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Induction of Interferon in Chick Embryo Cells Infected by Adenovirus Type 5 and Polyoma Virus.

by

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A dissertation submitted to the
UNIVERSITY OF GLASGOW
for the degree of
DOCTOR OF PHILOSOPHY

Institute of Virology

May 1973.

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ACKNOWLEDGEMENTS

I wish to thank Professor J.H. Subak-Sharpe for providing me with facilities in the Institute of Virology and for making useful suggestions relating to this work.

The most valuable advice, suggestions and encouragement of Dr. J.F. Williams throughout this work and during the preparatio of this manuscript is gratefully acknowledged.

I wish to thank Drs. N.M. Wilkie and J.F. Szilagyi for useful suggestions in the biochemical experiments, and also Dr. E.A.C. Follett for doing the electron microscopy.

I am thankful to Drs. C.S.H. Young and P. Austin for helping me by reading and criticising the manuscript.

The patience of Miss Margaret Cameron who typed this thesis is greatly appreciated.

In addition, I greatly appreciate the excellent facilities provided by the members of the Cytology, Media and Glasswashing divisions of the Institute.

This work was carried out with the help of a research scholarship from the Turkish Minintry of Education.

All the experimental work reported in this thesis was carried out by the author.

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CHAPTER I

GENERAL INTRODUCTION

1. **Interferon**
2. **Interferon induction and action.**
3. **Interferon inducers.**
4. **The use of conditional-lethal mutants to investigate viral gene functions.**
5. **Adenoviruses.**
6. **Polyoma viruses**
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<u>Interferon.</u>

Interferon is the biologically active protein discovered 16 years ago during a study of virus induced interference (Isaacs and Lindenman, 1957). It is a cellular protein produced in response to viral infection or to exposure to severa other non-viral biological and synthetic inducers *in vitro* and in vivo. It inhibits the replication of RNA and DNA viruses. The biological, physical and biochemical properties of interfer have been studied in detail and several reviews of this subject have appeared (Finter, 1966; Lockart, 1967; Wagner, Levy and Smith, 1968; Colby and Morgan, 1971; Kleinschmidt, 1972 Interferons from different souces are in general heat stable, stable to extremes of pH (pH 2 - 10), non-sedimentable becaus of low molecular weight (M.W. ranges between 25,000-120,000 non-dialysable, and active only on the species on which they ar produced (species-specific). In addition, interferons do not inactivate viruses directly, are not neutralised by specific antiserum to the inducer virus, and are sensitive to trypsin and other proteases. These properties are useful in assessing the relative purity of the interferon preparation.

Interferon has been purifeid by various physical and biochemical technqiues (reviewed by Wagner, Levy and Smith, 1968), but it is not yet available in 100% pure form. Recently, however, highly concentrated preparations have become available (Fantes, 1969; Fantes, 1970; Paucker et al. 1970).

Interferon does not appear to be toxic to cells, even at high levels. This characteristic is of advantage in the use of interferons over a wide range of doses in vivo and in vitro. In vivo the half life of interferon is very short because of its sensitivity to proteases present in the serum, therefore, high doses are essential. In vitro its activity can be easily measured even at low concentrations and many techniques are available for the titration of interferon. The most common titration methods are as follows; 1) Inhibition of virus yield (Gaunt and Lockart, 1966), 2) Inhibition of cytopathic effect (Sreevalsan and Lockart, 1962), 3) Plaque reduction assay (Wagner, 1961) 4) Dye-uptake test (Finter, 1960). The plaque reduction assay is extremely sensitive and perhaps the most accurate procedure for titration of interferon. The end point of the titration using the latter method is expressed as the

dilution of interferon at which plaque number is reduced to 50% of the control count (50% plaque depressing dose=PDD_{50}). Vesicular stomatitis virus, Semliki Forest virus, Sindbis virus, Chikungunya virus and vaccinia virus are all very sensitive to interferons, and since they replicate and readily form plaques on most cells they are most often used in interferon assays.

In addition to its antiviral activity, interferon treatment of cells produces two different effectson subsequent interferon induction by virus infection. High doses of interferon have been shown to inhibit subsequent synthesis of interferon (Lockart, 1963; Friedman 1966) while low doses potentiate the final yield (Isaacs and Burke, 1958). The latter non-viral phenomenon is called "priming".

Interferon induction and action.

Many viruses, both infectious and non-infectious can induce interferon in cells. The production of interferon depends upon cellular DNA-dependent RNA synthesis and protein synthesis and treatment of cells with actinomycin D (Heller, 1963; Wagner, 1963, Wagner and Huang, 1965, 1966) or the addition of a protein inhibitor (e.g. puromycin) throughout

induction depresses the level of interferon produced (Wagner and Huang, 1965). These inhibitors also prevent the antiviral action of interferon (Taylor, 1964; Lockart, 1964).

At the present time neither the induction nor the action of interferon is clearly understood, but several models have been proposed for both mechanisms and the intermediates involved in each step (Burke, 1970; Lockart, 1970; Kleinschmidt, 1972). The basic models proposed for the mechanism of interferon induction derive from the Jacob-Monod model for genetic regulation of protein synthesis in Bacteria (Jacob and Monod, 1961). In these models, the presence of a repressor molecule is postulated and this prevents the expression of the interferon gene. Derepression starts when the inducer invades the cell and the inducer itself may act directly as derepressor, or it may lead to some cellular events needed for the production of derepressor. Derepressor then attaches to the repressor at the operator gene or locus and activates the interferon gene. Transcription of the interferon gene results in the formation of a messenger-RNA which is subsequently translated to give interferon.

Virus replication on cells is almost certainly not inhibited directly by interferon itself but by a postulated protein called the antiviral protein (AVP). This conclusion is based largely on the fact that both messenger-RNA and protein synthesis are required for interferon action (Wagner, 1961, Heller, 1963, Taylor, 1964). In addition, the time sequence and the temperature dependence for interferon action suggest that certain metabolic activities are necessary before cells become fully refractory to virus infection (Lindenmann, Burke and Isaacs, 1957, Vilcek and Rada, 1962). In studies of the action of interferon in a cell-free system, it has been postulated that the actual inhibitory substance is translation inhibitory protein (TIP), which corresponds to the AVP. TIP has bean shown to associate with ribosomes and specifically inhibit the translation of viral messenger RNA (Marcus and Salb, 1966). More recent results also suggest that in interferon-treated cells, virus protein synthesis is inhibited at the translational level (Friedman et al, 1972).

Inducers of interferon.

There are many inducers of interferon, both viral and

non-viral which act both in vivo and in vitro. The nature of the inducers varies widely and inducers include viruses, bacteria, rickettsia, mycoplasma and protozoa, most of which share the common property of being intracellular parasites. In addition, bacterial products (endotoxins) and fungal products (Statolon, Helenine), naturally-occurring double-stranded RNAs and synthetic polymers are inducers of interferon (Kleinschmidt 1972).

Both RNA and DNA viruses are active interferon inducers in vivo and in vitro. Isaacs et al. (1963) hypothesized that the nucleic acid component of the virus is responsible for triggering the interferon response in the cell. This idea originally arose from the finding that chick liver RNA was a more effective induc on mouse cells than on chick cells and that mouse liver RNA was more effective on chick cells (Isaacs et al. 1963). It was thought that the cell responds to any foreign nucleic acid but recognises a specific sequence of bases (Rotem et al. 1963). More recently however, it has been shown that purified double-stranded RNAs from rabbit kidney cells and chick embryo cells induce interferon on both homologous and heterologous systems (Kimbal and Duesberg, 1971).

Synthetic double-stranded polynucleotides containing

homo-sequences of I, C, A, U, G and X have been shown to be active inducers (Field et al., 1967, Colby and Chamberlin, 1969, De Clercq and Merigan, 1969). These results suggest thatneither foreigness nor a special primary structure (base sequence) are necessary properties for interferon induction. (Field et al. (1967) first showed that the single-stranded polyribonucleotides such as poly (U), poly (I), poly (A) and poly (C) were inactive even at large concentrations while the homopolymer poly (I). poly (C) was extremely active in small quantities in the establishment of viral interference. These results conflict with the findings of Baron et al. (1969) who found that single-stranded polyribonucleotides were also active inducers of interferon. It has been suggested (Colby, 1971, De Clercq and Merigan, 1969) that the affinity of the inducing molecule for the intracellular recognition site and the stability of the conformation of the polyribonucleotides are more important than the rate of uptake of polyribonucleotides or their sensitivity to the intracellular nucleases (Colby and Chamberlin, 1969). It seems likely that a stable secondary structure is necessary for a polynucleotide to act as an inducer. Nevertheless, double-stranded

deoxypolynucleotide homopolymers; poly dA:dT, poly dI:dC and poly dG: dC were found to be inactive in inducing interferon or viral interference on chick embryo cells (Colby and Chamber 1969). However the study of De Clercq, Eckstein and Merigan (1970) has shown that under certain conditions polydeoxyribonucleotides also conferred definite resistance to virus infection, but with somewhat lower efficiency than double-stranded RNAs. Generally, synthetic ribo-deoxyribonucleotide hybrids were found to be inactive in i interferon induction (Nemes et al. 1969). In summary, double-stranded RNAs are very active inducers of interferon, while double-stranded RNA:DNA hybrids are inactive, and single-stranded polyribonucleotides and double-stranded DNAs are only active under certain conditions.

T_4 coliphage was found to be capable of inducing interferon in mice, and antiviral activity of primary rabbit kidney cells (Kleinschmidt, 1970). Although intact T_4 coliphage is active an an inducer, separate ghosts and phenol extracted DNA were both found to be incapable of inducing interf response. Since bacteriophages do not replicate on mammalia

cells, and the possibility of synthesis of viral specific double stranded RNA (see below) is unlikely, it is thought by thes authors that the configurationally active DNA of the input virus is responsible for induction of interferon.

The activity and apparent specificity of synthetic double-stranded RNAs suggested that they may play an important role in the induction of interferon in cells infected with RNA viruses. In fact a double-stranded replicative form or intermediate RNA is produced by most of the single and double-stranded RNA-containing animal viruses in infected cells. An attempt to test this possibility was made using temperature-sensitive (ts) mutants of Sindbis virus on chick embryo cells (Lockart et al. 1968). Wild type Sindbis virus produced significant amounts of interferon at both permissive (29°C) and non-permissive temperature (40°C). One class of ts mutant produced significant amounts of viral RNA at permissive temperature, but did not synthesize it at the non-permissive temperature (ts-RNA^{-}). The other class produced RNA equally well at both temperatures (ts-RNA^{+}). Neither was capable of producing interferon at non-permissive temperatures and it

looked as though accumulation of viral RNA in the cells was not enought to trigger the interferon response. The study was taken further by using purified virus preparations of *ts* mutants (Marcus, personal communication in Colby and Morgan, 1971) and it was found that wild type and ts-RNA$^+$ mutants induc interferon at both temperatures while ts-RNA$^-$ mutants induced interferon only at the permissive temperature. The latter study indicated that viral RNA production in cells infected with Sindbis virus is required for interferon induction.

The same approach was made using Semliki Forest virus *ts* mutants (Lomnicsi and Burke, 1970). The interferon induci ability of W. T., ts-RNA$^-$ and ts-RNA$^+$ mutants was further characterised, and it was found that the interferon inducing capacity of *ts* mutants was multiplicity dependent. At high multiplicities all three classes induce interferon at both temperatures indicating that RNA synthesis was not required for interferon induction and that input RNA acted as inducer. At lower multiplicities, however, the interferon response depended on the synthesis of viral RNA. This result supports previous findings in which Semliki Forest virus continued to

induce interfero n after heat inactivation; i. e. in the absence of viral RNA synthesis (Burke, Skehel and Low, 1967; Goorha and Gifford, 1970). Experiments in which it was found that hydroxylamine treatment of Semliki Forest virus reduced virus infectivity, hemagglutinating capacity, virus-induced RNA synthesis, viral RNA polymerase and interferon production all a the same rate indicate that intact virus nucleic acid is essential for induction of interferon. (Skehel and Burke, 1968).

It is known that infectivity is not always necessary for induction of interferon and viruses inactivated in various ways are capable of inducing interferon. UV-inactivated Newcastle disease virus is a very efficient inducer of interferon on mouse l cells without producing infectious progeny. In addition, UV-inactivated Newcastle disease virus (NDV) synthesizes ribonucle: resistant RNA on chick embryo cells (Huppert, Hillova and Gresland, 1969). It has been shown that NDV virion transcripta is more resistant to UV irradiation than virus infectivity (Bratt, personal communication in Colby and Morgan, 1971) which suggests that RNAse resistant RNA could well be the inducer of interferon (Colby and Morgan, 1971). Recently Sheaff,

Meager and Burke, (1972) have demonstrated that both infectivity and the virion associated RNA polymerase activity of NDV are destroyed at the same rate by heat and that virus with no polymerase activity was incapable of inducing interferon (UV-irradiated of β-propiolactone treated virus loses its infectivity more rapidly than polymerase activity and still induces IF). However there is evidence to suggest that single-stranded input NDV virus RNA may be capable of inducing interferon (Dianzani et al. 1970). The conclusion to be drawn from the work with RNA viruses mentioned above is that double-stranded RNA production is important, although structurally stable single stranded RNA's can induce interferon (De Clercq and Merigan, 1969). Both myxo and arbo-viruses have very stable RNA conformations (Tichchonenco et al. 1964; Sreevalsan et al., 1968) so that in some cases the single-stranded RNA of the input virus may act as an inducer.

While there is reasonable understanding of the viral components or products necessary for interferon induction by RNA viruses from the information available to date, it is still an open question as to how DNA viruses induce interferon. Many

DNA viruses including vaccinia (Nagano and Kojima, 1958, Glasgow and Habel, 1962), herpes virus (Lampson et al. 1965), polyoma virus (Allison, 1961, Talas, Weisfeiler and Batkai, 19 and human adenoviruses (Beladi and Pusztai, 1967) are able to induce interferon in vivo and in vitro.

Colby and Duesberg (1969) have demonstrated the formation of virus specific double-stranded RNA in vaccinia virus infected chick embryo cells. This double-stranded RNA induces interference against virus challenge, but its ability to induce interferon was not measured. On the basis of their evidence, however, they concluded that double-stranded RNA produced after infection of chick cells was the inducer. Recen however, this hypothesis was challenged by Bakay and Burke (1 who inactivated vaccinia with UV and measured the loss of infectivity add RNA polymerase activity, and correlated these with interferon induction. They found that no interferon was formed in cells until the virion polymerase activity was almost completely lost and there was no evidence of any loss of interfe inducing capacity of virus even after long periods of irradiatio as is the case for RNA viruses (Gandi and Burke, 1970). Chic

cells still produced interferon while there was no detectable virion associated polymerase activity and the authors suggest that virus-directed RNA synthesis is unnecessary for interferon induction on vaccinia virus infected chick embryo cells. However, no search for double-stranded RNA was made in cells infected with inactivated virus.

Human adenoviruses induce interferon on chick embryo cells (Beladi and Pusztai, 1967) which are non-permissiv for these viruses. It is generally assumed that in this system, either an input component(s) of the virus or a product(s) of incomplete virus replication is responsible for interferon inductio In this respect the finding concerning the effect of trypsin on adenovirus is of interest (Beladi and Pusztai, 1967, Ho and Kohler, 1967). Trypsin treatment of adenovirus has little or no effect on infectivity for human cells, but the virus no longer triggers the interferon response on chick embryo cells (non-permissive host). Because of its sensitivity to trypsin treatment (Pereira, 1958, Rowe et al. 1958) the penton antigen is regarded as a probable candidate for inducer. These

findings are supported to some extent by UV inactivation experiments, in which it was found that UV irradiation of adenovirus destroyed its infectivity faster than its interferon-inducing capacity, although these findings can be interpreted in other ways. On the other hand, purified adenovirus antigens including hexon, penton and fibre failed to induce an interferon response on chick embryp cells (Russell, W.C., personal communication). Of course it cannot be ruled out that there may not be sufficient uptake of these components in order to trigger the interferon response. Some interferon inducing activity is associated with the component characterized as trypsin sensitive incomplete hemagglutinin of adenovirus type 8 (dodecons) induced interferon in chick cells (Lengyel, et al, 1969) although the titres were very low. Thus the evidence for penton antigen being responsible for interferon induction still remains inconclusive and indirect.

It is possible that the adenoviruses induce interferon via virus specified double-stranded RNA as has been proposed for the vaccinia virus system. However, chick embryo cells infecte

with adenovirus type 12 showed no detectable double-stranded RNA (Bakay and Burke, 1972). In addition Markovits and Coppey (1972) reported no significant difference in the amount of double-stranded RNA in adenovirus type 7 infected and non-infected chick embryo cells. Interferon induction by adenoviruses on chick embryo cells is not affected by the DNA synthesis inhibitor (cytosine arabinoside) suggesting that DNA synthesis is not necessary for interferon induction (Bakay and Burke, 1972, Markovits and Coppey, 1971, 1972). Therefore it seems likely that some early functions expressed soon after infection, or alternatively a component of the input virus might be responsible for interferon induction.

<u>The use of conditional lethal mutants to investigate viral gene functions.</u>

The role of virus genes and their products in the intracellular development of viruses has been studied by using conditional-lethal mutants (reviewed by Fenner, 1969). Two main classes of conditional lethal mutants have been so far isolated and used in genetical analysis extensively. There are

two types of conditional-lethal mutant, suppressor-sensitive mutants and temperature-sensitive mutants. Suppressor-sensitive mutants are a class of host-dependent mutants whi are so far known only for bacteriophages. These mutants can grow in permissive cells containing a suppressor gene which "compensates" for the phage mutation (Garen, 1968) b fail to grow in suppressor-negative cells. In general the suppressor-sensitive mutants have very low leakiness and reversion rates and from this point of view they have been extremely useful for studying bacteriophage development and for carrying out detailed genetic mapping (Epstein et al., 19 Edgar and Wood 1966). To date there is no report on suppr sensitive mutants of animal viruses, although host-dependen mutants have been isolated and described for rabbit-pox virus (McClain, 1965). These mutants fail to grow on a line pig kidney cells but do so on chick embryo cells, while wild type virus grows equally well on both types of cell.

Another class of mutant, called "cytocidal" (cyt) muta some of which were host-dependent was isolated for human adenovirus type 12 by (Takemori, Riggs, and Aldrich (1968,

1969)). They were obtained either spontaneously or were induced by UV irradiation. These mutants form larger and clearer plaques than wild type does on human embryo kidney cells. In addition, most isolates were much less tumorigenic than parental virus when tested in newborn hamsters *in vivo*, and did not transform hamster embryo kidney cells *in vitro*. Thus, the authors hypothesized that the *cyt* gene may be responsible for the cytopathic effect and for tumoriginicity. No complementation was detectable between *cyt* mutants, and they may all be mutan in the same gene. Some of the *cyt* mutants are host dependent ("kb") in that they failed to grow in a particular line of KB cell (KB-1), but grew well in another line (KB-2). They proposed that the kb mutants could have nonsense mutations and they fail to grow in KB-1 cells because these cells lack certain suppressor(s) which are present in KB-2 line.

The other class of conditional-kethal mutants are called temperature-sensitive (*ts*) mutants and these have been isolated for many bacteriophages (Campbell, 1961, Epstein et al, 1962, Edgar and Lielausis, 1964, Edgar, 1966).

In animal viruses, ts mutants were first isolated for poliovirus type 1 (Cooper, 1964) and subsequently mutants have been isolated for many other RNA and DNA viruses (Fenner, 1969). The isolation of ts mutants of oncogenic DNA viruses is of special interest in investigating the role of viral genes in malignancy. Because of its oncogenic potential ts mutants of polyoma virus have been isolated and used to study the gene functions required for *in vitro* transformation (Fried, 1965, Eckhart, 1969, DiMayorca et al. 1969). Temperature-sensitive mutants have more recently been isolated for different types of human adenoviruses, including type 5 (Williams et al. 1971, Ensinger and Ginsberg, 1971, Takashashi, 1972), type 12 (Lundholm and Doerfler, 1971, Shiroki, Irisawa and Shimojo 1972), type 31 (Suzuki, Shimojo and Moritsugu, 1972) and avian adenovirus (Celo) (Ishibashi, 1971).

Human adenoviruses differ in their oncogenicity for rodents but most types transform rodent cells *in vitro* (Green, 1970). Therefore these mutants potentially provide a very good system to study non-permissive virus-cell interactions as well as the events taking place during replication of these viruses in permissive cells. The involvement of adenovirus genes and

their products in *in vitro* transformation (Williams and Ustacelebi, 1971) and interferon induction (Ustacelebi and Williams, 1972) is currently being investigated using *ts* mutants of adenovirus type 5.

Adenoviruses.

The adenovirus capsid is an icosahedral structure (Horne et al, 1959), containing 252 capsomeres. Of these, 240 make up the faces and edges of the icosahedron and are called hexons (each surrounded by 6 others) or non-vertex capsomeres (Wilcox, Ginsberg and Anderson, 1963). The 12 apical capsomeres, each surrounded by 5 capsomeres are called pentons or vertex capsomeres. Each penton is sub-divided into a spherical penton base and a projection called the fibre (Ginsberg et al. 1966). These capsid components are produced in excess amounts in infected cells, can be readily separated and purified by DEAE-column chromatography (Klemperer and Pereira, 1959), and of these, both hexon and fibre antigens have been crystallised (Pereira, Valentine and Russell, 1968; Moutner and Pereira, 1971). The genome of adenoviruses is a linear double-stranded molecule of DNA, of molecular

weight between 20 and 25 x 10^6 daltons. This is sufficient to code for some 25-50 average-sized proteins (Van der Eb and Van Kesteren, 1966; Green et al. 1967). It has been shown that the virion of type 2 contains at least nine distinct polypeptides by using acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) (Maizel, White and Scharff, 1968a). The molecular weights of these polypeptides have been characterized, and it has been calculated that these polypeptides account for one-third of the coding potential of the adenovirus genome. The hexon antigen appears to consist of three identical polypeptides each of a molecular weight of 120, Each penton base consists of single polypeptideofa molecular weight of 70,000, and the fibres also consist of a single polypeptide of a molecular weight of 60,000. Three other polypeptides of molecular weights of 44,000, 24,000 and 24,000 (Maizel, White and Scharff, 1968b), closely associated with viral DNA make up about 20% of the virion. However, a similar analysis of the protein components of purified adeno type 5 virions disrupted in 8 Mol urea and analysed by acrylamide gel electrophoresis in SDS reveals only 5 distinct

polypeptide bands (Russell and Skehel, 1972). These were identified as three capsid proteins (hexon, penton base and fibr and two core proteins (core 1 and core 2). Virion peptides of adenovirus would account for approximately 30% of the codin potential of the genome when infected cells were pulse labelled with ^{35}S-methionine and the extracts analysed on acrylamide gels, in addition to these 5 major peptide bands, a further 5 bands were observed. These are called infected cell specifi polypeptides (ICSP). Although it has not yet been determined whether these are virus-coded or virus-induced, if one assumes them to be virus coded, then on the basis of their molecular weights they would account for a further one-third of the adenovirus coding potential of the genome. (Russell and Skehel, 1972).

Following infection of susceptible cells, virus rapidly penetrates and uncoats (Philipson, Lonberg-Holm and Petterso 1968) and the adenovirus DNA moves to the nucleus without completely stripping off the viral protein (Lawrence and Ginsberg, 1967; Sussenbach, 1967; Lonberg-Holm and Philipson, 1969). One of the earliest antigens produced in lytically infected cells is called the "P" antigen and it shows

certain similarities with the tumor "T" antigen produced by oncogenic adenoviruses in abortively infected cells. Both are produced very early in the infectious cycle before viral DNA synthesis takes places and their production is not inhibited by cytosine arabinoside, an inhibitor of DNA synthesis (Russell et al. 1967). There is no real evidence that P antigen is virus coded; however it has been suggested that the early form of the P antigen could be a structural component (Russell 1971).

One-fifth of the adenovirus genome has been shown to be transcribed in the first 6 hours after infection, prior to DNA synthesis (early messenger-RNA), and 80% of the genome is transcribed by 18 hours post-infection in adenovirus type 2 infected cells (Thomas and Green, 1966, Fujinaga, Mak and Gree 1968). Viral DNA synthesis is detectable around 7-10 hours after infection and all capsid antigens can be detected after the appearance of viral DNA (Mäntyjärvi and Russell, 1969). The capsid proteins of adenovirus are synthesized in the cytoplasm of infected cells, and rapidly transferred to the nucleus for assembly of viral capsids and maturation (Thomas and Green,

1966; Horwitz, Scharff and Maizel, 1969; Velicer and Ginsberg, 1968, 1969).

Certain adenoviruses are oncogenic for rodents. On this basis and the G+C content of their DNA they have been classified into three groups; Group A, highly oncogenic (types 12, 18 and 31 containing 48-49% G+C), Group B, weakly oncogenic (types 3, 7 and 14 containing 50-52% G+C) and Group C, non-oncogenic (types 1, 2, 5 etc. containing 55-61% G+C) (Pina and Green, 1965). Oncogenic types induce tumours in newborn hamsters, rats and mice. In addition, most adenoviruses transfomr hamster and rat cells *in vitro* (Freema et al. 1962, McAllister et al. 1969). In abortive infection wit adenoviruses, cell DNA synthesis is usually induced and tumou antigen can be detected in these cells after infection using diffe immunological techniques (Hoggan et al. 1965, Gilead and Ginsberg, 1965). Synthesis of early adenovirus messenger-R in transformed cells is detectable by hybridization experiments (Green, 1970). However, the late gene functions are blocked. Recently, thesobservations of Petterson and Sambrook (1973) also provided some evidence for persistence of viral genes in r cells transformed by adenovirus type 2.

Polyoma virus.

Polyoma virus is a small oncogenic virus of mice, 40-45 mμ in diameter with a virus capsid consisting of 72 capsomeres (Caspar and Klug, 1962). It contains a circular duplex DNA of molecular weight approximately 3×10^6 daltons (Crawford, 1964). This, the genome of the polyoma virus consists of about 4500 nucleotide pairs, sufficient genetic information to code for 5-10 average-sized polypeptides. The structural components of the polyoma virus particle would account for one-third of the coding potential (Frearson and Crawford, 1972 Polyoma virus can be purified by density gradient centrifugation and "full" (complete) particles with a density of 1.32 and "empty" (incomplete) particles with a density of 1.29 can be readily obtained. (Crawford, 1962). Full particles are infectious and contain complete viral DNA, while empties are non-infectious with no viral DNA. DNA extracted from polyoma virions consists of three components; I. a circular duplex sedimenting at 20 S., II a circular duplex in which one strand is nicked, sedimenting at 16 S and III a linear host cell fragment which sediments at 12-14 S. Component I represents 70-90% of the total extracted DNA. Purified form I polyoma virus DNA has

been shown to be both infectious and oncogenic (Green, 1966).

Polyoma virus replicates in mouse kidney or mouse embryo cells in vitro and is assembled within the nucleus of the infected cells (Green, 1966). Polyoma induces synthesis of cellular DNA both in productively and abortively infected cel (Dulbecco, Hartwell and Vogt, 1965; Weil, Michel and Ruschmann, 1965; Winocour, Kaye and Stollar, 1965). In relation to DNA synthesis, the activities of certain enzymes are increased in infected cells (Kit and Dubbs, 1969). It still remains to be determined if these virus induced enzymes are coded for by viral or cellular genes but it seems likely that they are cell-coded in view of the limited coding capacity of the virus. Polyoma virus induces tumors in several rodents (Hamster, mice and rats) and transforms cells from hamsters, rats, mice and monkeys in vitro. Cells transformed by polyoma virus have altered cell morphology, grow to higher cell densities, grow in agar suspension and in some cases are malignant. In transformed cells polyoma viru "T" antigen and transplantation antigen "TSTA" are synthesize (Green, 1970)and it has been proposed that at least part of the viral genome is persistent in transformed cells. Cell

transformation induced by polyoma virus provides an excellent system for the analysis of the mechanism of oncogenesis. Genetical analysis of polyoma virus by using conditional-lethal, temperature-sensitive mutants provides a means of identifying the virus functions involved in transformation and oncogenesis (Di Mayorsa and Callendar, 1970; Eckhart, 1972).

Aims of this work.

It is probable that all known viruses are able to induce interferon in an appropriate host under appropriate conditions Viruses are generally active as inducers in both lytically and abortively infected cells *in vitro.* Therefore complete replication of the virus is not always necessary for interferon induction. Many DNA viruses are very efficient inducers, particularly in abortively infected, non-permissive cells. A present it is not known which viral functions are involved in interferon induction by DNA viruses. Previous studies by Beladi and Pusztai (1967) have suggested that the trypsin sens penton antigen of adenovirus is involved and may be responsib for induction. However, the evidence is indirect and in any

this would not explain how other DNA viruses induce interferon.

The main aim of this work was to investigate the mechanism by which DNA viruses can induce interferon synthes A non-permissive system (CEF cells) will be used in this study with human adenovirus types 1, 5 and 12, polyoma virus, all of which induce very good interferon yields in these cells. In this investigation with respect to adenovirus, two major approa have been taken. The first approach involved the isolation of temperature-sensitive mutantsxof adenovirus type 5 and the use these to identify the viral gene function(s) required for interfer induction. In the second approach, a viral inhibitor, rifampic which inhibits the replication of certain adenovirus types, was in an attempt to probe the viral events involved in interferon induction. In addition, interferon induction by polyoma virus on CEF cells was also studied. In this case, a first step was to determine which virus component was necessary for inductic determining the inducing ability of infectious "full" and non-infectious "empty" particles of polyoma virus. In addition the effect of priming on the ability of both adenovirus type 5 and polyoma virus to induce interferon was examined.

CHAPTER II

INTERFERON INDUCTION BY ADENOVIRUSES ON CHICK EMBRYO CELL

1. INTRODUCTION.

2. MATERIALS AND METHODS.

3. RESULTS.

a) Kinetics of interferon induction by adenovirus types 1, 5 and 12.

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4. DISCUSSION.

INTRODUCTION

Interferon can be induced on different host cells by many of the known RNA and some of the known DNA viruses under certain conditions. The DNA viruses are generally considered to be poorer inducers of interferon on their permissive hosts. For instance mouse embryo cells are permissive for both polyoma and vaccinia viruses, but the amount of interferon induced by these viruses on these cells is relatively low (Allison, 1962; Glasgow and Habel, 1963). Human adenoviruses have also been reported to be incapable of inducing interferon in permissive Hela, KB, Human embryo kidney and Hamster embryo cells (Ho and Köhler, 1967). On the other hand the adenovirus-chick embryo cell system first described by Beladi and Pusztai (1967) provided a new system in which high levels of interferon can be obtained.

The mechanism by which DNA viruses induce interferon is not known. Adenoviruses are structurally well-defined DNA viruses and their interactions with permissive and non-permissive cells have been studied in some detail (reviewed by Schlesinger, 1969; Green, 1970) and for this reason it could be useful as a model to identify the viral function(s) necessary for interferon induction. Adenoviruses do not replicate on

chick cells (Beladi and Pusztai, 1967; Ho and Kohler, 1967), therefore it is unlikely that progeny virus produced in cells responsible for interferon induction. Thus, either a component of the input virion or certain product(s) of abortive infection may play a role in inducing interferon. In this respect it has been shown that heat or trypsin treated adenovirus does not induce interferon (Beladi and Pusztai, 1967) and exposure to U.V. radiation inactivates adenovirus infectivity faster than its interferon inducing capacity (Pusztai et al, 1969). These results suggested that the trypsin sensitive penton antigen of the virus is involved in and perhaps responsible for interferon induction. The avian adenovirus (GAL), which replicates in CEF cells, also induces interferon. Trypsin treatment of GAL virus does not affect its infectivity and interferon inducing capacity and it has been suggested that newly synthesized penton antigen, rather than the input penton antigen is responsible for or involved in interferon induction in this virus (Beladi et al, 1970). Canine hepatitis virus induced interferon on CEF cells after trypsin treatment, under condit where virus did not produce any progeny and it was suggested penton antigen may be synthesized in these cells and be responsible for interferon induction (Beladi et al, 1970). The infectivity of human adenoviruses is resistant to trypsin action (Pereira, 1958; Ho and Kohler, 1967). Recentl the tryptic digestion of adenovirus type 5 penton and particularly hexon

antigen has been examined in detail (Pereira and Skehel, 1971). It has been shown that trypsin treatment causes breaks in a number of peptide bonds of both the hexon and penton antigen of virus. The polyacrylamide electrophoresis of trypsin treated hexons showed six distinct fragments. The molecular weight calculations of these fragments revealed three trypsin sensitive sites in the hexon polypeptide, and only one site thought to be accessible to tryptic action when hexon is incorporated into virion. Trypsin digestion caused a definite change in electrophoretic pattern of pentons. The total breakdown of the penton polypeptide resulted with the appearance of two new polypeptides with molecular weights of 48,000 and 35,000 (Pereira and Skehel, 1971). The fact that human adenoviruses are still infective for human cells after trypsin treatment suggests that the trypsin-sensitive antigen (s) is not involved in virus adsorption to or uptake into these cells. The effect of trypsin on the adenovirus adsorption to both KB and chick cells has been tested using radioactively labelled adenovirus type 5 (Ho and Kohler, 1967) and it was shown that trypsin does not affect virus adsorption to either type of cell.

Experiments to be describdd in this chapter were carried out in order to establish the conditions, in particular the kinetics of interferon induction by adenovirus types 1, 5 and 12 and the virus multiplicity requirements for interferon indcution by adenovirus type 5. The

biological and physicochemical properties of the interferon induced on chick cells by adenovirus was examined. The effect of trypsin treatment on the infectivity and interferon inducing capacity of adenovirus type 5 was examined and experiments were carried out to determine if adenovirus adsorption to both Hela and chick cells is altered by trypsin treatment. The growth potential of adenovirus type 5 was tested in chick cells under the experimental conditions used for interferon induction. In addition adenovirus was inactivated by hydroxylamine, a mutagen and inactivating agent, which is thought to act preferentially on nucleic acids (Freese and Freese, 1965), and interferon inducing capacity of this inactivated virus was examined.

MATERIALS AND METHODS

Medium. In all experiments Eagle's minimal essential medium (Glasgow modification) was used.

Cell cultures. Hela cells were originally obtained from Flow Laboratories and were grown in Eagle's medium containing 10% calf serum. The cells were passed every 4-5 days and routinely grown in rotating Winchester bottles (burrlers). Monolayers for plaque assay were made by seeding 1.5×10^6 cells in 5 ml. of growth medium in 50 mm. plastic petri dishes (NUNC or Sterilin) 2 days prior to use.

KB cells. These were also obtained from Flow Laboratories and were grown in 20 ounce bottles in Eagle's medium supplemented with 10% foetal bovine serum. KB cell monolayers were made in 50 mm. petri dishes by seeding cells at 1×10^6 in 5 ml. of growth medium 2-3 days prior to use.

Chick embryo fibroblasts (CEF). The standard procedure used for preparation of CEF cells was as follows. Unembryonated Newcastle disea virus and leucosis virus free eggs were obtained from the Edinburgh Poultry Research Station. Prior to use these were incubated for 10 days at 38°C in a humidified incubator. The 10 day old embryos were removed from eggs under sterile conditions, the heads removed, and

the whole body transferred into PBS. Embryos (usually 10-15) were washed 3 times in PBS, once in versene solution, then cut into small fragments. The fragments were washed twice in PBS, transferred into a dimple flask (500 ml. capacity) then trypsin (300 ml. of 0.25%) was added and the contents stirred for 2-3 hours at 37°C. After trypsinization the dispersed cells were decanted and 5% calf serum was added to stop the action of the trypsin. After trypsinization, cells were pelleted by centrifugation at 1000 rpm for 15 minutes, resuspended in Eagle's medium and counted. Finally cells were diluted in growth medium (Eagle's medium containing 10% calf serum and 10% tryptose phosphate broth) and seeded in 50 mm. plastic petri dishes at a concentration of 5×10^6 cells/dish. Monolayers formed within 2-3 days, and cells were used at this time. Burrlers were also seeded with 2×10^8 cells in 200 ml. growth medium, and these were later used for secondary cell cultures.

BHK 21 cells, clone C13 (Stoker and Macpherson, 1964). They were grown in burrlers in Eagle's medium supplemented with 10% calf serum. Monolayers were prepared by seeding 2×10^6 cells/50 mm dish in 5 ml. of growth medium and were confluent by about 2 days.

Viruses. Adenoviruses type 1 was obtained from Dr. R. McAllister and type 5 was obtained from Drs. H.G. Pereira and W.C. Russell. These viruses were grown in Hela cells (in burrlers) infected at a multiplicity of around 1 pfu/cell. Virus was adsorbed for 2 hours, and infected cultures were maintained at 37°C in Eagle's medium supplemented with 2% calf serum. A complete cytopathic effect was observed at 40-48 hours after infection and at this time infected cells were collected and pelleted by centrifugation at 2000 rpm for 15 minutes. The pellet from each burrler was resuspended in 5 ml. of Tris saline (pH 7.4) and this suspension was frozen and thawed three times to release virus from the cells. After centrifugation at 3000 rpm for 15 minutes the supernatant was collected and stored at -20° in small aliquots.

Adenovirus type 12 (Strain Huie was obtained from Dr. R. McAllister and strain 1131 from Dr. H.G. Pereira). These were cultivated in KB and human embryo kidney cells, respectively. Virus was added at a multiplicity of 1-5 pfu/cell, adsorbed for 2 hours, and the infected cultures were maintained in Eagle's medium containing 2% foetal calf serum. Complete cytopathic effect was usually observed 48-72 hours after infection, at which time infected cells were collected. These

were pelletted by centrifugation, suspended in Tris saline and frozen and thawed three times. Finally the extract was centrifuged at 3000 rpm for 15 minutes, the supernatant collected and stored at -70° C in small aliquots in the Revco.

Vaccinia virus. (McGregor strain, obtained from Dr. M.C. Timbury) was grown on either BHK 21, C13 cell line or primary chick embryo fibroblast cells in Winchester bottles. Cells were infected at 1 pfu/cell and after 1 hour for adsorption at 37° maintained in Eagle's medium supplemented with 2% calf serum. Infected cells were harvested 48 hours after infection, pelleted by centrifugation, frozen and thawed three times, sonicated for 5 minutes by using sonicator (Megason), centrifuged at 3000 rpm for 15 minutes and the supernatant stored at -20°.

Plaque assays. The infectivities of all virus stocks described above were assayed by plaque formation on appropriate cell cultures.

Plaque assays for adenoviruses. Adenovirus types 1 and 5 were assayed on confluent Hela cell monolayers in 50 mm. plastic petri dishes (a/s NUNC, Denmark). Serial logarithmic dilutions of virus were made in Eagle's medium and 0.1 ml. virus dilution was inoculated per dish. (Duplicate cultures were used for each dilution). Virus

was adsorbed at 37°C for 90 minutes, then monolayers were overlaid with 5 ml. of 0.65% Noble agar (Difco) in Eagle's medium supplemented with 2% calf serum, and incubated at 37°C in humidified air containing 5% CO_2. In most cases 25 mM-$MgCl_2$ was incorporated in the overlay medium (Williams, 1970). After incubation for 5 days, 2 ml. of additional agar overlay medium was added to the cultures and plaques were counted 5, 7 and 9 days after infection. With added $MgCl_2$ plaques first appeared at around 5 days and increased in size and number till 7 days after infection.

Adenovirus type 12. This was assayed on KB cell monolayers or on Human embryo kidney (HEK) cell monolayers in 50 mm. plastic petri dishes. Monolayers were infected with virus and after adsorption at 37°C for 90 minutes plates were overlayed with 5 ml. of overlay medium. The medium differed from that described above for adenovirus type 1 and adenovirus type 5 in that on KB cells no $MgCl_2$ was used, and foetal calf serum was used. For assay on HEK cells only 12 mM $MgCl_2$ was added. Plates were incubated at 37°C in an atmosphere of humidified air containing 5% CO_2. After 5-6 days incubation an additional 2 ml. of overlay medium was added and plaques were counted at 6, 7 and 9 days after infection.

Vaccinia virus. This was titrated both on BHK21/C13 cells and CEF

primary or secondary cells grown in 50 mm. plastic petri dishes. Virus diluted in Eagle's medium was adsorbed at 37°C for 30 minutes and overlaidd with Eagle's medium supplemented with 2% calf serum. Plates were incubated at 37°C in an atmosphere of humidified air containing 5% CO_2 for 48 hours. At this time cultures were fixed with formol saline and stained with Giemsa prior to counting plaques which were easily visible with the naked eye.

Radioactive labelling of adenovirus type 5 DNA with ^3H -thymidine.

The labelling procedure used for adenovirus type 5 was as follows: Hela cells growing in burrlers were infected with adenovirus at 10 pfu/cell. Six hours after infection 1 µci ^3H-thymidine/ml. (specific activity 18.4 ci/m Mol) was added to the maintenance medium which consisted of Eagle's medium supplemented with 2% calf serum and containing additional cold thymidine (1 mMol/ml.). .A complete cytopathic effect was observed 40-48 hours after infection at which time cells were harvested and pelleted by centrifugation at 1000 rpm for 15 minutes. The resuspended pellet was frozen and thawed three times, and the extract centrifuged at 3000 rpm for 15 minutes to remove large debris. The supernatant was then purified by Caesium chlroide density gradient centrifugation as described below.

Purification of adenoviruses by caesium chloride density gradient centrifugation.

The purification method used was that described by Russell et al, (1967) with some minor modifications as follows. Two different CsCl solutions were used; one of density 1.32 gr/cc. and the other of 1.45 gr/cc. in 0.05 Mol. tris Hcl buffer (pH 7.4). First, 1 ml. of CsCl, 1.45 density was added to a 5 ml cellulose nitrate tube (Beckman), then an additional 2 ml. of CsCl solution of 1.32 density was carefully added to avoid mixing the two different density solutions. 1 ml. of crude virus suspension was then layered on to the CsCl solution and the volume brought to 5 ml. by addition of tris-HCl buffer. The samples were centrifuged at 100.000Xg for 2 hours at 4°C using an SW 50 rotor. The clearly separated virus band was collected dropwise through the bottom of the tube. The band collected thus was diluted 4X in tris-HCl buffer and recentrifuged again overnight (15-18 hours) using the same procedure as described above. The final band was collected and dialysed against 2 changes of tris-HCl overnight at 4°C. The dialysed virus was assayed on Hela cells for infectivity and stored at -70°C in the Revco.

Interferon induction on CEF cells.

Primary CEF cells were infected with adenovirus at a multiplicity

of between 10-20 pfu/cell by adding 0.2 ml of the appropriate virus dilution. Virus was adsorbed to cells at 37°C for 2 hours, then excess virus was removed by washing the cells once with Eagle's medium The cultures were overlayed with Eagle's medium supplemented with 2% calf serum and incubated thereafter at 37°C. At different intervals after infection medium was collected from infected cultures and kept at 4°C till assayed. Prior to assay samples were centrifuged at 3000 rpm for 10 minutes to remove cell debris and the supernatant was heated at 56°C for 30 minutes to inactivate possible residual virus.

Interferon assay on CEF cells.

Interferon was assayed by measuring the inhibition of plaque formation by vaccinia virus on CEF cells. For titration, serial two fold dilutions of the test interferon sample were made in Eagle's medium supplemented by 2% calf serum and 2 ml of each dilution was added to each culture dish. Duplicate cultures were used for each dilution. These were incubated overnight (12-18 hours) at 37°C in a humidified atmosphere containing 5% CO_2. After this period medium was withdraw cultures were washed once with Eagle's medium, then challenged with 80-100 pfu of vaccinia virus/dish. The interferon titre was expressed as the PDD_{50} (plaque depressing dose 50-the reciprocal of the interferon dilution causing a 50% depression of the control plaque count).

*Culture medium. The medium used was the Glasgow modification of Eagle's medium supplemented with either calf or foetal calf serum and in some cases (for both CEF primary cells and BHK21 cell line) 10% tryptose phosphate broth.

*Phosphate Buffered Saline (PBS) The phosphate buffered saline, pH 7.2 described by Dulbecco and Vogt (1954) contains, 0.14 Mol NaCl, 0.034 MolKCl., 0.04Mol Na_2HPO_4 and 0.002 Mol KH_2PO_4. $CaCl_2 2H_2O$ and $MgCl_2 6H_2O$ were added to this mixture at a final concentration of 0.005 Mol.

*Tris Saline. Tris saline solution contains 0.14 MolNaCl., 0.005 MolKC 0.0007 Mol Na_2HPO_4 and 0.025 Mol Tris, pH 7.4., 0.003 Mol dextrose, 100 units/ml Penicillin and 100 ug/ml. streptomycin.

*Versene. A solution of the di-sodium salt of diamino ethanetetra-acetic acid (versene) at a concentration of 0.6 mMol in PBS without $CaCl_2 2H_2O$ and $MgCl_2 6H_2O$ and with 0.002% phenol red added was used.

*Trypsin (Difco 1:250), 0.25% Trypsin was prepared in tris saline and pH was adjusted to 7.7 at room temperature with N/I HCl.

*These medium and saline solutions were prepared by the media department of the Institute.

RESULTS

Kinetics of interferon induction by adenovirus type 1, 5 and 12.

The adenoviruses used in this study have been examined in detail for their optimum interferon inducing capacity in terms of time and virus concentration on CEF cells. To examine the kinetics, CEF primary cultures were infected with each adenovirus type at a multiplicity of infection of 20 pfu/cell as described in the materials and methods. At various intervals after infection medium was removed from petri dishes (four for each point) and pooled, and these samples were assayed for interferon titres on CEF cells as described in materials and methods. Figure IIa illustrates the experimental results obtained using adenovirus type 1, 5 and 12. The kinetics of interferon induction by these three adenoviruses were more or less identical. With all three types the interferon response was first detected in the medium between 12-15 hours after infection. Titres increased over the next 50-60 hours and maximum levels were usually attained by 72 hours after infection.

Multiplicities required for optimum interferon induction.

Primary CEF cells were infected with adenovirus types 1, 5 and 12 at different multiplicities in order to determine the optimum

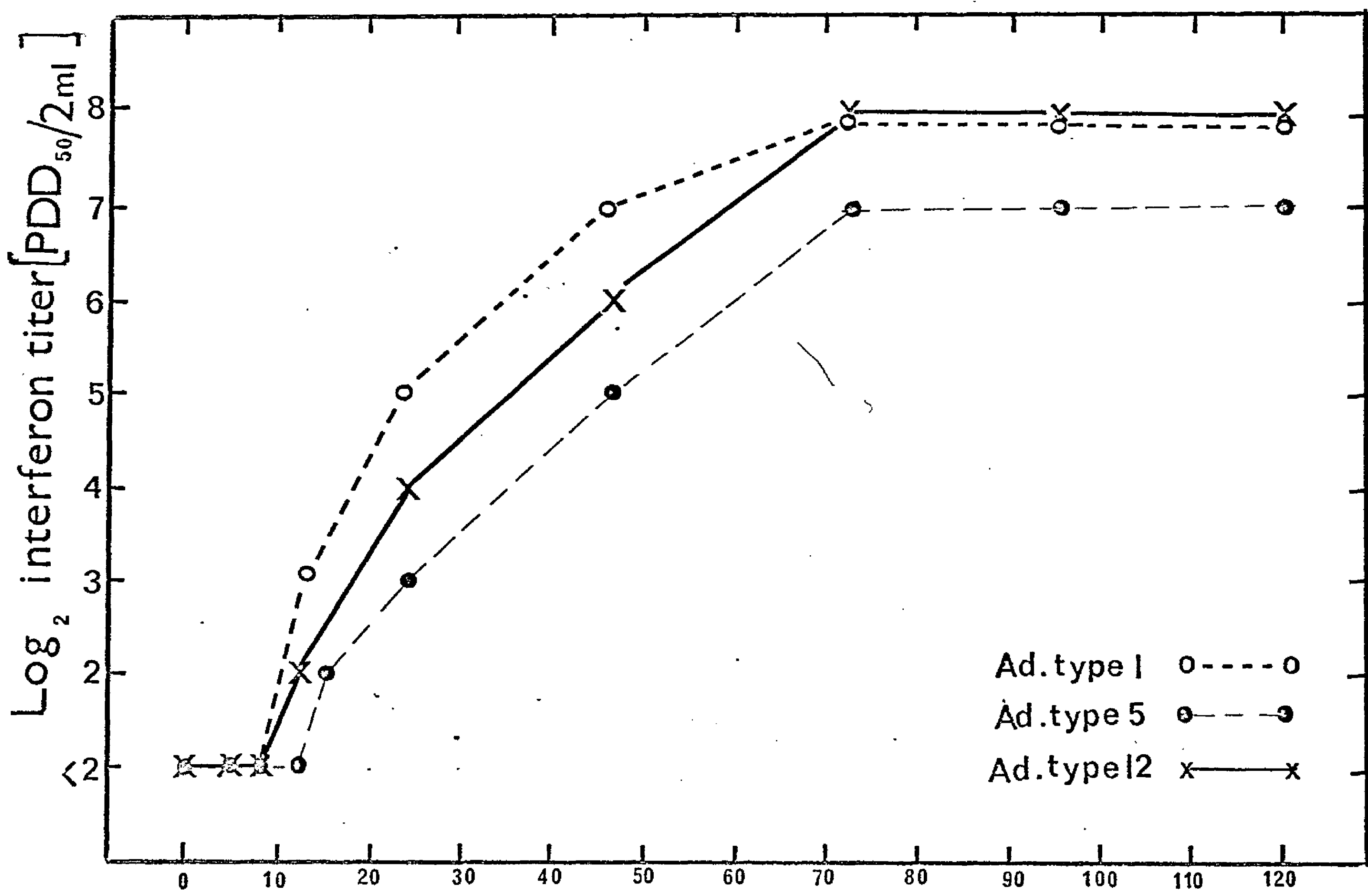


Figure IIa. Kinetics of interferon induction by adenovirus types 1, 5 and 12 at 37°C on CEF cells.

multiplicity of infection required for interferon induction. For interferon titration culture fluids were harvested 3 days after infection. The results obtained with adenovirus type 5 are shown in Figure IIb. As little as 0.5 pfu/cell caused some interferon induction but a multiplicity of 10-20 pfu/cell was essential for the maximum interferon response on CEF cells. Similarly, below a multiplicity of infection of 0.5 pfu/cell, adenovirus type 1 and a multiplicity of infection of 5 pfu/cell, adenovirus type 12 failed to induce interferon and maximum levels were obtained when multiplicities of 10-20 pfu/cell were used. At multiplicities above 50 pfu/cell adenoviruses usually have a cytotoxic effect on CEF cell monolayers. At this high multiplicity of infection the cell sheet invariably collapses within a day. In some instances as high a multiplicity as 100 pfu/cell induced much lower yields. (Figure IIb).

Characterization of interferon produced on CEF cells by human adenoviruses.

When high multiplicities (10-20 pfu/cell) of infection were used maximum interferon titres (128-512 PDD_{50}/2 ml.) were obtained on CEF cells following infection by adenovirus types 1, 5 and 12. To prove that the inhibitory substance(s) released into medium after

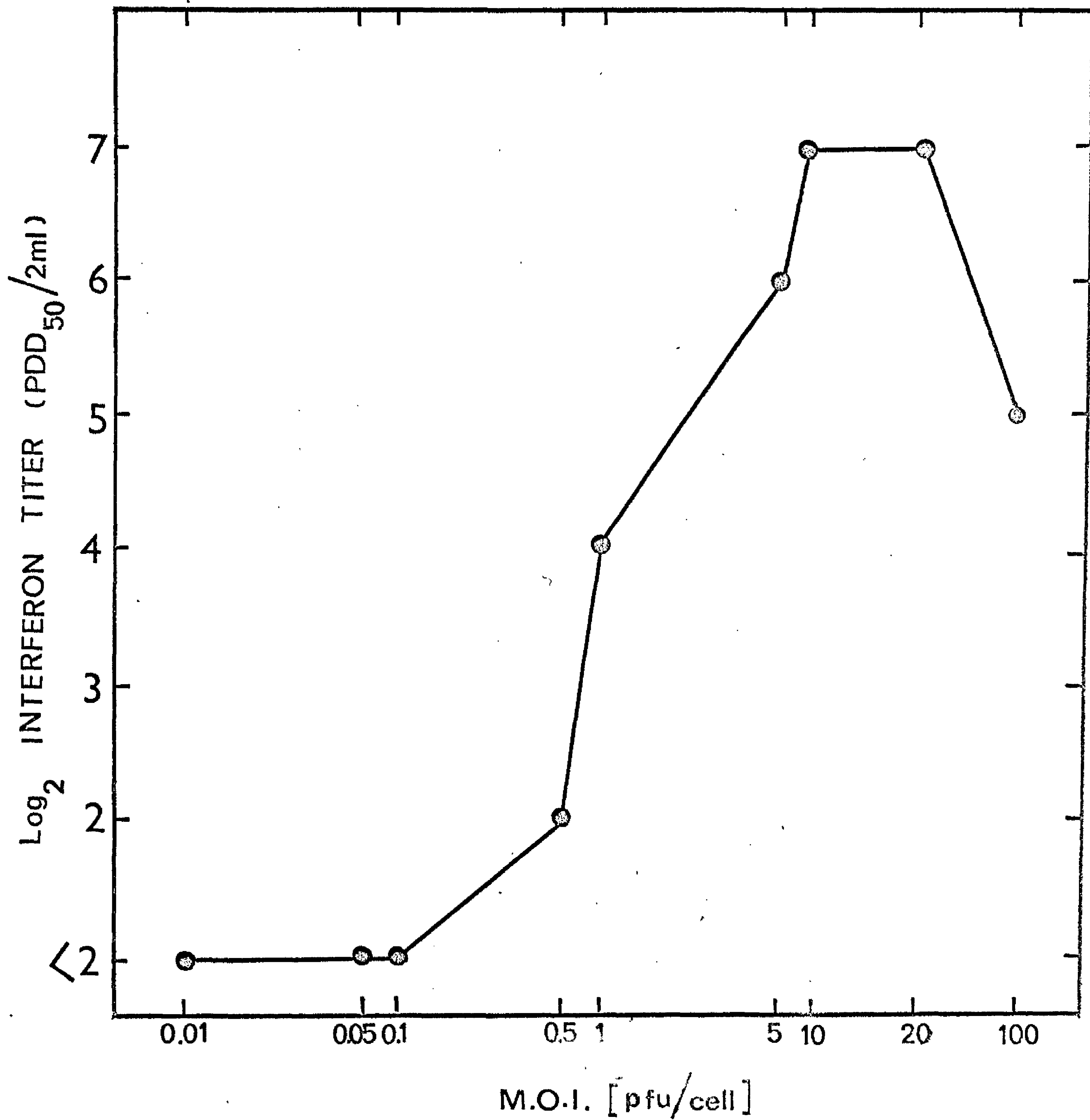


Figure IIb. Multiplicity dependence of interferon induction on CEF cells by adenovirus type 5.

infection with adenoviruses was really interferon rather than some non-specific inhibitor, a number of physical and biological tests were applied to the interferon preparations. These were as follows;

a) Ultracentrifugation. Medium from cells infected by adenovirus types 1 and 5 was centrifuged at 100,000Xg for 2 hours at 4°C. The supernatants were collected and assayed on primary CEF cells for interferon. There was no loss of activity in the supernatant after centrifugation (Table IIa) indicating that the inhibitory substance(s) has a low molecular weight.

b) Heat stability. Interferon samples were heated at two different temperatures, 56°C for 30 minutes and 70°C for 10 and 30 minutes. After heating the samples were assayed for interferon levels as described before. There was no significant loss of inhibitory activity after heating at these temperatures, indicating that the preparations were relatively heat stable (Table IIb).

c) pH stability. The pH of the interferon samples was adjusted to 2 with 1N HCl and they were left 48 hours at 4°C. Then pH was adjusted back to 7.4 with 1N NaOH and samples were assayed for interferon levels. There was no loss of inhibitory activity after such treatment (Table IIc).

VIRUS	Interferon titre (PDD_{50}/2ml)	
	Before centrifugation	After centrifugation
Ad. type 1	128	128
Ad. type 5	256	256

Table IIa. The effect of ultracentrifugation on the levels of interferon in samples obtained by induction with adenovirus types 1 and 5 on CEF cells.

VIRUS	Heating Temperature	Interferon titre (PDD_{50}/2ml.	
		Before heating	After heating
Ad. type 1	56°	128	128
	70°	128	64 128
Ad. type 5	56°	256	256
	70°	256	128

Table IIb. The effect of heat treatment on the inhibitory activity of an interferon sample induced by adenovirus type 1 and type 5.

VIRUS	Interferon titre (PDD_{50}/2 ml)	
	Neutral pH	Low pH
Ad. type 1	64	64
Ad. type 5	128	128

Table IIc. The stability of an interferon sample at low pH.

d) Species specificity. One of the major biological properties of interferons is that in general they act only on cells derived from homologous species of animals and not on cells from heterologous species. Here it was shown that chick interferon is only active on chick cells. Interferon samples were tested on BHK21/C13 hamster cells, but no activity could be detected on these cells (Table IId). The results suggest that the inhibitory substance (s) is only active on CEF cells, in which they were produced.

e) Treatment with type specific neutralising serum.

Interferons are considered to be cellular rather than viral-coded proteins. Accordingly, their activity should not be neutralised by virus-specific antiserum. To test this for adenovirus-induced interferon, serial dilutions of adenovirus type 1 induced interferon were mixed with an equal volume of adenovirus type 1 type specific neutralising antiserum (16 units/ml.). Mixtures were kept for 1 hour at 37°C, then tested for their inhibitory activity. The results (Table IIe) show that the inhibitory activity is non-neutralisable by virus specific antiserum.

f) Trypsin sensitivity. The biological activity of

Interferon dose (units/2ml)	Vaccinia virus plaque counts per plate	
	on CEF cells	on C13 cells
0	72, 74	70, 75
2	2, 5	79, 71
10	0, 0	80, 75

Table IId. The inhibitory activity of an interferon sample obtained from CEF cells induced by adenovirus types on CEF and BHK 21 C13 cells.

Interferon dilution	% of Vaccinia plaque reduction	
	CONTROL	ANTISERUM (16 units)
NONE	100	100
1/4	100	100
1/8	100	100
1/16	80	84
1/32	63	61
1/64	45	30

Table IIe. The effect of type specific antiserum treatment on the inhibitory activity of an adenovirus-induced interferon sample.

interferons are known to be destroyed by proteolytic enzymes, such as trypsin, pepsin, etc. In order to test the action of trypsin on adenovirus type 1 induced interferon, 0.2 ml of 1% 2X crystallised trypsin (Sigma) was added to 1 ml of 0.4x diluted interferon sample and the mixture was incubated at 37°C for 1 hour. Controls also were treated in the same way without trypsin. To stop the trypsin action, 0.2 ml of soybean trypsin inhibitor (Sigma) was added at the end of the incubation period. Samples were then tested for interferon activity, and it was clear that trypsin treatment destroys the virus inhibitory activity of the sample (Table IIf).

The experiments described above indicate that the inhibitory substance(s) produced on CEF cells in response to adenovirus infection is of low molecular weight, relatively heat stable, stable at low pH, species-specific, non-neutralisable with type specific viral antiserum, and trypsin sensitive. All these results confirm that the active substance is actually interferon rather than some non-specific viral inhibitor, since these are all well known properties of interferons (Finter, 1966).

Adenovirus growth on CEF cells.

CEF cells have been described to be non-permissive for the

Interferon dilution	% of Vaccinia plaque reduction	
	Control Interferon	Trypsin treated Interferon
1/16	100	20
1/32	100	10
1/64	79	0

Table IIf. The effect of trypsin on the inhibitory activity of an interferon sample induced by adenovirus type 1.

replication of human adenoviruses (Beladi and Pusztai, 1967, Ho and Kohler, 1967). The adenovirus type 5 used in the present interferon experiments was tested to determine if any virus replication takes place in the system. CEF cells were infected as described previously at a multiplicity of infection of 20 pfu/cell, and different times after infection cells from duplicate plates were harvested into 2 ml of tris buffer. The samples were frozen and thawed three times and assayed on Hela cells for infectivity. At the same times the medium from infected cultures was collected and the interferon levels were measured. It was found that the cell-associated infectivity of adenovirus type 5 gradually decreases until 5 days after infection (Figure IIc), although some cell-associated infectivity was still detectable when the interferon level reached a maximum at 72 hours post-infection. A similar experiment was also carried out with adenovirus type 1 and the same result was obtained.

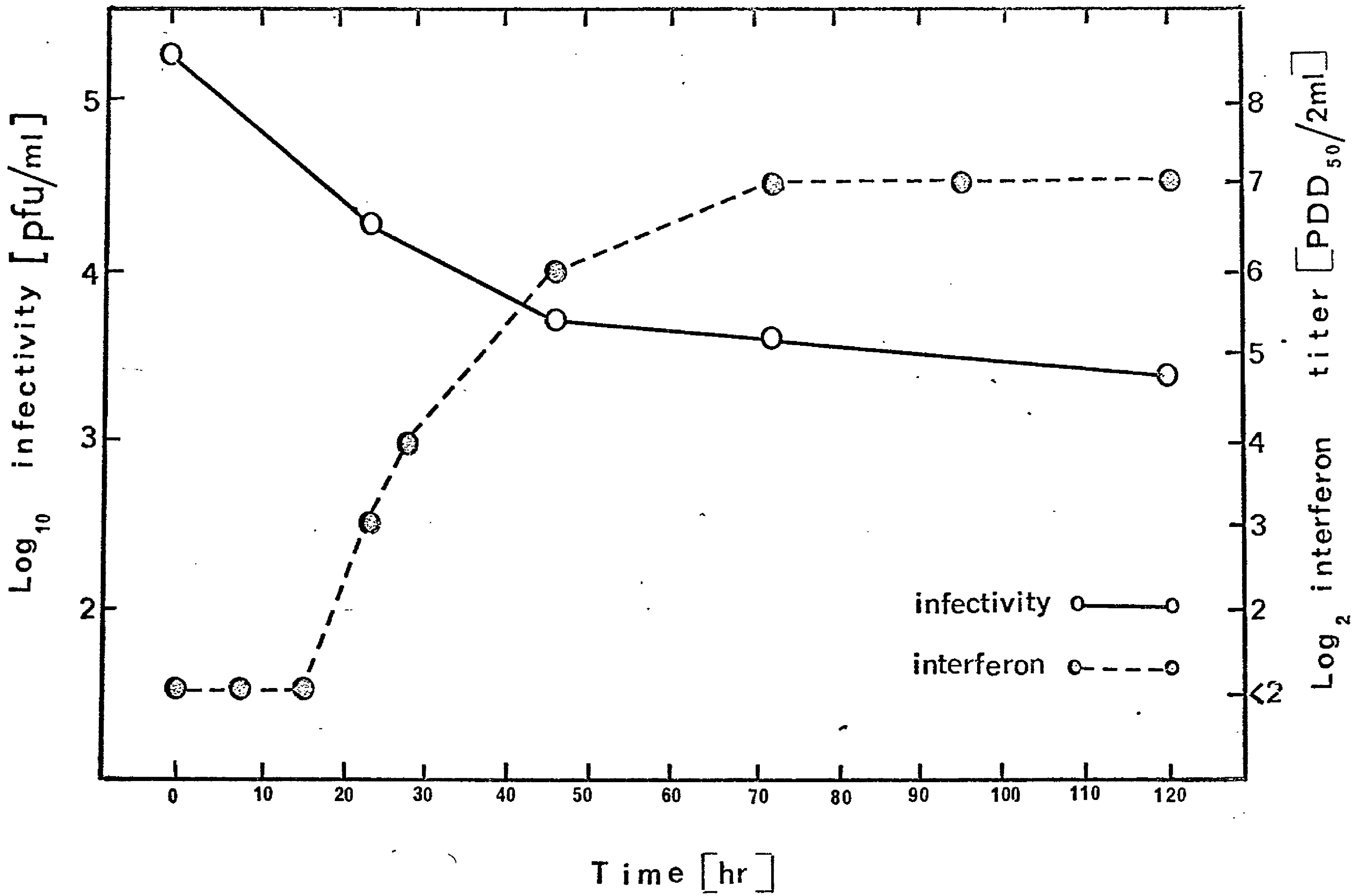


Figure IIc. Production of interferon and the loss of infectivity in CEF cells infected with adenovirus type 5.

The effect of trypsin on the infectivity and interferon inducing capacity of adenovirus types 1 and 5.

Adenovirus infectivity is resistant to trypsin treatment (Pereira, 1958, Ho and Kohler, 1967) while it has been shown that the interferon inducing ability of adenoviruses is greatly reduced after such treatment (Beladi and Pusztai, 1967). The trypsin sensitivity of virus infectivity and of the interferon inducing capacity of adenovirus types 1 and 5 has been tested here. Adenoviruses purified by caesium chloride were used in these experiments, and 1 ml of virus suspension was mixed with 0.1 ml of a 1% solution of 2X crystallised trypsin in tris-saline solution. The mixture was incubated for 1 hour at 37°C and the trypsin action was stopped by adding 0.1 ml. of 1% of Soybean trypsin inhibitor to each sample. A control virus suspension was also treated with 0.1 ml of tris-saline instead of trypsin. The infectivities of the samples were then tested on Hela cell monolayers by the plaque assay, and their interferon inducing capacities were tested on CEF primary cell cultures as described above. Table IIg and IIh illustrates the result of representative experiments for adenovirus types 1 and 5. The virus infectivities were not significantly affected by trypsin treatment but the interferon-inducing capacity of adenovirus

VIRUS	Infectivity titre (PFU/ml)	
	Control	Trypsin treated
Ad. type 1	5.0×10^9	4.3×10^9
Ad. type 5	3.4×10^9	1.9×10^9

Table IIg. The effect of trypsin on the infectivity of adenovirus types 1 and 5.

VIRUS	Interferon titre (PDD_{50}/2 ml)	
	Control	Trypsin treated
Ad. type 1	64	<4
Ad. type 5	128	4

Table IIh. The effect of trypsin on the interferon inducing capacity of adenovirus types 1 and 5.

types 1 and 5 is completely destroyed.

Effect of trypsin on adsorption of ^{3}H-thymidine labelled adenovirus type 5 on Hela and chick embryo cells.

It has been suggested previously that the penton antigen may be necessary for the adsorption of adenovirus to human cells (Valentine and Pereira, 1965). Since the penton antigen of adenovirus was shown to be sensitive to trypsin digestion (Pereira, 1958, 1960, Rowe et al, 1958) the effect of trypsin on the adsorption of radioactively labelled adenovirus type 5 on CEF and KB cells has been tested (Ho and Kohler, 1967). These experiments were also carried out here to test the adsorption capacity of ^{3}H - thymidine-labelled adenovirus type 5 on Hel and CEF cells after trypsin treatemnt. Hela and CEF cells were grown in 50 mm. plastic petri dishes containing sterile round coverslips (13 mm in diameter) and infected at confluency with a multiplicity of infection of 20 pfu/cell with either trypsinised or non-trypsinised ^{3}H -thymidine labelled adenovirus type 5. At intervals after addition of virus excess virus was removed by washing, monolayers were washed three times with PBS, once with formal saline, then fixed for 30 minutes with formal saline at room temperature or overnight at 4°C in the refrigerator. Fixed coverslip cultures were extracted with 5% ice-chilled trichloroacetic acid, washed twice with distilled

water, dried in ethanol and the radioactivity was determined by using a liquid scintillation counter. Two coverslips were counted for each time point. Experiments were carried out once at room temperature and twice at 37°C. No significant difference in the rate of adsorption of trypsinised and non-trypsinised ^{3}H-thymidine labelled adenovirus type 5 on either Hela or CEF cells was detected (Tables IIj). At room temperature it appears that untreated virus adsor more efficiently then trypsinised virus on CEF cells, but at 37°C there was no significant difference in the rates of adsorption of trypsinised and non-trypsinised virus to both Hela and CEF cells.

<u>Effect of Hydroxylamine on infectivity and interferon inducing capacity of adenovirus type 5.</u>

Hydroxylamine is a potent mutagenic and inactivating agent of nucleic acids (Freese and Freese, 1965). It has been shown to inactivate virus infectivity, presumably by reacting with viral nucleic acid, without affecting virus antigenicity (Schafer and Rott, 1962., Grossgebauer, 1966). It has been shown that Semliki Forest virus partially inactivated with hydroxylamine failed to induce interferon on CEF cells indicating that complete viral nucleic acid is necessary for interferon induction. Similar

Time (min)	HELA cells		CEF cells	
	C*	T**	C	T
5	249***	I36	I36	87
20	475	2I6	I92	I2I
45	525	365	26I	I47
60	7I5	433	393	I39

EXP.I.
(at room temperature)

Time (min)	HELA cells		CEF cells	
	C	T	C	T
5	69	64	49	40
10	I2I	97	83	74
20	I59	I25	I07	84
30	20I	I55	I49	98
60	233	I96	I60	I32
90	257	226	I98	I67

EXP.II.
(at 37°C)

Time (min)	HELA cells		CEF cells	
	C	T	C	T
5	I78	I7I	I74	I78
15	26I	283	284	273
30	736	68I	599	377
45	908	896	846	640
60	I099	I006	II3I	885

EXP.III.
(at 37°C)

* Control adenovirus type 5 .
** Trypsin treated adenovirus type 5.
*** Counts per minute (cpm).

Table IIj. The effect of trypsin on adsorption of ^{3}H - thymidine labelled adenovirus type 5 on Hela and CEF cells.

experiments were carried out here, in order to determine the interferon inducing capacity of adenovirus type 5 inactivated with hydroxylamine. The inactivation and mutagenesis of adenovirus type 5 by hydroxylamine is described in Chapter III and will not be repeated here. Samples treated for 0, 1, 2, 3, 4 and 5 hours were tested for their infectivity on Hela cells and 0, 3 and 5 hoursamples for their interferon-inducing capacity on CEF cells as described above. The results are shown in figure IId. Hydroxylamine treatment results in a 4.5 log reduction in infectivity titre in 5 hours and the interferon-inducing capacity of adenovirus type 5 is reduced to around 12.5% of the control by 3 hours and completely lost by 5 hours treatment. This strongly suggests that the interferon inducing capacity of adenovirus type 5 is directly relatedto the infectivity of the virus.

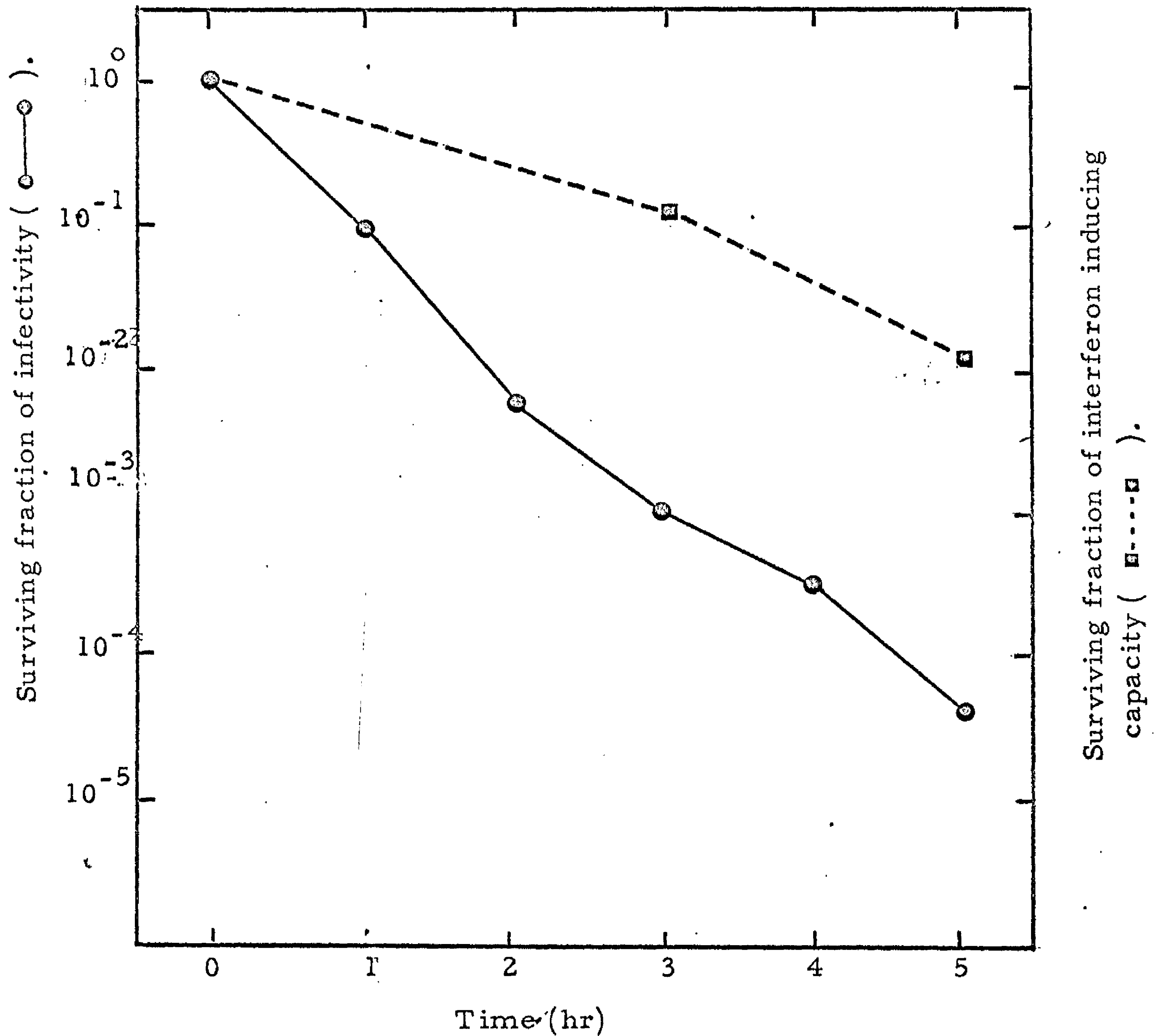


Figure IId. The effect of hydroxylamine on infectivity and interferon inducing capacity of adenovirus type 5.

DISCUSSION

Adenovirus types 1, 5 and 12 have been shown to induce reasonably high levels of interferon on chick cells. The kinetics of interferon induction by all three adenoviruses was found to be similar in that interferon appears in the culture medium between 12-15 hours after infection, increases in titre in the next 50-60 hours, and reaches a maximum level by 72 hours. This result indicated that certain time is required before the appearance of interferon in the culture medium of adenovirus-infected chick embryo cells. It is not known at present which virus activities are expressed in adenovirus-chick cell interaction. However, if the time required for the appearance of interferon in the culture medium is compared with the replication cycle of adenovirus in Hela cells in which virus production is first detected 15-18 hours post-infection, by this time the interferon production is well on the way in chick cells. As shown and discussed in Chapter III, the viral DNA synthesis does not take place on CEF cells or is not required for interferon induction. It is possible that this time period is needed for some early viral events (such as uncoating) or for the synthesis of certain virus coded early products. However, it cannot be ruled out that some time is required

for the external inducer to reach the target for stimulation of interferon production; for example the penetration of adenovirus into chick cells migh a slow process. Also experiments concerning the growth of adenoviruses on CEF cells indicate that adenovirus type 5 multiplication is not required for interferon induction.

The interferon induced by adenoviruses on chick cells has the same properties as those described for other chick interferons (Finter, 19 The minimum multiplicity required for an interferon response is approximately 0.5 pfu/cell and the optimum interferon responses were attained when multiplicities of 10-20 pfu/cell were used. Higher multiplicities of infection had usually a toxic effect on CEF cells, therefore the interferon levels were lower. Thus, the yield of interferon was related to multiplicity of exposure. In this respect adenoviruses behave like polyoma virus (Chapter V). A multiplicity dependence for interferon induction has also been described for the reovirus-chick cell system, where virus multiplies but interferon is induced. (Long and Burke, 1971).

It is worth noting that after infection of chick cells with adenovirus type 5 a mild cytopathic effect is observed parallel to interferon induction and the culture medium goes very acidic, indicating that virus causes

some changes in the cell metabolism. In addition, it is certainly possible that some virus functions are expressed in adenovirus infected chick cells, but the virus does not undergo a complete replication cycle.

Trypsin treatment of adenovirus type 1 and type 5 had no effect on infectivity, but the interferon inducing capacity of these two types was greatly reduced. These results are in agreement with the results of others who found that trypsin treatment destroyed the interfernn inducing capacity of adenoviruses (Beladi and Pusztai, 1967; Ho and Kohler, 1967). The primary action of trypsin has been shown to be on the penton antigen of adenovirus (Pereira, 1968; Rowe et al. 1958). In addition, however, trypsin has been shown to cause breaks in the penton and hexon polypeptides (Pereira and Skehel, 1971). Nevertheless, both hexon and penton antigen retained their antigenicity and morphological appearance after trypsin treatment. It is reasonable to think that the penton antigen of the virus is in some way responsible for interferon induction, because of its sensitivity to trypsin, but it has to be considered that both hexon and perhaps internal viral antigens of the virus may also be affected by trypsin. However, partially purified penton antigen from adenovirus type 8 was shown to induce a very low level of interferon on chick cells. (Lengyel et al, 1970) but, the

purified capsid components of adenovirus type 5 fail to induce interferon (W.C. Russell, personal communication). It has been thought that penton antigen may play a role in the adsorption of virus to cells (Valentine and Pereira, 1965). Therefore, the adsorptive capacity of ^{3}H - thymidine labelled adenovirus type 5 on chick cells after trypsin treatment was tested. Trypsinised and non-trypsinised viruses both adsorbed equally as well to Hela and to chick cells. These results are in agreement with a previous report (Ho and Kohler, 1967).

Treatment of adenovirus type 5 with hydroxylamine destroys virus infectivity faster than the interferon inducing capacity. Neverthele the interferon inducing capacity of adenovirus type 5 is reduced approxima 2 log after 5 hours by 1 Mol hydroxylamine. Differences in the rate of reduction of infectivity and interferon inducing capacity may be due to differences in their target size. Alternatively the physical integrity of th viral chromosome may need to be disrupted to affect the interferon inducing capacity. This would require greater chemical changes than would be the case for loss of infectivity.

The results presented in this chpater support the view that the penton antigen of the virus may play a role in interferon induction, but the evidence for this is both indirect and inconclusive. Extensions of the work described in this chapter will be presented in the following

chapters of this thesis. Different approaches, designed to determine the viral functions necessary for interferon induction by adenoviruses and polyoma virus will be outlined.

CHAPTER III

ISOLATION, PRELIMINARY CHARACTERISATION OF AND INTERFERON INDUCTION BY TEMPERATURE-SENSITIVE MUTANTS OF ADENOVIRUS TYPE 5.

1. INTRODUCTION
2. MATERIALS AND METHODS.
3. RESULTS.

A. **Isolation and preliminary characterisation of temperature-sensitive mutants of adenovirus type 5.**

Aa Inactivation and mutagenesis of adenovirus type 5 by hydroxylamine.

Ab Isolation of temperature-sensitive mutants of adenovirus type 5.

B. **Induction of interferon by temperature-sensitive mutants of adenovirus type 5 on CEF cells.**

Ba Interferon induction with *ts* mutants of adenovirus type 5.

Bb Kinetics of interferon induction with wild type, *ts* 18 and *ts* 19 on CEF cells at permissive and non-permissive temperat

Bc Effect of adsorption temperature on interferon induction by wild type, *ts* 18 and *ts* 19.

Bd Test for a possible inhibitor of interferon produced in *ts* 1 infected CEF cells.

C Further characterisation of ts 18 and ts 19.

Ca One-step growth experiments with wild type adenovirus 5 and ts 18 on Hela cells at permissive (31°C) and non-permissive (38°C) temperatures.

Cb Complementation between ts 18 and ts 19 on CEF cells at non-permissive temperature for interferon inducti

Cc Viral DNA synthesis by wild type adenovirus 5, ts 18 and ts 19 on Hela cells at non-permissive temperature.

Cd A search for viral DNA synthesis on CEF cells infect with adenovirus type 5 wild type at 37°C.

Ce Temperature shift-up experiments using ts 18 on Hela cells.

Cf Effect of temperature shift-up on the interferon induct by ts 18 on CEF cells.

Cg Virion heat sensitivity of adenovirus wild type, ts 18 and ts 19.

4. DISCUSSION.

INTRODUCTION

Temperature-sensitive (ts) mutants, a class of conditional-lethal mutants, have been very useful for studies of bacteriophage genetics and development (Epstein et al. 1963). Ts mutants of animal viruses grow adequately at a permissive temperature, but fail to grow at a restrictive temperature. The polypeptide specified by the mutated gene is synthesised, but its function is not expressed at the restrictive temperature. Ts mutations will occur spontaneously, but the frequency is very low. Ts mutations can be induced by treatment with a variety of different mutagens *in vivo* and *in vitro*. Ts mutations theoretically occur in all virus genes, but only functions necessary to virus replication will be isolated by the selective methods used such as, plaquing efficiency at permissive and restrictive temperatures. Several ts mutants for a number of animal viruses have been isolated and their genetics have been extensively studied (reviewed by Fenner, 1969).

Until recently, the genetic functions of adenoviruses have not been studied. Adenoviruses are average-sized DNA viruses; 80 mμ in diameter, containing linear double stranded DNA with a molecular weight of about 20-25 x 10^6 daltons, sufficient genetic

information to code for some 30-50 average-sized proteins. A few years ago, cytocidal mutants were isolated for adenovirus type 12 (Takemori, Riggs, and Aldrich, 1968, 1969). These mutants produced larger plaques than the wild type virus on KB cells, and were considerably less oncogenic than parental virus when inoculated into newborn hamsters (Takemori, Riggs, and Aldrich, 1968). Some of these mutants were found to be host-dependent, in that they grow only on certain lines of KB cells, but no complementation could be observed between these mutants, suggesting that they were defective in the same cistron. Therefor they would be of limited use for detailed genetical analysis.

Adenoviruses have not been studied by means of ts mutants until recently, principally perhaps because of poor and inefficient plaque assays. The plaque assay recently developed by Williams (1970) provides enhanced and rapid plaque formation by adenovirus type 5 on Hela cells. Within the last two years ts mutantshave bee isolated for the avian adenovirus, CELO (Ishibashi, 1970) and for human adenovirus type 5 (Williams et al. 1971; Ensinger and Ginsberg, 1971; Takashashi, 1972), adenovirus type 12 (Lundholm and Doerfler, 1971; Shiroki, Irisawa and Shimojo, 1972), adenovirus type 31 (Suzuki and Shimojo, 1971; Suzuki, Shimojo

and Moritsugu, 1972).

As mentioned above ts mutants have been used to explore viral genes and their function in the development of virus replicatio In addition, ts mutants have been used in order to identify certain viral functions necessary for permissive and non-permissive virus-cell interactions (such as transformation and interferon induction). Interferon can be induced by many RNA and DNA viruses in their permissive and non-permissive hosts. In permissive cells the virus replicates and may also induce interferon, detectable some hours after infection. Since synthetic double-stranded RNA polymers have been found to be active inducers (Field et al. 1967), it is thought that viral RNA production in cells infected with RNA viruses might be necessary for interferon induction. For this reason, the visal events necessary for the induction of interferon by RNA viruses in permissive cell systems have been investigated by using conditional-lethal, temperature-sensitive mutants. This was first attempted for Sindbis virus (Lockart et al. 1968). A class of ts mutants, which fail to synthesise viral RNA at the restrictive temperature (ts-RNA$^-$) and another class which synthe viral RNA equally well at both temperatures (ts-RNA$^+$), failed to

induce interferon synthesis at the restrictive temperature. Therefore, it was concluded that production of viral proteins is necessary, and accumulation of viral RNA is not sufficient for interferon induction in CEF cellsinfected with Sindbis virus. Subsequently it was found that purified preparations of Sindbis virus ts-RNA$^-$ mutants failed to induce, while ts-RNA$^+$ mutants were able to induce interferon at the restrictive temperature (Marcus, P.I., personal communication in Colby and Morgan, 197 suggesting that the production of viral RNA is necessary for interfe induction. It has also been found that interferon induction with ts mutants of Semliki Forest virus is multiplicity dependent (Lomnicz and Burke, 1970). At low multiplicities of infection of CEF cells, interferon induction with ts mutants of Semliki Forest virus is dependent on viral RNA synthesis, but at high multiplicities both ts-RNA$^-$ and ts-RNA$^+$ mutants were able to induce interferon, indicating that both newly synthesised and input viral RNA of Semliki Forest virus can act as an inducer of interferon.

Ts mutants have been used also to study non-permissive virus-interactions. DNA tumor viruses induce malignant transformatior in vitro in animal cells in which they can not undergo the complete replication cycle. The gene functions necessary for transformatic

have not been identified yet, and in order to help identify these functions ts mutants are currently being used; examples include ts mutants of polyoma (Fried, 1970; Eckhart, 1971) and adenovirus type 5 (J. F. Williams and P. Austin, unpublished results).

Another example of a non-permissive system, the chick embryo-adenovirus interaction in which interferon is produced, has been described in a previous chapter of this thesis. Human adenoviruses fail to replicate in chick embryo cells (Beladi and Pusztai, 1967; Ho and Köhler, 1967; Ustacelebi and Williams, 197 The adenovirus function(s) responsible for the induction of interfero in this system is (are) not known at the present. It seemed feasible that ts mutants of adenovirus type 5 could be used to identify the genes required for interferon induction provided functions essential to virus replication are involved in induction. In this chapter the isolation of ts mutants of adenovirus type 5 from hydroxylamine mutagenised stocks will be described. Preliminary characterisation and genetic analysis of these and other ts mutants has been published (Williams and Ustacelebi, 1971; Wilkie, Ustacelebi and Williams, 1972; Russell, Newman and Williams,

1972) and some details of this will be discussed in this chapter. The ts mutants of adenovirus type 5 belonging to 14 complementatio groups were used in an attempt to identify the viral function(s) necessary for the interferon response on CEF cells, and two mutants (ts 18 and ts 19) were found to be unable to induce interfer at the non-permissive temperature. These were further characterised in both Hela and CEF cells and the relation of their properties to interferon induction by adenovirus type 5 will be discussed.

MATERIALS AND METHODS

Cells. Hela cells and CEF cells were prepared as described in chapter II.

Viruses and infectivity assay. Preparation of wild type adenovirus 5 stocks, and plaque assays were described in Chapter II

Interferon induction and assay. As described in Chapter II

Preparation of ^{32}P-labelled adenovirus type 5. Confluent monolayers of Hela cells grown in Winchester bottles were infected with wild type virus at an input multiplicity of 5-10 pfu/cell. Viru was adsorbed on cells at 37°C for 90 min., excess virus was removed by washing with Eagle's medium, and Eagle's medium supplemented with 2% calf serum was added. The infected burrlers were kept at 37°C for 6 hours before addition of phosphate free medium supplemented with 2 µci/ml. (phosphorus 32-orthophosphate (sp. act. 2 ci/mMol) and 2% calf serum. After addition of radioacitve medium, cultures were kept at 37°C till complete cytopathic effect was observed. At this time infected cells were shaken into the medium, pelletted by centrifugation at 20 r.p.m. for 15 minutes, resuspended in tris saline, pH 7.4, and frozen and thawed three times. After centrifugation at 2000 r.p.m

for 15 minutes, the supernatant was collected and purified by density gradient centrifugation as described in Chapter II

Extraction of total DNA from infected and mock-infected cells.

The method of extraction is as described by Wilkie, Ustacelebi and Williams, (1972). Infected and mock-infected cells were washed three times with PBS, and cells were lysed by addition of 1 ml. of 0.15Mol NaCl, 0.015 Mol tri-sodium citrate 0.005 Mol EDTA, 0.5% SDS and 0.1 N tris-HCl, pH 8.00. Prior to lysis a small amount of ^{32}P-labelled purified wild type virus was added as a radioactive virion DNA marker. The lysate was then heated at 60°C for 10 minutes, cooled to room temperature an pre-incubated pronase was added to a final concentration of 500 ug/ml. The solution was further incubated at 37°C for 3 hours then dialysed against 0.005 Mol EDTA, 0.01 Mol tris-HCl, pH 7.5 The dialysed extracts were kept frozen at -20°C until analysed by caesium chloride density gradient centrifugation.

Spinco Model L centrifugation

Extracts were made up to 6 ml. by 0.05 Mol EDTA, 0.01 Mol tris-HCl, pH 7.5. Solid caesium chloride was added to adjust the density to 1.402 and after this each sample was transferred into 10 polyallomer tubes. All tubes were topped with mineral oil and

centrifugation was carried out at 40,000 r.p.m for 3 days at 20°C in the Ti 50 angle-head rotor. After centrifugation each gradient was collected in 60-80 fractions by using an LKB peristaltic pump (60 gear). The extruded drops were collected on to 2.5 cm diameter Whatman No. 1 paper discs (4 drops/disc). The refractive index of every tenth fraction was measured using a refractometer. Air-dried discs were transferred into scintillation liquid mixture of 2½ litres of toluene, 12.5 gr. of 2,5-Diphenyloxazolyl (PPO) and 0.75 gr. of 1,4-di 2-(5-phenyloxazolyl) benzene (POPOP), and samples were counted using an Intertechnique scintillation counter.

Buffers. B1 buffer, consists of 0.05 Mol $NaH_2PO_4 \cdot 2H_2O$, pH 7.5.

B2 buffer, consists of 0.05 Mol $NaH_2PO_4 \cdot 2H_2O$, 1 Mol. NaCl, pH 7.5.

RESULTS

A-ISOLATION AND PRELIMINARY CHARACTERISATION OF AND INTERFERON INDUCTION BY TEMPERATURE-SENSITIVE MUTANTS OF ADENOVIRUS TYPE 5.

Inactivation and mutagenesis of adenovirus type 5 by hydroxylamine

Hydroxylamine (Hx) was used as a mutagenic agent for adenovirus type 5in order to obtain temperature-sensitive mutants. Hx mutagenised stocks were prepared as follows. Hx (Koch-Light) was prepared at a concentration of 2 Mol in B2 buffer. The 2 Mol Hx solution was mixed with an equal amount of virus suspensio and left at room temperature. The pH of the mixture was adjusted to 7.5 before the reaction commenced. The 0 hour sampl or control was taken by mixing the virus preparation with an equal amount of B2 buffer. Samples were removed from 1 Mol Hx treate mixture at 1, 2, 3, 4 and 5 hours and immediately diluted 100X in cold B1 buffer. After this, they were dialysed overnight against B buffer. Dialysed samples were then immediately assayed on Hela cells monolayers for infectivity as described previously. The kinetics of inactivation of adenovirus type 5 by Hx is illustrated in Figure IId . In Chapter II . A 4.5. log. inactivation in infected titre was attained in 5 hours, and such stocks were kept frozen

at -70°C and used for isolation of temperature-sensitive mutants.

Isolation of temperature-sensitive mutants.

The Hx mutagenised stock was plated for plaque production on Hela cells at 31°C. Virus was diluted so that only 1-2 plaques formed on each dish. After incubation for 2-3 weeks, well isolated plaques were picked at random from the cultures with pasteur pipettes and suspendded in 1 ml. of tris-saline. These plaque isolates were frozen and thawed three times and retitrated at both 31°C and 38°C on Hela cell monolayers. Isolates showing a 50 fold or greater plaquing efficiency at 31°C than at 38°C were considered to be potential mutants. From these, a number of plaques were picked from the dishes at 31°C seeded with the highest dilutions. These were titrated again at 31°C and 38°C an[d] most showed a 1000-fold or higher plaquing efficiency at 31°C tha[n] at 38°C. The plaque forming abilities of these mutants at 31°C a[nd] 38°C are shown in Table IIIa. In the present work, a total of 26 temperature-sensitive mutants were isolated from 351 plaques tested (a frequency of 7.4%). After isolation, mutants were passaged at 31°C in Hela cells in order to obtain high titre stocks. After 2 to 3 passages, stocks with high infectivity titres were obtained for most mutants. Titres ranged from 4×10^8 to

Virus	Infectivity pfu/ml. 38°C	31°C	Ratio of plaque forming cap. (38°C/
5 ts 1	10	3.7×10^5	2.7×10^{-5}
5 ts 11	$< 10^2$	4.4×10^5	$< 2.3 \times 10^{-4}$
5 ts 12	$< 10^2$	3.9×10^4	$< 2.5 \times 10^{-3}$
5 ts 13	$< 10^2$	5.7×10^5	$< 1.8 \times 10^{-4}$
5 ts 14	$< 10^2$	5×10^4	$< 2 \times 10^{-3}$
5 ts 15	$< 10^2$	2.2×10^3	$< 4.5 \times 10^{-2}$
5 ts 16	$< 10^2$	3×10^5	$< 3.3 \times 10^{-4}$
5 ts 17	$< 10^2$	9.5×10^5	$< 1 \times 10^{-5}$
5 ts 18	$< 10^2$	7×10^5	$< 1.4 \times 10^{-4}$
5 ts 19	$< 10^2$	3×10^5	$< 3.3 \times 10^{-4}$
5 ts 20	$< 10^2$	3×10^5	$< 3.3 \times 10^{-4}$
5 ts 21	9.5×10^2	1.4×10^6	7×10^{-4}
5 ts 24	$< 10^2$	1.7×10^5	$< 6 \times 10^{-4}$
5 ts 25	$< 10^2$	5.6×10^5	$< 1.8 \times 10^{-4}$
5 ts 26	$< 10^2$	1.4×10^5	$< 7 \times 10^{-4}$
5 ts 27	$< 10^2$	8.2×10^5	$< 1.2 \times 10^{-4}$
5 ts 28	$< 10^2$	2×10^6	$< 5 \times 10^{-5}$
5 ts 29	$< 10^2$	2.7×10^5	$< 3.7 \times 10^{-4}$
5 ts 30	$< 10^2$	7×10^5	$< 1.4 \times 10^{-4}$
5 ts 31	$< 10^2$	2.9×10^5	$< 3.4 \times 10^{-4}$
5 ts 32	$< 10^2$	2.5×10^5	$< 4 \times 10^{-4}$
5 ts 33	$< 10^2$	2.2×10^5	$< 4.8 \times 10^{-4}$
5 ts 34	$< 10^2$	1.6×10^5	$< 6.2 \times 10^{-4}$
Wild type	4.0×10^6	4.2×10^6	1.0

TABLE IIIa The plaque forming ability of wild-type and ts mutants of adenovirus type 5 at 31°C and 38°C.

6×10^9 pfu/ml. when titrated at 31°C while at 38°C the infectivities were very low, in most cases around 10^3 pfu/ml. It is rather difficult to measure infectivity at 38°C accurately since at high concentrations many of these mutants were cytotoxic for Hela cells.

A measure of the stability of the mutants was obtained by comparing the yields from single growth cycles at 31°C and 38°C in Hela cells infected at a multiplicity of 10 pfu/cell. (Table IIIb). Yield experiments were done as follows; Hela cell monolayers were infected with each mutant at a multiplicity of 10 pfu/cell, and virus was adsorbed on cells for 90 minutes at 38°. After adsorption, monolayers were washed once with 3 ml. of Eagle's medium, treated with 1 ml of 1/150 dilution of adenovirus type 5 neutralising antiserum (neutralising titre, 1/10,000) for 10 minutes at 38°C, washed again with Eagle's medium, and finally overlaid with Eagle's medium supplemented with 2% calf serum. Infected cultures were incubated for 5 days at 31°C and for 40 hour at 38°C. At these times cells were scraped off into 1 ml of tris-saline, frozen and thawed three times and the extracts assayed for infectivity at 31°C and 38°C on Hela cell monolayers.

Mutant	Virus yield (p. f. u.)		Ratio of yields
	31°C	38°C	38°C/31°C
5 ts 1	2.8×10^9	1.0×10^2	3.6×10^{-8}
5 ts 13	1.5×10^8	2.8×10^5	1.8×10^{-3}
5 ts 14	1.3×10^8	2.0×10^2	1.5×10^{-6}
5 ts 17	9.0×10^7	4.5×10^2	5.0×10^{-6}
5 ts 18	5.0×10^7	$< 10^2$	$< 2.0 \times 10^{-6}$
5 ts 19	3.5×10^7	1.9×10^4	5.4×10^{-4}
5 wild-type	2.9×10^9	4.3×10^9	1.5

TABLE Ts mutant yields at 31°C and 38°C.

Examples of the titrations of virus yields at 31°C and 38°C are shown in Table IIIb, and it can be seen that with the exception of ts 13 all mutants are non-leaky and the reversion rate is low with all mutants tested (Williams and Ustacelebi, 1971). Wild type grows equally well at 38°C as at 31°C.

B - INDUCTION OF INTERFERON BY TEMPERATURE-SENSITIVE MUTANTS OF ADENOVIRUS TYPE 5 ON CEF CELLS.

Interferon induction with ts mutants of adenovirus type 5.

Wild type and ts mutants of adenovirus type 5 were tested for their interferon inducing capacity on CEF monolayers at 31°C and 38°C in the following way. Primary CEF monolayers were infected with wild type adenovirus 5 or ts mutants at an input multiplicity of around 20 pfu/cell. Virus was allowed to adsorb for 2 hours at 38°C, then cultures were washed once with Eagle's medium and overlaid with Eagle's medium supplement with 2% calf serum. Usually 4 replicate monolayer cultures were infected with each virus, with two subsequently incubated for 3 days at 38°C, and the other two for 4 days at 31°C. At the end of the incubation periods, the culture fluids were harvestec and assayed for interferon levels as described in the previous Chapter. The results of interferon induction by wild type and twenty-five ts mutants are shown in Table IIIc. Experiments were repeated several times with both wild type and ts mutants (both purified and non-purified) at both permissive and non-permis temperatures and consistent results were obtained. Wild type

Virus	Interferon titre(PDD_{50}/2 ml.)	
	31°C	38°C
5 wild type	128	128
5 ts 1	256	128
5 ts 2	128	128
5 ts 3	256	128
5 ts 4	128	64
5 ts 5	128	128
5 ts 6	128	64
5 ts 7	128	128
5 ts 8	128	64
5 ts 9	64	64
5 ts 10	256	128
5 ts 12	64	16
5 ts 13	256	128
5 ts 14	256	128
5 ts 17	256	128
5 ts 18	128	<4
5 ts 19	64	<4
5 ts 20	128	128
5 ts 22	64	64
5 ts 24	128	64
5 ts 29	64	32
5 ts 30	64	64
5 ts 31	64	64
5 ts 33	128	64
5 ts 36	128	128
5 ts 37	128	128

Table IIIc. Interferon induction with ts mutants of adenovirus type 5 at permissive (31°C) and non-permissive (38°C) temperatures.

virus and all ts mutants except ts 18 and ts 19 induced good interferon levels at both temperatures on CEF cells. The two exceptions failed to induce interferon at 38°C, but induced norm yields at 31°C, and were considered to be defective in their interferon inducing capacity at the non-permissive temperature. They were subsequently studied further in order to identify the defective viral function(s) responsible for interferon induction.

Kinetics of interferon induction with wild type, ts 18 and ts 19 on CEF cells at permissive and non-permissive temperatures.

In order to establish the kinetics of interferon induction with wild type, ts 18 and ts 19 at 31°C and 38°C, primary CEF cells were infected with 20 pfu/cell with each virus and at several time intervals after infection fluid was harvested for measurement of interferon levels. The induction kinetics obtained for wild type, ts 18 and ts 19 at 31°C and 38°C are shown in Figure IIIa . Wild type induces good yields of interferon at both temperatures. At 38°C, maximum levels were obtained by 3 days after infection while at 31°C, interferon levels reached a maximum 4 days after infection with slightly higher levels of interferon than at 38°C. As seen in Figure IIIa ts 18 and ts 19 did not induce any detectable

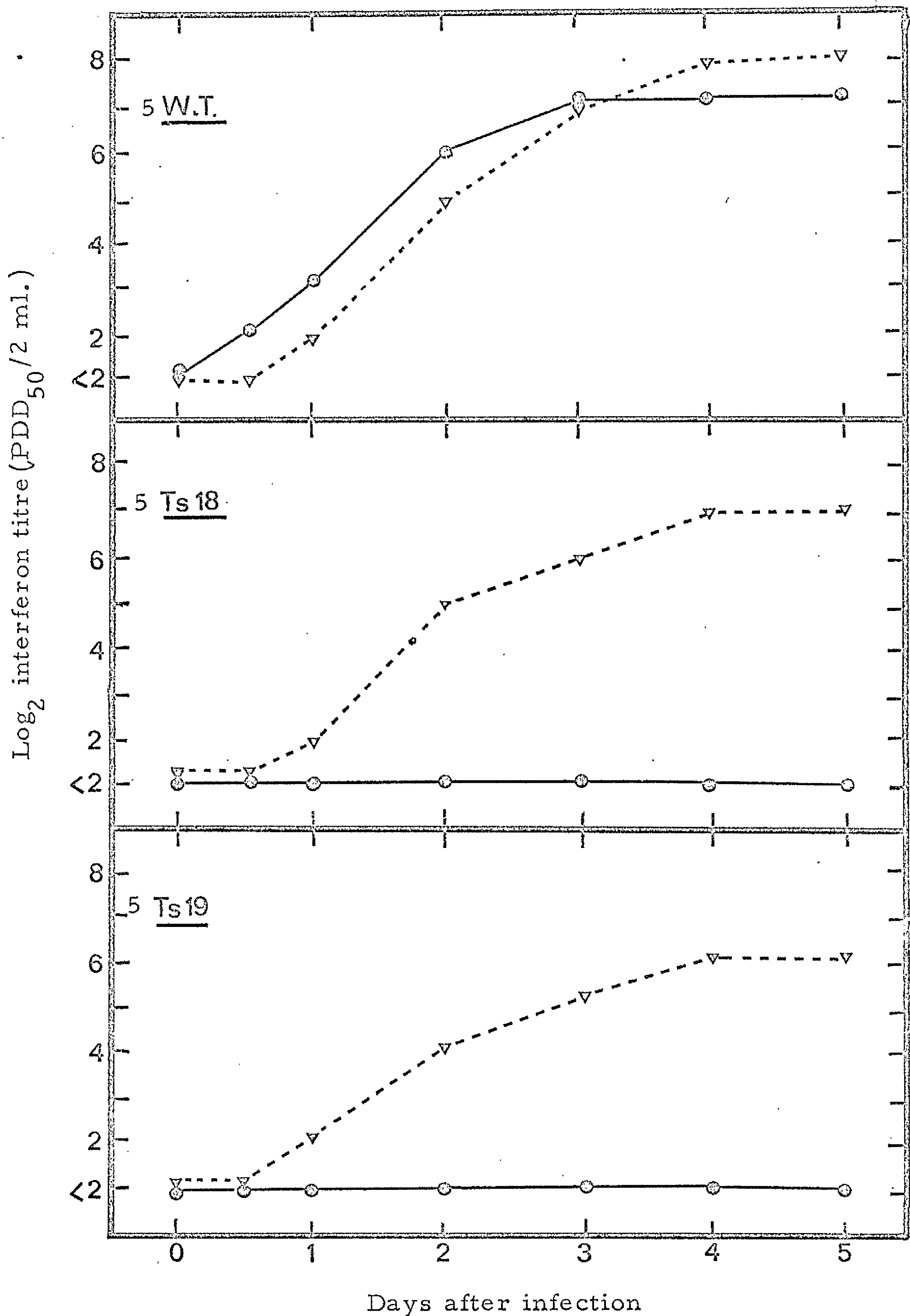


Figure IIIa. Kinetics of interferon induction with wild type adenovirus 5, ts 1[...] and ts 19 on chick embryo cells at permissive (31°C) (▶- - -▶) and non-perm[...] (38°C) (●——●) temperatures.

interferon on CEF cells during 5 days of incubation at 38°C, while they induced normal levels of interferon, reaching a maximum by 4 days after infection, at 31°C.

Effect of adsorption temperature on interferon induction by wild type virus, ts 18 and ts 19.

In all interferon induction experiments, ts mutants, including ts 18 and ts 19 were adsorbed on CEF cells at 38°C prior to incuba at 31°C or 38°C. In order to test the possibility that these mutan fail to adsorb at the non-permissive temperature, virus was adsorbed at both 31°C and 38°C prior to incubation at either 31°C or 38°C for interferon induction. In these experiments CEF cells were infected in the usual way with wild type adenovirus 5, ts and ts 19. One set of plates was incubated at 31° and the other at 38°C for 2 hours for adsorption and subsequently overlaid in the Eagle's medium supplemented with 2% calf serum. Half of each set was further incubated for 4 days at 31°C and the other half 3 days at 38°C and at the end of each incubation period medium was harvested and interferon levels measured. The results of these experiments are shown in Table IIId . After adsorption at either 31°C or 38°C, ts 18 and ts 19 are still unable to induce the interferon response on CEF cells at the non-

Virus	Adsorption temperature °C 2 hours	Interferon titre (PDD_{50}/2 ml) Following incubation temperature (°C)	
		31°C (4 days)	38°C (3 days)
5 wild type	31°C	64	64
	38°C	128	64
5 ts 18	31°C	64	<4
	38°C	64	<4
5 ts 19	31°C	32	<4
	38°C	32	<4

Table. IIId. Effect of adsorption temperature on interferon induction by wild type virus, ts 18 and ts 19.

permissive temperature (38°C) while they do so in cells grown at the permissive temperature (31°C). On the other hand, wild type induced good interferon yields at both temperatures after adsorption at either 31°C or 38°C. The results suggest that the defect involved in interferon induction by *ts* 18 and *ts* 19 is not expressed at the stage of virus adsorption.

Test for a possible inhibitor of interferon produced in ts 18 infected CEF cells.

Since *ts* 18 and *ts* 19 fail to produce an interferon response on CEF cells at the non-permissive temperature, one possible explanation for this is that infection with these mutants may lead to the synthesis of an inhibitor on CEF cells at non-permissive temperature which suppresses the induction of interferon. In order to determine if such an effect might occur, CEF cells were infected simultaneously with both *ts* 18 and wild type adenovirus 5. The experiment was carried out as follows. Primary CEF cells were infected with wild type virus or *ts* 18 singly, at an input multiplicity of 20 pfu/cell, or doubly with wild type virus and *ts* 18 together at an input multiplicity of 10 pfu/cell each. Infected cells were incubated for 3 days at 38°C and 4 days at 31°C, and

medium harvested for interferon assay at these times. The results are shown in Table IIIe . In theory the presence of such an inhibitor in ts 18 infected cells would inhibit interferon induction by wild type in the mixed infection at the non-permissive temperature (38°C). Nevertheless in mixedly infected cells, wild-type virus was still able to induce a normal interferon response at both 31°C and 38°C. In single infections both wild type and ts 18 induced the interferon response at the permissive temperature but ts 18 was unable to trigger the interferon response at the non-permissive temperature. These results suggest that induction of an inhibitor bythis mutant is unlikely.

Virus	m. o. i. (pfu/cell)	Incubation temperature	
		31°C	38°C
5 wild type	20	128 *	64
5 <u>ts</u> 18	20	64	<4
5 w.t. x 5 <u>ts</u> 18	10 x 10	64<128	64

* interferon titre PDD_{50}/ 2 ml.

Table IIIe. Test for a possible inhibitor of interferon produced in <u>ts</u> 18 infected CEF cells.

C - FURTHER CHARACTERISATION OF ts 18 and ts 19

One step growth experiments with adenovirus 5 wild type and ts 18 on Hela cells at permissive (31°C) and non-permissive (38°C temperatures.

The kinetics of viral growth in Hela cells at permissive (31 and non-permissive (38°C) temperatures was determined for wild type adenovirus 5 and ts 18. For this, confluent monolayers of Hela cells were infected at an input multiplicity of 10 pfu/cell with each virus. Virus was allowed to adsorb for 90 minutes at 38°C and then monolayers were washed with Eagle's medium, treated with 1 ml. of 1/200 dilution of neutralising antiserum (neutralising titre 1:10,000) for 15 minutes to remove unadsorbed infectious virus, washed again and finally overlaid with Eagle's me supplemented with 2% calf serum. For infectious centre assay, cells were trypsinised and dispersed cells were plated with 2.5×10^6 freshly trypsinised Hela cells in Eagle's medium supplemented with 5% calf serum. After overnight incubation at 37°C, medium was withdrawn and monolayers were overlaid with Eagle's medium containing 0.65% agar and 2% calf serum. Plates were incubated for 7 days at 37°C for wild type and 13 days at 31°C for ts 18. The results of the infectious centre assay

showed that 85% of the cells were infected. In the one-step growth experiment, samples were harvested at various times after infection by scraping the cells off into 2 ml of tris buffer At 31°C,t samples were taken at intervals until 120 hours after infection and at 38°C damples were taken until 45 hours after infection All samples were frozen and thawed three times, then plaque assayed on Hela cell monolayers. The growth curves obtained with wild type adenovirus 5 and ts 18 at both 31°C and 38°C are given in Figures IIIb and IIIc respectively. There is no significant difference in the growth rates of wild type and ts 18 at 31°C. They both have a latent period of approximately 40 hours and the cell-associated virus titre starts to rise at about 44-48 hours and reaches a maximum level about 96 hours after infection. At 38°C, ts 18 fails to replicate while wild type shows a 14 hour latent period before increasing in titre, to reach a maximum by 28-30 hours after infection.

Complementation between ts 18 and ts 19 on CEF cells at non-permissive temperature for interferon induction.

Previous experiments established that ts 18 and ts 19

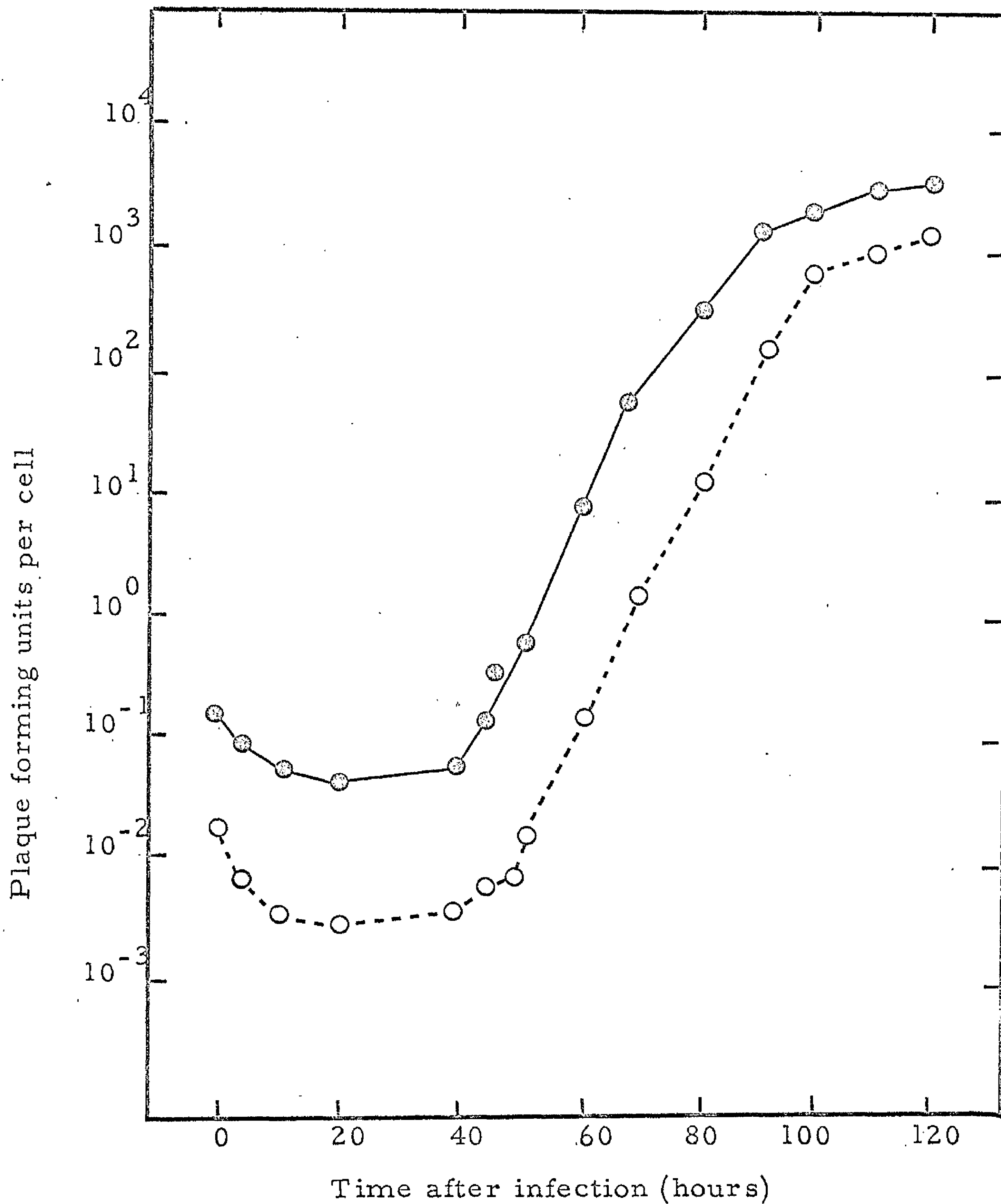


Figure IIIb. One-step growth curve of W. T. (●——●) and ts 18 (o- - -o) at 31°C on Hela cells.

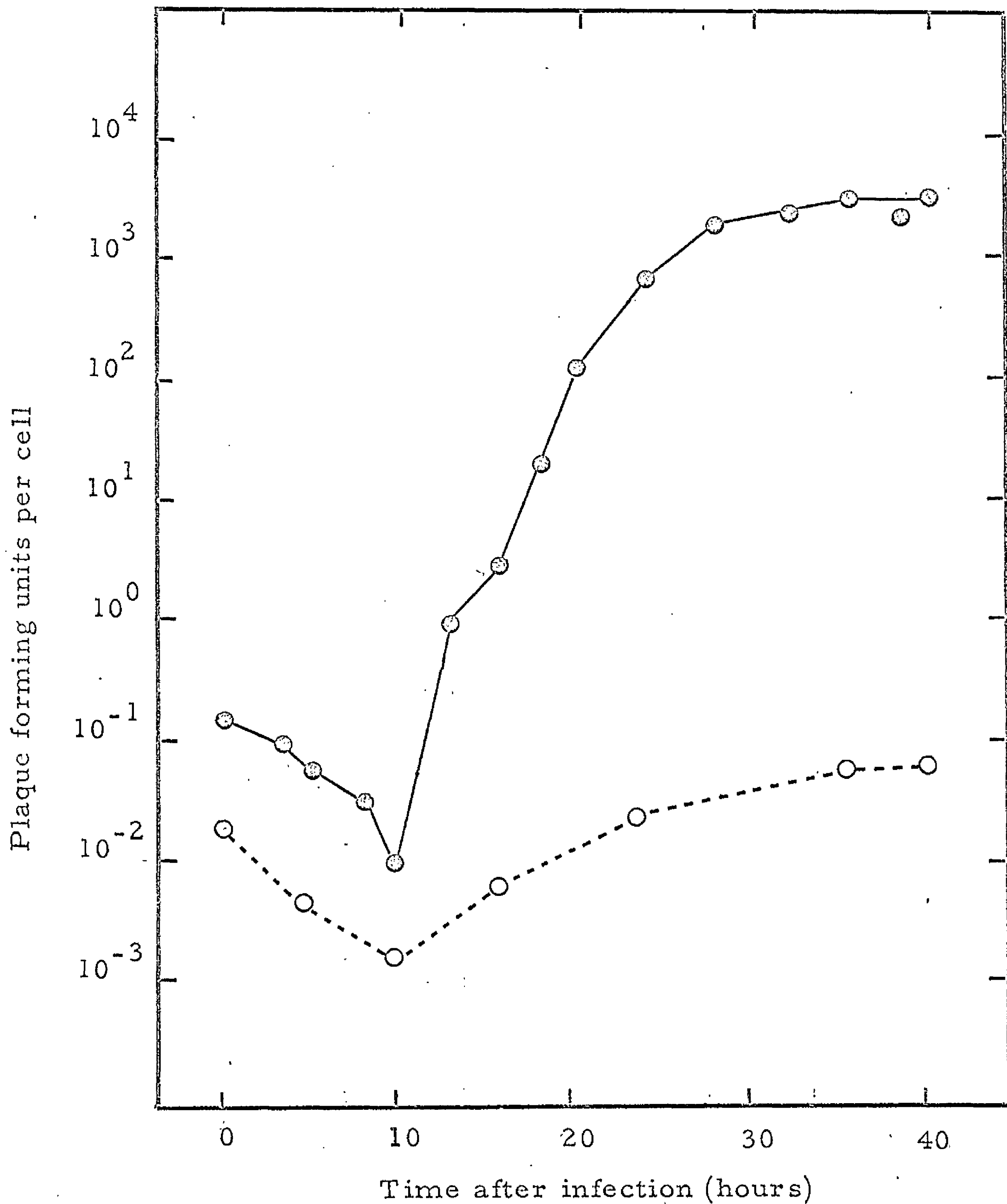


Figure IIIc. One-step growth curve of wild type (●——●) and *ts* 18 (○- - -○) at 38°C on Hela cells.

complement each other very well in mixed infection in Hela cells and the infective titre was increased about 2×10^3 fold (Williams and Ustacelebi, 1971); thus they were defective in separate functions. Experiments were carried out to determine if the defective functions of ts 18 and ts 19 complemented at 38°C on CEF cells and thus induced interferon after mixed infection with these viruses. In this experiment primary CEF cells were infected either with pairs of mutants each at an input multiplicity of 10 pfu/cell or with the single mutants or wild type at an input multiplicity of 20 pfu/cell and incubated either at 38°C for 3 days, or at 31°C for 4 days. The results of two representative experiments are shown in Table IIIf. Ts 18 and ts 19 fail to complement in CEF in that no interferon is induced at the non-permissive temperature, while they both produce normal amounts of interferon in cells singly infected with each mutant or doubly infected with both mutants at the permissive temperature. Therefore, while these two mutants complement each other in permissive Hela cells, they fail to do so in CEF cells.

Viral DNA synthesis by wild type adenovirus 5, ts 18 and ts 19 on Hela cells at the non-permissive temperature.

In order to characterise the DNA synthesising capacity of

Virus	m.o.i. (pfu/cell)	Exp. 1		Exp 2.	
		31°C	38°C	31°C	38°C
W.T.	20	32 *	32	64	128
5 ts 18	20	64	<4	64	<4
5 ts 19	20	16	<4	32	<4
5ts18x5ts19	10 x 10	64	<4	64	<4

* interferon titre, PDD_{50}/2ml.

Table IIIf. Complementation between ts 18 and ts 19 on CEF cells at non-permissive temperature for interferon induction.

wild type, ts 18 and ts 19 on permissive Hela cells, the following experiments were carried out. Confluent monolayers of Hela cells grown in 50 mm. plastic petri dishes were infected with wild type, ts 18 and ts 19 at an input multiplicity of 20 pfu/cell. Infected and mock-infected cultures were labelled between 16-24 hours post-infection by adding ^{3}H - thymidine (specific activity, 18.4 ci/mMol) to the medium at a final concentration of 2 μci/ml. At the end of the labelling period DNA was extracted and analysed by caesium chloride density gradient centrifugation using ^{32}P-labelled marker virus. The results of DNA analysis are shown in Figure IIId. Host DNA synthesis in cells infected with wild type, ts 18 and ts 19 was depressed greatly between 16-24 hours post infection (^{3}H - thymidine labelled cell DNA appeared at a density of 1.698). In each case a new peak of ^{3}H - thymidine labelled DNA coinciding with the ^{32}P-labelled marker virion DNA appeared (density of 1.714). These results indicate that ts 18 and ts 19 are able to synthesise viral DNA at non-permissive temperature to the same extent as wild type as shown also by Wilkie, Ustacelebi and Williams (1972).

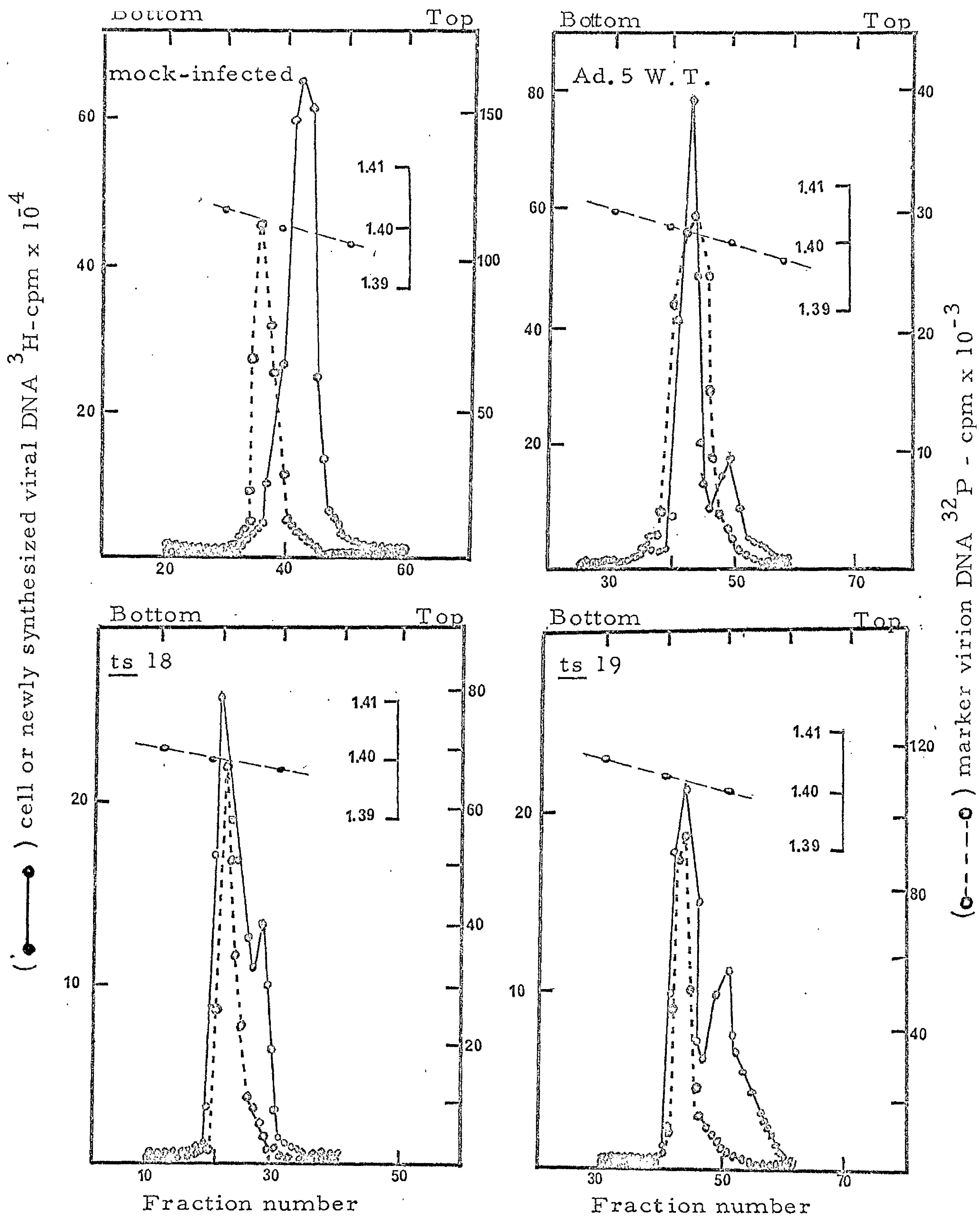


Figure IIId. Viral DNA synthesis by wild type adenovirus 5 ts 18 and ts 19 on Hela c
Caesium chloride buoyant density separation of DNA extracted from infected and non-infected Hela cells exposed to ^{3}H - thymidine from 16-24 hours after infectio
^{32}P - DNA extracted from adenovirus type 5 added as a marker.

<u>A search for viral DNA synthesis in CEF infected with adenovirus type 5, at 37°C.</u>

Human adenoviruses fail to undergo a complete replication cy in CEF, but it is possible that certain product(s) are synthesised in the infected cells, and that these might be responsible for inducing interferon. Therefore it was important to establish if viral DNA synthesis took place in CEF. Primary CEF cells were infected as described above for Hela cells. Cells infected with wild type adenovirus 5 were labelled with ^{3}H-thymidine (2 μci/ml) from 0-12, 12-24 and 0-24 hours post-infection and mock infected cells were labelled with ^{3}H-thymidine (2 μci/ml) for 24 hours. The DNA extraction and density gradient centrifugation for separation of viral and cellular DNA's were carried out as described in Methods. Replicate cultures were also set up for measurement of the interferon level. The results of an experiment are shown in Figure IIIe . It was found that cellular DNA synthesis is not depressed as much as in Hela cells after infection with wild type adenovirus 5 and there is no virus peak coinciding with the ^{32}P-labelled marker DNA; therefore und the conditions used in this experiment, viral DNA synthesis was

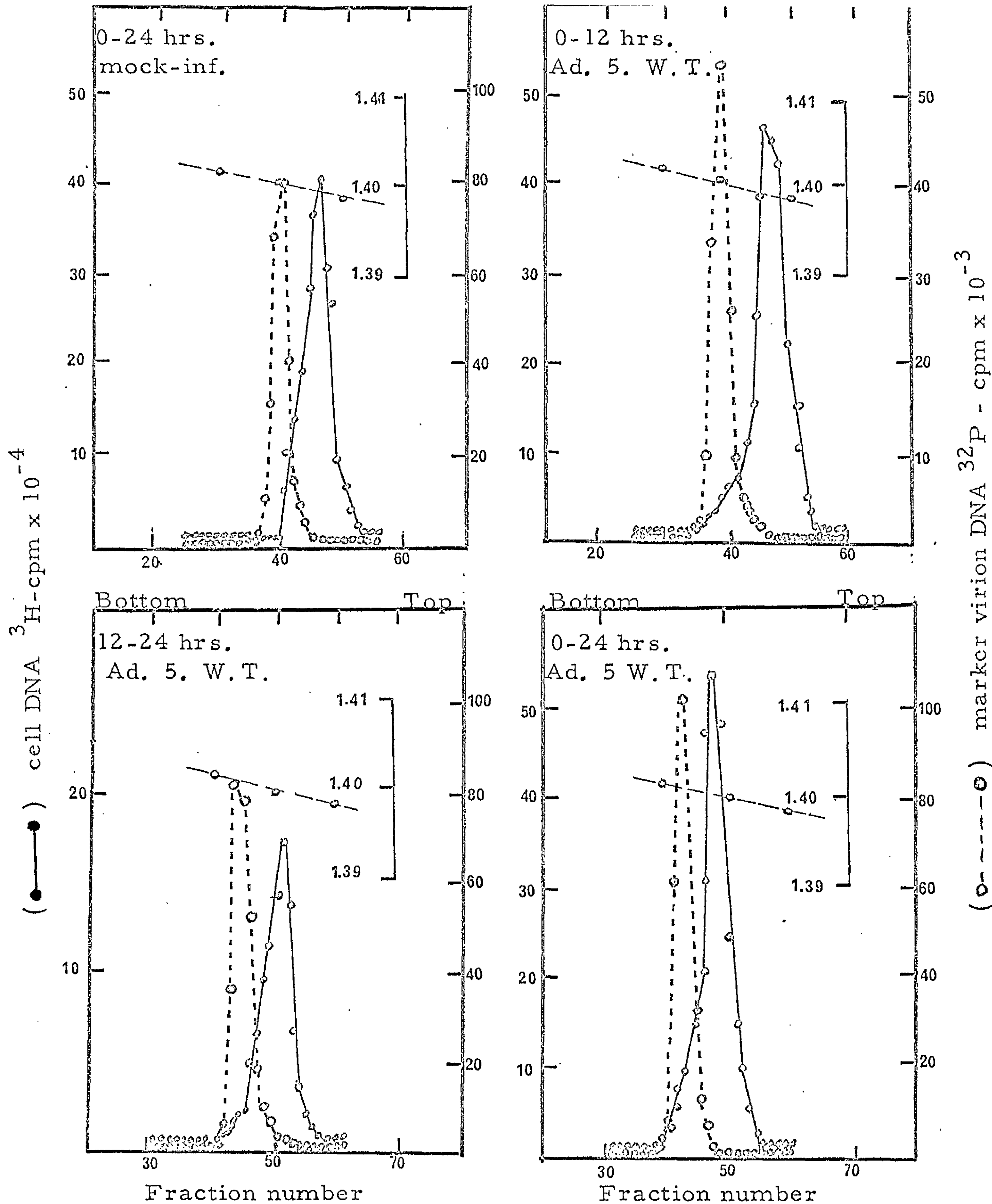


Figure IIIe. A search for viral DNA synthesis on CEF cells infected with wild type adenovirus 5. Caesium chloride buoyant density separation of DNA extracted f control cells exposed to ^{3}H - thymidine from 0-24 hours, and DNA extracted fr wild type adenovirus 5 infected cells exposed to ^{3}H-thymidine from 0-12, 12-24 and 0-24 hours. ^{32}P-DNA extracted from adenovirus 5 added as a marker.

not detected in CEF infected with wild type adenovirus 5. In this experiment, infected cells produced 128 PDD_{50}/2 ml interfer Despite the apparent lack of DNA synthesis, it can not be completely ruled out that a small but undetectable amount of viral DNA is synthesised in CEF or that viral DNA synthesis takes place later than 24 hours post-infection in CEF. If the latter is correct, then it is unlikely to be related to or important for interferon induction, because, interferon appears in the culture medium of adenovirus type 5 infected CEF cells by 12 hours post infection. Therefore it is likely that early virus functions are involved in the induction process.

Temperature shift-up experiment for ts 18 in Hela cells.

Having established the growth kinetics of wild type adenoviru 5 and *ts* 18 on Hela cells at permissive (31°C) and non-permissive (38°C) temperatures, temperature shift-up experiments were carried out in order to locate the time of synthesis or time of operation of the defective viral product of *ts* 18. Monolayers of Hela cells were infected either with wild type or with *ts* 18 as described for the one-step growth experiment. After adsorption the cells were overlaid with medium and incubated at 31°C. Fou plates were placed at 38°C immediately after adsorption for each

virus to measure the yield of wild type and ts 18 at the non-permissive temperature. A 0 hour sample was collected afte[r] adsorption and at different time intervals thereafter. At the times of shift-up, samples were harvested from two infected plates for each virus and two other plates were moved to 38°C for completion of the required incubation period. Samples were collected and shift-up was carried out at 0, 16, 40, 44, 48, 54, 64, 72 and 86 hours after infection. All samples were frozen and thawed three times and kept at -20°C till assayed. Figure IIIf shows the effect of temperature shift-up on wild type virus growth. It can been seen clearly that changes of temperatures at the different time intervals after infection havevery little effect on the final titres attained. However, there is a pronounced effect of temperature shift-up on ts 18 growth (Figure IIIg). Shifting the incubation temperature of ts 18 infected Hela cells up to 38°C immediately stops virus replication and no more increases in titre can be attained at 38°C. This effect occurs until at least 72 hours after infection and it seems likely that the defect expressed by this mutant is a late one. One possibility is that a defective viral gene product is synthesized

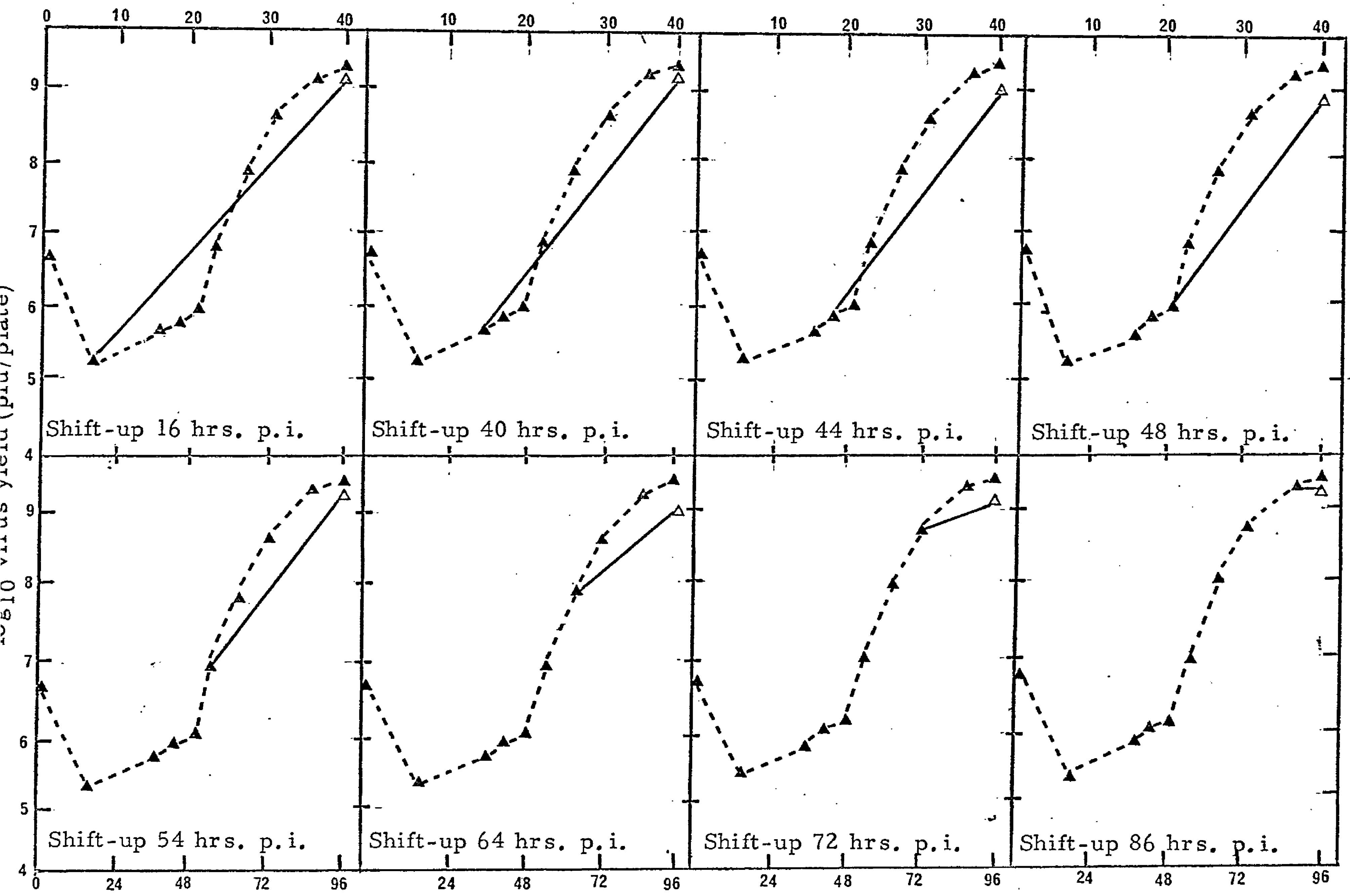


Figure IIIf. Growth of adenovirus type 5 (wild type) on Hela cells at 31°C and effect of temperature

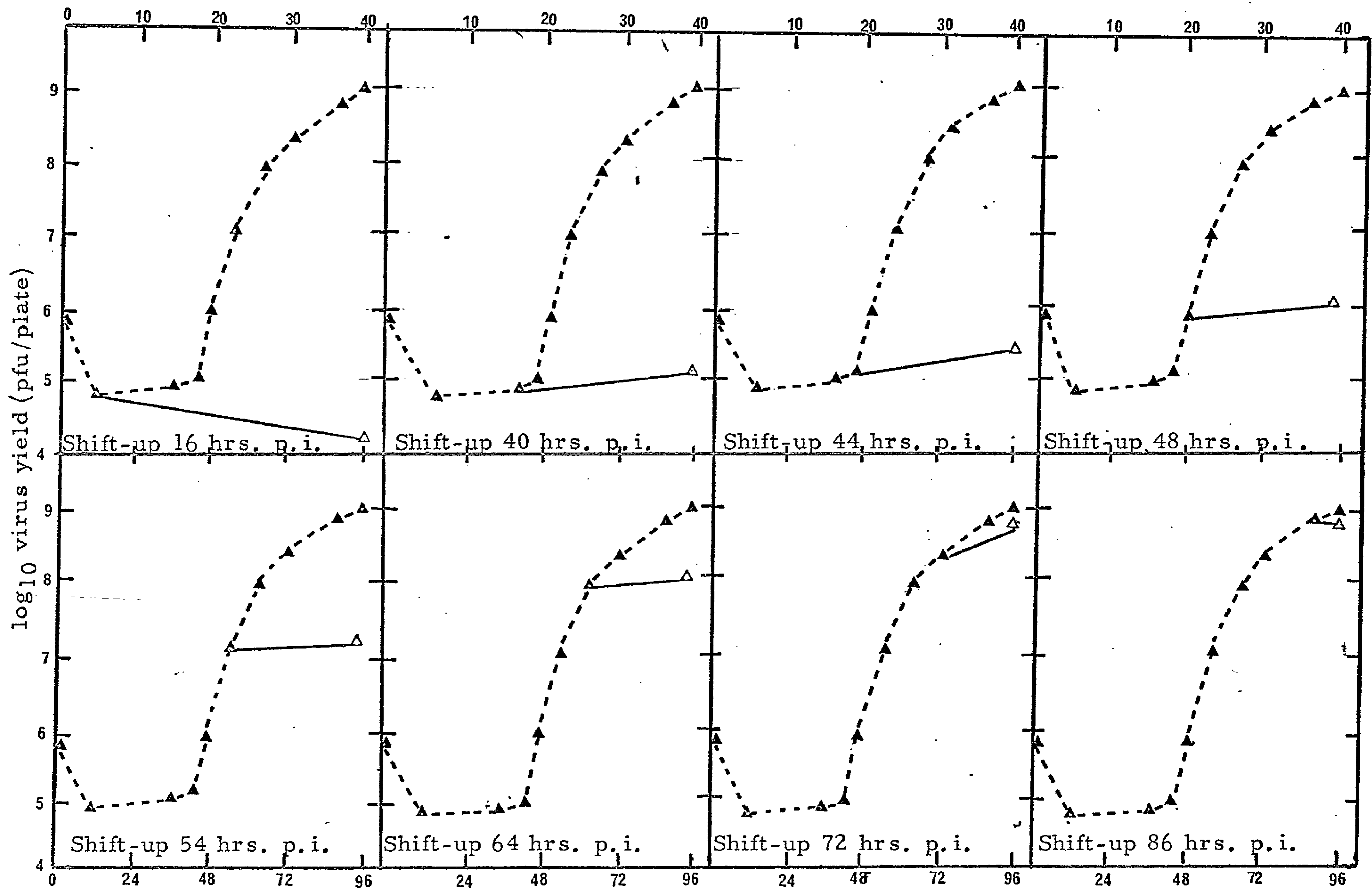


Figure IIIg. Growth of adenovirus 5 ts 18 on Hela cells at 31°C and the effect of temperature shift-up to 38°C on the final virus yield; (▲---▲) incubated at 31°C, (△——△) incubated at 38°C.

late in infection. Alternatively, the defect is in a product made earlier but required to act throughout the period of viral replication.

Effect of temperature shift-up on the interferon induction by ts 18 on CEF.

As shown previously, wild type adenovirus 5 is able to induce good yields of interferon at both 31° and 38°C, while ts 18 and ts 19 only induce the response at 31°C. On CEF cells infected with wild type, the first interferon can be detected in the medium 12-15 hours after infection at 37°C. This suggests that the first 12 hours after infection is a critical period for the inducer to work as a stimulus for the interferon response and subsequently synthesis of interferon messenger-RNA. At 31°C interferon is first detected in the medium and reaches the maximu level much later than at 38°C (Figure IIIa), and it is reasonable to suppose that the induction process is much slower at the lower temperature. In order to identifyythe temperature-sensitive period for interferon induction after infection of CEF cells with ts 18, temperature shift-up experiments were carried out. CEF cells were infected as described above for interferon induction an

immediately after addition of maintenance medium 4 plates were moved to 38°C and incubated for 3 days. This is the 0 hour sample. All other infected plates were incubated at 31°C and at various times two plates were shifted-up to 38°C and medium from two other plates was harvested to determine the interferon titre at the time of shift-up. After shift-up, infected cultures were incubated for a total of 3 days, then culture fluids were collected and interferon levels measured. At the same time shift-up experiments were carried out using wild type adenovirus 5. The results of two experiments are shown in Figure IIIh . In Experiment I , the infected plates were first shifted-up to 38°C at 4 hours after infection and no interferon was induced. At 5 hours shift-up there is a low level of interferon detectable and by 6-7 hours the interferon levels are normal after shift-up. In Experiment II a similar result was found. If the infected plates were shifted-up to 38°C in the first 5 hours after infection no interferon formed, but later shifts starting from 6 hours result in the production of interferon. Cultures infected with wild type were also carried out in parallel in these experiments as a control and in each case high yields

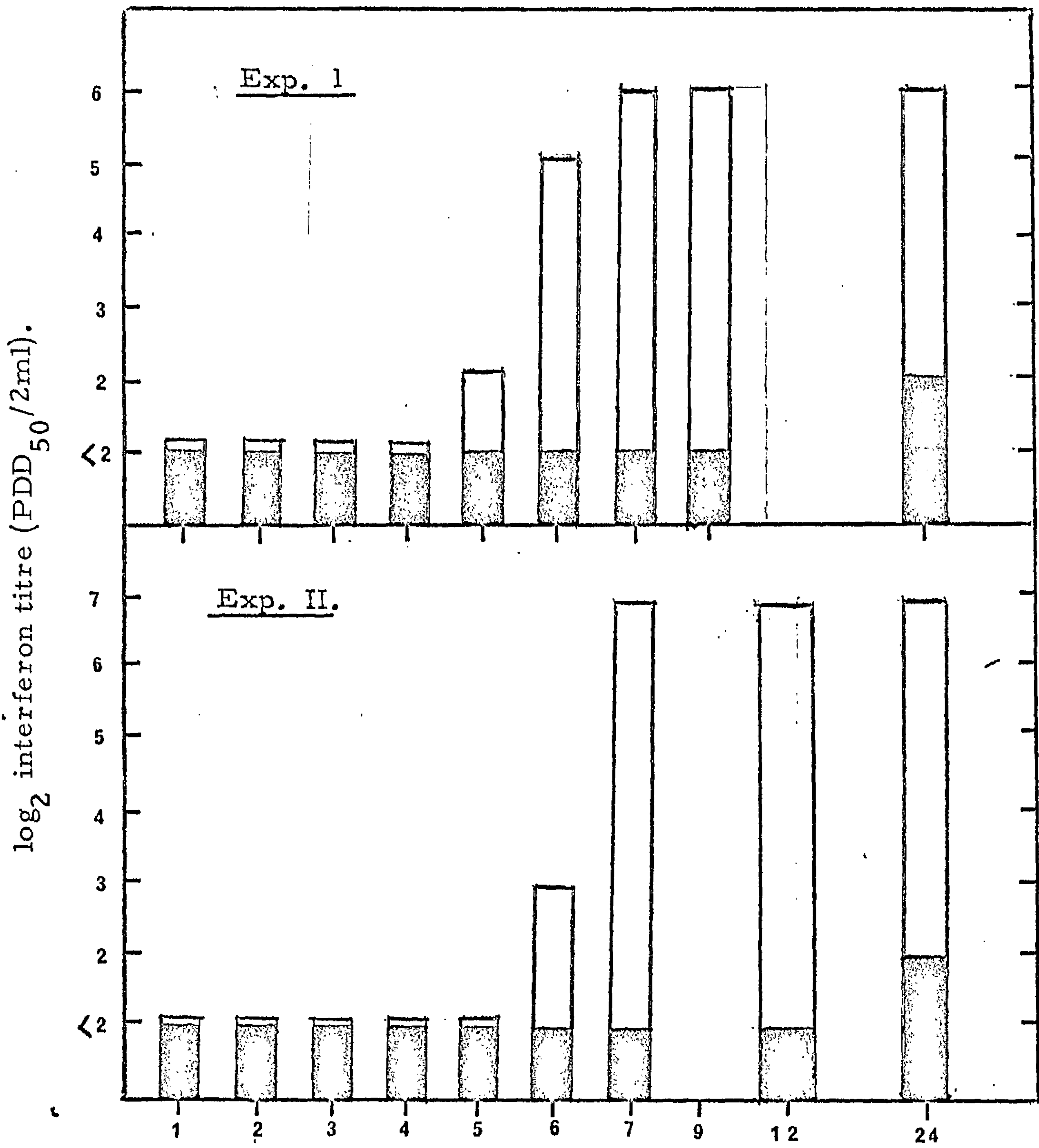


Figure IIIh. Effect of temperature shift-up (from 31°C to 38°C) on the final yield of interferon at 3 days after infection of primary chick embryo cells infected with ts 18. full blocks; interferon titres at the time of shift-up; empty blocks; final interferon titre after incubation at 38°C.

of interferon were produced, showing that induction by wild type virus is unaffected by temperature shift-up.

Virion heat sensitivity of wild type adenovirus 5, ts 18 and ts 19.

Adenoviruses are relatively heat stable at room temperature, but on heating purified virions at 56°C for only a few minutes, virus infectivity is completely destroyed (Russell et al. 1967). Since *ts* 18 and *ts* 19 failed to induce interferon at high temperature (38°C) but not at low temperature (31°C) it seemed possible that these mutants are inactivated more rapidly at the higher temperature as a result of their defect, or alternatively the virus remains infectious, but the inducer site (may be a structural component of the virion) is configurationally inactive so that it no longer induces interferon. It is generally accepted (Fenner, 1969) that if the defective protein of a mutant is a structural one, the virion is usually more heat labile than the corresponding wild type virus. For these reasons the heat sensitivities of wild type, *ts* 18 and *ts* 19 were tested *in vitro* at 31°C, 38°C, 50°C, and 52°C. Experiments were carried out as follows. Wild type, *ts* 18 and *ts* 19 virus stocks were diluted 10 times in 0.05 Mol tris-HCl buffer, pH 7.5 for all

inactivation experiments. Virus suspensions were then incubated at 31°C or at 38°C and at different times during incubation 0.5 ml samples were removed and frozen at -20°C until assayed for infectivity. Samples treated thus, were also tested for their interferon inducing capacities on CEF cells. With both wild type and ts mutants there was no significant drop in infectivity by incubation at either 31°C or 38°C for as long as 24 hours (Table IIIg). In addition after 24 hours incubation at 31°C and 38°C, wild type and both ts 18 and ts 19 were able to induce interferon on CEF at 31°C. Therefore it is unlikely that the interferon inducing capacity of these mutants is destroyed or inactivated by incubation at 38°C, up to 24 hours. Since the first 12-15 hours period after infection is important for interferon induction, therefore lack of interferon inducing capacity can not beedue to in vitro inactivation of these mutants at 38°C.

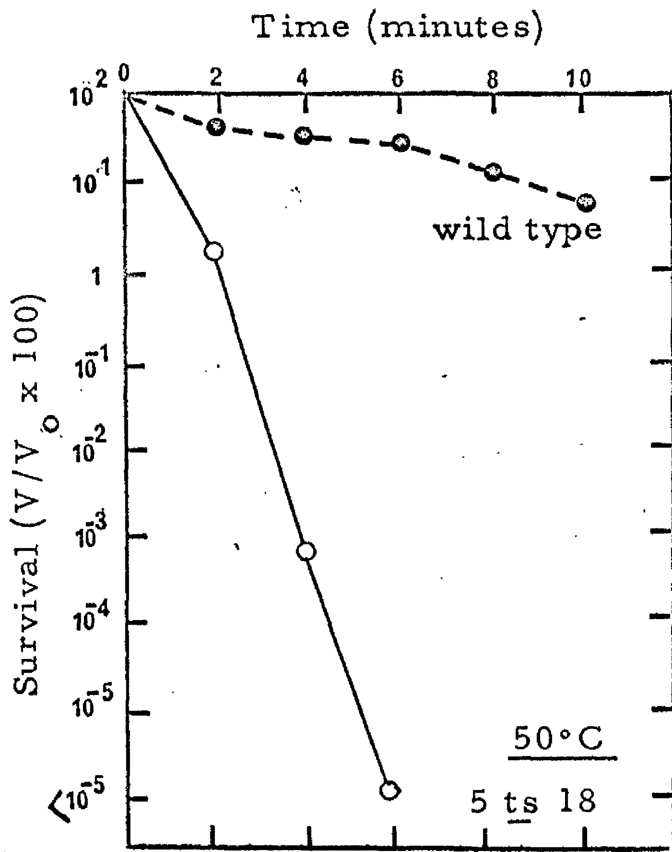
Heat inactivation of wild type adenovirus 5 is much more rapid at 50°C and 52°C, and a comparison was made between wild type and ts 18 and ts 19. The heat inactivation tests were carried out in the following way. Universal bottles containing

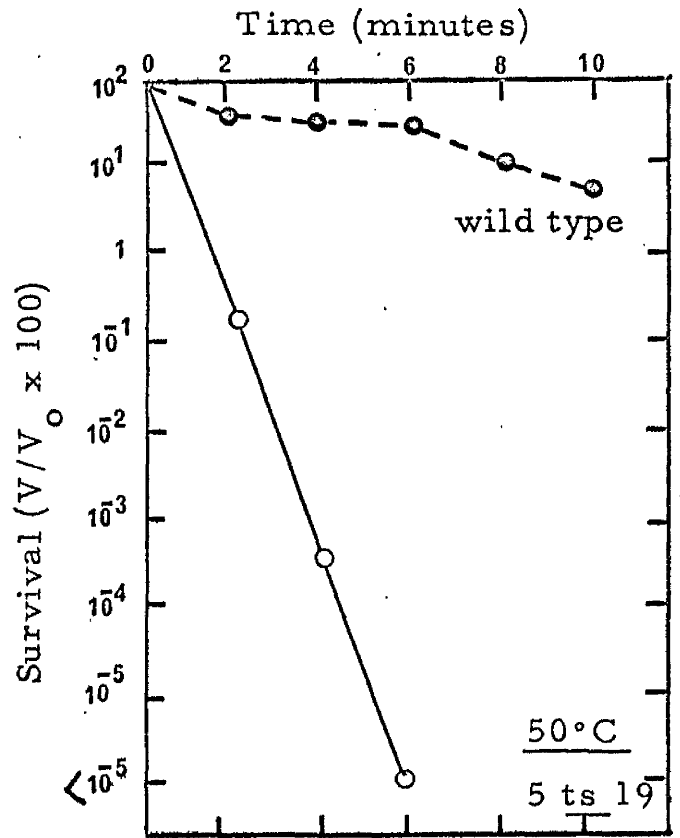
TABLE IV

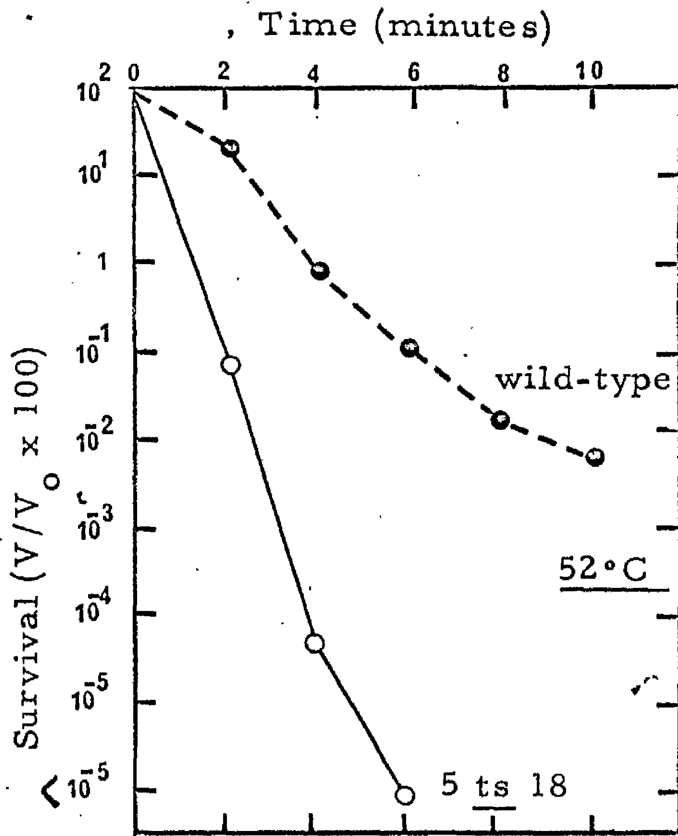
VIRUS	INCUBATION TEMPERATURE	TIME OF INCUBATION	INFECTIVITY TITRE PFU/ml. ($\log_{10}$)	Interferon titre; PDD_{50}/2ml.
5 Wild type	31°	24 hrs	8.74	128
	38°	0	8.87	
		30 min	8.76	
		60 min	8.67	
		120 min	8.69	
		24 hrs	8.65	128
5 ts 18	31°	24 hrs	8.05	64
	38°C	0	8.51	
		30 min	8.40	
		60 min	8.28	
		120 min	8.33	
		24 hrs	8.20	64
5 ts 19	31°C	24 hrs	8.18	32
	38°C	0	8.20	
		30 min	8.05	
		60 min	8.14	
		120 min	8.14	
		24 hrs	8.23	32

Table IIIg. Effect of incubation of wild-type adenovirus 5, ts 18 and ts 19 at 31°C and 38°C on their infectivity and interferon inducing capacity.

0.05 Mol tris HCl buffer, pH 7.5 were preheated in a water bath at 50° ∓ 0.5° or 52° $\pm$ 0.5° prior to adding $^1/_{10}$ volume of virus. During heating 0.5 ml samples of virus suspension were collected every 2 minutes for a total of 10 minutes and transferred immediately into a bottle in an ice-bath, then assayed for infectivity. The inactivation kinetics of wild type, ts 18 and ts 19 are shown in Figure IIIh . As shown, the wild type virus is inactivated approximately 4 logs. by heating at 52°C for 10 minutes while the infectivity of ts 18 decreased by 6 logs in 4 minutes and more than 7 logs in 6 minutes and ts 19 by 5 logs in 2 minutes and more than 7 logs in 4 minutes. The heat inactivation of wild type is much slower at 50°C, the titre of virus being reduced only slightly more than 1 log at this temperature after 10 minutes of heating. On the contrary the infectivity of both ts 19 and ts 19 decreased by more than 7 logs after 6 minutes of heating at this temperature. Therefore both ts 18 and ts 19 are much more heat sensitive than wild type virus suggesting that the temperature-sensitive defects in the mutants may be located in structural components of the virion. Nevertheless, it still remains to be determined if these two







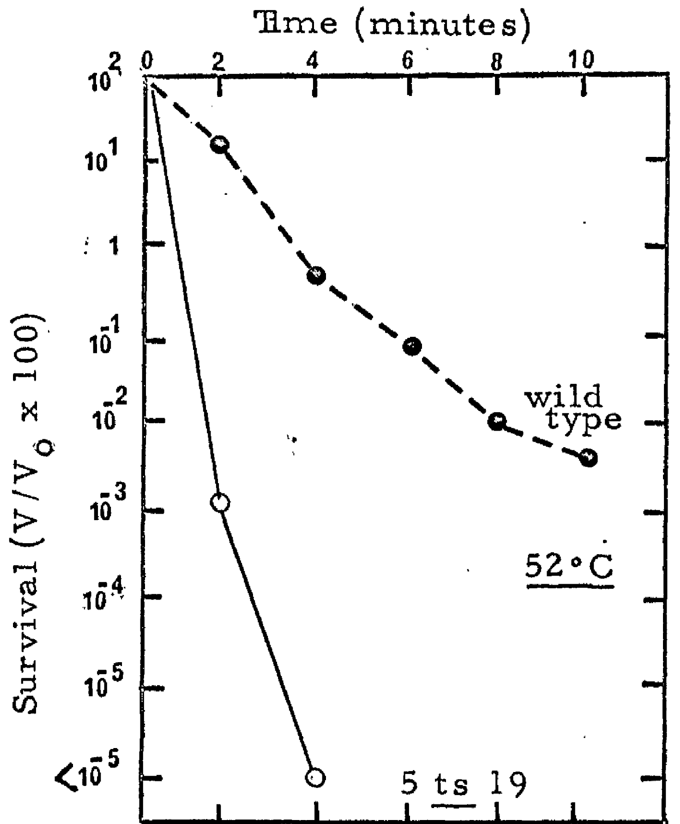


Figure IIIh. Heat inactivation of wild type adenovirus 5, ts 18, ts 19 at 50°C and 52°C.

phenotypes (temperature-sensitivity and heat sensitivity) are due to one mutation.

DISCUSSION

Adenovirus type 5 ts mutants have been isolated from stocks mutagenised by hydroxylamine. Treatment with 1 Mol hydroxylamine reduced the infectivity of adenovirus type 5 approximately 4.5 logs in 5 hours and after such inactivation the surviving fraction contained a frequency of 7.4% ts mutants. This value seems to be slightly higher than the frequency of ts mutants previously obtained from nitrous acid treated stocks (J.F. Williams, personal communication). These plaques were independently isolated from stocks mutagenised *in vitro*, therefore they cannot be clonally related. In fact 12 of these mutants have been classified into 9 of 14 complementation groups (Williams and Ustacelebi, 1971; Russell, Newman and Williams, 1972). Most of the mutants isolated showed very little leakiness, and had low reversion frequencies, and for this reason they were suitable for complementation and recombination experiments some of which have already been reported (Williams and Ustacelebi, 1971; Wilkie, Ustacelebi and Williams, 1972; Russell, Newman and Williams, 1972). As mentioned, there are 14 complementation groups (Russell

Newman and Williams, 1972), so that presumably there are still at least another 10 complementation groups remaining to be identified.

In the present investigation twenty-five temperature-sensitive mutants of adenovirus type 5 were tested for their ability to induce interferon on CEF cells at both permissive (31°C) and non-permissive (38°C) temperatures. Two mutants ts 18 and ts 19 were found to be defective in their interferon inducing capacity at the non-permissive temperature while inducing good interferon yields at the permissive temperature. In order to identify the function (s) of adenovirus type 5 which is responsible for interferon induction on CEF cells, these two mut were further studied. Ts 18 and ts 19, complement each other a the restrictive temperature on Hela cells after mixed infect ion (Williams and Ustacelebi, 1971) and are almost certainly defective in genes controlling different viral functions. However it cannot be completely ruled out that both mutations affect the same viral function and are located at different sites in the same gene, and we are observing intragenic rather than intergen complementation. The recombination frequency of ts 18 and ts is approximately 1% but such a frequency is obtained between

ts mutants of adenovirus type 5 which are in the same complementation group (C. S. H. Young and J. F. Williams, personal communication). Nevertheless, the complementation between these two mutants is very efficient and the increase over the single infection yield was increased 2 x 10^3 times at the restrictive temperature after mixed infection (Williams and Ustacelebi, 1971). Thus, it is most likely that they are mutant in separate genes.

On Hela cells, wild type adenovirus, ts 18 and ts 19 have be shown to replicate normally at the permissive temperature. Howev the mutants completely failed to grow at the restrictive temperature while wild type grew well. Subsequently, experiments were carrie out to measure the DNA synthesizing capacity of ts 18 and ts 19 at the restrictive temperature and the results indicated that they bot undergo DNA synthesis at the restrictive temperature to the same extent as wild type adenovirus 5. In connection with these findings it has been shown that both ts 18 and ts 19 are able to synthesize all the major structural antigens at the restrictive temperature (Russell, Newman and Williams, 1972). In addition, ts 18 is apparently partially assembled in the nucleus of infected Hela cells (E. A. C. Follett, personal communication), despite the fact that neither particles nor infectious virus can be detected in extracts

of cells (J. F. Williams, personal communication). These results suggest that the defective function (s) of mutant ts 18 and ts 19 is located at a late stage of the virus replication cycle and may affect the maturation of virus. These results also agree with the temperature shift-up experiments described in this chapter for ts 18 on Hela cells. Throughout the exponential growth phase at 31°C (44 to 72 hours after infection), if the temperature is shifted-up to 38°C no further increase in infectivity is attained. This makes it likely that either an essential viral polypeptide is continuously being synthesized during this period, or that a polypeptide made earlier is being required to function continuously during this growth period. This polypeptide could be either a maturation factor being incorporated into the virion or an enzyme or some other factor necessary for virus assembly.

It is generally accepted that if the defective protein of a ts mutant is a structural component of the virion, then virus produced at the permissive temperature may be considerably less heat stable *in vitro* than wild type virus (Fenner, 1969). Heat inactivation experiments with wild type adenovirus 5, ts 18 and ts 19 showed clearly that the mutants are much less heat stable than wild type. Therefore, this result suggested that possibly

the defects in ts 18 and ts 19 are located in structural cmmponents of the virion. However, from the experiments described in this cha it is not possible to determine whether or not these two phenotypes (temperature-sensitivity and heat sensitivity) are due to only one mutation. It is most likely that one mutation may cause both phenotypic changes, but it cannot be ruled out that these mutants car two or more mutations. One is the temperature sensitive mutation which allows virus to grow adequately at permissive temperature, b not at the restrictive temperature. The second mutation does not affect virus growth at either temperature, but it results in the virus being much more heat labile at higher temperatures (50°C or 52°C). These two possibilities can be distinguished by examining the heat sensitivity of wild type revertants. If the temperature-sensitivity (ts) and heat-sensitivity (hs) revert together to ts^+, hs^+ then the two phenotypes are caused by one mutation. If they revert independently, it suggests, but by no means proves, that it is a double mutation. Furthermore, the ts^+ revertants may also be checked for interferon inducing capacity. There is another possibility which has to be considered and that is that these two phenotypic characteristics are due to two separate ts mutations both of which affect growth on Hela but only one of which affects

interferon induction. However this possibility seems to be unlikely since ts 18 and ts 19 both complement each other, and complement the other known complementation groups (Williams and Ustacelebi, 1971; Wilkie, Ustacelebi and Williams, 1972). In addition, the recombination frequency of these mutants, with some others are reasonably high. Therefore, these mutants are unlikely to have double mutation. However, there is still a possibility that ts 18 and ts 19 could have double ts mutations, only one of which affects virus replication in Hela, the other does not, but it does affect interferon induction on CEF cells. Therefore, the second ts mutation would notnnecessarily show up in complementation and recombination analysis on Hela cells. The genetical analysis of ts 18 is now being carried out by Dr. C. S. H. Young and these studies will eventually distinguish the relationship of temperature sensitivity, heat sensitivity and interferon inducing capacity of ts 18.

Ts 18 and ts 19, both of which are defective in interferon inducing capacity, fail to complement in mixed infection of CEF cells at the non-permissive temperature despite the fact that they complement very well in Hela cells (Williams and Ustacelebi, 1971). The reason for this failure is not known at

present. However a number of possibilities for this failure can be put forward. It is perhaps that the mutations affect genes specifying different structural polypeptides both of which are necessary for interferon induction. Therefore the viral functions required for interferon induction will not be expressed after mixed infection. Alternatively, mutants may be defective in penetration or uncoating in CEF cells. If virus is not uncoated, transcription of certain early functions would not take place, therefore the mutants would fail to complement in the induction of interferon on CEF cells.

The experiments presented in this chapter established that the *ts* defect is not expressed at the adsorption step. *Ts* 18 and *ts* 19, adsorbed at both permissive and restricted temperatures,are sti able to induce interferon when incubated subsequently at the permissive temperature but not at the restrictive temperature. However, it cannot be ruled out that the mutants fail to penetrate or uncoat at the restrictive temperature. It is known that wild type adenovirus 5 is able to uncoat on CEF cells (B. Senior, personal communication) but it remains to be tested if ts 18 and ts 19 do so at the restrictive temperature. Of course

it should be pointed out that it is not known whether virus uncoating is essential in CEF cells in order to induce interferon response. It may be that viral DNA or some internal components ar directly required for interferon induction. Alternatively, the viral DNA may be the template for the transcription of viral specific double-stranded RNA which in turn is responsible for interferon induction. Previous reports suggested that no viral specific double-stranded RNA is detectable in CEF cells infected with adenovirus type 12 (Bakay and Burke, 1972) and the total double-stranded RNA level is unaltered in adenovirus 7 infected CEF cells (Markovits and Coppey, 1972). However, it has been found that adenovirus type 5 infection stimulates the production of total double-stranded RNA in CEF cells, although it remains to be shown if this double-stranded RNA is virus specific. (B. Senior, and P. Austin, personal communication).

The experiments described in this chapter also indicate that early viral functions are possibly involved in interferon induction on CEF cells. No adenovirus DNA synthesis could be detected in the first 24 hours after infection with adenovirus type 5 on CEF cells. Therefore it is unlikely that viral DNA synthesis is involved in the

induction of interferon. In relation to this finding, *ts* mutants defective in their DNA synthesising function on Hela cells, *ts* 36 and *ts* 37 (Wilkie, Ustacelebi and Williams, 1972) are both able to induce good interferon yields on CEF cells at permissive and restrictive temperatures. In addition, *ts* 18 and *ts* 19 are both able to synthesise viral DNA at the restrictive temperature in Hela cells, although both fail to induce interferon response at the restrictive temperature on CEF cells. Furthermore, it has been clearly shown that interferon is induced on CEF cells infected with adenovirus types 12 and type 7 in the presence of the DNA inhibitor, cytosine arabinoside (Bakay and Burke, 1972; Markovits and Coppey, 1972). These results are all in agreement with the view that viral DNA synthesis is not required for interferon inductinn by human adenoviruses on CEF cells.

Temperature shift up experiments carried out with *ts* 18 on CEF cells clearly showed that the defective function for interferon induction is expressed around 6-7 hours after infection at the permissive temperature. Of course, it cannot be completely ruled out that this defect might affect interferon induction indirectly. For example, if the penetration or uncoating of *ts* 18 is defective in CEF cells, the virus would fail to undergo subsequent viral

events which might be necessary for interferon induction.

It is unlikely that an inhibitor produced by ts 18 on CEF cells at the restrictive temperature is responsible for the lack of interferon inducing capacity, as cells mixedly infected with ts 18 and wild type produce normal amounts of interferon.

The evidence presented in this chapter does not rule out the possibility that the penton antigen of adenovirus is responsible for induction of interferon. The data are still consistent with the possibility that a viral structural component might be repomsible for or involved in interferon induction. Alternatively a structural component(s) might be involved indirectly by interfering with penetration or uncoating of the adenovirus so that subsequent functions are blocked. Since viral DNA synthesis is not requirdd for interferon induction however, it is most likely that viral functions expressed prior to viral DNA synthesis, are responsible for interferon induction on CEF cells. The further biochemical and genetical analysis and the identification of the specific defective function(s) of ts 18 and ts 1 will possibly reveal the adenovirus function(s) needed for interferon induction.

CHAPTER IV

DEPRESSION OF INTERFERON SYNTHESIS IN CEF CELLS BY RIFAMPICIN.

1. Introduction.
2. Materials and Methods.
3. Results.
   - a. Effect of rifampicin on adenovirus induced interferon synthesis.
   - b. Effect of rifampicin on Semliki Forest virus induced interferon and virus replication.
   - c. Effect of rifampicin on polyoma virus induced interferon synthesis.
   - d. Lack of effect of rifampicin on the action of interferon.
   - e. Inhibition of CEF cell growth by rifampicin.
   - f. Effect of rifampicin on macromolecular synthesis by CEF cells.
4. Discussion.

INTRODUCTION

The antibiotic rifampicin is a derivative of rifamycin, produced by fermentation of Streptomyces mediterranei (Sensi et al. 1960). The antibiotic blocks bacterial growth by inhibiting bacterial DNA-dependent RNA polymerase activity at the RNA chain initiation step (Wehrli et al. 1968; Sippel and Hartmann, 1968). Rifampicin and other derivatives have also been reported to inhibit the growth of certain mammalian DNA viruses, notably poxviruses, and certain adenoviruses (Heller et al. 1969; Subak-Sharpe, Timbury and Williams, 1969; Subak-Sharpe et al. 1970). In addition, rifampicin has an inhibitory effect on transformation by Rous sarcoma virus (Diggelmann and Weissmann, 1969; Robinson and Robinson, 1971). It has also been shown that growth of polyoma virus in permissive mouse cells is not inhibited but the transformation frequency on BHK21/C13 cel is greatly reduced (Williams et al. 1971). In the case of poxviruses still no definitive answer has emerged as to how rifampicin acts to inhibi virus replication. In vaccinia infected cells the antibiotic seems to have no effect on either early or late messenger-RNA synthesis or viral DNA synthesis (McAuslan, 1969; Moss, Katz and Rosenblum, 1969; Moss et al. 1969). It is well known that rifampicin binds to bacterial polymerase molecules, but it has been shown that the antibiotic does not inhibit the RNA polymerase activity of purified virus particles

(Moss, Katz and Rosenblum, 1969; McAuslan, 1969). The assembly of vaccinia virus is reversibly blocked (Moss et al. 1969). Since it is possible to obtain drug resistant mutants of vaccinia (Subak-Sharpe, Timbury and Williams, 1969), it is likely that the drug effectsthe virus-coded product of a single gene.

Except at very high doses, rifampicin has no effect on the activity of RNA polymerase from nuclei of mammalian cells (Wehrli et al. 1968; Jacob, Sajdel and Munro, 1968). There is evidence that rifampicin inhibits mitochondrial RNA synthesis in rat liver (Gadaleta Greco and Saccone, 1970; Shmerling, 1969) and bovine heart (Gamble and McCluer, 1970). However, there are conflicting reports concerning the effect of rifampicin on the growth of mammalian cells. Antiviral doses of the drug were reported to have no effect on the growth of normal mouse and chick embryo cells (Heller et al.; 1969; Heller, 1970; Vaheri and Hanafusa, 1971), but did inhibit the growth of Rous sarcoma virus transformed CEF cells (Vaheri and Hanafusa, 1971). Another report states that growth of both normal and Rous sarcoma virus transformed CEF cells is inhibited (Robinson and Robinson, 1971). In addition to the inhibitory effect of rifampicin outlined above, it has been shown that both the humoral and

the cellular immunological response *in vivo* and *in vitro* are depressed (Paunescu, 1970).

Certain adenoviruses are inhibited by rifampicin. Rifampicin at 100 µg/ml depressed the replication of adenovirus types 1, 2 and 5 in Hela cells (Subak-Sharpe et al. 1970), but the growth of adenovirus type 12 in Hela cells was apparently not affected by the drug (Ustacelebi and Williams, 1972). The reason for this is not known, but this differential inhibition of adenovirus growth in Hela cells suggested that these viruses might also exhibit exploitable differences with respect to interferon induction on CEF cells despite the fact that adenoviruses fail to replicate on CEF cells (Beladi and Pusztai, 1967; Ho and Kohler, 1967; Ustacelebi and Williams, 1972).

Experiments described in this chapter were carridd out in order to test the effect of rifampicin on interferon induction by adenovirus types 1, 5 and 12, and the RNA containing Semliki Forest virus. Another small DNA virus, polyoma, whose replication is not affected by rifampicin on mouse embryo cells, also induces interferon and fails to replicate on CEF cells (Chapter V). Experiments will also be described on the effect of rifampicin on interferon induction by polyoma virus onCEF cells. In addition, the effect of rifampicin on CEF cell growth and macromolecular synthesis was determined.

MATERIALS AND METHODS.

Cell cultures. CEF cells and BHK 21/C13 cells were prepared as described in Chapter II .

Viruses. Growth and assay of adenovirus types 1, 5 and 12 was describe in Chapter II .

Semliki Forest virus. This was obtained from Dr. G. Appleyard (M. R. E., Porton) and grown on BHK 21/C13 cells by infecting fresh monolayers of cells in rotating Winchester bottles. Infected cells usually showed cytopathic effect by 24-48 hours after infection, at which they were harvested, pelleted by centrifugation, resuspended in tris buffer, frozen and thawed three times, and centrifuged again at 3000 rpm for 15 minutes. The supernatant was collected and kept feozen at -20°C. Semliki Forest virus infectivity was measured by plaque formation on BHK 21/C13 and CEF. Virus dilutions were prepared in Eagle's medium and fresh confluent monolayers of cells on 50 mm. plast petri dishes wereinfected with 0.2 ml of virus dilution. Virus was adsorbed at 37°C for 1 hour, then plates were overlayed with Eagle's medium supplemented with 0.65% Noble agar and 2% calf serum. Monolayers were stained with neutral red 24-36 hours after infection and plaques were counted at 48 hours.

Semliki virus was purified by the method described by Cheng (1961) with minor modifications. The virus suspension was spun down at 3000 g for 30 minutes, and the supernatant maintained overnight with 5 mg/ml protamine sulphate. The suspension was then spun at 3000 g for 15 minutes and the sediment discarded. The protamine sulphate supernatant was then centrifuged for 60 minutes at 120,000 g (40,000 rpm) and the pellet resuspended in 1 ml 0.75% BSA in 0.1N NH_4Ac. This was the partially purified virus preparation.

Growth and infectivity assay of polyoma virus. This was described in Chapter IV .

Interferon production. Induction of interferon by adenoviruses was described in Chapter II . With Semliki Forest virus, interferon was induced at two different temperatures (37°C and 42°C) in CEF cells (Skehel and Burke, 1968). For induction of interferon at 37°C, primary CEF cells were infected at a multiplicity of infection of 0.5 to 1.0 pfu/cell. Virus was adsorbed at 37°C for 1 hour, then monolayers were washed with Eagle's medium, overlaid with Eagle's medium supplemented with 2% calf serum and incubated at 37°C for 18-24 hours. For induction of interferon at 42°C, primary CEF cells were infected at a multiplicity of infection around 80 pfu/cell. Virus was adsorbed at 37°C for 1 hour. Monolayers were then washed with Eagle's medium,

overlaid with Eagle's medium supplemented with 2% calf serum and incubated at 42°C for 18-24 hours. After incubation, the medium was collected, centrifuged at 100,000g for 1 hour and heated at 56°C for 30 minutes before assay on CEF cells.

Interferon assays. Adenovirus and Semliki Forest virus induced interferons were assayed as described in Chapter II .

Measurement of cell DNA, RNA and protein synthesis on rifampicin treated and non-treated CEF cells. The incorporation of ^{3}H-thymidine, ^{3}H uridine and ^{3}H-leucine into DNA, RNA and protein respectively was measured as follows; Primary CEF cells (5×10^{6} cells) were seeded in growth medium on 50 mm dishes containing 3 glass coverslips (13mm in diam.). After 24 hours the cells were treated with either 100 or 50 μg/ml rifampicin and radioactive precursors were added (10 μc/ml) for 1 hour intervals at various times from 0 to 72 hours after treatment. After each one hour period, the coverslip cultures were washed twice in phosphate buffered saline, fixed with formal saline for 20 minutes at room temperature, extracted with ice-cold 5% trichloroacetic acid for 10 minutes, washed twice with cold distilled water and dried in ethanol. The scintillant consisted of 2½ litres of toluene, 125 gr. of 2.5 Diphenyloxazolyl (PPO) and 0.75 gr. of 1,4-di 2-(5-Phenyloxazolyl) benzene (POPOP). (PPO and POPOP were both

obtained from Koch-Light Lab. Ltd.). Radioactivity was determined using an Intertechnique liquid scintillation counter.

Rifampicin. This was obtained from Lepetit Pharmaceutical Ltd., (Slough) and was prepared for use as s stock solution of 1 mg/ml in distilled water. It was stored frozen (-20°C) for periods up to three weeks prior to use.

Radiochemicals. ^{3}H-thymidine, specific activity 18.4 ci/m Mol, uridine -5-^{H}3 specific activity 39.0 ci/m Mol, leucine-4,5-^{H}3, specific activity 19.0 ci/m Mol were obtained from the Radiochemical Centre, Amersham.

RESULTS

Effect of rifampicin on adenovirus induced interferon synthesis.

Initial experiments were carried out to examine the effect of rifampicin on interferon induction by adenovirus type 5 on CEF cells. It was found that the induction of interferon by adenovirus type 5 was completely inhibited by rifampicin at 100 µg/ml. The kinetics of interferon production on CEF cells in the absence and the presence of rifampicin were also examined. Virus at a multiplicity of infection of 20 pfu/cell was adsorbed to cells for 2 hours at 37°C, cells were then washed with Eagle's medium, overlaid with Eagle's medium supplemented with 2% calf serum either with or without rifampicin at 100 µg/ml. Cultures were incubated at 37°C and medium was removed (from 4 plates for each point) at the times indicated, centrifuged at 2000 rpm. for 15 minutes to remove debris and heated at 56°C for 30 minutes prior to assay for interferon. As shown in Figure IVa interferon induction was completely inhibited by the presence of rifampicin at 100 µg/ml while normal levels were attained in the absence of the drug. Next, the relationship between the dose of rifampicin added and the interferon yield in CEF cells infected with adenovirus types 1, 5 and 12 was

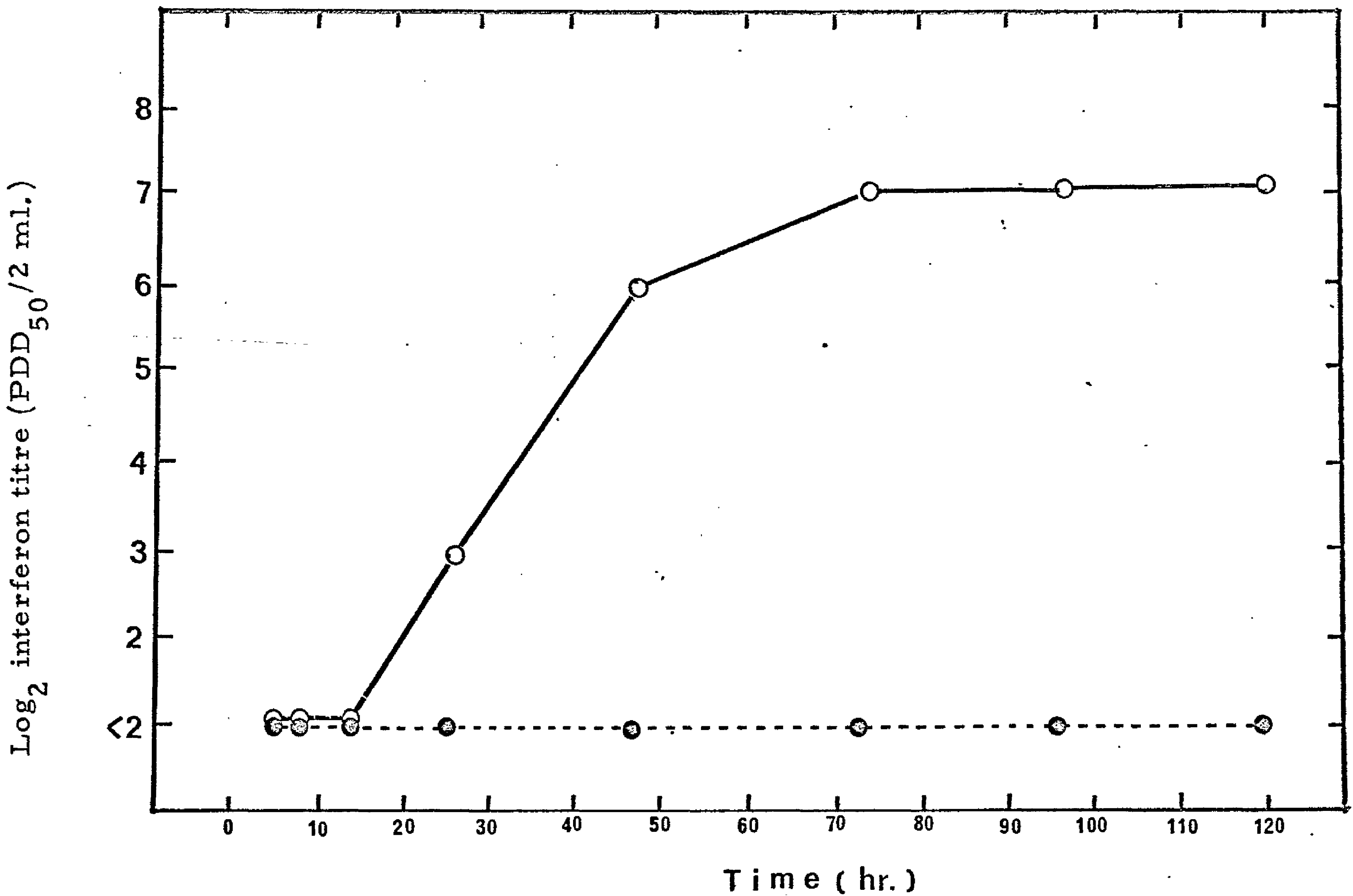


Figure IVa Production of interferon in absence (o———o) and presence of 100 ug /ml (●- - - -●) rifampicin in chick embryo cells infected with adenovirus type 5.

examined. In these experiments cells were again infected with each type at a m.o.i. of 20 pfu/cell as described above. After adsorption at 37°C, plates were overlaid with Eagle's medium supplemented with 2% calf serum and rifampicin added to some at concentrations of 100, 75, 50 and 25 µg/ml and for controls without rifampicin. Four replicate plates were used for each concentration. The cultures were incubated at 37°C and three days after infection, medium was collected and assayed for interferon levels on CEF cells. Figure IVb. illustrates the dose response curves for type 1, 5 and 12 adenoviruses. In the presence of 100 µg/ml rifampicin, interferon production by all three adenoviruses was completely inhibited. At 75 and 50 µg/ml the levels were greatly reduced and the production of interferon was affected even by 25 µg/ml rifampicin. Experiments were carried out to determine the effect of pretreatment of CEF cells with rifampicin on the subsequent induction of interferon by adenovirus type 5 in the absence of rifampicin. CEF cells were pretreated for 12 hours with Eagle's medium supplemented with 2% calf serum and containing either no rifampicin or 100 µg/ml rifampicin. After the incubation period at 37°C, medium was withdrawn and cells were infected with adenovirus type 5 at 20 pfu/cell. After adsorption at 37°C for 2 hours, cells were washed with Eagle's medium, overlaid with

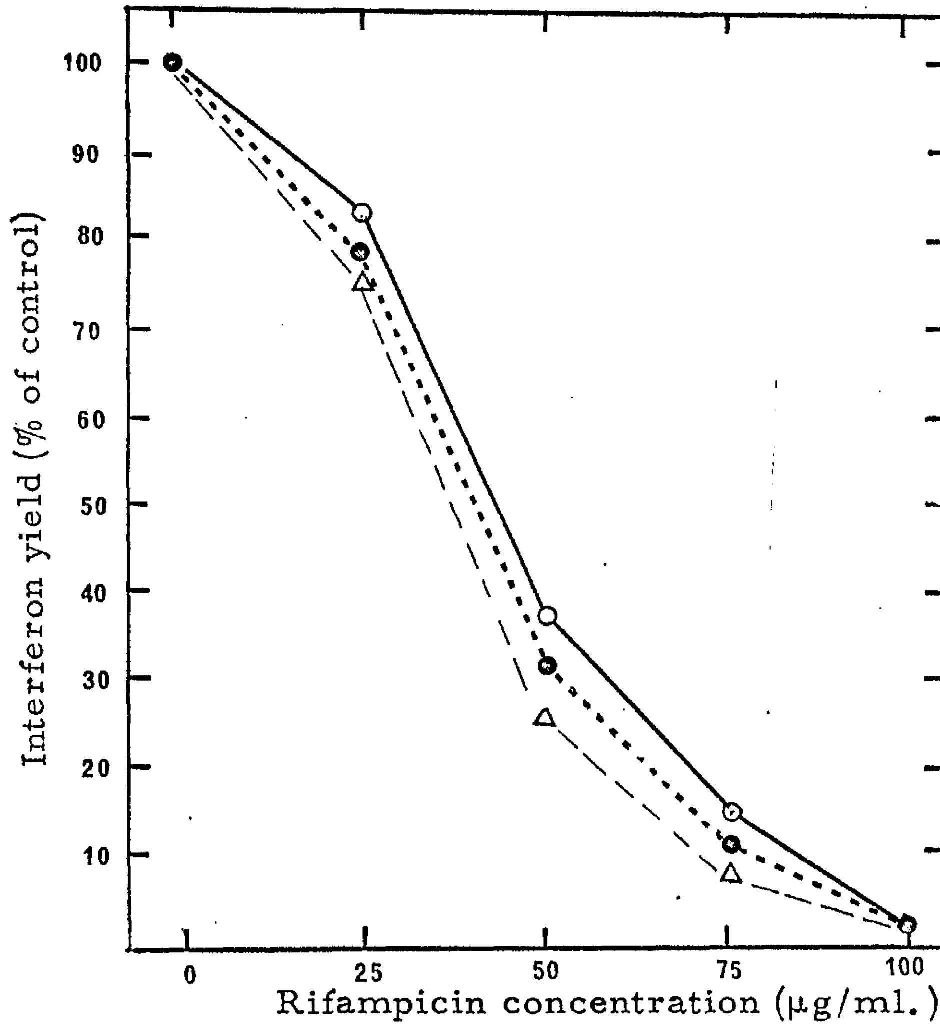


Figure IVb. Relationship between rifampicin dose and interferon yield in chick embryo cells infected with adenovirus type 1 . o——o , type type 5 ●------● , and type 12 △— — —△

Eagle's medium supplemented with 2% calf serum, and incubated at 37°C for 3 days. Medium was collected as above and interferon levels were measured. The results in Table IVa show that pretreatment of CEF cells with 100 ug/ml rifampicin has no effect on subsequent interferon induction.

As reported in a previous chapter of this thesis, pretreatment of CEF cells with interferon at 10-20 units/dish results in the enhancement (priming) of the interferon inducing capacity of adenoviruses. Experiments were carried out here to examine the effect of priming on subsequent interferon production in the presence of rifampicin. CEF cells were primed with 10 units of interferon as described in Chapter VI , and the primed cells were infected with adenovirus type 5 at a m.o.i. of 20 pfu/cell. Virus was adsorbed at 37°C for 2 hours, cells were washed with Eagle's medium, overlaid with Eagle's medium supplemented with 2% calf serum and incubated at 37°C for 3 days. At that time medium was collected and interferon levels measured. As shown in Table IVb priming does not prevent the depression of interferon production in CEF cells by rifampicin.

In order to examine the period of induction in CEF cells which is sensitive to inhibition by the drug, rifampicin was added to the

PRETREATMENT (12 hours)	POST TREATMENT (3 days)	INTERFERON TITRE (PDD_{50}/2 ml.)
None	None	128
Rifampicin (100 µg /ml.)	None	128
None	Rifampicin (100 µgr/ml.)	<4
Rifampicin (100 µgr/ml.)	Rifampicin (100 µgr/ml.)	<4

Table IVa. Rifampicin pre and post-treatment and interferon induction in CEF cells by adenovirus type 5.

Pretreatment	Interferon titre (PDD_{50}/2 ml.)	
	No rifampicin	100 μg /ml. rifampicin
None	128	<4
Interferon (10 units)	1024	4

Table IVb. Inhibition by rifampicin of induction of interferon by adenovirus type 5 in primed CEF cells.

medium at various times after infection with adenovirus type 5. Infection was carried out as described above and rifampicin (100 µg/ml) was added to the medium at various times up to 72 hours. Medium was collected 5 days after infection in every case. The results of such an experiment are shown in Table IVc. Rifampicin added at any time up to 24 hours after infection brings about complete suppression of interferon synthesis, while addition at later times results in a reduced effect.

Experiments were also carried out to examine the reversibility of the effect of rifampicin on interferon synthesis on CEF cells. CEF cells were infected with adenovirus type 5 as described above and rifampicin was added at 100 µg/ml immediately after infection. The drug was then withdrawn at different times after treatment, cells were washed with Eagle's medium after withdrawal, and Eagle's medium supplemented with 2% calf serum was added. Medium was collected 5 days after infection and interferon levels were measured. As shown in Table IVc withdrawal of the drug as late as 24 hours after addition resulted in complete recovery of interferon production indicating that the inhibition is completely reversible. The removal of the drug at 48 hours after treatment still

Addition time (hr. after infection)	Interferon titre (PDD_{50}) No rifampicin	100 µg. /ml. rifampicin	Interferon titres at times of addition
0	64	<4	-
5	64	<4	-
9	64	<4	-
18	64	<4	4
21	64	<4	4
24	64	4	8
28	32	8	16
45	32	16	32
72	32	32	64

Removal time (hr. after infection)	No rifampicin	100 µg. /ml. rifampicin	Interferon titre at time of removal: No rifampicin	100 µg. . rifampici
24	32	32	8	<4
24 *	256	128	-	-
48	16	8	32	<4

* Primed with 10 units of interferon.

Table IVc. The effect on interferon production of addition and removal of rifampicin at various times after infection with adenovirus type 5.

resulted in recovery of interferon but the amount was much less. The experiment was also carried out in primed cells and in those too, interferon production was resumed when the drug was removed 24 hours after infection (Table IVc).

<u>Effect of Rifampicin on polyoma virus induced interferon synthesis.</u>

A small oncogenic DNA virus, polyoma, is able to induce interferon on CEF cells but fails to replicate (Chapter V). It has been reported that replication of polyoma virus is not affected by rifampicin on mouse embryo cells (Williams et al, 1971). The effect of rifampicin on polyoma virus induced interferon synthesis was tested as follows. Primary CEF cells were infected with polyoma virus at a m.o.i. of 20 pfu/cell. After 2 hours of adsorption at 37°C cells were washed with Eagle's medium and overlaid with the Eagle's medium supplemented with 2% calf serum and rifampicin was added to the medium at concentrations of either 50 or 100 µg/ml. Control cells were maintained in medium without rifampicin. Media were collected 72 hours after infection and interferon levels were measured. Results in Table IVd shown that even 50µg/ml rifampicin depressed interferon production 8 fold and by 100 µg/ml of rifampicin the production was depressed 32 times.

Rifampicin concentration ($\mu g/ml$)	Interferon titre $PDD_{50}/2$ ml
None	128
50	16
100	<4

Table IVd . Inhibition by rifampicin of induction of interferons by polyoma virus on CEF cells.

Effect of rifampicin on Semliki Forest virus induced interferon and virus replication.

The effect of rifampicin (60 and 100 µg/ml) on Semliki Forest virus induced interferon synthesis and virus replication was examined at 37°C and 42°C. CEF cells were infected at 0.5-1 pfu/cell and incubated at 37°C for 20 hours. Medium was harvested for interferon assay, and cells collected for assay of infectivity. At 42°C, CEF cells were infected at a multiplicity of 80 pfu/cell and incubated for 20 hours, at which time the medium was harvested for interferon assay and the cells collected for infectivity assay. The results of these experiments are illustrated in Table IVe and it can be seen that at 37°C in the presence of 60 and 100 µg/ml. rifampicin, virus multiplication is not affected, while the interferon titre is reduced 4 fold in the presence of 60 µg/ml and 8 fold by 100 µg/ml. The same is true of interferon production at 42°C where the virus fails to replicate.

Lack of effect of rifampicin on the action of interferon.

Actinomycin D is a potent inhibitor of interferon induction, and it also inhibits interferon action by blocking DNA directed messenger RNA synthesis in cells (Taylor, 1965). As discussed in this chapter, rifampi inhibited induction of interferon synthesis by adenovirus, polyoma and

Rifampicin concentration (μg./ml.)	Virus titre ($\log_{10}$ p.f.u./ml.)	Incubation temp. for Interferon	Interferon titre (PDD_{50})
0	8.32	37°C	128
		42°C	128
60	8.25	37°C	32
		42°C	32
100	8.53	37°C	16
		42°C	16

Table IVe. Effect of rifampicin on interferon and Semliki Forest virus formation on chick embryo cells.

Semliki Forest virus. In order to determine if rifampicin had an effect on the antiviral action of interferon the following experiments were carried out. Primary CEF cells were treated with 10 units of interferon either in the presence or the absence of 100 µg/ml of rifam for 12 hours prior to challenge. Non-interferon-treated controls were also kept with or without 100 µg/ml. rifampicin for the same period. After treatment, medium was removed, cells were washed with Eagle's medium, and infected with either Semliki Forest virus or vaccinia virus at a multiplicity of infection of 5 pfu/cell. Virus was adsorbed for 1 hour at 37°C, then cells were washed with Eagle's medium and overlaid with Eagle's medium supplemented with 2% calf serum. Semliki Forest virus infected cultures were incubated for 24 hours and vaccinia virus infected cultures for 48 hours at 37°C. After incubation the cells were harvested, frozen and thawed three times and assayed for infectivity on CEF cells. The results illustrated in Table IVf show clearly that interferon depresses Semliki Forest virus and vaccinia virus growth on CEF cells to the same extent in the presence and absence of rifampicin at 100 µg/ml and it is concluded that the drug has no effect on the antiviral action of interferon.

Pre-treatment (12 hours)		Semliki Forest virus yield at 24 hr. $\log_{10}$ pfu/ml.)	Vaccinia virus yield at 48 hr. $\log_{10}$ pfu/ml.)
Interferon (10 units)	Rifampicin (100 μg. /ml)		
-	-	8.3	7.9
+	-	5.4	6.6
-	+	8.5	7.8
+	+	5.6	6.7

Table IVf Effect of rifampicin on the action of interferon on Semliki Forest virus and vaccinia virus growth in CEF cells.

Inhibition of CEF cell growth by rifampicin.

In the present experiments, the cells appear to remain viable after exposure of monolayers to rifampicin concentrations of 100 μg/ml for up to 5 days. However, conflicting results have been reported concerning CEF growth inhibition in the presence of rifampicin by Digglemann and Weissmann (1969) who found that cell growth was not affected by the drug, and by Robinson and Robinson (1971) who observed inhibition of CEF cell growth by the drug. Experiments were therefore carried out to determine if CEF cell growth was inhibited under the culture conditions used in the experiments described above. In order to test the growth of primary CEF cells in the presence and absence of rifampicin, freshly trypsinised CEF cells were resuspended in growth medium and seeded on 50 mm dishes at an initial concentration of 2×10^6 cells/dish (20-30% of these cells adhere). After 24 hours rifampicin was added to the medium at concentrations of either 50 or 100 μg/ml. and cell counts were taken at each 24 hours period until 4 days after seeding. Four separate petri dishes were counted for each point. The results are illustrated in FigureIVc and it can be seen that cell growth is partially depressed by 50 ug/ml rifampicin and greatly inhibited by 100 μg/ml. Experiment

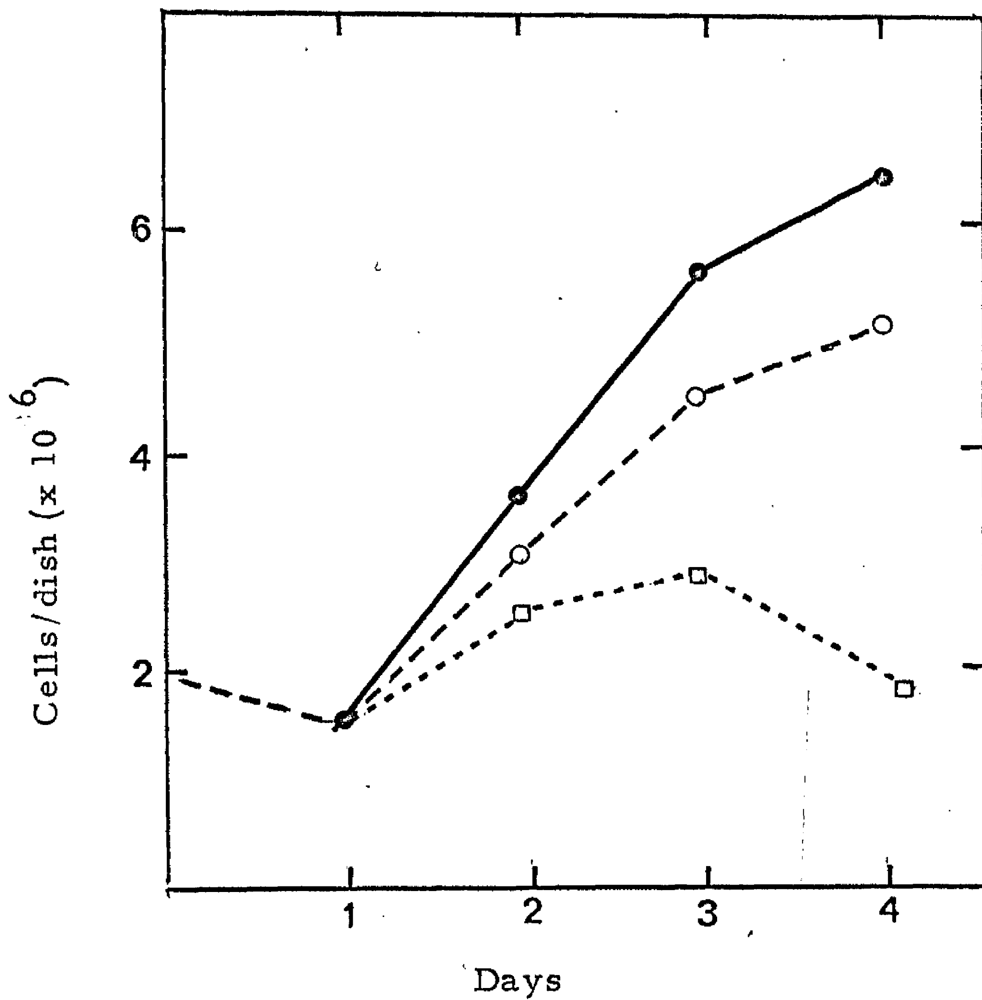


Figure IVc. Effect of rifampicin on CEF cell growth. ●——● , No rifampicin; ○----○ , 50 μg./ml.; □-----□ , 100 μg./ml.

were repeated with a lower density of cells in exactly the same way as described above. In this case 5 x 10^5 cells were seeded per dish and rifampicin (50 or 100 μg/ml) was added 24 hours after seeding. Cells were again counted every 24 hours and four plates were counted for each point. Figure IVd illustrates the results of such an experiment. The inhibition of cell growth is much more pronounced when a lower density of cells is exposed to the drug, and cell growth is completely inhibited by both 50 and 100 μg/ml of rifampicin. These results agree very closely with the findings of Robinson and Robinson (1971) who observed that the inhibition of CEF cell growth by rifampicin is inversely related to the density of cells in cultures. This also agrees with the fact that BHK21 cell growth is inhibited (Williams et al. 1971). Having established the inhibitory effect of rifampicin on CEF cell growth, the reversibility of this effect was investigated in two ways. First, in terms of the colony-forming ability (plating efficiency) of CEF cells, and second, in terms of the growth rate of the cells. Complete monolayers of CEF cells were washed with Eagle's medium, then overlayed with Eagle's medium supplemented with 2% calf serum, either with or without 100 μg/ml rifampicin and incubated at 37°C for 48 hours. After incubation, the cells were trypsinised and

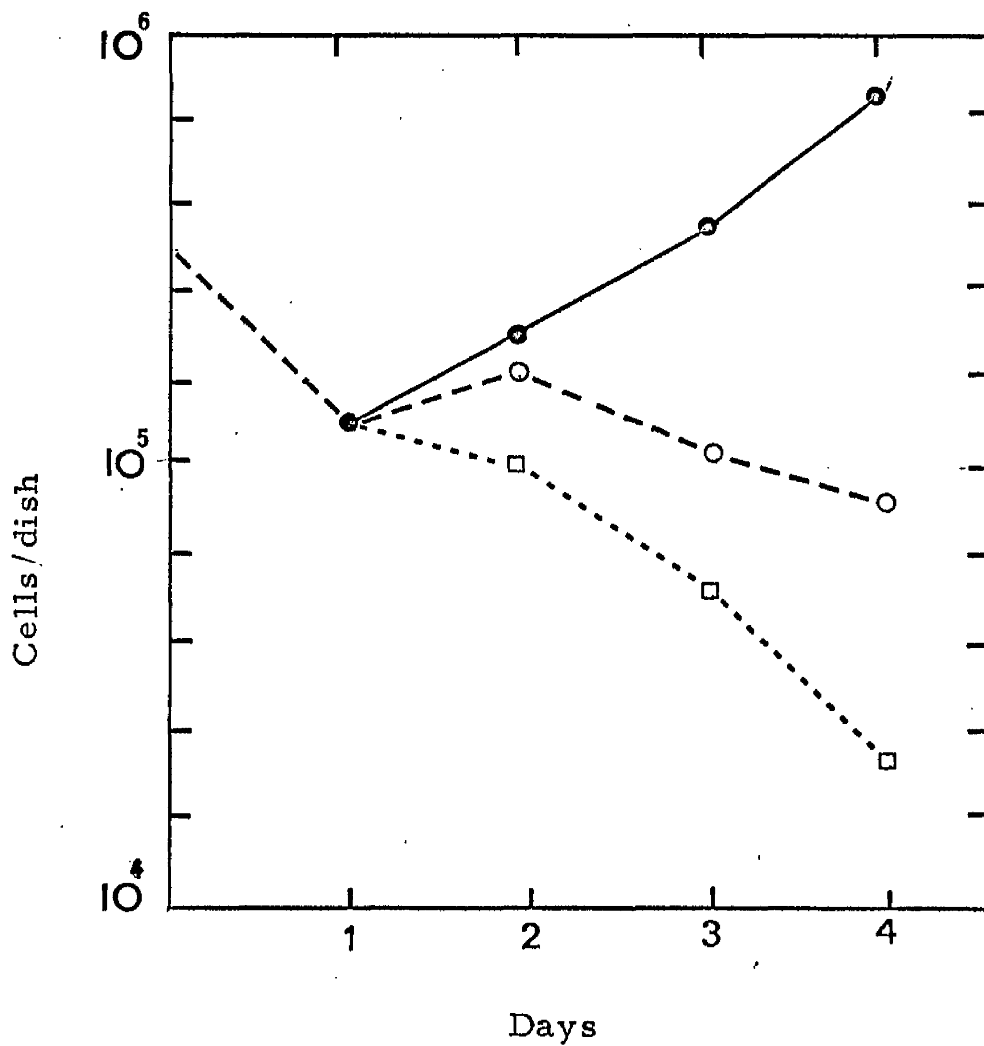


Figure IVd. Effect of rifampicin on CEF cell growth. ●——●, No rifampicin; o- - -o, 50 µg. /ml.; □- - - -□ 100 µg. /ml.

resuspended, and 10^4 cells were seeded on 50 mm petri dishes containing 10^5 irradiated rat embryo feeder cells in 5 ml of Eagle's medium supplemented with 5% calf serum. (Rat embryo cells were exposed to approximately 3000 rads of X-rays). These cultures were then incubated for 7 days at 37°C and stained with Giemsa for counting. As shown in Table IVg , the plating efficiency of CEF cells treated for 2 days with 100 µg/ml of rifampicin did not seem to dif very much from that of control non-treated cells. In one experiment it was higher and in the other lower.

The growth rates of rifampicin treated and non-treated cells were also tested. After treatment, which was the same as that described for the plating-efficiency experiment, trypsinised cells were seeded at 10^6 cells/dish in Eagle's medium supplemented with 10% calf serum. Cell counts were made every 24 hours (4 petri dishes being counted separately for each point). The growth rate of cells treated for 2 days with 100 µg/ml of rifampicin was about the same as non-treated CEF cells (Figure IVe). In addition, the CEF cells remained in healthy appearance for up to 5 days and collectively these results indicate that the treatment of CEF cells with 100 µg/ml rifampicin does not result in general cell death. Thus, one can not account for the depression of interferon synthesis simply in terms

Treatment	Plating efficiency	
	Expt. 1	Expt. 2
None	0.55	0.75
Rifampicin (100 μg./ml.)	0.85	0.44

Table IVg. The plating-efficiency of CEF cells after incubation for two days in the absence and presence of rifampicin.

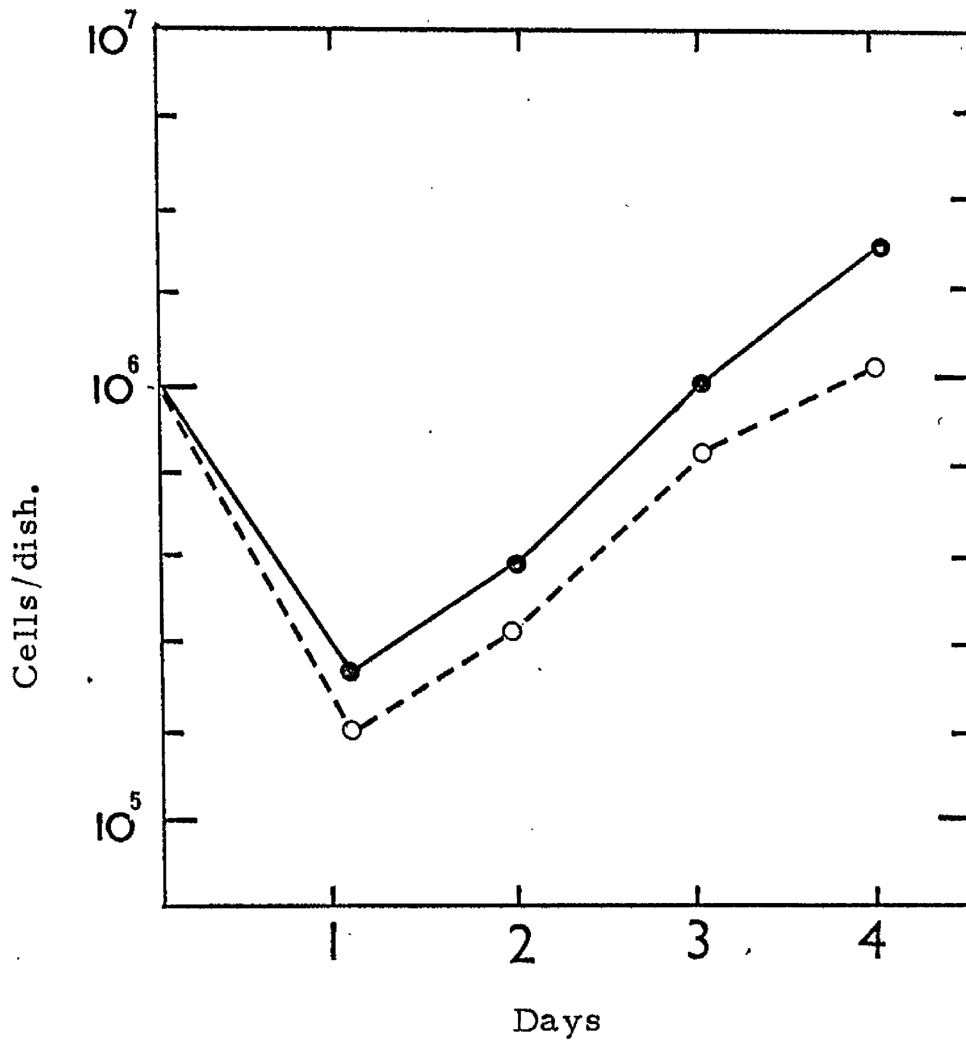


Figure IVe. Effect of pretreatment with rifampicin on the growth rate of CEF cells; ●——● , Non-treated; o- - -o , treated for 48 hours with 100 µg/ml.

of cell killing.

Effect of rifampicin on macromolecular synthesis by CEF cells.

Rifampicin, at doses which cause depression of interferon synthesis have been reported to have very little effect on the incorporation of radioactive precursors into cellular DNA, RNA and proteins of mouse cells (Heller et al. 1969) or CEF (Heller et al. 1970). On the other hand, incorporation of ^{3}H-thymidine into the DNA of partially synchronised BHK 21 cells is inhibited to some extent (Subak-Sharpe et al. 1970) and the experiments described above suggest that CEF cell growth is reversibly inhibited wit doses of rifampicin which inhibit interferon synthesis. Therefore experiments were carried out in order to establish the effect of rifampicin on the uptake of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine into cell DNA, RNA and protein respectively. The results of two separate experiments in which the relevant precursors were present for 1 hour periods at intervals up to 72 hours after the addition of rifampicin are shown in tables IVh and IVj. The greatest effect was on ^{3}H-thymidine incorporation, which was reduced 40 to 80% in the first 24 hours and 90% by 48 hours by 100 μg/ml rifampicin. The incorporation of ^{3}H leucine was depressed to a much lesser extent

Time interval (hr)	Rifampicin concentration (µg./ml.)					
	^{3}H - Thymidine		^{3}H (Uridine)		^{3}H - Leucine	
	50	100	50	100	50	100
0 to 1	0.93	0.55	0.89	0.90	0.91	0.80
5 to 6	0.87	0.87	1.00	0.85	1.23	1.06
9 to 10	0.84	0.70	1.17	0.91	1.27	1.20
15 to 16	0.90	0.31	1.05	0.96	1.21	1.10
21 to 22	0.69	0.22	1.50	1.52	1.28	1.12
27 to 28	0.83	0.27	1.06	0.88	1.00	0.70
35 to 36	0.73	0.12	0.96	0.72	0.87	0.48
47 to 48	0.40	0.09	1.14	0.60	0.90	0.61
57 to 58	0.45	0.07	1.28	0.52	-	0.15
71 to 72	0.41	0.12	0.60	0.26	0.73	0.13

The values in the table are the ratios of the average counts per minute per coverslip for the rifampicin treated cells compared with the non-treated control cells. All values represent means for two separate cover-slips, and the lowest average count/min./coverslip for the controls was 4107.

Table IVh. The effect of rifampicin on ^{3}H - thymidine, ^{3}H - uridine and ^{3}H - leucine incorporation in CEF cells.

	Rifampicin concentration (μg./ml.)					
	^3H - Thymidine		^3H - Uridine		^3H - leucine	
Time interval (hr.)	50	100	50	100	50	100
0 to 1	1.25	1.04	0.89	1.16	1.15	0.98
15 to 16	0.76	0.67	0.99	0.96	0.91	0.88
23 to 24	0.85	0.68	0.80	0.71	0.81	0.83
37 to 38	0.60	0.42	0.65	0.55	0.72	0.71
47 to 48	0.62	0.11	0.70	0.37	0.70	0.45
61 to 62	0.59	0.07	0.63	0.24	0.69	0.25
71 to 72	0.45	0.08	0.31	0.17	0.62	0.13

The values in the table are the ratios of the average counts per minute per coverslip for the rifampicin treated cells compared with the non-treated cells. All values represent means for two separate coverslips, and the lowest average count/min./coverslip for the controls was 3500.

Table IVj. The effect of rifampicin on ^3H -thymidine, ^3H - uridine and ^3H - leucine incorporation in CEF cells.

up to 48 hours even by 100 μg/ml but in the next 24 hour period it was depressed by as much as 85%. In the first experiment, 50 μg/ml rifampicin did not effect the ^{3}H-uridine incorporation up until 72 houss after treatment, however 100 μg/ml rifampicin had an effect from 28 hours and by 72 hours incorporation of ^{3}H-uridine was inhibited 74%. In the second experiment the depression was more pronounced, but still in the first 24 hours, depression was 20 and 29% with 50 and 100 μg/ml of rifampicin. By 72 hours, incorporation was depressed 70% with 50 ug/ml and 83% with 100 μg/ml rifampicin.

DISCUSSION

The mode of action of rifampicin on the replication of animal DNA viruses *in vitro* is not clear as yet, but it almost certainly differs from its antibiotic effect on bacteria. In addition to vaccinia and other poxviruses, the replication of certain adenoviruses is depressed to some extent by rifampicin (Subak-Sharpe et al. 1970). It is particularly interesting that all adenoviruses do not seem to behave in the same way with respect to inhibition by rifampicin in Hela cells; adenovirus types 1, 2 and 5 are inhibited (Subak-Sharpe et al. 1970), but adenovirus type 12 is not inhibited in the presence of the drug (Ustacelebi and Williams, 1972). The reason for this difference is not known at the present time. While we do not know how adenoviruses induce interferon production in non-permissive CEF cells, the assumption is made that certain viral functions may be expressed in these cells and that these are responsible for induction. In the experiments described above, rifampicin was used initially to determine if adenoviruses also show differential sensitivity with respect to interferon induction. However, it was repeatedly shown that the induction by adenovirus types 1, 5 and 12 was inhibited to the same

extent. Interferon induction by Semliki Forest virus in CEF was also shown to be inhibited by rifampicin at the permissive temperature (37°C) by concentrations which had no effect on virus replication, and also under conditions where Semliki Forest virus did not replicate, at 42°C, interferon induction was inhibited by rifampicin. Interferon induction by a small oncogenic DNA virus, polyoma, was inhibited on CEF cells to some extent, although the virus does not replicate on CEF cells and rifampicin has no effect on the replication of polyoma virus on mouse embryo cells (Williams et al. 1971). These results suggest that either a non-essential virus function, or alternatively a cellular function involved in interferon induction is inhibited and it is most unlikely that an essential viral functio[n] is involved in the inhibition.

The possibility that rifampicin acts by inactivating the interferon produced by cells in vitro was tested, although the results have not been presented. When interferon was mixed with 100 μg/ml rifampicin and incubated for either 30 minutes at 56°C or 60 minutes at 37°C prior to titration no drop in titre was observed. Therefore it is unlikely that the effect of rifampicin is simply due to the *in vitro* inactivation of preformed interferon.

While CEF cells remian viable during 3-5 days after treatment with 100 μg/ml rifampicin, they fail to divide, although the effect on cell growth is reversible in that when the drug is removed, interferon production resumes. Therefore it is not a lethal effect and the depression of interferon production is not simply due to cell killing. Of course, it is possible that only a small, special class of CEF, particularly sensitive to rifampicin, may be responsible for interferon induction. However, even allowing for the existence of such a class of cells, the possibility that rifampicin acts in a lethal way on them seems unlikely, in view of the fact that the rifampicin blocksis reversible and CEF cells treated for up to 24 hours produce normal amounts of interferon on removal of the drug. The inhibition of both cell growth and interferon systhesis in CEF cells treated with rifampicin could result from inhibition of cell macromolecular synthesis and to test this the incorporation of radioactive precursors into cellular DNA, RNA and protein was measured in the presence and absence of rifampicin. Two separate experiments were carried out and in both experiments the incorporation of ^{3}H-thymidine was found to be inhibited by 33-70% by 15-16 hours of treatment with 100 μg/ml rifampicin. During the same period, ^{3}H-uridine and ^{3}H-leucine incorporations were not affected but subsequently

incorporation of these precursors also dropped significantly. The messenger RNA for interferon induced by adenovirus and Semliki Forest virus is presumably synthesised by 15-16 hours after infection in infected cells yet during this period, the effect of even 100 μg/ml of rifampicin is very small on the incorporation of ^{3}H-uridine. Therefore inhibition is unlikely to be due to the lack of RNA synthesis, unless rifampicin prevents only transcription of certain classes of RNA. However, it has also been reported that rifampicin does not inhibit mammalian cell nuclear RNA polymerase activity (Wehrli et al. 1968; Jacob et al. 1968; Gadaleta, Greco and Saccone, 1970), but it inhibits mitochondrial RNA polymerase *in vitro* (Gadaleta, Greco and Saccone, 1970). This raises the possibility that interferon synthesis is directed by mitochondrial rather than nuclear DNA, but there is no evidence that rifampicin acts in the same way in CEF cells. However, a compound related to rifampicin (AF/ABDP) causes profound morphological changes in the mitochondria of CEF cells (E.A.C. Follett, personal communication). It is of course possible that mitochondrial damage relating to a mitochondrial function such as oxidative phosphorylation could be the cause of the depression of interferon synthesis.

In contrast to the action of actinomycin D, which inhibits both

production and action of interferon (Heller, 1963; Taylor, 1965), rifampicin was shown to inhibit only the induction but not the action of interferon. While the action of actinomycin D on interferon induction and action is irreversible, the inhibitory effect of rifampicin on the induction of interferon has been shown to be reversible. Therefore it is likely that these two antibiotics act in fundamentally different ways.

In addition to the inhibitory effect of rifampicin on interferon induction described here, another cellular function, the immune response, is inhibited in vivo (Paunescu, 1970). Rifampicin also inhibits the transformation of CEF cells by Rous sarcoma virus (Diggelmann and Weissmann, 1969) and BHK 21 cells by polyoma virus (Williams, et al. 1971). Considering all of these effects, one could speculate that rifampicin might be acting somewhat preferentially, on "induced" cell functions. While it is not yet known if rifampicin inhibits interferon induction in vivo, the experiments presented in this chapter and the results of Paunescu (1970) raise a serious issue concerning the use of of rifampicin and other derivatives on rifamycin in vivo. The doses which prevent virus multiplication may depress the natural defence mechanisms of the body against the invading virus amd this problem will have to be assessed in the future.

CHAPTER V

INTERFERON INDUCTION BY POLYOMA VIRUS ON CHICK EMBRYO CELLS.

1. INTRODUCTION.

2. MATERIALS AND METHODS.

3. RESULTS.

a. Interferon induction with polyoma virus preparations on CEF cultures.

b. Inhibition of polyoma virus interferon induction by treatment with specific neutralising anti-serum.

c. Kinetics of interferon induction by polyoma virus on CEF cells.

d. Polyoma growth on CEF cells.

e. Multiplicity requirement for interferon induction by polyoma virus.

f. Chacacteristics of interferon induced by polyoma virus on CEF cells.

g. Interferon inducing capacity of purified polyoma virus "full" and "empty" particles.

h. Effect of cytosine arabineside (CAR) treatment of cells on interferon production of cells infected with polyoma virus.

i.

i. Interferon induction on continuously growing cultures of CEF cells by polyoma virus and adenovirus type 5.

4. DISCUSSION.

INTRODUCTION

Polyoma virus induces interferon in productively infected mouse (Allison, 1961; Friedman, Rabson and Kirkham, 1963) and abortively infected hamster cells (Talas, We feiler and Batkai, 1968; Gotlieb-Stematsky and Vansover, 1970. However the level of interferon induced by polyoma virus on productively infected mouse cells is very low, and interferon can only be detected in the culture medium very late after infection (Allison, 1961; Friedman, Rabson and Kirkham 1963). In abortively infected hamster cells polyoma virus induced interferon to slightly higher levels than in mouse cells and it is produced earlier, (Talas, We feiler and Batkai, 1968; Gotlieb-Stematsky and Vansover, 1970).

While the exact relation between oncogenic potential and interferon inducing capacity of polyoma virus variants is not clear, it has been suggested that more highly oncogenic variants are more efficient interferon inducers than low oncogenic variants in hamster embryo cells (Gotlieb-Stematsky and Vansover, 1970). A result opposing this view was previously reported for mouse cells (Friedman, Rabson and Kirkham, 1963), in which less

oncogenic variants induced to a higher level. Polyoma virus may vary in its capacity to induce interferon response on different hosts, since the virus certainly does not express the same set of functions in abortively and productively infected cells. At present the mechanism by which polyoma virus induces interferon in the infected cells, is unknown.

Polyoma is a small DNA virus and its circular duplex genome, molecular weight of 3.0×10^6 (Crawford, 1964) can only code for a few viral-specified functions (maybe 5-8). Therefore polyoma could provide a good model to study the mechanism by which a DNA virus induces the interferon response. In addition the genetics of polyoma virus is being studied in detail by means of temperature-sensitive mutants (Eckhart, 1969). Ts mutants of polyoma virus have been used for identification of viral genes necessary for transformation in abortively infected cells (Di Majorca et al. 1969, Fried, 1970; Eckhart, 1971) and it feasible that a similar approach could be taken for the identificatio of the gene function involved in interferon induction in abortively infected cells. As discussed in previous chapters the same

approach has been used for adenovirus, an intermediate-sized DNA virus. Since adenoviruses fail to replicate on CEF cells, but induce good interferon levels, it seems possible that polyoma virus also may behave in the same way, because the CEF cells are known to be good interferon producing hosts. In addition, polyoma virus induces interferon response on hamster cells in which it fails to replicate. Therefore this possibility has been tested and experiments will be described in this chapter dealing with the kinetics and multiplicity requirement of interferon induction and the properties of interferon induced by polyoma virus in CEF cells. In addition, experiments will be described in this chapter concerning the interferon inducing capacity of physically separable infectious "full" and non-infectiou "empty" polyoma particles on CEF cells. The possible mechanis by which polyoma virus induces interferon on CEF will be discuss

There is no previous report dealing with the susceptibility continuous CEF lines to interferon induction by viruses. The interferon inducing capacity of polyoma virus and adenovirus type two continuous CEF lines will also be described.

MATERIALS AND METHODS

Cells. Mouse embryo fibroblasts (MEF) were prepared from 15-17 day old embryos by the cytology department using a standard procedure (Pregnant mice of the Porton strain were obtained from the Animal Virus Research Institute, Pirbright). They were grown in rotating Winchester bottles in Eagle's medium supplemented with 10% calf serum and 10% tryptose Phosphate broth. After 5-6 days growth at 37°C,cultures were passaged to provide secondary cultures. The procedure for the preparation of CEF is the same as described in Chapter The continuous lines of CEF cells were maintained in 20 ounce flat bottles and passed every 5-7 days. CEF-A, was supplied by Dr. J. F. Williams, while line CEF-B was established under the same conditions by myself.

Viruses. Two separate polyoma virus small plaque variants of the Toronto strain (McCulloch et al. 1959) were used. One variant originated in Glasgow (Crawford, 1962) and the other in Toronto (Stanners et al. 1963). These were grown on MEF primary or secondary cell cultures in burrlers by infecting at a multiplicity of infection of 5-10 pfu/cell. After adsorption

for 2 hours the infected cells were maintained in Eagle's medium supplemented with 2% calf serum. Complete cytopathic effect was usually observed at 6-8 days after infection, when the cells were shaken off into the medium. Extracellular virus in the suspension was allowed to adsorb to cells by keeping the suspension at 4°C for 24 hours. The cells were pelleted by centrifugation at 2000 rpm for 15 minutes, resuspended in tris-saline (pH 7.4), frozen and thawed three times, and finally sonicated for 2 minutes. At this stage RDE (receptor destroying enzyme prepared from Vibrio cholerae by the cytology department) was added (1 volume RDE to 5 volumes of virus suspension). The mixture was kept at 37°C overnight. Finally, the RDE treated preparation was centrifuged at 2000 rpm for 15 minutes and the supernatant was stored at -20°C in small aliquots.

Virus assays. Polyoma virus was assayed by plaque formation on MEF secondary monolayers as follows. MEF monolayers were infected with serial dilutions of polyoma virus and after adsorption at 37°C for 2 hours the monolayers were overlaid with 5 ml. of Eagle's medium containing 0.65% Noble agar (Difco) and supplemented with 2% calf serum. These were then incubated

at 37°C and at the 6th day of incubation, 2 ml. of additional overlay medium (sometimes with neutral red incorporated) was added to the cultures and plaques were counted at 9 and 14 days after infection.

<u>Interferon induction.</u> Confluent primary CEF monolayers, seeded 2-3 days previously, were infected at a multiplicity of infection of 10-20 pfu/cell. Virus was adsorbed at 37°C for 2 hours. Then 3 ml. of Eagle's medium supplemented with 2% calf serum was added to each plate and cultures were incubated at 37°C. At various times after infection, culture fluid was harvested, centrifuged at 3000 rpm for 10 minutes and kept at 4°C until it was assayed.

<u>Interferon assay.</u> As described in Chapter II

<u>Purification of polyoma virus.</u> The procedure previously described by Crawford, Crawford and Watson, (1962) was used. Virus suspension was layered on to caesium chloride solution mac up at a final density of 1.3 in 0.05 Mol tris-HCl buffer (pH 7.4) in 5 ml. cellulose nitrate tubes. Centrifugation was carried out for 18 hours at 30,000 rpm in an SW 39 rotor in the Spinco model L centrifuge. The two visible opalescent bands were

collected by puncturing the bottom of the tube and collecting drops. Virus bands were diluted 2 times in tris-HCl buffer and dialysed against this buffer overnight. Finally, after the addition of 0.1% bovine serum albumin the virus was stored at -20°C.

RESULTS

Interferon induction with polyoma virus preparations on CEF cultu

Primary CEF cells infected with non-purified polyoma virus preparations at a multiplicity of infection of approximately 10 pfu/cell produced high levels of interferon, detectable in the culture fluid 2-3 days post-infection. Initial experiments were carried out by using crude virus stock which of course contained both mouse cell components and RDE. To test the possible role of these contaminants in interferon induction the following preparations were used. 1. Virus stocks, prepared with and without RDE treatment. 2. Polyoma virus, purified by caesium chloride density centrifugation as described above. 3. Non-infected MEF cell extracts prepared both with and without RDE. CEF cells were infected with virus preparations at a multiplicity of infection of 10 pfu/cell or treated with non-infected cell extracts at approximately the same dilutions as the virus inocula. Culture fluids were harvested 3 days after infection and interferon levels were measured. The results given in Table Va illustrate the interferon titres obtained with different preparations of polyoma virus.

Inoculum	Interferon titre PDD$_{50}$/2 ml.)
Polyoma virus prepared with RDE	512
Polyoma virus prepared without RDE	512
Polyoma virus (purified)	1024
MEF extracted with RDE	<4
MEF extracted without RDE	<4

TABLE Va Interferon induction with different polyoma virus preparations on chick embryo cells.

Purified polyoma virus and virus prepared with or without RDE induced good interferon yields while non-infected MEF cell extracts prepared both with or without RDE completely failed to induce interferon response. It is concluded neither the RDE present in the crude preparations of polyoma virus, nor a mouse embryo cell factor is responsible for interferon induction.

Inhibition of polyoma virus interferon induction by treatment with specific neutralising anti-serum.

As a further control, a purified polyoma virus preparation was treated with antiserum specific to purified polyoma virus. Purified virus was mixed with an equal amount of neutralising antiserum (1/50 dilution of serum, neutralising titre 1/3000) and incubated at 37°C for 1 hour. Control non-serum treated virus was diluted with an equal amount of tris-saline and also incubated at 37°C for 1 hour. Cells were infected with neutralised and cont virus preparations for interferon induction as described before. The infectivities of both preparations wdre measured by plaque assay on MEF cells. A 240 fold decrease in infectivity of polyoma virus occurred as a result of treatment with antiserum,

and neutralised polyoma virus failed to induce interferon. (Table V

Control non-treated virus induced interferon in CEF cells.

Kinetics of interferon induction by polyoma virus on CEF cells.

In order to determine the time at which interferon is produc

by CEF cells infected with polyoma, experiments were carried out

as follows. Primary CEF cells were infected with polyoma

virus at a multiplicity of infection of 10 pfu/cell as described

before. Culture fluid was collected at various times after

infection, stored at 4°C, and assayed later for interferon levels

as described above. Interferon was first detected in the culture

fluid about 32 hours after infection and continued to increase in

titre for the next 40-50 hours, reaching a maximum level

approximately 72 hours after infection (Figure Va).

Polyoma growth on CEF cells.

Tests were carried out to determine if polyoma virus

replicates on CEF cells. CEF cells were infected with polyoma

virus as described above at a multiplicity of infection of 10 pfu/cel

At different intervals after infection, cells from two replicate

infected plates were scraped into 2 ml. of tris-buffer (pH 7.4).

Samples were frozen and thawed three times, then sonicated for 2

Inoculum	Infectivity (pfu/ml.)	Interferon titre (PDD_{50}/2 ml.)
Polyoma	1.1×10^8	64
Polyoma + Antisera	4.5×10^5	4

TABLE Vb. **Neutralisation of polyoma virus infectivity and interferon inducing capacity.**

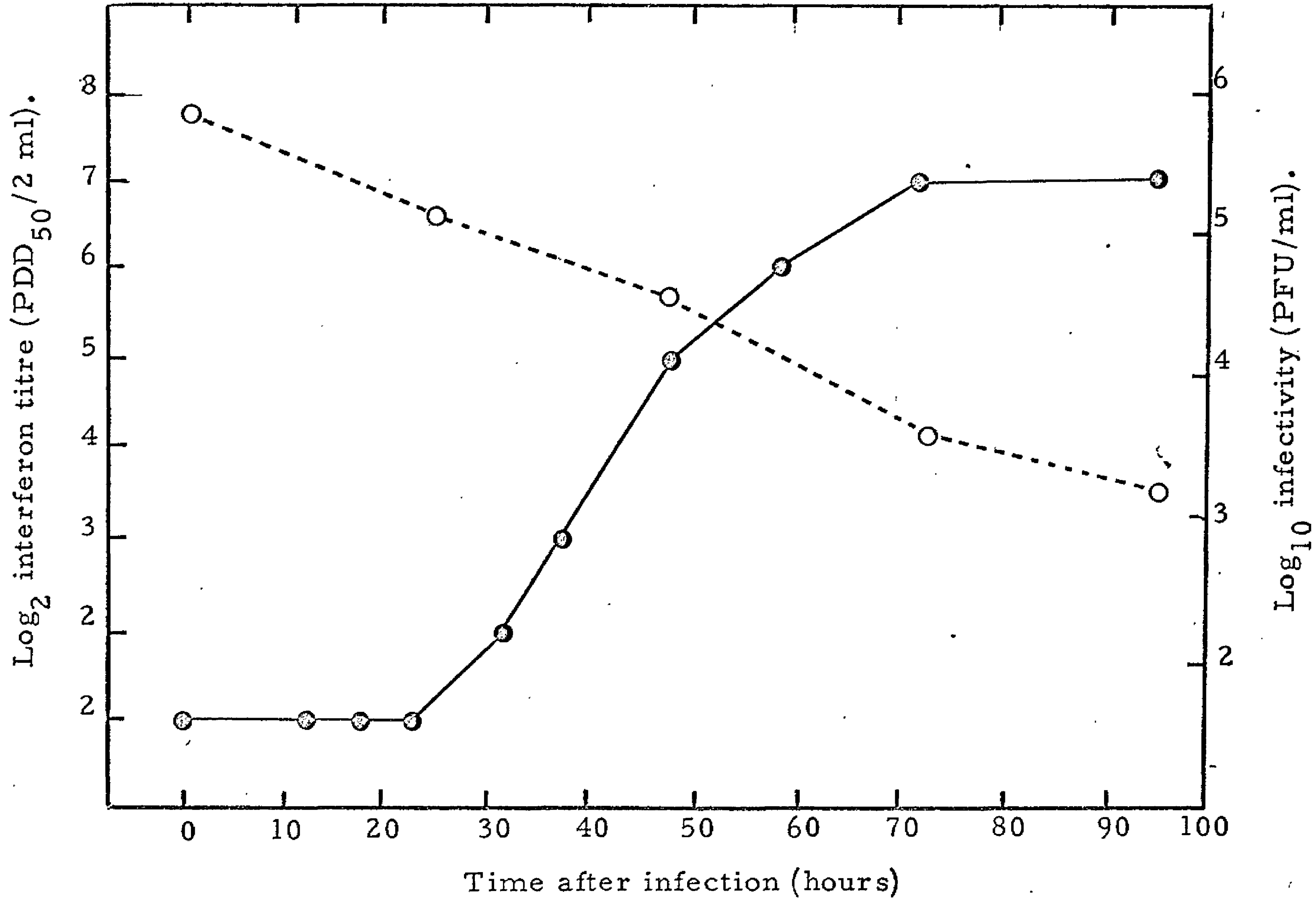


Figure Va.Kinetics of interferon induction (o——o) and loss of cell associated infectivity (o- - -o) on CEF cells infected with polyoma virus

minutes. The infectivity was assayed by plauqe formation on MEF monolayers as described before. The cell-associated infectivity of polyoma virus on CEF cells gradually decreased and only a small fraction was left 4 days after infection. (Figure Va This clearly indicates that polyoma virus does not replicate on CEF cells.

Multiplicity requirement for interferon induction by polyoma virus

In initial experiments, the multiplicity of infection used for interferon induction was relatively high. In order to determine the optimum multiplicity requirement for interferon induction by polyoma virus, multiplicities ranging from 0.01 to 20 pfu/cell were used. Infection was carried out at the indicated multiplicity as described above and culture fluid was collected 3 days after infection. It was determined that a multiplicity of at least 0.5 pfu/cell is necessary for detectable interferon production (Figure Vb). In primed cells, as is discussion later in Chapter VI. interferon is induced by a 10 fold lower multiplicity (0.05).

Characteristics of interferon induced by polyoma virus on CEF cel

The interferon induced on CEF cells by polyoma virus had the following properties. The activity of the preparations

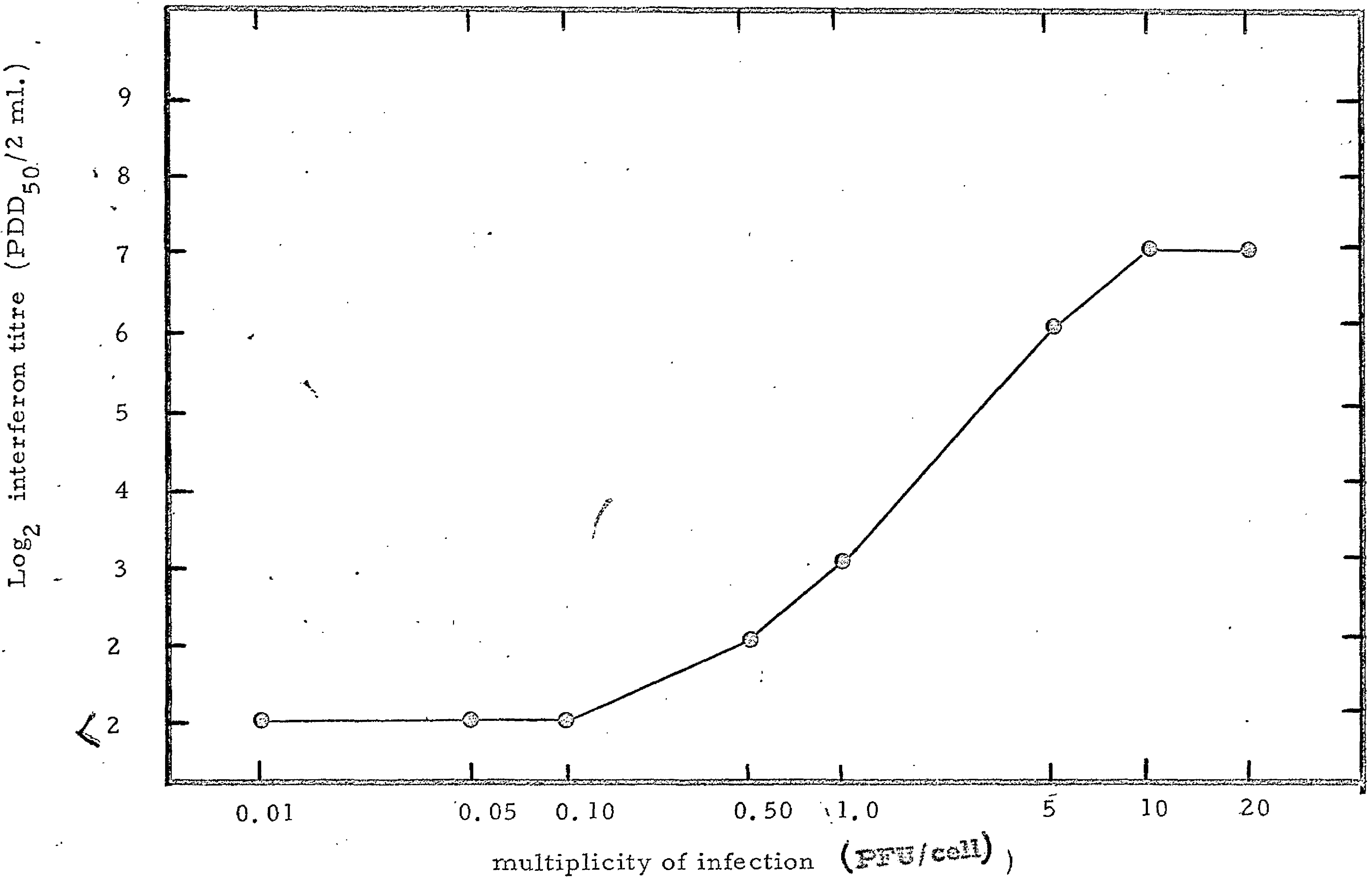


Figure Vb. Multiplicity requirement for interferon induction by polyoma virus.

was stable at pH2 for 48 hours, heat stable, non-sedimentable (100,000 g for 2 hours), not neutralised by polyoma specific antiserum, trypsin-sensitive and species specific, as described for the adenovirus induced interferon in Chapter II. These properties of the interferon are outlined in Table Vc.

Effect of cytosine arabinoside (CAR) treatment of cells on interferon production of cells infected with polyoma virus.

Addition of the DNA synthesis inhibitor, CAR, to CEF cell cultures infectddwith CELO virus (avian adenovirus) or human adenovirus type 7 or 12* (Markovits and Coppey, 1971, 1972; Bakay and Burke, 1972). These results indicated that no DNA synthesis is necessary for interferon induction with adenoviruses an CEF cells. In order to determine if interferon induction with polyoma virus required viral DNA synthesis, CAR was added at 20 and 40 μg/ml. The results of one experiment are given in Table Vc[1] and it can be seen that even at the dose where virus replication is generally inhibited (40 μg/ml) interferon is produced to the same level as in

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* does not inhibit interferon production

CYTOSINE ARABINOSIDE μg/ml	INTERFERON YIELD (PDD_{50}/2 ml.)
0	128
20	128
40	128

TABLE Vc[1]. Effect of cytosine arabinoside on interferon induction by polyoma birus on CEF cells.

non-treated cultures. The result suggests that polyoma DNA synthesis, if there is any in CEF is not necessary for interferon induction.

Interferon inducing capacity of purified polyoma virus "full" and "empty" particles.

After infection of MEF with polyoma virus, 4 different types of particles emerge in the yield (Yelton and Aposhian, 1972). Infectious polyoma virions which contain complete viral DNA, the so-called "full" particles, defective polyoma virions, containing only 50-70% of the viral genome, polyoma pseudovirions, containing fragments of cell DNA encapsidated within the polyoma capsid coat and finally "empty" particles which lack any DNA, and consist solely of polyoma capsid. The latter 3 types are non--infectious. "Empty" and "full" particles of polyoma virus can be separated by density gradient centrifugation on caesium chloride because of density differences (Crawford, Crawford and Watson, 1962). To determine if the empty shells of polyoma virus possess interferon inducing capacity on CEF cells "empty" and "full" particles of polyoma virus were separated and purified by twice

Treatment	Interferon titre (PDD_{50}/2 ml.)	
	Before treatment	After treatment
pH 2(48 hours at + 4°C).	128	64
Heat treatment, at 56°C for 30 min.	128	128
at 60°C for 1 hour.	128	128
at 70°C for 10 min.	128	64
Trypsin	256	<4
Ultracentrifugation (100, 000 x g, 2 hours)	64	64
Species Specificity	on CEF	on BHK21/C13
	256	<4

TABLE Vc. Properties of the polyoma virus induced interferon on chick embryo cells.

banding in caesium chloride density gradients. A total particle to pfu ratio of the crude non-purified polyoma preparation was around 50 to 70. The purified "full" and "empty" bands were examined using the electron microscope by Dr. E. A. C. Follett. Counts showed that "full" bands consisted of 99.5% "full" particles and "empty" bands were made up of 99.75% "empty" particles. (Table Vd.). These preparations were assayed for infectivity by plaque formation on MEF. The "empty" and "full" particle preparations were used for interferon induction on both primed and non-primed CEF cells. Interferon induction was carried out at particle multiplicities of 700 and 1400/cell which gave multiplicities of 20 and 40 pfu/cell respectively with the "full" preparation, and 0.002 and 0.004 pfu/cell with "empty" particles. The results of particle counts, infectivities and interferon inducing capacities of "full" and "empty" particles are shown in Table Vd . The empty particles completely failed to induce interferon even on primed cells, while the equivalent amount of full particles induced high levels of interferon on both primed and non-primed CEF cells. Therefore when polyoma virus particles are used only complete infectious polyoma virus particles can

induce interferon in CEF cells and "empty" particles do not.

Interferon induction on continuously growing cultures of CEF cells by polyoma virus and adenovirus type 5.

In the interferon induction experiments described so far in this Chapter and in other Chapters, either primary or secondary CEF cultures were used. While this work was in progress a continuously-passaged line of CEF cells was established by Dr. J. F. Williams (line CEF-A). These cells were tested for production of interferon after infection with polyoma and adenovirus type 5 and found to produce normal levels even after 12 passages. This continuously passaged CEF line grew successfully until around 20th passage, after which the cells tended to degenerate. Subsequently, I established another line from chick embryo, called CEF-B. Interferon was also produced by CEF-B in response to adenovirus type 5 and polyoma virus infection. Table Ve illustrates the yields of interferon obtained at certain passages of the two continuous lines of CEF cells infected by polyoma virus or adenovirus type 5. Both lines, produce normal levels of interferon, comparable to the levels usually found in CEF primary or secondary cultures.

Virus	Full particles/ml.	Empty particles/ml	Infectivity (p.f.u./ml)	Multiplicity of infection			Interferon titre *	
				pfu/cell	fulls/cell	empties/cell	primed CEF	non-primed CEF
Full band	7.0×10^{11}	3.5×10^{9}	2.0×10^{10}	20	700	3.5	256	64
	"	"	"	40	1400	7.0	1024	128
Empty band	1.5×10^{8}	6.0×10^{10}	2.0×10^{6}	0.002	1	700	<4	<4
	"	"	"	0.004	2	1400	<4	<4

*PDD_{50}/2 ml.

TABLE Vd Interferon induction with Full and Empty polyoma particles on CEF cells.

DISCUSSION

DNA viruses, with the exception of adenoviruses, are considered to be poor inducers of interferon. Polyoma virus, a small oncogenic mouse virus, replicates on cells derived from mouse embryos, but the amount of interferon induced in these cells is very low, and interferon appears in the medium of the infected cultures only several days after infection (Allison, 1962). The reason for the low interferon yields in mouse cells is not yet known. However, interferon induction on non-permissive hamster cells infected by polyoma virus has been reported to be relatively high in titre and appearance is slightly earlier (Talas, Weiszfeiler and Batkai, 1968; Gotlieb-Stematsky and Vansover, 1970). In relation to this, it is worth noting that adenoviruses fail to induce any detectable interferon in human and hamster cells (Ho and Kohler, 1967) in which they can readily replicate, but high titres of interferon are induced in CEF where human adenoviruses fail to replicate. It may be that non-permissiveness of the host cell allows for greater production of interferon. With this in mind, polyoma virus small plaque variants were tested for their capacity to induce interferon and high levels of interferon were obtained. Interferon was

Passage number	CEF - A		CEF-B	
	Ad. type 5	Py	Ad. type 5	Py
Primary			128 *	256
2				128
4				64
5				128
6				256
7	128	64		128
9	32			
10				64
12	64	128	64	128
14	64			
15				64
20			32	64

TABLE Ve. Interferon induction by polyoma virus in cultures of the continuous CEF-A and CEF-B lines.

* Interferon titre (PDD_{50}/2 ml.)

detectable in the culture fluid of infected cells 32 hours after infection and reached a maximum level by 72 hours after infection. Therefore, this sytem is potentially useful as a simple model to study the mechanism by which a DNA virus induces the interferon response.

Polyoma virus fails to replicate in CEF cells, and while there is no information at present concerning the interaction of polyoma virus with CEF cells, it does seem unlikely that DNA synthesis, even if it takes place in CEF cells is important to induction. Treatment of cells with cytosine arabinoside (CAR), which inhibits DNA synthesis in concentrations up to 40 μg/ml does not affect the interferon yields This was also found for interferon production by adenoviruses (Markovits and Coppey, 1971, 1972; Bakay and Burke, 1972).

Interferon induction by polyoma virus on CEF cells was found to be multiplicity dependent, and the amount of interferon induced is directly related to the input virus multiplicity. In this respect, adenovirus type 5 also shows a similar requirement for interferon response on CEF cells.

Thus, it is shown that interferon induction in this system does not require virus multiplication and most likely viral DNA

synthesis is not necessary. While it is not known whether or not polyoma virus uncoats in CEF cells, it is likely either an input virus component or viral function(s) expressed in these cells,are reponsible for interferon induction.

The "empty" and "full" particles of polyoma virus separated and purified by density gradient centrifugation were teste for their capacity to induce interferon in CEF. "Empty" shells are morphologically similar to "full" particles under the electron microscope, but do not contain viral DNA and are non-infectious. They failed to induce interferon, while an equal quantity of "full" infectious particles which possess viral DNA induced interferon on CEF cells. These results suggest that the protein coat of the virus is not directly involved, and complete infectious particles are required for interferon induction. Nevertheless, it cannot be excluded that "empty" shells no longer possess the same structural conformation necessa to stimulate the interferon response or that alternatively they may fail to adsorb to or penetrate into CEF cells. However, one must consider that infectious polyoma DNA may be necessary for interferon induction in CEF cells.

It may be that virus uncoats on CEF cells and certain early viral functions are expressed, such as viral specific double-stranded RNA synthesis. However, the inducer of interferon formation could be the viral DNA itself. There is evidence that T4 coliphage induces interferon (Kleinschmidt, 1970), while virus ghosts or DNA extracted from virus fail to do so *in vivo* and it has been suggested that configurationally active DNA may be needed for interferon induction. It remains to be tested if infectious polyoma virus DNA alone can induce interferon response, on CEF cells. In addition the temperature-sensitive mutants of polyoma virus are available (Eckhart, 1969) and can be used for identification of these functions.

Polyoma virus could be further studied for its interaction with CEF cells, and the establishment of the viral events taking place after infection which leads to the interferon induction may eventually help to determine which factors are involved in this process.

CHAPTER VI

PRIMING: EFFECT ON ADENOVIRUS TYPE 5 AND POLYOMA VIRUS INDUCED INTERFERON ON CHICK EMBRYO CELLS.

1) INTRODUCTION

2) MATERIALS AND METHODS

3) RESULTS

a) Enhancement of interferon yields by priming of CEF cells subsequently infected with adenovirus type 5 and polyoma virus.

b) Effect of different priming doses on interferon yields on CEF cells infected with adenovirus type 5 or polyoma virus.

c) Effect of priming on the kinetics of interferon production in CEF cells infected with adenovirus type 5 or polyoma virus.

d) Priming and minimum multiplicity requirement for interferon induction on CEF cells by adenovirus type 5 and polyoma virus.

e) Effect of priming on interferon induction by wild type adenovirus 5, ts-18 and ts-19 on CEF cells at permissive and non-permissive temperatures.

4. DISCUSSION.

INTRODUCTION

Pretreatement of cells with interferon results in the enhancement of their interferon producing capacity following subsequent virus infection and this enhancement is known as priming. The amount of interferon produced by primed cells is much higher than that produced by control cells which are not pretreated with interferon. Priming was first discovered by Isaacs and Burke (1958), when they found that interferon pretreated chick chorioallantoic membrane produced much greater interferon yields than non-treated membrane in response to live influenza virus. In addition to increasing interferon yields, priming brings about earlier production of interferon in chick embryo cells infected with Semliki Forest virus and Chikungunya virus (Friedman, 1966; Levy Buckler and Baron, 1966). The dose used to prime cells is important since pretreatment with very high doses of interferon inhibits it subsequent production (Vilcek, 1962; Vilcek and Rada, 1962). Priming also occurs on L cells and dose dependent responses have been reported by Lockart (1963) and Friedman (1966). More recently it has been shown that Encephalomyocarditis (EMC) virus MM strain does not produce any interferon on L cells unless the cells

are primed (Stewart, Gosser and Lockart, 1971b). It is not yet known how interferon operates in priming. One feature of priming is that it is species specific, a well-established feature of the antiviral effect of interferon. Thus, interferons can only prime the cells of those species in which they are produced. Cells exposed to optimum doses of interferon are fully primed in two hours, even in the presence of protein synthesis inhibitors, but antiviral activity also develops if the cells are exposed to interferon for longer than two hours (Stewart, Gosser and Lockart, 1971b). These findings support the view that priming is a non-antiviral function of interferon, but conflict with the results of Friedman (1966) who found that chick cells failed to become primed in response to Chikungunya virus in the presence of the protein synthesis inhibitor p-fluorophenylalanine (FPA). It may be that L cells and chick cells behave in a fundamentally different way in this respect, but the reason for this is not yet known.

A priming effect on adenovirus and polyoma virus induced interferon yields has not been reported before, and experiments will be described in this chapter concerning priming on the induction of interferon by these viruses. This study was extended to determine the optimum doses of interferon for the priming effect, kinetics

of interferon induction and multiplicity requirements for interferon induction on primed and non-primed cells infected with adenovirus type 5 and polyoma virus. The priming effect also has been tested on two ts mutants of adenovirus type 5, ts 18 and ts 19, which were not able to produce interferon at the non-permissive temperature (380), and they were found to produce reasonable levels of interferon at the non-permissive temperature (38°C) and enahced levels at permissive temperature (31°C) on primed chick embryo cells.

MATERIALS AND METHODS

Cell cultures. Primary CEF cells were prepared as described in Chapter II .

Viruses and virus assays. Wild type adenovirus 5 was grown and assayed as described in Materials and Methods in Chapter II For the growth and assay of *ts* 18 and *ts* 19 see Chapter II Polyoma virus was grown and assayed on MEF cells as described in Chapter V .

Interferon production and assay. As described in Chapter

Priming. Primary CEF cells were pretreated with priming doses (usually 20 units/dish)2 of interferon in 2 ml. of Eagle's medium supplemented with 2% calf serum, for 12-15 hours (usually overnight). They were then washed once with Eagle's medium, virus added at the indicated multiplicity (pfu/cell) and adsorbed for 2 hours at 37°C. Then the cells were washed with Eagle's medium again and Eagle's medium supplemented with 2% calf serum was added. Plates were incubated at 37°C (or 31°C and 38°C in the case of the *ts* mutants and several times after infection medium was harvested and later assayed for interferon on CEF cells.

RESULTS

Enhancement of interferon yields by priming of CEF cells subsequently infected with adenovirus type 5 and polyoma virus.

CEF cells were pretreated overnight with 20 units of interferon as described above. Controls were treated with Eagle's medium plus 2% calf serum. After treatment, medium was withdrawn, cells were washed with Eagle's medium, and infected with either adenovirus type 5 or polyoma virus at 20 pfu/cell. Virus was allowed to adsorb for 2 hours at 37°C, cells were washed once again with Eagle's medium and overlaid with Eagle's medium supplemented with 2% calf serum. Cultures were incubated for 3 days at 37°C, then fluids were harvested and interferon levels were measured. Table VIaillustrates the results of the priming effect on adenovirus type 5 and polyoma virus induced interferons. In primed cells as seen in the Table VIa, adenovirus induced 8 times more and polyoma virus induced 4 times more interferon than in control, non-treated cells.

Effect of different priming doses on interferon yields on CEF cells infected with adenovirus type 5 or polyoma virus.

CEF cells were incubated overnight with 0.001, 0.005, 0.01,

Pretreatment (18 hours)	Infecting virus (20 pfu/cell)	Interferon yield (PDD_{50}/2 ml.)
None	Adenovirus type 5	128
Interferon (20 units)	Adenovirus type 5	1024
None	Polyoma	512
Interferon (20 units)	Polyoma	2048

TABLE VIa **Effect of priming on adenovirus type 5 and polyoma virus induced interferons.**

0.05, 0.1, 0.5, 1, 5, 10, 20, 40 and 100 units of interferon as before. These primed cells were then infected with either adenovirus type 5 or polyoma virus at a m.o.i. of 20 pfu/cell. Infected plates were incubated at 37°C for 3 days, medium was collected and interferon levels were measured. The effect of different primingsdoses on interferon yields from CEF cells infected with adenovirus type 5 or polyoma virus is illustrated in Figure VIa. The minimum dose of interferon bringing about enhancement was found to be 0.5 units in CEF cells infected with either virus. The optimum dose for priming is around 10 units per dish; no further enhancing effect is obtained with higher doses. As shown in Figure VIa the maximum doses of priming interferon enhanced the final interferon yield to 16 times that of the control level in adenovirus type 5 infected and to 18 times that of the control in polyoma virus infected CEF.

Effect of priming on the kinetics of interferon production in CEF cells infected with adenovirus type 5 or polyoma virus.

CEF cells were primed with 20 units of interferon per dish for 18 hours before infection with either adenovirus type 5 or polyoma virus at a multiplicity of 20 pfu/cell. Several times after

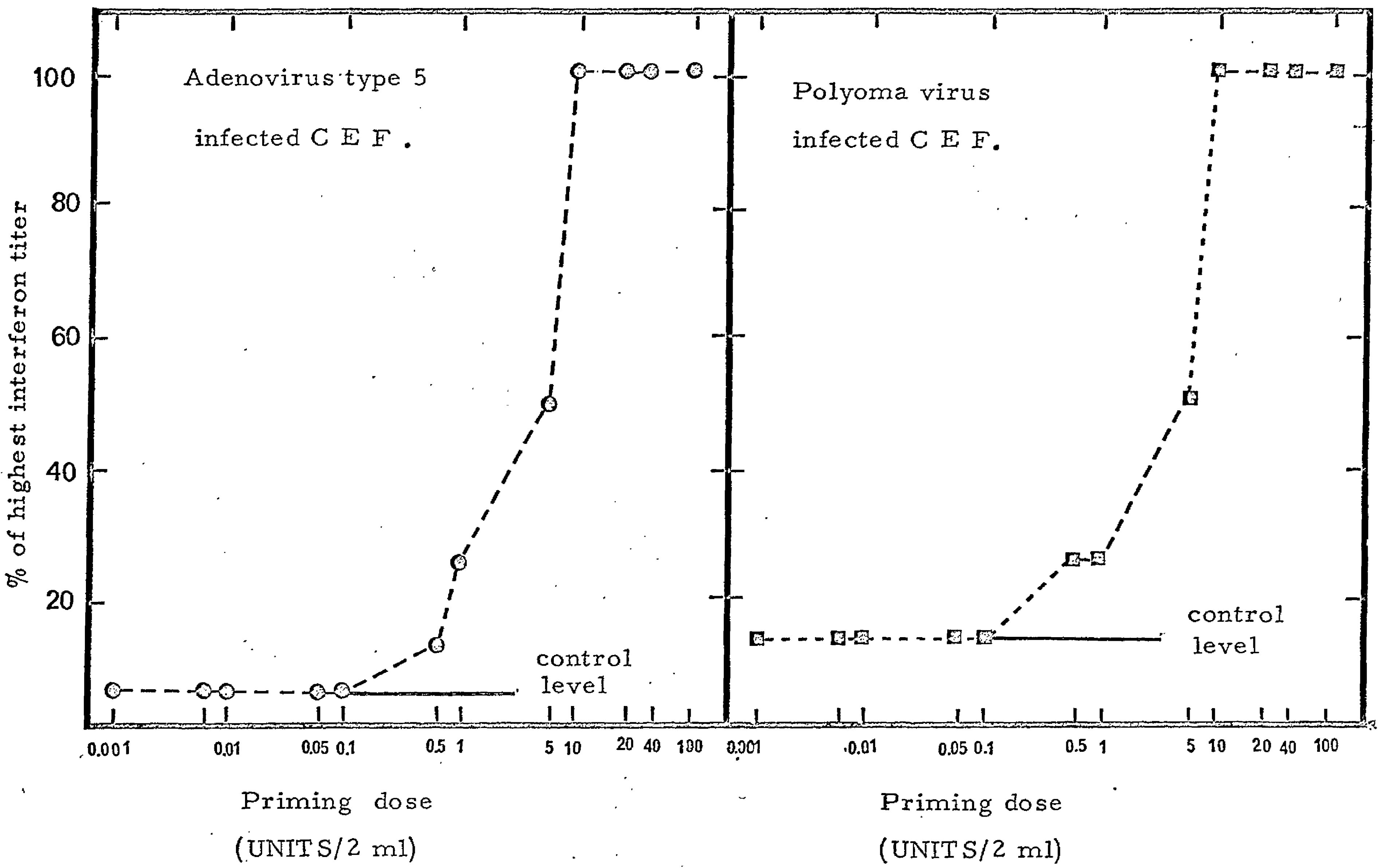

Figure VIa Effect of different priming doses on interferon yields on CEF cells infected with adenovirus type 5 or polyoma virus.

infection, medium was removed from infected cultures and interferon titres were measured. In non-primed adenovirus infected cells interferon first appeared about 15 hours after infection, while in primed cells interferon appeared about 8 hours after infection as is shown in Table VIb. In polyoma infected CEF the first interferon can be detected in the medium of infected cultures 28 hours after infection, but if the cells are primed interfer[illegible] can be detected as early as 12 hours after infection (Table VIc). As found previously the final interferon titres obtained in primed cells infected with adenovirus type 5 and polyoma virus were 4-8 fold highe[illegible] than the control interferon levels (Table VIa).

Priming and minimum multiplicity requirement for interferon inducti[illegible] on CEF cells by adenovirus type 5 and polyoma virus.

CEF cells were primed with 20 units of interferon per dish. Primed and non-primed cells were infected with different multiplicities of either adenovirus type 5 or polyoma virus and incubated at 37°C for three days. Medium from infected cultures was collected at this time and assayed for interferon. The results are given in Tables VId and VIe . In adenovirus type 5 infected CEF cells the lower limit of multiplicity for minimum interferon [illegible] induction in control cells is 0.5 pfu/cell, while in primed cells it is

Time after infection (hours)	Interferon titre (PDD_{50}/2 ml.)	
	Primed cells	Control cells
5	< 4	< 4
8	4	< 4
12	8	< 4
15	8 < 16	4
18	16	4 < 8
24	32	8
28	32 < 64	16
32	128	32
44	256	64
72	1024	128
96	1024	128

TABLE VIb Kinetics of interferon induction by adenovirus type 5 on primed and non-primed CEF cells.

Time after infection (hours)	Interferon titre (PDD_{50}/2 ml.)	
	Primed cells	Control cells
8	< 4	< 4
12	4	< 4
15	8	< 4
18	16	< 4
24	32	< 4
28	32 < 64	4
32	64	8
44	128	32
50	128 < 256	64
68	512	128
75	1024	128 < 256
96	1024	128

TABLE VIc Kinetics of interferon induction by polyoma virus on primed and non-primed CEF cells.

Multiplicity of Infection (p.f.u./cell)	Interferon Titre (PDD_{50}/2 ml.)	
	Primed Cells	Control Cells
0.01	<4	<4
0.05	4	<4
0.1	8	<4
0.5	32	8
1	128	16
5	512	64
10	1024	128
20	1024	128

TABLE VId Multiplicity requirement for interferon induction by adenovirus type 5 on primed and non-primed CEF cells.

Multiplicity of Infection (p.f.u./cell)	Interferon titre (PDD / 2ml.)	
	Primed cells	Control cells
0.01	<4	<4
0.05	$4<8$	<4
0.1	8	<4
0.5	16	4
1	64	16
5	512	64
10	1024	128
20	1024	128

TABLE VIe Multiplicity requirement for interferon induction by polyoma virus on primed and non-primed CEF cells.

0.05 pfu/cell. Also the lower limit for interferon induction on CEF cells with polyoma virus is 0.5 pfu/cell, while in primed cells it is 0.05 pfu/cell.

Effect of priming on interferon induction by wild type adenovirus 5, ts 18 and ts 19 on CEF cells at permissive and non-permissive temperatures

In these experiments CEF cells were primed with 20 units of interferon per dish prior to infection with wild type adenovirus 5, ts 18 or ts 19 at a multiplicity of 20 pfu/cell. Infected cultures were divided into two sets, one was incubated at 31°C for 5 days and the other at 38°C for 3 days. Medium was collected from infected cultures at these times and interferon levels were measured. TableS VI gives the results of a priming experiment on CEF cells with wild type, ts 18 and ts 19. At 31°C all three induced normal amounts of interferon on control cells, and enhanced levels of interferon on primed cells. At 38°C wild type induced a normal amount of interferon, but ts B and ts 19 failed to induce interferon in non-primed cells as previously found. In primed cells at 38°C on the other handm ts 18 and ts 19 induced yields of interferon comparable to those produced at 31°C without priming and wild type

Pretreatment	Wild type Adenovirus 5		*ts* 18	
	31°	38°	31°	38°
None	64	128	32	< 4
Interferon (20 units)	512	512	128	64

Pretreatment	Wild type Adenovirus 5		*ts* 19	
	31°	38°	31°	38°
None	128	128	64	< 4
Interferon (20 units)	512	512	256	64

TABLE VIf Interferon induction by wild type adenovirus 5, ts 18 and ts 19 on primed and non-primed CEF cells.

gave an enhanced yield. These experiments clearly showed that while *ts* 18 and *ts* 19 are defective in interferon induction at 38°C on CEF, if the cells are primed they do induce interferon at this temperature.

DISCUSSION

The experimental results presented in this chapter show that pretreatment of CEF cells with moderate levels of interferon (priming) experts a definite enhancing effect on subsequent induction of interferon by adenovirus type 5 and polyoma virus. There are many previous reports that different viruses (both active and inactivated) are able to induce enhanced interferon yields after priming (Burke and Isaacs, 1958; Ho and Brieng, 1962; Mandy and Ho, 1964). In addition, interferon yields induced by synthetic polynucleotides (poly rI; poly rC) are enhanced by priming (Stewart, Gosser and Lockart, 1971b). In this respect, it seems likely that priming operates on cells regardless of the type of virus or inducer used.

Under the conditions described in this chapter, the minimum priming dose was found to be 0.5 units/dish and optimum dose was 10 units/dish. Between these two doses the amount of interferon produced was directly related to the priming dose used. Priming doses higher than 10 units/dish and up to 100 units/dish do not change the final yield. However, although a certain input dose of inter feron is required for

optimum priming response, it is known that when larger amounts of interferon are used for pretreatment an inhibitory effect on subsequent interferon induction results in (Lockart, 1963; Friedman, 1966).

After priming, interferon is produced considerably earlier by cells, (Friedman, 1966; Levy, Buckler and Baron 1966), and the same effect has been found here in adenovirus type 5 and polyoma virus on CEF cells. In this reppect, this is the first time that there is a clear demonstration of shortening of t time required for interferon induction. It is difficult to assess from the present data how priming brings about the earlier interferon production, but it may be relevant that the messenger-RNA for interferon appears to be made earlier in primed cells (Friedman, 1966) on the basis of experiments involving actinomyc: D resistance of interferon production.

This is the first time it has been shown clearly that there is a reduction of multiplicity requirement in primed cells. The minimum multiplicity required for interferon induction by adenovirus type 5 and polyoma virus on CEF cells is 10 times lower than on control cells. These differences could be due

to one or more of several different factors. It is possible that priming enhances the uptake or penetration of the inducer virus particles. However, preliminary experiments carried out using ^{32}P-labelled adenovirus type 5 showed that the rate of uptake of virus is about the same in both primed and non-primed cells. Alternatively interferon may sensitize the receptor site in cells which recognise the inducer molecule even when it is in very small quantity in cells. Another possibility is that the affinity of inducer molecule for the receptor site is enhanced in primed cells.

Levy, Buckler and Baron (1966) have proposed that priming may work in one of two ways. First, after priming the cell is modified in a way which facilitates the steps required before the induction event, such as derepression. Secondly, primed cells may modify and process the infecting virus in some way so that the virus is a much more efficient induc of interferon formation. However, the proposals have not been proved experimentally. Another hypothesis put forward is that interferon induction is a process requiring multiple steps and that priming replaces or removes one or more of those steps,

so that the interferon induction period is shortened (Stewart, Gosser and Lockart, 1971). However this hypothesis does not explain the enhanced levels attained after priming.

The _ts_ mutants of adenovirus type 5, _ts_ 18 and _ts_ 19, are defective in interferon induction on CEF cells (Chapter III) at the non-permissive temperature, but are able to induce at the permissive temperature. It has been shown in this chapter that priming can overcome the failure of these two mutants to induce interferon at the restrictive temperature so that after priming they are able to induce interferon at the restrictive temperature. It is not possible to explain at present how priming compensates for the defective function(s) of _ts_ 18 and _ts_ 19 which are required for interferon induction. However, it is possible that the defective function of the _ts_ mutants may be required for one of the proposed multiple steps prior to derepression. It is hoped that the subsequent characterisation of the defective function(s) of _ts_ 18 and _ts_ 19 may reveal which viral factors are involved in both priming and interferon induction in primed cells.

From the present data, it is feasible to speculate that

priming may act on a purely cellular function(s) necessary for interferon induction. For example, it may act on inducer receptor or inducer recognition site which are presumably cellular. Therefore either more receptor sites are available for the inducer molecule or alternatively they are sensitised in some way in primed cells. It may be that priming acts indirectly on a viral function required for induction. For examples, it may replace or remove certain steps prior to induction, so that certain virus function (s) are not required prior to the depression event in primed cells.

CONCLUDING REMARKS

The work described in this thesis is concerned with the problem of how DNA viruses induce interferon in chick embryo cell Basically , the problem can be stated as an enquiry into the virus function(s) necessary for inductionmand a number of biological, biochemical and genetical approaches have been used to probe this problem. Two viruses of differeing complexity, human adenovirus type 5 and polyoma virus were chosen as models for investigation. These viruses were found to be efficient inducers of interferon in chick embryo cells *in vitro*, and were considered to be very suitabl for such an investigation.

The following concluding remarks are intended to sum up the findings reported in this thesis, to discuss the relevance of thes findings to the work of others, to the problem stated above, and to future studies in this field.

It is known that adenovirus does not complete a replication cycle in CEF cells, so that virus progeny is not required for interferon induction. In fact the block to replication is presumably prior to viral or at the initiation of viral DNA synthesis, since this does not occur in CEF cells. In addition, the time course of induction in either primed ornnon-primed cells indicates that the

events leading up to interferon induction are early in relation to the growth cycle on Hela cells. Further support for the view that early viral events are involved in induction comes from the temperature shift-up experiments with ts 18 on CEF cells.

Accepting that all the evidence points to an early virus function we still cannot say whether the inducer resides in a component of the input virion or in a product of early transcription of the adenovirus genome in CEF cells. Results presented in chapter III show that ts 18 and ts 19 are considerably less heat stable than wild type virus, which suggests that these mutants have a defect in a structural component of the virion. However, as pointed out in the discussion of that chapter, the ts and hs phenotypic characteristics of these mutants may not result from the same mutation, and further experiments are required to determine whether or not this is the case. Assuming for the moment that these characteristics do result from the same mutation then one can postulate either a direct or an indirect role of the structural component in induction.

The structural component might reside either in the capsid or may be an internal component of the virion. A possible candidate in the first category is the penton antigen of adenovirus, as first proposed by Beladi and Pusztai (1967). Their proposal

was based simply on the finding that the interferon inducing capacity of the virus is destroyed by trypsin treatment, and the present work (chapter II) confirms this result. However, as previously argued, components other than penton base are also trypsin sensitive, and this result alone cannot be taken as positive proof for penton involvement. In addition, the ts mutants defective in induction of interferon, ts 18 and ts 19 show no apparent lesion in the synthesis or structure of penton base antigen in Hela cells (Russell, Newman and Williams, 1972; W. C. Russell, personal communication), but one should bear in mind that only defects giving rise to immunological differences or altered behaviour on acrylamide gels would be detected by the methods used.

Of course, if the penton base, or some other capsid component were involved in induction, it may do so indirectly, rather than directly. It may be that a defect in such a component prevents either complete penetration or uncoating of the virus, so that some internal component can function in CEF cells. There is evidence that adenovirus type 5 penetrates and uncoats in CEF cell (B. Senior, personal communication) but as yet we do not know if ts 18 and ts 19 are defective in such events at 38°C, and we cannot really say if these events are necessary for induction to take place.

Temperature shift-up experiments in CEF cells suggest that adsorption is not the blocked event, although in the absence of evidence concerning viral events at 31°C in these cells, again one cannot rule out the possibility that some stage in either penetration or uncoating is blocked.

As an alternative to the proposals outlined above, one most also consider that an internal component of the virus is involved in interferon induction. Again, this could be a direct or an indirec involvement. The internal core proteins of the virion are candidates in this class, and it is conceivable that one of them might be the inducer. More likely, however, is the possibility that these act indirectly in connection with their probable role in stabilising the configuration of the viral DNA. A defect in one of these proteins could result in instability of the structure of the viral DNA, so that the latter does not act as an efficient inducer. Of course there is no solid evidence that viral DNA itself can act as an inducer, and it is more likely that RNA made on the viral DNA template is the inducer. In this case, certain configurations of the viral DNA may be essential for ealy transcription to take plac and it may well be that one or other of the core proteins is essentia to this process. It should be pointed out, however, that neither ts

nor ts 19 shows any alteration in the pattern of synthesis of any virion proteins, including core 1 and core 2 in Hela cells (W. C. Russell, personal communication), although of course, minor defec might be overlooked by the analytical methods used.

In summary, there is no direct evidence for involvement of any protein component of the virion in interferon induction, althoug this possibility cannot be completely ruled out at present. How about the nucleic acids of the virus, at either the viral DNA or the virus-specified RNA? It was suggested in the paragraph above that the DNA of the virus is perhaps the inducer. Evidence for direct involvement of viral DNA is not available, and one can simply speculate about this point at present. It has been shown that empty particles of T4 coliphage (Kleinschmidt et al. 1970) and empty particles of polyoma (chapter V this thesis) fail to induce which can be interpreted to mean that DNA is required for inductio However T4 DNA and synthetic deoxyribopolynucleotides induce interferon very inefficiently (Colby and Morgan, 1971) although this test has not yet been carried out with polyoma, DNA, nor with adenovirus DNA. However, even allowing for a positive result one would then have to consider the next possibility, namely that vir RNA is transcribed from the DNA and that the former acts as induc

As discussed previously in this thesis there is a considerable body of evidence to support the view that double-stranded RNAs (Field et al. 1967) and to a lesser extent single-stranded RNAs (Baron et al, 1969) are efficient inducers of interferon in eukaryotic cells. Synthetic polyribonucleotides are particularly good inducers (Field et al 1967). It has also been found that a virus-specific double-stranded RNA accumulates in Vaccinia virus infected CEF cells, and that this RNA promotes interference against Sindbis virus in these cells (Colby and Duesberg, 1969). Attempts to detect such virus-specific RNA in adenovirus infected CEF cells have been reported to be unsuccessful (Bakay and Burke, 1972), although confirmation of the vaccinia situation was obtained. More recently, in our laboratory, evidence has been found for significant increases in double-stranded RNA in CEF cells infected with boht adenovirus type 5 and polyoma virus (B. Senior and P. Austin, personal communication). It remains to be determined if this RNA is
communication). It remains to be determined if this RNA is
virus-specified and it must be shown that treatment of cells with it
ts 18 and ts 19 should prove to be extremely useful in analysing the ro
of this double-stranded RNA in interferon induction.

Metabolic inhibitors have been used by others, and in this work,

to probe interferon induction by viruses. It has been shown that actinomycin D (Heller, 1963, Taylor, 1964) and protein synthesis inhibitors such as puromycin and parafluorophenylalanine inhibit production of interferon, showing that interferon induction or production depends upon new RNA and protein synthesis in cells. On the other hand the DNA synthesis inhibitor, cytosine arabinoside has been shown to have no effect on either adenovirus induced interfe (Markovits and Coppey, 1971, 1972; Bakay and Burke, 1972), or polyoma virus induced interferon synthesis (chapter V, this thesis). Of course, these inhibitors act on both cell-directed and viral-directed processes, and are not too useful in dissecting out the viral events in induction. During the early part of the work reposted here it was hoped that the antiviral agent, rifampicin, might prove to be a useful biochemical probe in analysing interferon induction. Experiments using the drug were reported in chapter IV., and will not be outlined again here. It is sufficient to repeat that rifampicin appears to inhibit interferon production, either by acting on a viral function not essential to virus replication or on a cellular function. The exact mode(s) of action of rifampicin on virus replication or on cell growth is not known at present, and one cannot really say how it inhibits interferon production. However, as other specific inhibitors of viral synthesis (or inactivators of virions) become

available, they will undoubtedly be useful in investigating the problem of interferon induction.

During the progress of the work with adenoviruses, it was considered that a simple, small, DNA virus might provide a useful analytical model system for examining interferon induction. Accordingly, polyoma virus was examined in this respect, and turned out to be a very potent inducer (chapter V of this thesis). It is worth noting that another small papova virus, SV40 completely failed to induce interferon on CEF cells (J. F. Williams, personal communication). These two viruses are similar in size and chemistry, but they do differ very much in host range characterisatio and therein may lie the explanation for their different activities. SV4 may be inactive simply because CEF cells are a completely non-permissive host. Of course CEF cells are also a non-permissiv host for polyoma virus, but the block in the series of viral events may differ for the two viruses. Although there is no clear proof of this, I suggest that SV40 fails to penetrate or even adsorb to CEF cells, while polyoma virus proceeds past these steps and enters the cells.

The induction of interferon by polyoma virus and adenoviruses in CEF cells is somewhat similar. However, polyoma appears to be a more efficient inducer, in that lower multiplicities (of p. f. u.) induce, although interferon takes a little longer to appear in infected

cells. As pointed out above, and as is the case for the adenoviruses, polyoma DNA synthesis is not required for induction CEF cells. Thus, either a component of the input virus, or a product of early virus-specified synthesis is the likely inducer, much the same as is the situation for adenoviruses. A preliminar attempt to determine if a capsid component might be involved in sor way was made by comparing the inducing capacity of empty and full particles. The results indicate that only full particles can induce, and imply that an internal component, perhaps the DNA, is necessary for induction. Of course, an outer coat component may be necessary and sufficient, but in an unsuitable configuration in the empty particle. The experiments carried out to date cannot distinguish between these possibilities, and further work of both a biochemical and a genetic nature will be necessary. In this respec ts mutants (Eckhart, 1969) may be extremely useful.

I would like to terminate these concluding remarks with a few comments concerning the phenomenon of "priming" discussed already in chapter VI of this thesis. Priming, the enhancement of interferon production by prior treatment with low levels of interferon, has obvious practical advantages. Large amounts of interferon can be produced where previously there was only low

levels of production. Quite apart from this, however, priming is a separate property of interferon preparations which deserves study in its own right. It is unlikely to be simply a laboratory phenomenon and it is probable that it plays an role in the control of virus infection in the animal.

Studies involving the use of RNA and protein inhibitors indicate that the priming and antiviral activities of an interferon preparation are separate. However, there is no really solid evidence that the two activities reside in the same interferon molecule. The difficulties encountered in purifying interferon no doubt contribute to this problem, and it is quite possible that the two activities are properties of separate molecular populations.

The mechanism by which interferon preparations enhance subsequent induction by virus infection is not known. A number of alternative modes of action are possible. One can think of the induction process as a series of events, some viral and some cellular, and priming may act on one or more of these events, either modifying it to provide greater efficiency, or removing the need for such an event. 1) The priming activity may operate purely on cellular functions, independent of viral event so that they in turn operate more effectively subsequent to infectio

to produce more interferon. 2) The activity may operate on a cell process necessary to one or more of the viral events occurring in CEF cells and required for induction, in other words an indirect effect on a viral event.

3) The activity may operate directly on a viral event necessary for induction. At present it is not possible to determine which of these (or if all of these) possibilities operate. Further investigations with _ts_ 18 and 19 may provide clues to the mechanism of priming, since priming largely removes the temperature-sensitive block to induction observed for these mutants at 38°C.

In conclusion, it is fair to say that the methods used in this investigation provide suitable avenues of approach to some of the basic problems outlined in the introduction and in this discussion. Therefore, I hope that the system will be used in the future to provide further information about induction of interferon by DNA viruses.

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Page Number	Line	Correction	
2	17	Finter, 1960	Finter, 1969
8	17	Kleinschmidt, 1970	Kleinschmidt et al, 197(
19	11	Williams et al, 1971	Williams et al, 1971b.
20	2	Ustacelebi and Williams, 1972.	Ustacelebi and Williams, 19
23	6	Russell et al, 1967	Russell, Valentine and Pereira, 1967
24	2	Velicer and Ginsberg, 1969	Velicer and Ginsberg, 1
27	7	Eckhart, 1972	Eckhart, 1971
54	last line	Lengyel et al, 1970	Lengyel et al, 1969
38	17	Ishibashi, 1971	Ishibashi, 1970.
58	18	Williams et al, 1971	Williams et al, 1971a
61	10	Ustacelebi and Williams, 1972	Ustacelebi and Williams
98	15	Williams et al, 1971	Williams et al, 197
100	6	Ustacelebi and Williams, 1971	Ustacelebi and Williams,
100	16	Ustacelebi and Williams, 1971	Ustacelebi and Williams,
109	9	Williams et al, 1971	Williams et al 1971
113	12	Williams et al, 1971	Williams et al 1971
115	6	Heller et al, 1970	Heller, 1970
117	11	Ustacelebi and Williams, 1972.	Ustacelebi and Williams 1

118	9	Williams et al 1971	<u>Williams et al 1971a</u>
121	13	Williams et al 1971	<u>Williams et al 1971a</u>
149	7	Burke and Isaacs, 1958	<u>Isaacs and Burke 1958</u>
149	8	Mandy and Ho, 1964	<u>Mahdy and Ho, 1964.</u>
152	1	Stewart et al 1971	<u>Stewart et al 1971b.</u>

SUMMARY

The study presented in this thesis is primarily concerned with the induction of interferon by DNA viruses. Two DNA viruses, belonging to different taxonomic groups, adenovirus type 5 and polyoma virus were chosen for this study, since both were found to be very good inducers of interferon on chick embryo fibroblasts. (CEF). The conditions for peoduction, and the properties of this interferon are presented in chapters II and V. In an attempt to identify the viral functions involved in interferon induction by adenovirus type 5 and polyoma virus, a number of approaches were taken.

A genetic approach, was to use ts mutants of adenovirus type 5 isolated on Hela cells. These were tested for interferon induction on CEF cells at the permissive and non-permissive temperatures, and all except two mutants were found to be able to induce interferon at both temperatures. These two mutants, ts 18 and ts 19, belonging to separate complementation groups, were found to be unable to induce interferon at non-permissive temperature, while able to induce normal levels of interferon at the permissive temperature. Further characterisation of these mutants has been carried out both biologically and biochemically and this is dealt with in chapter III. It is clear that despite the fact that these mutants are in separate complementation

groups, they share some properties.

Investigations reported in this thesis and elsewhere indicate tha ts 18 and ts 19 both synthesize normal amounts of viral DNA on Hela cells, and synthesize all the major capsid components at both the permissive and the non-permissive temperature. Ts 18 and ts 1 are both found to be extremely heat labile relative to wild type adenovirus type 5 and other ts mutants at 50°C and 52°C, which suggests that the defect in these mutants is a strucutral one. Temperature shift-up experiments carried out on Hela cells with ts 18 suggest that the defective function is a late one. Temperature shift-u experiments carried out on CEF cells by using ts 18 suggested that th defective function is not expressed at the adsorption stage, although the defect is expressed early on CEF cells. It is considered that these mutants may be defective in penetration or uncoating on CEF cells at the non-permissive temperature. These findings are discussed in relation to interferon induction on CEF cells.

Another approach which has been taken in the use of viral inhibitors, in particular rifampicin. This drug has an inhibitory effe on the replication of adenovirus types 1 and 5, but does not inhibit the growth of type 12 or Semliki Forest virus on CEF cells. However as presented in chapter IV rifampicin depresses interferon induction o CEF cells by all four viruses to the same extent. The effect

of rifampicin on both interferon induction and cell growth was tested and it is concluded that rifampicin acts either by inhibiting a viral function which is r required for virus replication, or by affecting a cell function required for induction.

In order to test the involvement of polyoma virus components in inductio of interferon, empty and full virus particles were separated and examined for their ability to induce interferon in CEF cells. These results are presented in chapter V, and it is shown that only full (infectious) particles are able to induce interferon while empties completely fail to do so. The relationship of these findings to the possible involvement of viral DNA in induction is discussed.

Finally, the non-antiviral property of interferon preparations, namely the priming effect has been tested on induction of interferon on CEF cells by adenovirus type 5 and polyoma virus. Kinetics of induction, and the multiplic requirements for induction have been examined on primed and non-primed CEF cells, and the effect of priming on interferon induction by _ts_ 18 and _ts_ 19 at permissive and non-permissive temperature was determined. Priming results in increased levels of interferon production in cells infected with wild type adenovirus and overcomes the failure of both _ts_ 18 and _ts_ 19 to induce inteeferon. Thus priming compensates for the defective function(s) of ts 18 a ts 19 which are required for interferon production. These results are preser in chapter VI, where the possible mechanism of priming is discussed.