Studies on the Susceptibility of Ultraviolet Irradiated BHK 21 C13 Cells to Transformation by Polyoma Virus and Some Aspects of their Radiobiology.

by

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A Dissertation Submitted to the

UNIVERSITY OF GLASGOW

for the Degree of

DOCTOR OF PHILOSOPHY

Institute of Virology

September, 1968.

Summary of Thesis to be submitted to the Faculty of Science, University of Glasgow, for the degree of Doctor of Philosophy.

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The role played by viruses and radiation in the etiology of cancer is reviewed. The isolation and properties of one tumour causing virus, polyoma virus, are described.

When BHK21 C13 cells were irradiated with low doses of ultraviold light before infection with polyoma virus an enhancement of the transformation rate was observed. A two-fold rise in the proportion of transformed colonies to normal colonies was found after a radiation dose of 100 ergs/mm^2. This irradiation dose was sufficient to lower the plating efficiency of the cells by approximately 45%.

A method was developed by which the rise in the proportion of transformed cells could be more adequately observed. This method involved the delayed plating of irradiated, infected cells in agar suspension medium.

BHK21 C13 cells which were irradiated up to 24 hours before infection with polyoma virus did not show loss of the transformation enhancement. No enhancement was observed, however, when the cells were irradiated four or more hours after infection. The irradiation doses used did not significantly effect the transforming ability of the virus particles.

The results obtained when experiments were performed to test the effect of ultraviolet radiation on the interferon synthesising capacity of the cells suggested that reduced synthesis of interferon was not the mechanism by which transformation enhancement occurred.

Survival curves were determined for BHK21 C13 cells and two lines of polyoma virus transformed BHK21 C13 cells. The curves obtained were of the normal "S" form. The transformed cells were found to be more resistant to ultraviolet irradiation than the untransformed cells. All cell lines tested showed a greater resistance to ultraviolet irradiation when plated out for colony formation in the presence of mouse embryo feeder cells. Cycloheximide, an inhibitor of protein synthesis, did not significantly effect the survival curves.

Photoreactivation of ultraviolet radiation induced damage could not be demonstrated in BHK21 C13 cells. Repair of ultraviolet radiation induced sublethal damage in BHK21 C13 cells was demonstrated by a split dose technique, although the expression of this repair was

not observed until 8 hours after the initial irradiation.

Transformation of BHK21 C13 cells by ultraviolet irradiation alone could not be demonstrated.

The results are discussed with regard to a possible mechanism for the observed enhancement of transformation.

Acknowledgements

I am particularly grateful to Professor M. G. P. Stoker for the opportunity and facilities to carry out this research in the Institute of Virology and for his invaluable advice and encouragement during the course of the investigation and during the preparation of this thesis.

I also wish to thank Professor H. Subak-Sharpe for his interest and advice, Dr. J. F. Williams for his helpful comments on the text and all the other members of the Institute of Virology for their useful suggestions during the work.

I am also grateful to Dr. J. Zavada of the Institute of Virology, Czechoslovak Academy of Sciences, Czechoslovakia for the gift of a cadmium photocell, to Mr. D. Rowan and Mr. W. Stevenson of the Western Regional Hospital Board, Regional Department of Clinical Physics and Bio-Engineering, Glasgow (Director, Dr. J M. A. Lenihan) for assistance in redesigning the equipment from a basic plan which was kindly sent to me by Dr. Msena of the Czechoslovak Academy of Sciences and to Dr. J. M. A. Lenihan and his staff for building the equipment.

The encouragement of my wife and her assistance in the preparation of the figures is gratefully acknowledged.

I am indebted to Miss Dorothy Fraser for typing this thesis.

The financial support for this work was kindly provided as a research studentship of the Medical Research Council.

The experimental work reported in this thesis was carried out by the author, except when otherwise stated.

Contents

CHAPTER I

INTRODUCTION

The interaction of a mammalian cell with its environment is a highly complex and finely balanced phenomenon. If the balance is disturbed then the cell may become emancipated from the mechanisms which control normal cells. This can permit the unrestrained division and movement of cells which, in varying degrees, are characteristic of the cancer cell.

A variety of agents are known to be able to induce neoplasms in multicellular organisms. These agents include chemicals, hormones, physical irritation of tissues, parasites, radiation and viruses. The present study is concerned with the two latter causes and in particular with the carcinogenic effect resulting from the combination of the two agents.

The Virus Theory of Cancer

Tumour viruses were discovered soon after the turn of the century when Ellermann and Bang (1908) succeeded in transmitting the erythro-myeloblastic form of chicken leukaemia by cell free filtrates. Rous (1911) reported the transmission, by cell free filtrates, of the first solid tumour, a chicken sarcoma. For a long period there were no further reports of tumours which could

be transmitted by cell free material but in 1932 Shope succeeded in transmitting the rabbit fibroma and shortly thereafter (1933) the rabbit papilloma by cell free material. In 1936 Bittner reported cell free transmission of the mouse mammary cancer agent.

The next landmark in the history of tumour virology occurred when Gross (1951) reported that filtered extracts of leukaemic tissues from Ak mice would cause leukaemia in mice of other strains. This transmission was only successful if new-born mice were inoculated. This report was the start of a surge of work on viral oncology which has resulted in the discovery of many new tumour viruses.

More recent work has shown that certain viruses, for example, adenoviruses which normally infect man, can induce cancer in animals (Trentin, Yabe and Taylor, 1962; Huebner, Rowe and Lane, 1962; Girardi, Hilleman and Zwickey, 1964). Schmidt-Ruppin (1964) showed that certain strains of Rous sarcoma virus could induce tumours in adult hamsters. These results suggest that the action of tumour viruses is far from species specific.

The genetic material of viruses is known to be either Deoxyribonucleic Acid (DNA) or Ribonucleic Acid (RNA), but not

both. Viruses containing either type of nucleic acid can cause tumours although it would appear that two mechanisms are involved. There is viral replication accompanying transformation in the case of the RNA tumour viruses, whereas DNA tumour viruses either replicate in the infected cell or produce an abortive infection which may lead to transformation.

It has been estimated that the DNA of a mammalian cell could carry enough information for the synthesis of up to a million proteins if three DNA bases code for each amino acid of a protein (Crick, Barnett, Brenner and Watts-Tobin, 1961). The alteration of a normal to a neoplastic cell may require the alteration of only one, or of many, of these proteins.

The understanding of the information in the viral genome and how this information is involved in the transformation of the neoplastic cell may well be the key which will ultimately unlock the problems involved in neoplasia.

The Radiation Theory of Cancer

The induction of neoplasms by ionizing radiation was noted accidentally soon after the discovery of X-rays and radioactive

substances and the clinical observations were soon verified by experimental studies in animals. The first cancer arising in radiologists was described by Frieben in 1902). The observations in man were verified by Marie, Clunet and Raulot-Lapointe (1910, 1912) and Clunet (1910). These workers showed that sarcomas could be produced in rats by repeated administrations of X-radiation.

Extensive studies since these early reports have suggested two main steps in carcinogenesis by ionizing radiation which may work together or independently. Firstly, doses of ionizing radiation can cause considerable changes in a cell's environment which may be local or sytemic and, secondly, ionizing radiation can cause inheritable mutations to occur in individual cells. Either or both of these effects could lead to the production of a neoplasm.

An example of a change in a cell's environment can be seen in the work of Furth (1961) who reported that sustained interference of thyroid hormone production by radiation damage caused a continuous increase in production of the thyroid-stimulating hormone which gave rise to adenomas and later carcinomas of the thyroid. The effect of ionizing radiation on single cellshas been

reported by Borek and Sachs (1966) who transformed normal hamster cells in tissue culture with X-radiation. Of more interest in the current study is carcinogenesis by ultraviolet radiation. The first observation that ultraviolet light, in the form of sunlight, might be associated with skin cancer was made by Unna in 1894. Findlay (1928) found that mercury arc radiation would induce skin cancers in mice and rats and his results were confirmed by Putschar and Holtz (1930) and Herlitz, Jundell and Wahlgren (1931). In 1936, Funding, Henriques and Rekling found that only wavelengths below 3, 200Å were carcinogenic by using spectrally calibrated filters and large numbers of mice. It is of interest that both nucleic acids and unconjugated proteins absorb only wavelengths below 3, 200Å and that the main erythemal action also occurs below this wavelength.

Grady, Blum and Kirby-Smith (1943) found that repeated doses of ultraviolet light from an intermediate pressure mercury lamp produced predominantly dermal tumours in albino mice but that repeated doses of radiation from a low pressure source produced predominantly epidermal tumours (Blum and Lippincott, 1942; Kelner and Taft, 1956). These results were explained on the basis

that the chance of inducing cancer in a given tissue was related to the amount of ultraviolet radiation absorbed there.

Blum (1950, 1959) has shown that the magnitude of the ultraviolet dose and the interval between doses affect the rate of tumour development in mice. The time required to produce tumours is inversely related to the square root of the dose and directly related to the square root of the interval between doses. The results clearly indicate a cumulative character in carcinogenesis by ultraviolet light.

Although ultraviolet and X-radiations are both carcinogens they exert their biological effects by fundamentally different mechanisms. Due to the high energy of the X-ray quantum, the passage of X-rays through a cell leaves a track of ionised molecules whereas the ultraviolet quantum is only of the same order of magnitude as the energy of a chemical bond and its energy is absorbed in the excitation of individual molecules (Lea, 1946; Blum, 1959).

Synergistic effects in carcinogenesis

In recent years many workers have been concerned with the possibility that joint actions between different carcinogenic agents

may be responsible for the formation of some tumours. It would appear that viruses, chemicals and radiation can all interact with one another and in most cases produce more tumours than any of the agents alone.

Kaplan (1967) has shown that mice of strain C57 BL and other strains which show a low rate of "spontaneous" leukaemia normally harbour a latent leukaemogenic virus which is activated when the animals are exposed to appropriate doses of irradiation or to certain chemical agents. Stoker (1963a, 1964a) has found a two to three fold enhancement of the transformation rate amongst the surviving cells after a dose of X-radiation given immediately prior to infection of the cells with polyoma virus. Pollock and Todaro (1968) have shown that 3T3 and human diploid fibroblast cells also show an enhanced transformation rate if they are exposed to X-radiation prior to infection with Simian Virus (SV) 40.

Shellabarger (1967) found that when female rats were exposed to X-rays and 3-methylcholanthrene more mammary adenocarcinomas occurred than when either treatment was given alone. She suggested that the effects of 3-methylcholanthrene and X-rays were additive.

Epstein and Epstein (1962) and Epstein (1965) have clearly demonstrated that ultraviolet light carcinogenesis was significantly accelerated by prior application of a potent chemical carcinogen. The latent period was shortened, the tumours grew faster, and a larger number of tumours per mouse were produced. The type of tumour growth and the incidence of tumour-bearing mice were, however, not altered by chemical carcinogen initiation.

Rous and Kidd (1938) were among the first workers to suggest that a synergistic response could occur between tumour viruses and chemical carcinogens. They found such a response occurred with Shope papilloma virus and tar in rabbits. More recently Rawson, Roe, Ball and Salaman (1961) reported that when new-born mice were inoculated with polyoma virus and later treated with 9, 10 - dimethyl - 1, 2 - benzanthracene or with croton oil, they developed significantly more polyoma-type tumours than mice that received only the virus.

It also appears that viruses which are not known to be oncogenic by themselves can increase the incidence of chemically induced tumours. Duran-Reynals and Stanley (1961) reported that more

cortisone treated mice inoculated with vaccinia virus and treated topically with methylcholanthrene developed skin tumours than identically treated mice that were vaccinia immune. Similarly Martini, Magnusson, Gosciensk and Hansen (1961) found an increased incidence of lymphoma and myeloid leukaemia in polyoma virus free weanling mice injected with small amounts of a carcinogenic hydrocarbon or amine together with vaccinia, polio 2, Coxsackie B_4 or Echo 9 viruses.

It is unlikely, however, that all the known carcinogenic agents act by the same mechanism or that all neoplasms are caused by viruses which may be latent until activated by some other agent. For example any one chemical agent can give rise to a wide range of tumour antigens in transformed cells (Foley, 1953; Klein, Sjögren, Klein and Hellström, 1960) whereas a particular tumour virus will give rise to only one tumour specific antigen (Habel, 1961; Klein and Klein, 1962). Blum (1963) has worked out the dose response relationships for carcinogenesis by viruses, chemicals and ultraviolet light and these indicate that a different mechanism is responsible for carcinogenesis with each of the three types of agent.

Polyoma Virus

In the present study the tumour producing virus, polyoma virus, has been used and its properties are described below.

Whilst attempting to grow the Gross leukaemia virus in tissue culture, Stewart, Eddy, Gochenour, Borghese and Grubbs (1957) isolated a contaminant virus from their virus sample. This virus, designated polyoma virus, was identical to that originally described by Gross (1953) as the parotid tumour virus. It was soon realised that this virus could produce a large variety of tumours in new-born rodents, originating in both epithelial and connective tissues and appearing in many different organs and tissues. Only three years after the isolation of polyoma virus, Vogt and Dulbecco (1960) and Sachs and Medina (1961) showed polyoma virus could transform mouse and hamster cells in vitro. Polyoma virus is a member of the Papova-virus group which includes, amongst others, the rabbit papilloma virus, SV40 virus and human wart virus.

Polyoma virus has a diameter of 47 mμ (Crawford, Crawford and Watson, 1962) and consists of a protein capsid surrounding a core of DNA. The form of the viruses in the Papovavirus group

is basically icosahedral with 5-3-2 symmetry. The protein capsid probably consists of 72 capsomeres (Klug and Finch, 1965; Klug, 1965; Finch and Klug, 1965). The genome is a single molecule of double-stranded DNA, with a molecular weight of 3.0×10^6 daltons, which is arranged in a supercoiled circular form (Vinograd, Lebowitz, Radloff, Watson and Laipis, 1965).

Transformation by polyoma virus

Following the in vitro demonstration of transformation of mouse and hamster cells by polyoma virus, Stoker and Macpherson (1961) developed an assay system for quantitative estimations of the number of transformed cells occurring when cultures of hamster embryo or new-born hamster cells were infected with polyoma virus. In this technique cells were infected in suspension with polyoma virus and then plated out at a very low dilution so that individual cells grew to form colonies. These colonies could then be scored as being normal or transformed. This system, however, had several disadvantages. These cultures contained a mixed population of cells with low plating efficiency and there was a variation in transformation rate in cultures from different hamster litters (Stoker, 1962). This problem was

resolved by the isolation of a stable line of hamster fibroblasts designated BHK 21 cells (Macpherson and Stoker, 1962; Stoker and Macpherson, 1964). These cells were isolated from a culture of baby hamster kidney cells which had been in continuous culture for two months. They were composed of one morphological type of fibroblast which grew in orientated parallel sheets and showed a plating efficiency of 5-10% as opposed to approximately 1% for young baby hamster kidney cells. More than 98% of the cells were diploid (probably pseudo-diploid). The rate of cell division was rapid - during logarithmic growth the division time was approximately 12 hours.

Polyoma virus acts in two different ways depending on the host cell. In mouse cells the virus multiplies and most cells are killed. Some cells survive and it is among these that the transformed cells appear. When BHK 21 cells are infected the virus multiplies in only a few cells (Fraser and Gharpure, 1962) and there is very little cell killing. A small proportion of the surviving cells become neoplastic (Vogt and Dulbecco, 1960; Sachs and Medina, 1961). Hence the assay system, developed by Stoker and Macpherson (1961),

adapted to the BHK 21 line of cells (Macpherson and Stoker, 1962; Stoker and Abel, 1962) is an excellent system with which to study the transformation phenomenon. A disadvantage in using BHK 21 cells in studies of carcinogenesis is that these cells are, to some extent, transplantable in hamsters and fibrosarcomas may develop. When BHK 21 cells are grown for long periods of time, or deliberately exposed to selective conditions, cell lines with increased transplantability (Defendi, Lehman and Kraemer, 1963) or the ability to grow in agar (Montagnier, Macpherson and Jarrett, 1966) can be isolated. Jarrett and Macpherson (1968) have reported that this was due to a small proportion of highly tumorigenic variant cells present in populations of BHK 21 C13 cells.

An important refinement of the quantitative assay using BHK 21 cells was reported by Macpherson and Montagnier (1964) who developed a selective assay for polyoma virus transformed cells. They showed that the cells which had been transformed by polyoma virus would grow to form colonies in soft agar suspension medium, whereas the untransformed cells were unable to divide.

Abel and Crawford (1963) showed that transformation was

associated with DNA-containing particles and not with the protein capsid, suggesting that transformation was due to the viral DNA. This suggestion was confirmed by Crawford, Dulbecco, Fried, Montagnier and Stoker (1964) when they exposed BHK 21 cells to DNA extracted from polyoma virus and determined the proportion of cells growing to form colonies in soft agar. Bourgaux (1964) showed that a BHK21 cell can adsorb up to 10^5 polyoma virus particles, with about one third of these entering the cell and becoming associated with the nuclear fraction. One would therefore expect that 100% of the BHK 21 cells would be transformed with a high input multiplicity of polyoma virus. However, Stoker and Abel (1962) showed that only 5-10% of the cells become morphologically transformed, although transformation appeared to be due to the direct action of a single virus particle infecting a single cell. The explanation of this "ceiling" effect remains unknown although much investigative work has been carried out.

The low transformation frequency, even at very high input multiplicities of virus, may be related to the competence of the cells for transformation at the time of infection. Competence

may be controlled genetically, or it may derive from a transient physiological state of the cell, active around the time of infection. Studies on the genetic susceptibility of the cells by Macpherson and Stoker (1962), Sachs, Medina and Berwald (1962) and Black (1964) suggest, however, that the "ceiling" effect is not due to the presence of only a small proportion of genetically susceptible cells.

Basilico and Marin (1966) have reported that, although cell competence for transformation is not restricted to any portion of the cell cycle, the susceptibility increases with the progress of the cell through interphase, reaching a maximum in G2 cells.

One of the difficulties which occurs in these experiments is the problem of delayed transformation. Stoker (1963b) found that when single BHK 21 cells were exposed to polyoma virus and isolated in microdrops they gave rise to normal, transformed and mixed clones. The mixed clones were probably due to delayed transformation occurring up to seven or eight generations after exposure of the cell to the virus.

There have been a number of reports suggesting that the physiological state of the cells may affect the transformation

rate. Stoker and Abel (1962) showed that cells exposed to high concentrations of magnesium ions after infection gave a three to five fold increase in the transformation rate. Kisch and Fraser (1964) found that increasing the pH from 6.6 to 7.6 after infection increased the transformation frequency more than four fold. This was a direct effect on the initiation of transformation rather than a selection for transformed cells. Medina and Sachs (1963) have shown that holding infected cells at low temperature after infection increases the probability of transformation. Marin and Basilico (1967) showed that puromycin and p-fluorophenyl-alanine, both inhibitors of protein synthesis, gave a two to three fold enhancement of transformation when the cells were exposed to these drugs for a period of five hours starting at the end of the virus adsorption period.

The regular, orientated arrangement of normal untransformed tissue culture cells changes to a random, disorientated arrangement when cells are transformed. The change is usually completely stable and heritable and, in the case of polyoma virus and BHK 21 cells, polyoma virus particles cannot usually be detected in the

transformed cell lines. Not all the information that is carried in the polyoma genome appears to be necessary for transformation. Basilico and Di Mayorca (1965), Benjamin (1965) and Latarjet, Cramer and Montagnier (1967) have reported that the ability of a polyoma virus particle to complete a vegetative cycle of growth was more sensitive to inactivation by ultraviolet irradiation, X-irradiation or γ -irradiation than its ability to cause transformation. Latarjet et al (1967) deduce from this that transformation requires only about one-fifth of the viral genome needed for plaque formation. These results could, however, also be explained if multiplicity reactivation occurred in the infected cells.

There is some evidence that at least part of the viral DNA persists in transformed cells. This does not appear to occur by a method analogous to the lysogenic state of prophage in the bacterial cell, since treatments known to induce mature phage particles in lysogenic bacteria did not induce the formation of mature polyoma virus particles from transformed mouse cells (Vogt and Dulbecco, 1962). However, Gerber and Kirschstein (1962) showed that tumour cells containing no extractable virus

yielded SV40 virus when grown in contact with susceptible monkey cells. Virus specific complement fixing and transplantation antigens do persist in transformed cells (Sjögren, Hellström and Klein, 1961; Habel and Eddy, 1963; Habel, 1965) in which viral antigen has not been detected (Habel and Silverberg, 1960). The presence of these new antigens is often taken as evidence that the viral genome persists in transformed cells.

The most convincing demonstrations that at least some of the viral DNA persists in the transformed cell have come from the use of DNA/RNA hybridisation techniques (Hall and Spiegelman, 1961; Gillespie and Spiegelman, 1965). Benjamin (1966) found a small fraction of radioactive pulse-labelled RNA, from virus-free polyoma-transformed cells, which hybridised with polyoma virus DNA. The amount of RNA detected represented approximately one to five polyoma DNA equivalents per cell. More recently, Westphal and Dulbecco (1968) determined the amounts of viral DNA present in polyoma- and SV40-transformed cell lines by hybridising the cellular DNA with in vitro RNA complementary to viral DNA. They found five to seven viral DNA equivalents in the case of polyoma

transformed cell lines. They also found that the viral DNA was located in the nuclei of the cells.

Cells infected with polyoma virus show a stimulation of cellular DNA synthesis. This was demonstrated independently in three laboratories with contact inhibited mouse cells infected with polyoma virus (Dulbecco, Hartwell and Vogt, 1965; Weil, Michel and Ruschman, 1965; Winocour, Kaye and Stollar, 1965). Gershon, Hausen, Sachs and Winocour (1965) showed that polyoma virus could induce the synthesis of cellular DNA in X-irradiated rat embryo cells and that the cells almost doubled their DNA content before the DNA synthesis stopped. Nitrous acid viral inactivation experiments (Gershon, Hausen, Sachs and Winocour, 1965) showed that the inactivation curves for transformation and for the DNA synthesis inducing capacity for polyoma virus were identical, again suggesting that only about one-fifth of the viral genome was necessary for both transformation and induction of DNA synthesis. Sachs (1967) has suggested that an efficient carcinogen would be one which could induce both a change in the control mechanism of the cell, and the replication of the cellular

constituents to permit fixation of the transformation. It seems likely that integration of all or part of the viral genome would be most likely to occur during the replication of the cellular DNA.

The effect of ultraviolet radiation on mammalian cells

The present study is concerned with the interaction of ultraviolet radiation and polyoma virus in the transformation of mammalian cells. Some of the major effects of ultraviolet radiation on mammalian cells are reviewed below.

Although a great deal of work on the ultraviolet irradiation of bacteria and other micro organisms has been carried out, relatively few studies have been made on mammalian cells. It has been well established that there is a loss of proliferative capacity of mammalian cells exposed to ultraviolet light and that this is dependent on the phase of the cell cycle occupied by the cells at the time of irradiation. Eriksson and Szybalski (1963) using the D89/AG cell line derived from human sternal marrow, Sinclair and Morton (1965) using the V79 line of Chinese hamster cells, and Rauth and Whitmore (1966) using the L-60T line of L cells, all showed that the survival of cells after ultraviolet irradiation

decreased after mitosis, reached a minimum early in S and then increased to a maximum in late S or early G2.

Rasmussen and Painter (1964) and Cleaver (1965, 1966a) have studied the dose response curve for the inhibition of DNA synthesis by ultraviolet light and they have found that it is in the form of a biphasic curve. Cleaver (1965) found that the major cause of the inhibition in the rate of DNA synthesis in L-cells was the effect of ultraviolet light on the assembly of nucleotide triphosphates into DNA. He also found that the rate of DNA synthesis after irradiation with 240 ergs/mm^2 of ultraviolet light fell to a minimum after two hours and then began to recover. At higher doses however the rate of DNA synthesis fell to a very low level after 3 hours and no recovery was detected.

It is now well established that one of the main biological effects of ultraviolet radiation in mammalian cells is the production of pyrimidine dimers (Trosko, Chu and Carrier, 1965; Klímek, 1966). The number of dimers formed is dose dependent and at low doses (between 0 and 200 ergs/mm^2) the presence of these dimers does not completely block DNA synthesis in the irradiated cells although

such cells show a pronounced decrease in their ability to synthesize DNA immediately after irradiation (Klímek and Vlašínová, 1966). Cleaver (1967) has estimated that the efficiency of dimer formation at 2,537Å is approximately one dimer in 700 microns of DNA per erg per square millimeter. Klímek (1966) using L-cells and Trosko, Chu and Carrier (1965) using Chinese hamster cells could not detect dimer excision into the soluble fraction, implying that dimer excision as an expression of repair of radiation damage, analogous to dark repair in Escherichia coli, was not present in these cells. More recently, however, Regan, Trosko and Carrier (1968) have shown that ultraviolet irradiated human cells lost approximately 50% of the pyrimidine dimers from the DNA within 12-24 hours after irradiation. These dimers appeared in the TCA soluble fraction of the irradiated cells within 24 hours.

Rasmussen and Painter (1964, 1966) have reported a type of DNA synthesis in mammalian cells that was stimulated by the presence of 5-bromodeoxyuridine in the DNA molecule, was not semiconservative and occurred in cells that were not in the normal DNA synthetic phase. Djordjevic and Tolmach (1967) confirmed this result when they found ultraviolet radiation induced "unscheduled" DNA synthesis in G_1 and G_2

HeLa cells. Cleaver (1968) has shown that skin fibroblasts cells from patients with the rare hereditary disease, xeroderma pigmentosum, carry a mutation causing repair replication, of the type described above, to be completely absent or much reduced in comparison with normal human skin fibroblasts. Patients with xeroderma pigmentosum are extremely sensitive to sunlight or ultraviolet light and exposure to sunlight leads to the development of skin tumours.

In strains of E. coli lacking the dark repair mechanism, pyrimidine dimers act as blocks to DNA replication (Setlow, Swenson and Carrier, 1963). Dendy and Smith (1964) and Dendy, Smith and Aebi (1967) showed that the rate of DNA synthesis in mouse L-cells could be reduced when a micro-beam of ultraviolet light was directed into the medium surrounding the cells. This effect was due to the production of a peroxide and could be prevented if the cells were incubated in catalase for at least 4 hours before irradiation. At high doses of radiation the protection was only partial.

Photoreactivation of ultraviolet radiation damage in DNA is

usually absent from mammalian cells (Cleaver, 1966b) under conditions in which it can be demonstrated in many micro-organisms (Jagger, 1958; Blum, 1959). However, Rauth (1967) has reported a caffeine-sensitive repair system for ultraviolet damage in L cells. This effect was similar to the dark reactivation repair system reported in certain bacterial strains (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964).

Perry, Hell and Errera (1961) reported that two thirds of the labelling of cytoplasmic RNA in interphase cells is suppressed after irradiation of the nucleoli with an ultraviolet microbeam. De Maeyer-Guignard and De Maeyer (1965) and Burke and Morrison (1966) found an inhibition of RNA synthesis in ultraviolet irradiated cells. Interferon production was depressed at the same radiation doses which depressed DNA and RNA synthesis. This loss of interferon producing ability with increasing ultraviolet dose showed first order kinetics (Cogniaux-Le Clerc, Levy and Wagner, 1966; Burke and Morrison, 1966). Klímek and Vlašínová (1967) have shown, however, that the inhibition of DNA synthesis in ultraviolet irradiated cells was not due to the inhibition of RNA synthesis.

Protein synthesis appears to be little affected by ultraviolet radiation. Errera, Hell and Perry (1961) reported that protein synthesis was decreased as a result of inhibition of nuclear RNA synthesis by ultraviolet radiation. Takeda, Naruse and Yatani (1967) and Klímek and Vlašínová (1967) however, have reported that there was no significant effect on protein synthesis after ultraviolet irradiation of HeLa and L cells.

Ultraviolet light has been reported to inactivate various enzymes including succinate dehydrogenase (Barber and Ottolenghi, 1957), choline dehydrogenase (Ottolenghi, Bernheim and Wilbur, 1955), ATPase (Beyer, 1962), tyrosinase and acid phosphatase (Seiji and Iwashita, 1965).

Many of the effects that ultraviolet radiation has on mammalian cells are superficially similar to the effects of ionizing radiation. However it must be remembered that the passage of the X-radiation is non-specific and the high energy of the X-ray quantum can affect any part of the cell. Ultraviolet radiation will affect only those cellular constituents which have a high specific absorption for the particular wavelength used. In the case of a wavelength of 2, 537Å, the nucleic acids probably absorb most of the energy.

Purposes of the present study.

Stoker (1963a and 1964a) reported that when hamster cells were exposed to X-radiation prior to infection with polyoma virus the surviving cells were more sensitive to transformation. He also found that when irradiation was delayed for 24 hours after infection, this increased sensitivity to transformation still existed. Furthermore, this sensitivity to transformation remained high for two days after irradiation. More recently, Pollock and Todaro (1968) have found a similar enhanced sensitivity to transformation when 3T3 or human diploid fibroblast cells were exposed to X-radiation prior to infection with SV40 virus.

The primary aim of the present study has been to determine if irradiation of BHK 21 cells with ultraviolet light caused an increased sensitivity to transformation with polyoma virus, such as that observed with ionising radiation.

A secondary aim of the study was to investigate various aspects of the ultraviolet radiobiology of the polyoma virus-BHK 21 C13 cell system in an attempt to elucidate the role that radiation might play in the increased sensitivity to transformation. Stoker (1964a) and

Pollock and Todaro (1968) have suggested that the increased sensitivity to transformation in their systems was due to a direct action of X-radiation on the cells and the latter workers have suggested that if transformation depends on the integration of the viral genome into the host cell then transformation might be favoured if viral DNA was present during repair of the host cell DNA. In the light of these suggestions an investigation was carried out to determine if repair occurs in irradiated BHK 21 cells.

CHAPTER II

MATERIALS

1. Biological Materials

(a) Virus

The polyoma virus used throughout the present study was a small plaque strain derived from the Toronto strain, by plaque purification, by Diamond and Crawford (1964).

(b) Tissue culture cells

The tissue culture cells used in the experiments described in this Thesis were initially obtained from the Cytology Department of the Institute of Virology with the exception of 4n BHK 21 cells which were kindly supplied by Dr. S. Revell of the Chester Beatty Research Institute, London.

Cultures of whole mouse embryo cells were obtained as monolayer cultures in 80 oz. rotating bottles (House and Wildy, 1965).

BHK 21 C13 cells were a continuous line of hamster fibroblasts described by Macpherson and Stoker (1962).

Py H3 and Py H6 cells were clones of BHK 21 C13 cells which had been independently transformed by polyoma virus. These

clones were isolated by Professor M. Stoker from colonies growing in agar suspension culture.

4 n BHK 21 cells were a near tetraploid line of BHK 21 cells isolated by Dr. S. Revell of the Chester Beatty Research Institute, London.

(c) Enzymes

Trypsin was obtained from Difco Laboratories, Detroit, U.S.A.

Receptor Destroying Enzyme (RDE) was prepared by the method of House (1967). Its activity was determined by the method of Busby, House and Macdonald (1964).

(d) Calf sera and horse sera were sterilised by Millipore filtration.

(e) Tryptose phosphate broth, Bacto-Agar, Noble Agar and PPLO Agar were obtained from Difco Laboratories, Detroit, U.S.A.

(f) Antibiotics were obtained from Glaxo Laboratories, Greenford, England.

(g) Amino-acids and vitamins were obtained from the Koch-Light Laboratories, England.

(h) Brain heart infusion broth powder, sabouraud fluid medium base,

blood agar base powder and yeast extract were obtained from Oxoid Limited, London, England.

2. Composition of Media

The modification of Eagle's medium (Eagle, 1959) described by Busby, House and Macdonald (1964) was used for cell culture.

Tryptose phosphate broth was prepared by dissolving 147.5 g. of tryptose phosphate broth in distilled water to give a final volume of 5,000 ml. 20 ml amounts were autoclaved and checked for sterility.

ETC growth medium was composed of 80% Eagle's medium, 10% calf serum and 10% tryptose phosphate broth.

Cell Storage Medium was composed of 60% Eagle's medium, 10% tryptose phosphate broth, 25% calf serum and 5% glycerol.

DEAE dextran/Noble Agar was prepared by mixing 1 ml 0.5% DEAE dextran, 25 ml 4.8% Noble Agar and 5 ml calf serum with 75 ml Eagle's medium at 1.3 times the normal concentration and lacking phenol red.

Saline was composed of 0.9% W/V sodium chloride in distilled water.

Phosphate buffered saline (PBS) pH 7.4 had the composition described by Dulbecco and Vogt (1954).

Tris-saline (TS) had the composition described by Smith, Freeman, Vogt and Dulbecco (1960).

Versene, 0.02%, was prepared as a 0.02% solution in PBS lacking $CaCl_2$ and $MgCl_2.6H_2O$ and containing 0.0015% Phenol red.

Trypsin, 0.25%, was prepared as a 0.25% solution in TS, sterilised by Millipore filtration through a 0.22μ membrane and stored at -20°C.

Trypsin/versene solution consisted of one volume of trypsin to four volumes of versene giving a final concentration of 0.05% trypsin.

Neutral red stain was prepared as a 0.02% solution in TS.

ETC agar was composed of 60% Eagle's medium at 1.3 times the normal concentration plus 10% calf serum, 10% tryptose phosphate broth and 20% of 2.5% Difco Bacto agar. This gave an agar concentration of 0.5%.

EH agar was composed of 75 ml Eagle's medium at 1.3 times the normal concentration plus 2.5 ml horse serum and 25 ml of 3.6% Difco Bacto agar.

CHAPTER III

METHODS

This chapter describes procedures which have been used routinely throughout the present study. Special methods will be described in the relevant chapters.

1. Cell culture techniques

(a) Cell lines

The BHK 21 C13 clone of hamster cells, the two lines of polyoma virus transformed BHK 21 C13 cells, designated PyH3 and PyH6, and 4n BHK 21 cells were stored routinely in frozen stock suspensions. Cultures of cells which were just confluent were checked, by the method described by Fogh and Fogh (1964), to ensure that they were not contaminated by mycoplasma. They were then trypsinized with trypsin/versene solution and suspended in cell storage medium. Aliquots of the cell suspension, in small screw capped vials, were slowly cooled to -60°C in a deep freeze. For more permanent storage, some aliquots were sealed in glass ampoules and stored in a liquid nitrogen refrigerator. Cells were recovered by thawing rapidly in a water bath at 37°C.

The recovered cells were seeded into 8 oz. baby bottles with

10 ml ETC. The bottles were then flushed with 5% CO_2 in air and incubated at 37°C. When the resulting monolayer became confluent the cells were trypsinized with trypsin/versene solution and either sub-cultured into further baby bottles or resuspended in ETC for use in an experiment. Cell lines were recovered routinely every 14 days, so that no cells were used which had been in culture for more than two weeks from the time of recovery.

(b) Primary mouse embryo cultures

Minced near term mouse embryos were treated with 0.25% trypsin in Tris saline to give a suspension of cells. The cell suspension was washed in warm PBS and aliquots, each containing 2×10^8 cells, were added to 200 ml ETC in 80 oz. Winchester bottles. These bottles were flushed with 5% CO_2 in air and placed at 37°C on a roller culture rack (House and Wildy, 1965).

The cells grew and became confluent in about 5 days. They were then harvested by treatment with trypsin/versene solution and sub-cultured to give secondary cultures. Cells from the secondary cultures were used for plaque assays or in the production of feeder cells.

(c) Mouse embryo feeder cells.

The use of X-irradiated cells to assist colony formation by isolated cells in the same culture dish was first described by Puck and Marcus (1955). In the present study, feeder cells were prepared by the method of Stoker and Macpherson (1961). Secondary mouse embryo cells were irradiated with 1,700 r. of unfiltered X-rays from a Watson-type Mobilix X-ray apparatus. This apparatus was used at 81 kV and 3 mA and delivered 3.9 r/sec. at a target to cell distance of 12.7 cm. Following irradiation, the cells were centrifuged and resuspended in fresh ETC.

Feeder cultures were seeded with 10^5 cells 24 hours prior to their use. Alternatively, feeder cells were added to ETC agar so that 1.5 ml of the cell suspension in ETC agar contained 1×10^5 cells. All cultures in Petri dishes were incubated at 37°C in a humidified incubator flushed with 5% CO_2.

2. Production of polyoma virus.

(a) Virus growth and extraction

Polyoma virus, in an unpurified state, was prepared by a procedure based on those used by Crawford (1962) and Bourgaux (1964).

The medium was removed from newly confluent secondary mouse embryo monolayers in 80 oz. Winchester bottles and 10 ml of a polyoma virus suspension containing approximately 10 plaque forming units per cell was added to each bottle and the virus particles were allowed to adsorb to the cells. After the virus adsorption period, 200 ml of ETC was added to each bottle. The bottles were returned to the roller culture rack and incubated at 37°C for 5-7 days.

After 5-7 days, the cells were harvested and the resulting suspensions of cell fragments were placed at 4°C for 12 hours to allow any virus in the medium to attach to the cells. The suspension was then centrifuged at 4°C for 5-10 minutes at 2, 000 r. p. m. and the supernatant discarded. After checking that the pellets were free of bacterial contamination, they were pooled and resuspended in 10 ml of TS. The cells were disrupted by 3 cycles of freezing and thawing, centrifuged at 2, 000 r. p. m. for 10 minutes and the supernatant removed. Each pellet was resuspended in 10 ml TS and 2. 5 ml RDE and the suspension incubated at 37°C for 24 hours. The suspension was then centrifuged as before and the supernatant removed and stored at 4°C. The pellet was resuspended in 10 ml TS

and incubated at 37°C for a further 24 hours. The suspension was again centrifuged and the supernatant removed. This cycle was continued until the haemagglutination assay (see below) showed that the amount of virus remaining associated with the cell debris was too small to warrant further extraction.

(b) Haemagglutination Assay

The amount of virus in the RDE extracts and TS washes was estimated by assaying for haemagglutinating activity. Samples of the virus preparations were diluted and heated at 37°C for 15 minutes to release into the medium any virus adsorbed to the cell debris. Aliquots of 0.2 ml were further diluted in serial 2-fold steps in PBS at 4°C in perspex haemagglutination trays. Aliquots of 0.2 ml of a 1% suspension of guinea pig erythrocytes in PBS were added to each virus dilution and the trays were placed at 4°C for 3 hours. At the end of this period the end point was recorded. This was the dilution of the virus which caused agglutination of approximately half of the red cells.

(c) Density Gradient Centrifugation

The crude virus extracts were further purified by a method based

on that described by Crawford, Crawford and Watson (1962). The supernatants containing the crude virus extracts were pooled and centrifuged in a Spinco Model L ultracentrifuge with a 30 rotor at 30,000 r.p.m. for 3 hours to pellet the virus. The pellets, which contained most of the virus, were resuspended in 0.02M Tris HCl buffer (pH 7.5) containing $MgCl_2$ (5mM). A solution of caesium chloride was prepared by dissolving 4.8 g of caesium chloride in 7.8 ml Tris buffer (.0.05M pH 8). The gradient was prepared by layering 1 ml aliquots of the virus suspension onto 3 ml aliquots of the caesium chloride solution in Spinco SW39 tubes. This gave a final density of 1.3 g/ml. The tubes were centrifuged in a SW39 rotor at 10°C for 24 hours at 30,000 r.p.m. At the end of the centrifugation the gradient was at equilibrium and the virus was present as 2 bands - the lower band consisting of intact polyoma virus particles and the upper band consisting of particles lacking nucleic acid (Crawford, Crawford and Watson, 1962). Cell debris remained at the top of the tube. The lower band of "full" particles was collected, diluted in PBS solution A and centrifuged at 30,000 r.p.m. for 3 hours to pellet the virus. The supernatant, containing

the caesium chloride, was discarded and the virus pellet resuspended in PBS solution A. Samples were taken to measure the haemagglutination titre and the number of plaque forming units in the virus suspension. The remaining virus suspension was stored at -20°C in 1 ml aliquots.

(d) Plaque Assay

The number of plaque forming units in polyoma virus suspensions was determined by the method described by Dulbecco and Freeman (1959). Secondary mouse embryo monolayers were prepared and inoculated with 0.2 ml of a polyoma virus suspension. The infected monolayers were incubated at 37°C for 2 hours to allow the virus to adsorb to the cells. The plates were shaken at intervals to ensure an even spread of the virus over the cell sheet. After the incubation period, 6 ml of EH agar overlay were added and the cultures incubated at 37°C. On the 4th and 8th day of incubation 3 ml of EH agar overlay were added. On the 17th day 3 ml of neutral red stain were added and the plates incubated at 37°C overnight. Excess neutral red was then removed and the cell sheet fixed with formol saline. The agar overlay was shelled out and the plaques counted. One plaque forming unit (pfu) was the amount of virus which gave rise to one plaque.

3. Ultraviolet Irradiation

(a) Sources of ultraviolet radiation

Two types of bactericidal lamps were used as ultraviolet radiation sources. For a few preliminary experiments a 24 inch low pressure mercury vapour discharge tube with a Vycor envelope was used (Englehard Hanovia Lamps, Slough, England: Model 13A, Tube Type 3). This lamp operated at 30 watts and 45 mA and was used at a set tube to target distance of 40 cms. The dose at the target was 26 ergs/mm^2/sec.

For all other experiments a 12 inch low pressure mercury vapour discharge tube with a liquid filter jacket (Englehard Hanovia Lamps: Model 16, Tube Type 1) was used. The tube was mounted on an aluminium back plate and this was attached to a bench stand so that the dose rate could be varied by raising and lowering the tube relative to the target. The lamp operated at 15 watts and 30 mA. The incident dose at 40 cms was 17 ergs/mm^2/sec.

In both ultraviolet radiation sources 85% of the total energy output was at 2,537Å.

(b) Measurement of ultraviolet dose

A photometer was built to measure the incident dose. It was based on a cadmium photocell type 329 TUMC/B Cd V No. OD/0481 made by Pressler, East Germany. This photocell had a narrow wavelength specificity with a maximum output at 2, 537Å. The circuit diagram and the components of the photometer are shown in Fig. 1 and Table 1.

The photometer was calibrated by a uranyl-oxalate actinometer (Rose, 1961) and by inactivation curves of bacteriophage T2H. I am grateful to Dr. D. Ritchie of this department for carrying out the latter method of calibration.

The ultraviolet sources were checked at regular intervals to ensure that there was no deterioration in the output of the radiation. The sources were always switched on 30 minutes before any irradiations were performed to allow the radiation output to stabilize. Care was taken to ensure that the sources and reflectors were kept clean, as dust, grease etc. can lower the amount of energy emitted by a lamp.

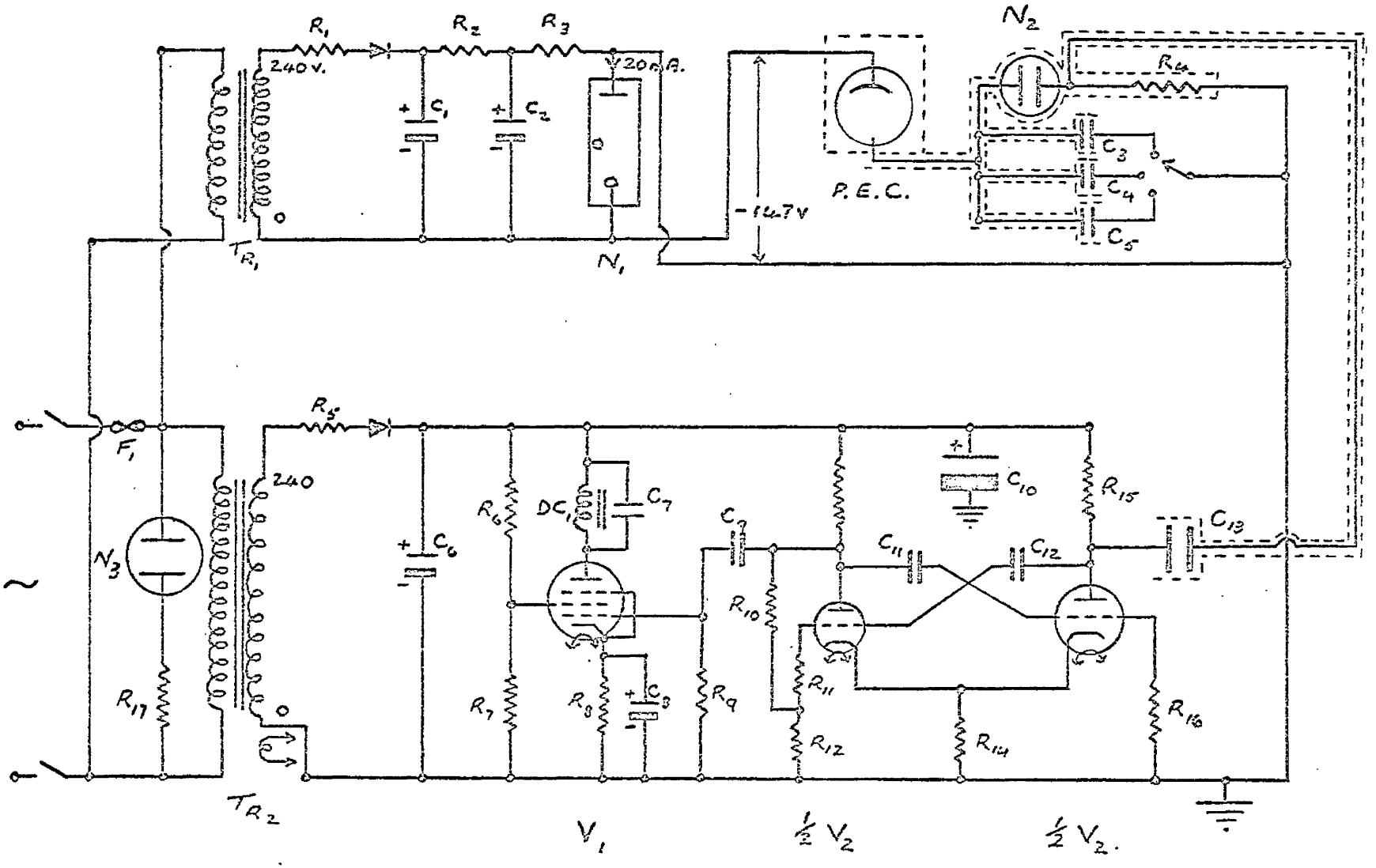

FIGURE 1. Circuit Diagram for the UV Photometer.

Resistances

R_1 = 22 Ω.
R_2 = 1 KΩ.
R_3 = 6.8 KΩ. 5 Watt
R_4 = 1.8 M. Ω.
R_5 = 22 Ω.
R_6 = 100 KΩ.
R_7 = 100 KΩ.
R_8 = 5.6 KΩ.
R_9 = 1 M. Ω.
R_{10} = 1 M. Ω.
R_{11} = 4.7 M. Ω.
R_{12} = 150 KΩ.
R_{13} = 47 KΩ.
R_{14} = 15 KΩ.
R_{15} = 47 KΩ.
R_{16} = 100 KΩ.
R_{17} = 220 KΩ.

Capacitors

C_1 = 16 μ. F. Electrolytic
C_2 = 16 μ. F. Electrolytic
C_3 = 2, 200 p. F.
C_4 = 0. 01 μ. F.
C_5 = 0. 1 μ. F.
C_6 = 50 μ. F. Electrolytic
C_7 = 1, 000 p. F.
C_8 = 50 μ. F. Electrolytic
C_9 = 0. 1 μ. F.
C_{10} = 2 μ. F. Electrolytic
C_{11} = 0. 02 μ. F.
C_{12} = 2 μ. F.
C_{13} = 0. 01 μ. F.

Valves

V_1 = 6 BW6
V_2 = 12 AU7

Neons

N_1 = 0 A2 or 15002
N_3/N_2 = Strike <150 v.

Photo Electric Cell

Pressler Type 329 TUMC/BCdV
Cadmium No. OD/0481

Transformers

TR_1 = 0/250 V Secondary
TR_2 = 0/250 V Secondary + 6. 3 v/1 Amp.

Decade Counter

DC_1 : V_{DC} = 98
R = 1 KΩ.

Fuse

F_1 1 Amp Anti-surge

Table 1. Components of UV photometer.

(c) Ultraviolet irradiation of cells

An appropriate number of cells (the number being dependent on the requirements of the experiment) were plated out into 60 mm glass Petri dishes with 5 ml ETC. The cells were incubated at 37°C in a humidified incubator flushed with 5% CO_2 for about 16 hours. This permitted the cells to stick to the glass, spread out on the glass and start to divide. After the incubation period, the medium was removed and the cells washed twice with PBS. As much as possible of the washing medium was removed and the cells were then irradiated. After irradiation the cells were washed twice - the washing medium being dependent on the following treatment. For example, if the cells were to receive no further treatment, the washing medium was ETC.

Cells which were to be used as unirradiated controls were treated in an identical way to the irradiated cells with the exception that they were not exposed to the ultraviolet radiation.

4. Infection of BHK 21 C13 cells with polyoma virus

As BHK 21 C13 cells are markedly aggregated by polyoma virus the cells were infected by the method described by Stoker (1963a).

Sparse monolayers of BHK 21 C13 cells were washed twice with TS plus 2.5% calf serum and infected by adding 0.2 ml of a polyoma virus suspension to each plate. This suspension was diluted from a stock solution with TS plus 2.5% calf serum to give an approximate input multiplicity of 600 pfu/cell. Uninfected controls were mock infected by the addition of 0.2 ml TS plus 2.5% calf serum. All the plates were incubated at 37°C for 1 hour to allow the virus to adsorb to the cells. During this incubation period the plates were shaken at 15 minute intervals to ensure an even spread of the virus over the monolayers.

After infection the monolayers were washed twice in ETC and 5 ml of ETC were added to each plate. The plates were then incubated at 37°C for 4 hours. This incubation period markedly reduced the rate of cell clumping due to the presence of the virus. This method of infection gave a somewhat lower transformation rate (Stoker, 1963a) than infection of cells in suspension but, on trypsinization of the monolayers, a cell suspension was obtained which usually consisted of over 90% single cells.

5. Plating of cells for colony formation

(a) Plating on glass

(a) Plating on glass

Cells were plated out for colony formation on glass by the method described by Stoker and Abel (1962).

Cells were trypsinized with trypsin/versene solution, suspended in ETC, and were then counted in a Hawksley Cristalite Improved Neubauer haemocytometer (the number of cell clumps were also counted to ensure that the majority of the cells were in a single cell suspension). A minimum of 200 cells in at least 2 haemocytometer chambers were counted for each estimate of the total cell number in any given cell suspension. After the total number of cells in a suspension had been counted, they were diluted in ETC to give a suspension containing the appropriate number of cells per ml for plating out for colony formation. For unirradiated BHK 21 C13 cells plated on feeder cells this number was 200 cells/ml ETC. One ml of the cell suspension, together with 4 ml ETC, were then plated per 60 mm glass Petri dish. Unless otherwise stated in the text, the cells were plated onto 10^5 mouse embryo feeder cells (Section 1(c)).

The cultures were then incubated at 37°C for 7-8 days before being fixed with formol saline and stained with Giemsa stain. Colonies were then counted and scored for transformation.

(b) Plating in agar

Cells were plated out for colony formation in agar suspension medium by the method described by Macpherson and Montagnier (1964).

Cells were trypsinized, counted and diluted as described in section 5(a). The cultures were prepared by pipetting 5 ml of ETC agar into 50 mm plastic Petri dishes, allowing the agar to solidify and then adding the experimental cells in a 1.5 ml layer of ETC agar - this layer having a final concentration of 0.3% agar.

When mouse embryo feeder cells were used in agar suspension cultures they were plated out on top of the basal layer of ETC agar. The experimental cells were plated on top of this feeder layer.

Agar suspension cultures were incubated at 37°C for 10 days in a humidified incubator flushed with 5% CO_2 in air.

6. Definitions in Transformation Assays

The definitions used in this study are based on those used by Stoker and Abel (1962).

Three basic parameters have been used in the measurement of transformation: the plating efficiency of the infected cells, the observed transformation rate per cells plated, and the observed transformation rate per total colonies counted. The absolute rate of transformation cannot be estimated directly as it would have to be expressed as the proportion of transformed to total cells in the original population exposed to the virus. As it is not possible with present techniques to obtain a plating efficiency of 100%, the actual number of newly transformed cells after infection with polyoma virus is unknown and therefore the absolute transformation rate cannot be measured.

The plating efficiency (PE%) is defined as the total colonies developing per total cells plated, expressed as a percentage.

The observed transformation rate per total cells plated (t per cell) is defined as the number of transformed colonies per total cells plated, expressed as a percentage.

The observed transformation rate per total colonies counted (t per colony) is defined as the number of transformed colonies per total colonies observed, expressed as a percentage.

The expression t per colony is useful in that it allows for differences in plating efficiency that occur, for example, when irradiated and unirradiated, infected cells are compared with regard to transformation. However, as there is no evidence that the plating efficiency of newly transformed cells is the same as that of normal cells, the value of t per colony may not be a reliable indication of the actual transformation rate.

The enhancement factor (Stoker, 1964a) has been taken as the ratio of the transformation frequencies (either t per colony or t per cell) of irradiated and unirradiated cells. This factor has been used as a measure of any change occurring in the transformation rate due to ultraviolet irradiation.

The results of transformation assays have been plotted as log (irradiated result/unirradiated result) against irradiation dose. The errors shown represent twice the standard error (2 x SE) of the mean for each result. The standard error of the mean has been calculated as the standard error of individual observations divided by the square root of the number of measurements that went into the measurement of this standard error. The value of 2 x SE gives approximately 90-95% confidence limits.

CHAPTER IV

RESULTS

I. Transformation Studies

1. The susceptibility of ultraviolet irradiated BHK 21 C13 cells to transformation by polyoma virus.

(a) Introduction

Stoker (1963a, 1964a) showed that when BHK 21 C13 cells were exposed to X-radiation immediately prior to infection with polyoma virus, the surviving cells were more sensitive to transformation. His results suggested that the increased sensitivity was due to a direct action of the X-radiation on the cells. More recently Pollock and Todaro (1968) have obtained similar results using SV40 with 3T3 and human cells. It seemed possible that ultraviolet (UV) radiation might also cause BHK 21 C13 cells to become more sensitive to transformation by polyoma virus. Experiments were performed to test this hypothesis.

(b) Transformation of irradiated, polyoma virus infected BHK 21 C13 cells plated on glass

Transformation of UV irradiated BHK 21 C13 cells was studied by scoring the number of transformed colonies which were produced when UV irradiated cells which were infected with polyoma virus were plated out with feeder cells in glass Petri dishes.

Table 2 and Figure 2 show the results of one experiment and Table 3 and Figure 3 the pooled results from a series of five experiments. The results are plotted in both figures as log (irradiated result/ unirradiated result) against irradiation dose. Twice the standard error is shown for each point. In the individual experiments the standard errors were derived from the variation between different plates in the experiment. Where results were pooled, the standard errors represent the variation between different experiments. The errors were large, due to the low transformation rate obtained by the method used and the errors increased at higher doses of UV radiation due to the decrease in the number of surviving colony forming cells. The results showed a significant rise in t per colony, the observed transformation rate per total colonies counted. There was a rise in t per cell, the observed transformation rate per total cells plated, which was just significant in Figure 2 but lost its significance when the results were averaged out in Figure 3. The maximum enhancement factor for t per colony, which is the ratio of the t per colony of the irradiated cells to the t per colony of the unirradiated cells was 2.1 at a 45% drop in plating efficiency. The

UV dose (secs. exposure to 26 ergs/mm^2/sec.)	No. of plates	P.E. % ± 2 x S.E.	t per colony ± 2 x S.E.	t per cell ± 2 x S.E.
0	8	44.3 ± 1.2	2.06 ± 0.67	0.91 ± 0.3
2	8	35.7 ± 1.2	4.13 ± 0.69	1.46 ± 0.23
4	8	20.1 ± 1.4	6.85 ± 1.22	1.38 ± 0.31
6	7	9.5 ± 0.6	3.79 ± 0.85	0.36 ± 0.08
8	8	3.5 ± 0.3	2.88 ± 2.36	0.10 ± 0.07

Table 2. Results from a single experiment showing an enhanced transformation rate by polyoma virus in BHK 21 C13 cells exposed to UV radiation and plated on glass.

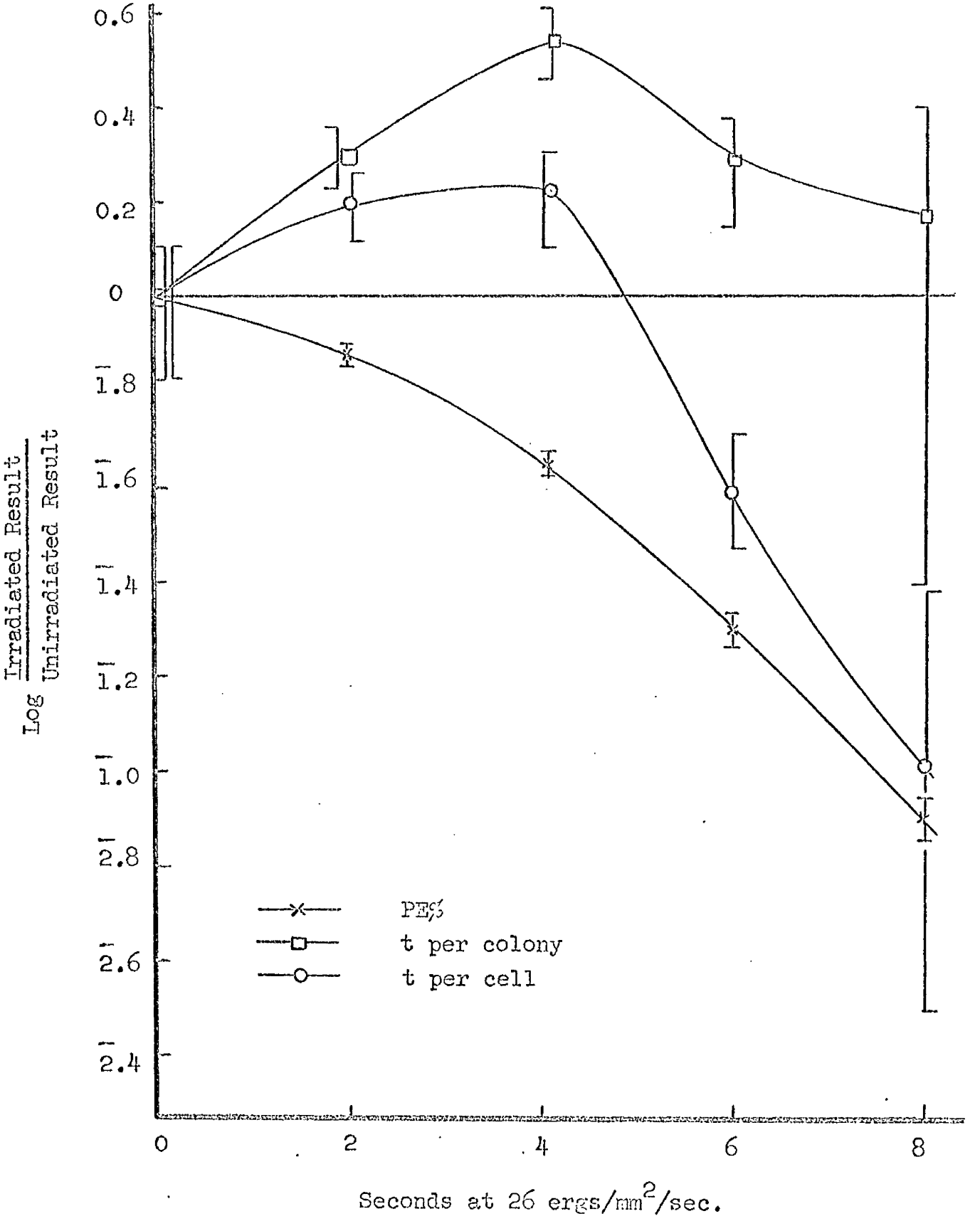

FIGURE 2. Result of a Single Experiment to Show an Increased Susceptibility of UV Irradiated Cells to Transformation by Polyoma Virus.

UV dose (secs. exposure to 26 ergs/mm^2/sec.)	P. E. % ± 2 x S. E.	t per colony ± 2 x S. E.	t per cell ± 2 x S. E.
0	45.7 ± 2.2	2.36 ± 0.33	1.08 ± 0.15
2	37.4 ± 2.8	3.52 ± 1.36	1.31 ± 0.2
4	27.2 ± 10.39	4.85 ± 1.59	1.26 ± 0.4
6	14.9 ± 7.9	4.23 ± 2.67	0.61 ± 0.46
8	12.1 ± 10.6	3.18 ± 0.39	0.38 ± 0.3

Table 3. Pooled results from 5 experiments showing an enhanced t per colony in BHK 21 C13 cells transformed by polyoma virus after exposure to UV radiation and plated on glass. The errors have been calculated from the means of the 5 experiments.

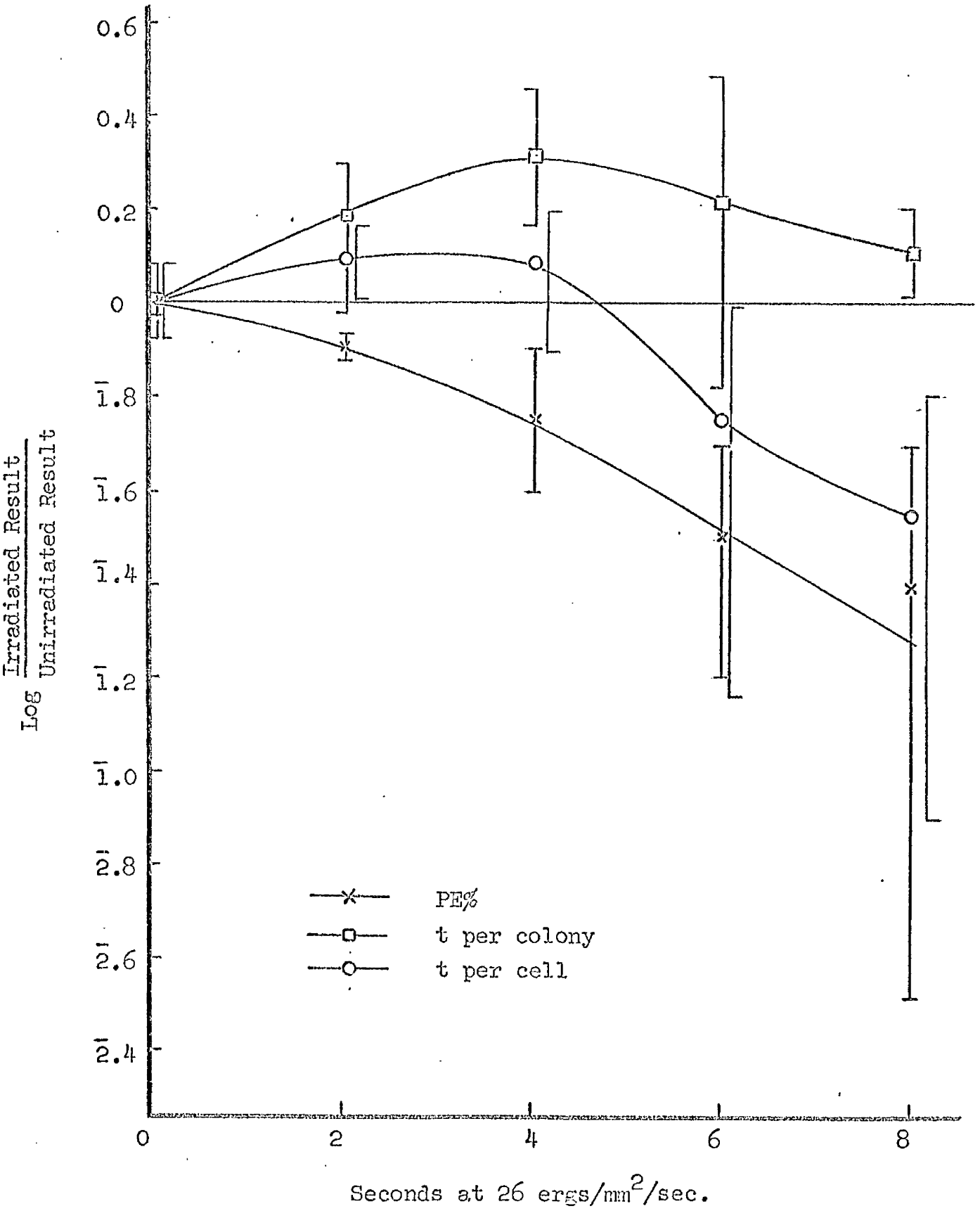

FIGURE 3. Pooled Results of 5 Experiments to Show an Increased Susceptibility of UV Irradiated Cells to Transformation by Polyoma Virus.

maximum enhancement factor for t per cell was 1.25 at a 15% drop in plating efficiency.

It therefore appeared that prior irradiation of BHK 21 C13 cells with UV light could enhance their susceptibility to transformation by polyoma virus, at least with respect to t per colony. Stoker (1963a, 1964a) also found that there was an increase in the t per colony values, but did not report any increase in the value for t per cell when the cells were X-irradiated prior to infection with polyoma virus. The value for t per cell is of importance in transformation enhancement studies as a rise in the t per cell value over that for the control cultures would show an absolute rise in the transformation rate. A rise in the value for t per colony over that for the control cultures only shows however, in the experiments discussed above, that more of the surviving cells gave rise to transformed colonies.

(c) Transformation of irradiated, polyoma virus infected BHK 21 C13 cells plated in agar.

Only a small percentage of polyoma virus infected BHK 21 C13 cells, plated out on glass, give rise to transformed colonies. The remaining cells give rise to normal colonies. Therefore, only a few

transformed colonies can be obtained on each plate because of the limitation in the total number of cells which can be plated to give well separated and identifiable colonies. Using the agar suspension technique (Macpherson and Montagnier, 1964) many more transformed colonies can be scored in individual cultures because the normal cells do not grow and so larger numbers of cells can be seeded. This allows greater precision in the assay.

When UV irradiated cells were infected with polyoma virus and plated on glass the main effect noted was a doubling of the value for t per colony (see previous section). Any absolute rise in the number of cells transformed was not always significant. Because of the increased precision of the agar suspension technique, experiments using this technique were performed in an attempt to investigate whether the rise in t per cell on glass was significant. As normal cells are unable to grow in agar, values for t per colony cannot be obtained directly. Irradiated cells, infected with polyoma virus, were therefore cultured using the agar suspension technique. To allow for cell killing, increasing numbers of cells were plated with increasing UV radiation doses. Increasing the cell number did not affect the results.

The rise in t per cell shown in Table 4 was again barely significant after 2 seconds irradiation (52 ergs/mm^2). Thereafter, the value for t per cell fell much more rapidly than might be expected from the data obtained by growing the cells in glass Petri dishes. The data again suggested that there might be a small rise in t per cell when the cells were infected with polyoma virus after low doses of UV radiation.

(d) <u>The effect of delaying the plating of irradiated, polyoma virus infected BHK 21 C13 cells in agar</u>.

The results in Table 4 suggested that there was little advantage to be gained by plating irradiated, infected cells directly into agar suspension medium. Experiments were carried out to investigate the effect of permitting the irradiated, infected cells to "recover" at 37^{o}C before being plated into agar suspension medium.

Sparse monolayers of approximately 10^5 irradiated BHK 21 C13 cells infected with polyoma virus were incubated at 37^{o}C for 24, 48, 72 and 96 hours before the cells were plated out in agar suspension medium without feeder cells. The medium was changed on all plates every 24 hours. Any plates which had not been used by 48

UV dose (secs. exposure to 26 ergs/mm^2/sec.)	No. of dishes	No. of infected cells plated/ dish	Total No. of colonies	Average No. of colonies/plate	Average t per cell $\pm$ 2 x S. E.
0	10	2×10^4	1184	118.4	0.59 ± 0.03
2	10	3×10^4	2179	217.9	0.73 ± 0.03
4	9	4×10^4	208	20.8	0.05 ± 0.01
6	10	5×10^4	98	9.8	0.02 ± 0.01

Table 4. Results of an experiment in which BHK 21 C13 cells were exposed to UV radiation and infected with polyoma virus and plated in agar suspension medium. No colonies were observed in the uninfected control plates.

hours were subcultured to ensure that the cells did not become fully confluent. This precaution was taken to ensure that contact phenomena did not affect the results as Stoker (1964b) has shown that hamster cells transformed by polyoma virus are inhibited from growing when in contact with stationary normal cells. As a control, cells were plated out 4 hours after infection (see Table 4).

The results are shown in Table 5 and Figure 4. They show that there was little change from the 4 hour pattern (Table 4) when the cells were plated out after 24 hours incubation. There was a small rise in the t per cell after 2 seconds irradiation (52 ergs/mm^2) which was barely significant in some experiments but not significant in others.

When the cells were plated out after 48 hours incubation on glass the slight rise in t per cell had completely vanished. The number of colony forming cells in the plates containing the irradiated cells remained approximately the same but the number of colony forming cells in the plates containing the unirradiated control cells had increased. After 72 hours incubation on glass the number of colonies in all the samples had decreased but the drop in the number of colonies was much

No. of hours incubation at 37°C	UV Dose (seconds exposure to 26 ergs/mm^2/sec.)	No. of cells plated	Total No. of colonies counted (10 plates)	t per cell ± 2 x S.E.
24	0	10^4	1861	1.86 ± 0.07
	2	10^4	1986	1.99 ± 0.08
	4	10^4	1273	1.27 ± 0.05
	6	10^4	643	0.64 ± 0.04
48	0	10^4	2474	2.47 ± 0.05
	2	10^4	1786	1.79 ± 0.07
	4	10^4	1149	1.15 ± 0.05
	6	10^4	627	0.63 ± 0.07
72	0	10^4	356	0.36 ± 0.02
	2	10^4	430	0.43 ± 0.02
	4	10^4	354	0.35 ± 0.01
	6	10^4	290	0.29 ± 0.03
96	0	10^4	207	0.21 ± 0.01
	2	10^4	392	0.39 ± 0.02
	4	10^4	170	0.17 ± 0.01
	6	10^4	105	0.105 ± 0.01

Table 5. The effects of incubating monolayers of UV irradiated, polyoma virus infected BHK 21 C13 cells for up to 96 hours at 37°C before plating into agar without feeder cells.

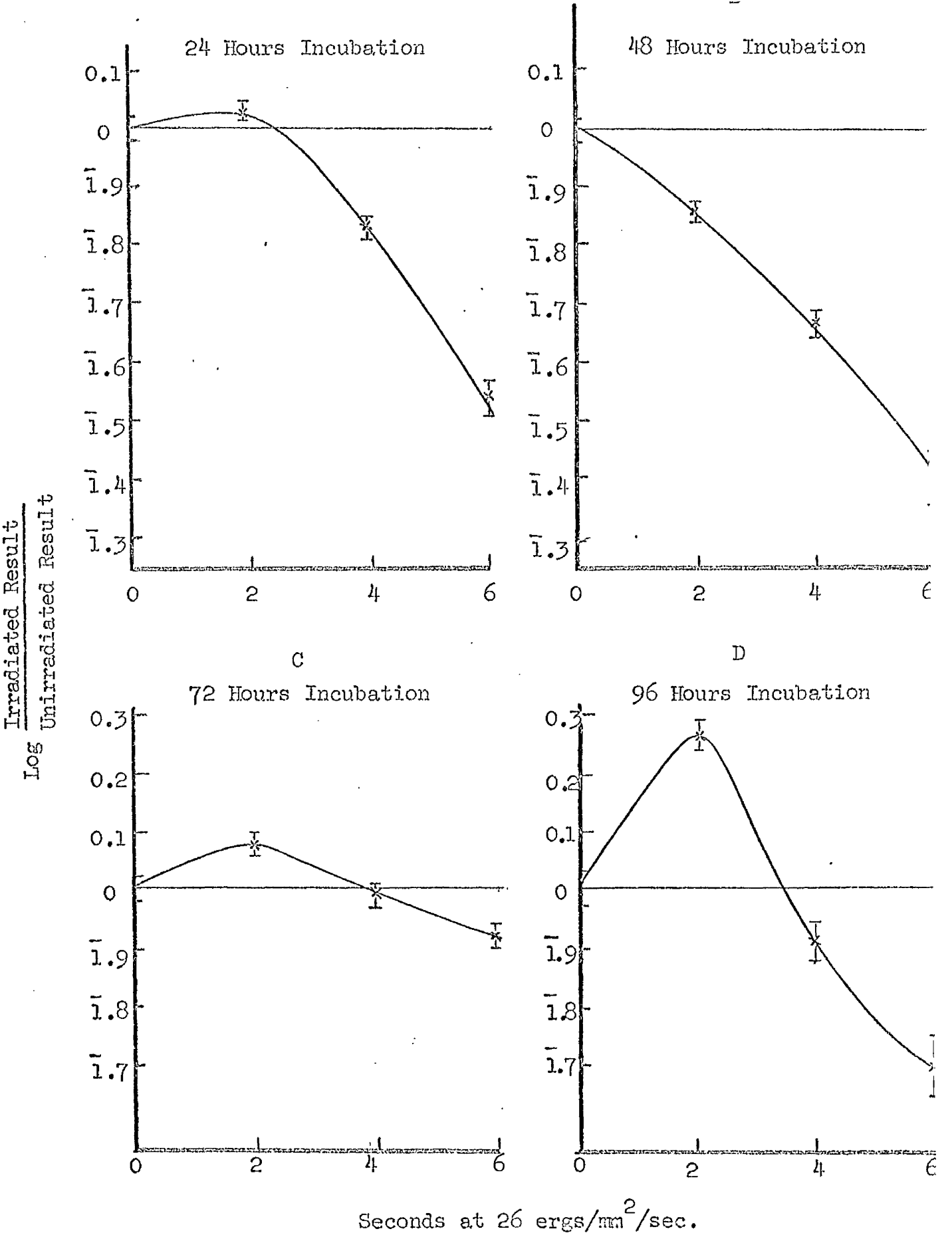

FIGURE 4. The Effect of Incubating Irradiated, Infected Cells at 37°C on Glass before Plating in Agar (—×— = t per cell)

more pronounced in the unirradiated plates. After 96 hours incubation on glass the number of colonies in all the samples was still smaller but the value after 2 seconds irradiation (52 ergs/mm^2) in all experiments worked out to be about double that in the unirradiated controls.

(e) <u>The effect of delaying the plating of irradiated polyoma virus infected cells in agar with feeder cells.</u>

Irradiated, polyoma virus infected cells were also plated in agar suspension medium in the presence of feeder cells in order to investigate whether an enriched agar suspension medium would affect the period before which the enhancement effect was seen in agar without feeder cells.

The results obtained are shown in Table 6 and Figure 5. Instead of the slight rise in t per cell observed 4 hours after two seconds irradiation (52 ergs/mm^2) when the cells were plated into agar suspension medium without feeder cells, there was a drop in the t per cell value from that obtained for the unirradiated controls. After 24 hours incubation this drop in the value for t per cell was not as pronounced as after 4 hours incubation. When the cells had

No. of hours incubation at 37°C	UV Dose (seconds exposure to 26 ergs/mm^2/sec.)	No. of cells plated/plate	Total No. of colonies counted (10 plates)	t per cell ± 2 x S.E.
4	0	10^4	1945	1.74 ± 0.04
	2	10^4	1265	1.26 ± 0.06
	4	10^4	450	0.45 ± 0.03
	6	10^4	373	0.37 ± 0.09
24	0	10^4	2094	2.1 ± 0.08
	2	10^4	1983	1.98 ± 0.13
	4	10^4	1389	1.39 ± 0.06
	6	10^4	1229	1.23 ± 0.06
48	0	10^4	476	0.48 ± 0.05
	2	10^4	789	0.79 ± 0.05
	4	10^4	285	0.28 ± 0.04
	6	10^4	128	0.13 ± 0.02

Table 6. The effect of incubating monolayers of UV irradiated, polyoma virus infected BHK 21 C13 cells for up to 48 hours at 37°C before plating into agar with 10^5 feeder cells.

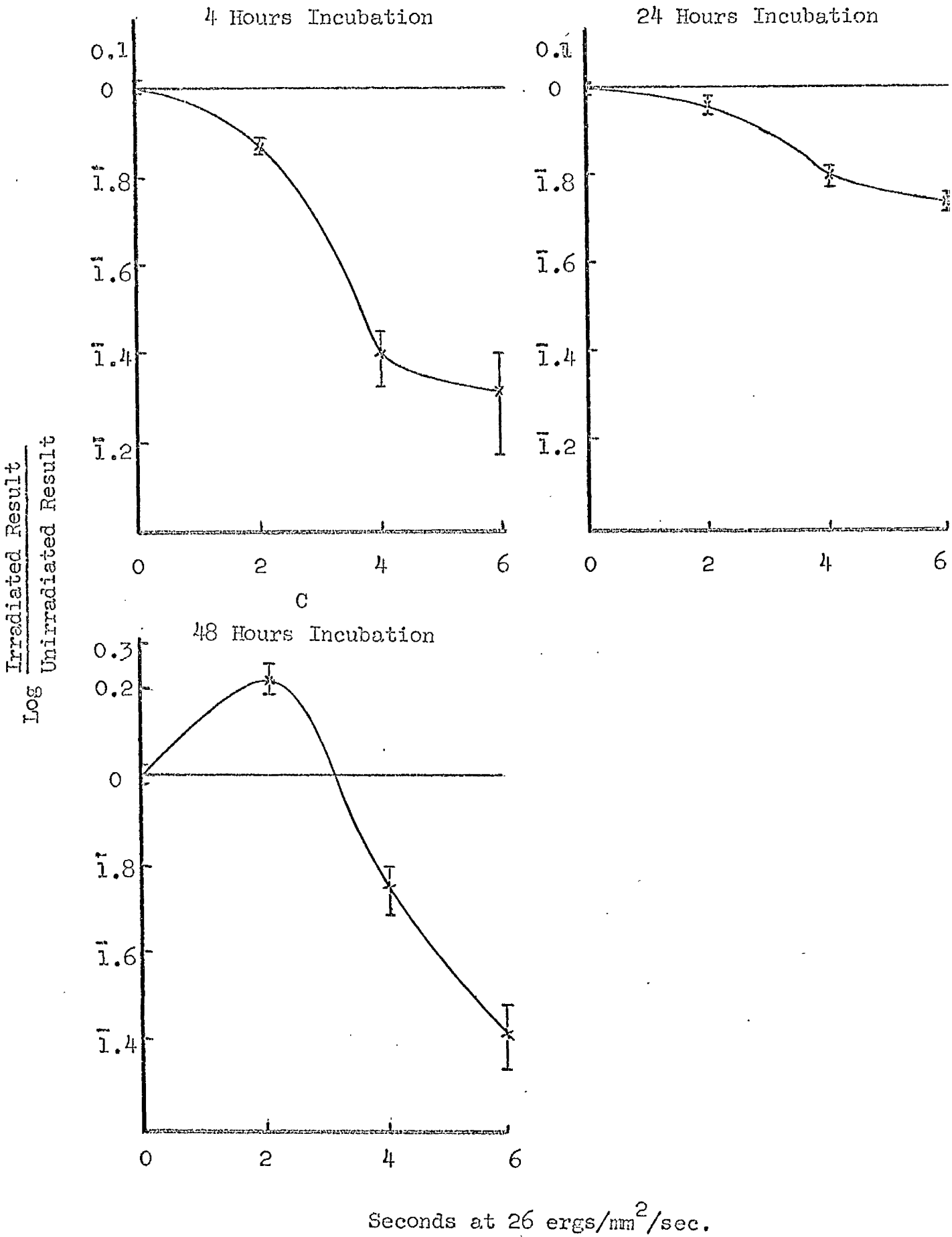

FIGURE 5. The Effect of Incubating Irradiated, Infected Cells at 37°C on Glass before Plating in Agar with Feeder Cells (—×— = t per cell)

been incubated at 37°C for 48 hours before being plated in agar, there was a marked rise in the value for t per cell compared to that for the unirradiated control cells incubated for the same period of time. This rise in the value for t per cell occurred after a marked drop in the number of colony forming cells. A similar drop in the number of colony forming cells was seen after 72 hours incubation when the cells were plated in the absence of feeder cells.

(f) Characterisation of the rise in the t per cell value after delayed plating of irradiated, infected cells in agar.

The increase in the value for t per cell, compared to the value for unirradiated control cells, was only observed over a very small dose range when there was a delay before infected cells were plated in agar suspension medium.

In order to further characterise the form of the enhancement of the value for t per cell, similar experiments were performed in which the UV radiation dose was lowered from 26 ergs/mm^2/sec to 9.5 ergs/mm^2/sec and the times of exposure were varied from 0 to 12 seconds. The cells were plated into agar without feeder cells after 96 hours incubation at 37°C, or into agar with feeder cells after 48 hours incubation at 37°C.

The results when cells were plated in agar without feeder cells, after 96 hours incubation at 37°C, are shown in Table 7 and Figure 6. The results when cells were plated into agar with feeder cells, after 48 hours incubation at 37°C, are shown in Table 8 and Figure 7.

The results obtained after 96 hours incubation on glass, when the cells were plated in agar, gave a consistent two-fold rise after 6-9 seconds irradiation (57-85.5 ergs/mm^2). When the cells were plated into agar together with feeder cells after 48 hours incubation on glass the maximum enhancement was not as marked, showing only a 1.5 fold rise, although this was significantly higher than that of the unirradiated controls. The curve was less steep than that obtained in the 96 hour experiment and differences between repeated experiments were more marked.

(g) <u>The applicability of the enhancement in agar as an assay system for irradiation-induced enhancement of transformation</u>

The results showed an enhancement of transformation when cells were plated in agar without feeder cells, after incubation at 37°C on glass. They suggested that the method might work as an assay system for irradiation induced enhancements of transformation. However, it

UV Dose (seconds exposure to 9.5 ergs/mm^2/sec.)	No. of cells plated/plate	Total No. of colonies counted in 3 experiments (30 plates)	Average t per cell + 2 x S.E.
0	10^4	1686	0.56 ± 0.05
3	10^4	1962	0.65 ± 0.06
6	10^4	3316	1.11 ± 0.08
9	10^4	3366	1.12 ± 0.1
12	10^4	1508	0.50 ± 0.05

Table 7. Characteristics of the enhancement of polyoma virus transformation in UV irradiated BHK 21 C13 cells after incubation of the cells for 96 hours at 37°C before plating into agar without feeder cells.

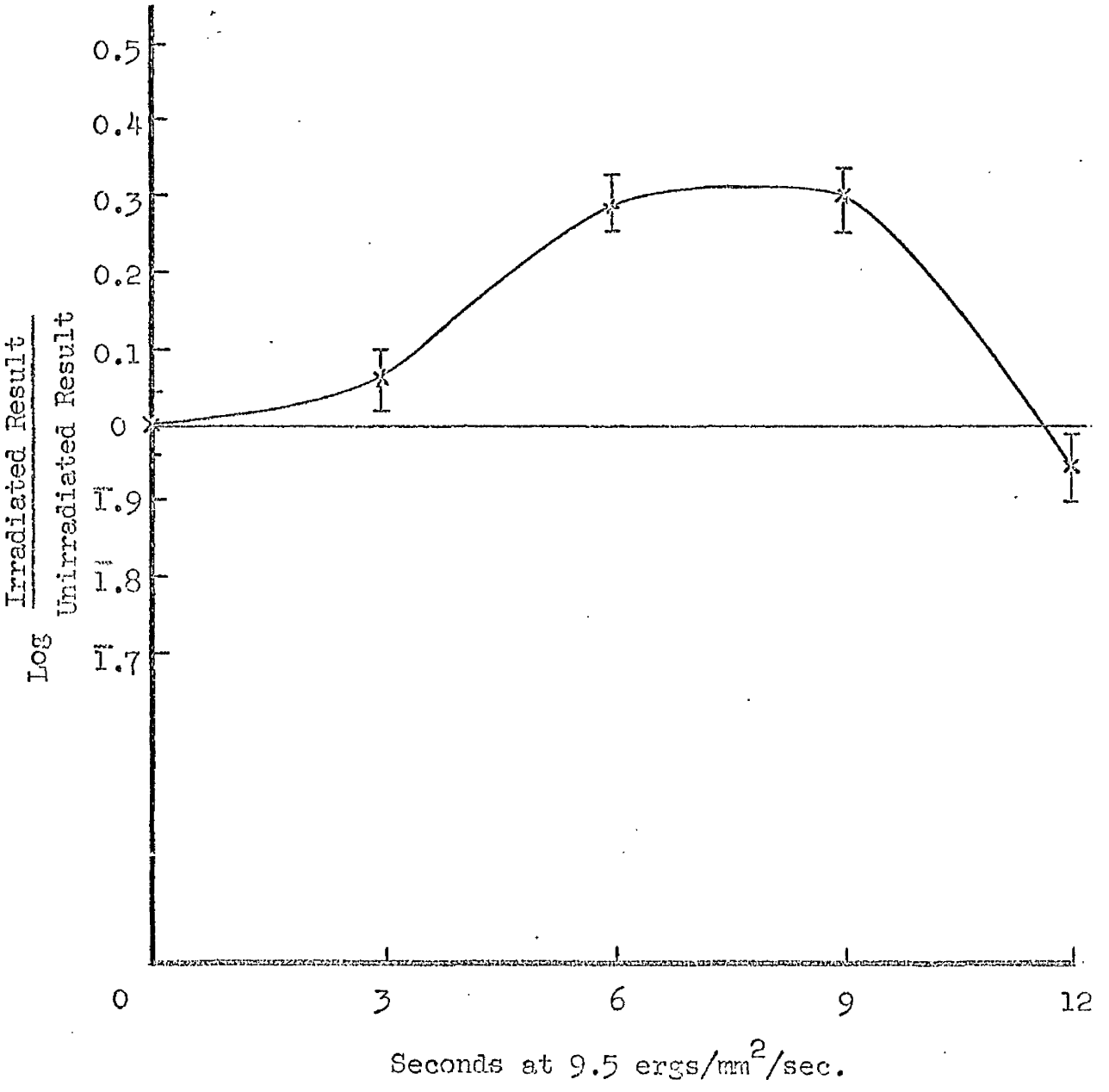

FIGURE 6. 96 Hours Incubation - No Feeder Cells (-⋇- =t per cell)

UV Dose (seconds exposure to 9.5 ergs/mm^2/sec.)	No. of cells plated/plate	Total No. of colonies counted in 3 experiments (30 plates)	Average t per cell + 2 x S. E.
0	10^4	1970	0.66 ± 0.06
3	10^4	2781	0.93 ± 0.07
6	10^4	3102	1.03 ± 0.04
9	10^4	2839	0.95 ± 0.07
12	10^4	1598	0.53 ± 0.05

Table 8. Characteristics of the enhancement of polyoma virus transformation in UV irradiated BHK 21 C13 cells after incubation of the cells for 48 hours at 37°C before plating in agar with feeder cells.

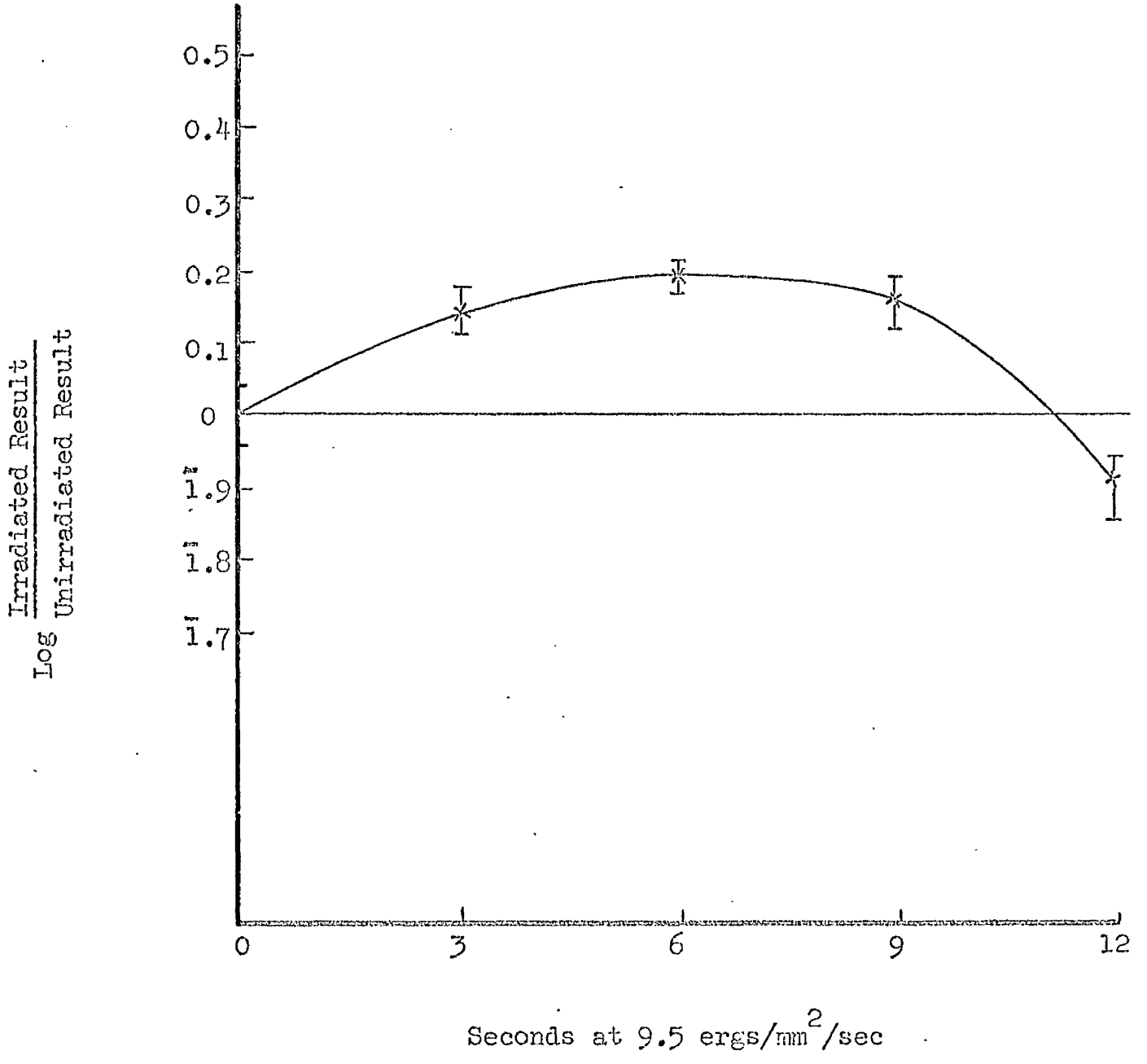

FIGURE 7. 48 Hours Incubation - 10^5 Feeder Cells/Plate

(⟶⟵ = t per cell)

was also possible that the results could be due to some form of selection - either through the subculturing (to keep the monolayers sparse) or through some effect(s) of the radiation itself. One possible effect could be a radiation induced selection process which gave transformed cells an advantage over the untransformed cells - for example, by enhancing the growth rate or by giving increased viability during subculture.

However, the experiments described above, in which the cells were plated in agar with feeder cells, ruled out selection due to the subculturing of the cells since an enhancement was seen when the cells were plated out after 48 hours - before subculturing became necessary.

In order to examine further whether or not a selection process was involved, "swamping" experiments were performed. Immediately after the cells were irradiated and infected, other cells were added to the cultures so that 5 "swamping" cells were present for every one irradiated, infected cell. The presence of a large excess of "swamping" cells in the cultures would have the effect of altering the environment and, therefore, any selection process occurring

in the experimental population of cells as these would now be a minority in the cultures instead of being the only cells in the culture. For example, if the presence of irradiated cells aided the selection of transformed cells, then the presence of excess irradiated cells in the experimental cultures should aid this selection process whereas the presence of an excess of unirradiated cells should have the opposite effect.

Cells treated in three different ways were used for swamping - (a) normal (unirradiated) BHK 21 C13 cells, (b) BHK 21 C13 cells which had been irradiated with UV light for 7 seconds at a dose rate of 9.5 ergs/mm^2/ sec. (the dose at which maximum enhancement of transformation was observed) and (c) BHK 21 C13 cells which had received a dose of UV radiation of 400 ergs/mm^2 - sufficient to reduce survival to less than 1% of the original population. One complication in these experiments was the problem of new infections in the swamping cells by virus particles remaining after washing. However, it can be assumed that a similar number of virus particles would remain in all the cultures and would, therefore, not affect the final result. The cells were plated in agar without feeder cells

after 96 hours - the medium being changed every 24 hours and the cells subcultured at 48 hours - or in agar with feeder cells after 48 hours - the medium being changed at 24 hours.

The results given in Table 9 show that even in the presence of 5 times the number of other cells which had been treated in different ways, there was still an approximate enhancement factor of 2 when the cells were plated in agar without feeder cells after 96 hours and an approximate enhancement factor of 1.5 when the cells were plated in agar with feeder cells after 48 hours.

These results suggested that selective forces were not involved in the enhancement of the value for t per cell when irradiated, infected cells were plated out in agar after a recovery period at 37°C. The presence of a large excess of different cells in the experimental cultures would ensure that selective forces within the cultures would be very different from those present when only irradiated, infected cells were present. The fact that the enhancement factor for t per cell was unaltered suggests that this type of selection force did not cause the observed results. It therefore appeared possible that the rise in the value for t per cell observed

Incubation period on glass	Treatment to swamping cells	UV dose in ergs/mm^2 (experimental cells)	No. of cells plated	Total No. of colonies counted in 3 experiments (30 plates)	t per cell $\pm$ 2 x S.E.
96 hrs. (without feeder cells)	None	0	10^5	2014	0.067 $\pm$ 0.012
		66.5	10^5	3987	0.133 $\pm$ 0.016
	66.5 ergs/mm^2	0	10^5	1986	0.066 $\pm$ 0.010
		66.5	10^5	4039	0.135 $\pm$ 0.014
	400 ergs/mm^2	0	10^5	2203	0.073 $\pm$ 0.016
		66.5	10^5	4106	0.137 $\pm$ 0.016
48 hrs. (with feeder cells)	None	0	10^5	2487	0.083 $\pm$ 0.015
		66.5	10^5	3721	0.124 $\pm$ 0.013
	66.5 ergs/mm^2	0	10^5	2765	0.092 $\pm$ 0.012
		66.5	10^5	3667	0.122 $\pm$ 0.011
	400 ergs/mm^2	0	10^5	2509	0.084 $\pm$ 0.016
		66.5	10^5	3843	0.128 $\pm$ 0.013

Table 9. The effect of swamping the experimental cells with 5 times their number of uninfected cells on the enhancement of transformation.

in agar could be a true reflection of the rise in the value for t per colony observed in the experiments in which irradiated, infected cells were plated out on glass for colony formation.

(h) Growth of irradiated infected cells on glass after delayed plating

The characteristics of the enhancement seen after delayed plating of irradiated, infected cells in agar were also studied by delayed plating of the cells on glass.

Cell monolayers were irradiated, at a dose rate of 9.5 ergs/mm^2/sec, and left on glass for 4, 48 and 96 hours before being plated out onto feeder cells on glass in order to determine how the survival curves for the cell population varied over this period. The results are shown in Figure 8 (see also Figure 11). Initially the survival curve was of the normal mammalian "sigmoid" or "type C" form - a curve composed of an initial shoulder region, followed by a region of exponential, or near exponential, decline (Alper, Fowler, Morgan, Vondberg, Ellis and Oliver, 1962). However, after 48 hours incubation, the survival curve was very much less steep, presumably due to repopulation of the culture by proliferation of the surviving cells. When the cells were plated out after 96 hours incubation the survival

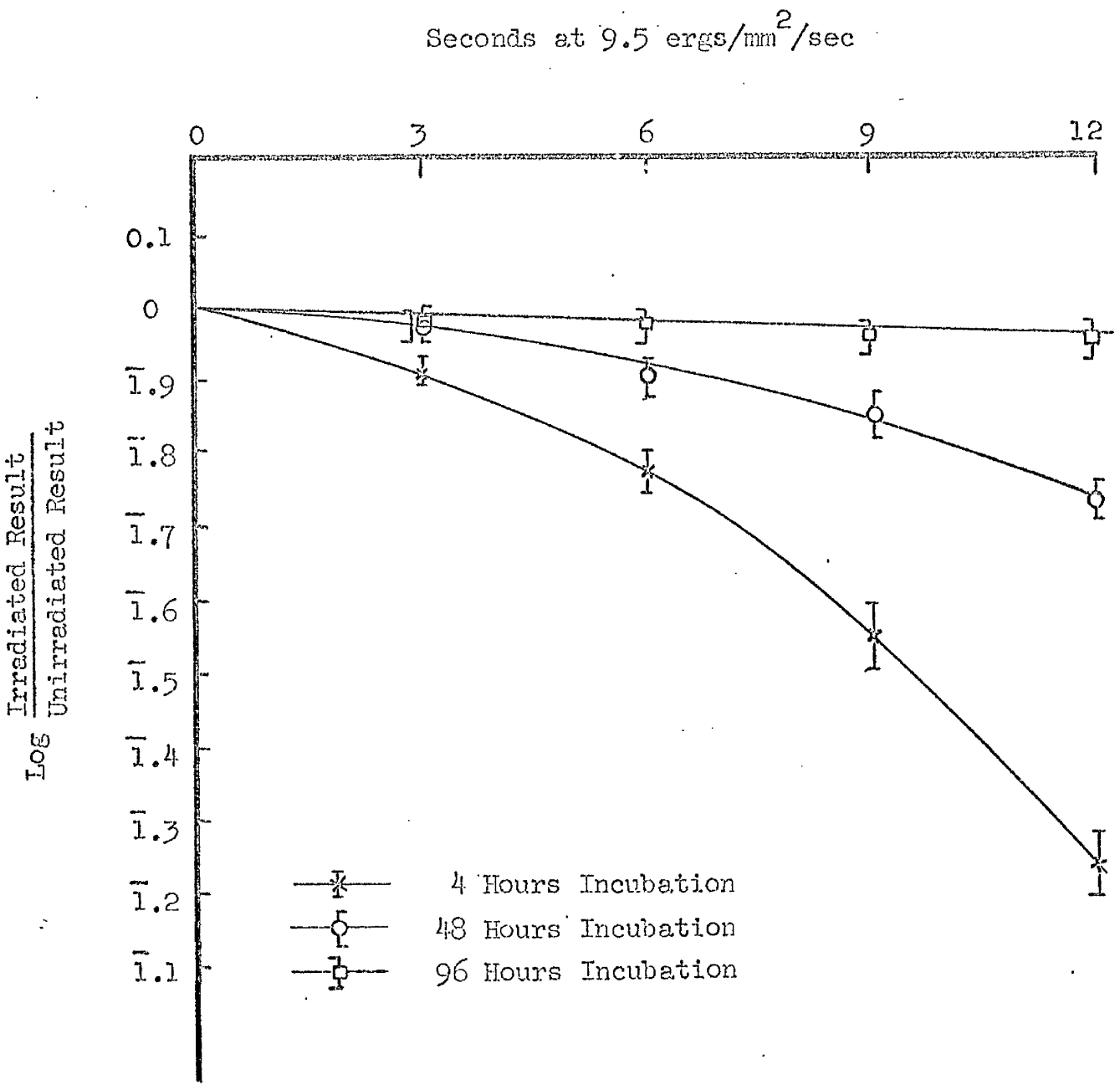

FIGURE 8. UV Radiation Survival Curves for BHK21 C13 Cells Plated onto 10^5 Feeder Cells after varying Periods of Incubation at 37°C.

curve did not vary significantly from the control level over the range of doses used. The 96 hour unirradiated control cultures contained, before the cells were plated out, approximately four times as many cells as the cultures irradiated for 12 seconds (114 ergs/mm^2).

Cells which had been irradiated, infected and left on glass for 48 hours, were plated out into 60 mm glass Petri dishes containing feeder cells for colony formation. The results - which are given in Figure 9 - show that the t per cell value has risen to that of the t per colony value after 3 seconds irradiation (28.5 ergs/mm^2).

Cells which had been irradiated and infected and left on glass for 96 hours were also plated out onto feeder cells in 60 mm glass Petri dishes. The results are shown in Figure 10. In these experiments the rise in t per cell closely parallels that for t per colony and, because more transformed colonies occur at the higher radiation doses, the level of significance of these points is better.

In both of the two previous series of experiments, test cells were also plated out into agar without feeder cells, after 96 hours on glass, as a control. In both series of experiments the rise in t per cell in

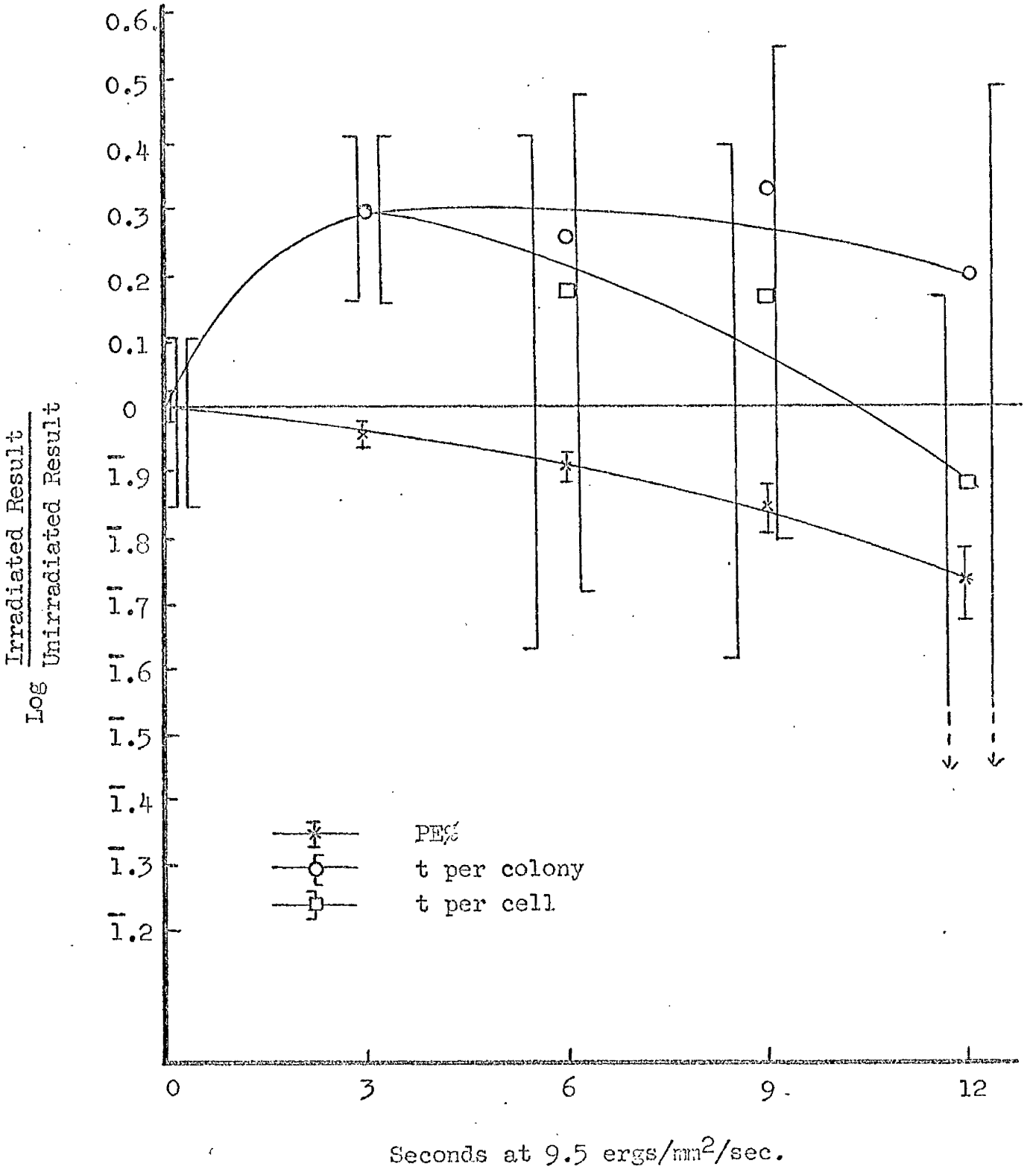

FIGURE 9. The Effect of Incubating Irradiated, Infected Monolayers of BHK21 C13 cells for 48 hours before Plating on Glass with Feeder Cells. Some of the Errors were very large due to the Low Number of Transformed Colonies on the Plates.

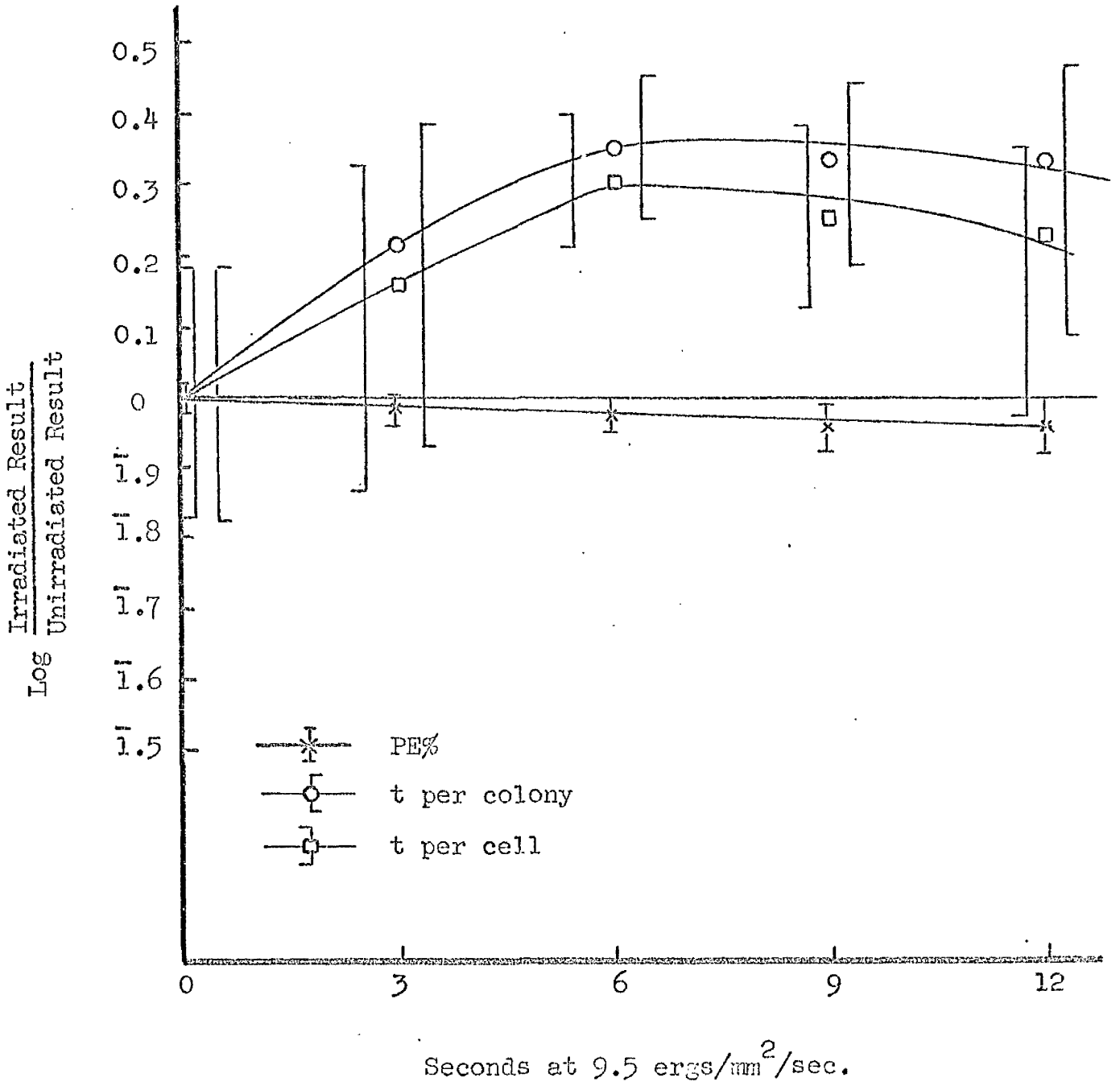

FIGURE 10. The Effect of Incubating Irradiated, Infected Monolayers of BHK21 C13 Cells for 96 hours before Plating on Glass with Feeder Cells. Control Infected Irradiated Cells in Agar without Feeder Cells gave Similar Results to Those Shown in Figure 6.

agar closely paralleled the rise in t per colony on glass, although the rise in t per cell in agar fell off more rapidly after 12 seconds irradiation (114 ergs/mm^2) than the value for t per colony on glass (see Figure 6).

2. The effects of altering the time of irradiation relative to infection

(a) Introduction

The experiments described up till now were based on near simultaneous irradiation and infection. Stoker (1964a) found that, when BHK 21 C13 cells were X-irradiated prior to infection with polyoma virus, the increased sensitivity to transformation remained high for 48 hours. Furthermore, when irradiation was delayed for 24 hours after infection, an increased sensitivity to transformation still existed. Therefore experiments were carried out, using the assay system described above, to determine the periods before and after infection with polyoma virus during which the BHK 21 C13 cells could be irradiated with UV radiation and still show a transformation enhancement effect.

(b) Enhancement of transformation after delayed infection

BHK 21 C13 cells were irradiated with UV radiation at a dose rate of 9.5 ergs/mm^2/sec. They were then incubated at 37^{o}C for 24, 48 or 72 hours before infection with polyoma virus. As a control, cells were also infected immediately after irradiation. The plates were then incubated for 96 hours at 37^{o}C. After the period of incubation they were plated in agar without feeder cells. The results are shown in Table 10. They show the enhancing effect of the irradiation to be unaltered at 24 hours, lower and only just significant at 48 hours and no longer present after 72 hours.

(c) Enhancement of transformation by irradiation after infection

Experiments were performed to investigate whether UV irradiation of cells, which had previously been infected with polyoma virus, showed a transformation enhancement effect similar to that which occurred when cells were irradiated before infection.

BHK 21 C13 cells were infected with polyoma virus 2 and 4 hours before irradiation and were plated out into agar after the usual incubation period of 96 hours.

Incubation period at 37° before infection	UV dose (secs. exposure to 9.5 ergs/mm^2/sec.)	No. of cells plated/plate	Total No. of colonies counted in 3 experiments (30 plates)	Average t per cell ± 2 x S.E.
24 hours	0	10^4	1784	0.59 ± 0.07
	3	10^4	2097	0.70 ± 0.06
	6	10^4	3371	1.12 ± 0.09
	9	10^4	3509	1.17 ± 0.09
	12	10^4	1670	0.56 ± 0.06
48 hours	0	10^4	1883	0.63 ± 0.08
	3	10^4	1727	0.64 ± 0.07
	6	10^4	2304	0.77 ± 0.06
	9	10^4	2513	0.84 ± 0.07
	12	10^4	1867	0.62 ± 0.05
72 hours	0	10^4	1862	0.62 ± 0.06
	3	10^4	1799	0.60 ± 0.07
	6	10^4	1873	0.62 ± 0.05
	9	10^4	1831	0.61 ± 0.06
	12	10^4	1906	0.63 ± 0.08

Table 10. The effect of incubating UV irradiated BHK 21 C13 cells at 37°C for varying periods before infection with polyoma virus.

The results given in Table 11 show that the enhancing effect was completely lost when the cells were irradiated 4 hours after infection. Control experiments showed that the doses of UV radiation used did not significantly affect the transforming ability of the virus particles - for example, after a dose of 250 ergs/mm^2 (more than double the highest dose used in the experiments) the t per cell value, in agar suspension medium, only dropped from 3.6 ± 0.1% to 3.3 ± 0.1% (see also Latarjet, Cramer and Montagnier, 1967).

3. Discussion

The experiments reported above showed that UV irradiation of cells, prior to infection with polyoma virus, caused an enhancement of the transformation rate. When the agar assay was used in an attempt to improve the precision of measurement, it was found that, when there was a delay before the cells were plated out, there was an apparent rise in the enhancement due to irradiation. This apparent rise occurred 96 hours after irradiation and infection when the cells were plated in agar without feeder cells and 48 hours after irradiation and infection when feeder cells were used.

Incubation period at 37° before irradiation	UV dose (secs. exposure to 9.5 ergs/mm^2/sec.)	No. of cells plated/plate	Total No. of colonies counted in 3 experiments (30 plates)	Average t per cell ± 2 x S.E.
2 hours	0	10^4	1397	0.47 ± 0.07
	3	10^4	1803	0.60 ± 0.08
	6	10^4	3004	1.00 ± 0.10
	9	10^4	2989	1.00 ± 0.09
	12	10^4	1433	0.48 ± 0.06
4 hours	0	10^4	1472	0.49 ± 0.06
	3	10^4	1503	0.50 ± 0.05
	6	10^4	1497	0.50 ± 0.07
	9	10^4	1521	0.51 ± 0.09
	12	10^4	1603	0.53 ± 0.05

Table 11. The effect of incubating polyoma virus infected BHK 21 C13 cells at 37°C for varying periods of time before irradiation with UV radiation.

These results are difficult to interpret. The rise in the enhancement factor following a delay before plating is based on t per cell. This value, but not t per colony, is likely to be affected during proliferation of an irradiated culture because of the dilution out of non-colony forming cells by colony forming cells as the survivors divide. A hypothetical example of this effect in Table 12 shows how the t per cell and therefore the enhancement factor would rise with succeeding cell divisions. A number of assumptions have been made in this example for the sake of simplicity, some of which would not be tenable in an actual culture. The example assumes that when the cells are first plated there is a PE% of 50 in the control cultures and 25 in the irradiated cultures and that the value for t per colony is higher in the irradiated cultures than in the unirradiated cultures - as observed in the experiments described above in which the irradiated, infected cells were plated on glass. The example also assumes that all the cells which are capable of division, in both the control and irradiated cultures, divide at the same time throughout and that there is no cell loss.

It will be noted that the final enhancement factor, based on t per

CONTROL (UNIRRADIATED) CELLS

Division cycle	Total No. of cells in the culture	Non-dividing cells	Viable cells in the culture		t per colony	Enhancement factor (t per colony)	t per cell	Enhancemen factor (t per cell)
			Untransformed	Transformed				
0	100	0	45	5	10	1.0	5.0	1.0
1	150	50	90	10	10	1.0	3.3	0.66
2	250	50	180	20	10	1.0	4.0	0.8
3	450	50	360	40	10	1.0	4.4	0.88
4	850	50	720	80	10	1.0	4.7	0.94
5	1650	50	1440	160	10	1.0	4.8	0.96
6	3250	50	2880	320	10	1.0	4.9	0.98

UV IRRADIATED CELLS

Division cycle	Total No. of cells in the culture	Non-dividing cells	Viable cells in the culture		t per colony	Enhancement factor (t per colony)	t per cell	Enhancemen factor (t per cell)
			Untransformed	Transformed				
0	100	25 (killed by UV)	20	5	20	2.0	5.0	1.0
1	125	75 (25+50)	40	10	20	2.0	4.0	0.8
2	175	75	80	20	20	2.0	5.7	1.14
3	275	75	160	40	20	2.0	7.3	1.46
4	475	75	320	80	20	2.0	8.4	1.68
5	875	75	640	160	20	2.0	9.1	1.82
6	1675	75	1280	320	20	2.0	9.6	1.92

Table 12. A hypothetical scheme to show that the value for t per cell could be affected during the growth on glass of an irradiated, polyoma virus infected population of cells and a control, unirradiated infected population, whereas the value for t per colony would be unaffected. The values for t per colony and t per cell are based on a PE% value of 50 for the viable cells in the culture (50% of the cells initially plated in the culture will not divide due to this 50% PE).

cell, in the irradiated culture rises towards the enhancement factor for t per colony. The value for t per colony cannot be measured directly in the agar assay but the later t per cell enhancement factors should show an equivalent value to the enhancement factor for t per colony.

Though the changes in proportion of colony forming cells during growth following irradiation and infection must affect the measurement of enhancement, and might be the whole explanation of the effect of delayed plating, there are other factors which suggest that the situation is a good deal more complex. It will be noted, for example, that there was a drop in the absolute number of transformed colonies when plating was delayed in either irradiated or unirradiated cultures. This drop might be explained if either the BHK 21 C13 cells divided more rapidly than the newly transformed cells and/or the newly transformed cells had a low plating efficiency in the agar medium.

The more rapid enhancement of transformation when the irradiated infected cells were plated in agar with feeder cells after a delay of 48 hours is also difficult to interpret. The results would appear to rule out the possibility that subculturing the irradiated,

infected cells during their incubation period on glass gave the transformed cells a selective advantage. The results suggest that the appearance of the enhancing effect in agar can be considerably speeded up by the presence of feeder cells in the agar.

There are two possible explanations for these results. The most likely one is based on the report by Stoker (1963b) that mixed clones of normal and transformed cells make up more than one third of the total colonies. Mixed clones are thought to be due to delayed transformation which may occur up to 7 or 8 generations after the exposure of the cells to the virus. If delayed transformation was also important in the present system, then cells plated into agar without feeder cells and before the necessary number of generations had occurred to permit the expression of delayed transformation, would be unable to form colonies in the agar. However, it was observed that when control, uninfected BHK 21 C13 cells were plated in agar with feeder cells, most cells, instead of remaining as single cells, appeared to be in groups of 4-8. This appeared to be due to the feeder cells causing the uninfected BHK 21 C13 cells to divide 2 to 3 times before stopping. This effect was

similar to that reported by Tjötta, Flikke and Lahelle (1968) who found that conditioned medium, in agar suspension medium, also caused BHK 21 C13 cells to form small colonies. It is therefore possible that delayed transformation could be expressed when infected cells were plated out into agar with feeder cells after 48 hours. However, if feeder cells were not present then the infected cells would not express delayed transformation until they had been able to complete the necessary divisions on glass - that being after 96 hours.

The other possible explanation of the results obtained when irradiated infected cells were plated into agar with feeder cells is that feeder cells can improve the plating efficiency of cells both on glass and in agar. It is possible that, under the conditions used in these experiments, the feeder cells can "rescue" irradiated, infected cells which would normally be incapable of further proliferation in agar suspension medium without feeder cells.

The results obtained when the irradiated infected cells were swamped with 5 times their number of irradiated or unirradiated cells suggest that a radiation induced selection process was not selecting for the transformed cells present in the population.

If this type of selection occurred then the presence of an excess of irradiated or unirradiated, uninfected cells would upset the conditions in the culture with the probable result that the conditions permitting the selection process would be altered and, therefore, the enhancement would not be expressed. The results show that the enhancement still occurred, in spite of the presence of an excess of uninfected cells.

The results obtained when BHK 21 C13 cells were irradiated at different times before infection showed that the sensitivity of the irradiated cells to transformation remained high for more than 24 hours after irradiation and were very similar to those obtained by Stoker (1964a) for polyoma virus transformation of X-irradiated BHK 21 C13 cells. However, there was a marked difference between the results reported by Stoker (1964a) and those obtained in the present study when the cells were irradiated after infection. Stoker (1964a) found that when polyoma virus infected cells were X-irradiated up to 24 hours after infection there was still a transformation enhancement similar to that obtained when the cells were irradiated immediately prior to infection. In the

present study with UV radiation a similar enhancement factor was obtained when infected cells were irradiated 2 hours after infection, but there was no apparent enhancement when the cells were irradiated 4 hours after infection. This discrepancy between the effects of UV radiation and X-radiation was rather surprising in view of the apparent close similarity between the two forms of radiation with regard to the enhancement of transformation by polyoma virus. Although control experiments in the present study showed that irradiation of virus particles with up to twice the maximum dose of UV radiation given to the cells did not significantly affect the ability of the virus to transform BHK 21 C13 cells, it is possible that virus particles (which may be uncoated) inside the cell are more susceptible to the action of the UV radiation. It is probable that intracellular virus is not inactivated by the UV radiation as the target size would still be very small compared to the target size of the cell. This would suggest that if residual virus was still present when the cells were irradiated after infection, radiation selection of cells genetically susceptible to transformation did not occur. An alternative interpretation of these results is that the early events leading to

transformation occur early after infection so that irradiation 4 hours after infection of the cells with polyoma virus was too late to influence the transformation rate.

The results reported in this chapter show that cells irradiated with UV radiation are more susceptible to transformation by polyoma virus. It seems likely from the results that this increased susceptibility is due to the direct action of the radiation on the cells. Possible mechanisms which might explain this effect are discussed in the General Discussion in the light of the more general radiobiological studies reported in the next chapter.

CHAPTER V.

RESULTS

II. Radiobiological Studies

1. General Introduction

The results reported in the preceding chapter showed that BHK 21 C13 cells which had been irradiated with UV light were more susceptible to transformation by polyoma virus.

The results reported in this chapter are concerned with more general radiobiological studies, performed in the hope that these would throw some light on the mechanism of UV induced enhancement of transformation. Several experiments were concerned with UV radiation induced damage, because it appeared possible that the viral genome might be integrated into the DNA of the cell more efficiently during repair of damage to the cellular DNA.

Many of the methods used in the following experiments differed from those used routinely and are therefore described in the relevant section rather than in the chapter on methods.

2. The effect of UV radiation on interferon production

(a) Introduction

Interferon, a potent inhibitor of viral nucleic acid replication, was first reported by Isaacs and Lindenmann (1957). Cells which

have been incubated with interferon show some degree of resistance to viral infection. Interferons produce their antiviral effects by inducing cellular processes which involve synthesis of both RNA and protein (Taylor, 1964; Lockart, 1964; Friedman and Sonnabend (1964).

De Maeyer-Guignard and De Maeyer (1965) showed that UV irradiation of rat embryo cells prior to viral infection decreased interferon yields and increased the size of Sindbis viral plaques. Cogniaux-Le Clerc, Levy and Wagner (1966) have related the UV dose to the interferon synthesising capacity of the cells. They found that UV inactivation curves for interferon production did not deviate significantly from "one-hit" kinetics. They have suggested that each effective UV "hit" damages a single interferon-synthesising target which must be located in the cellular DNA. This work has been confirmed by Burke and Morrison (1966).

Although the doses of UV radiation used in the experiments described above were larger than those used in the present study, it was possible that the doses of radiation given to the BHK 21 C13 cells, before infection with polyoma virus, might have reduced the

interferon-synthesising capacity of the cells. If interferon production was reduced, then the transformation of these cells might be expected to increase. Allison (1961) found that polyoma virus was susceptible to interferon and Todaro and Baron (1965) have shown that, although SV40 virus does not multiply in 3T3 cells, transformation of these cells by SV40 is inhibited by interferon. Experiments were performed to test the hypothesis that interferon production could be disrupted by UV radiation, resulting in an enhanced transformation rate by polyoma virus.

(b) Methods

Monolayers of 10^6 BHK 21 C13 cells were irradiated with a dose of 114 ergs/mm^2 (equivalent to the maximum dose used in the agar transformation experiments). Half of the irradiated plates and an equal number of control unirradiated plates were then infected with polyoma virus at an input multiplicity of 800 p.f.u. per cell. The remaining plates were uninfected controls. After the infection period 5 ml of ETC were added to each plate and all the plates were incubated at 37°C for 24 hours. At the end of the 24 hour incubation period the medium was removed from each of the four groups of

plates and pooled to give four samples of medium from (a) cells uninfected and unirradiated (b) cells infected and unirradiated (c) cells uninfected and irradiated (d) cells infected and irradiated.

The four samples of medium were then tested for the presence of interferon by a method similar to that first described by Wagner (1961). Fresh monolayers of approximately 10^6 BHK 21 C13 cells were washed twice in PBS and divided into five groups. 4.5 ml of from one of the batches of pooled medium was added to each plate in one of the groups of fresh monolayers. This was done for each of the batches of medium. The remaining group of plates received 4.5 ml of fresh ETC. All the plates were then incubated at 37°C for 24 hours.

At the end of the 24 hour incubation period the monolayers were assayed for resistance to plaque formation by a small plaque strain of encephalomyocarditis (EMC) virus, using the assay method developed by Dulbecco (1952) for Western equine encephalomyelitis. The medium on all the plates was discarded and the plates washed twice in TS with 1% calf serum. The plates which had received fresh ETC 24 hours before had 0.2 ml of TS with 1% calf serum

added to them. The remaining plates were inoculated with approximately 60 p.f.u. of EMC virus in 0.2 ml of TS with 1% calf serum. All the plates were then incubated at 37°C for 30 minutes to allow the virus to adsorb to the monolayer and they were shaken at 10 minute intervals during this period to ensure an even spread of the virus over the monolayer.

At the end of the adsorption period any excess virus was removed and the monolayers were overlaid with 5 ml of DEAE dextran/Noble agar. The plates were incubated at 37°C for 30 hours before being stained with neutral red, fixed with formol saline and the monolayers scored for plaques.

In addition, in order to investigate whether cells which had been treated with polyoma virus were resistant to plaque formation by EMC virus, monolayers of BHK 21 C13 cells which had been irradiated and/or infected were assayed for resistance to plaque formation by EMC virus.

(c) Results

The results of the first series of experiments are shown in Table 13. These were performed to test for the synthesis of interferon by the variously treated monolayers and showed that not

No. of Plates	Incubation with medium from monolayers treated with:- UV irradiation (114 ergs/mm^2)	Polyoma Virus	No. of EMC plaques/ plate ± 2 x S. E.
10	-	-	60.7 ± 4.5
9	+	+	53.5 ± 2.8
10	+	-	53.1 ± 3.6
8	-	+	61.5 ± 2.8
10	Control - No EMC virus		0

Table 13. Plaque inhibition by medium from monolayers of BHK 21 C13 cells treated with UV radiation and/or polyoma virus.

enough interferon was released into the medium to have any effect on the number of plaques formed by the EMC virus which had been produced in fresh monolayers, incubated in this medium. Furthermore, rather than there being a rise, when the monolayers were treated with medium from irradiated, infected monolayers, there was a barely significant fall in the number of plaques formed. This fall was identical to that found when the monolayers were treated with medium from cultures which had been irradiated only.

In the second series of experiments monolayers of BHK 21 C13 cells were irradiated with UV light and/or infected with polyoma virus and tested for resistance to plaque formation by EMC virus at 4 and 24 hours after the irradiation and/or infection. The results are given in Table 14.

When the monolayers were tested 4 hours after the initial treatment the main result observed was a marked inhibition in the number of plaques produced by the EMC virus in the cultures previously infected with polyoma virus. There was, however, no significant difference in the number of plaques formed in monolayers which had been either irradiated and infected or just infected. Similarly, there was no significant difference between irradiated

No. of Plates	Incubation before addition of EMC Virus	Treatment of monolayers		No. of EMC plaques/ plate ± 2 x S. E.
		UV Irradiation (114 ergs/mm^2)	Polyoma Virus	
9		-	-	63.8 ± 4.7
10		+	+	10.2 ± 2.3
10	4 hours	+	-	64.0 ± 2.4
10		-	+	10.4 ± 2.8
9		Control - No EMC virus		0
10		-	-	44.4 ± 2.3
10		+	+	26.8 ± 3.0
10	24 hours	+	-	37.2 ± 2.8
9		-	+	19.8 ± 4.4.
10		Control - No EMC virus		0

Table 14. Plaque inhibition on monolayers of BHK 21 C13 cells treated with UV radiation and/or polyoma virus.

and unirradiated, uninfected monolayers.

When the monolayers were infected with EMC virus 24 hours after treatment, the number of plaques in the control was slightly lower than at 4 hours - 44.4 ± 2.3 as opposed to 63.8 ± 4.7 plaques per dish. There was still fairly good agreement between the number of plaques obtained with EMC virus in the irradiated, polyoma virus infected cultures and the unirradiated polyoma virus infected cultures. The degree of viral inhibition was not as marked after 24 hours incubation as it had been after 4 hours incubation.

There was thus no evidence obtained to suggest that the irradiated cultures were any more sensitive to plaque formation by EMC virus than the controls under the experimental conditions used.

3. The sensitivity of normal and transformed BHK 21 C13 cells to UV radiation

(a) Introduction

Radiation damage in biological organisms is commonly measured by determining the proportion of cells which can proliferate to form colonies after various doses of radiation. The resultant survival curve gives a relative measure of the effect of the radiation. Survival curves for cells are usually of the "sigmoid" or "Type C" form in that the curve is composed of an initial shoulder region followed by a region of exponential or near exponential decline (Alper, Fowler, Morgan, Vondberg, Ellis and Oliver, 1962). When survival curves are of this type they can be characterised by three parameters:

(1) D_{37}, the dose required to reduce the surviving fraction by 63% from the value obtained in the unirradiated controls.

(2) D_0, the dose required to reduce the surviving fraction by 63% on the exponential portion of the curve.

(3) n, the observed extrapolation number, obtained by extrapolating the exponential part of the curve back to zero dose on the semi-logarithmic plot and reading off the fractional survival (Alper, Gillies

and Elkind, 1960).

D_{37} is a measure of the extent of the shoulder of the curve and D_0 measures the slope of the exponential region on a semilogarithmic plot.

Stoker (1963a) could detect no difference between the survival curves of polyoma virus transformed BHK 21 cells and untransformed BHK 21 cells after X-irradiation. However, Williams and Till (1966) found that some established lines of polyoma virus-transformed rat-embryo cells were less sensitive to X-radiation than either normal, untransformed rat embryo cells, or transformed cell lines which had been cultivated in vitro for less than 7 weeks.

Survival curves were determined for BHK 21 C13 cells and two lines of polyoma virus transformed BHK 21 C13 cells designated PyH3 and PyH6. The survival curves for all three lines were determined on glass with and without feeder cells. Survival curves for the transformed lines were also determined in agar suspension medium with and without feeder cells.

(b) Results

The results of the survival curve determinations for normal and polyoma virus transformed BHK 21 C13 cells are shown in Table 15

No. of experiments	Sample	Feeder Cells	Average PE %	Extra-polation No.	D_{37} (ergs/mm^2) ± 2 x S. E.	D_0 (ergs/mm^2) ± 2 x S. E.
10	BHK 21 C13 (Glass)	-	15.9	2.19	82.3 ± 8.3	39.6 ± 4.9
15	BHK 21 C13 (Glass)	+	66.6	2.29	114.1 ± 10.4	59.5 ± 5.6
10	Py H3 (Glass)	+	56.0	2.51	120.8 ± 10.7	73.4 ± 4.2
9	Py H3 (Agar)	+	33.6	1.66	107.7 ± 10.6	68.4 ± 8.3
10	Py H3 (Glass)	-	41.2	1.82	97.5 ± 7.8	54.2 ± 4.3
9	Py H3 (Agar)	-	23.6	1.66	86.2 ± 10.3	54.4 ± 4.9
8	Py H6 (Glass)	+	57.3	2.52	123.6 ± 10.9	75.3 ± 6.0
5	Py H6 (Agar)	+	35.1	1.68	109.9 ± 9.6	71.3 ± 6.3
8	Py H6 (Glass)	-	37.9	1.79	86.7 ± 8.7	52.7 ± 5.1
5	Py H6 (Agar)	-	22.7	1.64	85.1 ± 9.3	52.3 ± 4.3

Table 15. Values for the parameters of the survival curves for BHK 21 C13, Py H3 and Py H6 cells.

and Figures 11-14. The survival curves were of the normal "Type C" form. Those on glass were based on at least eight separate determinations of each curve and those in agar were based on at least five separate determinations.

BHK 21 C13 cells were more sensitive than PyH3 cells to UV light over the exponential portion of the curve either with, or without, feeder cells. The D_0 values for BHK 21 C13 cells were 59.5 ± 5.6 ergs/mm^2 in the presence of feeder cells and 39.6 ± 4.9 ergs/mm^2 in the absence of feeder cells. The equivalent D_0 values for PyH3 cells were 73.4 ± 4.2 ergs/mm^2 and 54.2 ± 4.3 ergs/mm^2. The D_0 values for both normal and transformed cells were significantly higher when the cells were plated onto feeder cells than when plated in their absence.

Similar results were obtained when PyH3 cells were plated on glass or in agar. The mean values for D_{37}, when the cells were plated in agar, appeared slightly lower than for those plated on glass but the twice standard error limits overlap. The values for D_0 were very similar.

In order to allow for the killing of the cells by the irradiation

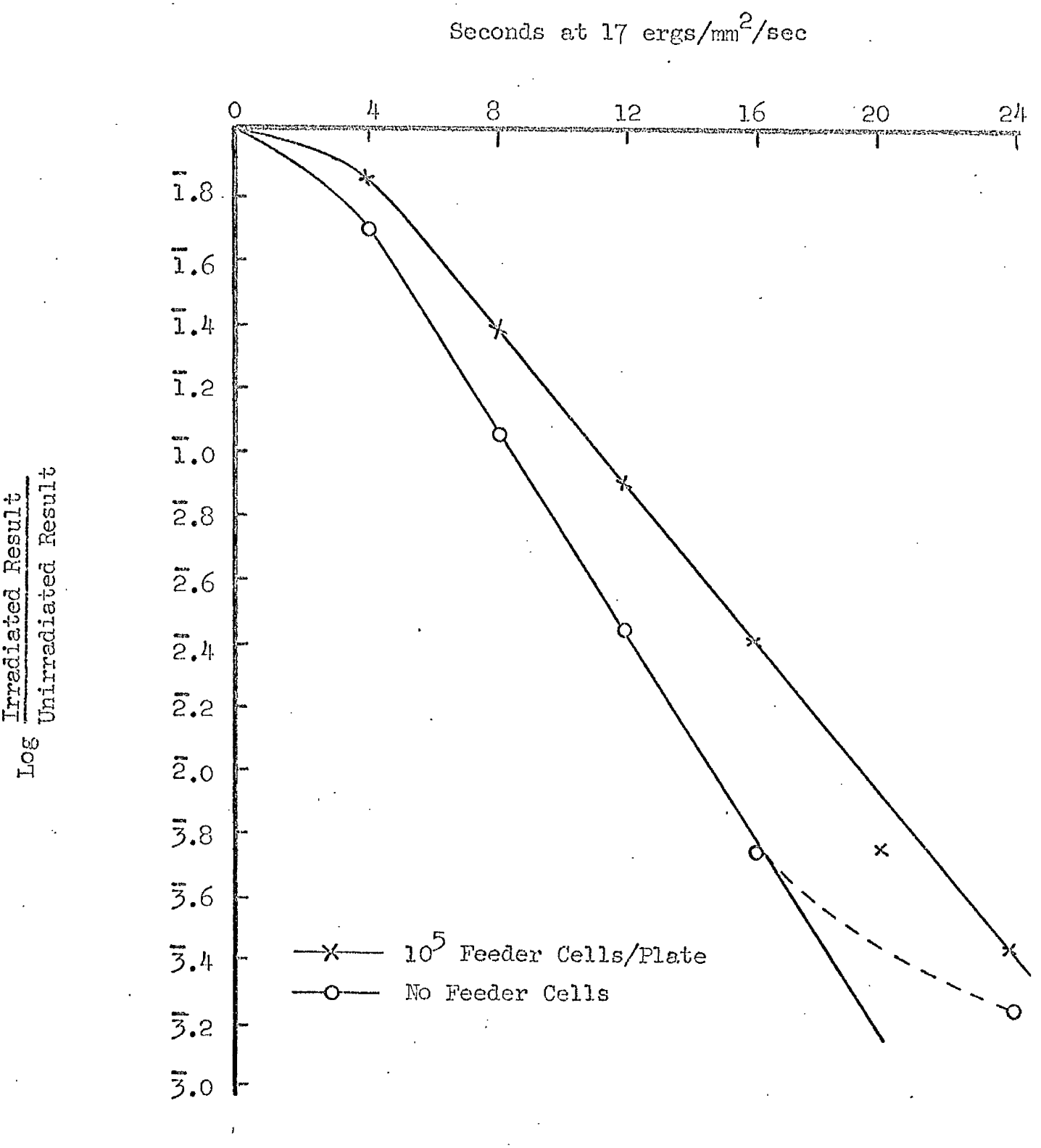

FIGURE 11. UV Radiation Survival Curves for BHK21 C13 Cells Plated, with and without Feeder Cells, on Glass.

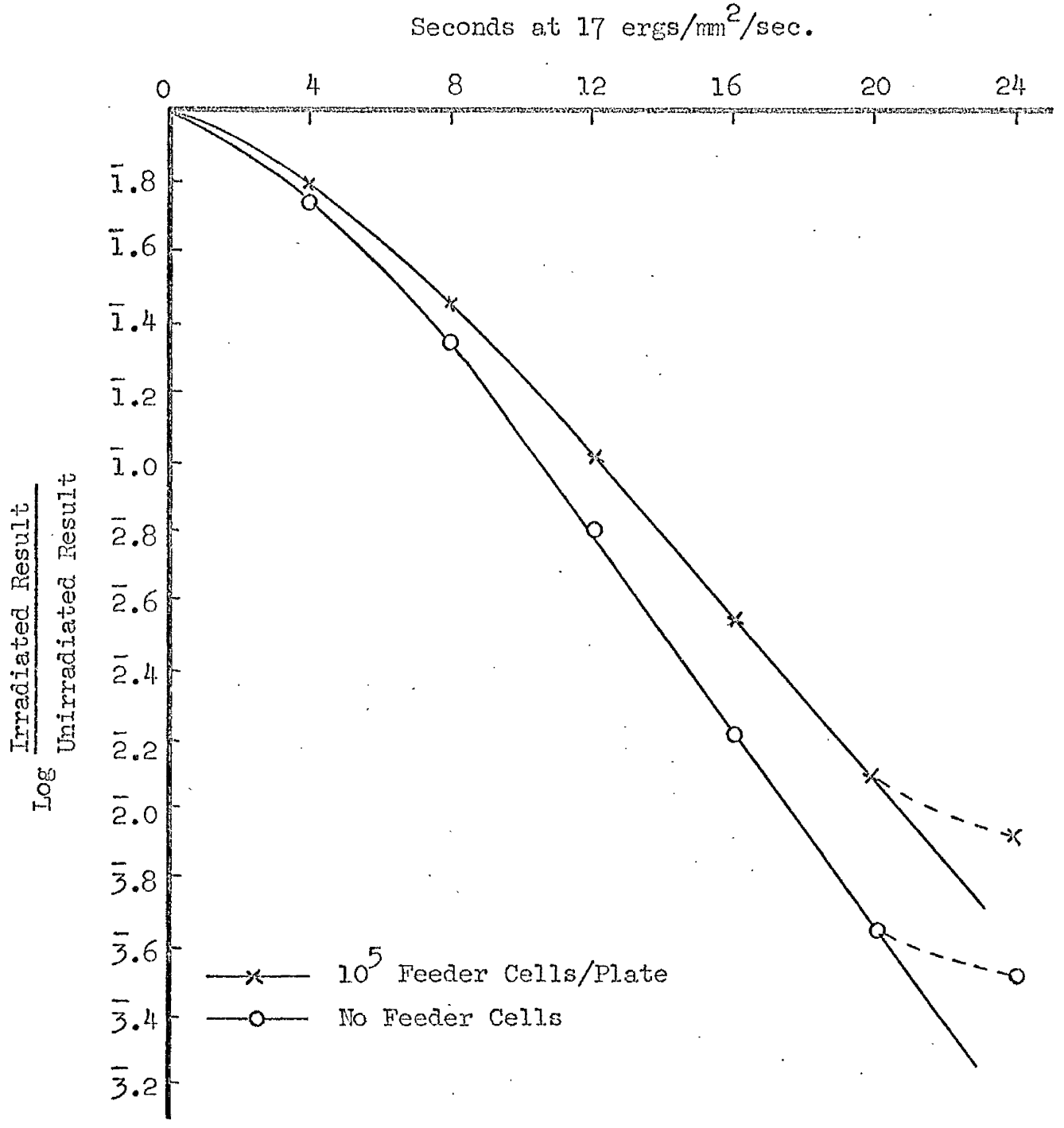

FIGURE 12. UV Radiation Survival Curves for PyH3 Cells Plated, with and without Feeder Cells, on Glass.

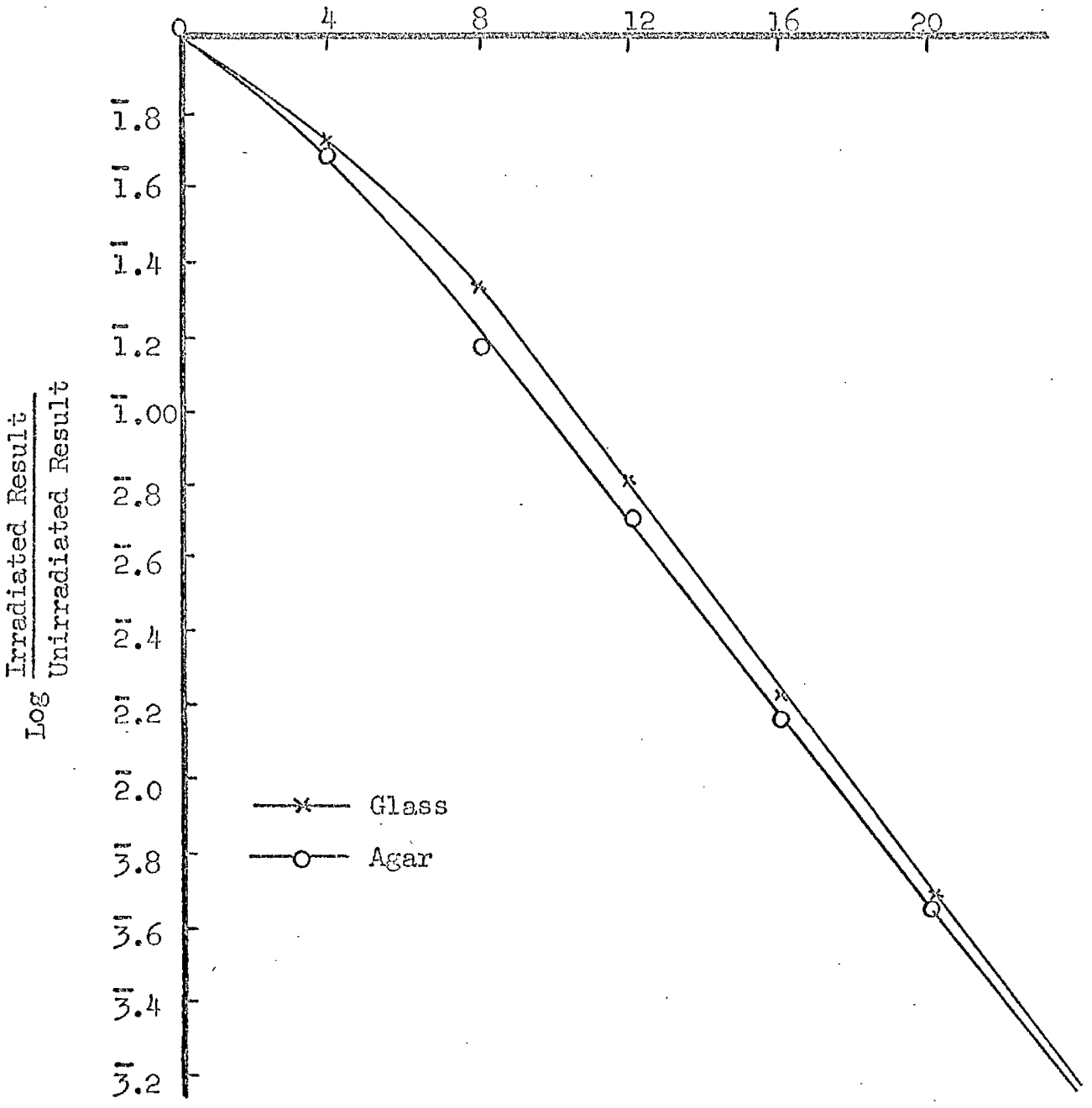

FIGURE 13. UV Radiation Survival Curves for PyH3 Cells Plated, without Feeder Cells, in Agar and on Glass.

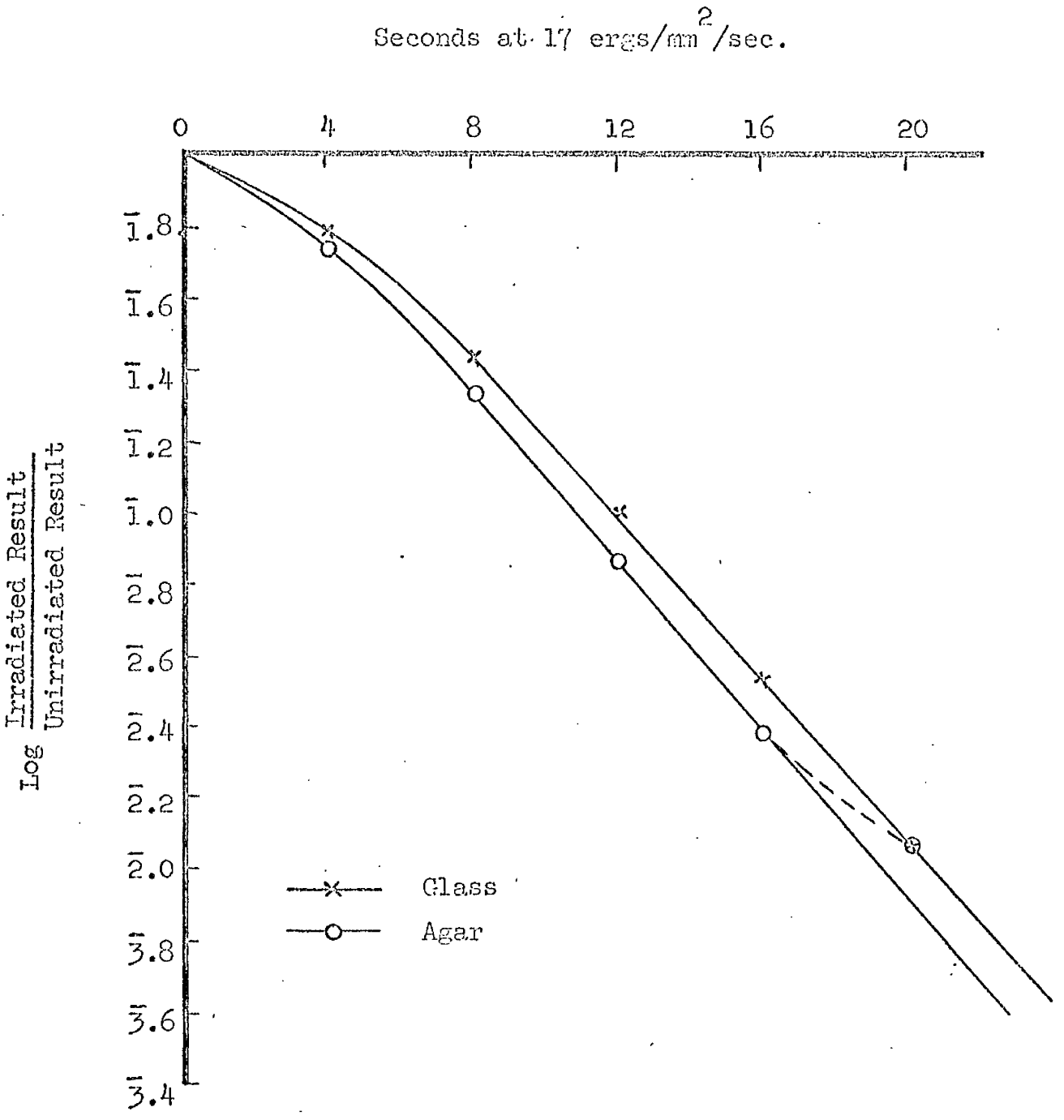

FIGURE 14. UV Radiation Survival Curves for PyH3 Cells Plated, with 10^5 Feeder Cells/Plate, in Agar and on Glass.

the number of cells plated out in these experiments had to be increased with increasing dosage. It was found that when more than 4, 000 test cells were plated per 60 mm Petri dish for colony formation the survival curve tended to deviate from the exponential part of the curve. This was possibly due to a very small proportion of the cells (less than 1%) which were partially protected from the irradiation dose by the sides of the dish in which they were irradiated. Alternatively, the greater number of cells per unit area could have produced a feeder effect resulting in a greater survival of the irradiated cells.

There was no significant difference between the survival curves obtained for PyH3 cells and PyH6 cells.

4. The sensitivity of a near tetraploid line of BHK 21 cells (4n BHK 21) to UV radiation

(a) Introduction

The BHK 21 C13 cells, transformed by polyoma virus, which were tested, were less sensitive to the effects of UV light than the untransformed cells. Macpherson (1963) has reported that a polyoma virus transformed clone of BHK 21 C13 cells - clone

C13-TC6 - had a tendency to accumulate chromosome abnormalities and tetraploid cells, although these abnormalities were not immediately associated with transformation. The lower sensitivity of the transformed cells tested could be due to their containing more DNA and hence more targets. The 4 n BHK 21 cells contain almost twice the amount of DNA present in normal BHK 21 C13 cells and survival curves of this line of cells were determined to investigate whether cells containing more DNA were less sensitive to the effects of UV radiation.

(b) Results

The results are shown in Figure 15. Five separate determinations of each curve were performed. When the irradiated 4 n BHK21 cells were plated with or without feeder cells the D_{37} values were higher than those obtained for BHK21 C13 cells. When the 4 n BHK21 cells were plated with feeder cells the D_{37} value was 139.6 ± 8.1 ergs/mm^2 and when plated without feeder cells the D_{37} value was 109.4 ± 9.0 ergs/mm^2.

The values for D_0 for 4 n BHK21 cells were 66.3 ± 4.7 ergs/mm^2 for cells plated with feeder cells and 56.1 ± 5.1 ergs/mm^2 for those plated without feeder cells. There was, therefore, an increase in the

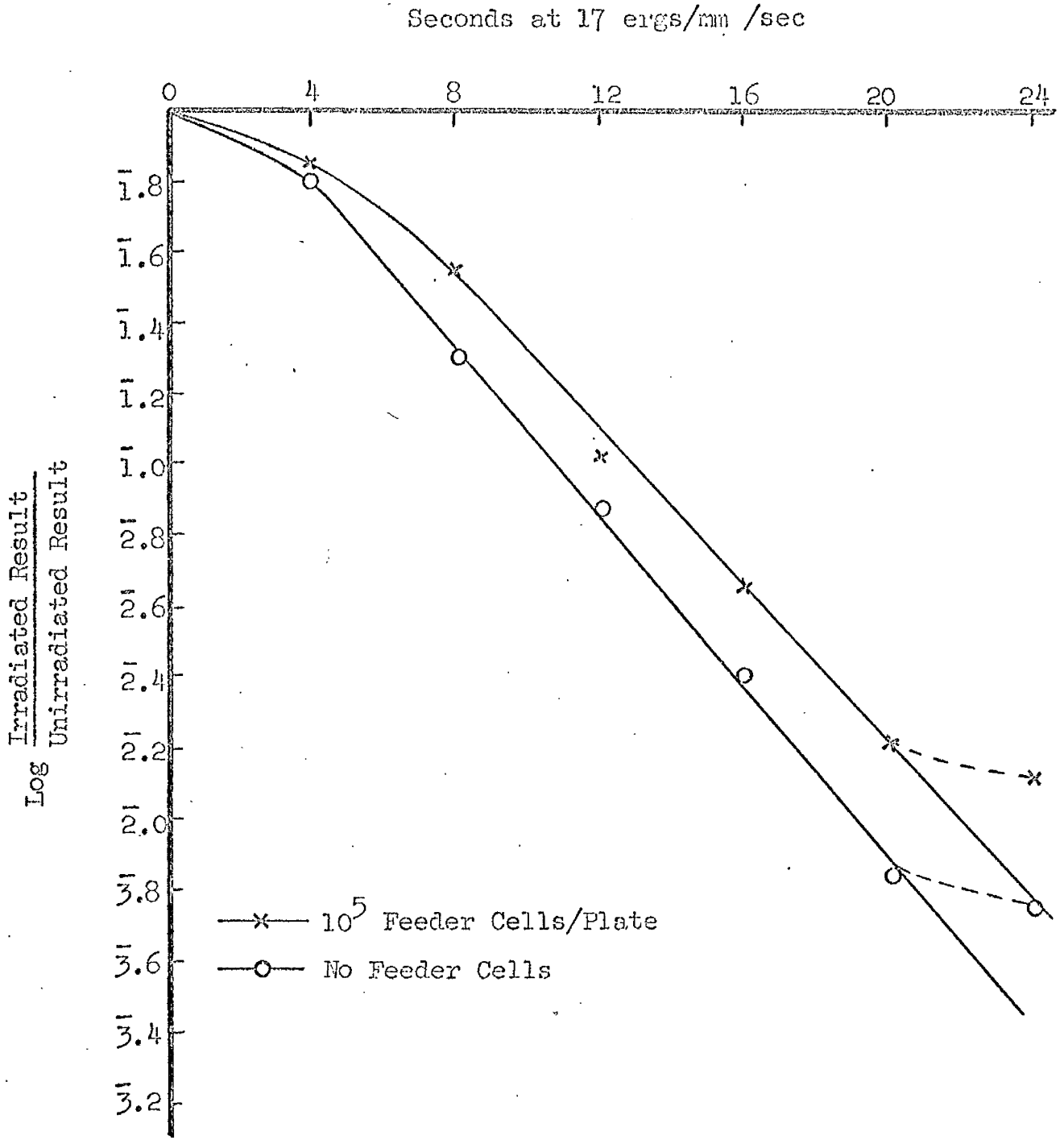

FIGURE 15. UV Radiation Survival Curves for 4n BHK21 Cells Plated, with and without Feeder Cells, on Glass.

D_{37} value compared to that for BHK 21 C13 cells but only a slight increase in the D_0 value.

5. Failure to isolate ultraviolet resistant BHK 21 C13 cells and failure to transform cells by ultraviolet irradiation alone.

(a) Introduction

Lassueur (1904) first reported that repeated doses of X-rays caused some tumours to become radioresistant. This result suggested that it might be possible to produce a population of BHK 21 C13 cells which would be resistant to UV radiation if a normal population were repeatedly exposed to UV radiation.

Repeated exposure of a population of cells to UV radiation might also lead to morphological transformation or to the ability to form colonies in agar. Blum (1959) has shown that UV light can cause skin tumours in mice and that the presence of cancer induction in mice by UV light is a cumulative process. Transformation *in vitro* by UV radiation has not been demonstrated but Borek and Sachs (1966) have reported that a single 300 r dose of X-radiation will transform about 0.5 - 2% of normal hamster cells irradiated *in vitro*.

Exposure of BHK 21 C13 cells to repeated high doses of UV radiation interspersed with periods during which the cells were permitted to recover at 37°C could lead to mutagenesis of some of the cells. For example, they might acquire the ability to form colonies in agar. Repeated doses of UV radiation could also lead to selection of pre-existing or induced mutants which were resistant to UV radiation.

A series of experiments was performed to investigate the two following possibilities: (a) that repeated exposures of a population of BHK 21 C13 cells to UV radiation would give rise to a cell line which was resistant to the effects of UV radiation and (b) that some cells which had been repeatedly irradiated with UV light might show morphological transformation or the ability to form colonies in agar.

(b) Method

Monolayers of BHK 21 C13 cells were irradiated with a dose of 140 ergs/mm^2 every 24 hours for up to 7 days. After each treatment all the monolayers were incubated at 37°C for 24 hours. At the end of each incubation period samples of the cells were irradiated for survival curve determinations and plated with feeder cells on glass.

Alternatively they were plated in agar without feeder cells to investigate whether any of the cells were transformed and therefore could grow in agar suspension medium.

Borek and Sachs (1967, 1968) have shown a fixation of the transformed state in cells exposed to X-rays, if the irradiated cells were incubated at 24°C. In two experiments the treated cells were incubated at either 4°C or 32°C for 24 hours immediately after the last irradiation treatment. They were then plated out in agar at 37°C for colony formation as an index of transformation. This was to determine whether incubation at different temperatures would have any effect on transformation due to UV irradiation alone.

(c) Results

The final populations of cells, even after 7 days irradiation treatment showed survival curves which did not differ significantly from those obtained using unirradiated control cells. For example, the D_{37} and D_0 values, from 5 separate determinations, for cells treated for 7 days with 140 ergs/mm^2 were 108.1 ± 13.7 ergs/mm^2 and 36.4 ± 9.1 ergs/mm^2 respectively, whereas for the controls they were 111.2 ± 8.2 ergs/mm^2 and 39.7 ± 4.0 ergs/mm^2. The

plating efficiency however for this cell population was 8.7 ± 2.9% as opposed to 69.2 ± 4.8% in the controls. Therefore repeated doses of UV radiation did not appear to cause a population of BHK 21 C13 cells to become more resistant to UV radiation in this system. There was no evidence to suggest that there was any mutation of cells giving UV resistant cells or selection of these mutated cells resulting in a resistant population.

Irradiated cells did not show random growth when plated out on glass and those cells which had been plated in agar either with or without temperature treatment did not show a significantly different number of colonies growing when compared with the unirradiated control cells plated in agar. It appeared therefore that, in this system, repeated irradiations of BHK 21 C13 cells did not lead to transformation of those cells.

6. Attempted photoreactivation of BHK 21 C13 cells after UV irradiation

(a) Introduction

Photoreactivation, a repair mechanism concerned with the recovery of organisms from damage due to UV irradiation, was

discovered by Kelner (1949). Jagger (1958) has defined photoreactivation as "the restoration of UV radiation lesions in a biological system with light of wavelength longer than that of the damaging radiation". The most common lesions in the DNA are pyrimidine and, in particular, thymine dimers (Beukers and Berends, 1960). It was shown by Wulff and Rupert (1962) that thymine dimers are eliminated from DNA by a photoreactivating enzyme. This enzyme acts by splitting the dimers in the presence of light. It has been demonstrated in many micro- and higher organisms (Jagger, 1968; Blum, 1959; Setlow, 1966). It has not been clearly demonstrated in mammalian cells.

Cleaver (1966b) looked for photoreactivation in three established mammalian lines and in one primary mammalian culture. He failed to find any trace of photoreactivation in the cells which he used. He reviewed the data previously published and concluded that, in all probability, mammalian cells did not have a photoreactivation mechanism.

It appeared possible that if photoreactivation occurred in BHK 21 C13 cells, integration of polyoma virus DNA might be affected either

by the presence of pyrimidine dimers in the DNA or by their being split. All the transformation experiments were performed under normal conditions of laboratory lighting and these conditions might have permitted a certain amount of photoreactivation to occur. A series of experiments was performed to find out if BHK 21 C13 cells showed photoreactivation or not.

(b) Method

Monolayers of BHK 21 C13 cells were irradiated for survival curve determinations. All irradiations were carried out in the absence of any light, other than that from the UV source. Half the plates were kept in darkness and the other half were exposed, for periods of time extending from one minute to two hours, to either a Warm White strip light (Phillips - MCFE 65/80w/29) or to bright sunlight. Both types of irradiation were carried out in 60 mm glass Petri dishes so that any UV light - particularly from the sun - would be filtered out by the glass lid of the Petri dish.

All the plates were returned to the dark. All manipulative procedures were carried out using a Kodak Safe Light filter ("Wrattan" series OB Lime-Yellow) as the only source of illumination. This filter did not allow light of wavelength shorter than

5, 000Å to pass through it. This is well above the most efficient wavelength for photoreactivation which is 3, 800Å (Jagger, 1958).

The cells were then plated out with feeder cells in order to determine the survival curves. The plates were stacked in new, sealed biscuit tins, which had been lined with metal foil to prevent light reaching the cells during colony growth. The tins were gassed with a mixture of 5% CO_2 in air before sealing.

(c) Results

There was no significant difference between the survival curves obtained when cells were exposed to light or kept in darkness after irradiation. For example, cells exposed to the strip light for 2 hours before being returned to the dark had plating efficiency, D_{37} and D_0 values of 67.7 ± 3.0, 113.6 ± 8.0 ergs/mm^2 and 61.7 ± 3.9 ergs/mm^2. The equivalent values obtained for cells kept completely in the dark were 66.9 ± 4.0, 117.2 ± 9.2 ergs/mm^2 and 57.6 ± 5.2 ergs/mm^2. These values are derived from 5 separate determinations for each curve.

No evidence was obtained to suggest that BHK 21 C13 cells have a photoreactivation mechanism. This result is in agreement with

the conclusion drawn by Cleaver (1966b) that such a mechanism is absent from mammalian cells.

7. Repair of sublethal radiation damage in BHK 21 C13 cells

(a) Introduction

Elkind and Sutton (1960) demonstrated the repair of sublethal damage in Chinese hamster cells after acute X-radiation doses, by fractionating the dose of X-radiation given to the cells. The normal survival curve for mammalian cells, after X-irradiation, is a threshold type curve. This is observed both with asynchronous populations (Elkind and Whitmore, 1967) and with synchronous populations (Terasima and Tolmach, 1963) irradiated at different periods during their growth cycles. The presence of a shoulder on the survival curves means that damage must be accumulated for a lethal effect. Surviving cells must, therefore, be sublethally affected (Elkind and Sutton, 1960) and probably the only difference between lethal and sublethal damage is degree.

Elkind and Sutton (1960) showed that, during the interval between the time of irradiation and the expression of surviving

proliferative ability, cells could repair sublethal damage. They used a fractionated dose technique by which they gave the cells an initial dose of radiation sufficient to overcome the threshold effect and followed this by a second dose after varying periods of time. When the second dose was given shortly after the first, before sublethal repair could have an effect, the inactivation of the proliferative ability of the cells continued to show the same kinetics as those in the control cells. However, when the second dose was given after longer periods of time, the survival curve, instead of following the control curve, showed the presence of a new threshold. These experiments were performed before any cell division occurred. For Chinese hamster cells the sublethal repair took approximately 1.5 hours to have a 50% effect (Elkind, Sutton-Gilbert, Moses and Kamper, 1967). A series of experiments based on a fractionated dose technique similar to that used by Elkind and Sutton (1960) was performed to investigate whether sublethal repair of UV radiation damage occurred in BHK 21 C13 cells as it seemed possible that transformation by polyoma virus could be enhanced as a result of the viral DNA being integrated into the cellular DNA more efficiently during repair of UV radiation damage.

(b) Method

Monolayers of BHK 21 C13 cells were irradiated at a dose rate of 17 ergs/mm^2/second. The control monolayers were irradiated with UV light and plated out with feeder cells to determine the survival of colony forming ability. The remaining plates received a dose of 136 ergs/mm^2 - sufficient to lower the level of cell survival to the top of the exponential region of the survival curve. These plates were then incubated at 37^{o}C. After various times of incubation, plates were removed from the incubator, given the same graded doses of UV radiation as the controls, and plated out on feeder cells for survival curve determinations. The control survival curve was expressed as colony forming ability relative to unirradiated cells. The experimental survival curves were expressed as the colony forming ability relative to the colony forming ability of cells which had received an initial dose of 136 ergs/mm^2.

Growth of cells during the time course of the experiment (0-24 hours) was followed by making cell counts, both for unirradiated control plates and plates which had received 136 ergs/mm^2.

(c) Results

Figure 16 shows the number of cells present in unirradiated cultures and in cultures receiving 136 ergs/mm^2 during the course of the experiment. The number of cells has been plotted relative to the number present at the start of the experiment. The number of cells present when the experiments were started has been taken as one.

The lower curve in Figure 16 shows the number of cells in the irradiated cultures from time zero to 24 hours. No significant change in cell number was noted during the first 16 hours after irradiation.

The upper curve in Figure 16 shows the growth curve for the unirradiated cells. It is clear from these results that the UV radiation causes a marked delay in the onset of proliferation by BHK 21 C13 cells.

Figure 17 shows the plating efficiency of BHK 21 C13 cells which had received an irradiation dose of 136 ergs/mm^2 at various times before plating out onto feeder cells. The results are plotted relative to the plating efficiency of the control cells which has been taken as one. The results show that there has been a marked

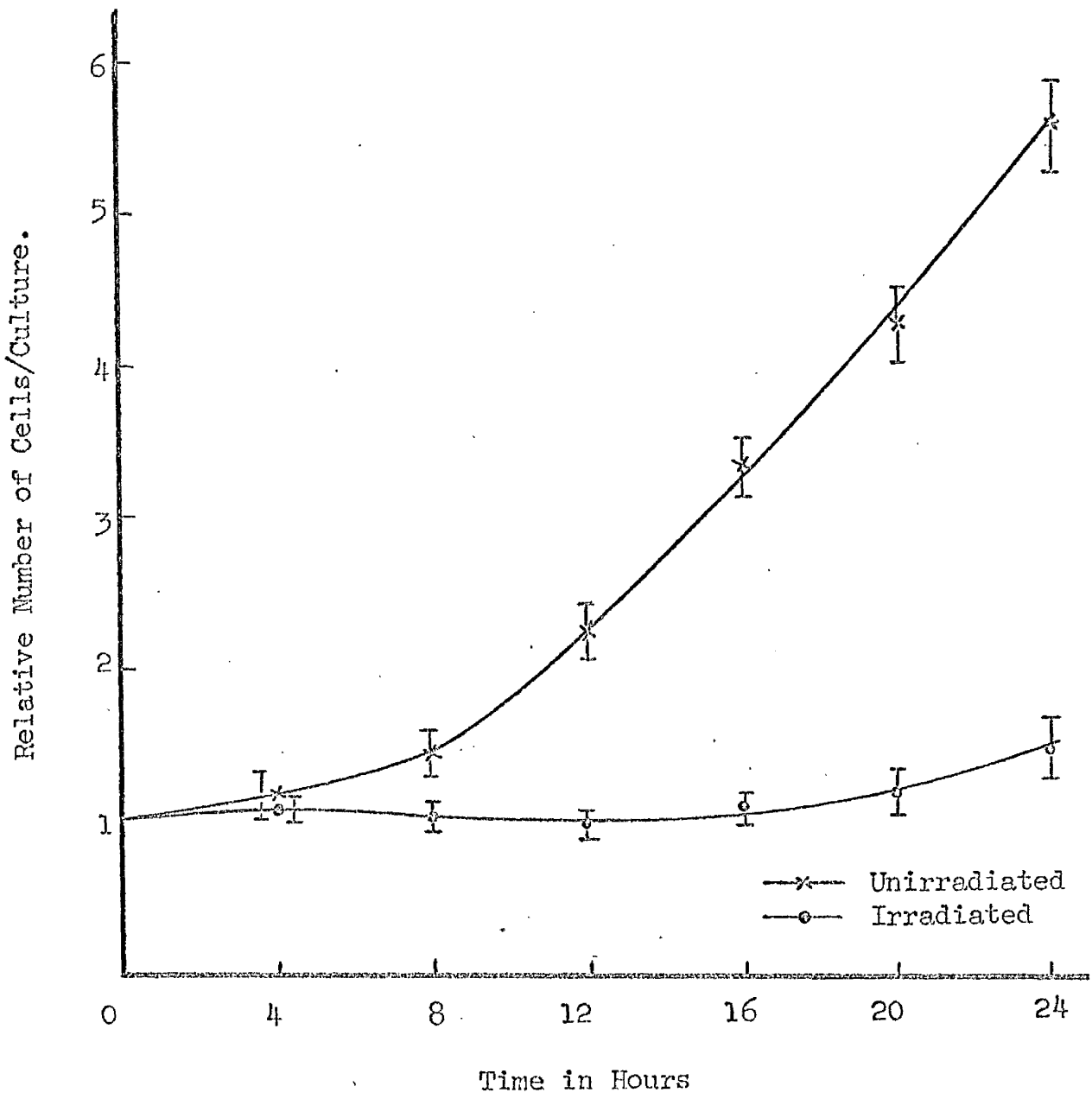

FIGURE 16. Growth Curves for Unirradiated and UV Irradiated (136 ergs/mm^2) BHK21 C13 Cells.

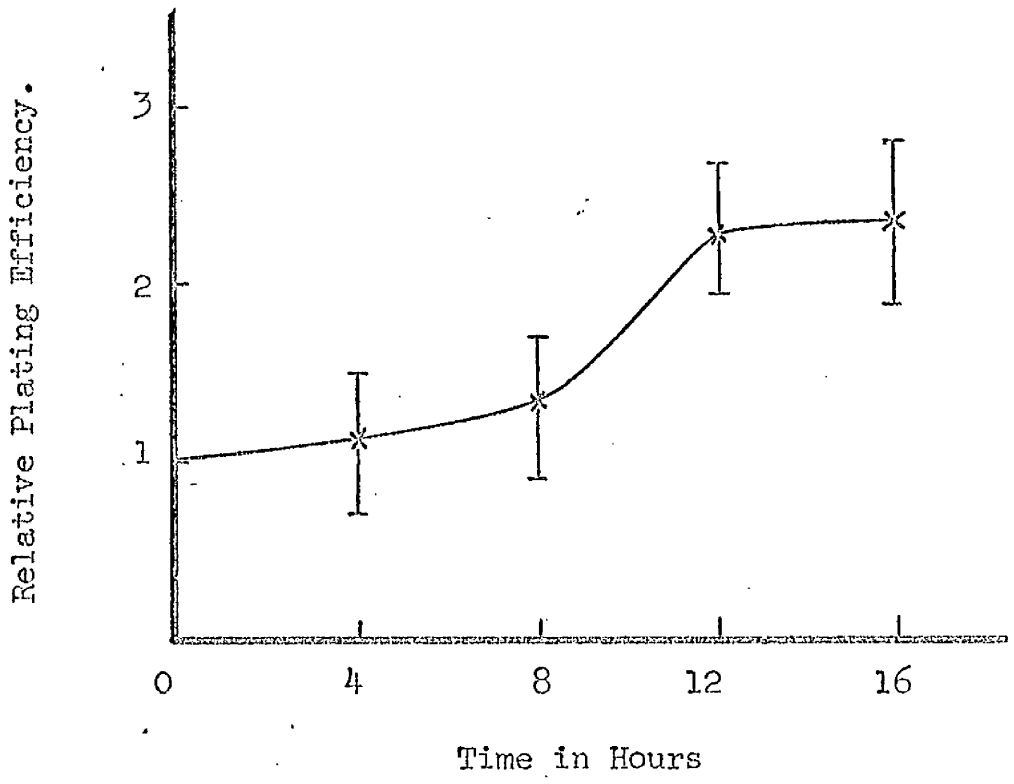

FIGURE 17. Plating Efficiency of UV irradiated BHK21 C13 Cells after Incubation at 37°C following a Dose of 136 ergs/mm^2.

increase in the number of cells which can form colonies after a dose of 136 ergs/mm^2 by 12 hours after the initial dose was given. The main increase in plating efficiency occurred when the cells were plated out between 8 and 12 hours after irradiation. The process which increased the ability of the cells to form colonies appeared to be complete by 12 hours after the irradiation as there was no significant difference in plating efficiency between the cells plated out 12 hours and 16 hours after irradiation. The data in Figure 16 show that no increase had occurred in the number of cells present in the irradiation cultures by 12 hours.

Figure 18 and Table 16 show the results of the survival curves of BHK 21 C13 cells which had been given graded doses of radiation after various periods of incubation at 37^{o}C, following an initial radiation dose of 136 ergs/mm^2. When the graded doses were given after 4 hours incubation the survival curve of the cells did not differ significantly from the exponential part of the control curve. There was no appearance of a shoulder on the curve to indicate the return of a threshold. When the second series of doses was given after 8 hours incubation there were signs of a

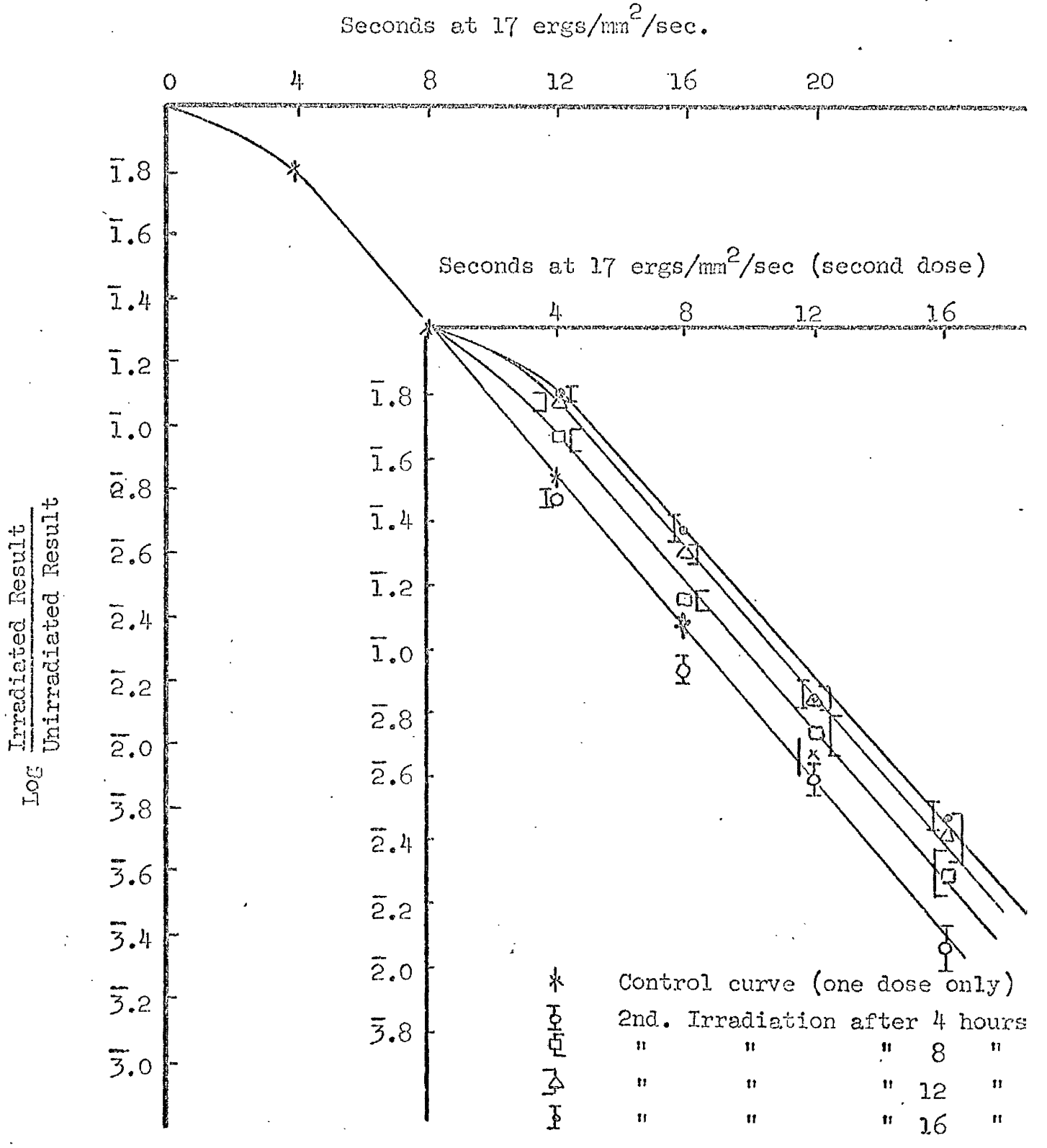

FIGURE 18. Split Dose Experiment to Show Repair of Sublethal Damage in UV Irradiated BHK21 Cl3 Cells. Ten Plates were used in the Determination of Each Point.

Incubation time before 2nd UV dose	D_{37} (ergs/mm^2)	D_0 (ergs/mm^2)
Controls	106.0	62.8
4 hours	62.8	62.8
8 hours	78.7	63.2
12 hours	94.0	63.3
16 hours	97.4	66.7

Table 16. D_{37} and D_0 values from Figure 18, showing an increase in the D_{37} value during the repair of sublethal damage.

shoulder to the curve returning. This shoulder was much more pronounced when the cells were given the second series of doses after 12 hours incubation. By 12 hours the D_{37} value had increased from 62.8 ergs/mm^2 at 4 hours to 94.0 ergs/mm^2 (Table 16). There was little difference between the survival curve after 12 hours incubation and that after 16 hours incubation. There was only a very slight increase in the value of D_0 during the period up to 16 hours after the initial dose of UV radiation was given.

It would therefore appear that at least some UV radiation induced sublethal damage can be repaired in BHK 21 C13 cells although the expression of this repair was not seen until 8 hours after the initial irradiation under the conditions used in these experiments.

8. The effect of cycloheximide on the survival curves of BHK 21 C13 and PyH3 cells.

(a) Introduction

Phillips and Tolmach (1966) reported that cycloheximide, ((β [2(3, 5-dimethyl-2-oxycyclohexyl)-2-hydroxyethyl] glutarimide)

Sigma Chemical Company, St. Louis, Missouri, U.S.A.), an inhibitor of protein synthesis, increased survival of HeLa S3 cells when administered to cultures at 8 μg/ml after X-irradiation. They suggested that the cycloheximide might enhance a repair process, perhaps by inhibiting a competing process by which the potentially lethal damage is ordinarily expressed in the cell.

A series of experiments was performed to determine whether cycloheximide, at the concentration used by Phillips and Tolmach (1966), would affect the survival curves of BHK 21 C13 or PyH3 cells after UV irradiation. If some form of repair which was enhanced by cycloheximide existed in these cells then it was possible that integration of the polyoma virus genome during transformation would be enhanced.

(b) Method

Cycloheximide was made up as a stock solution in ETC at a concentration of 8 μg/ml. Monolayers of BHK 21 C13 or PyH3 cells were irradiated with graded doses of UV radiation and 5 ml of the cycloheximide solution were added to each plate. Control plates received 5 ml of ETC. All the plates were then incubated at 37°C for 3 hours.

At the end of the incubation period the monolayers were washed twice in ETC and then incubated under 5 ml fresh ETC for 30 minutes to reverse the inhibition due to the cycloheximide. The treated cells were then plated out for colony formation on glass with and without feeder cells.

(c) Results

The dosage of cycloheximide used was not found to be toxic to the cells and hence did not affect the initial plating efficiency of the unirradiated control cells.

The results are shown in Table 17. In no experiment did the survival curve for cycloheximide treated cells differ significantly from that obtained for the untreated control cells. There was, therefore, no evidence obtained that cycloheximide improved survival under the conditions used in these experiments.

9. Discussion

The results reported in this chapter relate to the ultraviolet radiobiology of BHK 21 C13 cells. The experiments have been designed to try to elucidate the effect of UV radiation on the cells

Sample	Feeder cells	Cycloheximide	Average PE%	D_{37} ergs/mm^2 ± 2 x S. E.	D_0 ergs/mm^2 ± 2 x S. E.
BHK 21 C13	+	-	68.2	112.1 ± 7.2	61.7 ± 5.6
BHK 21 C13	-	-	17.1	80.4 ± 8.0	40.2 ± 4.6
BHK 21 C13	+	+	68.6	116.1 ± 9.0	59.9 ± 4.3
BHK 21 C13	-	+	16.8	81.3 ± 10.4	38.6 ± 4.7
Py H3	+	-	59.2	123.6 ± 6.1	76.3 ± 4.6
Py H3	-	-	38.4	101.2 ± 9.2	56.2 ± 4.6
Py H3	+	+	58.7	126.7 ± 10.0	77.1 ± 6.0
Py H3	-	+	37.6	96.9 ± 8.8	54.4 ± 4.4

Table 17. The effect of cycloheximide on the survival curves of BHK 21 C13 and Py H3 cells plated on glass. These results were derived from 5 separate determinations for each sample.

which resulted in an enhanced transformation rate following infection with polyoma virus.

The first possibility to be considered was the effect that doses of UV radiation would have on the ability of the cells to produce interferon. De Maeyer-Guignard and De Maeyer (1965) showed that UV irradiation of rat cells decreased interferon yields which led to the cells being more susceptible to viral infection. Allison (1961) showed that polyoma virus was susceptible to interferon and would itself produce interferon in mouse cells. Todaro and Baron (1965) have shown that the transformation of 3T3 cells by SV40 is inhibited by interferon, although SV40 does not multiply in 3T3 cells. Rotem, Berwald and Sachs (1964) showed that primary hamster cells infected with Newcastle Disease Virus produced an interferon. This information suggests that polyoma virus is sensitive to interferon and that interferon production can be induced in hamster cells by certain viruses. The results obtained when an attempt was made to induce interferon in BHK 21 C13 cells with polyoma virus are, therefore, somewhat surprising in that no free interferon could be demonstrated in this system. The results

suggest that polyoma virus is unable to induce the synthesis of interferon in BHK 21 C13 cells or that the technique used was not sensitive enough to detect a low level of interferon released into the medium by the cells. It seems unlikely, therefore, that UV irradiation affects transformation by reducing interferon production.

Viral interference, possibly due to interferon, was seen when polyoma virus infected BHK 21 C13 cells were infected with EMC virus. UV radiation, at the doses used in the experiments, did not significantly affect this interference and it is therefore possible that the cells were able to produce sufficient interferon to prevent some of the EMC virus particles from successfully infecting the cells. However, little interferon could have been released into the medium as it could not be detected in the experiments discussed above. It is also possible that some form of interference which was not due to interferon could be responsible - for example - competition for sites of viral attachment or alteration of the sites of attachment by the polyoma virus particles.

Before studying possible repair of UV damage, survival curves of normal and polyoma virus transformed BHK 21 C13 cells were

determined. They were of the normal type described for mammalian cells. The D_0 values for the two transformed lines tested were significantly higher than those for the untransformed cells under identical experimental conditions. This result was in agreement with that of Williams and Till (1966) who showed that established lines of polyoma virus transformed rat cells were, in general, more resistant to X-radiation than the normal rat embryo cells from which they were derived. The reason for the observed decrease in sensitivity of transformed cells to radiation is unknown although it is possible that the size of the cells may be of importance. Stoker, O'Neill, Berryman and Waxman (1968) have reported that the polyoma virus transformed BHK 21 C13 cells of Clone PyH3 were larger than the normal BHK 21 C13 cells from which they were derived. The PyH3 cells had twice the volume of BHK 21 C13 cells. The larger cell size could possibly help to shield the DNA from the effects of the UV radiation.

The presence of mouse embryo feeder cells in cultures during survival curve determinations caused a significant increase in both the D_0 and the D_{37} values. This result suggested that the presence

of feeder cells improved the chance of survival of some of the irradiated cells and might be analogous to a system described by Delaporte (1956) for X-irradiated bacteria. He reported that cell division was more likely to occur in an irradiated population of Escherichia coli B if the cells were tightly grouped on an agar surface. This phenomenon was termed "neighbour restoration". Adler, Fisher, Hardigree and Stapleton (1966) confirmed this result and extended the findings to UV irradiated bacteria. They found that an extract of the bacteria had the same effect and concluded that it was due to a diffusible substance leaking from groups of cells and stimulating cell division.

In mammalian cell systems, isolated cells have a low probability of proliferation in culture. A higher proportion of such cells can proliferate in the presence of feeder cells (Puck and Marcus, 1955). It appears that a feeder effect in both bacteria and mammalian cells can stimulate cell division in radiation damaged cells.

One possible way by which this feeder effect might work in the present system is by metabolic co-operation - a process whereby the metabolism of cells in contact is modified (perhaps controlled)

by exchange of material (Subak-Sharpe, Bürk and Pitts, 1966, 1968). These workers have found that when cells of a genetic variant of the BHK 21 line which lack inosinic pyrophosphorylase activity, and therefore cannot normally incorporate ^{3}H hypoxanthine, were grown in mixed culture with BHK 21 cells having inosinic pyrophosphorylase activity, they could, when in contact with the cells possessing inosinic pyrophosphorylase activity, incorporate ^{3}H hypoxanthine. It is therefore possible that, in the present system, the feeder cells might, in exchanging material with the UV irradiated cells assist the UV irradiated cells in their recovery from the effects of the radiation.

It is well established that chromosomes are the principal target for radiation-induced loss of proliferative ability of mammalian cells. It might therefore be expected that survival curves would be affected by changes in the chromosomal complement. Most of the work on these lines has been carried out with X-radiation and the results have fallen into two categories. Till (1961) and Lockart, Elkind and Moses (1961) have reported that there is no systematic change in the survival curve with increasing chromosome number. However,

Silini and Hornsey (1962) and Berry (1963) have reported an increase in the shoulder of the survival curve with little change in slope. The results in the present study when 4n BHK 21 cells were irradiated with UV radiation appear to fall into the latter category. There was an increase in the D_{37} value with only a slight increase in the D_0 value. This increase in the D_0 value was more pronounced when the survival curve was determined in the absence of feeder cells.

These results suggested that the amount of sublethal damage that could be absorbed by the cells was greater in the 4n BHK 21 cells than in the BHK 21 C13 cells. It is possible that the extra chromosomes, and hence the extra DNA, cause more duplication of the information in the DNA. Therefore more targets would have to be destroyed by the UV irradiation before the cells were prevented from further division.

Once the extra sublethal damage had been absorbed the 4n BHK 21 cells were prevented from further growth at approximately the same inactivation rate as the diploid BHK 21 C13 cells. The D_0 values suggest that the accumulation of lethal damage is not markedly affected by the presence of the extra chromosomes.

The D_0 values for the 4n BHK 21 cells suggest that the higher D_0 values, obtained for the PyH3 cells, compared with those obtained for the BHK 21 C13 cells, were not due either to any difference in the amount of DNA in the transformed cell or to a larger target size. The greater resistance to the effect of UV irradiation shown by the PyH3 cells must be a property which the cells acquire sometime after transformation by polyoma virus.

Attempts to produce lines of BHK 21 C13 cells which were resistant to UV radiation and to transform cells by UV radiation alone both failed. Repeated doses of UV radiation did not significantly affect survival curves and no signs of transformation were observed. These results differ from those obtained by other workers for X-irradiation of mammalian cells. Balmukhanov, Yefimov and Kleinbock (1967) obtained radio-resistant strains of Ehrlich ascites cells by repeated X-irradiation of the cells and Borek and Sachs (1966) have reported transformation of freshly isolated hamster embryo cells by a single dose of X-radiation. There has been no report of BHK 21 C13 cells being transformed by either X-radiation or chemical carcinogens. The negative

result obtained for transformation *in vitro* by UV light alone might be explained by reference to work *in vivo*. Blum (1959) found that the process of cancer induction in mice by UV light was cumulative. He found that regularly repeated doses of UV radiation must be given over several months before cancers appeared at the site of irradiation. The time required to produce tumours was inversely related to the square root of the dose and directly related to the square root of the interval between doses. The cumulative effect of UV radiation in the malignant transformation of cells suggests that some time may have to elapse before the malignant transformation could be expressed.

Photoreactivation of UV irradiated BHK 21 C13 cells could not be demonstrated. This result was in agreement with the conclusion drawn by Cleaver (1966b) for mammalian cells. It is probable that photoreactivation does not occur in mammalian cells although it has been reported in vertebrate cells - for example, Regan and Cook (1967) demonstrated photoreactivation in a line of fish cells. Cook and McGrath (1967) failed to detect any photoreactivating enzyme in a variety of mammalian tissues and it is probable that this enzyme is absent from mammalian cells.

The interpretation of the results obtained when split dose experiments were performed to measure the repair of UV radiation induced sublethal damage in BHK 21 C13 cells was difficult. One of the main problems is that some surviving cells may divide during the time interval necessary to perform these experiments. In the present results there did not appear, however, to be sufficient proliferation by 12 hours after the initial dose to account for the two-fold rise in plating efficiency observed at this time. The main increase in the value for D_{37} had also occurred by 12 hours - that is, a clearly defined shoulder had appeared in the survival curve of the cells receiving the second dose.

The results imply that some repair of sublethal damage had occurred during the time course of the experiment. There is now evidence suggesting that repair replication of UV radiation damage occurs but the evidence applies mainly to human cells. Cleaver and Painter (1967) and Djordjevic and Tolmach (1967) have demonstrated "repair replication" in HeLa cells which, unlike normal semi-conservative replication, was not inhibited by hydroxy-urea. Regan, Trosko and Carrier (1968) have shown that about 50% of

the UV radiation induced pyrimidine dimers were lost from the DNA of human cells and found in the TCA-soluble fraction within 24 hours of the irradiation. Although Trosko, Chu and Carrier (1965), Steward and Humphrey (1966) and Trosko and Kasschau (1967), have been able to demonstrate pyrimidine dimers in Chinese hamster cells, they have not been able to demonstrate loss or "excision" of the dimers from the DNA. However, caffeine is known to inhibit dimer excision in bacteria (Setlow and Carrier, 1967) and Rauth (1967) has suggested that there is a caffeine sensitive repair mechanism in mouse cells analogous to dark repair in bacteria. Rasmussen and Painter (1966) have reported that several lines of mammalian cells also show non-semiconservative replication after irradiation. The present results suggest that a repair system, at least for sublethal damage, exists in BHK 21 C13 cells.

A series of experiments was performed to investigate whether cycloheximide would affect the repair of UV induced damage in BHK 21 C13 cells. The increased survival of X-irradiated HeLa S3 cells after treatment with cycloheximide, reported by Phillips and Tolmach (1966) occurred in synchronised cell cultures. The

presence of the drug had little effect on the cells X-irradiated in the G_1 part of the cell cycle but the effectiveness of the treatment had increased significantly by the end of G_1 and its greatest effect was on cells X-irradiated in the early and middle phases of S. Its effect on cells in the latter portions of the cell cycle was not determined.

The cells used in the present study were asynchronous and irradiated with UV light. However, it is probable that there would have been enough cells in the stages which Phillips and Tolmach (1966) reported as being affected by the cycloheximide to have had some effect on the survival curves. No such effect was observed. The present results suggest that cycloheximide does not affect cells irradiated with UV light in the way reported by Phillips and Tolmach (1966) for the X-irradiated cells. Therefore, a repair system sensitive to cycloheximide treatment was unlikely to be responsible for the enhanced transformation rate of polyoma virus infected BHK 21 C13 cells. Possible mechanisms to explain the observed enhancement of the transformation rate when BHK 21 C13 cells were infected with polyoma virus will be discussed, in the light of the findings in this chapter, in the General Discussion.

CHAPTER VI

GENERAL DISCUSSION

In Chapter IV it was shown that UV-irradiation of BHK 21 C13 cells made them more sensitive to transformation by polyoma virus, and in Chapter V some studies on the ultraviolet radiobiology of BHK 21 C13 cells were reported. Although the results reported in Chapter V did not point directly to a mechanism by which the BHK 21 C13 cells were sensitised to transformation, they did exclude a number of possible mechanisms.

The enhanced transformation rate could not have been due to the selection of pre-existing, genetically susceptible cells. If genetically susceptible cells were selected for, then their sensitivity to transformation should remain indefinitely after irradiation. This was not so as the results showed that any enhancing effect was lost when the cells were irradiated more than 24 hours before infection.

It was possible that synchronisation of the cells by UV radiation could have led to an enhanced transformation rate as Basilico and Marin (1966) reported that BHK 21 C13 cells became more susceptible to transformation by polyoma virus with the progression of the cells through interphase, reaching a maximum in G_2. Although there

appear to be no reports suggesting that UV irradiation can lead to cell synchrony, Harrington (1960) and Whitmore, Stanners, Till and Gulyas (1961) have reported that there is a block in the cell cycle at the end of the G_2 phase after X-irradiation of mammalian cells. Yamada and Puck (1961) and Whitmore, Stanners, Till and Gulyas (1961) have reported that when mitotic figures appear in an X-irradiated population or when cell division recommences, there may be a transient overshoot in the rate of entry of cells into mitosis or division leading to a small degree of synchrony in the population. If either a block in G_2 or synchrony occurred after UV irradiation then the enhancement of the transformation rate could be due to the cells being held in the phase in which they are most susceptible to transformation by polyoma virus.

Cleaver (1965) has reported a block in the G_2 phase after UV irradiation in mouse L cells. However he used very large doses of radiation - a minimum of 240 ergs/mm^2. When lower doses of UV radiation are given, no such block has been seen (Humphrey, Dewey and Cork, 1963; Djordjevic and Tolmach, 1967). Djordjevic and Tolmach (1967) used HeLa S3 cells and doses up to

70 ergs/mm^2 (maximum enhancement in the present study occurred at approximately 60-80 ergs/mm^2). They found that these doses only inhibited DNA synthesis and did not delay the cells in G_2. This result was consistent with that reported by Humphrey, Dewey and Cork (1963) for Chinese hamster cells.

As no G_2 block occurs with low doses of UV radiation, the cells in the present study would not be held in this phase for a longer than normal period and, therefore, the period when they were most susceptible to transformation by polyoma virus would not be extended. Similarly, in the absence of a block in the cell cycle, there would not be any synchronous division and hence this also could not be the cause of the observed enhancement of transformation.

The present results could be explained if UV radiation had some inhibitory effect on the protein synthesis of the BHK 21 C13 cells leading to an enhancement of the transformation rate. Marin and Basilico (1967) have reported that puromycin, at a concentration of 10^{-4}M caused a drastic, but fully reversible, inhibition of protein synthesis in BHK 21 C13 cells. A two to three fold enhancement of the polyoma virus induced transformation rate was obtained when

the cells were exposed to puromycin for a period of 5 hours, starting at the end of the virus adsorption period. No transformation enhancement was observed when the cells were exposed to the drug, prior to infection. Klímek and Vlašínová (1967), however, have shown that the rate of protein synthesis in L cells, as measured by incorporation of ^{14}C-d, l-valine and ^{3}H-l-lysine, was not influenced by up to 200 ergs/mm^2 during the 3 hour period after the irradiation which they studied.

The results reported above also show that an enhancing effect only occurred when the cells were irradiated before the virus was added and that this effect was soon lost when the cells were irradiated after infection. This was the exact opposite to the conditions reported by Marin and Basilico (1967) for an enhancement due to an inhibition of protein synthesis by puromycin.

Interferons are proteins and hence, although large doses of UV radiation can decrease yields of interferon (Cogniaux-Le Clerc, Levy and Wagner, 1966), the doses used in the present experiments were lower than those reported, by Klímek and Vlašínová (1967), to have no effect on protein synthesis in L cells. The results obtained when

an interferon assay was performed showed that the amount of free interferon produced by BHK 21 C13 cells was undetectable. Therefore, because of the low UV radiation doses used in the experiments and the insignificant amount of free interferon produced by the BHK 21 C13 cells , an inhibition of protein synthesis and/or inhibition of interferon synthesis was unlikely to be responsible for the observed enhancement of the transformation rate.

Another hypothesis is that some form of DNA repair mechanism may be involved in the enhancement of the transformation rate. There are now a number of reports suggesting that non-semiconservative repair can occur in UV irradiated mammalian cells. Most of these reports are concerned with human cells (Rasmussen and Painter, 1964, 1966; Cleaver, 1967; Djordjevic and Tolmach, 1967; Regan, Trosko and Carrier, 1968). However, Rasmussen and Painter (1966) have reported that several lines of mammalian cells show non-semiconservative repair, although in some of the cell lines which they tested, the presence of 5-bromodeoxyuridine in the DNA was required before the repair could be demonstrated by autoradiography.

Two reports may have a bearing on a possible mechanism by which viral transformation might be enhanced after UV irradiation of the cells. One, by Westphal and Dulbecco (1968), showed that polyoma DNA is present in polyoma virus transformed lines. These ranged from 5 to 7 for polyoma virus DNA equivalents. They were also able to show that the viral DNA was located in the nuclei of the cells. It is very probable that this viral DNA is integrated into the DNA of the cell. The other, by Dulbecco, Hartwell and Vogt (1965); Weil, Michel and Ruschman (1965); Winocour, Kaye and Stollar (1965) showed that polyoma virus can induce the synthesis of cellular DNA after DNA synthesis in normal cells has been repressed by contact inhibition or by a very large dose, up to 5,000 r, of X-radiation (Gershon, Hausen, Sachs and Winocour, 1965; Gershon, Sachs and Winocour, 1966).

Using the information which has been described in the preceding paragraph, it is possible to put forward a hypothesis by which transformation of BHK 21 C13 cells may be enhanced by prior irradiation of the cells with UV light. It is assumed that, for transformation, all or part of the viral DNA must be integrated into the cellular DNA.

The virus particle would be adsorbed to and enter the cell in the normal way. After uncoating, the viral DNA must move into the nucleus so that it can come into contact with the DNA of the cell. If this DNA has been damaged by UV radiation and some form of repair is taking place, then it is possible that there will be more sites in the cellular DNA at which the viral DNA could be incorporated. It could well be incorporated into a site where the DNA of the cell, damaged by the UV radiation, has been cut out preparatory to the replacement of the damaged nucleotides by new nucleotides. In effect, a sequence of nucleotides from the polyoma viral DNA would be incorporated in place of the new nucleotides which would normally have been incorporated during repair. (It is, however, difficult to visualise incorporation occurring in this way unless there are specific sites on the cellular DNA where a high degree of base pairing can occur with the viral DNA). A ligase would probably be necessary for closing the final gap between the 3′ hydroxyl and the 5′ phosphate. A ligase with this ability to catalyse the repair of "nicked" DNA has been purified from E. coli by Gefter, Becker and Hurwitz (1967). They suggest that this enzyme may play

an important role in the repair of radiation damaged DNA, as the enzyme activity is present in relatively high concentrations in those radiation-resistant bacterial strains which have been tested. P. Beard (personal communication) has some preliminary evidence suggesting that a ligase exists in mammalian cells. He has found such an enzyme in polyoma virus infected mouse cells.

The alteration in the cellular DNA due to the inclusion of the viral DNA could then be fixed by the doubling of the DNA which is stimulated by the virus. Thus the observed enhancement of the transformation rate of BHK 21 C13 cells after UV irradiation could be a result of incorporation of the viral DNA during repair replication in the DNA of cells damaged by the UV radiation.

Although it is possibly dangerous to extrapolate results from bacteria to mammalian cells it is of interest that Jacob (1954) and Fraser (1957) have reported that UV irradiation of bacteria before infection with bacteriophages λ and T3 can lead to an increase in the frequency of host-range mutants. It is possible that these arose by a process of recombination between the infecting bacteriophages and the genome of the bacterial host cell (Stent, 1958).

One problem concerned with this hypothesis is an apparent discrepancy between the time taken for repair to occur and the time after irradiation during which the cells can be infected with polyoma virus without loss of the enhancement of the transformation rate. The only form of repair which has been demonstrated is sublethal repair of UV damage and this occurred within 12 hours after the irradiation. An enhancement of the transformation rate, however, was still observed when the cells were irradiated 24 hours before infection with polyoma virus. Unfortunately little is known about the repair of radiation damage in mammalian cells but it is possible that repair may continue in the UV radiation damaged cells for long periods. For example, Regan, Trosko and Carrier (1968) have reported that, in human cells, approximately 50% of the pyrimidine dimers formed after UV radiation of the cells, were lost after 12-24 hours. They could not demonstrate the removal of further dimers. It is therefore possible that this apparent discrepancy may be due to repair continuing, possibly at a slower rate, for considerably longer than the 12-16 hour period observed in the repair of sublethal damage.

It has been reported that a high proportion of polyoma virus

infected BHK 21 C13 cells are "abortively" transformed (Stoker, 1968). It is possible that this could be due to the viral DNA failing to become integrated into the cellular DNA and that both X-radiation and UV radiation give the viral DNA a greater chance of becoming integrated into the cellular DNA and therefore cause more of the "abortively" transformed cells to show full transformation.

The hypothesis that the repair of radiation damage can aid integration of the viral genome, although attractive, has, at present, no evidence to support it. It is probable that this hypothesis will only be proved, or disproved, when the repair of radiation damage in mammalian cells is clearly understood at molecular level. One conclusion which can be drawn from the work reported above, however, is that a synergistic effect can exist between UV radiation and oncogenic viruses. This factor may well be of importance in the ultimate understanding of the cause of skin cancer in people exposed to high levels of sunlight.

CHAPTER VII

SUMMARY

The role played by viruses and radiation in the etiology of cancer is reviewed. The isolation and properties of one tumour causing virus, polyoma virus, are described.

When BHK 21 C13 cells were irradiated with low doses of ultra-violet light before infection with polyoma virus an enhancement of the transformation rate was observed. A two-fold rise in the proportion of transformed colonies to normal colonies was found after a radiation dose of 100 ergs/mm^2. This irradiation dose was sufficient to lower the plating efficiency of the cells by approximately 45%.

A method was developed by which the rise in the proportion of transformed cells could be more adequately observed. This method involved the delayed plating of irradiated, infected cells in agar suspension medium.

BHK 21 C13 cells which were irradiated up to 24 hours before infection with polyoma virus did not show loss of the transformation enhancdment. No enhancement was observed, however, when the cells were irradiated four or more hours after infection. The irradiation doses used did not significantly affect the transforming ability of the virus particles.

The results obtained when experiments were performed to test the effect of ultraviolet radiation on the interferon synthesising capacity of the cells suggested that reduced synthesis of interferon was not the mechanism by which transformation enhancement occurred.

Survival curves were determined for BHK 21 C13 cells and two lines of polyoma virus transformed BHK 21 C13 cells. The curves obtained were of the normal "C" form. The transformed cells were found to be more resistant to ultraviolet irradiation than the untransformed cells. All cell lines tested showed a greater resistance to ultraviolet irradiation when plated out for colony formation in the presence of mouse-embryo feeder-cells. Cycloheximide, an inhibitor of protein synthesis, did not significantly affect the survival curves.

Photoreactivation of ultraviolet radiation induced damage could not be demonstrated in BHK 21 C13 cells. Repair of ultraviolet radiation induced sublethal damage in BHK 21 C13 cells was demonstrated by a split dose technique, although the expression of this repair was not observed until 8 hours after the initial irradiation.

Transformation of BHK 21 C13 cells by ultraviolet irradiation alone could not be demonstrated.

The results are discussed with regard to a possible mechanism for the observed enhancement of transformation.

CHAPTER VIII.

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