EFFECTS OF RADIATION

ON ADENOVIRUS EXPRESSION

A CORRELATION OF MOLECULAR DAMAGE AND BIOLOGICAL FUNCTIONS OF HUMAN ADENOVIRUS FOLLOWING UV AND GAMMA IRRADIATION

By

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SCOPE AND CONTENTS :

The expression of several different functions of the oncogenic human adenovirus type 12 and the non-oncogenic human adenovirus type 2 were examined after infection of human KB cells. Also, following UV and gamma irradiation of the virus, the molecular damage induced in the viral DNA was correlated with the biological functions expressed by the radiation induced defective virus particles. The results were discussed in relation to the current ideas of viral oncogenesis and the mechanisms of radiation DNA damage and repair in other organisms.

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INTRODUCTION

Viruses represent the simplest form of a self replicating system and their study has become a very fruitful branch of pure and applied biology. After virus infection, the viral genome functions to alter the metabolism of the host cell. An understanding of the biosynthetic processes induced by infection with animal viruses is important to the progress in several areas of science.

It has been estimated that about 10% of all illnesses are the result of viral infection (Horsfall 1965) and for this reason an understanding of the virus-cell interaction would bring new horizons to the field of medical science. Furthermore, an understanding of the cancer cell and the neoplastic process in molecular terms is now a realistic research goal for those studying tumorigenic viruses and the mechanism of virus induced cell transformation. Viruses can also be used as tools for the analysis of cell functions. The mammalian cell, containing more than a million genes (Green 1966), with thousands of different proteins being synthesised at any one time, presents an extremely difficult system for the analysis of transcription and translation of specific macromolecules. Virus infection affords the opportunity to introduce a defined segment of genetic material into a cell. Since viruses contain only a limited number of genes, it is technically feasible to analyse in detail, the transcription and translation of virus specific macromolecules, and the intracellular

controls of these processes, employing the virus infected cell for experimental analysis. More than 500 animal viruses of various sizes and degrees of complexity have been described (Green 1966), containing either DNA or RNA, and multiplying or maturing in different parts of the cell. In this framwork, the human adenoviruses as a group, offer a singularly rich field for learning.

The 31 serotypes of human adenoviruses (Wilner 1965) have a medium complexity with a particle size of 60 to 85 mμ. Some of these serotypes, notably adenovirus types 12, 18,and 31 are highly tumorigenic in animals (Crawford 1969, Huebner 1962 and Pereira 1965). The nucleic acid is double stranded DNA with a molecular weight of 21×10^6 to 25×10^6 daltons, and is able to code for at least 9 proteins (Maizel 1968 and White 1969). One important advantage in using this viral group is that the adenoviruses and the adenovirus-cell interaction have been widely investigated and are the subject of many excellent review articles (Crawford 1969, Ginsberg 1962, Ginsberg 1965, Ginsberg 1967, Green 1966, Rapp 1969 and Schlesinger 1969).

The Virus-Cell Interaction

Viral infection of a susceptible host can lead to two different types of cellular response. The permissive response leads to the production of new infectious virions, whereas non-permissive or abortive does not. However, in either form of infection, alterations in the normal cell morphology and metabolism can be found. Metabolic alterations may range from an inhibition of macromolecular synthesis (Green 1966 and Levine 1968) to the induction of cellular DNA and

cellular enzyme synthesis (Bresnick 1968, Green 1966, Kit 1967 and Ledinko 1967). Morphological alterations include the intranuclear inclusions found after infection with adenovirus (Boyer 1957, Ginsberg 1962, Ginsberg 1965 and Pereira 1963).

Although the overall response of a cell culture is usually described as either productive or abortive, the viral expression in each individual cell may vary throughout the infected culture. This variation in the response of each individual cell may be due to several factors such as the heterogeneity of the virus population (Huang 1970), the heterogeneity of the cell population (Sheinin 1965), the multiplicity of infection (Finter 1955 and Koprowski 1962) and the physiological state of the infected cells (Isaacs 1963).

The permissive cellular response, at the level of the single cell, may be defined as those events of virus infection which lead to the reproduction of the infecting virus. This is usually accompanied by cytopathic effects and may culminate in cell death. In a cell culture, such events may either lead to death of the population, or, in the case of a limited response, it may lead to an equilibrium of cell growth and cell death (Belyavin 1963 and Isaacs 1963). Virus replication can be divided into a series of steps : adsorption, penetration, uncoating of the nucleic acid, induction of early products, replication of the nucleic acid, synthesis of late products, assembly and release of infectious progeny.

For the adenoviruses, attachment to the host cell surface probably occurs at specific sites (Philipson 1967 and Philipson 1968) and may involve specialised components of the virion such as the pentons

and fibers (Valentine 1965). Pentons and fibers are also involved in haemagglutination by adenoviruses (Norby 1966 and Pereira 1962). Following penetration and transport of the infectious virions to the synthesis sites (Morgan 1969), specific regions of the parental genome are transcribed (Green 1970, Fujinaga 1968 and Mak 1968). The newly synthesised RNA forms a complex with the ribosomes (Fujinaga 1966) for the production of viral specific "early" enzymes (Green 1964). Some of these enzymes are concerned with the transcription and replication of the viral DNA (Green 1962 and Polassa 1965), while the functions of others, such as the neoantigens are still not known (Kit 1967, Kalnins 1967, Hoggan 1965, Tockstein 1968 and Weiss 1968). These events are followed by the replication of viral DNA (Ginsberg 1967 and Green 1962) and somewhat later by the production of "late" viral structural proteins (Ginsberg 1967, Green 1966 and White 1969). Finally, the viral components are assembled into mature infectious progeny virions.

In the abortive response, no infectious virus is produced, although viral constituents in varying degrees and proportions may be found (Rapp 1969). In general, the abortive response may be divided into two classes. One class of the abortive response is host dependent and results from the infection of an unnatural host. For example, human adenoviruses can produce abortive infection of canine cells (Carmichael 1965), simian cells (Rapp 1967) and various rodent cells (Hoggan 1965, Levinthal 1965, Pope 1964 and Schell 1968) with different degrees of expression.

A special consequence of abortive infection by animal viruses is cell transformation and the induction of tumors. Several human adenoviruses are capable of transforming suitable host cells in tissue culture (Freeman 1967, McBride 1964, Reed 1967 and van de Noordaa 1968) and human adenovirus types 12, 18 and 31 have been shown to be highly oncogenic in hamsters (Crawford 1969, Huebner 1962 and Pereira 1965).

Another class of abortive infection can occur in a normally permissive host and is due to infection by genetically defective virions. Such defective virions have been described with increasing frequency in animal virus systems, suggesting that they may be more common than had previously been expected (Huang 1970). These biologically active defective viral particles have the following properties : they contain normal viral structural protein, contain an incomplete viral genome, can replicate in the presence of helper virus and interfere specifically with the intracellular replication of non-defective homologous virus. Defective virions with the properties mentioned above occur in many and possibly all animal virus systems. Von Magnus (Von Megnus 1964) first described incomplete virus particles of influenza virus and since then such particles have been described for many different animal viruses (Haung 1970). Such particles are produced more efficiently upon infection with high multiplicities or "undiluted passage" virus.

A study of the abortive response helps towards an understanding of the virus-cell interaction and relates to the problem of viral oncogenesis. Furthermore, such a study also provides insight into cellular control mechanisms, since the abortive response may result

in a permanent modification of cellular functions.

Several approaches have been used to understand the mechanism of the virus-cell interaction. Cell fusion techniques, between permissive and non-permissive cells, have been used to examine the cellular factors which control the expression of viral functions (Weber 1969). Pretreatment or coinfection of cells with different viruses has been found to alter the course of a virus infection (Padget 1970). In this way, a normally permissive infection can be blocked by preinfection (Hillman 1968 and Padget 1970), a normally abortive cycle changed to a permissive one by "stimulon" (Chany 1967) or "helper" virus (Atchison 1966, Casto 1967, Easton 1966, Hanafusa 1964 and Mayor 1967). Coinfection has also been used to study modifications in the transcription and replication of viral DNA (Mak 1969).

In a productive system, the virus-cell interaction is relatively effective, leading to the production of mature infectious virus. The abortive system is characterised by the interruption of viral replication at a certain point (Rapp 1969). This allows examination of the events that have preceded the blocked step and a determination of what is required to continue the process of viral replication. In a system in which a wide variety of defective virions can be obtained, the entire range of events required for the production of infectious virus can be examined (Uchida 1968, Burge 1967 and Sambrook 1966). Defective virions may occur naturally during virus production or be produced by irradiation of the virions (Defendi 1967).

The Expression of Irradiated Virus

It has been shown that irradiated virions not capable of producing infectious virus particles are still capable of carrying out other limited viral functions, such as neoantigen production (Gilead 1966 and Stich 1968), chromosome breakage (Stich 1968 and zur Hausen 1968), DNA polymerase activity (Decker 1969), inhibition of host cell cloning (Rainbow 1970), cell transformation (Latarjet 1967 and Finklestein 1969) and tumor induction (Defendi 1967). This amounts to radiation induced defectiveness, which results from damage to the viral genome. By comparing the radiosensitivity of various viral functions, it is possible to determine the relative size of the genome responsible for each of the different functions. In this way, the amount of functional genome can be related to the degree of defectiveness of the virions.

The type of radiation damage produced in the viral DNA depends greatly on the characteristics of the radiation employed. The vast majority of damage induced by ultraviolet light (UV) is in the form of pyrimidine dimers (Smith 1969), whereas after gamma irradiation, double and single strand chain breakage is more common (Bohne 1970, Lytle 1968, and van de Schans 1970). These structural defects may inhibit normal viral replication. On this basis, the expression of the viral genome following irradiation should be jointly determined by the amount of damage produced and the subsequent repair of such damage.

Several reports suggest that a host mediated repair mechanism may be active following infection by irradiated virus (Kozinski 1967,

Zavadova 1968, Kalab 1970 and Sauerbier 1964). However, the evidence is not conclusive in all cases, and the exact mechanism is unknown. If present, the host mediated repair mechanisms may be identical to those reported for animal and bacterial cells (Haynes 1966, Cleaver 1969 and Rupp 1970). Consequently, a study of the expression of irradiated virus may also be used to understand the fundamental mechanism of radiation damage and repair in higher organisms. The importance of cellular repair mechanisms which repair radiation induced DNA damage cannot be overestimated since it is probably these mechanisms which function in the normal cell to maintain the genetic stability of the DNA. Irradiation of a cell may affect a variety of different biochemical systems. Many of these systems are closely related, so that damage to one may be manifest ultimately as damage to another. This is especially important when the effects of radiation damage to DNA and repair of DNA damage are being studied (Elkind 1967).

This problem does not arise during irradiation of viruses, since the cellular enzyme systems and the damaged viral genome are separate. Furthermore, the conditions of viral irradiation have potentially wider limits than those for higher organisms. Viral preparations can be dehydrated or kept at low temperatures during irradiation. For this reason, the virus is a very attractive biological entity for radiobiological investigation. Irradiated virus can be used to infect a suitable host cell and the subsequent expression of the damaged genome examined. In this way, the damage induced in the viral DNA can be correlated with the viral functions expressed by the defective virions (Bohme

1970, Lytle 1968, Wulff 1963 and Sauerbier 1964).

This investigation was carried out to examine several functions expressed by both naturally occuring and radiation induced defective virions of human adenovirus, in human KB cells. Also, following UV and gamma irradiation of the virus, the molecular damage in the viral DNA was correlated with the biological functions expressed by the irradiated virions.

MATERIALS AND METHODS

A. TISSUE CULTURE TECHNIQUES

1. Glassware for Cell Cultures : Monolayer stock cell cultures were grown on the surface of 32 oz. "Saniglass" screwcap prescription bottles (Brown Glass and Supplies, Montreal, Quebec). Stock cell suspension cultures were grown in standard chemical reagent bottles and the suspension of growing cells was maintained by rotation of a teflon magnetic stirring bar.

2. Media : Media for the cell cultures was purchased from Grand Island Biological Company, Grand Island, New York. MEM (Catalogue Number F-12) was used for monolayer cultures and MEM, Joklik modified (Catalogue Number F-13) was used for cell suspension cultures. The media were prepared by dissolving the powdered media in glass distilled water as directed and sterilized by filtration through a Millipore membrane, type GS, with a pore size of 0.22 microns. Generally, antibiotics (2×10^2 units/ml of penicillin and 40 ug/ml of streptomycin) were added to the MEM before use.

3. Cell Line : Human epithelial cheek carcinoma cell, KB, (Eagle 1965) were used throughout this investigation. The established line, originally obtained from Dr. M. Green, St. Louis, Missouri, was propogated in suspension culture or in monolayer. Generally, suspension cultures were used for the growth of purified adenovirus stock, whereas, monolayer cultures were used for experimental purposes.

Suspension cultures were kept in logarithmic growth phase at a cell desity between 2×10^5 and 4×10^5 cells/ml. by daily two fold dilutions in warm MEM (Joklik modified) containing 5% horse serum. Before cell monolayer cultures became confluent, cells were scraped from the glass surface with a soft rubber policeman and aliquots were seeded in fresh bottles. Warm MEM containing 5% fetal calf serum was than added and the cell culture incubated at 37°C in 5% carbon dioxide and 95% air.

B. PREPARATION OF VIRUS

Purified preparationsoof adenovirus type 2 (Ad 2) were made using the following technique. Approximately 3×10^8 cells of a suspension culture, in their logarithmic phase, 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 was resuspended in 30 ml. of fresh prewarmed MEM (Joklik modified) containing 1% fetal calf serum and then infected with purified Ad 2 at an input multiplicity of infection of between 50 and 100 plaque forming units (PFU) per cell. The virus inoculum was allowed to adsorb to the cells

for 60 min. at 37°C, with the cell suspension continuously agitated by a magnetic stirring bar. Following adsorption, the infected cells were diluted with fresh prewarmed MEM (Joklik modified) containing 5% horse serum to a concentration of 2×10^5 to 3×10^5 cells/ml. The infected culture was incubated for 48 hours at 37°C, at which time, the infected cells were collected by centrifugation at 350 x g for 15 min. at 4°C using an IEC Model PR-2 centrifuge. Following removal of the supernatant culture medium, the cell pellet was resuspended in 10 ml. of Tris buffer (TRIZMA, Sigma Chemical Company, St. Louis, Missouri), 0.01 M, pH 8, and frozen at -45°C until purification.

Virus purification was carried out using the methods of Green and Pina (Green 1963) with some modifications. Disruption of the infected cells was accomplished by ultrasonic vibrations generated from a Biosonic III system's needle probe, Model BP III 40 T (Bronwill Scientific, Rochester, New York). Sonication was performed for 2 min. at 30% power output (300 watts acoustical energy at full power) while the sample was held in an ice bath to minimise heating.

Cellular material was further dissociated from the virus by homogenisation with 50 ml. of cold Freon 113 (triflourotrichloroethane, Matheson Chemicals) using a Sorval 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 an IEC, model RP-2 centifuge was sufficient to separate the Freon 113 and the cellular debris from the pink aqueous , supernatant layer, which contains the virus. This homogenisation of the cellular material was repeated to

ensure that maximum recovery of the virus was achieved. The pooled aqueous layers were added to a graduated burette and slowly layered on top of a cushion of Tris buffer, 0.01 M, pH 8, containing CsCl to give a final density of 1.44 gm./ml. The virus was sedimented onto the top of the cushion by centrifugation in an SW 27 rotor at 20,000 rpm for 120 min. at 4°C in a Beckman L2-65B preparative ultracentrifuge.

The upper portion of the supernatant was then removed and the opalescent band, just on top of the CsCl cushion, containing the virus was collected using a pipette. The virus solution was mixed with sufficient CsCl powder to give a density of 1.34 gm./ml. and then centrifuged in a Beckman L2-65B centrifuge at 35,000 rpm and at 4°C, for 20 hours using a 65 rotor. After this time a density gradient was formed in the tube and the virus was concentrated in a narrow band.

The virus band was collected by piercing the bottom of the centrifuge tube and further purified by a second density gradient centrifugation. The virus collected from the second CsCl gradient was diluted 10 fold with tris buffered saline (see below) plus 20% glycerol (Winocour 1963 and Slonin 1969) and stored at -45°C in a Revco Freezer (Revco Inc., West Columbia, South Carolina) until used. Storage under these conditions produced no significant loss in infectivity.

A similar method was used in the preparation of adenovirus type 12 (Ad 12), except that the culture was infected with about 0.3 PFU/cell and harvested for virus 72 hours after infection.

Purified radioactive virus was prepared in the same manner as for unlabelled Ad 2 except that radioactive thymidine was added to the

cell culture 9 hours after infection. For ^{3}H labelled virus, 1 mC of ^{3}H labelled thymidine (specific activity 20 C/mM) together with 150 µgm. of cold thymidine was added to the infected culture. For ^{14}C labelled virus, 50 uC of ^{14}C labelled thymidine (specific activity 58 mC/mM) was added to the infected culture without the addition of cold thymidine.

Tris Buffered Saline (TBS) : TBS was prepared using a method similar to that of Winocour (Winocour 1963) in a 5 fold concentrated form :

Sodium Chloride	80.0 gm.
Potassium Chloride	3.8 gm.
Disodium Hydrogen Phosphate	1.0 gm.
Tris Buffer (TRIZMA), 1 M, pH 7.4 .	300.0 ml.
Glucose	10.0 gm.
Double distilled water to a final volume of	2000 ml.

The 5 fold concentrated stock solution was sterilised by filtration and stored at 4°C until used.

C. ASSAY FOR VIRAL FUNCTIONS

To ensure that cells in the same physiological state and under identical infection conditions were used for the assay of the different viral functions, all assays were carried out with the same infected cell culture.

Stock cells were scraped from the glass surface using a soft rubber policeman and dispersed with a 10-ml. pipette. 10^6 cells were sedimented by low speed centrifugation and mixed together with an appropriate amount of virus in 1 ml. of MEM without serum in a 12 ml. plastic tube. The suspension was rotated in a roller wheel at 37°C for 90 min. Then the cells were diluted 5 fold with prewarmed MEM plus 5% fetal calf serum and 5% pooled human serum, which was to prevent possible reinfection of the cells by progeny virus. In some experiments, dilute type specific antiserum (obtained from Grand Island Biological Company, Grand Island, New York) was added. Then samples of the cells were removed for a determination of cell concentration, infectious centers, and cloning efficiency. The remainder of the cells were further incubated at 37°C for an assay of nuclear inclusion bodies. A cell count was also performed at the end of this incubation period.

1. Plaque Formation : The infectivity was assayed by plaque formation using the method described by Green et al (Green 1967). 10^6 exponentially growing KB cells were suspended in 10 ml. of MEM supplemented with 10% fetal calf serum and pipetted into a 60 ml. plastic petri dish (Falcon

Plastic, Los Angeles, California). After 24 hours of incubation at 37°C in 5% carbon dioxide, 95% air and 90 to 100% humidity, a confluent cell monolayer was formed. The growth medium was poured off and the cell monolayer was inoculated with 0.2 ml. of virus, appropriately diluted in MEM without serum. After adsorption 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 made up immediately before use from complete overlay medium (see below) diluted 1 to 1 (volume/volume) with 1.8% (weight/volume) noble agar warmed at 45°C. After 5 days of incubation the plates were overlaid with an additional 5 ml. of white nutrient agar. At the 9 th day for Ad 2, and the 12 th for Ad 12, the plates were again overlaid with nutrient agar containing 0.0043% of neutral red. Plaques were observed 2 days after the addition of neutral red and the plaque number recorded daily until constant.

2. Formation of Infectious Centers : Infectious centers were assayed using the method of Green and Daesch (Green 1961) with slight modifications. After adsorption (see beginning of this section), the unadsorbed virions were inactivated by treatment with type specific viral antiserum. The infected cell suspension was then diluted 200 fold with medium containing no antiserum and samples of 0.1 or 0.2 ml., containing 100 to 1000 cells were then added to confluent cell sheets grown on 60 ml. plastic petri dishes (Falcon Plastics, Los Angeles, California). After the suspension had spread evenly, the cells were carefully overlaid with 1 ml. of

nutrient agar. When the agar had hardened, an additional 4 ml. of nutrient agar was added and the cells were further treated for plaque production.

Overlay Plaquing Medium : Complete overlay medium was prepared by adding aseptically the following ingredients :

EMEM (2x)	82 ml.
Amino Acid Mixture (100X) (Gibco Cat. # 13602)	2 ml.
Vitamin Solution (100X) (Gibco Cat. # 104)	2 ml.
L-Glutamine (2mM)	2 ml.
Arginine (2.1%)	0.7 ml.
Sodium Hydroxide (1N)	0.4 ml.
Horse Serum	12 ml.
Fetal Calf Serum	12 ml.
Sodium Bicarbonate (1M)	6.0 ml.
Fungizone (100X), Amphotericin B 250 mcg/ml. (Gibco Cat. # 529)	2 ml.
Antibiotic Solution (6×10^6 units/ml. penicillin and 7.4×10^3 ug/ml. streptomycin)	2 ml.

3. Cloning of Cells : The technique used was essentially that of Puck and Marcus (Puck 1955). Infected cell (see beginning of this section) were seeded on 60 ml. Falcon plasic petri dishes containing 6×10^4 gamma irradiated (5000 rads) KB cells as feeders. A dose of 5,000 rads resulted in no surviving feeder cells. MEM containing 5% fetal calf serum plus 5% pooled human serum was used as the cloning medium. Under these conditions, the colony count represents the true fraction of cells escaping the lethal action of the virus, since non-infected KB cells plated together with or without heavily infected cultures (10 PFU/cell) showed no difference in colony counts. The number of clones was determined using 5 X magnification after 7 to 9 days incubation at 37°C in 5% carbon dioxide and 95% air. Under these conditions, the efficiency of plating uninfected cells varied from 50 to 70% .

4. Examination of Nuclear Inclusion Bodies and Cells in Mitosis : Cells from the roller tube cultures (see beginning of this section) were sedimented by low speed centrifugation and the cell pellet resuspended in 1 ml. of 1% sodium citrate solution for 10 min at 37°C. After swelling, the cells were again sedimented and the cell pellet fixed with 0.5 ml. of acetic acid-alcohol mixture (1:3). A drop of cell suspension was air dried on a slide, stained with orcein and mounted for microscopic examination. Orcein stain was prepared by dissolving 2 gm. of orcein (British Drug House, Toronto) with 100 ml. of 45% acetic acid in water and then refluxing for either 2 or 24 hours.

The stain was cooled and filtered through Whatman Number 1 paper before use. Usually, 500 to 1,000 cells were examined for the appearance of metaphase plates and the presence of nuclear inclusion bodies.

5. Adsorption of Virus to Host Cell : 10^6 cells were sedimented by low speed centrifugation and then mixed together with an appropriate amount of ^3H labelled virus in 1 ml. of MEM without serum in a 12 ml. plastic tube. The infected suspension culture was rotated in a roller wheel at 37°C for 90 min. The cells were than diluted 5 fold with warm MEM plus 10% fetal calf serum and incubated for a further period of time. At an appropriate time after infection, 0.1 ml. samples of the infected cell suspension were spotted onto glass fiber filters (Sartorious Membrane filter GmbH, Cat. # SM 13400, British Drug House, Chemicals Division, Toronto). The cells from the infected roller culture were then sedimented by low speed centrifugation and 0.1 ml. samples of the supernatant fluid were spotted onto glass fiber filters. Each filter was dried in a glass scintillation vial, and radioactivity was counted in 5 ml. of toluene containing 0.02 gm. of Omnifluor (New England Nuclear Company, Cat # NEF-906) using a liquid scintillation spectrometer. The fraction of virus adsorbed to the cells was determined by subtraction of the radioactivity in the supernatant from the total radioactivity in the cell suspension.

6. Haemagglutination : The method used was essentially that of Pereira and de Figueiredo (Pereira 1962). Haemagglutination titrations were carried out using rat erythrocytes. Complete agglutination of rat erythrocytes by some some adenovirus types is only achieved upon addition of heterotypic virus specific antiserum to the red blood cells (Rosen 1960). In order to obtain red blood cells, black hooded rats (Sprague Dawley strain) were bled from the heart into a syringecontaining 1 ml. of a 3.8% solution of sodium citrate. Usually, about 10 ml. of blood could be collected. The erythrocytes were washed 3 times with normal saline (0.85% sodium chloride in double distilled water) and stored at 4°C as packed cells for up to 1 week.

Before use, a volume of packed cells was transferred to a graduated centrifuge tube, and washed with saline by repeated cycles of centrifugation and resuspension until no colour was seen in the washing fluid. The volume of packed cells was determined from the tube graduation and an equal volume of saline was added. For use in the haemagglutination tests, this was further diluted to a final concentration of 1%. Heterotypic virus specific antiserum (adenovirus type 1, specific antiserum, Gibco, Cat. # V.R. 3004) was added to the 1% red cell suspension at a dilution of 1 in 100.

Consecutive 2 fold dilutions of the virus were made in normal saline. Then 0.2 ml. of the red cell suspension was added to 0.5 ml of each dilution in a small test tube. The tubes were gently shaken and then left undisturbed at 37°C for a few hours, after which time the red blood cells in the saline control tubes had formed a visible cell pellet. Haemagglutination titers were then determined (see results).

D. IRRADIATION TECHNIQUES

1. Gamma Irradiation of Virus : Gamma irradiation was carried out using a 2,000 Curie ^{137}Cs source. ^{137}Cs produces a 660 Kev gamma ray, although after scattering and absorption in the source itself, some lower energy components would also be present. The irradiation technique is shown in Figure 1. Using this apparatus, six 1.5 ml. samples of virus could be irradiated simultaneously. Virus was suspended in MEM containing 5% fetal calf serum and kept at dry ice temperature (-75°C) during irradiation. One sample was removed every 24 hours and replaced by a dummy sample containing 1.5 ml. of medium. Several control samples underwent similar treatment except for the radiation. The irradiated virus samples were stored at -45°C before being assayed for the different viral functions.

The dosimetry of the source was carried out using a standard Fricke chemical dosimeter. The Fe^{++} solution contained the following ingredients :

0.2 gm. $Fe(NH_4)_2 6H_2O$

0.03 gm. NaCl (added to reduce the effect of impurities)

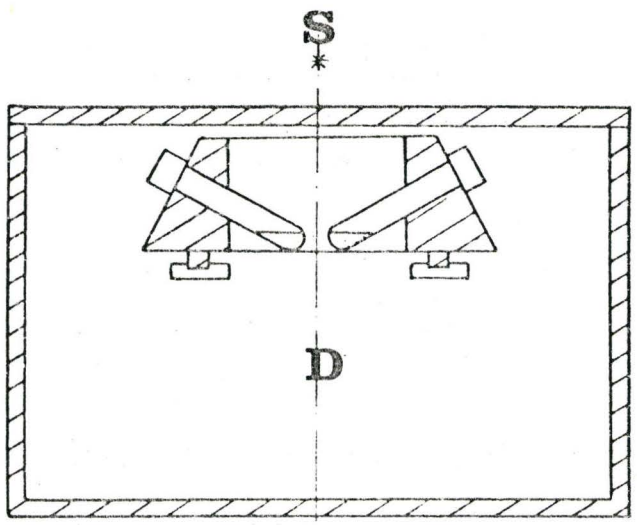
11.0 ml. analytical grade H_2SO_4

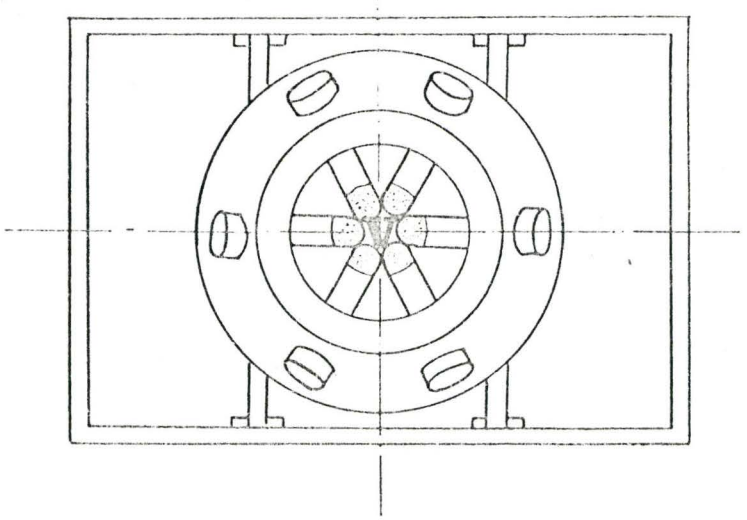
made up with glass distilled water to 500 ml. in a volumetric flask. Using similar conditions as for the virus samples, the solution was irradiated for different times and the yield of Fe^{+++} was calculated from the absorbance of the solution at a wavelength of 305 mu. The

FIGURE 1

Gamma Irradiation of Virus

Plan and side elevation of apparatus used for gamma irradiation of virus showing 2,000 Curie ^{137}Cs source, S, dry-ice chamber, D, and virus samples, V.





GAMMA IRRADIATION

absorbed dose of gamma irradiation was then calculated using the following relationship :

$$\text{DOSE IN RADS} = (\text{ABSORBANCE AT 305 mu}) \times 2.875 \times 10^4$$

Under the conditions employed the absorbed dose of gamma irradiation was 24.3 Krads/hour.

2. UV Irradiation of Virus : The UV irradiation technique is shown in Figure 2. Virus was suspended in 1.5 ml. of MEM without serum and irradiated at a distance of 10 cm. from an 8 watt General Electric Germicidal Tube, number G8T5. During irradiation, the virus suspension was stirred continuously with a small teflon magnetic bar and kept cool with running water at about 10°C through a metal plate supporting the petri dish. The incident dose rate under these conditions was determined by a UV intensity meter (Blak-Ray short wave UV meter, model J-225, Ultra-violet Products, Inc., San Gabriel, California).

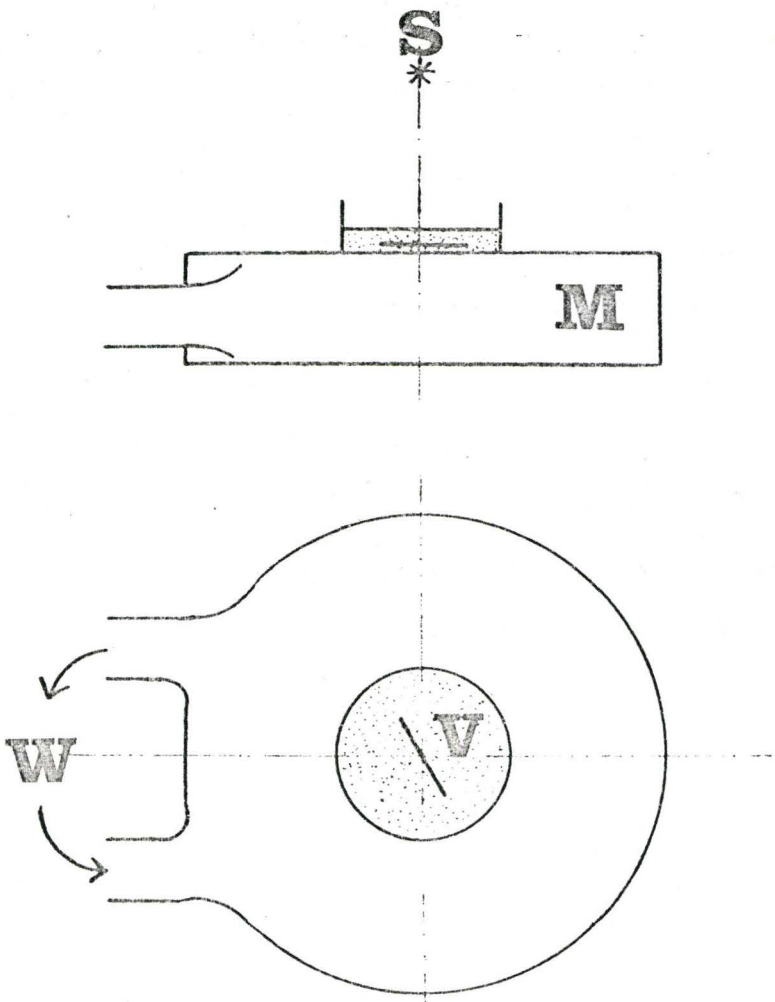
E. ASSAY FOR DNA DAMAGE

1. DNA strand breakage : Radiation induced DNA breaks were determined from the size distribution of the viral DNA fragments obtained on sucrose density gradients. At neutral pH, the hydrogen bonding between the single stranded DNA molecules of the viral DNA, is stable. Consequently, neutral sucrose gradients were used to assay radiation induced double

FIGURE 2

UV Irradiation of Virus

Plan and side elevation of apparatus used for UV irradiation of virus showing UV source, S, metal support, M, cold water flow, W, and virus sample V.



UV IRRADIATION

strand DNA breaks.

Samples of Ad 2, containing ^{3}H labelled DNA, were given various doses of irradiation (see previous section) and an aliquot of 50 lambda from each sample was suspended in 0.2 ml. of neutral buffer (see below). Unirradiated Ad 2, containing ^{14}C labelled DNA, was added to each sample and used as a marker. Pronase solution (20 mg/ml., previously self digested at 37°C for 2 hours) was added to give a final concentration of 40 ul/ml. and the mixture incubated at 37°C for 1 hour. After this time, 10 lambda of 5% sodium dodecyl sulphate (SDS) in 45% ethanol, was added to give a final concentration of about 1% SDS. This was left at room temperature for about 30 min. and then 0.2 ml. of the solution was layered carefully on the top of a previously prepared 5 to 20% neutral sucrose gradient. A 1 ml. plastic pipette (Falcon Plastics, Los Angeles, California, Cat # 7506), having a large bore, was used to avoid shearing of the DNA. The gradient was then centrifuged at 48,000 rpm and 15 to 20°C, for 2 hours, using an SW 50 or an SW 50.1 rotor in a Beckman L2-65B centrifuge.

After centrifugation, the cellulose nitrate tube was pierced at the bottom and 10 drop fractions were collected on glass fiber filters contained in scintillation vials. The filters were than dried and the radioactivity was counted in 5 ml. of toluene containing 0.02 gm. of Omnifluor (New England Nuclear Company, Cat # NEF-906) using a liquid scintillation spectrometer (Beckman Instruments Inc., model LS-250). Standards for ^{3}H and ^{14}C radioactivity were prepared in the following manner. Small aliquots of ^{3}H and ^{14}C labelled virus were treated with

pronase, suspended in sucrose solution and dripped onto glass fiber filters. The filters were then dried and the radioactivity counted under the same conditions used to count the tube fractions. In this way, an appropriate spill over value between the ^{3}H and ^{14}C channels of the scintillation spectrometer could be determined.

Double stranded DNA is denatured at high alkaline pH and separates into its constituent single stranded molecules. Consequently, alkaline sucrose gradients were used to assay single strand DNA breaks. The assay technique was essentially the same as that used for the neutral sucrose gradients. Aliquots of 20 lambda from each irradiated viral sample, containing ^{3}H labelled DNA, were mixed with 20 lambda of unirradiated virus, containing ^{14}C labelled DNA, and treated with 0.2 ml. of highly alkaline lysing solution (0.5 M NaOH and 0.01 M Ethylenedinitrilotetra--acetic acid, EDTA) at pH 12.8. This was left at room temperature for about 2 hours and then layered carefully onto a previously prepared 5 to 20% alkaline sucrose gradient (see below). The alkaline gradient was then treated as described above for the neutral gradients.

The DNA samples layered onto the sucrose gradients were never greater than 1 μg , when viral DNA was used, and never greater than 5 μg when infected cells were used. These amounts of DNA did not cause a significant overloading of the gradients, since the S values obtained were in agreement with those calculated from the accepted molecular weight for adenovirus type 2 DNA.

Neutral Buffer : This contained the following : 0.15 M Sodium Chloride, 0.015 M Sodium Citrate, 0.1 M Tris Buffer (TRIZMA) pH 7.5, 0.005 M EDTA, 0.3 M Sodium Trichloroacetate.

Sucrose Gradients : 5 to 20% sucrose gradients were prepared in 5 ml. cellulose nitrate tubes (Beckman, Cat. # 302232) using a Buchler Polystaltic Pump (Buchler Instruments). The gradient solutions were layered onto a 0.2 ml. cushion of 70% sucrose. Alkaline gradient solutions were made up using 0.3 M NaOH and 0.001 M EDTA, and neutral gradient solutions were made up in neutral buffer (see above).

2. Assay for Thymine Dimers : The method used was that of Rauth (Rauth 1970). Virus, containing ^{3}H labelled thymine, was hydrolysed in evacuated sealed glass tubes (Fisher Scientific, Cat. # 11-370) at 175°C for 90 min. using trifluoroacetic acid (TFA). Usually, 0.5 ml. of TFA was added to a 0.2 ml. sample of virus. After hydrolysis, the glass tubes were opened and the TFA evaporated off by heat treatment at 100°C for a few hours. 0.1 ml. of water was then added to dissolve the solutes and the hydrolysate spread carefully on 1.25 inch strips of Whatman's number 1 paper and allowed to dry.

Descending strip paper chromatography was carried out in a glass chromatography tank. The composition of the solvent used was n-butanol:water:glacial acetic acid (200:72:30, by volume). Using this system, the R_f values of thymine and thymine dimer are 0.6 and 0.3 respectively (Rauth 1970 and Setlow 1966), where R_f is defined as :

$$R_f = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent front}}$$

At the end of the chromatography run, usually 20 hours, the strip was removed from the tank and the solvent front marked. The strip was allowed to dry completely at room temperature and then cut up into 0.5 inch fractions. The radioactivity of each fraction was determined by liquid scintillation counting.

RESULTS

The expression of several different viral functions of human adenovirus type 2 in human KB cells was examined for both naturally occuring and radiation induced defective virus particles.

A. EXPRESSION OF ADENOVIRUS FUNCTIONS IN HUMAN KB CELLS

With animal viruses, the ratio of physical particles to infectious units is generally greater than unity (Wildy 1962). For the human adenoviruses, these ratios have been shown to vary depending on the serotype (Green 1967). Therefore adenovirus preparations may contain many non-plaque producing particles. It has been shown that irradiated viral particles, not capable of forming a plaque can still carry out other limited viral functions (Gilead 1966, Decker 1969, Stich 1968 and Zur Hausen 1968). It was therefore of interest to examine whether the non-plaque producing particles in the adenovirus preparations are capable of carrying out some other limited viral functions. Human adenovirus type 12 (Ad 12) has been shown to induce tumors in new born hamsters (Trentin 1968), whereas, adenovirus type 2 (Ad 2) is non-oncogenic, but has been shown to transform cells in vitro (Freeman 1967). Since it is thought that viral oncogenesis may result from the limited expression of a non-plaque producing particle (Defendi 1967), it was of interest to compare the viral functions expressed by these 2 adenoviruses.

FIGURE 3

Photomicrograph Showing Adenovirus-Induced Inclusion Bodies in KB Cells

(a) Inclusion body by Ad 2

(b) Infected by Ad 12, but no dense inclusion

(c) Inclusion body by Ad 12

(d) Non-infected cell

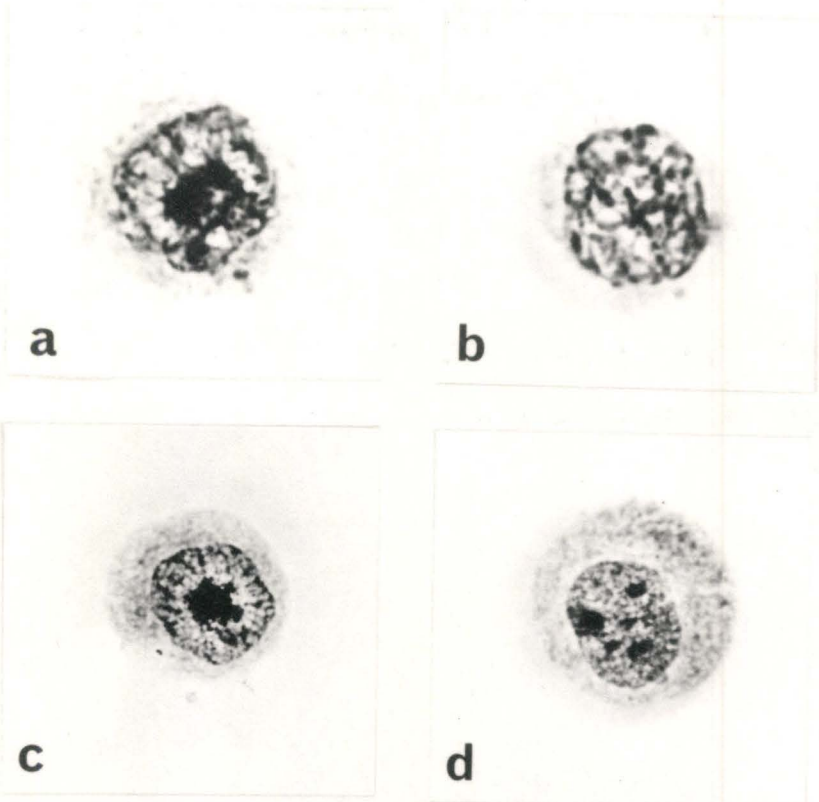
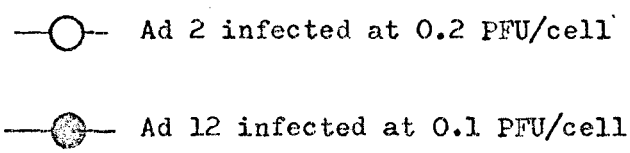
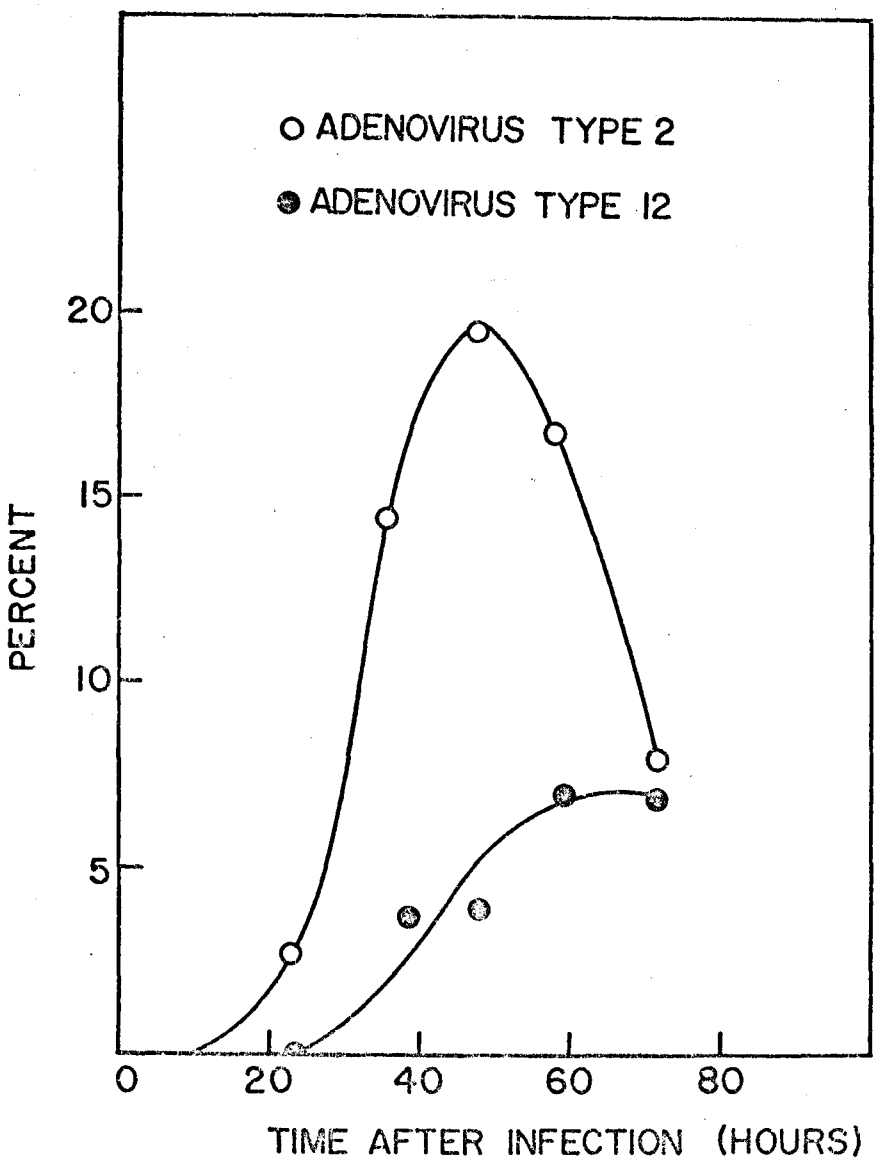

a
b
c
d

FIGURE 4

Time Course of Inclusion Body Formation in Ad 2 and Ad 12 Infected Cultures




O ADENOVIRUS TYPE 2
● ADENOVIRUS TYPE 12
PERCENT
20
15
10
5
0
20
40
60
80
TIME AFTER INFECTION (HOURS)

Ad 2 and Ad 12 were assayed for their ability to form plaques, infectious cell centres, nuclear inclusion bodies and to prevent host cell cloning in human KB cells.

1. Time Course of Inclusion Body Formation : KB cells were infected with either Ad 2 or Ad 12. At various times after infection, the cells were examined cytologically and found to display varying degrees of nuclear change. These signs of infection ranged form the appearance of dense nuclear inclusion bodies, chromatin condensation and micronuclei in the interphase cells, to contraction and pulverisation of chromosomes in the cells undergoing mitosis. Although at higher input multiplicities all cells showed varying degrees of infection,only those cells displaying a dense nuclear inclusion were scored (see Figure 3).

The percentage of cells displaying an inclusion body at the various times after infection is an underestimate for this viral function due to the mutiplication of the non-infected cells. Consequently, the following correction was applied to the observed percentage of inclusion bodies (IB).

$$(IB)_{corrected} = (IB)_{observed} \times \frac{\text{cell number at time of observation}}{\text{cell number at time of infection}}$$

The time course of the corrected percent inclusions is shown in Figure 4. The percentage of cells displaying an inclusion was a maximum at about 48 hours after infection for Ad 2 and 72 hours for Ad 12. The decline with time after 48 hours for the Ad 2 curve is probably due to a

selective loss of cells displaying dense inclusions, since these cells may be more fragile.

2. Effects of Input Multiplicity on Viral Functions : KB cells were infected with different multiplicities of either Ad 2 or Ad 12, and the percentage of cells capable of forming clones, producing infectious centres, and forming an inclusion body was determined. These results are shown in Table 1 and Figure 5.

It can be seen that, for the same input multiplicity (MOI), more cells were being prevented from cloning than were forming infectious centres or inclusion bodies. The effect was much more pronounced in the Ad 12 infected cultures than in those infected with Ad 2. The viral function of clone prevention followed one-hit kinetics predicted from the Poisson distribution (Marcus 1958). The formation of dense inclusions was consistent with one-hit kinetics for low values of input multiplicity (below 0.1 for Ad 12 and 0.5 for Ad 2), but with higher multiplicites the percentage of cells with an inclusion was substantially less than that predicted by the Poisson distribution. The percent inclusion reached a maximum of 40 to 60% for Ad 2 and 10 to 15% for Ad 12 for different experiments. It is possible that, at high input multiplicities, the large number of non-plaque producing particles infecting a cell interferes with inclusion body formation.(see section D4)

As shown in Figure 5, the percentage of infectious centres was substantially greater for Ad 2 than for Ad 12 when cells were infected with the same input of plaque forming units (PFU). For Ad 2

TABLE 1

Effects of Input Multiplicity of Adenovirus Types 2 and 12 on the Formation of Inclusion Bodies, Production of Infectious Centres and Inhibition of Host Cell Cloning in KB Cells.

TABLE 1

	Input multiplicity PFU / cell	Percent inclusions (b)	Final cell concentration $x10^5$ / ml. (a)	Percent inclusions corrected	Percent infectious centres	Percent surviving clones
Adenovirus Type 2	0	0	6.90	0	0	100
	.09	3.8	5.64	10.2	5.5	87.5
	.85	26.8	2.99	38.0	28.2	19.9
	4.25	21.5	2.41	21.5	45.3	1.8
	85.0	16.0	1.87	16.0	73.0	<0.1
Adenovirus Type 12	0	0	5.15	0	0	100
	.12	8.4	2.18	10.7	1.3	7.5
	1	6.6	1.80	7.0	4.1	<0.1
	4	4.6	1.79	4.8	4.9	<0.1
	16	7.6	1.72	7.6	5.3	<0.1

(a) Initial cell concentration 2.1 x 10^5 cells/ml. for Ad 2 and 1.7 x 10^5 cells/ml. for Ad 12

(b) 48 hours for Ad 2 and 72 hours for Ad 12.

FIGURE 5

Effects of Input Multiplicity of Adenovirus Types 2 & 12 on the Formation of Inclusion Bodies, Production of Infectious Centres & Inhibition of Host Cell Cloning in KB Cells

Clone prevention

Inclusion body formation

Infectious centres

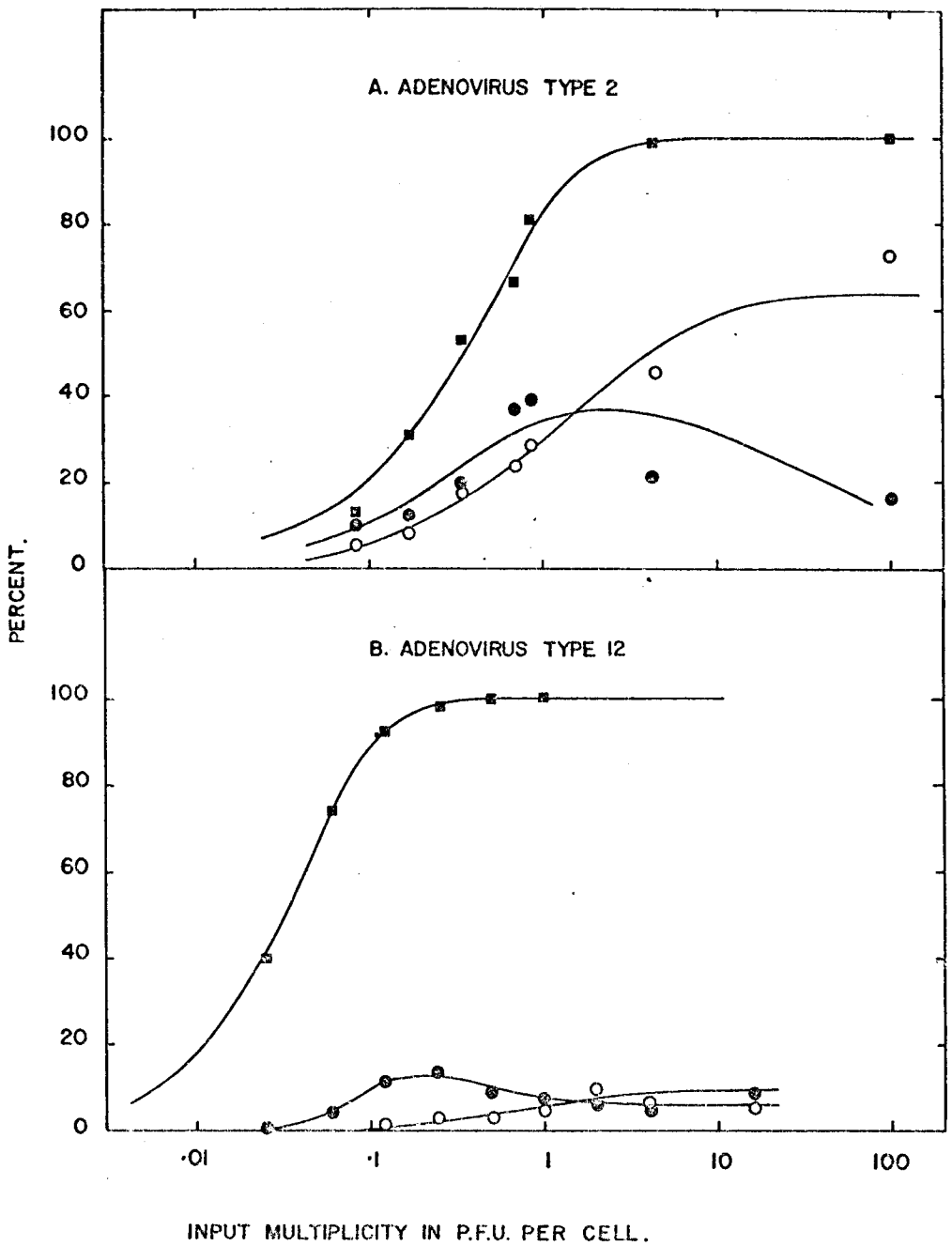

A. ADENOVIRUS TYPE 2
B. ADENOVIRUS TYPE 12
PERCENT.
100
80
60
40
20
0
·01
·1
1
10
100
INPUT MULTIPLICITY IN P.F.U. PER CELL.

FIGURE 6

Replot of Data from Figure 5 to Obtain a Quantitative Comparison of the Different Viral Functions

Clone prevention

Inclusion body formation

Infectious centres

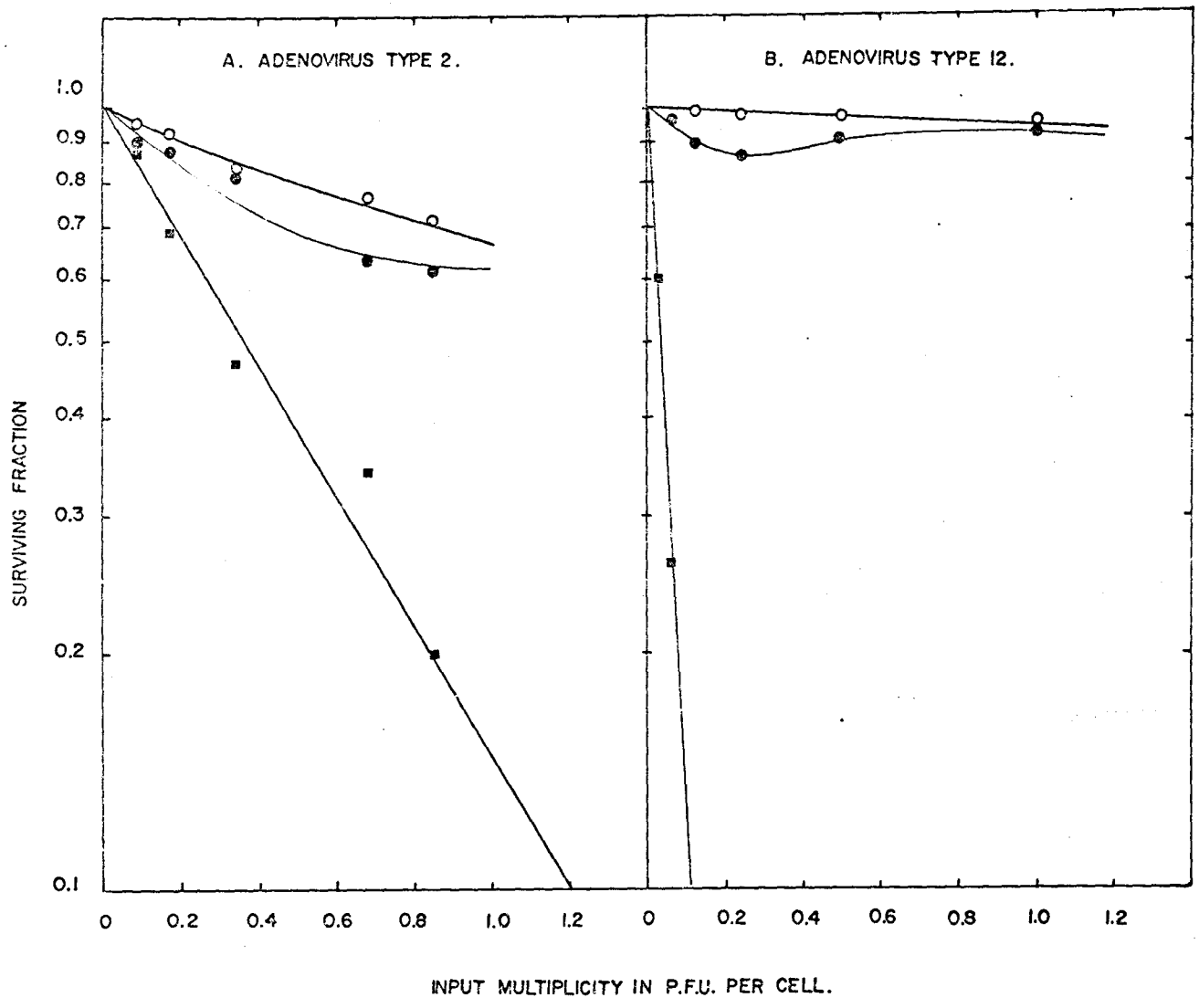

A. ADENOVIRUS TYPE 2.
B. ADENOVIRUS TYPE 12.
SURVIVING FRACTION
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.2
0.4
0.6
0.8
1.0
1.2
INPUT MULTIPLICITY IN P.F.U. PER CELL.

TABLE 2

Relative Ratios of Virus Particles Capable of Different Functions in Adenovirus Types 2 and 12

Ratio (a)	Ad 2	Ad 12
Particle/infectivity (Green 1967)	33	320
CIU / input PFU	1.1-1.9	21-85
IBU / input PFU (low MOI)	0.65-0.85	0.8-3.7
CIU / IBU (low MOI)	1.7-2.4	17.5-26
ICU / input PFU (b)	< 1	< 1

(a) Range for three experiments

(b) Infectious centre forming units (ICU)

it reached a maximum of 35 - 73%, similar to values reported previously (Green 1961 and Ledinko 1965) ; however, for Ad 12, it was only 5 to 10%. To obtain a more quantitative comparison of the different viral functions, part of the data are replotted in Figure 6, with the fraction of cells surviving that particular viral function plotted versus input multiplicity. Viral functions consistent with one-hit kinetics yielded exponential survival curves with a slope equal to the ratio of the number of particles capable of that viral function to the input multiplicity in PFU. These ratios are shown in Table 2. The ratio of clone inhibiting units (CIU) to input PFU had a range of 1.1 to 1.9 for Ad 2 and 20 to 85 for Ad 12. The ratio of infectious centre units (ICU) to input PFU was less than 1 for both viruses. For low input multiplicities, the ratio of inclusion body units (IBU) to input PFU had a range of 0.6 to 0.85 for Ad 2 and 0.8 to 3.7 for Ad 12. The spread of these ratios reflects, in part, the variation in adsorption efficiency and uncertainty in the determination of input PFU in different experiments. When these variations are eliminated by taking the ratio of CIU to IBU, the reproducibility is substantially better as shown in Table 2. These ratios are not the result of storage, since fresh virus preparations gave similar results.

Thus purified preparations of Ad 2 and Ad 12 contain some defective virus particles capable of preventing a cell from cloning but unable to induce inclusion bodies or form plaques. The proportion of such defective particles in Ad 12 was about 10 times that in Ad 2.

B. EFFECT OF GAMMA IRRADIATION ON VIRAL FUNCTIONS

Some of the viral functions expressed by non-irradiated Ad 2 in human KB cells were examined in the previous section. In this section, the effect of gamma irradiation on the survival of several viral functions is examined. Ad 2 was given graded doses of 0.58, 1.18, 1.75, 2.35, 2.94 and 3.52 Mrads of gamma rays and assayed for the viral functions of adsorption, haemagglutination, clone inhibition, inclusion body formation and plaque formation.

1. The effect of Gamma Irradiation on Adsorption of Virus to KB Cells :

Ad 2, containing ^{3}H labelled DNA, was assayed for virus adsorption. An aliquot of either 0.1 or 0.2 ml. from each irradiated sample was used to infect a 1 ml. suspension of 10^{6} KB cells. After incubating at 37°C for 90 min., using a roller wheel, the infected culture was diluted with warm nutrient medium and incubated for a further period of time. The amount of radioactivity in the cell suspension and in the cell free supernatant was assayed at either 2, 5 or 18 hours after infection. The amount of virus adsorbed to the cells was determined from the difference of these 2 measurements.

Typical values for the radioactivity of the cell suspension and the cell free supernatant are given in Tables 3A, B and C. It can be seen that under these conditions, the adsorption efficiency of unirradiated virus ranged from 50 to 90%. Highest adsorption efficiencies were obtained when cultures were incubated for at least 5 hours after

TABLE 3

Effect of Gamma Irradiation on Viral Adsorption

Samples of Ad 2, containing 3×10^{10} PFU/ml. and having an ^{3}H radioactivity of 1.3×10^{6} cpm/ml., were given graded doses of gamma rays and subsequently assayed for adsorption.

3A	Expt. I :	7×10^{6} cells infected with 0.1 ml virus and assayed 2 hours after infection.
3B	Expt. II :	2×10^{6} cells infected with 0.2 ml. virus and assayed 5 hours after infection.
3C	Expt. III:	10^{6} cells infected with 0.2 ml. virus and assayed 18 hours after infection.

TABLE 3A

Effect of Gamma Irradiation on Viral Adsorption

Radiation dose (10^6 rads)	Radioactivity (cpm / 0.1 ml.)			% Virus adsorbed (b)
	Cell suspension	Cell-free supernatant	Cell associated (a)	
0	3776	1757	2019	53.5
0.58	4077	2206	1871	46.0
1.18	3715	1760	1955	52.5
2.35	4354	3250	1100	25.0
2.94	3875	3173	702	18.7
3.52	3867	3519	348	9.0

(a) Column 2 - Column 3

(b) $\frac{\text{Column 4}}{\text{Column 2}} \times 100$

TABLE 3C

Effect of Gamma Irradiation on Viral Adsorption

Radiation dose (10^6 rads)	Radioactivity (cpm / 0.1 ml.)			% Virus adsorbed (b)
	Cell suspension	Cell-free supernatant	Cell associated (a)	
0	4887	537	4350	89.0
0.58	4321	876	3445	79.3
1.18	4228	1656	2572	60.8
1.75	2700	1826	874	32.5
2.35	4568	3973	595	13.0
2.94	6016	3957	2059	35.0
3.52	4515	4235	280	6.0

(a) Column 2 - Column 3

(b) $\frac{\text{Column 4}}{\text{Column 2}} \times 100$

TABLE 3B

Effect of Gamma Irradiation on Viral Adsorption

Radiation dose (10^6 rads)	Radioactivity (cpm / 0.1 ml.)			% Virus adsorbed (b)
	Cell suspension	Cell-free supernatant	Cell associated (a)	
0	5055	732	4323	85.5
0.58	5842	2542	3300	36.5
1.18	5656	4061	1595	28.2
2.35	6263	5285	978	15.7
2.94	6097	5008	1089	16.6
3.52	5869	4983	886	15.0

(a) Column 2 - Column 3

(b) $\frac{\text{Column 4}}{\text{Column 2}} \times 100$

infection.

The surviving fraction was calculated relative to the adsorption efficiency of unirradiated virus and is shown in Table 4. Adsorption measurements made at 2, 5, or 18 hours after infection all gave similar results. Mean values for the pooled results of several experiments are plotted in Figure 7. It can be seen that this viral function is consistent with an exponential inactivation at the doses of gamma irradiation employed giving a D_{37} value of 1.7×10^6 rads.

2. Effect of Gamma Irradiation on Haemagglutination (HA) : The adsorption of Ad 2 to KB cells was found to be inactivated by gamma irradiation. This is thought to indicate a destruction of viral attachments components by the radiation. Since these viral functions may also be involved in haemagglutination, it was thought of interest to determine whether the haemagglutinating ability of Ad 2 is inactivated by similar doses of gamma irradiation.

The ability of irradiated Ad 2 to haemagglutinate red blood cells was assayed. The photograph in Figure 8 shows the results of a typical experiment. It can be seen that there is a marked change in the sedimentation pattern after gamma irradiation of the virus, especially after 3.52×10^6 rads, indicating that this viral function is inactivated by gamma irradiation. The haemagglutination titre was taken as the reciprocal of the highest dilution causing partial haemagglutination. In Figure 8, the HA titres obtained were 64, 64, 16 and 8 for Ad 2 given doses of 0, 1.18, 2.35 and 3.52×10^6 rads respectively.

TABLE 4

Survival of Viral Adsorption after Gamma Irradiation

Radiation dose (10^6 rads)	Surviving Fraction (a)		
	Expt. I	Expt. II	Expt. III
0	1	1	1
.58	0.87	0.43	0.89
1.18	0.98	0.33	0.68
1.75			0.37
2.35	0.47	0.18	0.15
2.94	0.35	0.19	0.39
3.52	0.17	0.18	0.067

(a) The surviving fraction for the viral function of adsorption was calculated for the 3 experiments in Table 3A, B and C.

FIGURE 7

Survival of Viral Functions Following Gamma Irradiation

Each curve represents the pooled data from several experiments

Adsorption

Haemagglutination

Clone inhibition

Inclusion body formation

Plaques

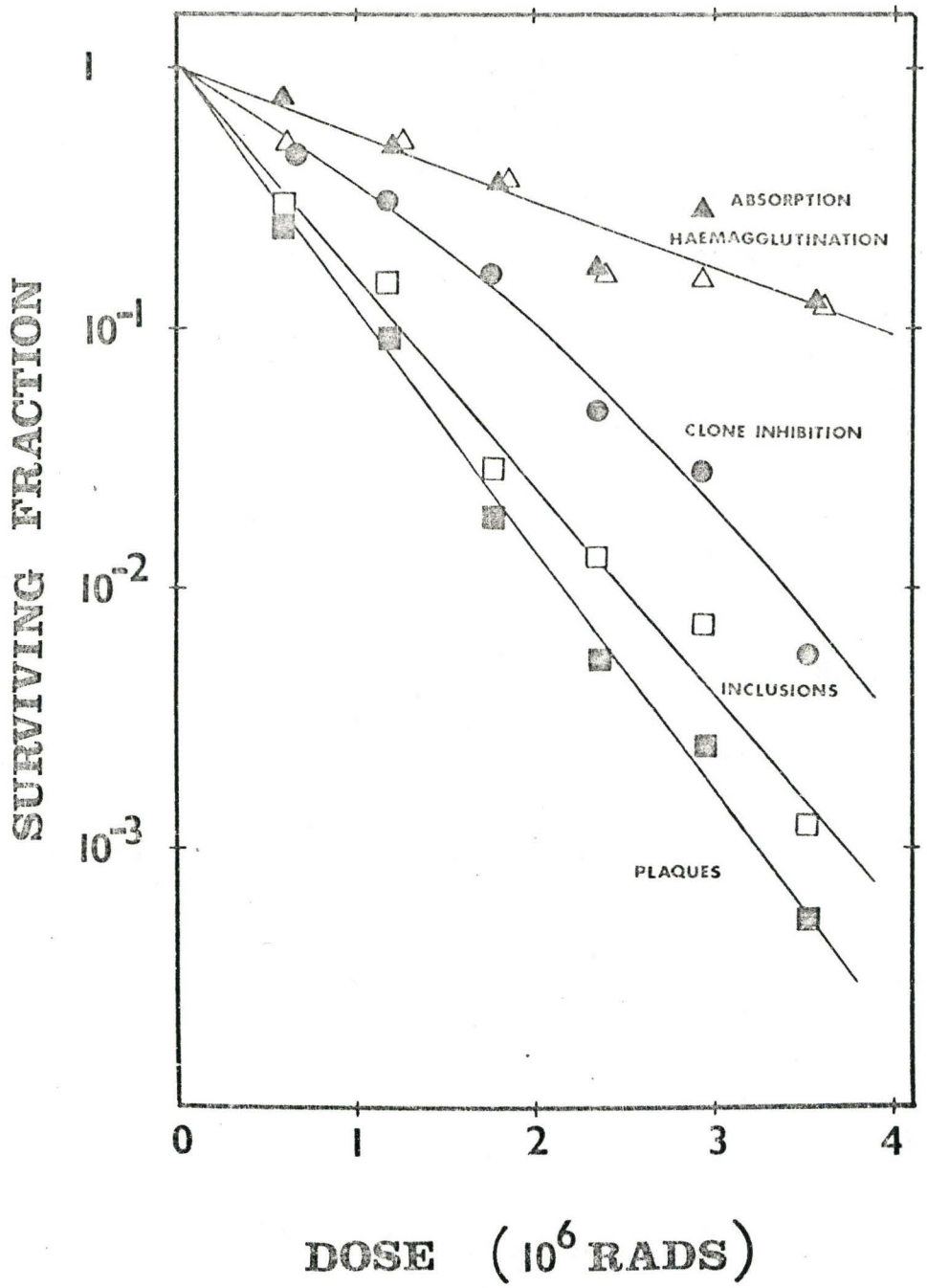
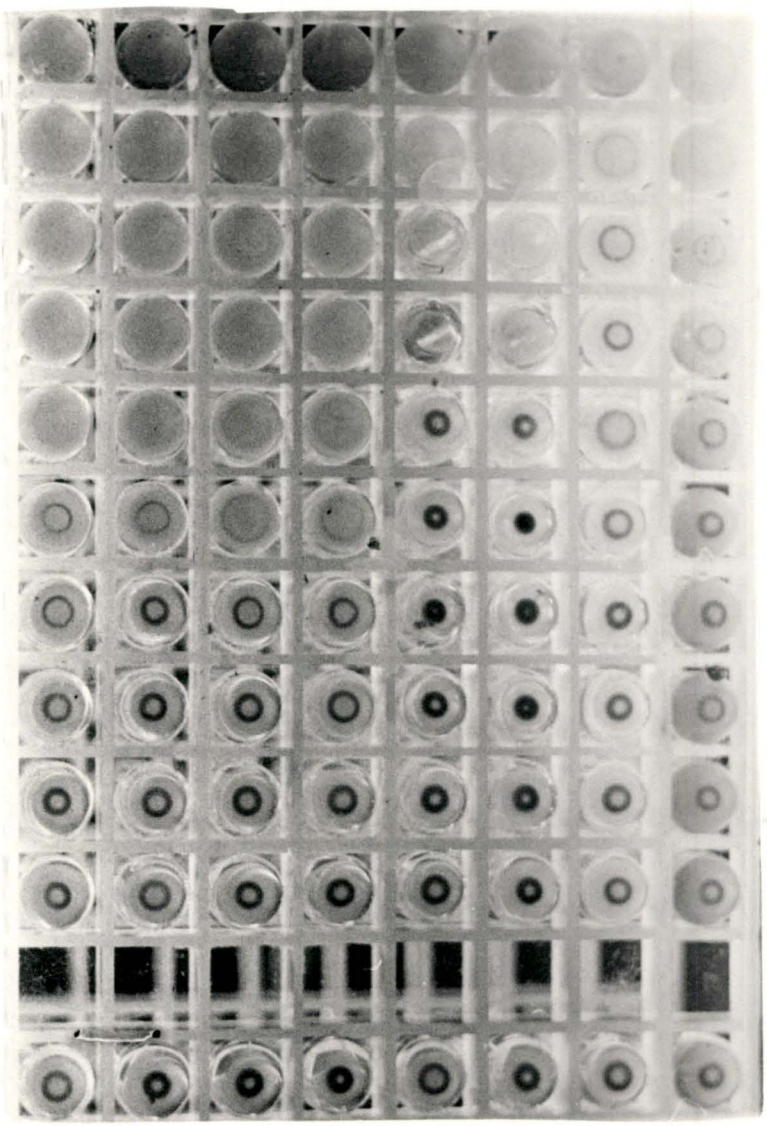

SURVIVING FRACTION
1
10^{-1}
10^{-2}
10^{-3}
ABSORPTION
HAEMAGGLUTINATION
CLONE INHIBITION
INCLUSIONS
PLAQUES
0
1
2
3
4
DOSE (10^6 RADS)

FIGURE 8

Haemagglutination Assay

Photograph showing a typical sedimentation pattern obtained in the haemagglutination assay. The results in this figure are from experiment IV shown in Table 5. Consecutive two fold viral dilution increases from top to bottom. The first tube of each column shows complete haemagglutination, whereas, the last tube of each column, containing no virus, shows a negative pattern of sedimentation. The haemagglutination titre was taken as the reciprocal of the highest dilution causing partial haemagglutination. From left to right, the HA titres obtained were 64, 64, 64, 64, 16, 16, 8, 8, for Ad 2 given doses of 0, 0, 1.18, 1.18, 2.35, 2.35, 3.52 and 3.52 Mrads respectively.



The results of 4 experiments are shown in Table 5 and the mean values for the survival of this viral function plotted in Figure 7. Results are consistent with an exponential inactivation for haemagglutination with a D_{37} value of 1.7×10^6 rads. The fact that the viral functions of adsorption and haemagglutination both have the same radiosensitivity suggests that the viral components required for adsorption may be the same as those components involved in haemagglutination.

3. Effect of Gamma Irradiation on Inhibition of Host Cell Cloning : 10^6 KB cells were infected with different amounts of virus and the percentage of cells capable of forming clones was determined. The fraction of cells surviving was then plotted against the amount of virus added, in a similar manner to that shown in Figure 6. The amount of virus which would give 37% surviving clones was then calculated for each virus sample. From this value the viral titre in CIU/ml. was calculated as described in Appendix 1. The results of a typical assay are shown in Table 6A.

The ability of irradiated Ad 2, to inhibit host cell cloning is shown in Table 6B for several experiments. Data from these and several other experiments were pooled and the mean values for the survival of this viral function were plotted in Figure 7. Although the results are consistent with an exponential inactivation curve which has a D_{37} value of 8.7×10^5 rads, the points fit better to a curve having a slight shoulder.

TABLE 5

Effect of Gamma Irradiation on Haemagglutination

Radiation dose (10^6 rads)	Expt. I		Expt. II		Expt. III		Expt. IV		Mean surviving fraction
	HA titre	Surviving fraction	HA titre	Surviving fraction	HA titre	Surviving fraction	HA titre	Surviving fraction	
0	64	1	64	1	32	1	64	1	1
0.58	32	0.5	32	0.5					0.5
1.18					4	0.13	64	1	0.56
1.75	16	0.25	32	0.5					0.38
2.35					4	0.13	16	0.25	0.19
2.94	4	0.063	16	0.25					0.15
3.52					4	0.13	8	0.13	0.13

TABLE 6A

Assay for Clone Inhibiting Units in Ad 2

Treatment	Assumed titre CIU / cell	Amount of virus CIU / cell (a)	Percent surviving clones	Calculated MOI giving 37% survival (a)	True titre CIU / ml. (b)	Suriving fraction
Ne gamma rays	$2x10^{10}$	0.25	36.3			
		0.50	18.2	0.31	$6.5x10^{10}$	1
		1.00	7.5			
2.35 Mrads	10^9	0.25	59.5			
		0.50	29.5	0.43	$2.3x10^9$	$3.6x10^{-2}$
		1.00	9.7			

(a) MOI, multiplicity of infection, see Appendix I

(b) $\frac{\text{Column 2}}{\text{Column 5}}$

TABLE 6B

Effect of Gamma Irradiation on Inhibition of Host Cell Cloning

Radiation dose (10^6 rads)	Expt. I		Expt. II		Expt. III	
	CIU per ml.	Surviving fraction	CIU per ml.	Surviving fraction	CIU per ml.	Surviving fraction
0	$3x10^{10}$	1	$6.5x10^{10}$	1	$5.7x10^{9}$	1
0.58	$1.9x10^{10}$	$6.6x10^{-1}$			$2.9x10^{-1}$	$5.0x10^{-1}$
1.18			$2.0x10^{10}$	$3.1x10^{-1}$	$3.6x10^{9}$	$6.3x10^{-1}$
1.75	$4.6x10^{9}$	$1.5x10^{-1}$			$1.0x10^{9}$	$1.76x10^{-1}$
2.35			$2.3x10^{9}$	$3.6x10^{-2}$	$3.3x10^{8}$	$5.8x10^{-2}$
2.94	$8.3x10^{8}$	$2.8x10^{-2}$			$1.5x10^{8}$	$2.6x10^{-2}$
3.52			$3.3x10^{8}$	$5.2x10^{-3}$		

4. Effect of Gamma Irradiation on Inclusion Body Formation :
The assay used was similar to that used for the viral function of clone inhibition (see section B3). Since the formation of dense inclusions was not consistent with one-hit kinetics at high input multiplicities, it was necessary to extrapolate a value for the amount of virus giving 37% cells without IB from the values obtained at low input multiplicities. This introduces uncertainties in the assay for inclusion bodies especially in the irradiated samples. The results obtained for 2 experiments are shown in Table 7 and the mean values for the survival of this viral function are plotted in Figure 7. It cab be seen that inclusion body formation is consistent with an exponential inactivation with a D_{37} value of 5.4×10^5 rads.

5. Effect of Gamma Irradiation on Plaque Formation : The effect of gamma irradiation on plaque formation was studied. Typical results for 3 experiments are shown in Table 8. Data from these and several other experiments were pooled and the mean values for the survival of plaque formation were plotted in Figure 7. This viral function was consistent with an exponential inactivation with a D_{37} value of 4.2×10^5 rads.

6. Radiosensitivity of Different Viral Functions Following Gamma Irradiation : For comparison purposes, the D_{37} values have been summarised in Table 9. It can be seen that the most radiosensitive viral function is that of plaque formation with a D_{37} of 4.2×10^5 rads,

TABLE 7

Effect of Gamma Irradiation on Inclusion Body Formation

Radiation dose (10^6 rads)	Expt. I		Expt. II	
	IBU per ml.	Surviving fraction	IBU per ml.	Surviving fraction
0	3.3×10^9	1	2.5×10^{10}	1
0.58	9.5×10^8	2.9×10^{-1}		
1.18	5.5×10^8	1.6×10^{-1}	3.3×10^9	1.3×10^{-1}
1.75	9.5×10^8	2.9×10^{-2}		
2.35	4.0×10^7	1.2×10^{-2}	3.3×10^8	1.3×10^{-2}
2.94	2.4×10^7	7.1×10^{-3}		
3.52			2.5×10^7	1.2×10^{-3}

TABLE 8

Effect of Gamma Irradiation on Plaque Formation

Radiation dose (10^6 rads)	Expt. I		Expt. II		Expt. III	
	PFU per ml.	Surviving fraction	PFU per ml.	Surviving fraction	PFU per ml.	Surviving fraction
0	3.8×10^{9}	1	3.1×10^{9}	1	2.8×10^{9}	1
0.58	5.8×10^{8}	1.5×10^{-1}			9.2×10^{8}	3.3×10^{-1}
1.18			2.9×10^{8}	9.3×10^{-2}	6.0×10^{8}	2.1×10^{-1}
1.75	8.0×10^{7}	2.1×10^{-2}			4.5×10^{7}	1.6×10^{-2}
2.35			1.1×10^{7}	3.6×10^{-3}	2.3×10^{7}	8.2×10^{-3}
2.94	9.0×10^{6}	2.4×10^{-3}			1.0×10^{6}	2.6×10^{-3}
3.52			1.6×10^{6}	5.1×10^{-4}		

TABLE 9

D_{37} Values Obtained for Various Viral Functions and DNA Damage after Gamma Irradiation

Viral function / DNA damage	D_{37} (rads)
Adsorption to host cell	1.7×10^6
$H_a$emagglutination of R.B.C.'s	1.7×10^6
Inhibition of host-cell cloning	8.7×10^5
Inclusion body formation	5.4×10^5
Plaque formation	4.2×10^5
Double stranded molecules with no double stranded breaks	4.6×10^6
Single stranded molecules with no single stranded breaks	1.7×10^5
Double stranded molecules with no breaks	8.7×10^4

followed by inclusion body formation with a D_{37} of 5.4×10^5 rads. These 2 functions are only just separable at the doses of gamma irradiation employed. The function of clone inhibition was more radioresistant than that of inclusion body formation with a D_{37} of 8.7×10^5 rads. The most radioresistant functions were those of adsorption and haemagglutination which both had the same D_{37} value of 1.7×10^6 rads.

Assuming target theory (Lea 1962), the amount of the Ad 2 genome required for clone inhibition and inclusion body formation was 43% and 78% respectively.

C. MOLECULAR DAMAGE TO THE VIRAL DNA AFTER GAMMA IRRADIATION

It is generally accepted that the primary target for radiation induced lethal damage in microorganisms is the DNA molecule (Haynes 1966). The type of damage occuring depends greatly on the characteristics of the radiation employed. A large amount of DNA damage following gamma irradiation is expressed in the form of single and double strand breaks in the DNA molecule.

The effect of gamma irradiation on several functions of Ad 2 was described in the previous section. In order to correlate the radiosensitivity of these viral functions with the molecular lesions sustained by the virus, Ad 2, containing ^3H labelled DNA, was given graded doses of gamma irradiation and assayed for both single and double strand DNA breaks. The number of radiation induced

breaks was determined from the size distribution of the viral DNA fragments obtained on sucrose gradients.

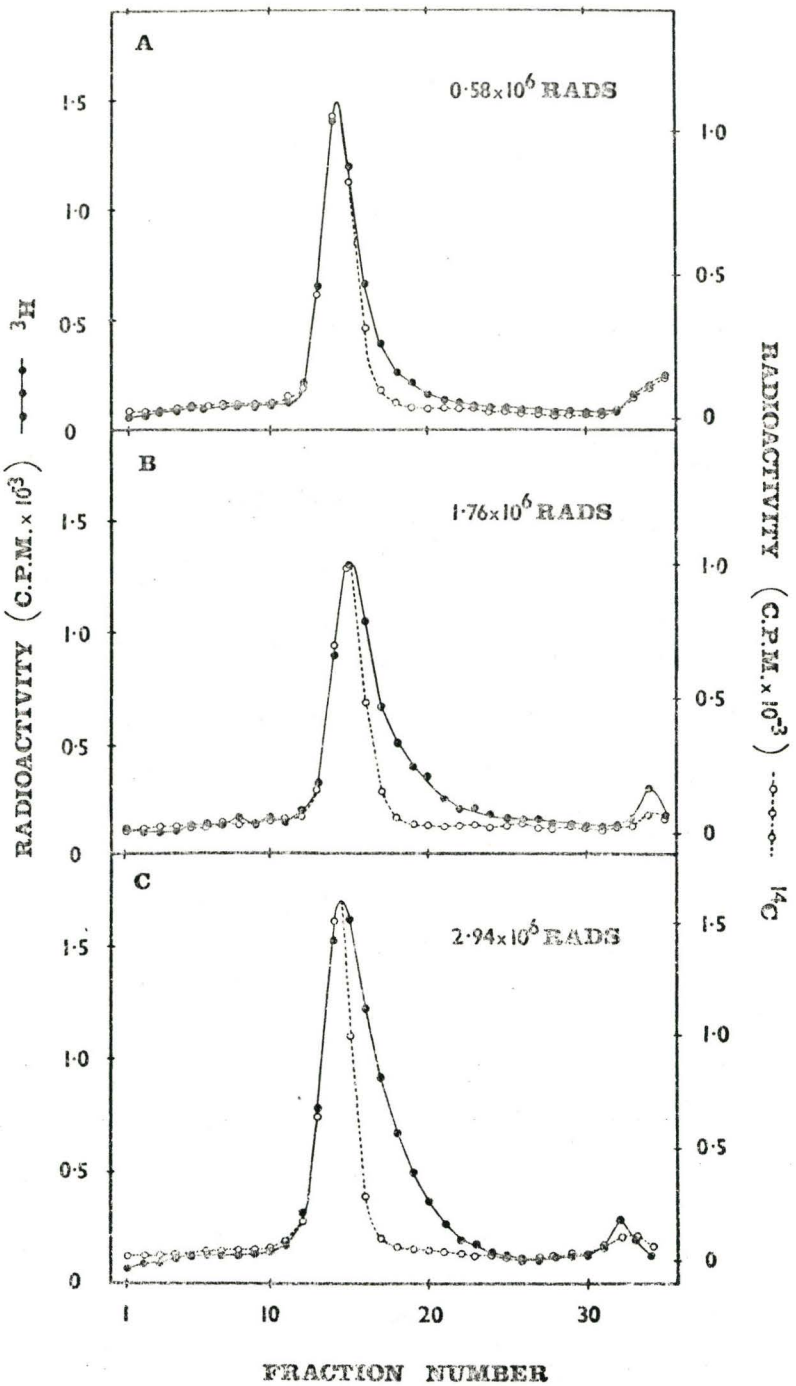
1. Assay for Double Strand Breaks : Irradiated Ad 2, containing ^{3}H labelled DNA, was disrupted by treatment with pronase and SDS and centrifuged on a 5to 20% neutral sucrose gradient. Typical radioactivity profiles obtained are shown in Figure 9. It can be seen that the distribution of the ^{14}C radioactivity from unirradiated marker virus was the same for all profiles, whereas the ^{3}H radioactivity from irradiated virus showed a more heterogenious profile. The ^{3}H radioactivity sedimenting to the right of the ^{14}C marker peak is a measure of the decrease in S value of the ^{3}H labelled DNA from irradiated virus. Assuming a similar conformation for all DNA molecules, these decrease in S value represents a reduction in molecular size and corresponds to an increase in double strand chain breakage after gamma irradiation. In calculating the number of radiation induced double strand breaks, it was assumed that the ^{3}H radioactivity sedimenting with that of the ^{14}C marker represents those double stranded molecules with no double strand breaks. From Figure 9, it can be seen that the proportion of molecules with no breaks decreases with irradiation dose, corresponding to an increase in double strand chain breakage after gamma irradiation.

The nature of the small radioactivity peak found at the top of the gradients, representing a few percent of the total radioactivity, is unknown. It may be due to artifacts resulting from the method of

FIGURE 9

Double Strand DNA Breakage after Gamma Irradiation

Samples of Ad 2, containing H^3 labelled DNA, were given graded doses of 0.58, 1.76 and 2.94 Mrads of gamma rays. Aliquots from each sample were disrupted by treatment with pronase and SDS. Unirradiated Ad 2, containing C^{14} labelled DNA was added to each sample as a marker. Samples were carefully layered onto a 5-20% neutral sucrose gradient. After centrifugation, tube fractions were collected from the bottom and the radioactivity of each fraction was counted.


A
0·58×10⁶ RADS
B
1·76×10⁶ RADS
C
2·94×10⁶ RADS
RADIOACTIVITY (C.P.M.×10³) —●— ³H
RADIOACTIVITY (C.P.M.×10⁻³) ---○--- ¹⁴C
1·5
1·0
0·5
0
1
10
20
30
FRACTION NUMBER

collecting fractions.

From the areas under the normalised radioactivity profiles, the fraction of DNA molecules with no double strand breaks was calculated as follows :

$$\text{Surviving fraction of molecules unbroken} = \frac{\text{Area under } ^{14}C \text{ profile from marker virus}}{\text{Area under } ^{3}H \text{ profile from irradiated virus}}$$

From the fraction of double stranded molecules unbroken, the average number of double strand breaks per molecule was calculated from the Poisson distributions as follows :

$$\text{Average number of double strand breaks per molecule} = \text{Log}_e \text{ (Surviving fraction)}$$

Pooled results for the average number of double strand breaks per molecule are shown in Table 10 and plotted as a function of dose in Figure 10B. Results are consistent with a linear relationship yeilding a DNA radiosensitivity of 0.01 double strand breaks/rad/10^{12} daltons. The surviving fraction of unbroken molecules is plotted against dose in Figure 11 and yields a D_{37} value of 4.6×10^6 rads.

The average number of double strand breaks per molecule may be an underestimate, since some of the broken molecules may sediment within the profile of unbroken molecules. However, this underestimate is not considered large enough to invalidate the interpretation of the

TABLE 10

Double Strand DNA Breakage after Gamma Irradiation

Radiation dose (10^6 rads)	Fraction of double stranded molecules unbroken (S.F.)	Average number of double stranded breaks / molecule ($= -\log_e$ S.F.)
0	1.0	0
0.58	0.86	0.15
1.18	0.70	0.35
1.75	0.63	0.46
2.35	0.64	0.44
2.94	0.54	0.62
3.52	0.56	0.58

FIGURE 10

Average Number of Breaks Induced in the Viral DNA after Different Doses of Gamma Irradiation

A. Single strand breaks

B. Double strand breaks

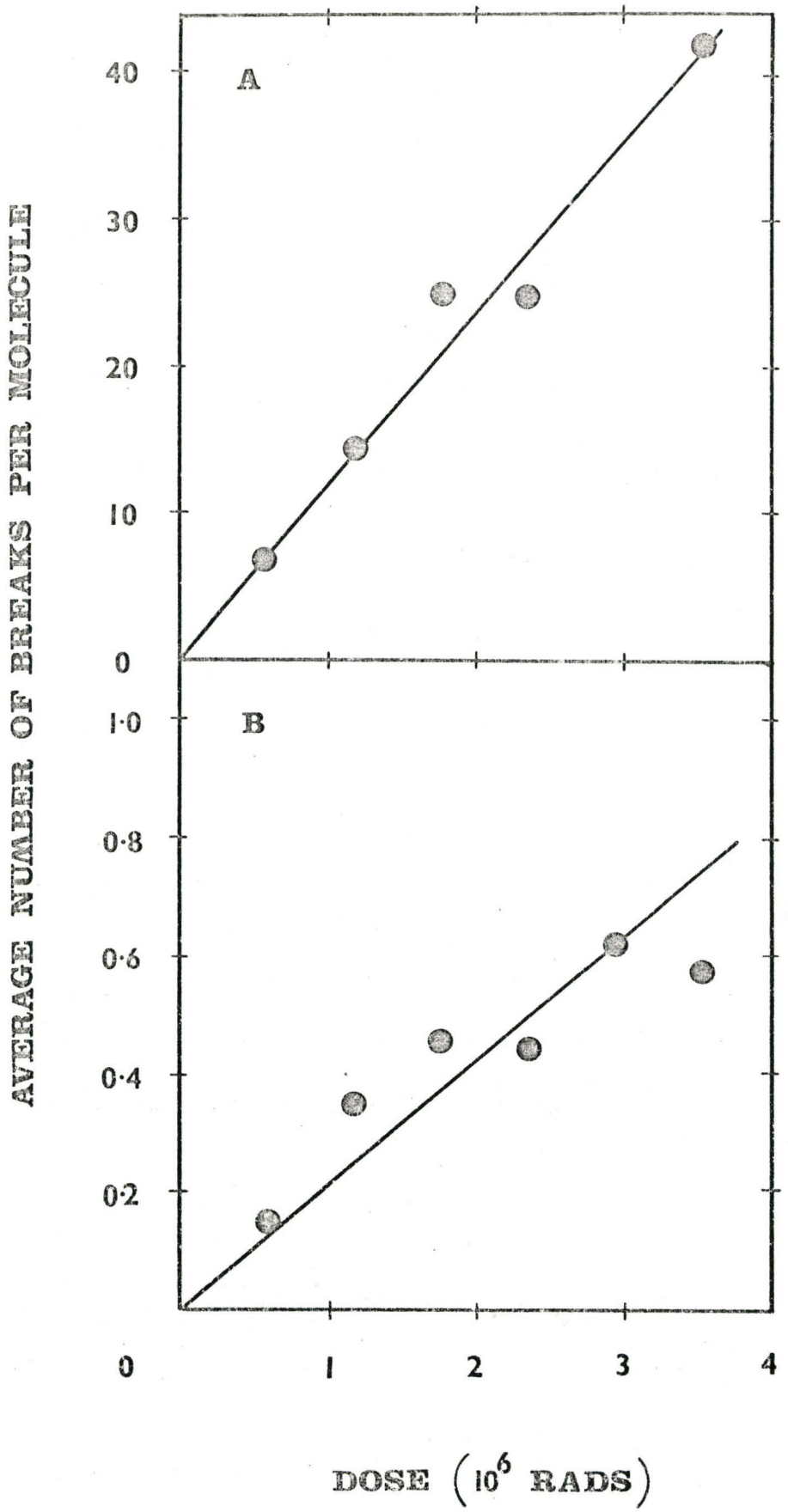

AVERAGE NUMBER OF BREAKS PER MOLECULE
A
B
40
30
20
10
0
1·0
0·8
0·6
0·4
0·2
0
1
2
3
4
DOSE (10^6 RADS)

FIGURE 11

Survival of DNA from Molecular Damage after Gamma Irradiation

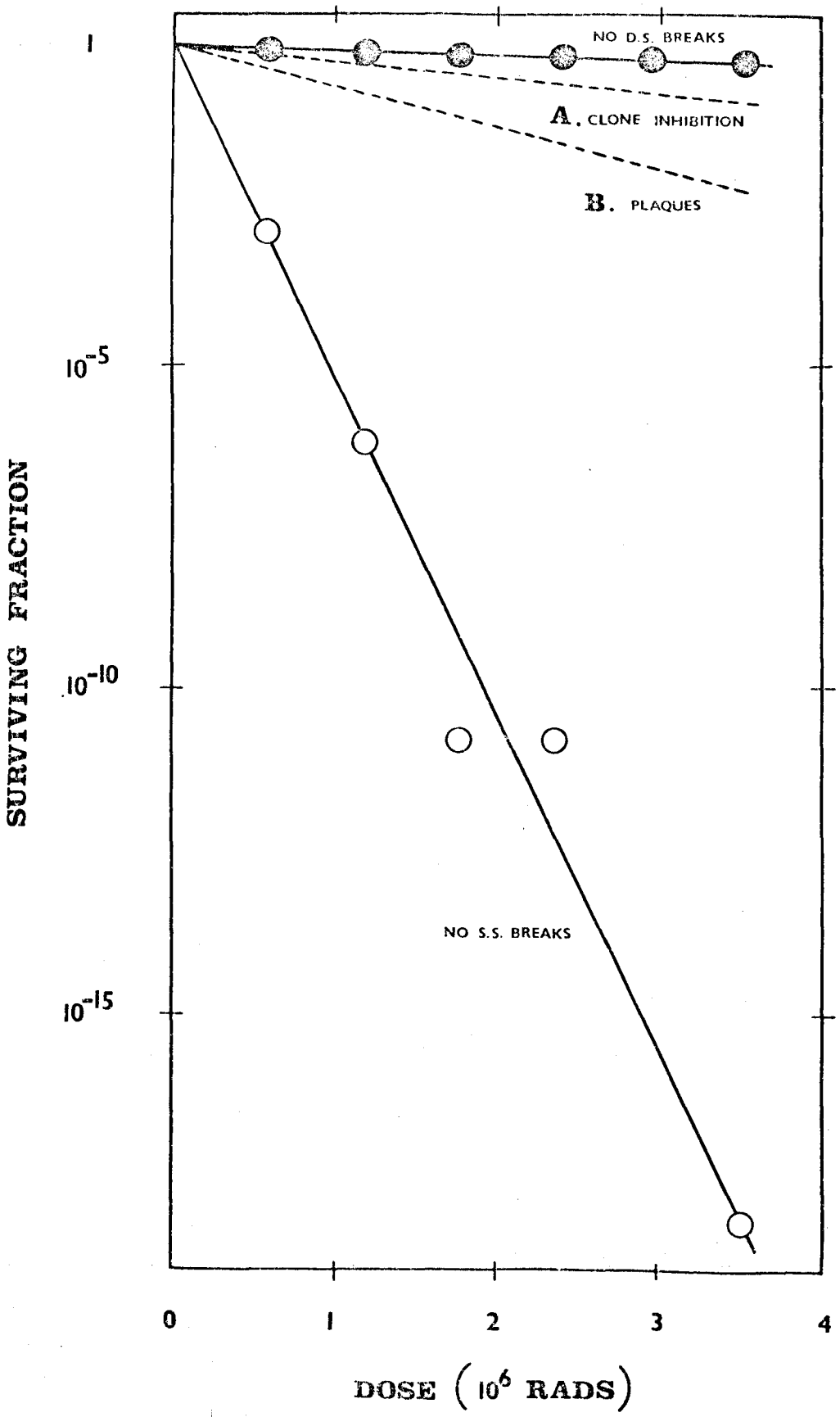
Replot of data from Figure 10 to show the fraction of DNA molecules containing no breaks after gamma irradiation. The survival of several biological viral functions are also shown for comparison purposes.

Fraction of viral DNA molecules with no double strand breaks

Fraction of viral DNA molecules with no single strand breaks

A — Fraction of virions capable of plaque formation, corrected for adsorption

B — Fraction of virions capable of clone inhibition, corrected for adsorption


NO D.S. BREAKS
A. CLONE INHIBITION
B. PLAQUES
NO S.S. BREAKS
SURVIVING FRACTION
1
10^{-5}
10^{-10}
10^{-15}
0
1
2
3
4
DOSE (10^6 RADS)

results for the following reason. The distribution of fragments resulting from an average number of 1 break per molecule has been calculated by Litwin (Litwin 1969). Using this distribution, the fraction of molecules sedimenting with the profile for unbroken molecules was 0.56 corresponding to an average number of 0.58 breaks per molecule. This suggests that the method used is most likely an underestimate by less than 100%.

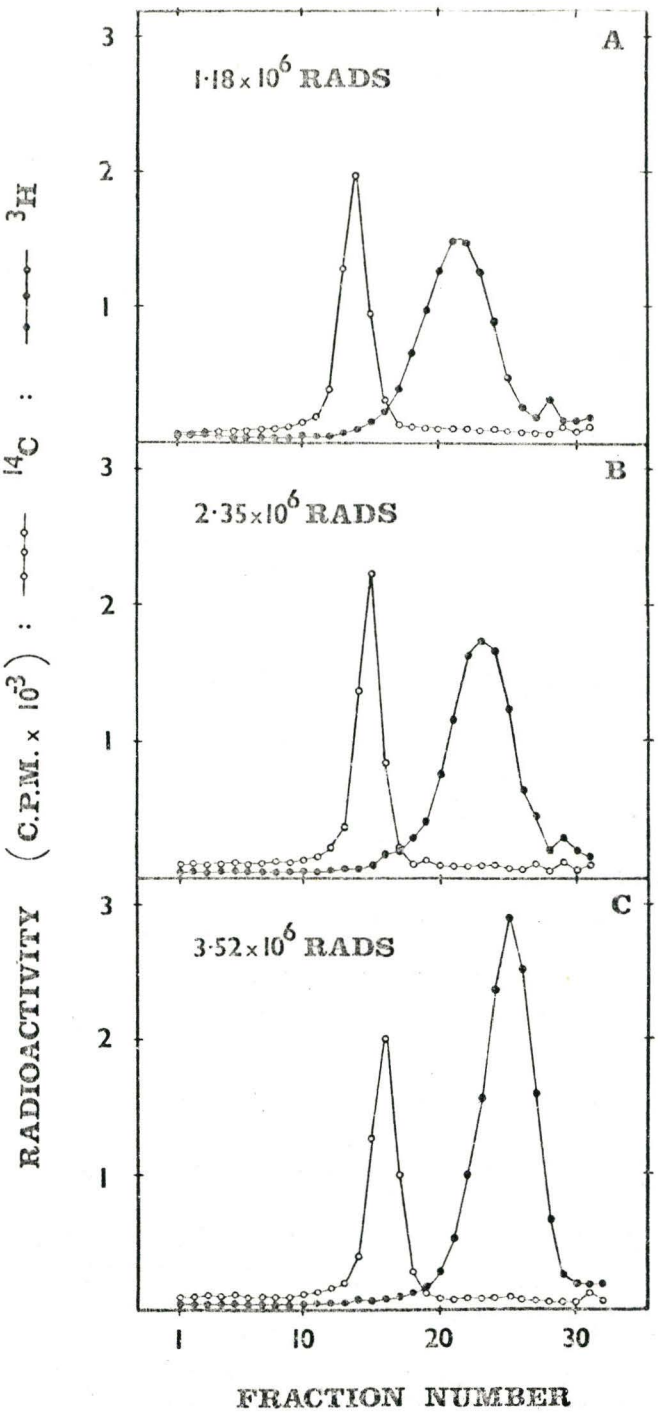
Furthermore, the collection of tube fractions from the bottom of the gradient produced a distortion in the radioactivity profiles, especially just above the radioactivity peak (Van der Schans 1969). Consequently, the assumptions used by Litwin (Litwin 1969), to calculate the distribution of fragments after random breakage may not be strictly true in this experimental situation. Errors due to gradient distortion are reduced when the fraction of broken molecules is calculated as described above using a ^{14}C labelled marker.

2. Assay for Single Strand Breaks : Double stranded DNA is denatured at high alkaline pH and separates into it consituent single stranded molecules. Consequently, the number of radiation induced single strand DNA breaks can be determined from the size distribution of single stranded DNA fragments obtained on alkaline sucrose gradients. Irradiated Ad 2, containing ^{3}H labelled DNA, was treated with a lysing solution at pH 12.8 and centrifuged on a 5 to 20% alkaline sucrose gradient, pH 12.4. Typical radioactivity profiles obtained are shown in Figure 12. It can be seen that the distribution of the ^{14}C

FIGURE 12

Single Strand DNA Braekage after Gamma Irradiation

Samples of Ad 2, containing H^3 labelled DNA, were given graded doses of 1.18, 2.35, and 3.52 Mrads of gamma rays. Aliquots from each sample were treated with a lysing solution at pH 12.8. Unirradiated Ad 2, containing C^{14} labelled DNA, was added to each sample as a marker, and samples were carefully layered on a 5-20% alkaline sucrose gradient. After centrifugation, tube fractions were collected from the bottom and the radioactivity of each fraction was counted.


A
1·18 x 10^6 RADS
B
2·35 x 10^6 RADS
C
3·52 x 10^6 RADS
3
2
1
1
10
20
30
RADIOACTIVITY (C.P.M. x 10^-3) : —o— 14C : —•— 3H
FRACTION NUMBER

radioactivity from unirradiated marker virus was the same for all profiles. However, the ^{3}H radioactivity from irradiated virus showed a more heterogen ous profile and a peak shift to the right. This indicated a decrease in molecular size corresponding to an increase in single strand DNA breaks after gamma irradiation. From Figure 12, it can be seen that after increasing doses of gamma irradiation, the ^{3}H radioactivity peak, representing the distribution of single strand DNA fragments, sedimented more slowly and sharpened. This was interpreted as being due to a decrease in size and an increase in homogeneity of the distribution of single strand DNA fragments. Both of these results are predicted by the calculation of Litwin et al (Litwin 1969).

From the radioactivity profiles, a value of D_2/D_1 was obtained (see below) and the average number of breaks per molecule was determined from the fcllowing relationship (Litwin 1969) :

$$\text{Average number of breaks per molecule} = \left[- B - (B^2 - 4AC)^{1/2} \right] \Big/ 2A$$

where

$$A = \alpha t^{\alpha} (1 - t^{\alpha})$$

$$B = t^{\alpha} (5\alpha - 1) + (1 - 2\alpha)$$

$$C = 2 (1 - 2\alpha)$$

$$\alpha = 1/0.35$$

and t is the normalised distance D_2/D_1, sedimented at peak recovery of the sucrose gradient.

D_1 is the distance sedimented by DNA from unirradiated virus

D_2 is the distance sedimented by DNA from irradiated virus

The fraction of molecules remaining unbroken was calculated using the Poisson distribution as follows :

$$\text{Fraction of molecules unbroken} = e^{-(\text{Average number of breaks/molecule})}$$

The results of these calculations are shown in Table 11 and Figure 10A. It can be seen that the average number of single strand breaks per molecule increases linearly with dose and yields a DNA radiosensitivity of 0.5 single strand breaks/rad/10^{12} daltons.

In Figure 11, the surviving fraction of unbroken molecules is plotted against dose and gives a D_{37} value of 8.7×10^{4} rads. In the same figure, the survival of several biological viral functions have been plotted for comparison purposes. It can be seen that viral particles containing several single strand breaks in their DNA were still capable of plaque formation.

3. The Fate of DNA from Gamma Irradiated Virus after Infection : In the previous section it was seen that gamma irradiated virus particles containing several single strand DNA breaks were still capable of plaque formation. It was therefore thought of interest to examine the fate of the viral DNA after infection in order to determine whether these breaks are repaired by the host cell after infection.

Irradiated Ad 2, containing ^{3}H labelled DNA, was used to infect a suspension of KB cells at several hundred PFU/cell. High input multiplicities were required in order to obtain sufficient radioactivity

TABLE 11

Single Stranded DNA Breakage after Gamma Irradiation

Radiation dose (10^6 rads)	D_2 / D_1 (a)	Average number of s.s. breaks per s.s. molecule	Average number of s.s. breaks per d.s. molecule	Fraction of s.s. molecules with no breaks	Fraction of d.s. molecules with no breaks
0	1	0	0	1	1
0.58	0.72	3.4	6.8	3.3×10^{-2}	1.1×10^{-3}
1.18	0.58	7.1	14.2	8.2×10^{-4}	6.7×10^{-7}
1.75	0.48	12.4	24.8	4.1×10^{-6}	1.7×10^{-11}
2.35	0.48	12.4	24.8	4.1×10^{-6}	1.7×10^{-11}
3.52	0.4	21.0	42.0	7.6×10^{-10}	5.8×10^{-19}

(a) D_1 is distance sedimented by unbroken single stranded DNA molecules

D_2 is distance sedimented by fragments of broken DNA molecules at peak recovery

in the 5 X 10^5 cells used for a sucrose gradient. Larger amounts of cells were found to overload the gradient. After infection at 37°C for 90 min., using a roller wheel, cells were diluted with warm nutrient medium and the infected culture incubated for a further period of time. At various times after infection, a sample of infected cells was pelleted by low speed centrifugation. Unirradiated Ad 2, containing ^{14}C labelled DNA was then added as a marker and the sample treated with alkaline lysing solution at pH 12.8, before being carefully layered onto a 5 to 20% alkaline sucrose gradient.

Typical radioactivity profiles obtained from cells infected with unirradiated virus are shown in Figure 13 and for gamma irradiated virus in Figure 14. The profiles were analysed as described in the previous section for single strand breaks. It can be seen that, even at 61 hours after infection, very few single strand breaks (less than 0.2 breaks/molecule) were found in the DNA from unirradiated virus. However, for gamma irradiated Ad 2, there was a substantial increase in the number of single strand DNA breaks after infection. The average number of single strand breaks in the viral DNA at various times after infection are shown in Table 12. It can be seen that there is a 6 fold increase in the number of single strand breaks found in the DNA from gamma irradiated virus, 30 hours after infection.

Unfortunately, there is no straight forward interpretation of these results. No rejoining of the single strand breaks could be detected after infection. Instead, a degradation of the viral DNA from irradiated virus, was observed. It is possible that the inability to detect repair results from inadequacies of the technique (see discussion).

FIGURE 13

The Fate of DNA from Unirradiated Ad 2 after Infection

10^6 KB cells were infected with Ad 2, containing ^3H labelled DNA, at an input multiplicity of about 3000 PFU/cell. At various times after infection, a sample of 5×10^5 cells was treated with lysing solution and unirradiated Ad 2, containing ^{14}C labelled DNA was added as a marker. The sample was then centrifuged on a 5 to 20% sucrose gradient. Fractions were collected from the bottom of the centrifuge tube and their radioactivity determined.

A. Intracellular virus DNA 44 hours after infection

B. Intracellular virus DNA 61 hours after infection

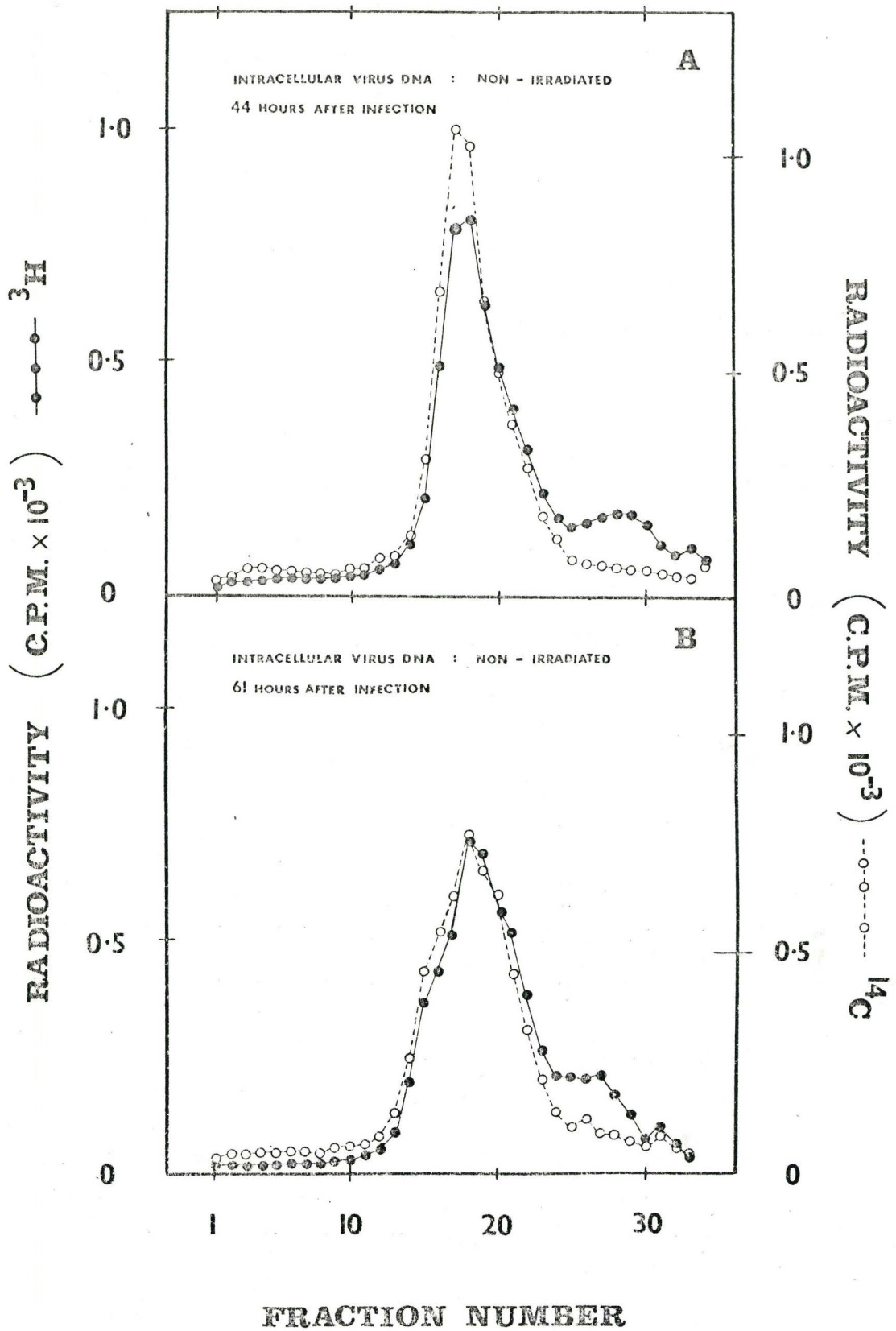

A
INTRACELLULAR VIRUS DNA : NON - IRRADIATED
44 HOURS AFTER INFECTION
B
INTRACELLULAR VIRUS DNA : NON - IRRADIATED
61 HOURS AFTER INFECTION
RADIOACTIVITY (C.P.M. × 10⁻³) —●— ³H
RADIOACTIVITY (C.P.M. × 10⁻³) --o--o-- ¹⁴C
1·0
0·5
0
1
10
20
30
FRACTION NUMBER

FIGURE 14

The Fate of DNA from Gamma Irradiated Ad 2 after Infection

A sample of Ad 2, containing ^{3}H labelled DNA, was given 0.58 Mrads of gamma irradiation and an aliquot used to infect 10^{6} KB cells at an input multiplicity of about 600 original PFU/cell. At various times after infection, a sample of 5×10^{5} cells was treated with lysing solution and unirradiated Ad 2, containing ^{14}C labelled DNA, was added as a marker. The sample was then centrifuged on a 5-20% alkaline sucrose gradient. Fractions were collected from the bottom of the centrifuge tube and their radioactivity determined.

A. Free virus DNA

B. Intracellular virus DNA 5 hours after infection

C. Intracellular virus DNA 30 hours after infection.

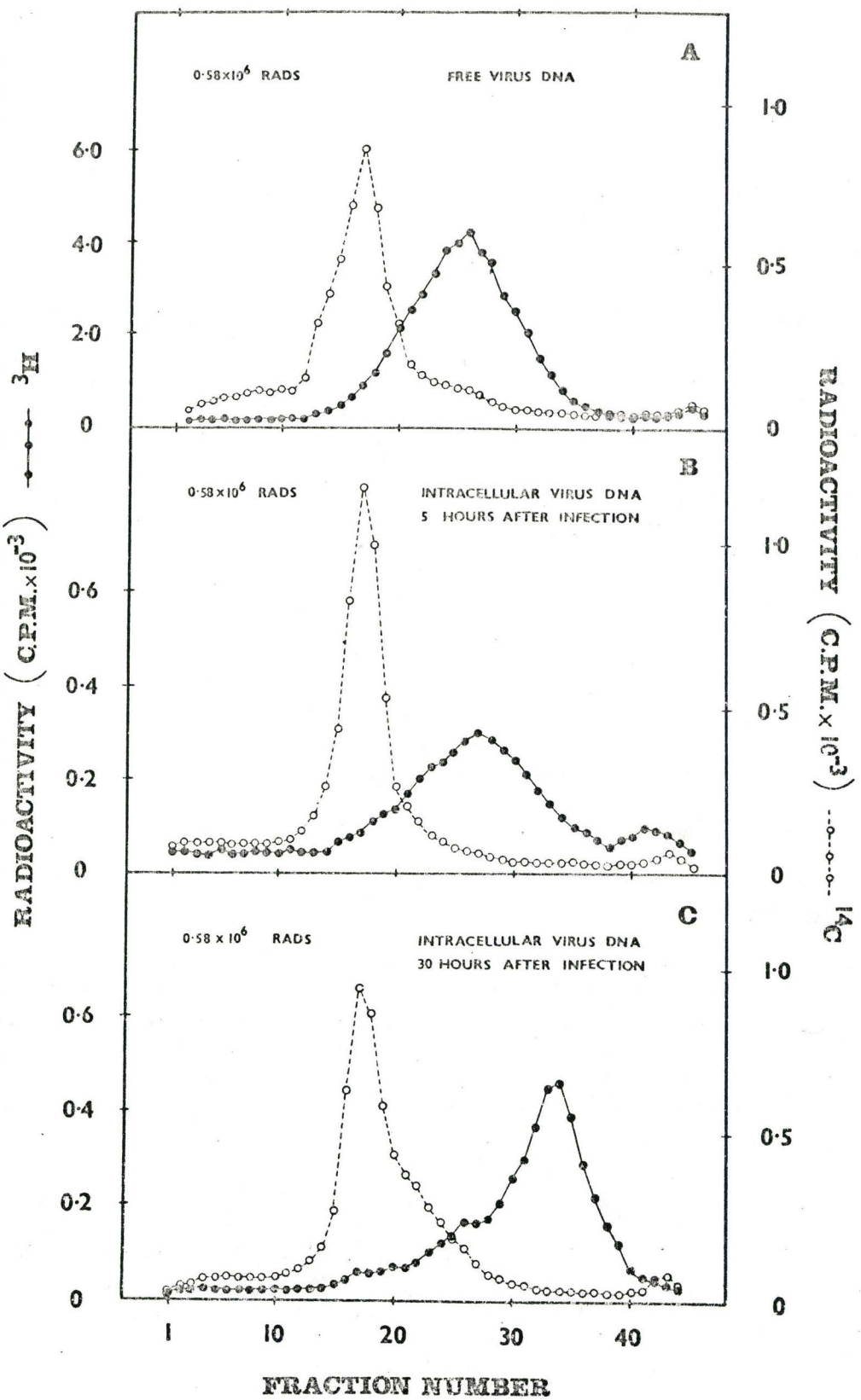

A
0·58×10⁶ RADS
FREE VIRUS DNA
B
0·58×10⁶ RADS
INTRACELLULAR VIRUS DNA
5 HOURS AFTER INFECTION
C
0·58 x 10⁶ RADS
INTRACELLULAR VIRUS DNA
30 HOURS AFTER INFECTION
RADIOACTIVITY (C.P.M.×10⁻³) — ³H
RADIOACTIVITY (C.P.M.× 10⁻³) - - - ¹⁴C
FRACTION NUMBER
1
10
20
30
40

TABLE 12

Average Number of Single-strand Breaks in the DNA from Gamma Irradiated Ad 2 at Different Times after Infection

Time after infection (hours)	Calculated number of breaks/ds molecule			
	Non-irradiated Ad 2		Ad 2 given 0.58×10^6 rads of gamma irradiation	
	Expt. I	Expt. II	Expt. I	Expt. II
0	0	0	5.6	8.0
5	0.19			11.2
18	0.20		34.0	
30	0.35			48.0
44		0.26		
60		0.19		

D. EFFECTS OF UV IRRADIATION ON VIRAL FUNCTIONS

The effect of gamma irradiation on the survival of various functions of Ad 2 was examined in section B. Also, following gamma irradiation, double and single strand chain breaks were found in the viral DNA. After UV irradiation, many different types of photolesions are produced in the DNA such as thymine dimers, DNA cross-links and single strand breaks (Smith 1969). It is not known which photolesion is of most biological importance in virus systems. Possibly, it is a combination of different types of DNA damage which lead to viral inactivation by UV irradiation.

It was therefore considered of interest to examine the effects of UV irradiation on the survival of several functions of the virus. Ad 2 was given graded doses of UV irradiation and assayed for the viral functions of adsorption, clone inhibition, inclusion body formation and plaque formation.

1. Effect of UV Irradiation on Adsorption of Virus to KB Cells :

Irradiated Ad 2, containing ^{3}H labelled DNA, was assayed for adsorption as previously described in section B1 for gamma irradiation. The adsorption efficiencies obtained at 5 hours after infection are shown in Table 13 and the survival of this viral function is plotted against dose in Figure 15. It can be seen that virus adsorption is not significantly affected by the doses of UV radiation employed.

TABLE 13

Effect of UV Irradiation on Adsorption (a)

Radiation dose (10^4 ergs/mm^2)	^3H Radioactivity (cpm / 0.1 ml.)			% Adsorbed (c)	Surviving fraction
	Cell suspension	Cell-free supernatant	Cell associated (b)		
0	1339	622	717	54	1
0.8	1434	847	587	41	0.75
1.6	1503	823	680	46	0.83
2.4	1444	881	563	39	0.72
3.2	1427	823	604	43	0.74

(a) 2×10^6 KB cells infected with ^3H Ad 2 at 1,500 PFU / cell

(b) Column 2 - Column 3

(c) $\frac{\text{Column 3}}{\text{Column 2}} \times 100$

FIGURE 15

Survival of Viral Functions Following UV Irradiation

Each curve represents the pooled data from several experiments

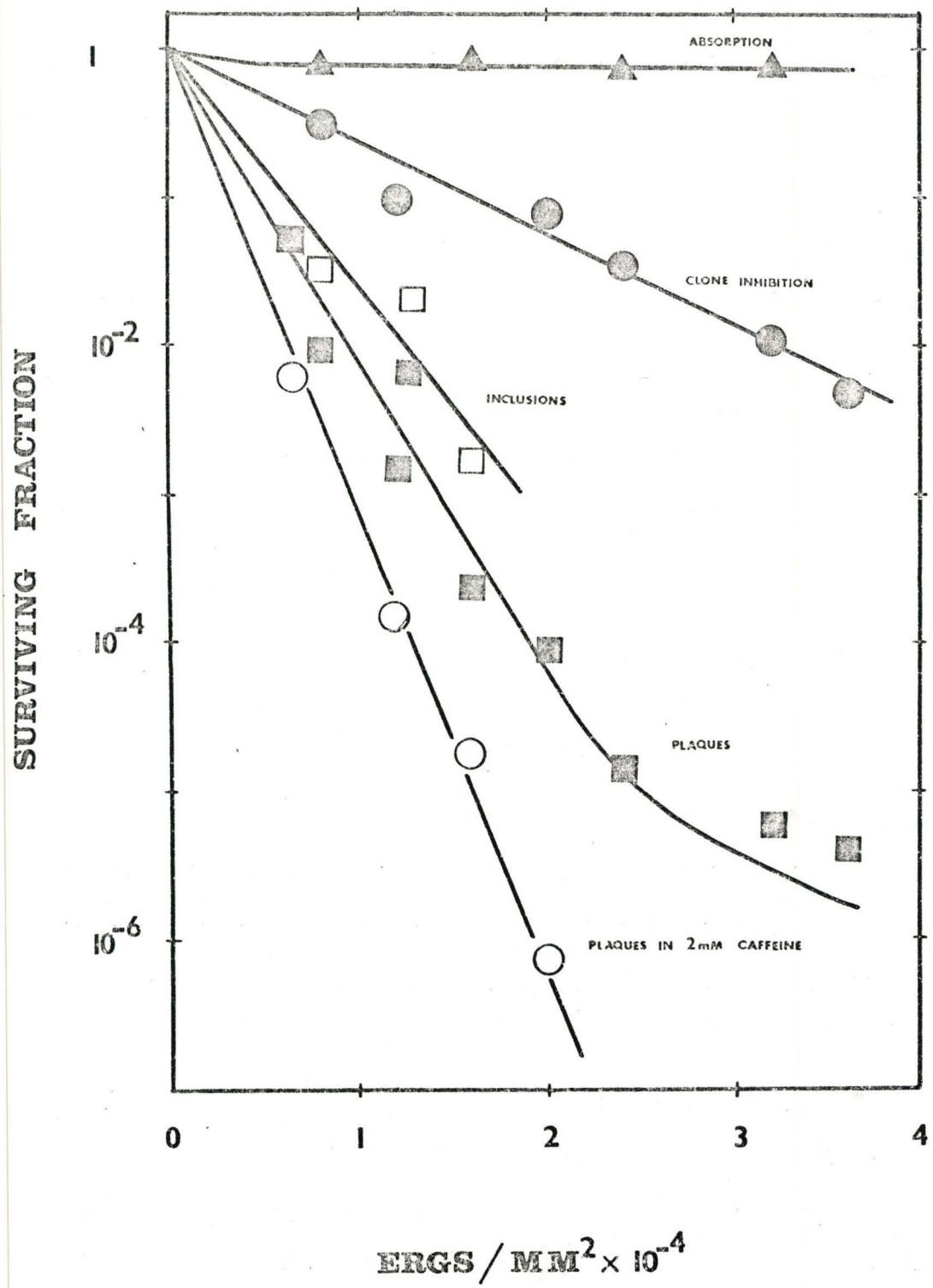
Adsorption

Clone inhibition

Inclusion body formation

Plaque formation

Plaque formation in 2mM caffeine


ABSORPTION
CLONE INHIBITION
INCLUSIONS
PLAQUES
PLAQUES IN 2mM CAFFEINE
SURVIVING FRACTION
1
10^{-2}
10^{-4}
10^{-6}
0
1
2
3
4
ERGS / MM^2 × 10^{-4}

2. Effect of UV Irradiation on the Inhibition of Host Cell Cloning : UV irradiated samples of Ad 2 were assayed for their ability to inhibit host cell cloning as described in section B3 and Appendix 1. Results for 2 experiments are shown in Table 14. Data from these and several other experiments were pooled and the mean values for the survival of the viral function were plotted in Figure 15. It is seen that the viral function of clone inhibition is consistent with an exponential inactivation with a D_{37} value of 7×10^3 ergs/mm^2.

3. Effect of UV Irradiation on Plaque Formation : Irradiated samples of Ad 2 were assayed for plaque formation. Typical results are shown in Table 15. Data from these and several other experiments were pooled and the mean values for the survival of this function plotted in Figure 15. It can be seen that plaque formation is consistent with an exponential inactivation up to a dose of 2.5×10^4 ergs/mm^2, which reduces the surviving fraction to 10^{-5}. The tail in the survival curve found at higher doses of UV may be the result of a multiplicity reactivation (Kim 1968) or a saturation of DNA damage (Johns 1962). (see section E). The D_{37} value for plaque formation was 2.1×10^3 ergs/mm^2.

4. Effect of UV Irradiation on Inclusion Body Formation : The assay method used was similar to that described in section B4 (see Appendix 1). Results of a typical assay are shown in Table 16A and the survival of this viral function shown in Table 16B and Figure 15. Inclusion body formation was consistent with an exponential inactivation up to a dose

TABLE 14

Effect of UV Irradiation on the Inhibition of Host Cell Cloning

Radiation dose (10^4 ergs/mm^2)	Expt. I		Expt. II	
	CIU per ml.	Surviving fraction	CIU per ml.	Surviving fraction
0	4.8×10^9	1	3.5×10^9	1
0.8	9.6×10^{-1}	2.0×10^{-1}	1.4×10^9	4.0×10^{-1}
1.2	4.3×10^8	9.0×10^{-2}		
1.6	4.3×10^8	9.0×10^{-2}		
2.0			1.9×10^8	5.6×10^{-2}
2.4	1.2×10^8	2.5×10^{-2}		
3.2			3.5×10^7	1.0×10^{-2}
3.6	2.4×10^7	5.0×10^{-3}		
4.4			4.9×10^6	1.4×10^{-3}
4.8	1.5×10^6	3.1×10^{-4}		

TABLE 15

Effect of UV Irradiation on Plaque Formation

Radiation dose (10^4 ergs/mm^2)	Expt. I		Expt. II		Expt. III	
	PFU per ml.	Surviving fraction	PFU per ml.	Surviving fraction	PFU per ml.	Surviving fraction
0	1.0×10^{10}	1	2.1×10^{10}	1	1.4×10^{9}	1
0.8	2.8×10^{7}	2.8×10^{-3}	2.9×10^{8}	1.4×10^{-2}		
1.2					2.0×10^{6}	1.4×10^{-3}
1.6			4.6×10^{6}	2.2×10^{-4}		
2.0	8.9×10^{3}	8.9×10^{-5}				
2.4			1.8×10^{5}	8.6×10^{-6}	2.5×10^{4}	1.8×10^{-5}
3.2	1.2×10^{4}	1.2×10^{-6}	2.5×10^{4}	1.2×10^{-6}		
3.6					1.1×10^{4}	7.9×10^{-6}
4.4	3.1×10^{3}	3.1×10^{-7}				

TABLE 16A

Assay for Inclusion Body Formation in UV Irradiation Ad 2

(a) Amount of virus given in working input multiplicity of IBU/cell (see Appendix 1)

(b) $\frac{\text{Column 2}}{\text{Column 5}}$

(c) $\frac{\text{Column 3}}{\text{Column 5}}$

TABLE 16A

Assay for Inclusion Body Formation in UV Irradiated Ad 2

Treatment	Assumed titre IBU / ml.	Amount of virus IBU / cell (a)	Percent inclusion bodies	Working MOI giving 63 % inclusions (a)	True titre IBI / ml. (b)	True MOI in IBU/cell (c)
No UV	10^{10}	0.12	19.5	0.7	1.4×10^{10}	0.18
		0.25	25.0			0.35
		0.50	49.5			0.71
		1.00	55.6			1.43
8×10^{3} ergs/mm^2	5×10^{8}	0.12	9.1	1.1	4.5×10^{8}	0.11
		0.25	22.0			0.23
		0.50	26.6			0.45
		1.00	26.0			0.91
1.6×10^{4} ergs/mm^2	2×10^{7}	0.12	13.1	0.9	2.2×10^{7}	0.14
		0.25	16.0			0.28
		0.50	12.3			0.55
		1.00	7.5			1.11

TABLE 16B

Effect of UV Irradiation on Inclusion Body Formation

Radiation dose (10^4 ergs/mm^2)	IBU per ml.		Surviving fraction
	Expt. I	Expt. II	
0	1.4×10^{10}	1.0×10^{9}	1
0.8	4.5×10^{8}		3.2×10^{-2}
1.2		2.0×10^{7}	2.0×10^{-2}
1.6	2.2×10^{7}		1.6×10^{-3}

of 1.6×10^4 ergs/mm^2, which reduced the survival to 10^{-3}. The D_{37} value was 2.4×10^3 ergs/mm^2. At higher doses of UV irradiation, the assay for inclusion bodies became masked by interference from the increased number of defective virus particles induced by the UV irradiation (see section D5 below).

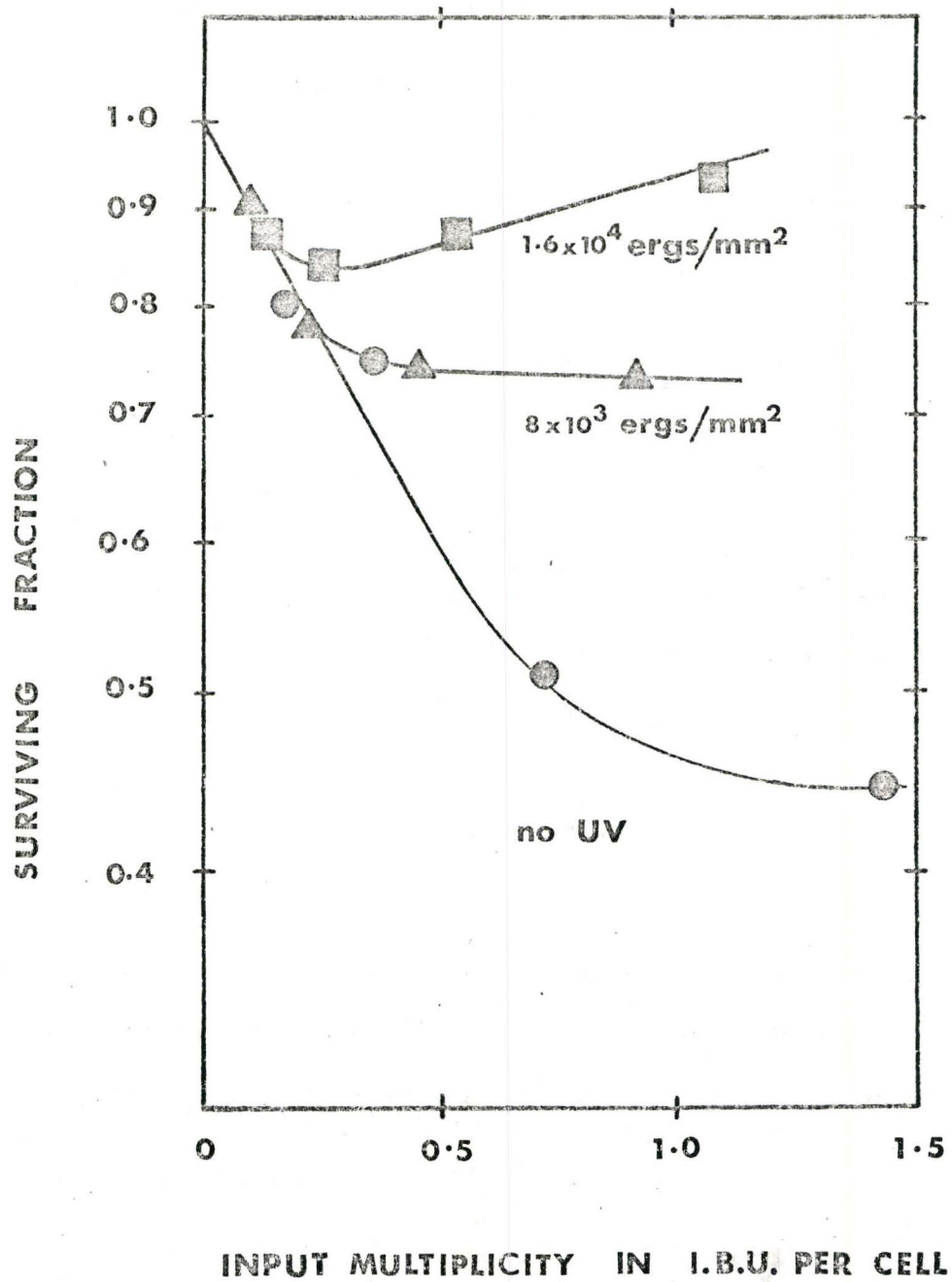
5. Inhibition of Inclusion Body Formation by UV Irradiated Virus :

The results of the inclusion body assay given in Table 16A were plotted in Figure 16, with the fraction of cells surviving inclusion body formation versus the true input multiplicity in IBU/cell. In this type of plot, viral functions consistent with one-hit kinetics, derived from the Poisson distribution, yield exponential survival curves. It can be seen that inclusion body formation was consistent with one-hit kinetics at low input multiplicities. At higher input multplicities, the percentage of cells with an inclusion is substantially less than that predicted by the Poisson distribution for both irradiated and unirradiated Ad 2. Moreover, the reduction in inclusion body formation was most marked in the irradiated preparations. The percent inclusions reached a maximum of 56% for unirradiated Ad 2, 26% for Ad 2 irradiated with 8×10^3 ergs/mm^2 and 16% for Ad 2 irradiated with 1.6×10^4 ergs/mm^2. It was suggested in section A2, that the reduction of inclusion body formation at high input multiplicities was due to interference by large numbers of non-plaque producing particles infecting a cell. In the same way, the greater reduction found with the UV irradiated samples may result from the increased proportion of non-plaque

FIGURE 16

Effect of Input Multiplicity on Inclusion Body Formation for UV Irradiated Ad 2

Fraction of cells not displaying an inclusion body plotted against the input multiplicity in true IBU/cell. Data obtained from Table 16A.


1·0
0·9
0·8
0·7
0·6
0·5
0·4
SURVIVING FRACTION
1·6x10⁴ ergs/mm²
8x10³ ergs/mm²
no UV
0
0·5
1·0
1·5
INPUT MULTIPLICITY IN I.B.U. PER CELL

producing particles induced by the UV irradiation.

Since some non-plaque producing particles may interfere with inclusion body formation, the addition of UV inactivated virus should inhibit inclusion body formation by non-irradiated virus. Ad 2 was irradiated with 3.2×10^4 ergs/mm^2, to a survival of 10^{-5} PFU and 10^{-2} CIU. KB cells were infected with different amounts of UV-inactivated virus together with a constant amount of non-irradiated Ad 2. At 48 hours after infection, the cells were examined for the presence of an inclusion body and the results are shown in Table 17. It can be seen that the inclusion body formation by the non-irradiated virus was greatly reduced when UV-inactivated virus was added.

6. Radiosensitivity of Different Viral Functions Following UV Irradiation : For comparison purposes, the D_{37} values obtained after UV irradiation are summarised in Table 18. It can be seen that the most radiosensitive viral function is that of plaque formation, with a D_{37} of 2.1×10^3 ergs/mm^2, followed by inclusion body formation with a D_{37} of 2.4×10^3 ergs/mm^2. These 2 functions are only just separable at the doses of UV employed. The function of clone inhibition was more radioresistant than that of inclusion body formation, with a D_{37} of 7×10^3 ergs/mm^2 and the virus adsorption was not significantly affected by the doses of UV irradiation employed.

The relative radiosensitivities of the different viral functions to UV irradiation were similar to those found using gamma irradiation (see section B6).

TABLE 17

Inhibition of Inclusion Body Formation
by UV-irradiated Adenovirus Type 2 (a)

Non-irradiated virus PFU/cell	UV-irradiated virus (b)		% inclusions
	Surviving PFU/cell	Surviving CIU/cell	
2	0	0	47.5
2	$2x10^{-5}$	$2x10^{-2}$	49.2
2	2.10^{-4}	$2x10^{-1}$	37.9
2	$2x10^{-3}$	2	32.6
2	$2x10^{-2}$	20	13.1
0	$2x10^{-2}$	20	13.4

(a) 10^6 KB cells were infected with 2 x 10^6 PFU of non-irradiated Adenovirus Type 2 and different amounts of UV irradiated Ad 2.

(b) UV treatment with $3.2x10^4$ ergs/mm^2

TABLE 18

D_{37} Values Obtained for Various Viral Functions and DNA Damage after UV Irradiation

Viral function / DNA damage	D_{37} (ergs/mm^2)
Inhibition of host-cell cloning	7.0×10^3
Inclusion body formation	2.4×10^3
Plaque formation	2.1×10^3
Plaque formation in 2 mM caffeine	1.3×10^3
Double stranded DNA molecules with no breaks	2.1×10^3
Double stranded DNA molecules with no thymine dimers	72.1

7. Effect of UV Irradiated Virus on Host Cell Mitosis : When human cells are lytically infected with several different types of adenovirus, the cultures show no detectable increase in chromosome aberrations (Cooper 1968 and Cooper 1968a). In contrast, when hamster cells are abortively infected by adenoviruses, a wide variety of chromosome aberrations are observed. In the previous sections it was found that infection of human KB cells by UV irradiated virus can lead to an abortive type of infection. It was thought of interest to determine whether infection of human KB cells by UV irradiated virus would produce the chromosome aberrations characteristic of abortive adenovirus infection.

Samples of Ad 2 were given different doses of UV irradiation and an aliquot from each sample was used to infect a 1 ml. suspension of 10^6 KB cells. After incubating at 37°C for 90 min., using a roller wheel, 4 ml. of warm nutrient medium was added and the infected culture incubated for a further period of time. At various times after infection, cells were examined cytologically for the appearance of metaphase plates.

The mitotic index obtained at different times after infection is shown in Figure 18. It can be seen that in the cultures infected with unirradiated virus, the mitotic index decreased with time after infection. The decrease was most marked for cells infected at a high input multiplicity. However, for cultures infected with UV irradiated virus, the mitotic index increased with time after infection. At the input multiplicity of 10^3 original PFU/cell, the mitotic index in cells infected with UV irradiated virus reached a maximum at 48 hours

FIGURE 17

Photomicrograph Showing Metaphase Plates from KB Cells Infected with UV Irradiated Ad 2

A. Metaphase plate from uninfected cell

B. Abnormal metaphase plate from a cell infected with UV irradiated Ad 2 showing chromosome contraction

C. and D. Abnormal metaphase plates from cells infected with UV irradiated Ad 2 showing fragmentation of chromosomes

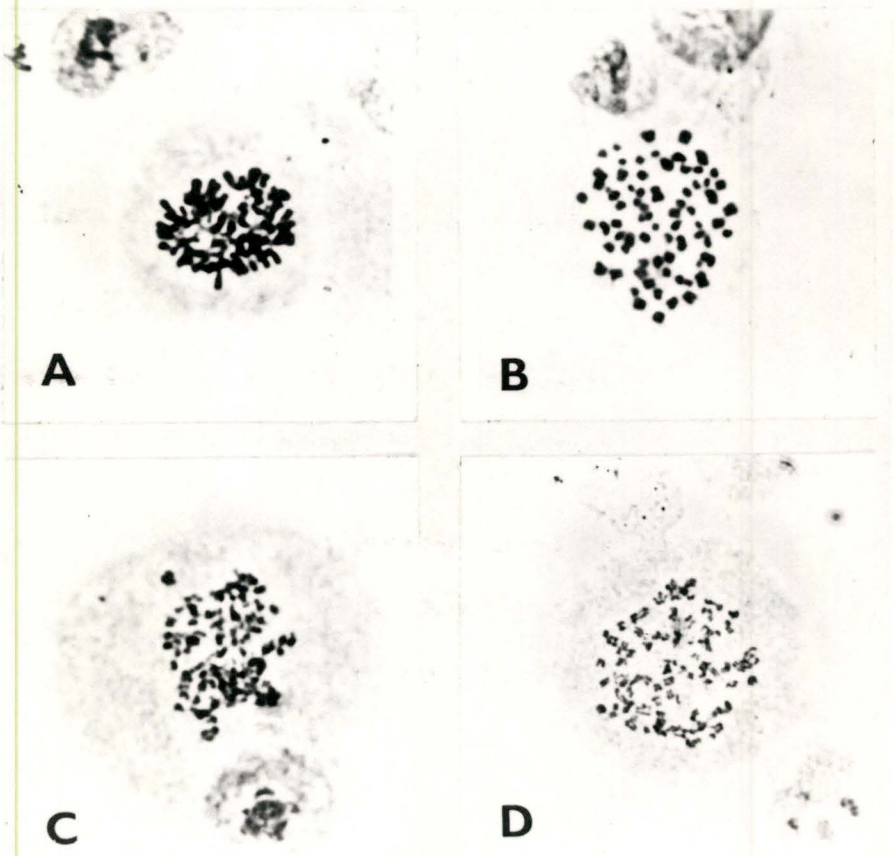

A
B
C
D

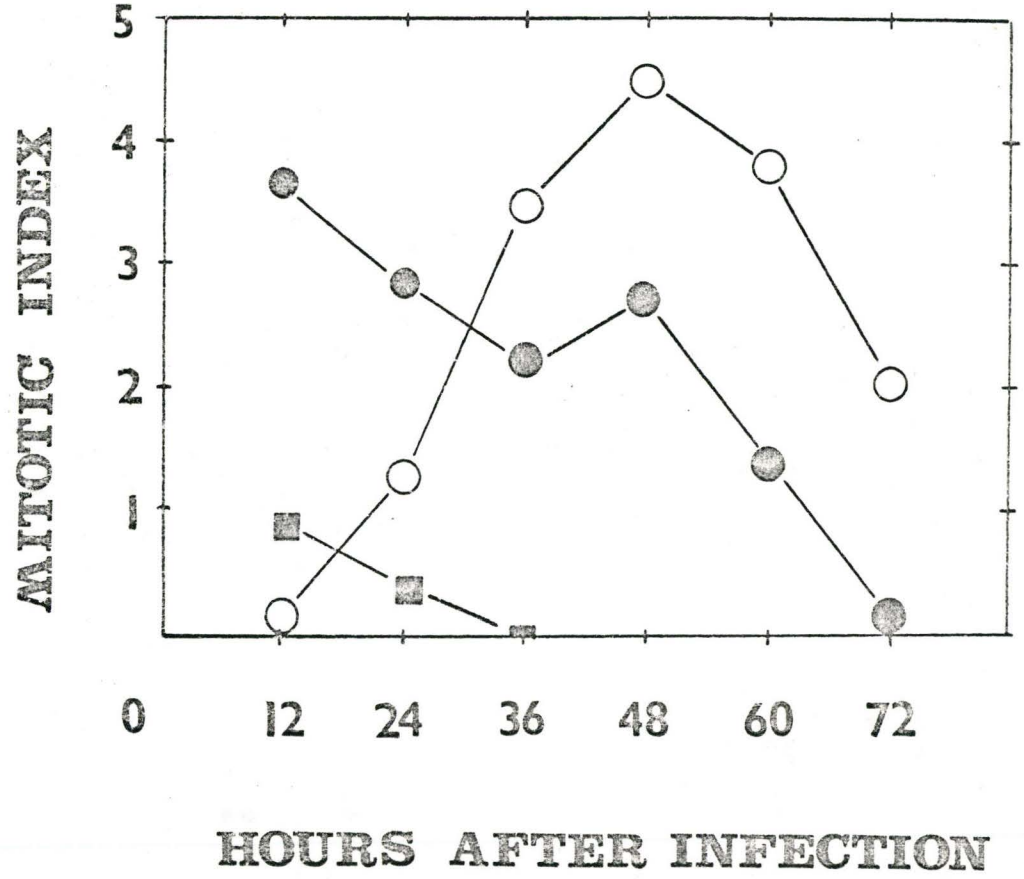
FIGURE 18

Mitotic Index at Different Times after Infection with UV Irradiated and Non Irradiated Ad 2

Non-irradiated Ad 2 infected at 100 PFU/cell

Non irradiated Ad 2 infected at 0.1 PFU/cell

UV irradiated Ad 2 with 3.6×10^4 ergs/mm^2. Infected at 10^3 original PFU/cell and 2×10^3 original CIU/cell. (Surviving Fraction 10^{-6} for PFU and 5×10^{-3} for CIU)


MITOTIC INDEX
5
4
3
2
1
0
12
24
36
48
60
72
HOURS AFTER INFECTION

after infection. This increase in the mitotic index after infection is thought to indicate that some of the cells are being arrested at metaphase. Metaphase arrest has also been observed after the abortive infection of hamster cells by several adenoviruses (Cooper 1968a).

A large proportion of the metaphase plates from cultures infected with UV irradiated virus showed various types of chromosome aberrations, such as chromosome pulverisation, contraction and fragmentation (see Figure 17), which are characteristic of abortive adenovirus infections (Cooper 1968a). It is probable that these chromosome aberrations result from infection of a defective virus particle and lead to an arrest of the cell at metaphase.

Larger doses of UV irradiation should produce a greater proportion of defective virus particles and result in a larger proportion of the infected cells arrested at metaphase. Furthermore, an increase in the input multiplicity of UV irradiated virus should also produce an increase in the metaphase arrest of the infected culture. In order to test this hypothesis, the following experiment was performed.

KB cells were infected with different input multiplicities of UV irradiated Ad 2, and the mitotic index determined at 48 hours after infection. Typical results are shown in Table 19. It can be seen that for cultures infected with unirradiated virus, the mitotic index decreased with input multiplicity from 2 to 0.1 . Although there was very little cell growth for the cultures infected with UV irradiated virus, the mitotic index increased with input multiplicity to a value substantially greater than that of the controls. For the same input

TABLE 19

Effect of UV Irradiated Ad 2 on the Formation of Metaphase Plates

(a) Initial cell concentration :

A. 2.1×10^5 cells/ml.

B. 1.8×10^5 cells/ml.

C. 2.0×10^5 cells/ml.

D. 2.1×10^5 cells/ml.

(b) $$\text{Mitotic Index} = \frac{\text{Number of cells in mitosis}}{\text{Total number of cells}} \times 100$$

TABLE 19

Treatment	Input multiplicity			Final cell conc. $x10^5$/ml. (a)	Mitotic Index (b)
	Before UV PFU/cell	After UV			
		PFU/cell	CIU/cell		
A. Ad 2 no UV	0.23	0.23	0.31	4.33	2.9
	0.46	0.46	0.63	4.08	2.2
	1.9	1.9	2.5	2.76	1.4
	3.8	3.8	5.0	2.15	0.4
	19.3	19.3	25.0	1.66	0.1
B. Ad 2 $2.4x10^4$ ergs/mm^2	$8.8x10^1$	$9.4x10^{-4}$	5.25	2.12	1.3
	$3.5x10^2$	$3.8x10^{-3}$	21.0	1.80	2.6
	$7.0x10^2$	$7.5x10^{-3}$	42.0	1.38	3.9
	$1.4x10^3$	$1.5x10^{-2}$	84.0	1.41	6.1
C. Ad 2 $3.6x10^4$ ergs/mm^2	$6.5x10^1$	$2.8x10^{-5}$	0.63	2.57	0.9
	$1.3x10^2$	$5.6x10^{-5}$	1.25	3.25	3.8
	$4.5x10^2$	$1.1x10^{-4}$	2.5	1.64	3.5
	$9.0x10^2$	$2.3x10^{-4}$	5.0	1.81	12.6
	$1.8x10^3$	$4.5x10^{-4}$	10.0	1.64	12.3
D. No virus	0	0	0	6.41	1.6
	0	0	0	5.60	1.9

multiplicity of original PFU/cell, the mitotic index was highest for Ad 2 treated with the highest dose of UV irradiation. At an input multiplicity of approximately one thousand original PFU/cell, the mitotic index for cells infected with virus given 3.6×10^4 ergs/mm^2 was 12.3 , whereas for cells infected with virus given 2.4×10^4 ergs/mm^2 it was 6.1 .

These results lend support to the hypothesis that metaphase arrest is the expression of a UV induced defective virus particle.

E. MOLECULAR DAMAGE TO THE VIRAL DNA AFTER UV IRRADIATION

Correlations have been made between biological survival after UV irradiation and the production of certain types of photolesions in the DNA such as thymine dimers and DNA chain breakage (Sauerbier 1964, Smith 1966, Rauth 1970 and Wulff 1963).

The effect of UV irradiation on several viral functions of Ad 2 was described in the previous section. In order to correlate the radiosensitivity of these viral functions with the molecular lesions sustained by the viral DNA, samples of Ad 2, containing ^{3}H labelled DNA, were given graded doses of UV irradiation and subsequently assayed for both thymine dimers and DNA chain breaks. The number of thymine dimers was determined using paper chromatography. The number of radiation induced DNA breaks was determined from the size distribution of the viral DNA fragments obtained on sucrose gradients.

1. Assay for Thymine Dimers : Irradiated samples of Ad 2 were assayed for thymine dimers. When ^{3}H labelled thymine is used, the presence of both thymine-thymine ($\widehat{TT}$) and thymine-cytosine ($\widehat{CT}$) can be detected (Setlow 1966). UV irradiated samples were subjected to acid hydrolysis and the hydrolysate run on a paper chromatogram.

Typical results for the chromatography of thymine and thymine dimers are shown in Figure 19. It can be seen that all profiles showed a peak in radioactivity between fractions 20 and 30 (R_f = 0.6), corresponding to thymine. Profiles from irradiated samples also showed a radioactivity peak between fractions 8 and 19 (R_f = 0.3) correspoding to thymine dimer. The dimer peak was not detected in the profile for unirradiated virus. With doses of UV up to 3.2×10^{4} ergs/mm^{2} the dimer peak contained a small shoulder around fractions 8 and 9 corresponding to thymine-uracil dimer ($\widehat{TU}$), the hydrolysis product of thymine-cytosine dimer. The small peak in radioactivity seen on all profiles between fractions 1 and 5 is probably due to a small proportion of the sample which is not completely hydrolysed.

From the radioactivity profiles, the fraction of thymine molecules present as dimer was calculated as follows :

$$\text{Fraction of thymine molecules present as thymine dimer} = \frac{\text{Counts in dimer peak}}{\text{Counts in thymine peak + Counts in dimer}}$$

The pooled results of 3 experiments are shown in Figure 20. It can be seen that the production of $\widehat{TT}$ dimers is linear with dose up to about

FIGURE 19

Production of Thymine Dimers after UV Irradiation

Radioactivity Profiles Obtained on Strip Paper Chromatography

Samples of Ad 2, labelled with H^3 thymidine, were given UV doses of 1.6×10^4 and 3.2×10^4 ergs/mm^2. Aliquots from each sample were subjected to acid hydrolysis and the hydrolysates run on a strip paper chromatogram. Strip fractions were cut and their radioactivity determined in a scintillation spectrometer.

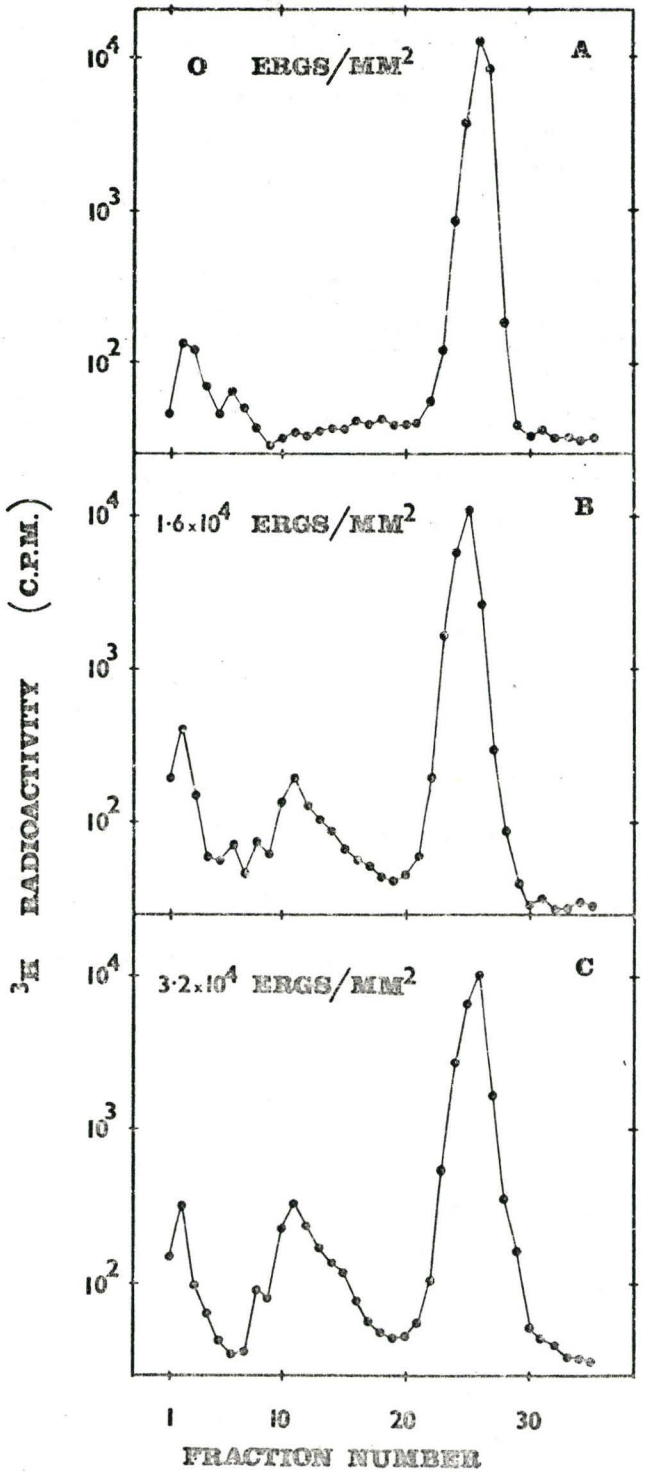

0 ERGS/MM2
A
1·6×10^4 ERGS/MM2
B
3·2×10^4 ERGS/MM2
C
10^4
10^3
10^2
3H RADIOACTIVITY (C.P.M.)
1
10
20
30
FRACTION NUMBER

FIGURE 20

Fracrion of Thymine Molecules Present as Thymine Dimer after UV Irradiation

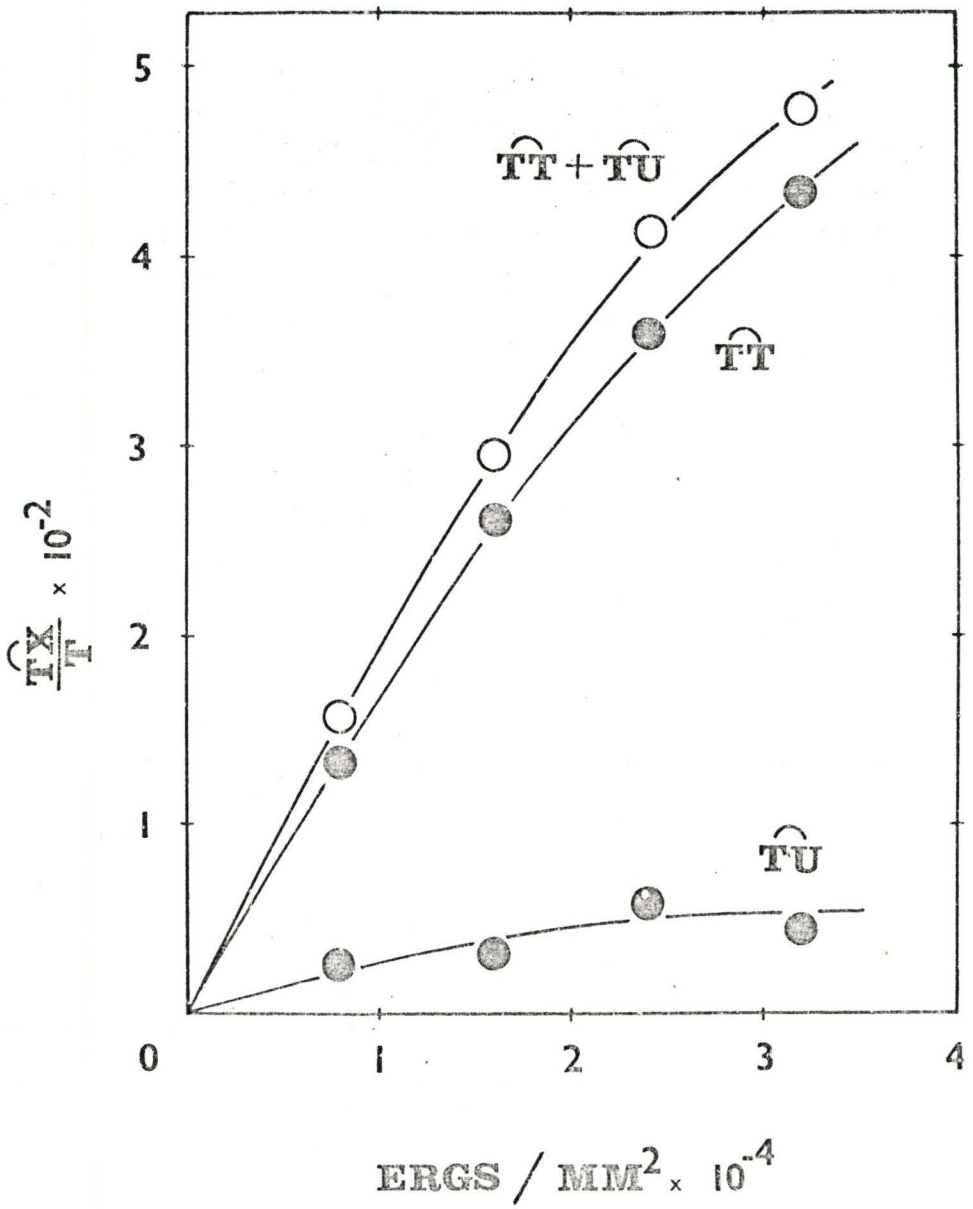
Plot of data from Table 17.

$\frac{\overset{\frown}{T\ X}}{T}$: Fraction of thymine present as thymine dimer

$\overset{\frown}{T\ U}$: Thymine-uracil dimer (the hydrolysis product of thymine-cytosine dimer)

$\overset{\frown}{T\ T}$: Thymine - thymine dimer

$\overset{\frown}{T\ T}+\overset{\frown}{T\ U}$: Thymine-uracil plus thymine-thymine dimer


5
4
3
2
1
0
1
2
3
4
$\widehat{TT} + \widehat{TU}$
$\widehat{TT}$
$\widehat{TU}$
$\frac{\widehat{TX}}{\widehat{TT}} \times 10^{-2}$
ERGS / MM^2 × 10^{-4}

2×10^4 ergs/mm^2. At higher UV doses of about 4×10^5 ergs/mm^2, the production of dimers saturated when the fraction of thymine molecules present as dimer was about 0.1, as shown in Table 20. The production of CT dimer (observed as TU dimer) was about 10 fold lower than the production of TT dimer and saturated at a lower UV dose of about 2×10^4 ergs/mm^2. This saturation may result from an equilibrium being established between dimer formation and dimer reversal (Johns 1962). For bacteria, this saturation value has been found to be a function of the wavelength of the UV light and the nearest neighbour frequency of pyrimidine pairs in DNA (Setlow 1966 and Wulff 1963a).

2. Assay for DNA Breaks after UV Irradiation : It is very unlikely that UV irradiation produced a localised energy absorption of sufficient magnitude to result in double strand chain breakage. However, a change in the neutral sucrose gradient profile may result from 2 single strand breaks occuring on opposite strands within the space of a few nucleotide pairs.

UV irradiated samples of Ad 2 were disrupted by treatment with pronase and SDS and centrifuged on a 5 to 20% neutral sucrose gradient. No double strand breaks were detected after 1.4×10^4 ergs/mm^2, and less than 0.25 breaks/molecule were found after 2.8×10^4 ergs/mm^2.

The number of UV induced single strand DNA breaks was determined using alkaline sucrose gradients as previously described in section C2 for gamma irradiated Ad 2. Typical radioactivity profiles obtained for UV irradiated virus on alkaline sucrose gradients are shown

TABLE 20

Production of Thymine Dimers after UV Irradiation

UV dose (10^4 ergs/mm^2)	$\frac{\hat{UT}}{T} \times 10^{-3}$ (a)	$\frac{\hat{TT}}{T} \times 10^{-3}$ (b)	$\frac{\hat{TX}}{T} \times 10^{-3}$ (c)	Dimers/molecule $\times 10^{-2}$ (d)
0	0	0	0	0
0.8	2.6	13.2	15.7	1.2
1.6	3.0	26.1	29.6	2.2
2.4	5.9	35.8	41.5	3.1
3.2	4.3	43.3	47.6	3.6
28.8	2.8	90.0	92.8	6.9
43.2	4.1	110.5	114.6	8.7

(a) $\hat{UT}$, uracil-thymine dimer (the hydrolysis product of cytosine-thymine dimer)

(b) $\hat{TT}$, thymine-thymine dimer

(c) $\hat{TX} = \hat{TT} + \hat{TU}$

(d) $\frac{\text{Thymine molecules per virus particle}}{2}$ X Column 4

$= 7.49 \times 10^{3}$ X Column 4

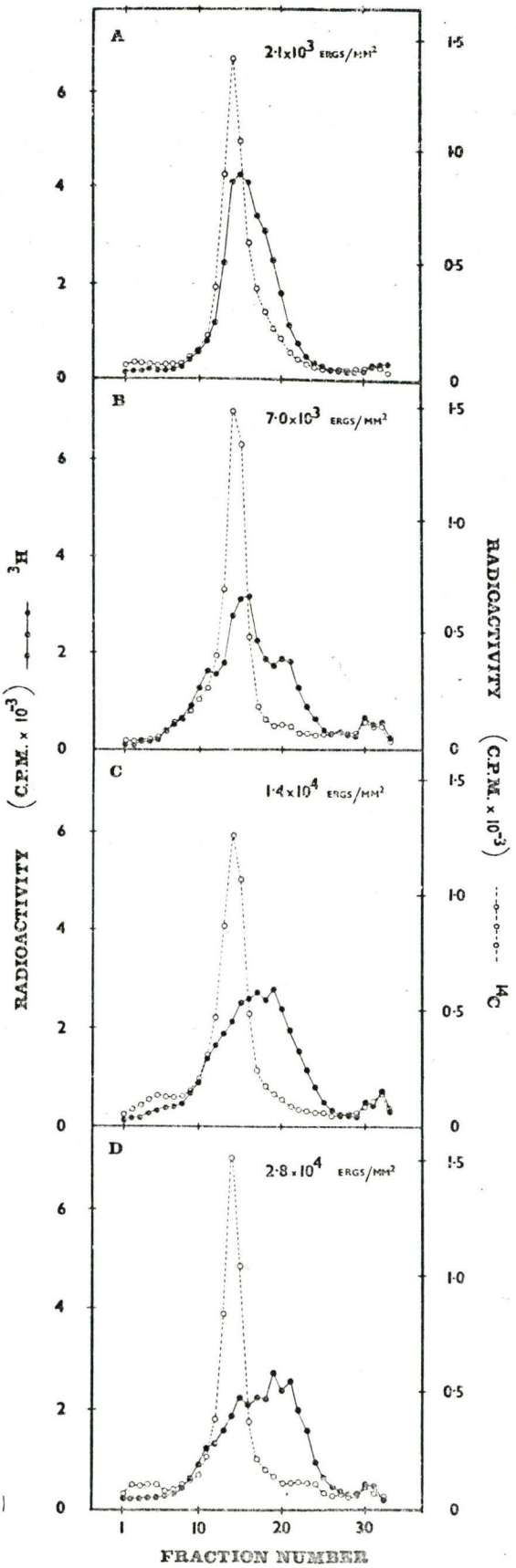
in Figure 21. It can be seen that the distribution of the ^{14}C radioactivity from unirradiated marker virus was the same for all profiles. However, the ^{3}H radioactivity from the UV irradiated virus showed a more heterogenous profile and a decrease in molecular size corresponding to an increase in single strand DNA breaks. According to Litwin et al (Litwin 1969), the distribution of single stranded DNA fragments after random breakage should sediment to the right of the marker peak from unirradiated virus, and sharpen as the number of breaks increases. This was not found for the profiles from UV irradiated virus, In fact, the radioactivity profiles became broader with increasing dose and some radioactivity sedimented to the left of the ^{14}C marker. Radioactivity sedimenting to the left of the marker is especially prominent in Figure 21B and probably corresponds to 'larger molecules' which result from interstrand dimerisation (cross-linking). Similar cross-links, also found after UV irradiation of T_4 phage (Kozinski 1967) would not be removed by alkaline treatment.

Consequently, the radioactivity profiles from UV irradiated virus on alkaline sucrose gradients are believed to result from a combination of single strand chain breaks and interstrand cross-links in the viral DNA. From the radioactivity profiles, the average number of single strand DNA breaks per molecule was calculated using the methods described in section C1 or C2, which ever was applicable. Since the radioactivity profiles are not the result of random breakage alone, but also involve cross-linkage, the number of single strand breaks per molecule may be an underestimate, especially at UV doses which produce

FIGURE 21

Single Strand DNA Breakage and Cross-linking after UV Irradiation

Samples of Ad 2, containing H^3 labelled DNA, were given graded doses of 2.1×10^3, 7.0×10^3, 1.4×10^4 and 2.8×10^4 ergs/ mm^2 of UV irradiation. Aliquots from each sample were treated with lysing solution and unirradiated Ad 2, containing C^{14} labelled DNA, was added as a marker. Samples were then carefully layered on a 5-20% alkaline sucrose gradient. After centrifugation, tube fractions were collected from the bottom and the radioactivity of each fraction was determined.


A
2·1x10³ ERGS/MM²
B
7·0x10³ ERGS/MM²
C
1·4x10⁴ ERGS/MM²
D
2·8x10⁴ ERGS/MM²
RADIOACTIVITY (C.P.M. x 10⁻³) —●— ³H
RADIOACTIVITY (C.P.M. x 10⁻³) --o--o-- ¹⁴C
FRACTION NUMBER

a substantial amount of cross-linkage. The results are shown in Table 21 and Figure 22. It can be seen from Figure 22, that the average number of single strand breaks per molecule increases linearly with dose up to 2 X 10^4 ergs/mm^2. In Figure 23, the surviving fraction of unbroken molecules is plotted against dose and gives a D_{37} value of 2.1 X 10^3 ergs/mm^2. In the same figure, the survival of various biological viral functions, and the fraction of molecules with no thymine dimers have been plotted for comparison purposes.

3. Effect of Caffeine on the Plaque Formation of UV Irradiated Virus :
A reduced survival for some UV irradiated bacteria and mammalian cells has been reported when cells were incubated in the presence of caffeine. A concentration of 2mM caffeine was found to produce a marked reduction in the survival of mouse L cells after UV irradiation (Rauth 1967). It has been suggested that caffeine binds to the locally denatured regions in the DNA which are induced by UV irradiation, and during subsequent DNA synthesis, abnormalities are induced in the DNA which lead to a reduced survival (Doman 1970). It was therefore of interest to examine the effects of caffeine on the plaque forming ability of UV irradiated Ad 2.

It should be pointed out that the appearance of plaques in 2 mM caffeine was different from those without caffeine. In 2 mM caffeine, the cell monolayer showed only a weak neutral red stain on the 10 th day after infection, and plaques were seen as rings of bright red stain at the periphery of the plaques.

TABLE 21

Single Stranded DNA Breakage after UV Irradiation (a)

Radiation dose (10^4 ergs/mm^2)	D_2/D_1	Fraction of ss molecules with no ss breaks	Breaks per ss mol. (b)	Breaks per ds mol.	Fraction of ds molecules with no breaks
0.2		0.54	0.6	1.3	2.9×10^{-1}
0.7		0.17	1.8	3.6	2.7×10^{-2}
1.4	0.71		3.6	7.2	7.5×10^{-4}
2.1	0.69		3.8	7.5	5.0×10^{-4}
2.8	0.62		5.5	11.0	1.7×10^{-5}

(a) An underestimate due to DNA cross-linking

(b) Rows 1 and 2 calculated using method described in C1:
Rows 3,4 and 5 calculated using method described in C2.

FIGURE 22

Average Number of Single Strand Breaks in the Viral DNA after UV Irradiation

Data from Table 22

AVERAGE NUMBER OF BREAKS PER MOLECULE
12
10
8
6
4
2
0
1
2
3
ERGS / MM^2 × 10^{-4}

FIGURE 23

Survival of Viral DNA from Molecular Damage after UV Irradiation

Replot of data from Figure 22 to show the fraction of DNA molecules containing no breaks after UV irradiation. The survival of several biological viral functions and the fraction of DNA molecules with no dimers are shown for comparison purposes.

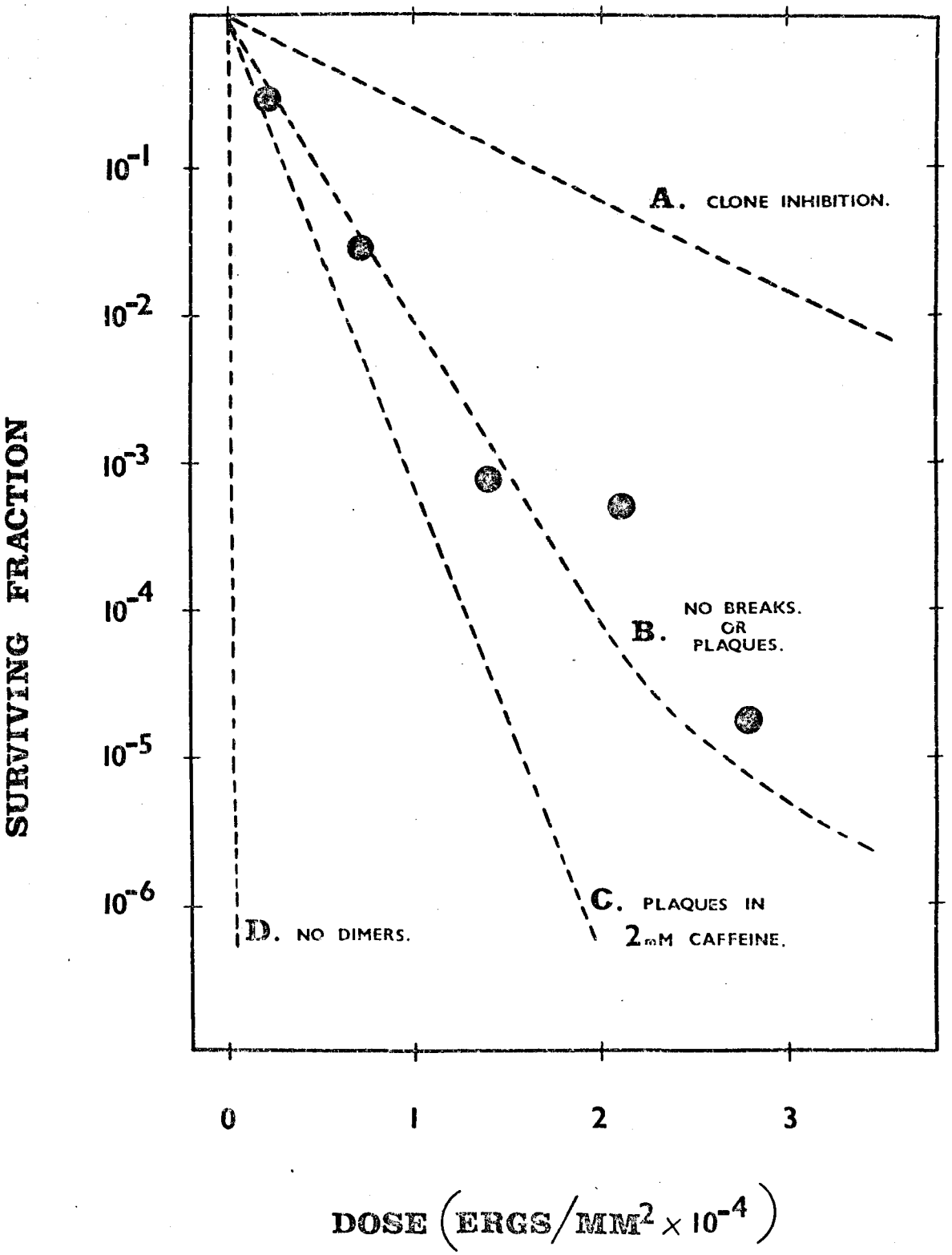
Fraction of viral DNA molecules with no single strand breaks

A — Fraction of virions capable of clone inhibition

B — Fraction of virions capable of plaque formation

C — Fraction of virions capable of plaque formation in 2 mM caffeine

D — Fraction of viral DNA molecules with no thymine dimers (extrapolated using data from Table 20)



The results from 2 pooled experiments are shown in Table 22, and the survival of plaque formation in 2 mM caffeine after UV irradiation is shown in Figure 15. It can be seen that the plaque titre for unirradiated virus in 2 mM caffeine was 35% less than the titre without caffeine. Due to the inherent uncertainty in the plaque assay, this difference is not considered significant. However, the surviving fraction of plaque formers was significantly lower for virus plaqued in the presence of caffeine. The survival of plaque formation in 2 mM caffeine after UV irradiation is consistent with an exponential inactivation with a D_{37} of 1.3×10^3 ergs/mm^2.

4. The Fate of DNA from UV Irradiated Virus after Infection : It was seen in section E, that virions containing many thymine dimers were still capable of plaque formation. It is possible that thymine dimers are not of biological importance to the expression of viral functions. Alternatively, these photolesions are biologically important, but can be repaired by the host KB cell. It was therefore considered of interest to examine whether a host mediated nicking of DNA with a subsequent repair, could be detected after infection of KB cells with UV irradiated Ad 2.

UV irradiated Ad 2, containing ^{3}H labelled DNA, was used to infect a suspension of 10^6 KB cells at an input multiplicity of several hundred PFU/cell. At various times after infection, the viral DNA from the infected cells was examined for single strand breakage on alkaline sucrose gradients as described in section C3. As shown in

TABLE 22

Effect of Caffeine of the Plaque Formation of UV Irradiated Virus

Radiation dose (10^4 ergs/mm^2)	PFU per ml.		Surviving Fraction	
	No caffeine	2mM caffeine	No caffeine	2mM caffeine
0	3.4×10^{10}	2.2×10^{10}	1	1
0.64	31.7×10^{9}	1.7×10^{8}	5.0×10^{-2}	7.7×10^{-3}
1.2		3.2×10^{6}		1.4×10^{-4}
1.6	2.1×10^{6}	3.9×10^{5}	6.2×10^{-5}	1.8×10^{-5}
2.0		1.5×10^{4}		6.8×10^{-7}

Table 23, the number of single strand breaks in the DNA of UV irradiated Ad 2 was found to increase after infection.

At the dose of 1.4×10^4 ergs/mm^2 employed, an average of 200 thymine dimers per viral DNA molecule are produced (see Table 20). Thus complete removal of dimers would require an average of 200 single strand breaks per viral DNA molecule. However, the maximum number of single strand breaks per molecule found in the viral DNA after infection was only 15.2. Consequently these results are difficult to interpret in terms of the current ideas of DNA repair mechanisms (Haynes 1966, Cleaver 1969 and Rupp 1970).

It is possible that the observed number of host mediated single strand breaks observed in the DNA from UV irradiated Ad 2 is the result of thymine dimer excision. The low number of breaks observed may mean that few dimers are excised. Alternatively, most of the dimers may be excised, but the true number of host mediated breaks in the DNA is masked by a subsequent repair of the broken ends.

The interpreation of these results is further complicated by inadequacies of the experimental technique (see discussion).

TABLE 23

Average Number of Single Strand Breaks in the DNA from UV Irradiated Ad 2 at Different Times after Infection

Time after infection (Hours)	Calculated number of s.s. breaks/ds molecule after 1.4 x 10^4 ergs/mm^2 of UV irradiation	
	Expt. I	Expt. II
0	7.6	7.4
5		7.0
18		10.8
30		15.2
44	11.2	
61	10.2	

DISCUSSION

A study of the expression of defective adenovirus particles in human cells is important to an understanding of the virus-cell interaction and bears relevance to the mechanism of viral oncogenesis. Furthermore, when the defective virus particles are induced by UV and gamma irradiation, such a study can also be used to investigate the biological effects of radiation induced DNA damage. For this reason, a discussion of this investigation falls conveniently into two sections. The first section concerns the virus-cell interaction before the introduction of radiation, whereas the second section is concerned mainly with the biological effects of UV and gamma irradiation.

A. THE EXPRESSION OF DIFFERENT ADENOVIRUS FUNCTIONS IN HUMAN KB CELLS

Infection of human cells by human adenoviruses normally results in a permissive response which leads to the production of infectious virus particles. This investigation has shown that infection of human KB cells by adenovirus can lead to an abortive response in which only a limited viral function is expressed and no infectious virus is produced. A possible consequence of the abortive type of cell response is cell transformation and the induction of tumors. The human adenoviruses represent a group of widely distributed viruses affecting large segments of the population and including serotypes

which have been shown to be oncogenic in animals. Consequently, man's natural exposure to adenoviruses is potentially of great importance in his exposure to possible oncogenic agents.

Within the group of adenoviruses, some are highly oncogenic in newborn rodents, such as Ad 12 (Green 1962), and others are not oncogenic, such as Ad 2. However, both Ad 2 and Ad 12 have been shown to produce cell transformation in tissue culture (Freeman 1967). All adenovirus-induced tumors, as well as adenovirus transformed cells, are positive for 'T' antigen, indicating the expression of a limited viral function (Trentin 1968). Furthermore, it has been suggested that expression of the whole viral genome is not necessary for cell transformation, and that viral oncogenesis is the property of a defective virus particle (Defendi 1967).

Purified preparations of Ad 2 and Ad 12 were found to contain some defective particles capable of preventing a cell from cloning, but unable to induce inclusion bodies or form plaques. The proportion of such defective particles in Ad 12 was about 10 times that in Ad 2. It is of interest to note that the particle to PFU ratios found by Green et al (Green 1967) also indicate that Ad 12 contains more non-plaque producing particles than Ad 2. These results suggest that at least some of the non-plaque producing particles are capable of clone prevention. Furthermore, it is possible that the oncogenic potential of Ad 12 is in some way related to the large proportion of non-plaque producing particles, some of which may produce tumors. Defective virus particles of other virus systems have been found to be capable of cell

transformation and tumor production (Uchida 1969).

The capacity to inhibit host cell cloning is a true viral function, resulting from expression of the viral genome, since it can be inactivated by UV irradiation. After infection of baby hamster kidney cells (BHK) by Ad 12, it was found that all cells being prevented from cloning showed 'T' antigen synthesis (Strohl 1969). This suggests that all particles capable of clone inhibition are capable of 'T' antigen synthesis. A single particle appears to be sufficient for this viral function, similar to the situation found for Newcastle disease virus (Marcus 1958).

Purified preparations of Ad 2 and Ad 12 also contained defective particles which were capable of interfering with the replication of non-defective virus particles. Again, Ad 12 contained a greater proportion of these defective interfering particles than Ad 2. These defective particles may also be capable of clone inhibition. Other non-plaque producing particles have been detected in adenovirus preparations which can act as 'helper' virus (Butal 1966).

Using CsCl density gradients, the clone inhibiting particles of Ad 12 were found to have a lower density that that of the non-defective standard virions (Mak 1970). Defective particles of SV_{40} have also been found with a lower density than that of standard virus particles (Uchida 1968). It is therefore possible that some of the defective particles are deletion mutants containing only a part of the viral genome.

Similar biologically active defective viral particles have

been described in many virus systems (Huang 1970) Specific interference by defective particles may be an important mechanism in virus-cell interactions, and have a role in determining the outcome of natural viral infections and the evolution of viral diseases.

Ad 2 and Ad 12 also differ in their plaque morphology on KB cells. For the production of a plaque, initially, one standard plaque producing particle infects a cell and produces more standard particles which then infect the surrounding cells. Occasionally, a defective particle may arise in an infected cell. Once the defective particle appears, it may begin to replicate with the standard particles and eventually defective particles are the predominant component of the viral progeny. The defective particles may then result in a termination of infection by interference and lead to a termination in the growth of the plaque. Thus the smaller plaque size for Ad 12 on KB cells may result from its ability to produce a large proportion of defective interfering particles.

The development of intranuclear inclusions is characteristic of an adenovirus infected cell (Boyer 1957). Cytochemical analyses of these inclusion bodies, based on the positive Feuglen staining feature, have been used to suggest that inclusion bodies contain a large amount of DNA (Boyer 1957 and Bloch 1957). Intranuclear inclusions have also been shown to contain viral structural antigens (Weber 1969). This is thought to indicate that virus replication is occuring in those cells showing an inclusion body.

At low input multiplicities, the formation of inclusion

bodies by adenovirus may be used for virus titration as for Shope Fibroma virus (Hodes 1968). The failure of inclusion body formation to follow single hit kinetics at high input multiplicities is thought to be due to interference by large numbers of non-plaque producing particles. This is consistent with the observation that Ad 12 infected cultures produced a lower maximum percentage of cells containing an inclusion body than Ad 2, since Ad 12 has a much higher proportion of defective particles. Furthermore, the inhibition of inclusion body formation by UV-inactivated virus lends support to this hypothesis. Interference by UV-inactivated virus has also been reported in other virus systems (Levy 1964, Watson 1950 and Watson 1952) resulting in both a reduction in the number of cells producing virus (exclusion) and a decrease in the yield of cells which can produce viral progeny.

It is not known whether the low maximum percentage of infectious centres in Ad 12 infected cultures is due to the same interference phenomenon, or due to a difference in the biological efficiency of infectious centre formation. High multiplicities of virus infection have been shown to reduce the yield of total infectious adenovirus particles (Boyer 1957 and Chany 1961) and infectious centres (Ledinko 1965). The interference may be the result of competition or a destruction of the host capacity to support virus growth by defective particles.

Defective virus particles which possess limited viral functions have also been found in other animal viruses (Haung 1966, Uchida 1968 and H ng 1970). It is most probable that the large particle to

infectivity ratios found in animal viruses (Wildy 1962) may, in part be the result of a heterogeneity of the virus particles. However, the biological inefficiency of plaque formation cannot be completely ruled out. It is possible that in a homogeneous population of virus particles, only a fraction can complete the complex process of plaque formation.

B. THE EFFECT OF RADIATION ON THE EXPRESSION OF DIFFERENT ADENOVIRUS FUNCTIONS IN HUMAN KB CELLS

Several viral functions of Ad 2 were found to be inactivated by UV and gamma irradiation. The inactivation of plaque formation by UV irradiation gave a D_{37} value of 2.1×10^3 ergs/mm^2. Similar doses of UV have been found to inactivate the plaque forming ability of several other adenoviruses (Wasserman 1962). The D_{37} for plaque formation of Ad 2 after gamma irradiation was 5.4×10^5 rads as compared to 7×10^4 rads found for Ad 5 (Ginoza 1967 and Kaplan 1964). The high value for Ad 2 probably results from the low temperature (-75°C) used during irradiation. Low temperatures have been found to reduce the indirect effects of ionising radiation as a result of the reduced yields of free radicals at low temperatures (Hart 1961).

The radiosensitivity of the viral functions of adsorption and haemagglutination were found to be the same for Ad 2 after gamma irradiation. This was thought to indicate that those viral components required for adsorption are the same as those involved in

haemagglutination, possibly the pentons and attached fibers (Norrby 1968, Petterson 1968 and Schlesinger 1969). Inactivation of viral adsorption and haemagglutination have been described for other viruses after ionising radiation (Setlow 1964b). This investigation showed that the adsorption of Ad 2 was not significantly affected by UV irradiation at the doses employed. It has also been found by other workers that doses of UV irradiation which caused a reduction of viral infectivity as measured by plaque formation, did not significantly affect viral adsorption (Gilead 1966 and Zur Hausen 1968). This is consistent with the view that UV absorption from a germicidal lamp emitting at 2537 Å, occurs in the viral nucleic acid rather than the protein coat (Jagger 1967 and Smith 1969).

The viral functions of inclusion body formation and clone inhibition were found to be more radioresistant than that of plaque formation. Assuming that the viral DNA represents the sensitive target, these results suggest that the expression of the viral functions of clone inhibition and inclusion body formation require less genetic information than the expression of plaque formation. Using target theory (Lea 1962), the fraction of the Ad 2 genome required for clone inhibition and inclusion body formation was 0.43 and 0.78 respectively, as determined by gamma ray inactivation. Similar values of 0.3 and 0.87 respectively , were obtained from UV inactivation. The different values obtained for UV and gamma irradiation may reflect the different types of molecular damage induced by these two types of radiation. Also target theory may not be applicable after UV irradiation, since

damage may not be random, or after gamma irradiation if repair mechanisms are present.

The fraction of the Ad 2 genome required for inclusion body formation is probably between 0.78 and 0.87. A complete Ad 2 genome is capable of coding for at least 9 proteins (Maizel 1969 and White 1969), so that the defective particle capable of inclusion body formation probably lacks the correct genetic expression of just one or possibly 2 late proteins. The formation of inclusion bodies has been correlated with the production of viral structural proteins (Weber 1969), although it is not clear whether mature virus progeny are present. The results of this investigation suggest that the presence of an inclusion body does not necessarily indicate that infectious progeny are produced, especially when cells are infected with irradiated virus. It is possible that the defective inclusion body forming particles are capable of producing virus progeny containing mutated protein components, which are not infectious. Alternatively, these defective particles may lack the ability to assemble the viral components into infectious progeny. Similar defective particles of SV_{40} have been described which can induce 'V' antigen synthesis but not form plaques (Uchida 1968).

The fraction of the Ad 2 genome required for the inhibition of host cell cloning is probably between 0.3 and 0.43. Available data on the fraction of the adenovirus genome required to carry out other limited viral functions is summarised in Table 24. The results of Gilead and Ginsberg (Gilead 1966) suggest that 'T' antigen synthesis

TABLE 24

Fraction of the Adenovirus Genome Required for the Expression of Several Different Viral Functions (a)

(a) Calculated assuming target theory (Lea 1962)

(b) Ratio of D_{37} values obtained for the viral function assayed and the viral function of infectivity.

Virus and type of radiation	Infectivity assay	Viral function assayed	Ratio of D_{37} values (b)	Reference
Ad 1 UV	PFU in primary human embryo kidney	Transformation of rat embryo cells	0.2	Finklestein 1969
Ad 12 UV	PFU in human embryo kidney (HEK)	Chromosome aberrations in hamster NIL	0.2-0.25	Zur Hausen 1967
Ad 12 UV	PFU in human KB	T antigen in KB	0.25	Gilead 1966
Ad 18 UV	Inclusions in human epidermoid, Hep2 or $TCID_{50}$ in HEK	T antigen in Hep2	0.1-0.5	Stich 1968
		V antigen in Hep 2	0.55	Stich 1968
Ad 2 UV	PFU in human KB	Inhibition of host KB cell cloning	0.3	This work
Gamma	"	"	0.43	"
UV	"	Nuclear inclusion body in human KB	0.87	"
Gamma	"	"	0.78	"

requires about 0.2 of the viral genome required for plaque formation of Ad 12 in human KB cells. Similar results were found by Stich et al (Stich 1968) for 'T' antigen synthesis by Ad 18 in human epidermoid cells (Hep2). These results suggest that slightly more of the viral genome is required for the inhibition of host cell cloning than for 'T' antigen synthesis.

The fraction of the adenovirus genome for the transformation of rat embryo cells was 0.2, also less than that required for the inhibition of host cell cloning (Finklestein 1969). This is to be expected since transformation results in survival of the host cell. Consequently, the clone inhibiting partcles found in adenovirus preparations and also induced by radiation, are not those defective virus particles responsible for viral oncogenesis in humans, since such particles result in death of the host cell. If a defective adenovirus particle is capable of oncogenesis in humans it would necessarily contain less genetic information than that of the clone inhibiting particles.

Ad 2 is non-oncogenic in newborn hamsters, possibly because it contains few defective particles carrying less genetic information than that of the clone inhibiting particles. Ad 12 was found to contain a large proportion of clone inhibiting particles, which suggests it may also contain other types of defective virus particles which are responsible for its high oncogenicity.

An enhancement of cell transformation and tumor induction has been observed after irradiation of other viruses (Dubbs 1968 and Defendi 1967). The results of this investigation showed that irradiation

of Ad 2 resulted in an increase in the proportion of defective particles. Also, the infection of human KB cells by UV irradiated Ad 2 resulted in various types of chromosome aberrations together with an arrest of some infected cells at metaphase. This response is characteristic of the abortive response of hamster cells after infection with adenoviruses (Cooper 1968a). Since it is this type of response which is associated with cell transformation and tumor induction, it would be very interesting to examine the oncogenic potential of UV irradiated Ad 2.

C. CORRELATION OF THE MOLECULAR DAMAGE AND THE EXPRESSION OF VIRAL FUNCTIONS AFTER GAMMA IRRADIATION

Ionising radiation has been shown to induce single and double strand chain breaks in the DNA of many different organisms (Veatch 1969). These breaks probably play a role in biological inactivation by radiation.

The production of DNA strand breakage after gamma irradiation of Ad 2 is consistent with a linear relationship with dose, which has a radiosensitivity of 0.01 double strand breaks/rad/10^{12} daltons and 0.5 single strand breaks/rad/10^{12} daltons. The production of DNA breaks by ionising radiation has been studied for several organisms (Bohne 1970, van der Schans 1970, Rupp 1970, Palcic 1970 and Lett 1970). In all the systems studied, the production of DNA breaks was consistent with a linear increase with radiation dose. The number of single strand breaks in the DNA varied from 0.2 to 3 breaks/rad/10^{12} daltons,

and the ratio of single to double strand breaks varied from 10 to 50, for the various organisms examined. Representative values for the production of radiation induced DNA breaks in several organisms are given in Table 25. The low values for the production of DNA breaks found for Ad 2, may result from the low temperature (-75°C) used during gamma irradiation. It has been shown that the indirect effects of ionising radiations are much reduced at low temperatures. The yield of radiation induced chemical reactions in ice is a factor of 3 or 4 lower than in liquid water (Hart 1961). Van der Schans (van der Schans 1970) found a reduced number of radiation induced breaks for bacteriophage in the presence of nitrogen. Other factors, such as post-irradiation treatment, may also affect the observed number of radiation induced breaks. The available data thus indicate that the radiosensitivity of DNA to single strand breakage is of the same order of magnitude in all organisms including viruses, bacteria and cultured mammalian cells.

In order to correlate the molecular damage in the viral DNA with the biological functions expressed by the virus particles, the number of radiation induced lesions per inactivation of the plaque forming ability of Ad 2, was calculated. It was found that gamma irradiation of Ad 2 in a frozen suspension medium results in an average of 4.8 single strand breaks and 0.1 double strand breaks per inactivation of the plaque forming ability of one virus particle (lethal hit). Similar correlation have been made after gamma irradiation of bacteriophages T1 (Bohne 1970) and T4 (van der Schans 1970). Correlations of biological function and radiation induced lesions are shown in

TABLE 25

Production of DNA Strand Breaks after Ionising Radiation for Several Different Organisms.

(a) D_{37} for colony forming ability in mammalian cells and bacteria, and for plaque formation in viruses.

References :

(1) Lett 1967

(2) Veatcl 1969

(3) McGrath 1966

(4) This work

(5) Bohne 1970

(6) Van der Schans 1970

(7) Van der Schans 1970

Organism and Treatment			DNA content (daltons)	D_{37} (rads) (a)	Number of breaks /rad/10^{12} daltons		Number of breaks /D_{37} organism	
					Single	Double	Single	Double
L5178Y	(1)	Suspension (saline)	5.2×10^{12}	65	1.5	-	290	-
L5178Y	(2)	Suspension (growth medium)	5.2×10^{12}	180	0.6	0.06	560	60
E. coli B/r	(3)	Suspension (growth medium)	2.8×10^{9}	1.5×10^{4}	0.2	-	10	-
Ad 2	(4)	Suspension (growth medium + serum) frozen -75°C	2.3×10^{7}	4.2×10^{5}	0.5	0.01	4.8	0.1
Phage T1	(5)	Suspension (buffer + histidine) ice	3.1×10^{7}	9.5×10^{4}	1.1	0.05	3.5	0.17
Phage T4	(6)	Suspension (buffer + 20mM guanylate) O_2	1.3×10^{8}	6.0×10^{4}	1.2	0.08	9.2	0.62
"		" N_2	1.3×10^{8}	5.0×10^{4}	0.8	0.08	5.3	0.27
Phage T7	(7)	" O_2	2.8×10^{7}	1.1×10^{5}	1.5	0.1	4.7	0.33
"		" N_2	2.8×10^{7}	9.0×10^{4}	1.2	0.04	3.1	0.1

Table 25 for several different organisms.

Although the radiosensitivity of DNA to single strand chain breakage was found to be of the same order of magnitude for all the organisms examined, the number of breaks per D_{37} was not. The number of single strand breaks per D_{37} ranged from 3 to 560, and the number of double strand breaks varied from 0.1 to 60. The above results indicate that a single break in the DNA molecule is not in itself sufficient to cause lethality.

In bacterial cells, the number of radiation induced double strand breaks is roughly equal to the number of lethal hits, except when mutants with a reduced capacity to repair single strand breaks are irradiated (Kaplan 1966). In E. coli B/r, single strand breaks are repaired to a large extent (McGrath 1966), but double strand breaks are not. These data suggest that bacterial cell death by ionising radiation is largely due to double strand breaks. According to Dean et al (Dean 1966), Micrococcus radiodurans derives its radioresistance from the capacity to repair double strand breaks. It is thought that for bacterial inactivation, single strand breaks and base damage are in general much less effective.

For mammalian cells, the number of DNA breaks per D_{37} is considerably higher than the number found in bacteria and viruses. In order to account for the large amount of DNA damage, several assumption can be made (Veatch 1969). Most of the breaks are repaired and the remaining few unrepaired cause the biological effect, or improperly joined breaks cause the biological effect. Alternatively,

most breaks occur in an inactive part of the DNA and are not lethal, whereas breaks occuring in an active part are lethal. It may also be that breaks in the DNA are not lethal in themselves, but have a certain probability of progressing to lethal damage. All of these possibilities remain to be explored.

It is interesting to note that the number of double strand breaks per D_{37} are all of the same order of magnetude for the viruses tested, ranging from 0.1 to 0.6. This suggests that radiation lesions other than double strand breaks are of biological importance in the inactivation of viruses. Other damage such as single strand breaks, base damage, cross-linking and protein damage may also play a role (Friefelder 1966).

For Ad 2, the viral coat protein was damaged after gamma irradiation as manifest by a reduced viral adsorption. However, protein damage cannot account completely for the inactivation of the plaque forming ability of Ad 2 by gamma rays. A reduced viral adsorption after ionising radiation has been reported in other systems (Watson 1952 and Setlow 1964). Protein damage has been reported in some systems after suspension of virus in a previously irradiated buffer (Heine 1968 and Watson 1952).

The production of DNA-DNA and DNA-protein cross-linkage has been correlated with other radiation induced damage after gamma irradiation of bacteriophage T1 (Bohne 1970). After irradiation of an aqueous suspension of the virus in ice, an average of 3.5 single strand breaks, 0.17 double strand breaks, 0.03 DNA-DNA cross-links and 0.06 protein-DNA

cross-links were found per phage lethal hit. This indicated that under the conditions of irradiation used, DNA-DNA and protein-DNA cross-links were of relatively little importance in viral inactivation by gamma rays.

For double stranded DNA viruses, the number of single strand breaks per lethal hit has always been found to be greater than one. This suggests that one single strand break in the viral genome does not result in lethality. This investigated ruled out the possibility of a multiplicity reactivation (Kim 1968) for Ad 2. It is possible that single strand breaks are repaired by the host cell after infection. The host mediated repair mechanism may be identical to those reported in bacterial and animal cells (Sawada 1970, Rupp 1970 and Haynes 1966). Howver, no repair of single strand breaks could be detected after infection. On the contrary, the viral DNA from irradiated virus was further degraded after infected, whereas, the DNA from unirradiated virus remained intact. This may result from a specific degradation of the radiation damaged DNA. Radiation induced DNA degradation has also been reported for some bacteria (Achey 1967 and Djordjevic 1970). From bacteria, crude extracts have been isolated which degrade X-irradiated DNA at a higher rate than unirradiated DNA .

The inability to detect repair after infection with gamma irradiated Ad 2 may result from inadequacies of the technique used. At the high input multiplicities used, repair of one viral genome may be sufficient for the commencement of viral synthesis, and one repaired molecule in several hundred would not be detected. Also, the molecular events occuring after high input multiplicities of infection, are not

necessarily those which occur during plaque formation, where only a single viral particle infects a cell, since high input multiplicities have been shown to inhibit viral multiplication.

D. CORRELATION OF THE MOLECULAR DAMAGE AND THE EXPRESSION OF VIRAL FUNCTIONS AFTER UV IRRADIATION

The biological effects produced by gamma rays and UV irradiation are often similar, but at the molecular level the effects are very different. Many different photolesions are induced in the DNA molecule after UV irradiation. Correlations have been made between the survival of UV irradiated organisms and certain types of photochemical damage in their DNA, such as thymine dimers, cross-links and single strand chain breaks. (Smith 1966, Sauerbier 1964 and Wulff 1963). The intrinsic sensitivity of DNA to alteration by UV can be affected by base composition, substitution by analogues and changes in the environment or physical state of the DNA during irradiation. Although a given photochemical lesion has been shown to be of biological importance under a certain set of conditions, it is not expected it should enjoy a similar importance under all conditions. The biological importance of any photoproduct depends not only upon whether it is formed under a particular set of conditions, but also upon whether or not the particular system under investigation is capable of repairing the lesion.

After UV irradiation of Ad 2, thymine dimers, cross-links and single strand breaks could be detected in the viral DNA.

Thymine dimers were produced linearly with dose in the low exposure range used to inactivate the plaque forming ability of the virus. At higher doses, the fraction of thymine present as dimer levels off. This levelling off is presumably due to an equilibrium being established between dimer formation and dimer reversal (Johns 1962). For bacteria, the value at which levelling off occurs, has been shown to be a function of the wavelength of the UV light and the nearest neighbour frequency for pyrimidine pairs (Wulff 1963 and Setlow 1966). The fraction of thymine present as dimer was $1.9 \times 10^{-6}/erg/mm^2$ for Ad 2 over the linear portion of the dose response curve (see Figure 20). The production of thymine dimers have been reported for a wide variety of mammalian cell lines (Klimek 1966, Klimek 1966a, Steward 1966, Trosko 1965 and Regan), bacteria (Setlow 1963, Wulff 1963 and Smith 1966) and bacteriophage (Sauerbier 1964 and Wulff 1963a). Available data for the production of thymine dimers in bacteria and mammalian cells has recently been summarised by Rauth (Rauth 1970). Representative values for several different organisms are given in Table 26.

The differences observed in the fraction of thymine present as dimer, shown in Table 26, may be due to differences in the organisms themselves or the conditions of irradiation and dosimetry. The high value shown for bacteriophage probably results from the different wavelength of UV irradiation used and a difference in the method of dosimetry. Sauerbier and Wulff used a measure of absorbed dose

TABLE 26

Production of Thymine Dimers after UV Irradiation of Several Different Organisms.

(a) V^- allele causes a UV radiosensitivity increase by a factor of 2.2 compared to V^+.
X^- allele causes a UV radiosensitivity increase by a factor of 1.67 compared to X^+.

(b) D_{37} for colony forming ability in mammalian and bacterial cells, and for plaque formation in viruses.

(c) Calculated by the author from published data.

Organism	Treatment	Nucleic acid content (daltons)	D_{37} (ergs/mm^2) (b)	Fraction of thymine present as dimer/erg /mm^{2}x10^6	Number of dimers/D_{37}	Reference
Mouse L cells	2537 Å	5.2x10^{12}	90	1.1	190,000 (c)	Rauth 1970
E.coli B/r T$^-$	2537 Å, 21°C	2.8x10^9	240	2.2	560 (c)	Smith 1966
Ad 2	2537 Å	2.3x10^7	2,100	1.9	30	This work
Bacteriophage (a)						
$T_4V^-X^-$	2600 Å	1.3x10^8	6.23	6.1	2.4	Sauerbier 1964
$T_4V^+X^+$	2600 Å	1.3x10^8	28.30	6.1	10.1	Sauerbier 1964
$T_4V^-X^+$	2600 Å	1.3x10^8	12.0	6.1	4.8	Wulff 1963

(Wulff 1963), whereas the incident dose may have been used by other workers as was the case for this investigation. Furthermore, Wulff has shown in E.coli DNA, that for the same UV exposure, the fraction of thymine molecules present as dimer increases with the wavelenth of the UV light (Wulff 1963a). Thus the radiosensitivity of DNA to thymine dimer production is of the same order of magnitude for all the organisms examined, including viruses, bacteria and mammalian cells. Measuring the production of thymine dimers in biological systems may be one way of actually measuring the absorbed dose, thus eliminating the dosimetry problems encountered with UV irradiation (Rauth 1970).

Unfortunately, simple generalisations cannot be made as to which photoproduct in DNA is the most biologically important to all organisms under all experimental situation (Smith 1969). In order to correlate the molecular damage induced in the viral DNA with the biological functions expressed by the UV irradiated virus particles, the fraction of particles with no radiation induced lesions was calculated (see Figure 23). These calculations showed that virus particles containing many thymine dimers in their DNA were still capable of clone inhibition, inclusion body formation and plaque production.

UV irradiation of Ad 2 suspended in growth medium at 10°C resulted in an average of 30 thymine dimers and one single strand DNA break per lethal hit, as measured by plaque formation. A correlation of biological function and UV induced thymine dimers has been reported for several other organisms. Representative values for the number of thymine dimers per lethal hit are shown in Table 26 for several

different organisms. For all the organisms studied, many thymine dimers were induced in the DNA per lethal hit.

Recently, there has been an increased awareness of the role played by repair systems in modifying the nature of the initial UV damage. Present evidence indicates that thymine dimers can be enzymatically excised in some systems (Setlow 1964a) and repair replication occurs using the intact strands of the DNA helix so that the double stranded region having the original base sequence is restored (Pettijohn 1964). In a final step the continuity of the repaired strand is restored by enzymatic closure of the single strand break using enzymes similar to polynucleotide ligase (Becker 1967).

After a given exposure to UV irradiation, the number of dimers present in E. coli B and E. coli B_{s-1} is the same (Setlow 1963). In E. coli B_{s-1}, 1 to 2 dimers per cell appear to be lethal (Swenson 1966) and the dimers are not removed from the DNA. However, in E. coli B several hundred dimers are produced per lethal hit and repair of these dimers occurs. Thus E. coli B, unlike E. coli B_{s-1} contains the enzyme systems capable of dimer excision and repair. Furthermore, UV irradiated phage shows a greater survival in E. coli B than in E. coli B_{s-1} (Sauerbier 1964c). This is thought to indicate the presence of host cell reactivating enzymes (HCR) in E. coli B, but not in E. coli B_{s-1}. The same enhancement of phage survival was found when UV irradiated T1 phage was plated on E. coli K12 hcr^+ and E. coli C syn^+ as compared to E. coli K12 hcr^- and E. coli C syn^- respectively (Sauerbier 1964c).

Kozinski et al (Kozinski 1967) have studied the intracellular events after infection with UV irradiated T4 phage. When the UV resistant E. coli B was infected with UV irradiated T4, single strand DNA chain breaks followed by a subsequent repair were mediated by host coded enzymes . The UV sensitive mutant E. coli B_{s-1} efficiently produced single strand breaks in the UV damaged DNA, but the process of repair was significantly hindered. However, even when repair was present, it did not restore viability to the phage. There are a number of problems which arise in correlating the existence of repair mechanisms with the survival of biological functions. The existence of a repair mechanism is usually detected from an examination of the molecular events occuring after irradiation of either a cell culture or a virus preparation. For example, the excision of thymine dimers followed by repair may be detected (Kozinski 1967 and Pettijohn 1964). Since the radiation dose used in the detection of such repair mechanisms is of sufficient magnitude to result in a much reduced biological survival, the molecular events being investigated will be predominantly those of the cell or virus population which do not survive. Consequently, the repair processes occuring in such irradiated populations may not be those which lead to biological survival.

Bacteriophage infection of cells not possessing host cell reactivation results in several thymine dimers per phage lethal hit (Sauerbier 1968). Thus the question arises as to why the majority of thymine dimers are not lethal in the double stranded DNA bacteriophage. Possibly, there exist mechanisms, different from host cell reactivation

(Sauerbier 1961) and v gene reactivation (Sauerbier 1968) wich remove thymine dimers from DNA. It has been calculated that not more than one out of three hits in bacteriophage can be due to thymine dimers (Sauerbier 1964a). Also, it has been found that the majority and possibly all, of the UV induced thymine dimer , is transfered from parent to progeny DNA, in phages T1 and T2 using host cells with or without HCR (Sauerbier 1968). Thus the high ratio of dimers to lethal hits found in bacteriophage is not caused by removal of dimers from the DNA. Possibly, thymine dimers can be bypassed during transcription and replication of the phage genome. Alternatively, at the site of thymine dimers and other UV induced DNA lesions, transcription and replication may be blocked or occur at a reduced rate. It has been suggested that for bacteriophage T4 V^-X^-, the effect of UV irradiation on transcription can account for a large amount of biological damage (Sauerbier 1970).

It was found that the presence of caffeine reduced the survival of UV irradiated Ad 2. A similar effect of caffeine has been described in many other systems (Domon 1969, Zavadova 1968 and Rauth 1970) after UV irradiation and in some cases after gamma irradiation (Sauerbier 1964c). It has been suggested (Domon 1970), that the sensitising effect of caffeine treatment after UV irradiation results from a binding of the caffeine molecule with denatured regions of the DNA which are induced by the radiation. These denatured regions are thought to occur at the site of pyrimidine dimers and other base alterations in the DNA. This binding of caffeine may inhibit repair mechanisms

(Rauth 1970). Alternatively, some localised UV lesions may only become a block to transcription and replication, when bound to caffeine. Thus the sensitising effect of caffeine may result from an inhibition of repair or an enhancement of damage.

The number of single strand breaks induced in the viral DNA after UV irradiation of Ad 2, was consistent with a linear increase with UV dose, yielding a radiosensitivity of 21 breaks/erg/mm^2/10^{12} daltons. Since the radioactivity profiles used to calculate the amount of DNA breakage did not result from random breakage alone, but also involved cross-linkage, this value is an underestimate.

Unfortunately, there is very little available data concerning UV induced single strand DNA breakage in other systems. Moroson and Alexander (Moroson 1961) found a decrease in the viscosity of native DNA after UV, suggesting chain breakage. Single strand chain breaks were detected by Marmur (Marmur 1961) from a decrease in the sedimentation constant of denatured DNA after UV irradiation of DNA suspensions obtained from several different bacteria. Marmur's results were consistent with a linear increase of single strand breaks with UV dose. From the decrease in sedimentation constant reported by Marmur after UV irradiation, the number of single strand breaks breaks per molecule was calculated using the method of Litwin et al (Litwin 1969). These calculations are shown in Table 27. In the same table, some preliminary results for UV induced single strand breaks in mouse L cells are given (Palcic 1970). The number of UV induced breaks was 13 single strand breaks/erg/mm^2/10^{12} daltons for L cells and varied from 2.7 to 6.7

TABLE 27

Production of Single Strand DNA Breaks after UV Irradiation of Several Organisms

Organism	Treatment of UV irradiation 2537 Å	Number of single strand breaks/erg/mm^2/10^{12} daltons (a)	Reference
B. subtilis	DNA in solution	2.7	Marmur 1961
Serratia marcescens	"	4.2	"
Shigella dysenteraie	"	4.2	"
D. pneumoniae	"	6.7	"
L cells	cell suspension	13.0	Palcic 1970
Adenovirus type 2	virus suspension	21.0	This work

(a) Calculated by the author from the change in S value of the DNA after UV irradiation using the method of Litwin et al (Litwin 1969)

single strand breaks/erg/mm^2/10^{12} daltons for DNA from bacteria. Although, these values are lower than the value found after UV irradiation of Ad 2, they are of the same order of magnitude.

Approximately one single strand break per lethal hit was found in the DNA of Ad 2 after UV irradiation. It is therefore possible that single strand breaks may be of importance in the inactivation of adenoviruses by UV irradiation. Gamma irradiation of Ad 2 suggested that one single strand break was not in itself sufficient to cause viral inactivation. At most only 1 in 5 single strand breaks could account for the loss of biological function. Assuming that the nature of the breaks induced by UV and gamma irradiation are similar, then single strand breaks in the viral DNA can only account for about 20% of the lethal damage. Consequently, although single strand chain breakage may play a role in the inactivation of adenovirus by UV, other types of photolesions such as thymine dimers and DNA cross-links must also be biologically important.

After infection with UV irradiated Ad 2, the number of single strand breaks in the viral DNA was found to increase. Due to several inadequacies in the technique, it is not certain whether these results are the expression of a host mediated repair mechanism (see section C3).

It was suggested by earlier workers that DNA breaks after UV doses in the biological range were not of a sufficient magnitude to to biologically important (Setlow 1966a, Setlow 1964, Jagger 1967 and Smith 1969). However, the inability to detect DNA breaks may have been due to a lack of adequate techniques. Using the techniques now available

for the detection of single strand DNA breaks (McGrath 1966 and Litwin 1969) it would be interesting to determine the radiosensitivity of DNA to UV induced single strand breaks in other organisms. The radiosensitivity of DNA to dimer production was found to be of the same order of magnitude for all the organisms examined. It is reasonable to expect that the radiosensitivity of DNA to UV induced single strand breaks would also be of the same order of magnitude in all other organisms. If this assumption is correct, then it can be calculated that the number of UV induced single strand breaks per lethal hit would be less than one for bacteriophage, but considerably greater than one for bacteria, mammalian cells and animal viruses. For this reason, it is possible that single strand chain breakage may play a role in the biological inactivation of bacteria, mammalian cells and animal viruses.

From this investigation, it is seen that the mechanisms of radiation damage and its biological expression found for Ad 2, are similar to those found in other organisms. Consequently, animal viruses are very attractive biological entities for a study of mechanisms in radiobiology.

SUMMARY

This investigation has shown that infection of human KB cells by adenovirus can lead to an abortive response in which only limited viral functions are expressed and no infectious virus is produced. The expression of several different functions of an oncogenic adenovirus (Ad 12) and a non-oncogenic adenovirus (Ad 2) were examined after infection of human KB cells. These two viruses were assayed for their ability to form plaques, nuclear inclusions and to prevent host cell cloning.

Purified preparations of Ad 2 and Ad 12 were found to contain some defective virus particles capable of preventing a cell from cloning but unable to induce inclusion bodies or form plaques. Preparations also contained some defective particles capable of interfering with the replication of non-defective standard virus. The proportion of such defectives in Ad 12 was about 10 times higher than in Ad 2. At high input multiplicities the proportion of cells showing an inclusion displayed a negative deviation from the Poisson prediction. This deviation was interpreted as due to multiple infection by non-plaque producing particles interfering with inclusion body formation, such a hypothesis being supported by the fact that infection with UV-inactivated virus inhibited inclusion body formation. It is suggested that the functional heterogeneity of Ad 12 may be related to its high oncogenicity.

Following UV and gamma irradiation, Ad 2 was assayed on human KB cells for the expression of the viral functions of adsorption, haemagglutination, clone inhibition, inclusion body formation and plaque production. It was calculated using target theory, that the fraction of the adenovirus genome required for clone inhibition is probably between 0.3 and 0.43, and the fraction required for inclusion body formation is probably between 0.87 and 0.78. These results were compared to the fraction of the adenovirus genome capable of carrying out other limited viral functions. The relevance of these finding to the current ideas in viral oncogenesis was discussed.

The irradiated Ad 2 was also assayed for DNA damage. After gamma irradiation of Ad 2 in a frozen suspension of growth medium, 0.5 single strand breaks/rad/10^{12} daltons and 0.01 double strand breaks/rad/ 10^{12} daltons were produced in the viral DNA. An average of 4.8 single strand breaks and 0.1 double strand breaks were induced per lethal hit as determined by plaque formation. This suggested that single strand breakage in the Ad 2 DNA does not generally result in lethality. Some inactivation may result from double strand breakage although other types of lesions such as protein damage, base damage and singlestrand breakage may also play a role in the inactivation of Ad 2 by gamma irradiation.

After UV irradiation of Ad 2 suspended in growth medium at 10° C, thymine dimers, cross-links and single strand breaks were detected in the viral DNA. The fraction of thymine present as dimer was 1.9×10^{-6}/erg/mm^2 over the linear portion of the dose response curve. The number of UV induced single strand breaks was 21 single strand

breaks/erg/mm^2/10^{12} daltons, and this may be an underestimate due to the technique used. An average of 30 thymine dimers and 1 single strand break were induced per lethal hit as measured by plaque formation. Consequently, although it is possible that single strand breaks play a role in viral inactivation by UV, other types of lesions such as cross-links and thymine dimers must also be important.

APPENDIX

Assay of a Viral Function

Virus preparations express a variety of different viral functions such as inclusion body formation and inhibition of host cell cloning. It is convenient to express the titre of a preparation in terms of the ability of the virus to express a particular viral function. Throughout this investigation, the following method was used in order to determine the number of functional units/ml (FU/ml) of a virus preparation.

Firstly, an assumed titre of X FU/ml is assigned to the preparation. Twofold dilutions of the virus are then made and cells are infected at different input multiplicities. The fraction of cells surviving the particular viral function under investigation is then plotted against the working input multiplicity, as in Figure 21.

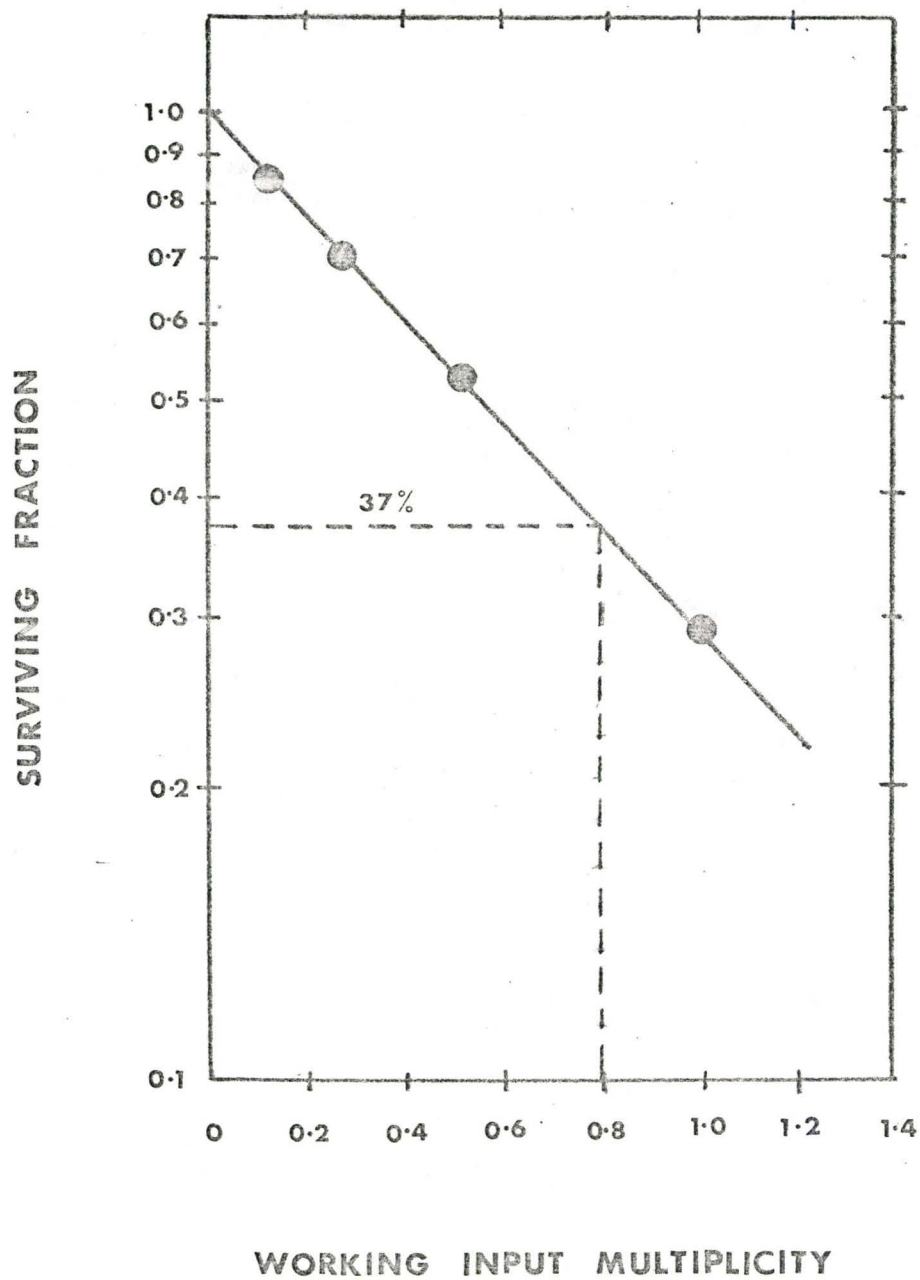
If the viral function under investigation results from infection of a single virus particle, then, from the Poisson distribution, the fraction of cells not expressing this viral function is given by:

$$\text{Surviving Fraction} = e^{-M} \qquad \text{.....(1)}$$

where M is the average number of particles, capable of expressing the viral function, infecting each cell. Thus M is the true MOI in FU/ml.

FIGURE 24

Assay of a Viral Function


SURVIVING FRACTION
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
37%
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
WORKING INPUT MULTIPLICITY

Viral functions consistent with one hit kinetics will consequently yield exponential survival curves.

From equation (1), when M = 1, then the surviving fraction of cells is 0.37. Consequently, when the fraction of cells which do not express the viral function is 0.37, then the input multiplicity is 1 true FU/ml. Experimentally, a straight line is drawn through points consistent with an exponential inactivation. From this line, the working input multiplicity giving 0.37 survivors, is extrapolated. In Figure 21, the working MOI of 0.8 corresponds to 1 true FU/ml. If the assumes titre was X FU/ml, then the true corrected titre is given by:

$$(FU/ml)_{TRUE} = \frac{(FU/ml)_{ASSUMED}}{\text{(Working MOI which gives 37\% survival)}}$$

$$= \frac{X}{0.8} = 1.25X \quad FU/ml$$

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