there is an increase in these enzyme activities after infection.

The radioautographic studies also revealed the site of active DNA synthesis within the infected cell at late times to be somewhat localized over the distinct Feulgen staining intranuclear structures although fewer silver grains were detected per labelled cell as opposed to the uninfected labelled cell (98). The nature of this DNA made at later times after infection will be examined later.

The acute changes in DNA biosynthesis, studied by ^{3}H-thymidine incorporation, induced by viral agents have been well studied (47). Polyoma virus (25) SV40 (51, 89) and Adenovirus (91,107) have all been shown to induce cellular DNA synthesis in specific confluent monolayer cell cultures. In contrast, adenovirus infection of growing human cells although showing a stimulation of DNA synthesis have been reported to shut off cellular DNA synthesis (40,52). Similar inhibition of host cell macromolecular synthesis has been demonstrated for pseudorabies (5), frog virus 3 (67) and vaccinia virus (62).

Attempts were made to see if some of the cells which were originally in DNA synthesis at the time of infection would be shut off after infection. The DNA content of individual cells, Feulgen stained, that were not in "S" phase were measured by the technique of combined radioautography with microspectrophotometry. The data obtained showed an increase in the "non-S phase cells"

having a DNA content greater than G_1 but less than G_2 phase DNA content values. This finding, along with the evidence from the rate of DNA synthesis which is discussed later would seem to indicate that host cell DNA is shut off in some of the infected cells even before viral DNA synthesis has been initiated. The exact mechanism(s) and function of induction or inhibition of host DNA synthesis in various cell systems during the early events of viral infection is as yet unknown (5,75,82,40).

An examination of the infection of human KB cells by adenovirus, type 2, at the cellular level has revealed many interesting features, but, it is not possible to ascertain the exact relationship of the synthesis of viral DNA and cellular DNA.

On the biochemical level, it can be seen that infection of an exponentially growing culture with Ad 2 results in a drastic inhibition of the total rate of DNA synthesis. It is important to note that the total rate of DNA synthesis was determined on the basis of only those cells from the infected culture that were labelled "S" phase cells from radioautographs. In an effort to further elaborate the nature of the viral-induced DNA synthesis in an infected cell culture the technique of DNA-DNA hybridization was employed. The annealing experiments conducted on labelled whole cell extracted DNA showed that viral DNA synthesis begins about 6 hours after infection

and cell DNA synthesis is gradually shut off. This observation is consistent with the assumptions made from the cytological observation concerning the increased DNA activity of the infected cell to be of a viral nature. The hybridization studies thus made it clear that Ad 2 infected KB cells do not resume the synthesis of host cell DNA after infection and also the viral DNA is made continuously although at a very low rate throughout the viral maturation cycle. The unusual properties of viral DNA (97) such as that of intracellular viral DNA synthesized at late times after infection as detected by DNA-DNA hybridization studies was unfortunately not resolved in this investigation. One might speculate that the viral genome is replicated in such a fashion as to render the viral DNA more susceptable to liquid self-hybridization than that synthesized earlier or after infection. A general conclusion that can perhaps be drawn from the general isotope incorporation studies, is that Adenovirus infection does interfere with the normal DNA metabolism even at a time when no specific viral DNA is being made within the infected cell.

Once the kinetics of viral DNA synthesis have been established by means of DNA-DNA hybridizations, it was interesting to examine its onset with the production of infectious intracellular virions. Green (43) has shown using 5-fluorodeoxyuridine inhibition of DNA synthesis

in KB cells at various times after infection and assaying the total virus yield per inhibited cell at 38 hours after infection, that viral DNA synthesis began about 6 hours after infection and 8 hours prior to mature infectious virus production. The results of DNA-DNA hybridization studies confirms this data as to the onset of viral specific DNA synthesis. The data of the rate of viral multiplication per cell as assayed by the plaque method is in agreement also with the intracellular growth of Ad 2 in suspension cultures of KB cells. It has also been confirmed that the average "burst size" of Ad 2 within an infected KB cell is equivalent to 10^4 PFU.

Having the information available on the quantitative nature of viral specific DNA synthesized within an infected cell at various times after infection and also the amount of viral DNA assembled into infectious virions per cell at various times after infection, it was possible to calculate the efficiency of encapsulation of naked intracellular viral DNA. The naked viral DNA made from 6-7 hours after infection to the time mature virions are first detectable forms a pool awaiting the synthesis of the structural components. The size of this naked viral DNA pool is equivalent to about 5000 Ad 2 virions per cell of 5% of the total "burst size" of Ad 2 virions per cell. However, once the synthesis of Adenovirus structural proteins has been initiated (79), the naked viral DNA synthesized is rather efficiently encapsulated into infectious virions

within the cell.

Ben-Porat and Kaplan (5) have examined the fate of pseudorabies virus DNA in rabbit kidney cells where there is inhibition of cellular DNA synthesis and, concurrently an increase in the rate of synthesis of viral DNA; analogous to the situation present in Ad 2 infected KB cells. Chemical analysis of the infected cells and the mature virion produced by each cell revealed that only 20% of the total DNA synthesized was incorporated into mature pseudorabies virions. Green (41,42) similarly analysed Ad 2 from a KB infected cell and proposed that only 6% of total DNA and 19% of total protein synthesized can be accounted for in the mature virions.

Although this investigation has confirmed the observation (41,50) that the DNA content of an Adenovirus infected cell is nearly doubled during infection, it has shown that much more naked viral DNA synthesized can be accounted for in the mature virions that has been reported previously.

The data suggest at later times during the viral maturation process within an infected cell, a form of aberrant viral DNA and/or viral protein are found which fail to be combined into infectious virions. If this should prove to be the case, it would be extremely interesting to examine the physical characteristics of such viral macromolecules, and the nature of the "assembly factor(s)" involved with

respect to possible viral, cellular, or environmental control mechanisms acuated in the permissive and non-permissive host systems.

BIBLIOGRAPHY

1. Ashkenazi, A., and Melnick, J. L. Tumorigenicity of simian papovavirus SV40 and of virus transformed cells. J. Nat. Cancer Inst. 30: 1227-1265, 1963.

2. Atchison, R. W., Castro, B. C., and Hammon, W. M. Electron microscopy of adenovirus-associated virus (AAV) in cell cultures. Virology 29: 353-357, 1966.

3. Bachmann, K. A cytochemical kinetic investigation of the Feulgen hydrolysis of fixed chromatin. Histochemie 16: 287-293, 1968.

4. Ben-Porat, T., and Kaplan, A. S. The synthesis and fate of pseudorabies virus DNA in infected mammalian cells in stationary phase of growth. Virology 20: 310-317, 1963.

5. Ben-Porat, T. and Kaplan, A. S. Mechanism of inhibition of cellular DNA synthesis by pseudorabies virus. Virology 25, 22-29, 1965.

6. Bloch, D. P., Morgan, C., Godman, G. C., Howe, C., and Rose, H. M. A correlated histochemical and electron microscopic study of the intranuclear crystalline aggregates of Adenovirus in HeLa cells. J. Biophys. Biochem. Cytol. 3: 1-18, 1957.

7. Boyer, G. S., Leuchtenberger, C., and Ginsberg, H. Cytological and cytochemical studies of HeLa cells infected with Adenovirus. J. Expt. Med. 105: 195-219, 1957.

8. Brenswick, E., and Rapp, F. Thymidine kinase activity in cells abortively and productively infected with Human Adenovirus. Virology 34: 799-802, 1968.

9. Castro, B. C., Atchison, R. W., and Hammon, W. M. Studies on the relationship between adeno-associated virus type 1 (AAV-1) and Adenoviruses. I. Replication of AAV-1 in certain cell cultures and its effect on helper Adenovirus. Virology 32: 52-59 (1967).

10. Castro, B. C. Adenovirus transformation of hamster embryo cells. J. Virology 2: 376-383, 1968.

11. Cleaver, J. E. (ed.). Thymidine metabolism and cell kinetics, in Frontiers of Biology, Vol. 6., J. Wiley and Sons, Inc., New York, 1967. pp 120.

12. Cohen, E. P. The appearance of new species of RNA in the mouse spleen after immunization as detected by molecular hybridization. P.N.A.S. 57: 673-680, 1967.

13. Cohen, S. S. Biochemical studies on multiplication of bacterial viruses. Fed. Proc. 10: 585-593, 1951.

14. Cohen, S. S. On the control of polymer synthesis in virulent phage infection, in Perspectives in Virology III, Pollard, M. (ed.), Harper and Row, Publishers Inc., New York, 1963, pp. 1-11.

15. Cohn, M., and Monod J. Specific inhibition and induction of enzyme biosynthesis. Symp. Soc. gen. Microbiol. 3: 132-154, 1953.

16. Cooper, J. E. K., Stich, H. F., and Yohn D. S. Viruses and mammalian chromosomes. VIII. Dose response studies with human adenoviruses type 18 and 4. Virology 33: 533-541, 1967.

17. Cooper, P. D. A chemical basis for classification of animal viruses. Nature 190: 302-305, 1961.

18. Crawford, L. V., and Crawford, E. M. A comparative study of polyoma and papilloma viruses. Virology 21: 258-263, 1963.

19. Crawford, L. V. Nucleic acids of tumor viruses, in Advances in Virus Research, Vol. 14, Smith, K. M. and Lauffer, M. A. (eds.), Academic Press, 1969. pp. 89-152.

20. Dales, S. J. An electron microscope study of the early association between two mammalian viruses and their hosts. J. Cell Biol. 13: 303-322, 1962.

21. Denhardt, D. T. A membrane filter technique for detection of complementary DNA. B.B.R.C. 23: 641-650, 1966.

22. DeCosse, J., and Aiello, N. Feulgen hydrolysis; effects of acid and temperature. J. Histochem. Cytochem. 14: 601-612, 1966.

23. Deitch, A. D., Wagnew, D., and Richard, R. M. Conditions influencing the intensity of the Feulgen reaction. J. Histochem. Cytochem. 16: 371-379, 1968.

24. DeRobertes, E. D. P., Nowinski, W. W., and Saez, F. A. In Cell Biology, 4th ed., W. B. Saunders Co., Philadelphia, 1965. pp. 82-89.

25. Dulbecco, R., Hartwell, L. H., and Vogt, M. Induction of cellular DNA synthesis by polyoma virus. P.N.A.S. 53: 403-410, 1965.

26. DuPraw, D. J. In Cell and Molecular Biology, Academic Press, New York, 1968. pp. 459-460.

27. Dyachenko, N. S., Nasach, L. N., Gushcha, K. P., and Nazarenko, V. G. Cytophotometric study of DNA accumulation in cells in the course of development of intranuclear inclusions caused by Type 1 Adenovirus. Acta Virol. 13: 81-87, 1969.

28. Eagle, H. Amino acid metabolism in mammalian cell cultures. Science 130: 432-437, 1959.

29. Eagle, H. Progagation in a fluid medium of a human epidermoid carcinoma strain KB. Proc. Sci. Expt. Biol. Med. 89: 362-364, 1965.

30. Eddy, B. E. Tumors produced in hamsters by SV40. Fed. Proc. 21: 930-935, 1962.

31. Eddy, B. E. Comparison of properties of two viruses - SV40 and polyoma - oncogenic in hamsters, in Perspectives in Virology III, Pollard, M. (ed.), Harper and Row Publishers, Inc., New York, 1963. pp. 138-158.

32. Fenner, F. The biological characters of several strains of vaccinia, cowpox, and rabbit pox viruses. Virology 5: 502-529, 1958.

33. Fishman, M. M., and Schiff, H. F. The use of nitrocellulose membrane filters for DNA samples. Biochim. Biophys. Acta 157: 419-420, 1968.

34. Fogh, J., and Fogh, H. A method for direct demonstrations of pleuropneumonia-like organisms in cultured cells. Proc. Soc. Biol. & Expt. Med. 117: 899-901, 1964.

35. Fox, D. P. Some characteristics of the cold hydrolysis technique for staining plant tissues by the Feulgen reaction. J. Histochem. Cytochem. 17: 266-272, 1968.

36. Freeman, A. E., Black, P. H., Vanderpool, E. A., Henry, P. H., Austin, J. B., and Huebner, R. J. Transformation of primary rat embryo cells by adenovirus type 2. P.N.A.S. 58: 1205-1212, 1967.

37. Fuginaga, K., and Green, M. The mechanism of viral carcenogenesis by DNA mammalian viruses: viral specific RNA in polyribosomes of adenovirus tumor and transformed cells. P.N.A.S. 55: 1567-1574, 1966.

38. Fuginaga, K., Mak, S., and Green, M. A method for determining the faction of the viral genome transcribed during infection and its application to adenovirus infected cells. P.N.A.S. 60: 959-966, 1968.

39. Gershon, D., Sachs, L., and Winocour, E. Induction of cellular DNA synthesis by simian virus 40 in contact-inhibited and X-irradiated cells. P.N.A.S. 56: 918-925, 1966.

40. Ginsberg, H. S., Bello, L. J., and Levine, A. J. Control of biosynthesis of host macromolecules in cells infected with adenoviruses, in The Molecular Biology of Viruses, Colter, J. S. and Paranchych, W. (eds.), Academic Press, New York, 1967. pp. 547-573.

41. Green, M., and Daesch, G. E. Biochemical studies on adenovirus multiplication. II. Kinetics of nuclear acid and protein synthesis in suspension cultures. Virology 13: 169-176, 1961.

42. Green, M. Studies on the biosynthesis of viral DNA. Cold Spr. Harbour Symp. on Quant. Biol. 27: 219-236, 1962.

43. Green, M. Biochemical studies on adenovirus multiplication. III. Requirement for DNA synthesis. Virology 18: 601-613, 1962.

44. Green, M., and Pina, M. Biochemical studies on adenovirus multiplication. IV. Isolation, purification and chemical analysis of adenovirus. Virology 20: 199-207, 1963.

45. Green, M., Pina, M., and Chagoya, V. Biochemical studies on adenovirus multiplication. V. Enzymes of deoxyribonucleic acid synthesis in cells infected by adenovirus and vaccinia virus. J. Biol. Chem. 239: 1188-1197, 1964.

46. Green, M., and Pina, M. Biochemical studies on adenovirus multiplication. VI. Properties of highly purified tumorigenic human adenovirus and their DNA's. P.N.A.S. (U.S.) 51: 1251-1258, 1964.

47. Green, M. Biosynthetic modifications induced by DNA animal viruses. Ann. Rev. Microbiol. 20: 189-222, 1966.

48. Green, M., Pina, M., Kimes, R., Wensink, P. C., MacHattie, L. A., and Thomas, C. A. Adenovirus DNA. I. Molecular weight and conformation. P.N.A.S. 57: 1302-1309, 1967.

49. Green, M., Pina, M., and Kimes, R. C. Biochemical studies on adenovirus multiplication. XII. Plaquing efficiencies of purified human adenoviruses. Virology 31: 562-565, 1967.

50. Green, M., Pina, M., Fujinaga, K., Mak, S., and Thomas, D. Transcription of viral genes in adenovirus infected and transformed cells. Perspectives in Virology, VI. (In press)

51. Hatanaka, M., and Dulbecco, R. Induction of DNA synthesis by SV40. P.N.A.S. 56: 736-740, 1966.

52. Hodge, L. D., and Scharff, M. D. Effects of adenovirus on host cell DNA synthesis in synchronized cells. Virology 37: 554-564, 1969.

53. Huebner, R. J., Rowe, W. P., and Lane, W. T. Oncogenic effects in hamsters of human adenovirus types 12 and 18. P.N.A.S. 48: 2051-2058, 1962.

54. Joklik, W. K. Some properties of pox virus deoxyribonucleic acid. J. Mol. Biol. 5: 265-274, 1962.

55. Joklik, W. K., Jungwirth, C., Oda, K., Woodson, B. Early and late functions during the vaccinia virus multiplication cycle, in The Molecular Biology of Viruses, Colter, J. S., and Paranchych, W. (eds.), Academic Press, New York, 1967. pp. 473-495.

56. Kit, S., Dubbs, D. R., and Piekarski, L. J. Enhanced thymidine phosphorylating activity of mouse fibroblasts (strain L-M) following vaccinia infection. Biochem. Biophys. Res. Commun. 8: 72-75, 1962.

57. Kit, S., Dubbs, D. R., and Frearson, P. N. Enzymes of nucleic acid metabolism in cells infected with polyoma virus. Cancer Res. 26: 638-646, 1966.

58. Kit, S. Induction of enzymes of DNA metabolism by simian virus 40, in Monograph of the 1st International Symposium on Tumor Viruses, 1967. pp. 116-143.

59. Kit, S. Enzyme induction in cell cultures during productive and abortive infection by papovavirus SV40, in The Molecular Biology of Viruses, Colter, J. S., and Paranchych, W. (eds.), Academic Press, New York, 1967. pp. 495-526.

60. Klamerth, O. Separation of high molecular deoxyribonucleic acids and ribonucleic acids. Nature 208: 1318-1319, 1965.

61. Kopriwa, B. M. and Leblond, D. P. Improvements in the coating technique of radioautography. J. Histochem. Cytochem. 10: 269-284, 1962.

62. Koziorowska, J., and Wlodarski, K. The effects of crude preparations of vaccinia on mitosis and DNA synthesis of KB cells. J. Expt. Med. 124: 199-208, 1966.

63. Maizel, J. V., White, D. O., and Scharff, M. D. Polypeptides of Adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A and 12. Virology 36: 115-125, 1968.

64. Maizel, J. V., White, D. O., and Scharff, M. D. Polypeptides of Adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. Virology 36: 126-136, 1968.

65. Mak, S. Personal communication.

66. Marmur, J. A procedure for isolation of deoxyribonucleic acids from microorganism. J. Mol. Biol. 3: 208-218, 1961.

67. McAuslan, B. R., and Smith, W. R. Deoxyribonucleic acid synthesis in FU-3 infected mammalian cells. J. Virology 2: 1006-1015, 1968.

68. McBride, W. D., and Wiener, A. In vitro transformation of hamster kidney cells by human adenovirus type 12. Proc. Soc. Exp. Biol. & Med. 115: 870-874, 1964.

69. Mellema, J. E., van Bruggen, E. F., and Gruben, M. An assessment of negative staining in the electron microscopy of low molecular weigh proteins. J. Mol. Biol. 31: 75-82, 1968.

70. Melnick, J. L., Khara, K. S., and Rapp, F. Papovavirus SV40: Failure to isolate infectious virus from transformed hamster cells synthesizing SV40-induced antigens. Virology 23: 430-432, 1964.

71. Mendelshon, M. L. A microspectrophotometer and tests a model system. J. Biophys. Biochem. Cytol. 4: 407-414, 1958.

72. Mendelshon, M. L. Two wavelength method of micro-spectrophotometry. II. Set of tables to facilitate the calculations. J. Biophys. Biochem. Cytol. 4: 415-424, 1958.

73. Parks, W. P., Melnick, J. L., Rongey, R., and Mayor, H. D. Physical assay and growth cycle studies of a defective adeno-satellite virus. J. Virology 1: 171-180, 1967.

74. Patau, K. Absorption microspectrophotometry of irregular-shaped objects. Chromosoma 5: 341-362, 1952.

75. Pène, J. J. Host macromolecular synthesis in bacteriophage infected B. subtilis. Bacteriological Rev. 32: 379-386, 1968.

76. Pereira, M. S., Pereira, H. G., and Clarke, S. K. R. Human adenovirus type 31, a new serotype with oncogenic properties. Lancet., Jan. 2, 21-23, 1965.

77. Pina, M., and Green, M. Biochemical studies on adenovirus multiplication. IX. Chemical and base composition analysis of 28 human adenovirus. P.N.A.S. 54: 547-552, 1965.

78. Pizer, L. I., and Cohen, S. S. Virus-induced acquisition of metabolic function: Purification and properties of the deoxycytidylate methylase and studies of its origin. J. Biol. Chem. 237: 1251-1258, 1962.

79. Polasa, H., and Green, M. Biochemical studies on adenovirus multiplication. VII. Analysis of protein synthesis. Virology 25: 68-79, 1965.

80. Leuchtenberger, C., Leuchtenberger, R., and Davis, A. M. A microspectrophotometric study of deoxyribose nucleic acid (DNA) content in cells of normal and malignant human tissues. Am. J. Path. 30: 65-85, 1954.

81. Leuchtenberger, C., Boyer, G. S., and Strain, J. J. Quantitative cytochemical investigation on the effect of virus on cells. Ann. N. Y. Acad. Sci. 81: 73-83, 1959.

82. Levine, A. J., and Ginsberg, H. S. Role of adenovirus structural proteins in the cessation of host cell biosynthetic functions. J. Virology 2: 430-439, 1968.

83. Rainbow, A. J. Personal communication.

84. Reed, S. A. Transformation of hamster cells in vitro by adenovirus type 12. J. Gen. Virology 1: 405-412, 1967.

85. Rightsel, W. A., Greiff, D. Freezing and freeze drying of viruses. Cryobiology 3: 423-431, 1967.

86. Rouse, H. C., and Schlesinger, R. W. An arginine-dependent step in the maturation of type 2 adenovirus. Virology 33: 513-522, 1967.

87. Russell, W. C., and Crawford, L. V. Some characteristics of deoxyribonucleic acid from herpes simplex virus. Virology 21: 353-361, 1963.

88. Russell, W. C., and Becker, Y. A maturation factor for adenovirus. Virology 35: 18-27, 1968.

89. Seemayer, N., and Maass, G. Autoradiographische untersuchungen des DNS-stoffwechsels von affennieren - gewebekulturen nach infection mit dem virus SV40. Zentralblatt fur Bakteriologie, Parasitenkunde infektionskrankheiten und Hygiene 209: 434-446, 1969.

90. Shenin, R. Deoxyribonucleic acid synthesis in cells infected with polyoma virus, in The Molecular Biology of Viruses, Colter, J. S., and Paranchych, W., (eds.), Academic Press, New York, 1967. pp. 627-644.

91. Shimjo, H., and Yamashita, T. Induction of DNA synthesis by adenoviruses in contact-inhibited hamster cells. Virology 36: 422-433, 1968.

92. Slonin, D., and Roslerova, V. Preservation of vaccinia virus with glycerin. J. of Hygiene, Epidemiology, Microbiology and Immunology 13: 43-44, 1969.

93. Smith, A. U. Effects of low temperature on living cells and tissues, in Biological Applications of Freezing and Drying, Harris, R. J. C. (ed.), Academic Press Inc., New York, 1954.

94. Stoker, M., and Abel, P. Conditions affecting transformation by polyoma virus. Cold Spring Harbour Symp. Quant. Biol. 27: 375-386, 1962.

95. Sueoka, N., and Cheng, T. Fractionation of nucleic acids with methylated albumin columns. J. Mol. Biol. 4: 161-172, 1962.

96. Swift, H. Cytochemical techniques for nucleic acids, in The Nucleic Acids, Charoff, E. C., and Davidson, J. N., (eds.), Academic Press Inc., Volume II, New York, 1955. pp. 51-92.

97. Szybalski, W., Erikson, R. L., Gentry, G. A., Gafford, L. G., and Randall, C. C. Unusual properties of fowlpox virus DNA. Virology 20: 586-589, 1963.

98. Takahashi, M., Van Hoosier, G. L., and Trentin, J. J. Stimulation of DNA synthesis in human and hamster cells by human adenovirus types 12 and 5. Proc. Soc. Exp. Biol. & Med. 122: 740-746, 1966.

99. Thomas, C. A., Berns, K. I., and Kelly, J. J. Jr. Isolation of high molecular weight DNA from bacteria and cell nuclei, in Procedures in Nucleic Acid Research, Cantoni, G. L., and Davis, D. R. (eds.), 1966. pp. 535-540.

100. Todaro, G. L., and Green, H. High frequency of SV40 transformation of mouse cell line 3T3. Virology 28: 756-759, 1966.

101. Ursprung, H., Smith, K. D., Sofer, W. H., and Sullivan, D. T. Assay system for the study of gene function. Science 160: 1075-1081, 1968.

102. van der Noordaa, J. Transformation of rat cells by adenovirus types 1, 2, 3. J. Gen. Virology 3: 303-304, 1968.

103. Watson, J. D. In The Molecular Biology of the Gene, W. A. Benjamin Inc., New York, 1965, pp. 297.

104. Warnaar, S. O., and Cohen, J. A. A quantitative assay for DNA-DNA hybrids using membrane filters. B.B.R.C. 24: 554-558, 1966.

105. White, D. O., Scharff, M. D., and Maizel, J. V. Polypeptides of adenovirus. III. Synthesis in infected cells. Virology 38: 395-406, 1969.

106. Winocour, E. Purifications of polyoma virus. Virology 19: 158-168, 1963.

107. Yamashita, T., and Shimojo, H. Induction of cellular DNA synthesis by Ad 12 in human kidney cells. Virology 37: 351-355, 1969.