VIRUS GROWTH IN PRE-HEATED CELLS.

by

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VIRUS GROWTH IN PRE-HEATED CELLS.

The results reported in this dissertation are concerned mainly with the effect of supraoptimal temperatures on BHK-21, C13 cells and on their ability to support the growth of various viruses.

The term "supraoptimal temperature" was defined as any temperature above the optimal temperature of 37°C and below 47°C, above which most cells became stainable by trypan blue.

The effect of supraoptimal temperatures on the multiplication of C13 cells was tested by comparing the plating efficiency of C13 cells, after exposure to higher temperature, with that of normal cells. The plating efficiency of the cells fell gradually with the rise in temperature. Nearly 80 to 100% of cells had ceased to divide between temperatures of 45°C and 46°C.

Before studying heated cells, the characteristics of the growth cycle of a small series of DNA and RNA viruses were established for BHK-21, C13 cells. Some of these had first to be adapted to the cells.

The capacity of heated BHK-21, C13 cells to support synthesis of these DNA and RNA viruses was tested by the formation of infectious centres, by the examination of one-step growth curves, and occasionally by serological test

for synthesis of virus protein and when advantageous, by haemadsorption.

Exposure of the cells to supraoptimal temperatures made the cells insensitive to the DNA viruses. The decline in sensitivity began at about the same temperature and fell at about the same rate as the plating efficiency of BHK-21, C13 cells.

On the other hand, BHK-21, C13 cells, after exposure to supraoptimal temperatures, continued to synthesize the RNA viruses with their normal efficiency.

The findings were not limited to BHK-21, C13 cells but held also for human, chicken and mouse cells tested with one representative virus of each type.

It is suggested that one heat-sensitive biochemical process in all species could be responsible for all these findings.

ABBREVIATIONS

C13	-	BHK-21, C13 cells
EMC	-	Encephalomyocarditis
NWS	-	Neurotropic strain of Influenza A
FPV	-	Fowl plague virus
NDV	-	Newcastle disease virus
pfu	-	Plaque forming unit
HA unit	-	Haemagglutinating unit
C.F. unit	-	Complement fixing unit
ETC	-	Eagle's medium (8 parts) Tryptose phosphate buffer (1 part) Calf serum (1 part)
ETHu	-	Eagle's medium (8 parts) Tryptose phosphate buffer (1 part) Human serum (1 part)
ETHr	-	Eagle's medium (8 parts) Tryptose phosphate buffer (1 part) Horse serum (1 part)
ETCm	-	1.3 Eagles (75 ml.) Calf serum (10 ml.) Carboxy methyl cellulose (15 ml.)
I.C.	-	Infective centres
C	-	Colony
N	-	Normal
H	-	Pre-heated

PREFACE

The work reported in this dissertation was carried out by the author herself except when clearly stated. This work was carried out at the Institute of Virology, University of Glasgow, from October, 1961 to September, 1964. The author is grateful to Professor Stoker for the permission to work in this department, for his supervision and for his introduction to this field of work.

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CHAPTER 1.

INTRODUCTION

The ability of animal viruses to multiply only in living tissue has been known for a long time. It is only with the achievement of Enders and his group (1949) in cultivating poliomyelitis virus in tissue culture that the task of analysing the reproduction of animal viruses within the cell became susceptible to accurate and sensitive experiments. The greatest impetus for this development came when Dulbecco (1952) most clearly demonstrated that a plaque counting technique, comparable to those employed with bacteriophage, was also applicable to animal viruses. Since then the most profitable attempts made to investigate animal virus-cell relationship have followed the lines of phage research.

The quest has been so successful that there has been, even concerning animal viruses and their interactions with the cells, a shift from biological to chemical investigations. Many important biological questions have been rephrased as chemical problems. It is likely that the work of this thesis ends similarly in a biochemical explanation, but the principal experiments belong to the series of biological questions as comparison will now show.

Viruses are smaller in size and chemically less

complex than the cells they infect. It has been shown that the infectivity of various viruses resides in their nucleic acid component and the infection of host cells can be initiated by the nucleic acid alone (Ellem and Colter, 1960; Colter et al., 1957; Sanders, 1960; Bellett et al., 1962). Thus, virus multiplication depends on the host metabolism and it is the host enzymes and nucleic acid acting in unison which produce new virus particles. It is reasonable to believe that any attack on known host functions which is found to affect virus growth will lead to knowledge of pathways for virus synthesis. The following account of the main avenues of experimental attack such as, change in the nutritional requirements, energy requirements and pH of the environment, the effect of inhibitors, radiation and temperature of incubation, on virus growth will indicate to which category the present work belongs.

Nutritional requirements:

The nutritional requirements of tissue in culture and of the virus multiplication have been worked out in detail by Eagle (1955). It is possible to determine the effect of omission of certain essential compounds from a certain completely defined medium which supports virus mutliplication. Eagle and Habel (1956) traced the importance of exogenous glucose and glutamine in the optimal production of poliomyelitis

in HeLa cells. Heggie and Morgan (1956) discovered that if selected single compounds such as phenylalanine or tryptophan were omitted from the medium, multiplication of psittacosis virus did not occur.

Energy requirements for virus multiplication:

It is natural that viruses depend on host cells for energy required for their multiplication. Conversely, it can be expected that the inhibition of major routes of energy metabolism will affect the virus production.

Ackermann(1951) measured the respiration of the chorioallantoic membrane and suspension of HeLa cells infected with influenza and poliomyelitis viruses respectively. He reported that the respiration of the cells remained fixed at the proliferation rate throughout the period of viral development and release. Similar results were obtained in tissues infected with Newcastle disease virus (MacLiman's et al., 1950); and vaccinia virus (Overman and Tamm, 1957). In a number of infections anaerobic glycolysis of infected tissue is stated to be unchanged. But an increase in anaerobic glycolysis in monkey kidney cells infected with poliovirus was noticed by Levy and Baron (1957). Many workers have reported a diminution of virus production in anaerobic conditions. Gifford and Syverton (1957) showed the production of poliovirus in HeLa cells to be the same under anaerobic as well as aerobic conditions.

Compounds like sodium malonate, arsenite, cyanide, azide, etc., which inhibit respiratory activity of tissues, inhibit multiplication of influenza virus (Ackermann, 1951) and vaccinia virus (Thompson, 1947). The fluoroacetate which blocks an important step in Kreb's cycle inhibits the multiplication of poliomyelitis (Ainslie, 1952) and Semliki forest virus (Watanabe et al., 1952). It has been suggested that de-oxygenation in infected tissue may be one factor in determining recovery from virus infection (Baron, 1963).

The exploration of energy metabolism and the use of inhibitors has shown that the over-all metabolism of tissues, after infection with viruses remains unchanged but has not been of great help in approaching the mechanisms involved in viral synthesis. This is mostly because both aerobic and anaerobic pathways seem to be utilized.

Hydrogen-ion concentration (pH).

There is little evidence that the fluctuation in hydrogen-ion concentration, which ordinarily occurs in cultures, markedly affects the growth of most agents. Vogt et al. (1957) showed that the cytopathic effect of variants of poliovirus, characterized by low pathogenicity for monkeys, is delayed in slight acid media as indicated by the slower formation of plaques in agar overlay cultures of monkey kidney cells. Baron and Karzon (1957) found that cytopathic changes induced by certain strains of Echo and Coxsackie viruses are

depressed or delayed when the pH of the medium is allowed to become acid.

It is possible that slight pH changes, acting in accord with other changes in inflammation of infected tissue, for example, may have a distinct influence on limiting virus infections (Baron, 1963).

Effect of inhibitors:

Various inhibitory compounds and antibiotics were discovered in the search for suitable antiviral drugs. This in turn pointed to the desirability of analyzing the specific effect of inhibitors on virus-cell complexes at different developmental stages.

Ackermannand Maassab (1954, 1955) showed that the compounds p-fluorophenyl alanine and methoxinine, which presumably act by interfering with normal protein synthesis, interfere, not with the adsorption of virus, but by blocking the multiplication of influenza virus and poliovirus. The inhibitory action of p-fluorophenyl alanine was released by addition of phenylalanine, which permitted immediate rapid release of virus, indicating that the inhibitor permits certain essential reactions to continue. On the other hand, the release of inhibition produced by methoxinine requires a lag of at least 2 hours before virus release. It is evident that these two compounds affect virus multiplication at different points.

Analogues of known constituents, such as purine and pyrimidine, have been used. A pyrimidine analogue, 5-bromo-2-deoxyuridine, a potent inhibitor of the synthesis of normal cellular DNA, inhibits the production of vaccinia (Thompson et al., 1949; Simon, 1961; Easterbrook & Davern, 1963) and Herpes Simplex (Siminoff, 1962) but has not action on RNA viruses, Newcastle disease virus and influenza virus A strain (Salzman, 1960).

Antibiotics such as puromycin, Actinomycin D, Mitomycin C, etc., with a specific inhibitory action have been largely proved useful in distinguishing the chronology of events in the formation of virus-specific material in infected cells.

Effects of UV and X-ray radiations:

In early works on viruses these radiations were employed to inactivate the viruses. There are a few characteristics of radiation which render it interesting in a study of virus-host relationship. It penetrates with minimal or no damage to the whole structure and it has localized action. Primarily radiobiological methods were used in the study of bacterial viruses. Eventually methods used to analyse the complex mechanism of bacteriophage infection served as a model for examining animal virus-cell interactions.

Dulbecco (1957) by performing experiments similar to

Luria and Laterjet (1947) on phage, showed that the ability of pollovirus infected cells to survive as infective centres after irradiation with UVL, increases progressively with time, and that the survival curves remain of a single-hit type during the latent period, as well as during the period of virus release. Similar results were reported for vesicular stomatitis virus - chick embryo cell complexes irradiated with X-rays (Franklin, 1958).

The radiations, UV and X-rays, have been known to prevent mitosis and arrest deoxyribonucleic acid synthesis in tissue culture cells (Klein, G. and Forssberg, A. 1954; Whitfield et al., 1959; and Powell, W.F. 1962).

These experiments show that the biosynthetic capacity of tissue culture is extremely radioresistant, although the reproductive capacity of the cells is lost much more readily.

Temperature of incubation:

Virologists have for long been aware of the adverse effects of higher temperature on virus multiplication and the existence of an optimal temperature for virus development.

Before the advent of tissue culture, remarkable results of hyperpyrexia in various infections turned the attention to its application in a great variety of disorders. Wolf (1934) studied the effect of hyperpyrexia on monkeys infected with poliomyelitis. He showed that it was possible to abort the development of poliomyelitis in monkeys by

hyperpyrexia. Thompson (1938) demonstrated the influence of temperatures upon the proliferation of infectious fibroma and myxoma viruses *in vivo*. According to him the relatively low temperature of the skin was the important factor for the multiplication of dermotropic virus, as the proliferation of fibroma was inhibited when the temperature of the skin was raised to the same value as the deeper tissue.

Enders and Pearson (1941) noted that the Melbourne strain of influenza virus failed to multiply in chick-embryo tissue cultures maintained at 40°C, although the cells were not affected appreciably.

Dubés and Chapin (1956) noted that poliovirus grown at low temperature loses its original capacity to grow at 37°C. Similar results were obtained by Bang *et al.* (1957) who showed that the cytopathic effect of Eastern equine encephalitis virus may also be modified by change in temperature. As a result of these observations many attempts were made to establish the relationship between the high temperature and virulence of viruses. Dubés and Wenner (1957) studied the effect on poliovirus grown at low temperatures. By selecting poliovirus able to develop at low temperatures, they obtained a strain which was inhibited at 37°C. The cold strain did not produce any paralysis whereas the original strain was neurovirulent. The relative avirulence of cold adapted strain appeared to be due to its reduced capacity to propagate

at 37°C or above. On the contrary, strains selected for their ability to grow at higher temperatures were found to be highly virulent (Sabin and Lwoff, 1959). Perol Vauchez et al. (1961) have found a similar kind of relationship between high temperature and virulence for the viruses of encephalomyocarditis.

Recent work on poliovirus by Lwoff (1962) indicates that there is a thermosensitive step involved in synthesis of this virus.

The effect of higher temperatures on tissue cultures has been studied for some time. The method of these investigations was to observe cell multiplication at sublethal and lethal temperatures in the hope of ascertaining the biochemical or physical processes involved. The commonest type of experiments had been to determine the exact lethal exposure time at temperatures just above normal.

Friedgood (1928) and Pincus and Fischer (1931) studied the effect of lethal temperatures on tissue cultures but failed to ascertain any one heat sensitive function responsible for cell death or injury.

The application of temperature shock to cultures for effective synchronization of cell division in tissue culture has been considered. Brief exposure to low temperatures is found to be more effective for this purpose (Gey, Bang and Gey, 1954; Wildy and Newton, 1958) than the supraoptimal temperatures.

The effect of incubation at temperatures higher than 37°C has not been carefully studied.

The methods mentioned in the above account have all yielded significant information about virus growth, although biological methods have provided less precise information than, for example, studies with chemical analogues and antibiotics. The effects of heat, particularly heat-sensitive step in the synthesis, have not yet been satisfactorily explained chemically. Heating the virus-host system is more complex than heating either virus or host separately. This last form of investigation does not seem to have been adopted and forms the subject of this thesis. The reasons for chosing this subject will be apparent in the following chapters.

The results of my experiments are probably more informative than the preceding accounts in the fields of nutrition and energy requirements, and sensitivity to pH changes; as informative as those of growth of virus at elevated temperatures and after irradiation of virus-cell complexes; but less instructive than biochemical findings with specific metabolic inhibitors and analogues.

CHAPTER 2.

GENERAL METHODS

This chapter includes an outline of procedures which have been routinely used throughout this work. Special methods will be described in the relevant chapters.

BHK-21, C13 cells:

The BHK-21, C13 clone of hamster cells (Macpherson and Stoker, 1962) were grown in ETC from frozen stock suspension. As a routine, cells were grown in 8 oz baby bottles, but whenever a considerable number of cells was required, they were propagated in 80 oz bottles laid on their sides, revolving about their long axis at approximately one revolution per minute at 37°C. About 8×10^7 to 1×10^8 cells suspended in 200 ml ETC were seeded in each bottle, yielding approximately 4×10^8 cells per bottle in 48 hours. The cells were harvested using 0.05% trypsin in versene solution. Cells were washed once with ETC and then resuspended in fresh ETC and stored at +4°C until used. Cells were used immediately or not later than 48 hours after being stored.

Refrigeration of cells:

Just confluent cultures of C13 cells were trypsinized with 0.05% trypsin versene, spun and resuspended in the medium

containing 25% calf serum and 5% glycerol. Aliquots of the cell suspension in small bottles were slowly cooled to -60°C in a deep freeze. Cells were recovered by thawing rapidly in a water bath at 37°C.

Exposure of cells to temperatures:

The logarithmically growing BHK-21, C13 monolayer cell cultures grown for 24-48 hours were trypsinized with 0.05% trypsin versene, centrifuged at 1000 rpm for 5 minutes, and resuspended in warm ETC to give a monodispersed cell suspension of approximately 1 x 10^6 cells per ml. The cells were distributed in 1 ml quantities in 4 x $\frac{1}{2}$ siliconed tubes. The tubes were tightly corked and placed in a water bath adjusted to the required temperature. The tubes were immersed in the water bath deep enough to keep the cell suspension well below the level of the water in the bath. It took between 90 - 120 seconds for the temperature of the fluid inside the tubes to equilibriate with that of the water in the bath. The temperature of the water bath was maintained within $\pm$ 0.05°C of the desired temperature. Cells were generally exposed to a particular temperature for a period of 15 minutes unless otherwise stated. Cell suspensions were occasionally shaken to prevent settling.

CHAPTER 3.

EFFECT OF TEMPERATURES ON BHK-21, C13 CELLS.

Optimal and Supraoptimal temperatures:

BHK-21, C13 cells are regularly cultured at 37°C, hence 37°C is considered as the optimal temperature for this cell line. The growth rate of C13 cells was compared at temperatures 37°, 39° and 41°C to check the assumption and to define the range of supraoptimal temperatures. Some alteration of growth was, in fact, observed at 39° and 41°C; the terms "supraoptimal", "higher temperature" and "heat" were used to denote one and the same meaning, i.e. any temperature above 37°C, the optimal temperature.

The growth rate at 37° and 39°C:

Replicate cultures were prepared in a number of plastic bottles seeded with 5×10^4 cells in 5 ml of ETC. The bottles were gassed with 5% CO_2 in air and incubated at 37° and 39°C in the air incubators. The duplicate cultures from each batch were taken at an interval of 24 hours. The cells from individual bottles were harvested and counted in a haemocytometer. The minimum number of cells counted was between 150 - 200 cells per sample. The growth rate was followed for 96 hours without replenishing the medium or the gas phase.

Fig. 1 shows that the growth rates of C13 cells at 37°C and 39°C for the first 72 hours are the same, the mean doubling time during the log phase being 12 hours, as noted by Macpherson (1963) for 37°C. After 72 hours at 39°C the growth rate of C13 cells seemed to slow down, and appeared to reach stationary phase, while cells at 37°C continue to grow at normal rate.

The growth rates were not followed after 96 hours, as the cells incubated at 39°C appeared unhealthy and granular.

The comparative curves of C13 cells at 37° and 41°C are shown separately (Fig. 2), because of one difference in the experimental method for the 39°C cultures. BHK-21, C13 cells do not adhere to plastic or glass at 41°C, therefore, all the cultures were loosely capped and placed in humidified CO_2 incubator at 37°C for the first 6 hours. When equilibration and settling had occurred at the end of this period, bottles were tightly capped and divided into two batches, one being placed in an incubator at 37°C, the other at 41°C. Cell counts were done on duplicate cultures from each batch at the end of 18 hours and every 24 hours thereafter. The growth rate, at both temperatures, remained the same for the first 24 hours and thereafter there was a gradual drop in the total cell population grown at 41°C. By 72 hours, the total cell population grown at 37°C was three times more than that grown at 41°C.

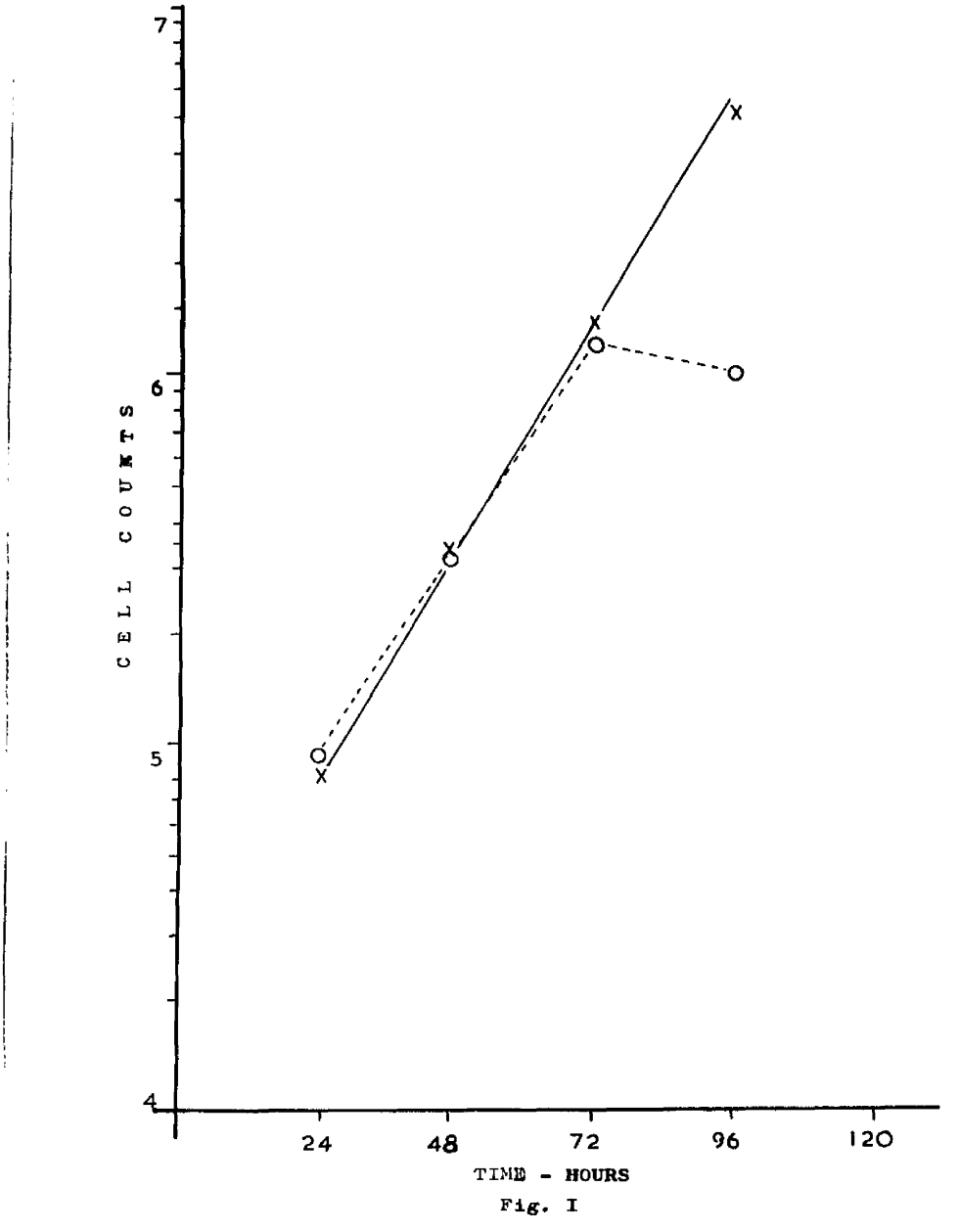

Fig. I

Fig. 1 The rate of multiplication of C13 cells at 37°C (x x) and 39°C (o --- o).
Each point is the arithmetic mean of two counts.

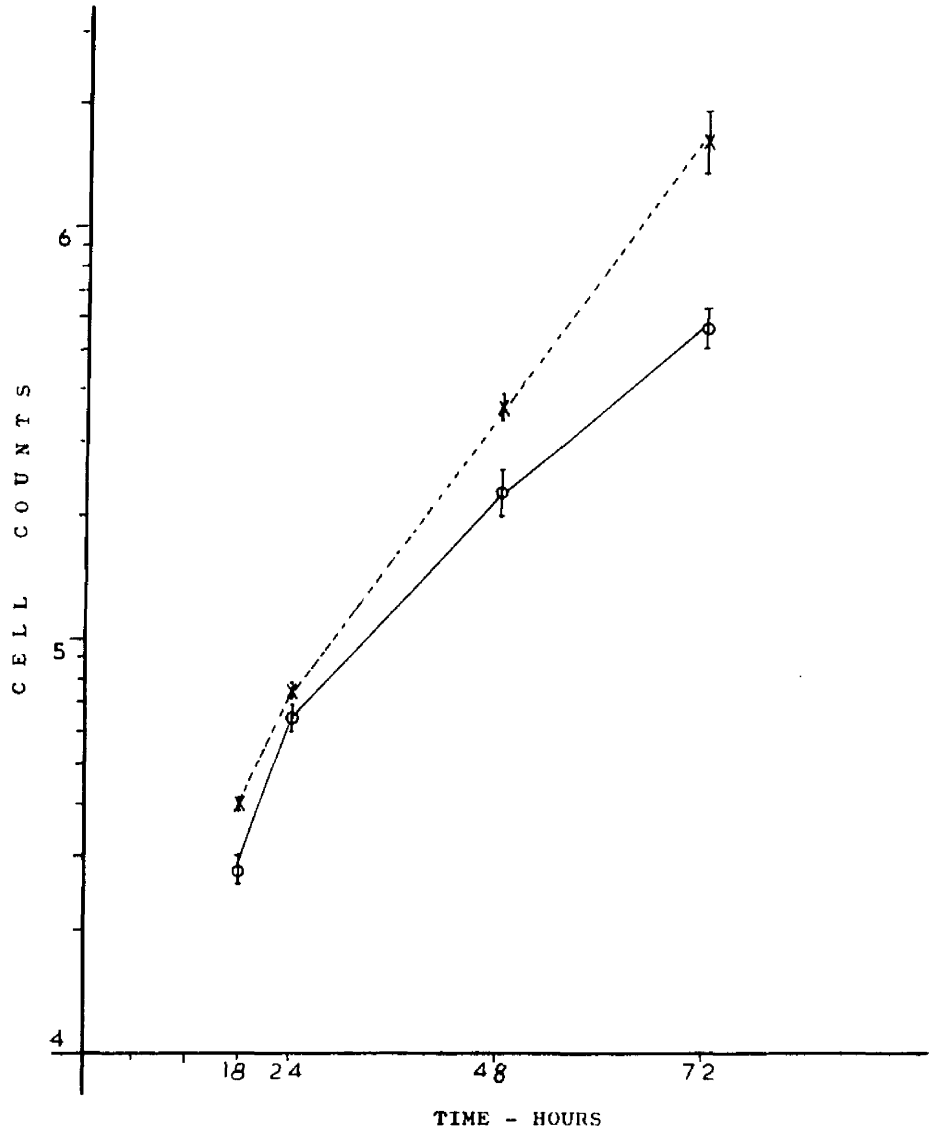

Fig. 2

Fig. 2 The rate of multiplication of Cl3 cells at 37°C (x ——— x) and 41°C (o - - - o).

Each point is the arithmetic mean of two counts.

These results indicated that the growth of C13 cells was retarded at temperatures of 39°C and above, which were considered supraoptimal temperatures.

Lethal effect of heat:

The effect of higher temperatures on tissue culture is known to be lethal (Pincus, G., and Fischer, A., 1931; Kokott, W., 1930a, 1930). The lethal effect of supraoptimal temperatures on C13 cells was judged by their increasing sensitivity to trypan blue stain. The stain trypan blue is generally used to distinguish dead organisms or tissue culture cells from living ones (Evans and Schulemann, 1914; Pappenheimer, A.M., 1917). Since the aim was to study the effect of supraoptimal temperatures on the metabolic activity of BHK-21, C13 cells, it was necessary to ascertain which of these were non-lethal.

The C13 cells in suspension were exposed to temperatures ranging from 39° to 50°C in a water bath for various lengths of time and then stained with 0.5% trypan blue. The counts on stained and unstained cells were compared to estimate the death rate in a cell population after exposure to higher temperatures. Uptake of stain was dependent on temperature of exposure and the time of exposure. Heating to 48°C and above was accompanied by a critical increase in the permeability of the cells to the dye (Fig. 3).

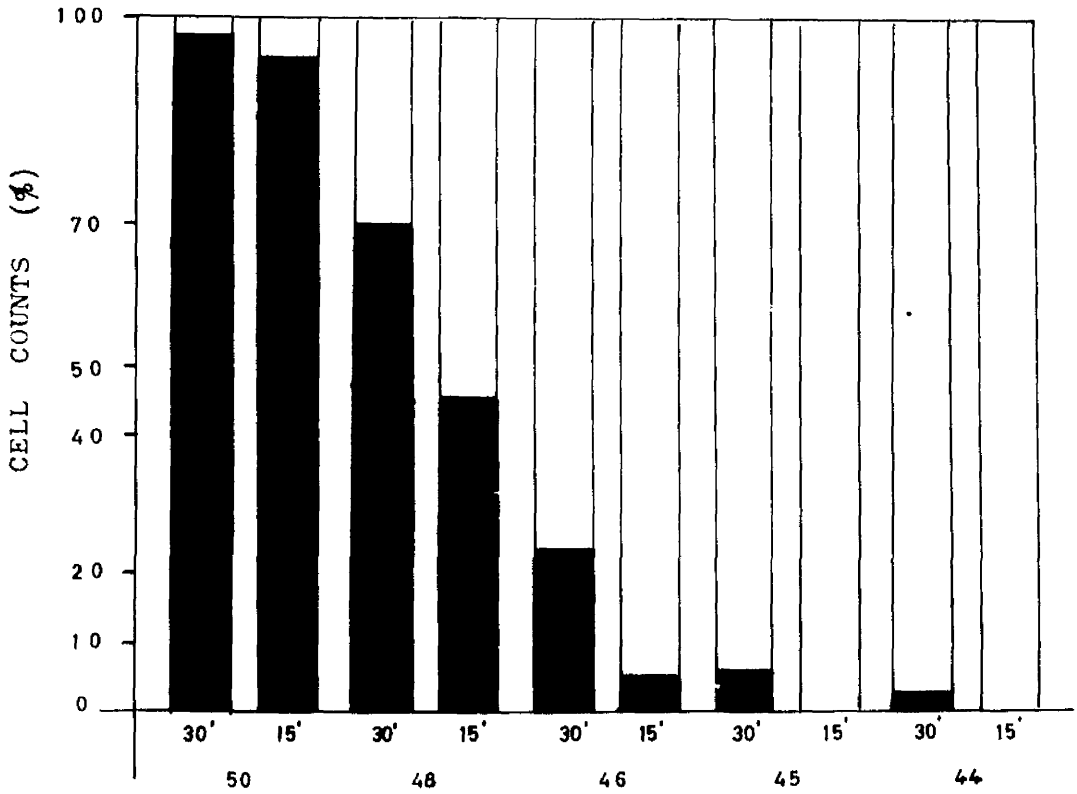

Fig. 3

Fig. 3 The cell counts represent the percentage of unstained □ and stained ■ cells in a cell population exposed to various temperatures for a duration of 15 and 30 minutes.

The shape of survival curve:

The terms "survival" or "viable" are used in the sense which has been standard in microbiology, i.e. referring only to the ability of the individual cells to multiply into a macroscopic colony.

The survival rate of the cells was determined by plating a dilute suspension of single cells on to the feeder layers for the formation of macroscopic colonies. The feeder layers were prepared as described by Stoker and Macpherson (1961). The plating efficiency of BHK-21, C13 cells under normal conditions was 60-80%. The effect of heat on the survival rate of cells was estimated by comparing the plating efficiency of normal and heated cells.

The logarithmically growing C13 cells were suspended in warm ETC to give approximately 1 x 10^6 cells per millilitre. Aliquots of 1 ml amounts were distributed in siliconed tubes. The cells were exposed to supraoptimal temperatures by placing a tube, containing cells, in a water bath, maintained at the required temperature, for 15 minutes (technique on page 12). The cells after exposure to heat were spun down and resuspended in 1 ml of ETC to give a suspension of single cells. The cells were then counted and diluted in ETC to give 100-200 cells per plate. The minimum number of 8 plates was used for each sample in order to attain 90% confidence limits. Plates were incubated in

a CO_2 incubator at 37°C for 7 days, then fixed with 10% formol-saline and stained with Leishmann's stain.

The survival curves of three different experiments are presented in Fig. 4. In each experiment, the effect of five different temperatures on the plating efficiency was compared with that of normal cells. The survival rate of cells appeared to drop gradually with the rise of temperature. The results varied from experiment to experiment. The variation in the survival rate at 41° and 43°C extended over a wide range, between 50 to 80%. The percentage of cells forming colonies when treated at 45°C varied between 0 to 20%, but the cells exposed to 46°C never formed colonies in any experiment. Various environmental and host factors might have been responsible for this variation. It was, therefore, necessary to control the conditions of assays. The external factors, such as medium and sera, were controlled by using one batch of medium and serum.

The effect of the duration of exposure at various supraoptimal temperatures on the survival rate of C13 cells was studied. The maximum time of exposure at any one temperature was 60 minutes. The results are shown in Fig. 5. The survival rate of the cells at all temperatures decreased with the increase in the time of exposure. The survival curves obtained were of multi-hit type.

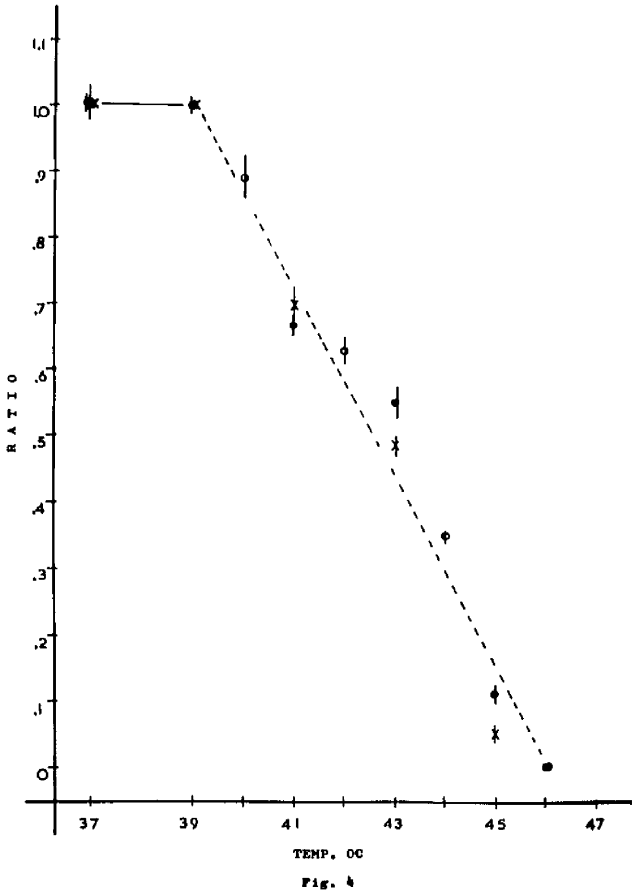

Fig. 4 Colony plating efficiency : the results of three different experiments are shown in this figure. Each point is a ratio of number of colonies formed by preheated cells to the number formed by normal cells.

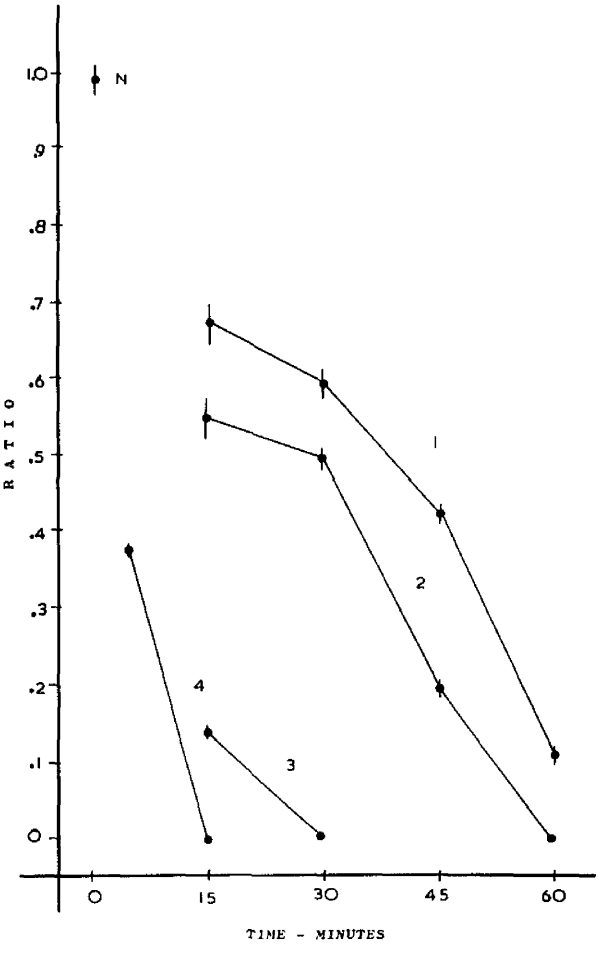

Fig. 5

Fig. 5 Each point is a ratio of number of colonies formed by preheated cells to number formed by normal cells.

1 - 41°C; 2 - 43°C; 3 - 45°C; 4 - 46°C.

Fate of the cells inactivated by heat:

Failure of cells to form macroscopic colonies could be due to one of the following reasons:

a) the heated cells may divide for a limited number of divisions and form microcolonies, or

b) the reproductibility of cells is completely inhibited so that they remain as single cells and disappear.

Limitations in number of divisions was ruled out by the absence of microcolonies at any stage. At higher temperatures, cells which had retained their ability to divide, formed colonies of normal size. This left complete inhibition of division as the cause of decreased survival rate.

Morphology of cells:

The cells exposed to temperatures up to 47°C for 15 minutes appeared normal when examined under ordinary optical microscope. The only obvious effect of heat on the C13 cells was the rounding of the cells. It was noticed that when heated cells were plated, with the rise of temperature an increasing number of cells remained rounded and failed to stick to the glass. At temperatures of 45°C and 46°C, a majority of the cells had rounded up and were found either floating in the medium or loosely attached to the glass. Rounding of the chick osteoblastic cells was observed by Pincus and Fischer (1931) when cells were exposed

to higher temperatures, but they attributed this change to the death of the cells. In the experiments described here, the cells were impermeable to trypan blue and were considered metabolically active.

Recoverability of heated cells:

Recovery of C13 cells after exposure to supraoptimal temperatures was tested by storing heated as well as unheated cells at +4°C for 24 hours. The stored cells were then plated for plating efficieney and the results were compared with that of unstored normal and heated cells plated 24 hours earlier. The effect of heat was irreversible. The results are shown in the Table 1.

The results of the experiments discussed above suggested that supraoptimal temperatures affect cell division and the process is irreparable with time. The nature of the action of heat is difficult to ascertain. Nothing can be deduced from the curves of the survival rate. In particular one cannot decide between single and multiple-hit effects. Some factors which might influence the nature of the curve cannot be ignored. The sensitivity of the cells to higher temperatures is shown to depend on the mitotic phase of the cells at the time of exposure (Thormar, 1959; Zeuthen, 1964). The variability alone in the sensitivity of the cell population to supraoptimal temperatures could explain the nature of survival curve. In that case

TABLE 1

Effect of storage on plating efficiency
of unheated and heated cells.

Temperature ^{o}C	Duration of exposure in minutes	Colony plating efficiency (%)	
		Fresh cells	Stored cells +4°C for 24 hours
Normal cells		87.60	63.07
41	15	71.71	42.0
43	"	52.59	28.11
45	"	10.13	1.27
46	"	-	-

action of heat may not be a multiple-hit phenomenon, but may have one single action on the cells depending on the state of the cells.

The site of action in the cells can be either structural or biochemical. Electron microscopic examination of normal and heated cells had shown no obvious structural damage (p.109). That does not rule out the possibility of damage without distortion or fragmentation, e.g. coagulation.

The only other obvious effect could have been either an inactivation or destruction of the essential metabolism required for the cell multiplication. The process of cell division is a complex one whose regulation is still a matter of speculation, and inhibition of any one of the many processes involved may result in the prevention of cell division, but not necessarily causing other effects on the metabolism. The experiments reported in the following chapters were designed to determine the effect on other functions of the host cell. Of these, I chose to test the capacity of the preheated cells to support virus reproduction, since several publications have appeared about the relationship between cell division and virus growth, e.g. Herpes Simplex (Stoker and Newton, 1959), NDV and NWS (Wheelock and Tamm, 1959) and Reo (Spendlove et al., 1963).

The following viruses were selected from the list of the viruses known to multiply and produce cytopathic

effect in BHK-21, C13 cells (Stoker and Macpherson, 1964).

Herpes viruses	:	Herpes Simplex
		Pseudorabies
Pox viruses	:	Vaccinia
Encephalomyocarditis	:	A small plaque variant of EMC
Myxoviruses	:	NWS strain of Influenza A
		Fowl plague virus
		Newcastle disease virus
Reo viruses	:	Reo type 3.

CHAPTER 4.

GROWTH OF DNA VIRUSES IN NORMAL C13 CELLS.

It was necessary to obtain precise information about the processes of viral infection of cells and multiplication of Herpes Simplex virus, Pseudorabies virus and Vaccinia virus in BHK-21, C13 cells before the sensitivity of preheated C13 cells to these viruses was tested.

The reasons for discussing these three viruses together will become obvious.

Herpes Simplex virus has been studied in embryonated eggs and many tissue culture cell lines. It contains DNA for its nucleic acid (Ben-Porat and Kaplan, 1962; Russell, 1962) and its site of multiplication in infected cells is in the nucleus of the host cells (Crouse et al., 1952; Morgan et al., 1954; Lebrun, 1956).

Pseudorabies virus has been placed in the Herpes group of viruses because both are pantropic, neuroinvasive and produce similar intranuclear inclusions (Sabin, 1934). Morphologically virus particles are indistinguishable under electron microscope (Morgan et al., 1954). Pseudorabies is a DNA virus (Ben-Porat and Kaplan, 1962).

Vaccinia virus belongs to the Pox group of viruses. It has been studied in embryonated eggs as well as in various tissue culture cell lines. It is a DNA virus but the group of poxviruses has been considered unique because the synthesis

of viral DNA is induced in the cytoplasm of infected cells (Cairns, 1960 and Sheek and Magee, 1961).

MATERIALS AND METHODS.

Viruses:

Herpes Simplex:

The HFEM strain of Herpes Simplex virus was kindly supplied by Dr. Wildy. The virus had been derived from a single pock on the chorioallantoic membrane of an egg and was adapted to BHK-21, C13 cells by serial passages. The virus seed was prepared in monolayers of BHK-21, C13 cells growing at 32° in ETC (Farnham and Newton, 1959). Two days after infection, cells were scraped off the glass and were disrupted in a small volume of medium using a Dawe Soniclean ultrsonicator. The cell debris was removed by centrifugation at 1000 rpm for 5 minutes. The supernatant fluid was dispersed in 1.0 ml quantities and stored at -70°C. The infectivity titre was 2×10^8 pfu/ml.

Pseudorabies:

Pseudorabies virus grown in HeLa cells was obtained by courtesy of Dr. Russell. The virus was then adapted to C13 cells by three passages and the seed was prepared from the culture fluid of the fourth passage which had the infectivity titre of 2.4×10^9 pfu/ml. The seed was stored at -70°C in 1 ml aliquots.

Vaccinia:

The Lister strain of Vaccinia, (kindly supplied by

Dr. Ross), derived from a single pock on the chorioallantoic membrane, was grown in human amnion cells for two passages. It was then adapted to C13 cells by three passages in monolayers, incubated at 37°C for 48 hours. Two days after infection of the monolayers with undiluted inoculum from a third passage tissue culture fluid, cells were harvested and disrupted in a small volume of medium using a Dawe Soniclean ultrasonicator. The suspension was spun at 1000 rpm for 5 minutes to remove cellular debris. The supernatant was titrated for infectivity and had the titer of 1.4×10^8 pfu/ml. The seed was stored at -70°C in 1 ml quantities.

Infectivity assay:

The sensitive plaque technique used was devised by Russell (1962a)who established the validity of this technique by showing a linear relationship between the concentration of virus and the number of plaques with Herpes Simplex, Pseudorabies and Vaccinia viruses.

Aliquots of C13 cells containing $4n \times 10^6$ cells in ETC (where n is the number of petri dishes used per dilution) were spun down and the pellet of cells resuspended in n ml of an appropriate virus dilution in ETC. This cell-virus suspension was shaken at 37°C for 30 minutes for Herpes Simplex and Pseudorabies virus and for 60 minutes for Vaccinia titrations. Then a further 4n ml of ETH or ETCm was added and 5 ml quantities of suspension were plated in n number of 60 mm petri dishes. These plates were then

incubated in a 5% CO_2 incubator at 37°C. The presence of human serum containing antibodies to Herpes Simplex in ETH prevented secondary plaque formation. Inhibition of secondary plaques in the absence of specific anti-serum for Pseudorabies and Vaccinia virus was achieved by adding Eagle's medium containing carboxy methyl cellulose (ETCm). Care was taken not to move the assay plates during incubation. Plaques 1 - 2 mm in diameter appeared in 2 - 3 days. Plates were fixed with 10% formol-saline and stained with Leishmann's stain. Plaques were counted with a hand lens (x4).

Adsorption rate:

The adsorption rate of viruses on monolayers, as well as on cells in suspension was studied, as both methods were used in subsequent experiments.

On monolayer:

A series of monolayer cultures was washed once with tris-calf solution and then inoculated with 0.1 ml of virus inoculum diluted in ETC to give a known number of plaques per plate. The infected cultures were incubated in a humidified 5% CO_2 incubator at 37°C. Every 30 minutes 4 plates were removed from the incubator, the monolayers were washed 3 times with tris-calf solution and then overlaid with agar medium and incubated at 37°C. The plates were overlaid with a second layer of overlay containing .004% neutral red at the end of 4 days incubation period. Plaques were visible as unstained areas.

In suspension:

A number of 4 x ½ inch siliconed test tubes containing 4×10^6 cells in each, plus 1 ml of virus inoculum diluted in ETC to give a known number of pfu/ml, were incubated at 37°C. The test tubes were tightly corked with silicone bungs. The virus-cell suspension was gently stirred on a small magnetic stirrer. One tube per sample was taken at different time intervals. Cells were washed three times with tris-calf solution to get rid of unadsorbed virus, then the cells were mixed with 1.2×10^7 normal C13 cells suspended either in 20 ml of ETH (for Herpes Simplex) or ETCm (for Pseudorabies and Vaccinia). The mixture of cell suspension was distributed into four 60 mm petri dishes in 5 ml quantities per plate. The plates were incubated in a CO_2 incubator at 37°C for 2 - 3 days and then fixed with formol-saline and stained with Leishmann's stain. Plaques were counted with a hand lens (x 4).

Single step growth curve:

To study the one-step growth curve, the method adopted was saturation of the cells by infection at high multiplicity, to ensure that every cell in the suspension was infected. The virus-cell mixture was incubated at 37°C. Input virus was removed by thorough washing, and in the case of Herpes Simplex, by neutralizing antibody. An equal number of cells was dispensed in separate bottles which were harvested at appropriate time intervals. In each case

infective centres were determined, by plating infected cells during the latent period, and intracellular and extracellular virus was measured together, by disruption of infected cells by freezing and thawing three times.

RESULTS:

Herpes Simplex virus:

Adsorption rate:- (a) On monolayer - A series of monolayers of C13 cells was incubated with 0.1 ml of virus inoculum containing 50 pfu/0.1 ml. Plates were rocked every 20 minutes to facilitate the even distribution of inoculum. Fig. 1 shows that more than 50% of virus inoculum was adsorbed within the first 30 minutes and the rate of adsorption gradually increased till it reached the maximum level in one and half hour where nearly 80% of the virus was adsorbed. At 2 hours it was difficult to measure the rate of adsorption because of thermal inactivation.

(b) In suspension - Results in Fig. 2 indicate that when 4×10^6 cells were infected in suspension with 1 ml of virus inoculum containing 400 pfu/ml, the adsorption rate was faster. A maximum was achieved in the first 30 minutes of incubation. The amount of virus adsorbed was significantly greater than on the monolayer.

Single-step growth curve:

In order to determine the eclipse period, duration of single growth cycle and an average yield of virus per

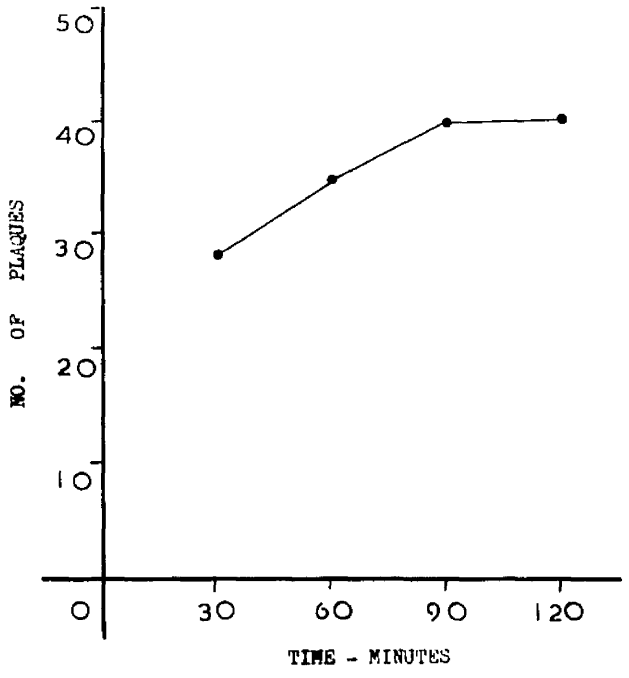

Fig. 1.

Fig. 1 Adsorption of Herpes Simplex to Cl3 monolayers.

Fig. 2 Adsorption of Herpes Simplex to Cl3 in suspension.

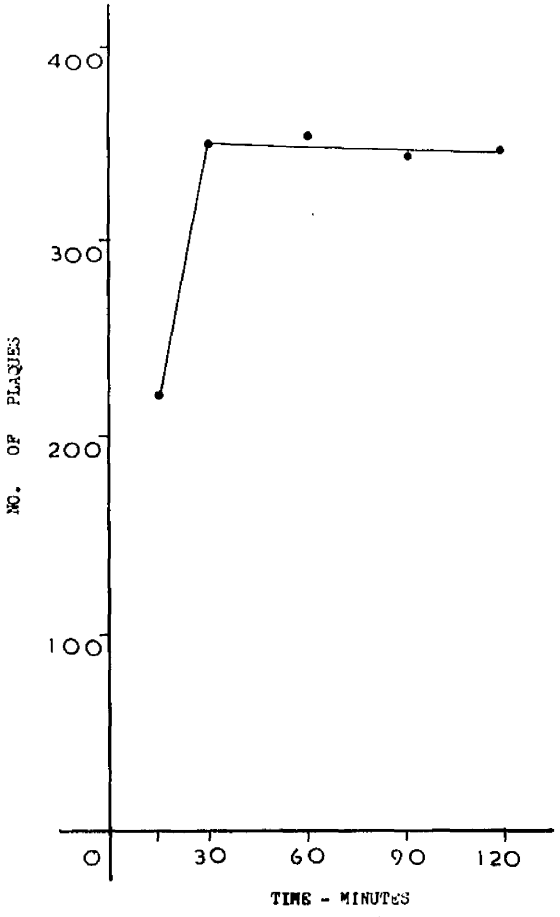

Fig. 2.

infected cells, it was necessary to use a high multiplicity of infection. Approximately 3 x 10^7 cells were infected with 1 ml of virus inoculum containing 4 x 10^8 pfu/ml to give an input multiplicity of about 12 pfu/cell. The virus-cell mixture was incubated for 30 minutes at 37°C by shaking on an automatic shaker. After incubation, the virus-cell mixture was treated with medium containing human serum with specific anti-herpes antibodies, for 20 minutes at 37°C. At the end of this incubation, cells were washed twice with tris-calf solution to get rid of antibodies. Cells were resuspended in ETC to give approximately 1 x 10^6 cells per ml. Aliquots of 2 ml quantities were dispensed into a series of 4 oz tissue culture bottles which were incubated at 37°C. An aliquot of cell suspension was further diluted, to give approximately 1 x 10^2 cells/plate, and were plated for infective centres.

The number of infective centres obtained was 62% in this experiment. Nearly 40% of cells had failed to produce infective virus or to make infective centres. By a Poissonian distribution, 100% of cells should have been infected. If all the cells were susceptible, which may be a wrong assumption, then the failure of infected cells to produce plaques must be due to loss of infected cells during the manipulations. In that case, the plating efficiency will never be 100 per cent.

Fig. 3 shows that, following washing and treatment

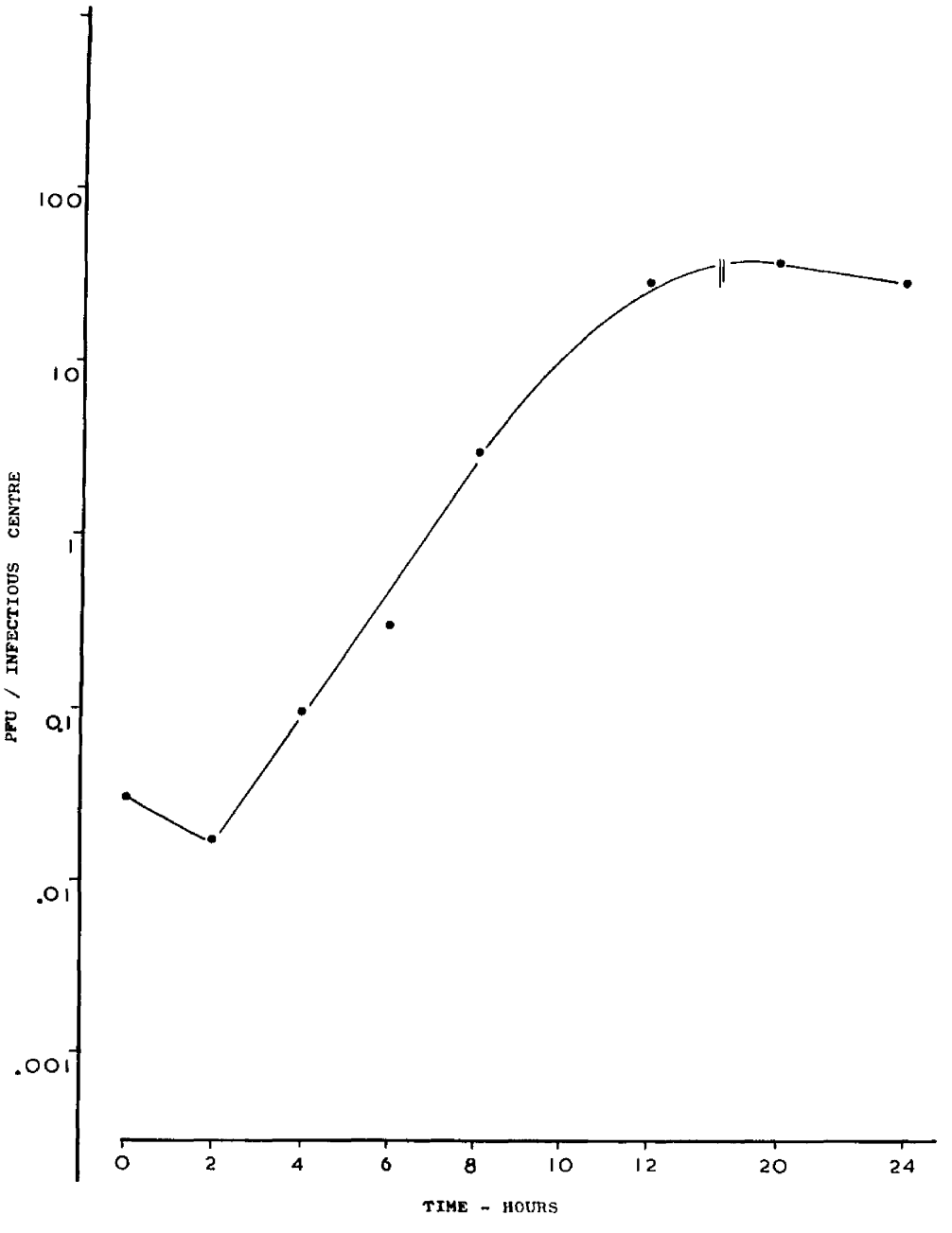

Fig. 3

Fig. 3 Herpes Simplex : single step growth cycle in Cl3 cells.

with immune serum, some virus still remained in the medium. The latent period extended to between 2 and 4 hours and during that period there was a slight fall in residual virus. Following the latent period, virus increased steadily to reach the maximum by 12 hours. The drop in the final yield at 24 hours could be due to thermal inactivation. The average yield per infected cell was 80 pfu/cell. This growth curve is now known to be characteristic of herpes virus in C13 cells (Gold et al., 1963).

Pseudorabies:

Adsorption rates: (a) On monolayer - A series of monolayers was infected with 0.1 ml virus inoculum containing 50 pfu/0.1 ml. Samples were taken every half an hour.

(b) In suspension - Approximately 4×10^6 cells were infected with 1 ml of virus dilution containing 480 pfu/ml. Samples were taken at various time intervals.

Figs. 4 and 5 indicate that the adsorption rate in suspension, is faster than it is on monolayers. Nearly 75% of virus inoculum was adsorbed when infected in suspension, as compared to 65% adsorption on monolayers.

Single-step growth curve:

The quantities used in the single-step growth curve were 2×10^7 cells and 1 ml of virus containing 2.4×10^8 pfu/ml, this gave a multiplicity ratio of 12 ; 1. The virus-cell mixture was incubated for 30 minutes at 37°C. The cells were

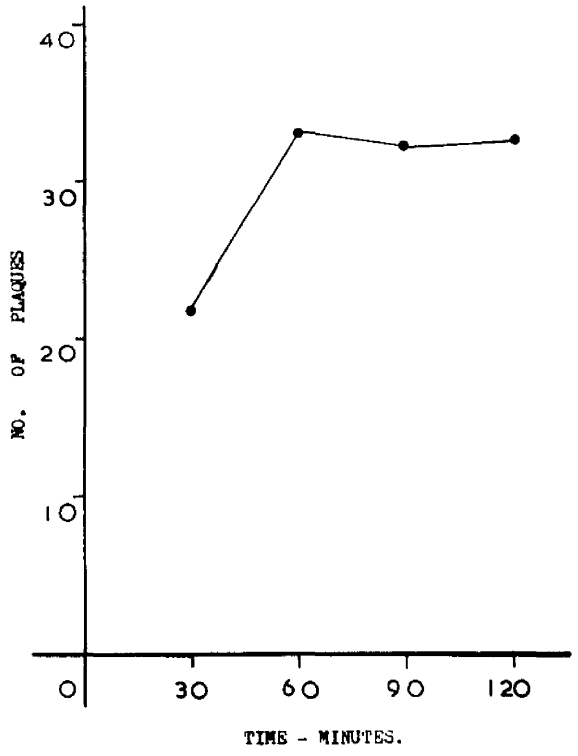

Fig. 4.

Fig. 4 Adsorption of Pseudo-rabies to Cl3 monolayers.

Fig. 5 Adsorption of Pseudo-rabies to Cl3 in suspension.

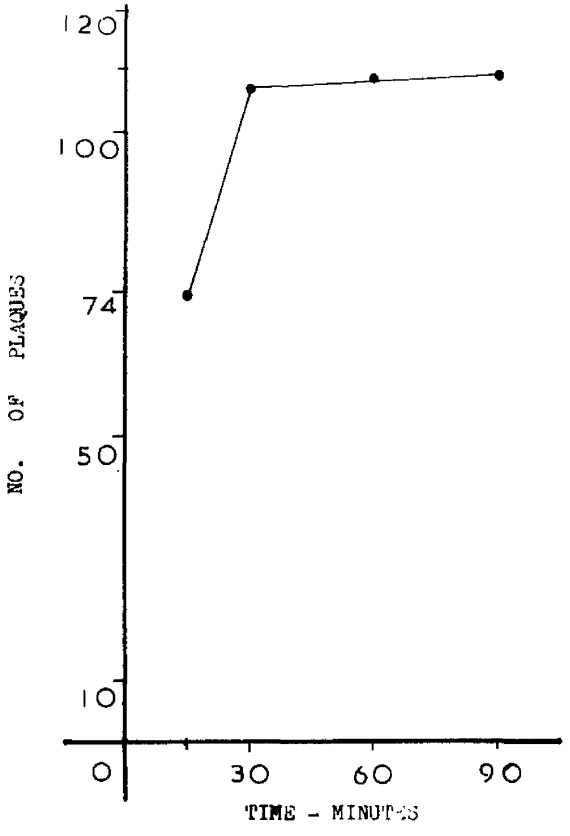

Fig. 5.

washed 4 times with tris-calf solution to get rid of unadsorbed virus. Cells were resuspended in fresh ETC and dispensed in 2 ml amounts containing approximately 1.4×10^6 cells/ml in tissue culture bottles. An aliquot of cells was used for the estimation of the number of cells yielding virus. Nearly 140 cells per plate were plated.

Fig. 6 shows that the latent period extended over 2 hours; during that period residual virus remained at the same level. After 2 hours, virus multiplication increased rapdily and maximum growth of virus was obtained by 10 hours, after which further incubation did not have any adverse effect on the infectious titre of the virus. In this experiment, 35% of cells produced infective centres. The yield per infected cell was nearly 800 pfu/cell.

Herpes Simplex virus grew at a slower rate in BHK-21, C13 cells and had a lower yield per infected cells as compared to Pseudorabies virus in BHK-21, C13 cells. Similar results were obtained in rabbit-kidney cells by Kaplan (1959).

Vaccinia:

Adsorption rate : (a) On monolayer - Monolayers were infected to give 110 plaques per monolayer. Fig. 7 indicates that maximum adsorption is achieved by 60 minutes and approximately 63% of virus inoculum is adsorbed.

(b) In suspension - Approximately 4×10^6 cells were infected

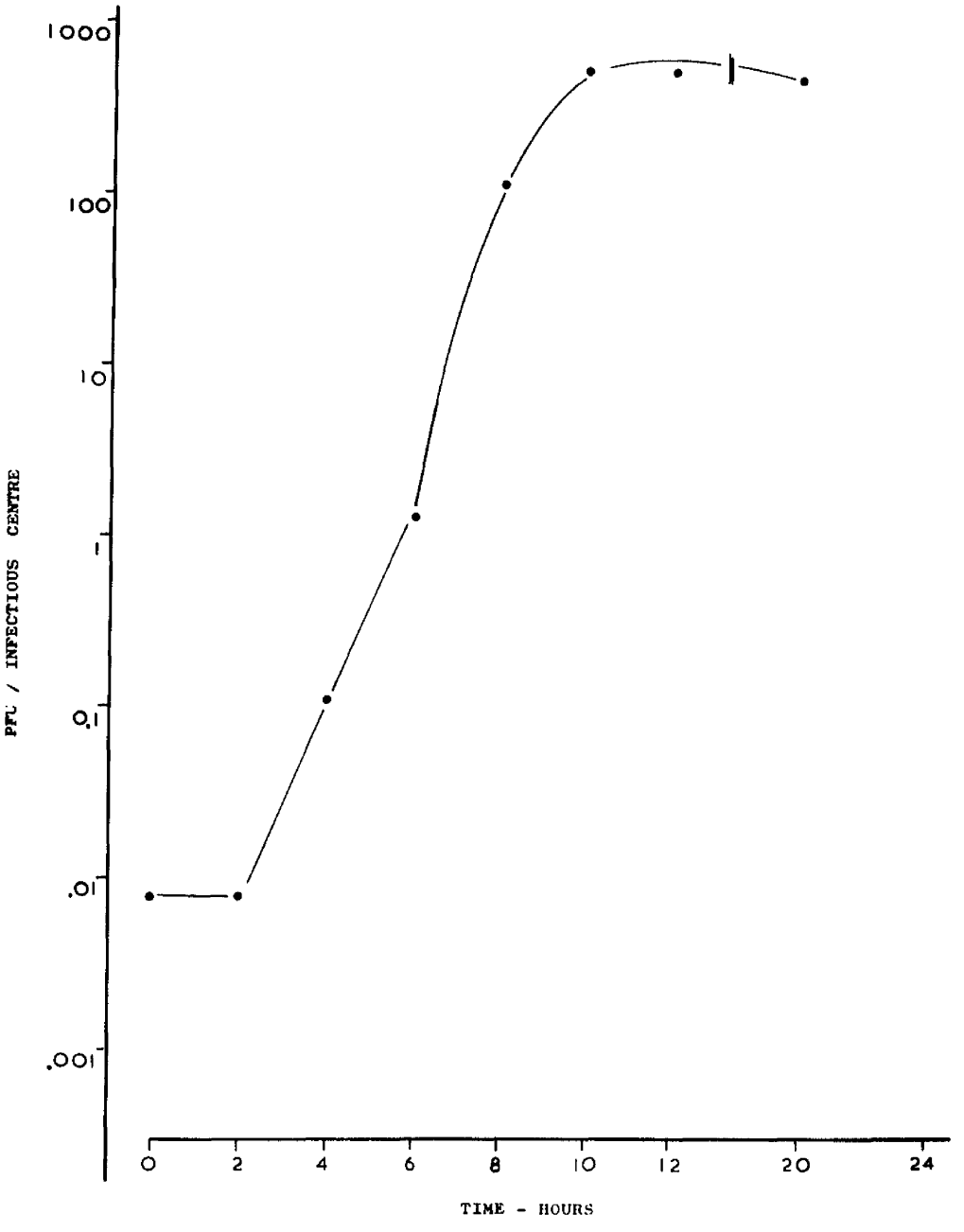

Fig. 6

Fig. 6 Pseudorabies : single step growth cycle in C13 cells.

with 1 ml of virus containing 550 pfu/ml. Although within the first 30 minutes the adsorption rate was faster than that on monolayer, the maximum adsorption was only reached by 60 minutes, as will be seen from Fig. 8.

Single-step growth cycle :

Briefly, the procedure was as follows. Approximately 3×10^7 cells were infected with 1 ml amount of virus inoculum containing 1.4×10^8 pfu/ml, which gave a multiplicity ratio of 5 : 1 approximately. Higher multiplicities were avoided as C13 cells were found to form clumps when mixed with vaccinia virus. The clumps formed were large and could not be broken by pipetting. At lower multiplicities, cells showed less tendency to clump and cells could be separated easily by pipetting.

The virus-cell mixture was incubated for 60 minutes at 37°C. The rate of adsorption of vaccinia is slower as compared to Herpes Simplex and Pseudorabies. Hence, a longer incubation period was used. After incubation, cells were washed with tris-calf solution at least three times to remove extracellular virus. Cells were resuspended in ETC to give approximately 1×10^6 cells/ml and distributed in tissue culture bottles in 2 ml amounts. At the same time cells were plated for infective centres. The number of infective centres estimated was 52%. The growth cycle of vaccinia is represented in Fig. 9 which indicates that vaccinia grows at only a slightly slower rate in BHK-21, C13

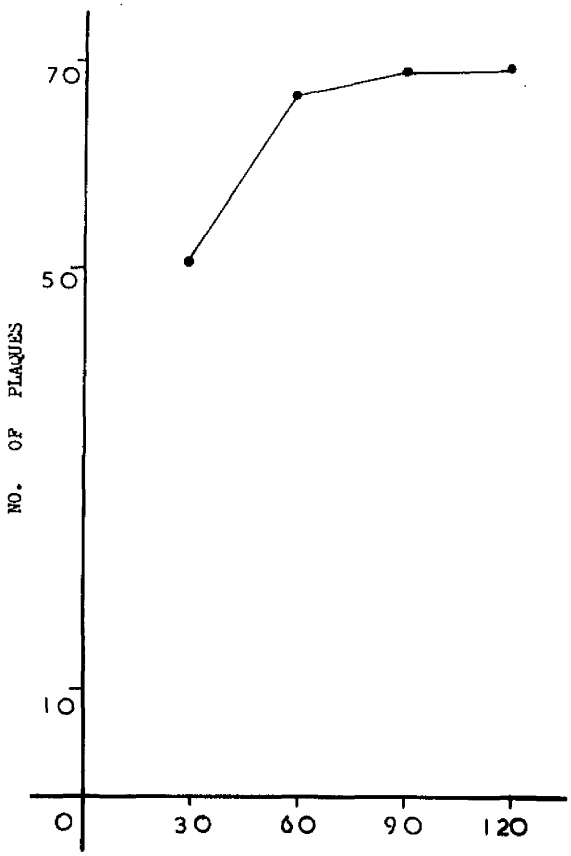

Fig. 7.

Fig. 7 Adsorption of Vaccinia to Cl3 monolayers.

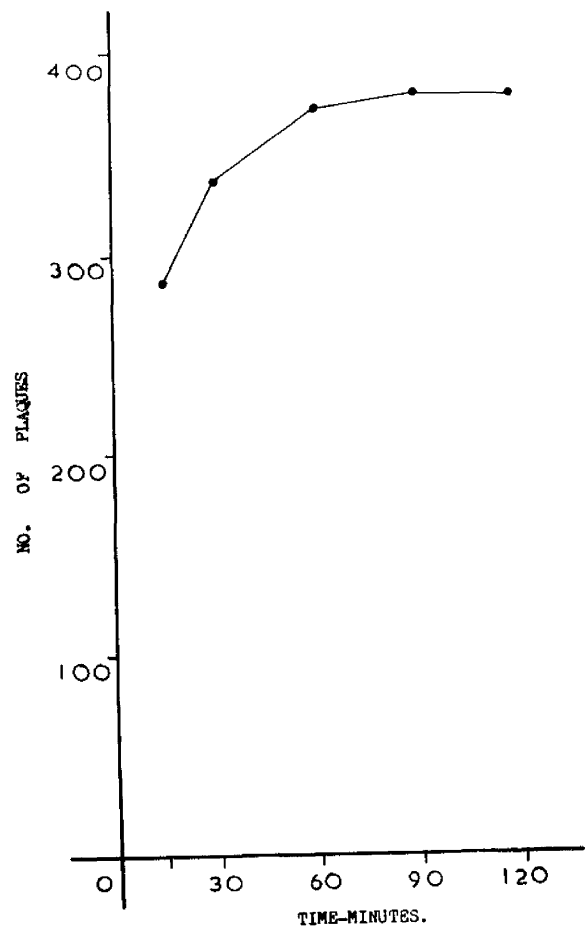

Fig. 8.

Fig. 8 Adsorption of Vaccinia to Cl3 in suspension

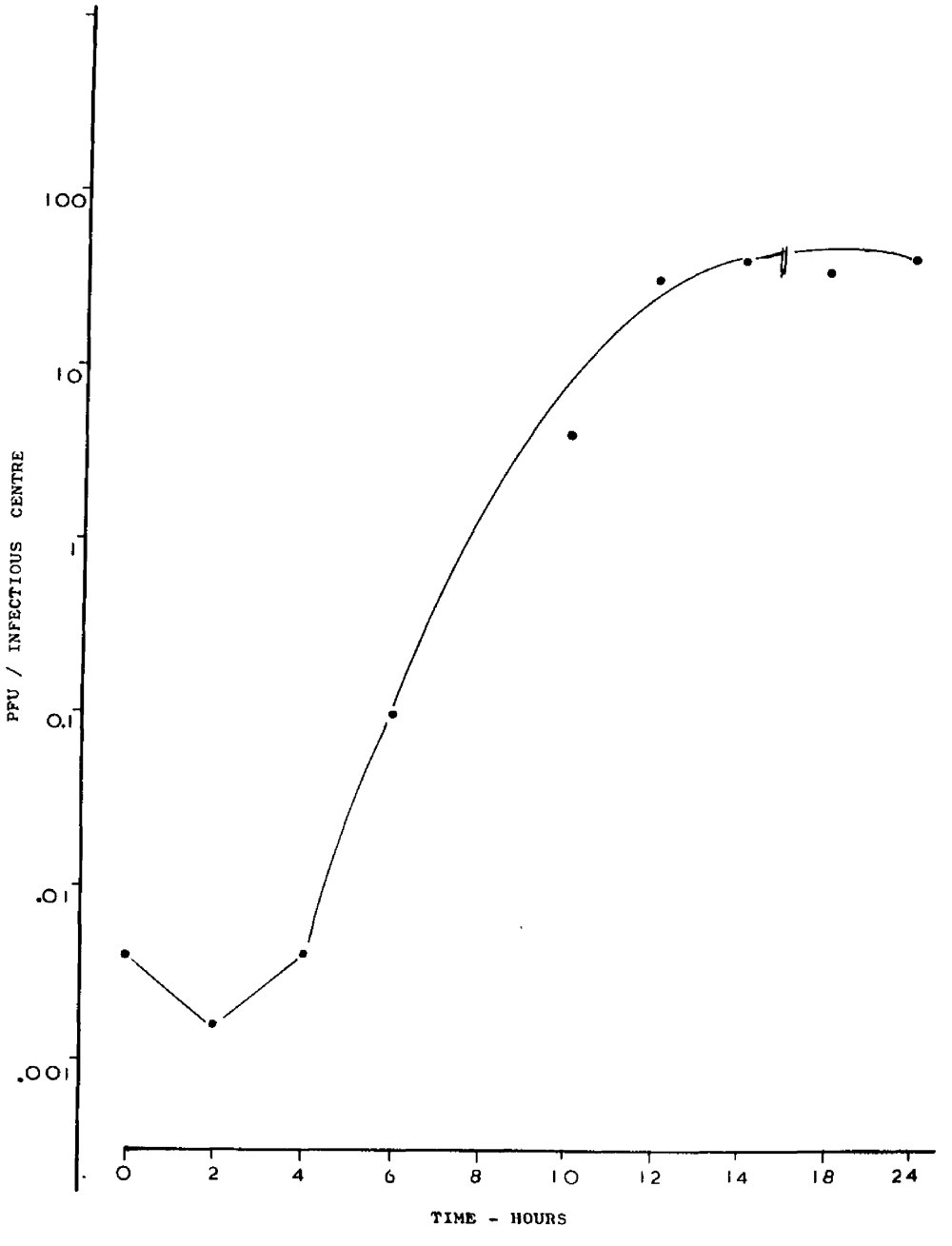

Fig. 9

Fig. 9 Vaccinia : single step growth cycle in C13 cells.

cells as compared to Herpes Simplex and Pseudorabies. The latent period was about 4 hours and after 4 hours virus continued to grow till 14 hours when the maximum yield of virus was obtained. Once the maximum level was reached, it remained stable on further incubation up to 24 hours, indicating that virus is not greatly inactivated by heat. The yield per infected cell calculated from the number of infective centres, was 75 pfu per infected cell.

CHAPTER 5.

GROWTH OF DNA VIRUSES IN HEATED CELLS.

The ability of BHK-21, C13 cells after exposure to heat, to support the growth of Herpes Simplex, Pseudorabies and Vaccinia virus, was determined by the formation of infective centres. Infective centres are the plaques formed by single infected cells and give a proportionate estimation of the fraction of infected cells capable of producing new virus in a cell population.

METHODS:

Infective centre assay by the suspension method:

A single cell suspension from actively growing C13 cells, was prepared and counted. Aliquots of 1 ml quantities, containing about 1 x 10^6 cells/ml, were dispensed in a number of 4 x ½ siliconed test tubes. Cells were exposed to supraoptimal temperatures for 15 minutes by placing the tubes in a water bath adjusted to the required temperature. The cells were then sedimented by centrifuging at 1000 rpm for 5 minutes, and resuspended in 1 ml virus diluted in ETC to give a high input ratio. The cell-virus complex was incubated at 37°C. The cells were kept in suspension during the adsorption period by stirring on a magnetic stirrer. After incubation, the cells were washed 4 times with ETC to remove unadsorbed virus, and resuspended in 1 ml of ETC.

0.5 ml of it was further diluted to give 2 ml of cell suspension containing approximately 800-1000 cells per millilitre. One millilitre of diluted suspension was used for infective centres, and the remainder was frozen and thawed three times, and titrated for any cell-associated or unadsorbed virus. Infected cells were mixed with $4n \times 10^6$ normal cells suspended in 5n ml of Eagle's medium (1.3 times normal strength), containing carboxy methyl cellulose, and plated in 5 ml amount per plate (n stands for the number of petri-dishes). The plates were incubated at 37°C in a CO_2 incubator. Care was taken not to disturb the plates to prevent the formation of secondary plaques. At the end of 3 days, plates were fixed with 10% formol saline and stained with Leishmann's stain. Infective centres were counted with a hand lens.

Cell counts - Cell counts were done in a haemocytometer. An equal amount of 0.5% trypan blue was added to the remaining 0.5 ml of undiluted cell suspension, and only unstained cells were counted. Doublets were counted as one and triplets were counted as two, which formed less than 1% of the total cell suspension. At least 200-400 cells were counted per sample.

Complement fixation test:

Antisera - (a) A pool of human herpes-immune serum was used in titration of Herpes Simplex C.F. antigen.

(b) Anti-vaccinial serum prepared in the rabbit was kindly

supplied by Dr. Ross and was used to measure Vaccinia C.F. antigen.

For the complement fixation tests, serial two-fold dilutions of the antigen were prepared in veronal buffer using 0.1 ml volumes. To the respective series of dilution were added 0.1 ml of complement (2 units) diluted in veronal buffer and 0.1 ml of diluted specific antiserum. After primary incubation at 4°C for 24 hours, 0.1 ml of sensitized sheep red cells were added to each dilution and the test was read after further incubation at 37°C for 1 hour. The last dilution of antigen giving complete haemolysis was taken as the end point. The results were expressed as antigen units per millilitre.

RESULTS:

Herpes Simplex:

The cells exposed to supraoptimal temperatures for 15 minutes were infected in suspension with the HFEM strain, to give a multiplicity ratio of 14 pfu/cell. The virus-cell mixture was incubated for 30 minutes at 37°C. The minimum number of 800 infected cells per sample were plated for infective centres. Infective centres were read after three days of incubation at 37°C in a CO_2 incubator. The frozen and thawed controls were included in all experiments. This gave less than 1 plaque per petri dish, and this excluded the possibility of formation of infective centres due to free virus.

It was found that the number of cells forming infective centres fell with the rise in temperature to which cells were exposed. The results of two experiments are shown in Fig. 1. In one of the experiments, cells were heated to five different temperatures and the ability of these cells to form infective centres was compared with that of normal cells. 67% of the normal cells produced infective centres, as against 100% by theoretical estimation. Cells heated at 39°C gave the same number of infective centres, suggesting that the sensitivity of C13 cells to support virus growth was resistant to a temperature of 39°C. In the second experiment only 52% of normal cells formed infective centres. The reasons for this discrepancy are not known. The number of infective centres obtained with the heated cells are compared with that of normal cells and are plotted on a relative scale in Fig. 1. It indicates that nearly 20 to 50% of cells exposed to temperatures between 41°C and 43°C failed to induce plaques, and nearly 80-100% of cells became resistant to infection when heated at 45°C and 46°C.

It is important to recollect that the number of cells unable to stick to a glass or plastic surface, increases with the rise in temperature of exposure (page 21). The reduction in number of infective centres could have been due to failure of cells to get attached or settle on the plates. Herpes virus is unstable at 37°C and this instability

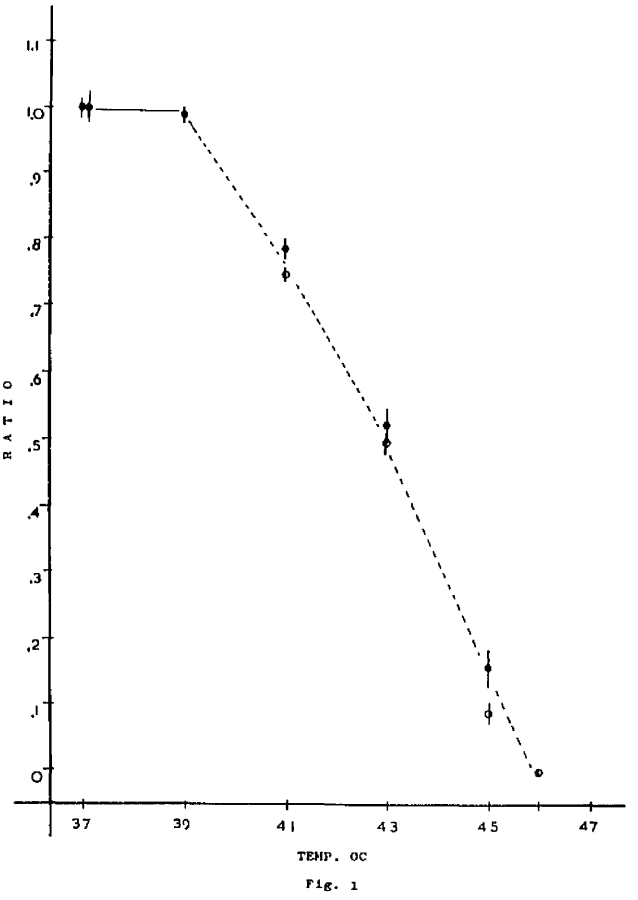

Fig. 1

Fig. 1 Herpes Simplex : The ratio of infective centres formed by preheated cells to the number formed by normal cells.

is probably responsible for the absence of infective centres, since virus released from infected cells can become inactivated by the time it has diffused through the medium containing carboxy methyl cellulose. To overcome this difficulty, a monolayer method was tried (described on page 66). The infected cells were plated on to the monolayers. A small amount, about 0.5 ml, of agar overlay was gradually delivered on to the surface of the monolayers and allowed to harden, and then 4.5 ml of agar overlay was poured onto the thin layer which prevented cells from swirling into the agar at various distances from the monolayers. At the same time, infected cells were plated by the suspension method. Results in Table 1 indicate that reduction in the formation of infective centres is not due to the failure of cells to stick to the surface. It also shows that suspension method is a more sensitive technique than the monolayer method.

Virus attachment:

Perhaps heated cells were not infected because heat had so altered the surface as to prevent virus attachment. The adsorption rates of Herpes Simplex virus to normal and heated cells, heated to 45°C, were compared. A series of siliconed tubes, containing normal and heated cells suspended in 1 ml of virus inoculum containing 400 pfu/ml, was incubated at 37°C. At various time intervals the cells of two tubes, one containing a normal

TABLE 1

Method of plating of heated cells compared in Herpes Simplex infection.

Temperature °C	Duration of exposure in minutes	Percentage of Infectious Centres	
		Monolayer	Suspension
Normal cells		27.1	33.1
39	15	28.0	40.2
41	"	19.4	28.5
43	"	11.6	18.6
45	"	1.1	7.1
46	"	-	-

cell-virus mixture and the other heated cells and virus, were spun down and the supernatant fluids were titrated for the residual virus. Data presented in Fig. 2 demonstrates that the adsorption rate in both cells is the same, thus ruling out the possibility of cells being not infected because of non-adsorption of virus.

The steps subsequent to attachment, penetration and eclipse were not specially studied. The absence of cell-associated virus in the frozen and thawed controls of infective centres, indicated that adsorbed virus was non-infectious during the latent period, that is, it had gone into eclipse.

A comparison of viral growth in normal and heated cells was made. Since 80-90% of cells failed to produce infectious centres when heated at 45°C, it was considered as a suitable temperature for comparison.

Single step growth curve:

Single step growth curves were performed and assays were made on the total infective centres, total intracellular virus and the antigen production.

Cells exposed to a temperature of 45°C for 15 minutes in number of siliconed test tubes, were pooled together to give approximately 3×10^7 cells. A pool of fresh cells containing about the same number of cells, along with the pooled heated cells were infected with Herpes Simplex virus

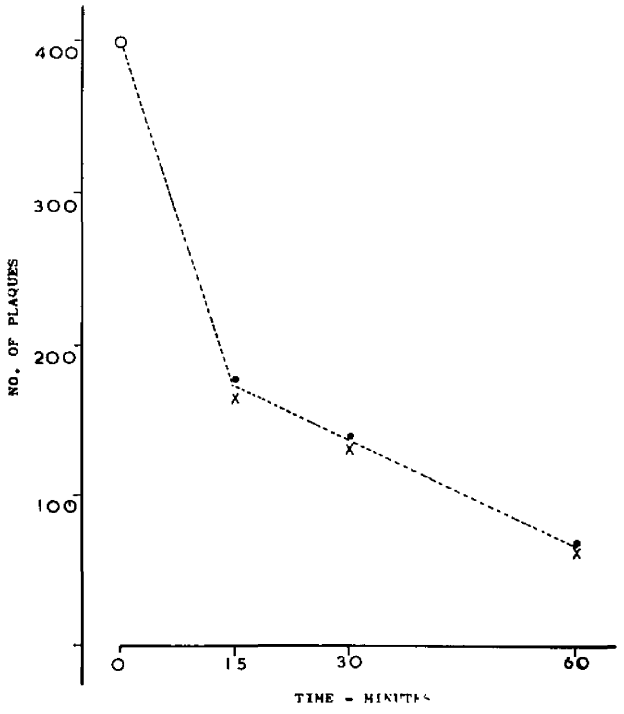

Fig. 2

Fig. 2 Herpes Simplex : Adsorption rate in normal (x --- x) and preheated cells (● --- ●).

at a high input multiplicity of infection. The virus-cell mixtures were incubated at 37°C for 30 minutes on an automatic shaker. At the end of incubation, cells were washed 4 times to get rid of unadsorbed virus. The cells were resuspended in ETC and distributed in small tissue culture bottles, in 2 ml amounts, containing approximately 1 x 10^6 cells/ml. An aliquot of cells was taken for estimation of infective centres. Samples from both sets were taken at various time intervals; cells were harvested and spun down at 1000 rpm for 5 minutes. The pellet of cells was suspended in 1 ml of distilled water and disrupted by treatment in a Dawe Soniclean bath. The completeness of disruption was determined by microscopic examination. Cellular debris was sedimented by centrifugation and the supernatant fluid was used for assay. The results of both curves are summarised in Fig. 3.

Normal cells:

After a 4 hour latent period, the titre of infectious virus rose, to give a maximum yield at 12 hours; 40% of the cells plated for infective centres formed plaques. The yield per cell was calculated from the total amount of virus and the total number of infected cells. The yield of infectious virus per infected cell rose from .004 pfu/cell at 2 hours to 200 pfu/cell at 12 hours. Antigen, measured by the complement fixation test, was detected by 4 hours and reached its maximum level by 12 hours.

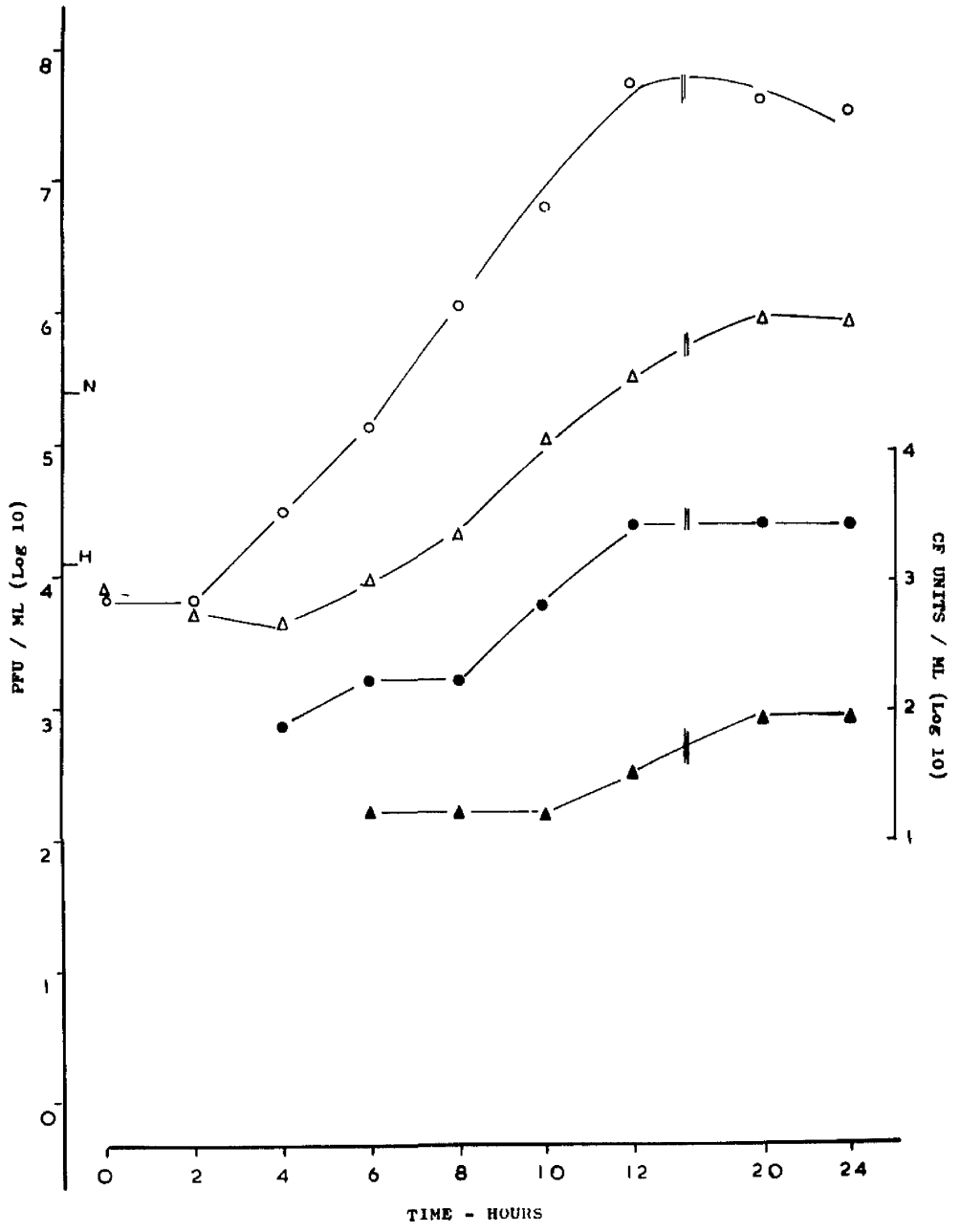

Fig. 3

Fig. 3 The multiplication of Herpes Simplex in normal cells and pre-heated cells (45°C for 15 min.).

N and H represent the number of infectious centres obtained with normal and pre-heated cells respectively.

o —— o yield of infectious virus in normal cells.
● —— ● yield of antigen in normal cells.
Δ —— Δ yield of infectious virus in preheated cells.
▲ —— ▲ yield of antigen in preheated cells.

The antigen and infectivity curves were not parallel and the difference expressed as a ratio between the highest yield of infectivity (pfu/ml) and antigen (C.F. unit/ml) was $10^{4.51}$.

Heated cells:

The infectious virus was not detected until 6 hours, and then gradually rose to reach its maximum by 20 hours. Only 2% of the cells plated for infective centres were recorded to have formed plaques. The yield per infected cell was 50 pfu/cell. The complement fixing antigen first appeared by 6 hours, two hours later than that of normal cells. The synthesis of antigen was completed by 20 hours. Since the antigen and infectivity curves were not parallel, the difference was expressed as ratio between the two and was $10^{4.10}$.

The appearance of infectious virus and antigen was delayed in heated cells, and the time required for the completion of a single cycle was more than that required in unheated cells. When the growth curves of infectious virus and antigen in unheated and heated cells were compared, as seen in Fig. 3, it became evident that the slopes of the curves were the same, which meant that the rate of virus multiplication in normal and heated cells was the same. The prolonged latent period might be due to undetectable quantities of virus in the early part of the cycle. As only 2% of heated

cells took part in the virus production as against 40% normal cells, the amount produced by these cells was probably too small to be detected by the present method of titration, and it was two more hours before measurable amounts were synthesized. The yield per unheated infected cell was 200 pfu/cell and for the heated infected cell was 50 pfu/cell, about 75% less than the normal cell.

The infectivity to antigen ratios in normal and heated cells were $10^{4.51}$ and $10^{4.10}$ respectively. The difference in the two ratios is about 0.4 log. These results indicated that, although heated cells made less infectious virus, they produced more antigen.

It is known that the most of the antigen is situated in protein which is not incorporated with the particle. The excess of antigen may thus represent protein which was excluded from particles by chance or else was never destined to become incorporated. It was possible that the heated cells which still produced virus were partially damaged and needed to make more antigen than normally required before the infectious virus was formed.

Another possibility is, that more cells took part in viral synthesis than produced infectious virus. The cells producing antigen alone could have been detected by the fluorescent staining technique. But because of technical difficulties of staining rounded cells, this technique could not be used.

Pseudorabies:

The sensitivity of heated cells to Pseudorabies virus was similarly tested by formation of infectious centres. The results obtained were similar to that of Herpes Simplex and are shown in Fig. 4. Further information regarding attachment of virus, nature of multiplication and production of antigen and infectious virus was not obtained.

Vaccinia:

The similarity in results between Pseudorabies and Herpes Simplex was not surprising, as these two belong to the same group of viruses and are similar to each other in many ways. Vaccinia virus differs from these two viruses except in one respect, that all three have DNA for their nucleic acid. The behaviour of heated cells towards vaccinia was a matter of speculation. Hence, synthesis of vaccinia in heated cells was investigated in much the same manner as with Herpes Simplex. All the experiments were done under similar conditions.

Estimation of infectious centres:

Normal and heated cells were infected with vaccinia with an input multiplicity of 5 to 1, and cells were incubated at 37°C for 60 minutes. The cells were maintained in suspension using a magnetic stirrer. After the adsorption period, cells were washed thoroughly to get rid of unadsorbed virus. The cell suspension was further diluted and counted.

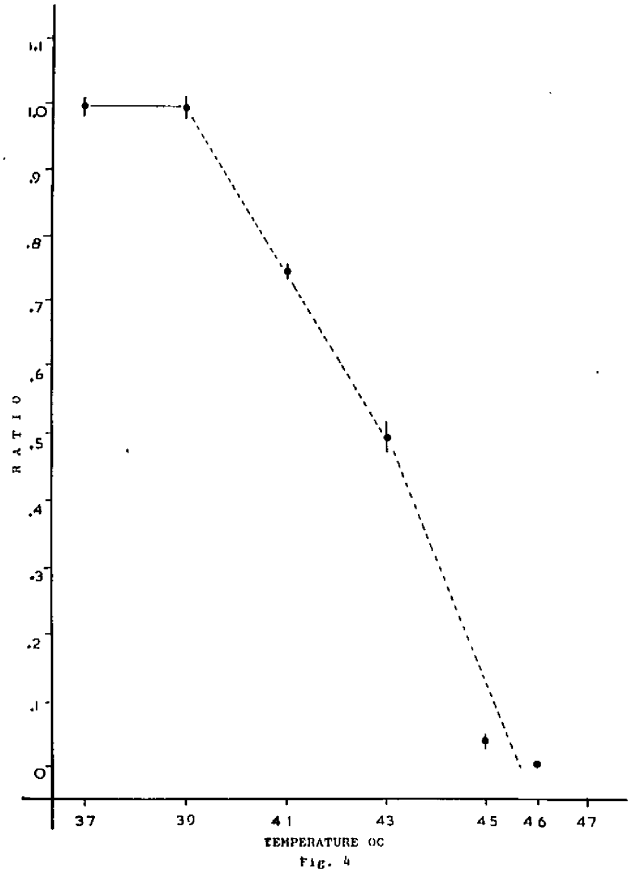

Fig. 4

Fig. 4 Pseudorabies : The ratio of infective centres formed by preheated cells to the number formed by normal cells.

About 800 to 1000 cells per sample were plated for the formation of infectious centres. Frozen and thawed controls were included in every experiment. The percentage of cell-associated and free virus was never more than 1%. The results of such two experiments are shown in Fig. 5. In both the experiments the percentage of infective centres was 62.1% and 55.4% respectively, which was less than theoretical expectations. Fig. 5 shows that the number of cells forming infective centres had decreased as the temperature of exposure had increased. On exposures to temperature 41°C and 43°C nearly 20 to 50% of cells lost their capacity to grow vaccinia, and at and above 45°C 90% to 100% of cells were unable to synthesize vaccinia.

Virus attachment:

The failure in formation of infective centres was not due to the failure in virus adsorption, as shown in Fig. 6.

Single-step growth curves at two different temperatures:

The growth curves in normal cells and in those heated to a temperature of 45°C for 15 minutes, were compared and summarized in Fig. 7.

Normal cell:

The growth curve obtained with normal cells was characteristic of vaccinia growth in Cl3 cells, previously described on page 39. The antigen production was

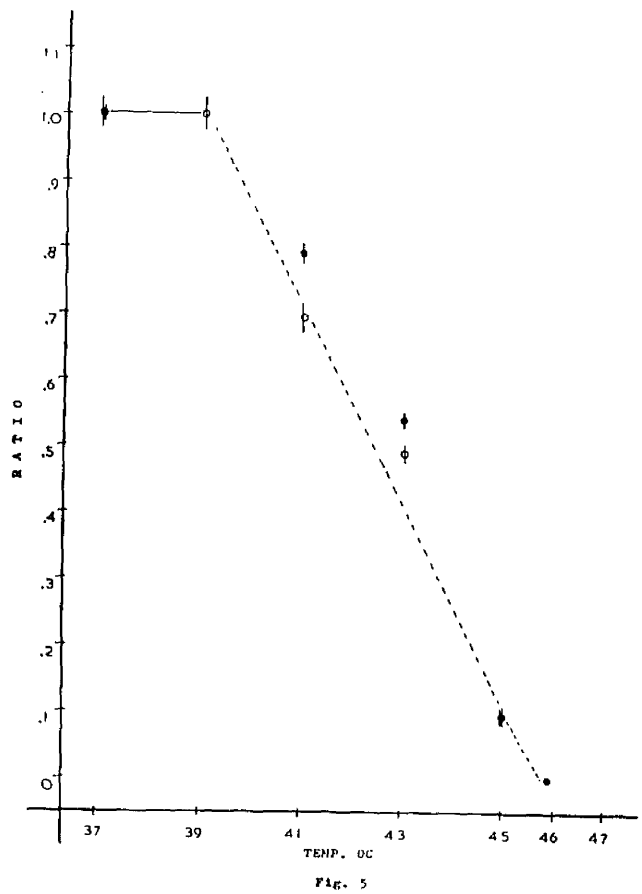

Fig. 5

Fig. 5 Vaccinia : the ratio of infective centres formed by preheated cells to the number formed by normal cells.

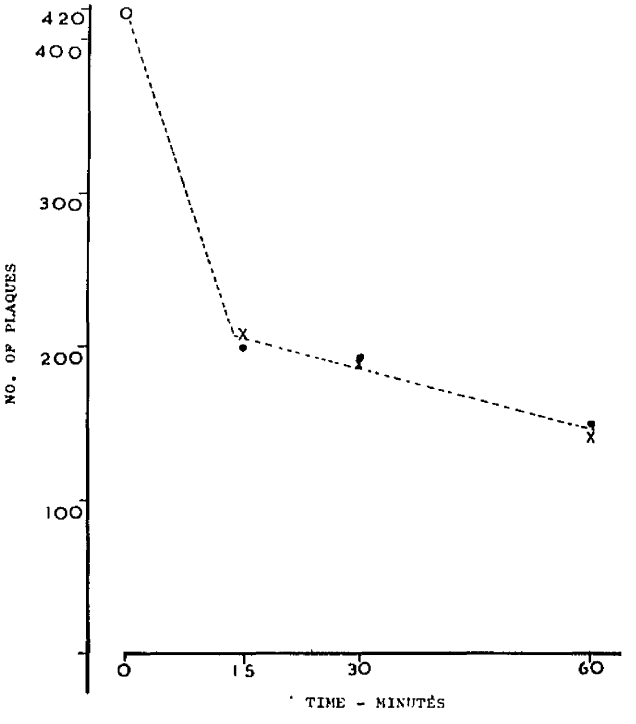

Fig. 6

Fig. 6 Vaccinia : adsorption rate in normal (x —— x) and preheated cells (● —— ●).

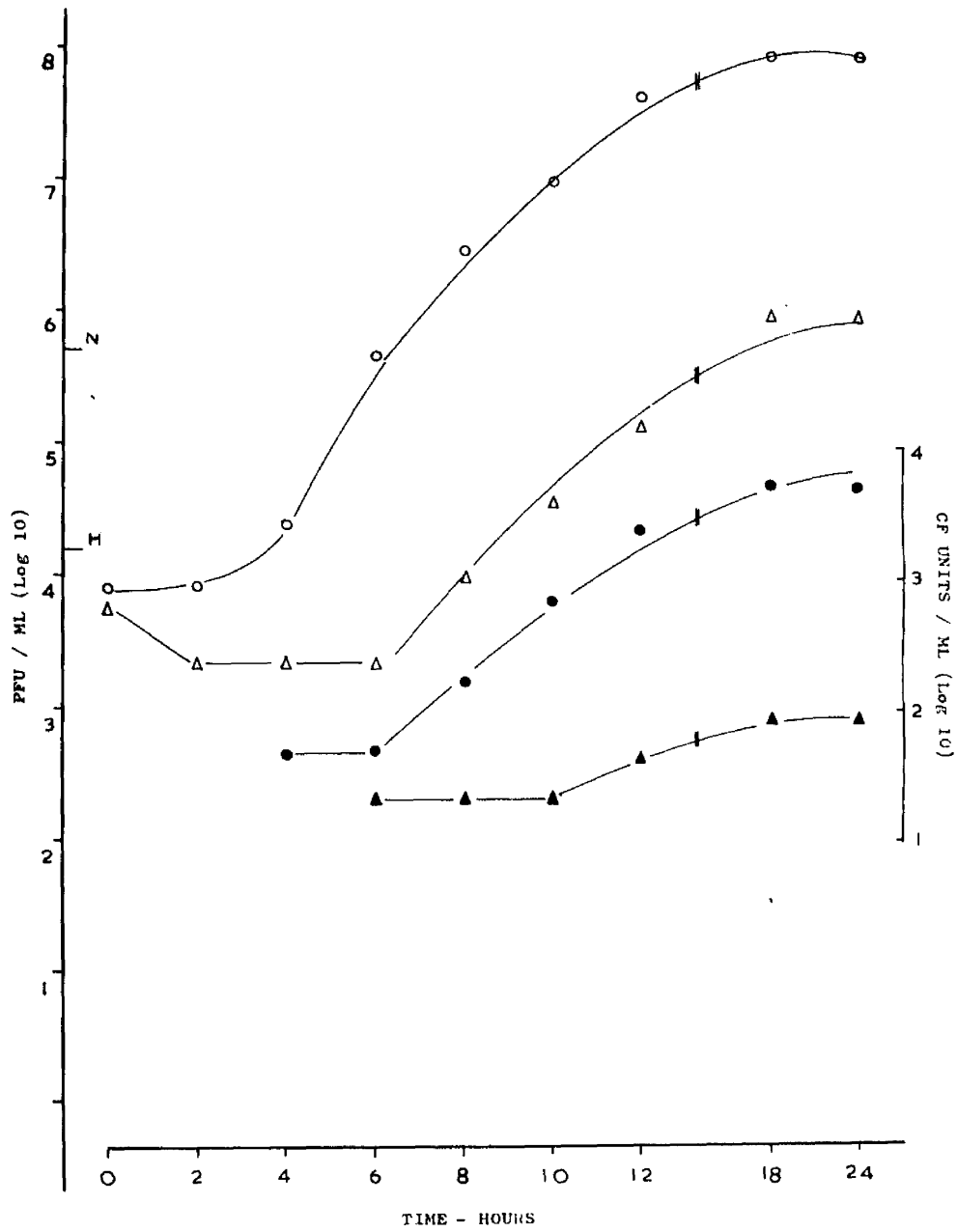

Fig. 7

Fig. 7 The multiplication of vaccinia in normal cells and preheated cells (45°C for 15 min.).

N and H represent the number of infectious centres obtained with normal and preheated cells respectively.

o —— o yield of infectious virus in normal cells.
● —— ● yield of antigen in normal cells.
△ —— △ yield of infectious virus in preheated cells.
▲ —— ▲ yield of antigen in preheated cells.

detectable by 4 hours and reached its maximum synthesis about the same time as infectious virus by 18 hours. The number of infectious centres obtained, was 70% and the yield per infected cell was 143 pfu/cell. The infectious virus and antigen was expressed as the ratio of the two and was $10^{4}.61$.

Heated cells:

The production of infectious virus and antigen was apparently delayed. The slopes of the growth in normal and heated cells were the same, indicating that the rate of virus multiplication in both cases was probably the same. Only 2% of heated cells formed infectious centres and the yield per infected cell was 50 pfu per cell. The ratio between infectious virus and antigen was $10^{4}.10$.

Once again, the results indicated that the heated cells produced more antigen and less infectious virus as compared to normal cells. A similar difference in the behaviour of heated and normal cells was observed with Herpes Simplex. The possible explanations have already been considered.

The results discussed above show that the exposure of cells to supraoptimal temperatures makes them insensitive to DNA virus infection. The decline in sensitivity begins at about the same temperature, and falls at about the same rate as the plating efficiency of BHK-21, C13 cells.

CHAPTER 6.

GROWTH OF RNA VIRUSES IN NORMAL C13 CELLS.

It was essential to examine the growth of Encephalomyocarditis, Myxoviruses - a neurotropic strain of Influenza A, NWS, Fowl plague virus and Newcastle disease virus - and Reo virus type 3 in normal C13 cells, prior to their study in cells exposed to supraoptimal temperatures.

Encephalomyocarditis virus belongs to the group of Mengo viruses. It is one of the small RNA viruses. The site of its synthesis in the infected cell was a matter of controversy, but recently Eason and Smellie (1964) have confirmed its synthesis in cytoplasm. It has one advantage over the other two groups to be discussed, that its RNA nucleic acid has been isolated and shown to be infectious in C13 cells (Milliken, unpublished). In addition Breeze (1964), in concurrent studies, has examined its growth in C13 cells in detail.

Myxoviruses:

There are two main subgroups of the myxoviruses which are known to be RNA viruses (Ada, 1957; Ada and Perry, 1954) but from which infectious RNA has never been satisfactorily isolated. (For a possible exception see Maassab, 1959; Portocala et al., 1961.) However, all are

characterised by the synthesis of a soluble antigen, i.e. ribonucleo-protein, which becomes enclosed in an envelope that incorporates particle antigen, i.e. haemagglutinin. Only in the influenza viruses the soluble antigen is synthesized in the nucleus and the haemagglutinin in the cytoplasm, while in the other subgroup, the mumps, Newcastle disease virus, para-influenza viruses, both antigens are synthesized in the cytoplasm. Because of these differences, it was decided to examine the viruses of influenza and Fowl plague from one subgroup and Newcastle disease virus from the other subgroup, lest heat should react differently on the nuclear and cytoplasmic synthesis of virus components. Fowl plague virus and Newcastle disease virus were already known, in this laboratory, to form plaques on monolayers of Cl3 cells. NWS, the mouse-neurotropic strain of influenza A virus, was added when Milliken (unpublished) found that it also, but not other influenza A strains, produced plaques on Cl3 monolayers.

Reo viruses are recently isolated and very little is known about them. They have characteristic inclusion bodies which are always located around the nucleus. The synthesis of new virus in the infected cell is said to take place along the fibres of the mitotic spindles (Spendlove et al., 1963; Dales, 1963). There are speculations regarding the infectious ribonucleic acid. There are

reasons to believe that the RNA of Reo virus is double stranded (Gomatos and Tamm, 1963a, 1963b).

MATERIALS AND METHODS:

Viruses:

Encephalomycarditis (EMC):

A small plaque variant of encephalomyocarditis virus isolated from a single plaque and passed several times in L-cells, was kindly supplied by Dr. Breeze. It was then grown in BHK-21, C13 cells. It grew readily in C13 cells and the stock virus was prepared from the tissue culture fluid of the second passage which gave a titre of 2×10^8 pfu/ml. The stock was titrated by the plaque method. The plaques appeared in 24 hours in the presence of DEAE dextran. Virus stock was stored at -70°C in 1 ml quantities.

Myxoviruses:

NWS, Fowl plague virus and Newcastle disease virus, grown in eggs, were kindly provided by Dr. Fraser.

NWS strain of Influenza A (NWS):

The seed of NWS was prepared by suballantoic inoculation of 11 day old eggs with 0.05 ml of infected allantoic fluid diluted to 10^{-5} to 10^{-6} in ETC. The eggs were incubated for 48 hours at 37°C. The allantoic fluids were collected and stored at -70°C. The stock was assayed for infectivity by the plaque technique and for haemagglutinin activity by the serial two-fold dilution method. The plaques

appeared in four days. The infectivity and HA titres were 5×10^8 pfu/ml and 2.5×10^3 HA units/ml, respectively. The pfu/HA ratio of the stock virus was of the order of $10^{5.30}$. The ratio of infectivity titre in eggs and in C13 cells was nearly unity, indicating that one infectious unit was needed to initiate plaque formation in C13 cells.

Fowl plague virus (FPV):

The stock of FPV was prepared in the same manner as above and stored at -70°C. The seed was titrated on C13 monolayers and plaques were scored at the end of 4 days. The titre of the seed was 1.1×10^8 pfu/ml. The HA titre was 1.2×10^3 HA units/ml. The ratio of egg infectivity titre (EID50) and plaque forming units (pfu/ml) in C13, was almost unity, which indicated that one infectious unit formed one plaque in C13 cells.

Newcastle disease virus (NDV):

Egg grown NDV was adapted to C13 cells by four passages. The tissue culture fluid of the fifth passage had the titre of 1.5×10^8 pfu/ml and was stored at -70°C. The haemagglutinin titre was not determined as it was produced in very small quantity and gave variable results at different temperatures.

Reo virus:

Reo type 3 grown in monkey kidney cells was obtained by courtesy of Dr. Ross. It was then passed in BHK-21, C13

cells several times. It grew slowly in C13 cells and took 3 to 4 days before the majority of cells showed cytopathic changes. BHK-21, C13 cells were generally grown in the medium containing 10% calf serum (ETC). Since calf serum inactivates Reo virus (Rhim and Melnick, 1961), cells were grown in a medium containing 10% horse serum, inactivated at 56°C for 10 minutes (ETHr). The cells were subcultured twice in ETHr before being used for the serial passages. The stock was prepared from 8th passage material. Cells were harvested on the fourth day after infection and suspended in a small amount of the supernatant fluid. The cells were disrupted in a Dawe Soniclean ultrasonicator, cellular debris was spun down at 1000 rpm for 5 minutes and the supernatant was stored at -70°C. The infectivity titre was assayed by the plaque technique and was 2×10^7 pfu/ml.

Plaque method for assay:

The virus was titrated routinely on C13 monolayers, prepared by plating 3.5×10^6 cells/5 ml of ETC in 50 mm petri dishes and incubating at 37°C in 5% CO_2 incubator for 24 hours. Plates were then washed once with tris-calf solution, and 0.1 ml of virus inoculum diluted in ETC was added to the monolayers. Adsorption of virus to the monolayers was allowed to proceed at 37°C in a CO_2 incubator for a specific length of time, depending on the virus used. At the end of adsorption, virus inoculum was removed and

5 ml of agar overlay were added to each plate. In some experiments, e.g. adsorption tests, monolayers were washed to remove excess of inoculum before addition of overlay, but it was later found to be unnecessary because the same plaque counts were obtained in both washed and unwashed plates. The overlay consisted of 1.3 Eg. saline, 5% calf serum, 0.9% Noble agar and 10 µg/ml DEAE dextran. This overlay medium was used for all viruses except Reo virus. DEAE dextran was routinely included in the overlay medium. EMC small plaque variant grew rapidly and attained a large sized plaque in the presence of DEAE dextran.

The growth of myxoviruses was not hastened in the presence of DEAE dextran, but the plaques formed were more regular in shape and distinct than those grown in the absence of DEAE dextran or with Difco Bacto agar overlay.

The second overlay containing 0.004% neutral red was added to the plates in 2.5 ml amounts, at the end of the incubation period, i.e. at the end of 24 hours for EMC, 4 days for NWS, FPV and NDV and 6 days for Reo virus. The plates were incubated for a further period of 6 hours and then plaques were scored as visible unstained areas.

The overlay used for Reo virus contained 1.3 Eg. saline, 1.2% Noble agar, 1% yeast extract and 0.1% bovine albumen.

Infective centre assay:

Sufficient virus was added to a suspension of C13 cells in ETC to insure that every cell was infected. The

cell-virus mixture was incubated at 37°C and kept in suspension by continuous stirring on a magnetic stirrer. The test tubes containing virus-cell complex were tightly corked with silicone bungs. At the end of the adsorption time the cells were centrifuged and washed in ETC three times to remove unadsorbed virus. The cells were resuspended in 1 ml ETC of which 0.5 ml was further diluted, and to the remainder, 0.5 ml of 1% trypan blue was added and used for counting. A known number of cells in a volume of 0.1 ml was gradually added to once washed C13 monolayers and allowed to spread. The plates were then replaced in a CO_2 incubator at 37°C for half an hour, which allowed cells to settle on the sheet. After half an hour 0.5 ml of overlay medium (same as the one used for plaque assay) was poured on to the monolayers and was allowed to harden. This immobilized the cells and prevented them from swirling into agar when the remaining 4.5 ml was added. The plates were incubated at 37°C in a 5% CO_2 incubator. Generally four plates per sample were used. The remainder of the cell suspension was frozen and thawed four times during latent period, and titrated for unadsorbed and cell-associated virus. The plates were stained with a second overlay containing 0.004% neutral red at the end of incubation period. The incubation time for each virus was the same as that for plaque assay. The infective centres appeared as unstained areas and were counted by the naked eye.

Quantitative haemagglutination:

Titrations were done in plastic HA trays.

EMC - Serial 2-fold dilutions in glucose/PBS/gelatine buffer were made of the virus inoculum to be tested. 0.4 ml of 0.1% suspension of sheep's red blood cells in glucose/PBS/gelatine buffer were added to 0.4 ml of each dilution. The titrations were left at +4°C overnight and read in terms of the pattern formed by the red blood cells at the bottom of the tubes. The last dilution of the virus preparation giving definate evidence of agglutination was taken as the end point. The results are expressed in terms of haemagglutinating (HA) units/ml.

NWS and FPV - Serial 2-fold dilutions of 0.25 ml of virus inoculum in normal saline were made to which 0.25 ml of 0.5% guineapig red blood cells were added to each dilution. The trays were incubated at room temperature. The readings were taken at the end of 1 hour, the end point being the last dilution showing evidence of agglutination. The results were expressed as HA units per millilitre.

RESULTS:

Encephalomyocarditis:

To establish the most convenient interval for adsorption of virus to CI3 cells on monolayers and in suspension, the adsorption rates were studied.

Monolayers were infected with 0.1 ml of inoculum

containing 50 pfu. The plates were incubated at 37°C in a CO_2 incubator and, at intervals, a set of four plates was withdrawn and washed three times with tris-calf solution. An agar overlay was added to the plates and incubated for 24 hours at 37°C. As Fig. 1 shows, 80% of virus was adsorbed in 60 minutes. A standard interval of 60 minutes was allowed for adsorption of virus in the plaque assay.

The adsorption rate by suspended cells was determined by titrating the supernatant fluid at intervals for residual virus. The cells were suspended in 1 ml of virus inoculum containing 240 pfu/ml. Fig. 2 indicates that about 88% of the virus was adsorbed in 30 minutes, and there was very little further adsorption.

Single-step growth curve:

Information about the growth cycle of EMC virus was obtained from the single-step growth curve. A pellet of logarithmically growing Cl3 cells containing approximately 4×10^7 cells were suspended in 2 ml of neat virus to give an adsorbed multiplicity of 10 pfu/cell. The virus-cell mixture was incubated at 37°C for 30 minutes. The cells were kept in suspension by shaking the mixture automatically. The infected cells were centrifuged and washed four times in ETC and resuspended in ETC at a concentration of approximately 1×10^6 cells/ml. Aliquots of 3 ml quantities were dispensed into 4 oz tissue culture bottles. The bottles were gassed

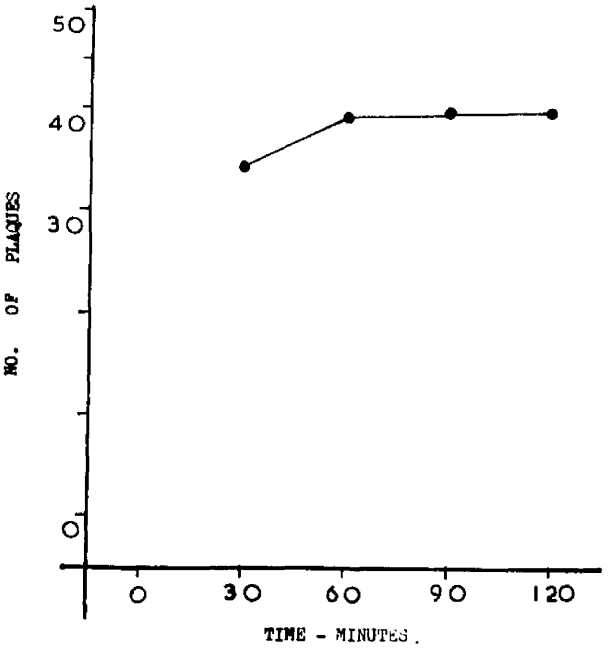

Fig. 1.

Fig. 1 Adsorption of EMC to Cl3 monolayers.

Fig. 2 Adsorption of EMC to Cl3 in suspension.

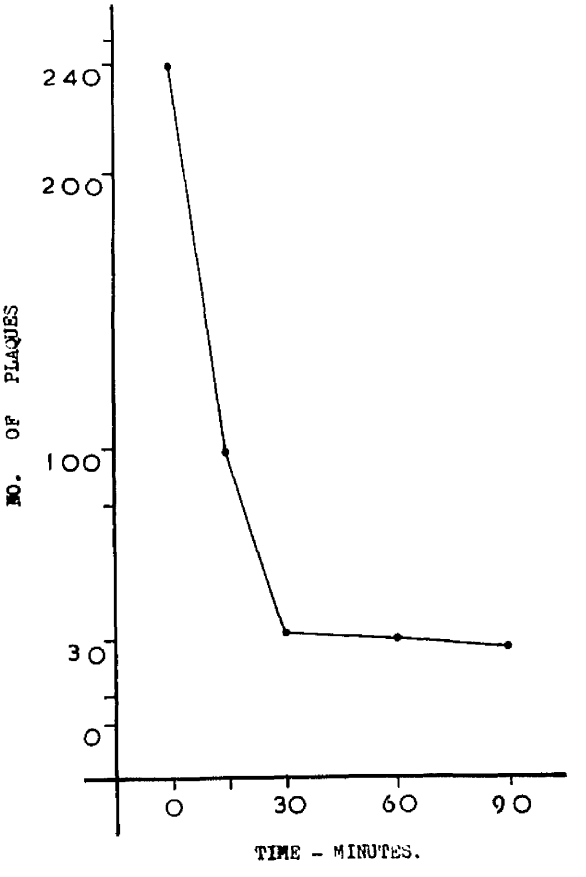

Fig. 2.

with CO_2 and incubated at 37°C. During the latent period a known number of cells was plated for infective centres. Samples were taken at different time intervals and the total yield of virus was determined.

The results are shown in Fig. 3. The solid line represents total infectious virus, while the broken line indicates haemagglutinin activity. The virus formation began somewhere between 2 and 4 hours and was complete by 10 hours. Haemagglutinin activity appeared at the same time as infectious virus and reached a maximum level by 8 hours. The plating efficiency of the infective centre was 41% in this experiment. The yield per cell was calculated based on the total yield of virus and the number of infected cells as judged from the percentage of infectious centres. The burst size was about 280 pfu/infected cell.

NWS :

The rate of virus adsorption to monolayers and to the cells in suspension, was determined in order to choose a convenient adsorption period for growth studies. Figs. 4 and 5 show that maximum adsorption by both methods was achieved by 30 minutes.

Growth curve:

The observation by Milliken (unpublished) that NWS produced plaques in C13 cells, provided an opportunity to examine the relation between input multiplicity and incomplete virus formation. Milliken (unpublished) showed that

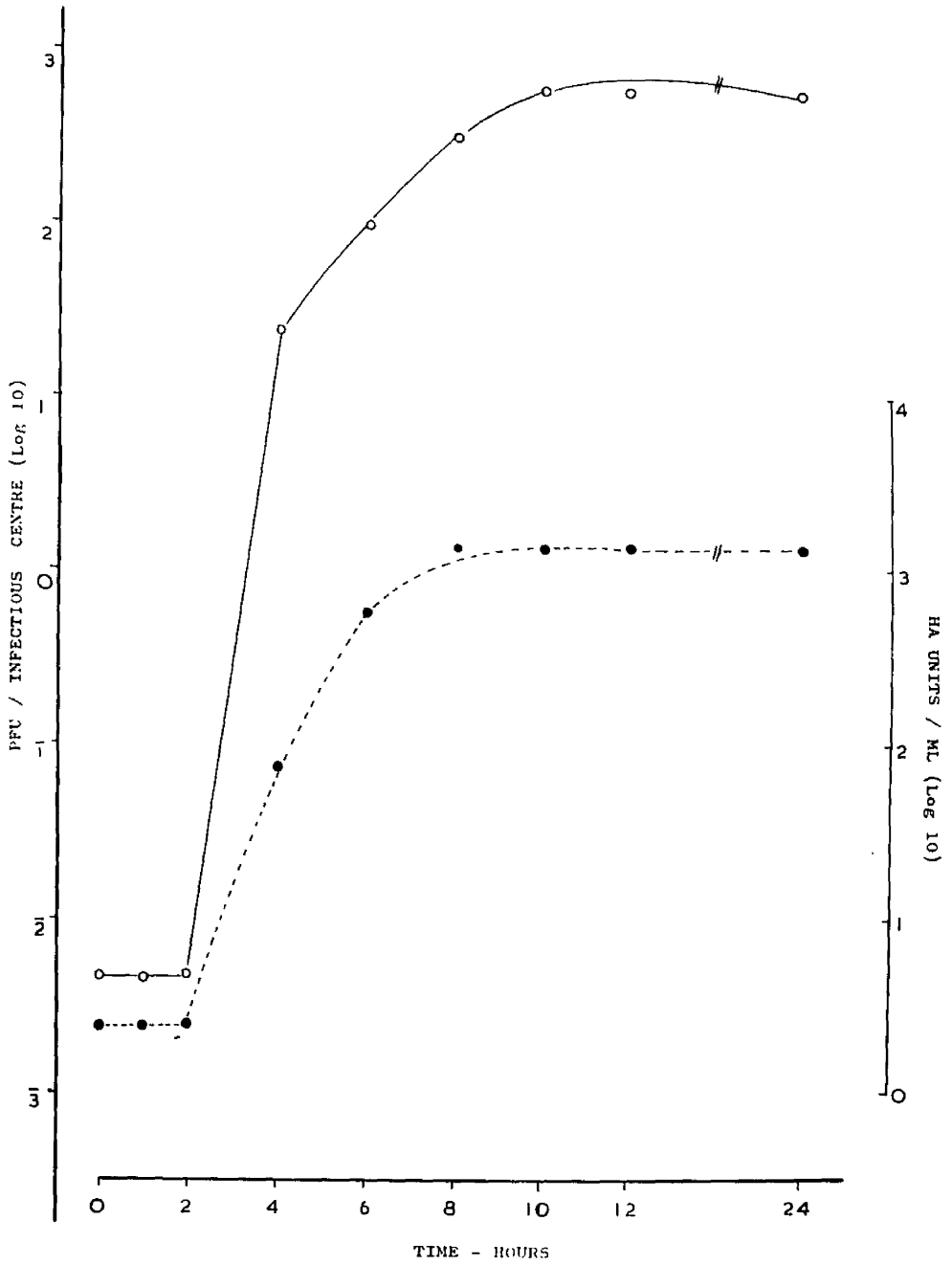

Fig. 3

Fig. 3 EMC : Single step growth cycle.

o —— o yield of infectious virus/infectious centre.
● —— ● yield of haemagglutinin/ml.

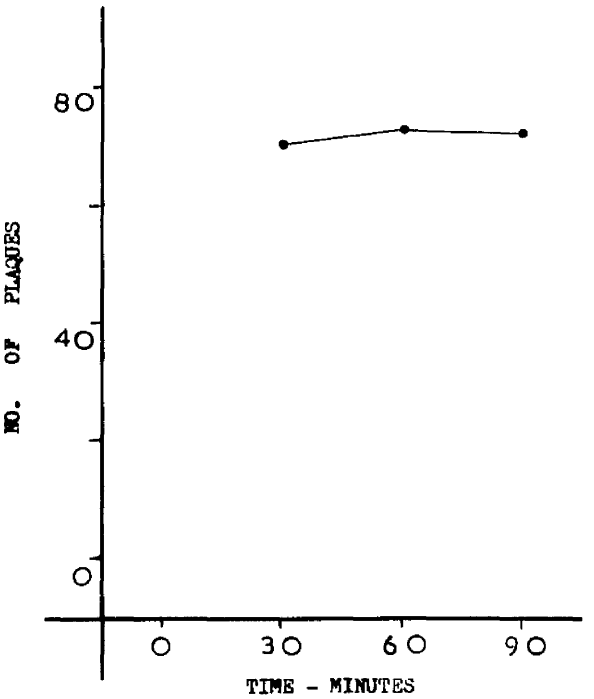

Fig. 4.

Fig. 4 Adsorption of NWS to Cl3 monolayers.

Fig. 5 Adsorption of NWS to Cl3 in suspension.

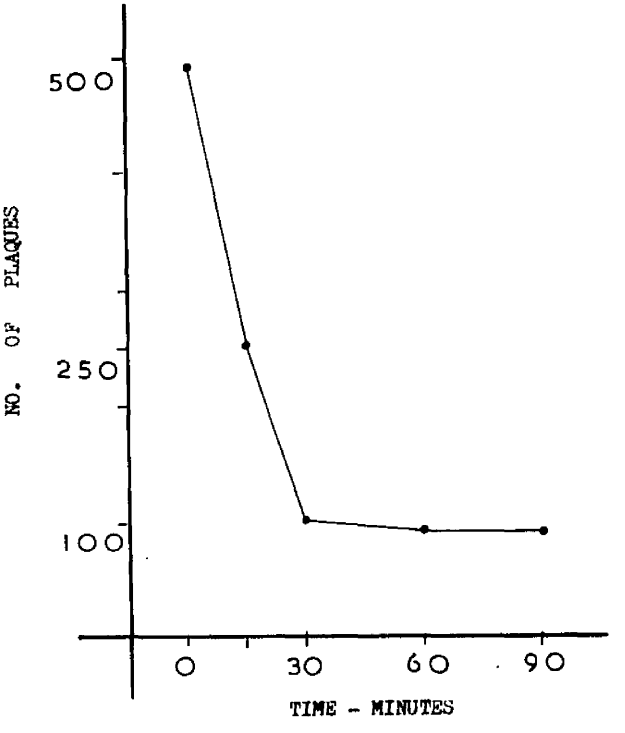

Fig. 5.

multiplication of NWS in this system was dependent on the multiplicity of infection, higher multiplicities accompanied by incomplete virus production. She also showed that the maximum complete virus is produced when there is a multiplicity of one particle per cell.

The first cycle yields were determined and compared at two different multiplicities. Multiplicity in the sense adopted here represents the number of plaque forming units per cell. Two sets of C13 cells containing 4×10^7 cells were suspended in 2 ml amounts of virus inoculum, to give multiplicities of approximately 10 pfu/cell and 1 pfu/cell respectively. Following an adsorption period of 30 minutes at 37°C, the cells were washed four times in ETC and resuspended in ETC and counted. The cell suspension containing about 1×10^6 cells/ml was distributed in $\frac{1}{4}$ oz tissue culture bottles in 3 ml amounts. The bottles were gassed with CO_2 in air and incubated at 37°C. An aliquot of cells was plated for infective centres. The samples were harvested at different time intervals and titrated for infectivity as well as haemagglutinin activity.

The infectivity titrations revealed that the rate of growth at a multiplicity 10 to 1 was slightly more rapid than that at 1 to 1 multiplicity ratio, as shown in Fig. 6. The maximum virus production at 1 to 1 multiplicity was reached by 12 hours, 2 hours later than that at a high input

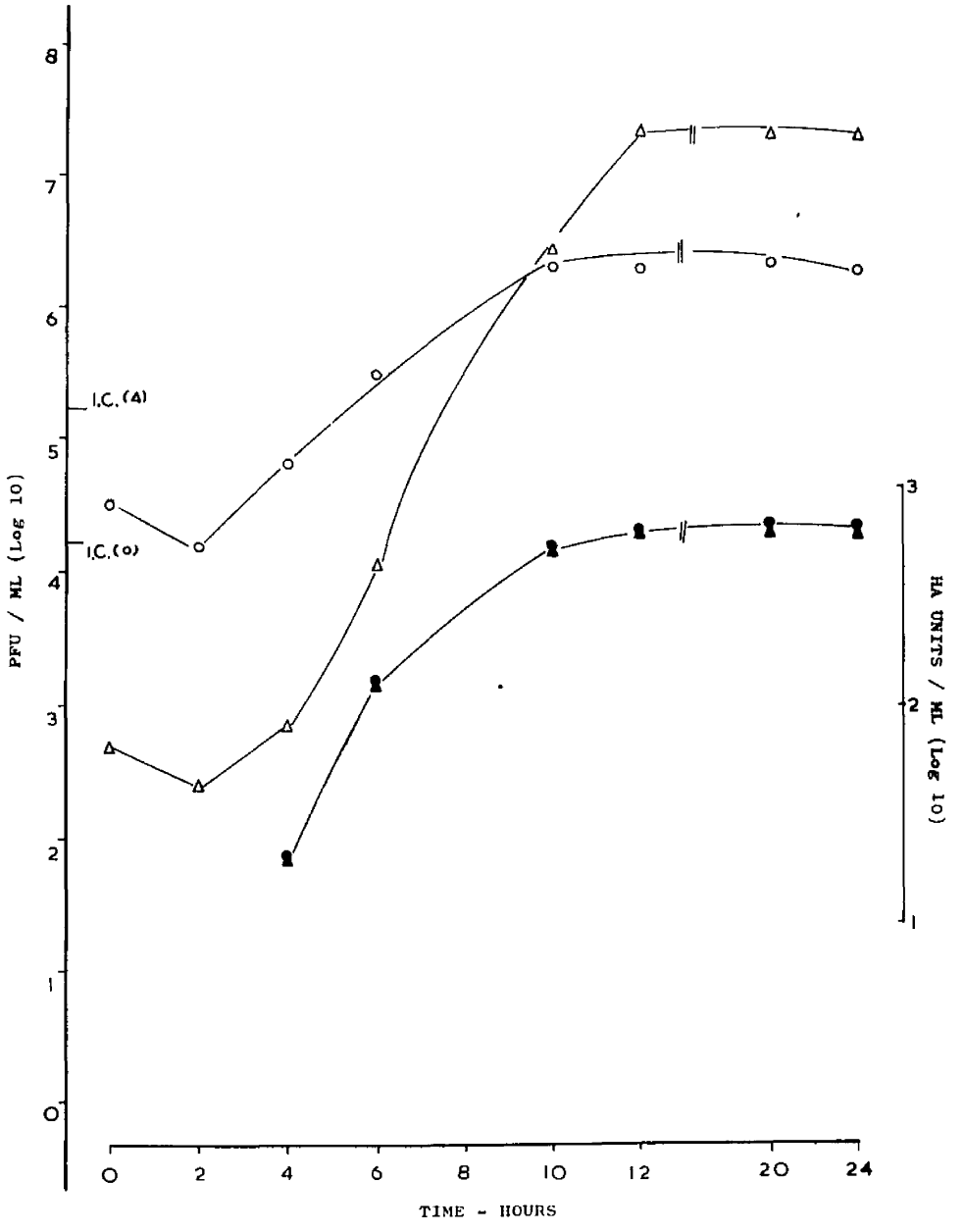

Fig. 6

Fig. 6 NWS : Single step growth cycle at two different multiplicities of infection.

o —— o yield of infectious virus at 10/1 multiplicity
o —— o yield of haemagglutinin " " "

Δ —— Δ yield of infectious virus at 1/1 multiplicity.
▲ —— ▲ yield of haemagglutinin " " "

ratio. The haemagglutinin became detectable by 4 hours and the rate of production was the same in both cases.

At the multiplicity of infection of 10 to 1 and 1 to 1 the pfu/HA/ml ratios were, $10^{3.40}$ and $10^{4.60}$ respectively, indicating that, at the higher multiplicity, infected cells contained a lower proportion of infectious virus. The haemagglutinin content was the same at both multiplicities. These results were in accordance with the results obtained by Milliken (unpublished).

The number of infectious centres obtained were 2% at multiplicity of 10 to 1 and 18% at the ratio 1 to 1. As will be shown later, that multiplicity was to prove an important factor in obtaining infectious centres. The burst size based on number of infected cells was approximately 225 and 208 pfu/cell respectively.

Fowl Plague Virus:

The adsorption rate of FPV on the monolayer and to the cells in suspension was the same. The maximum adsorption was achieved by 30 minutes, Figs. 7 and 8.

Single-step growth curve:

The growth of FPV in C13 cells was found to be dependent on the multiplicity of infection. If the infective dose of virus was large, predominantly non-infectious haemagglutinin was formed, whereas with a small inoculum more infectious virus was produced. This phenomenon was not studied in detail.

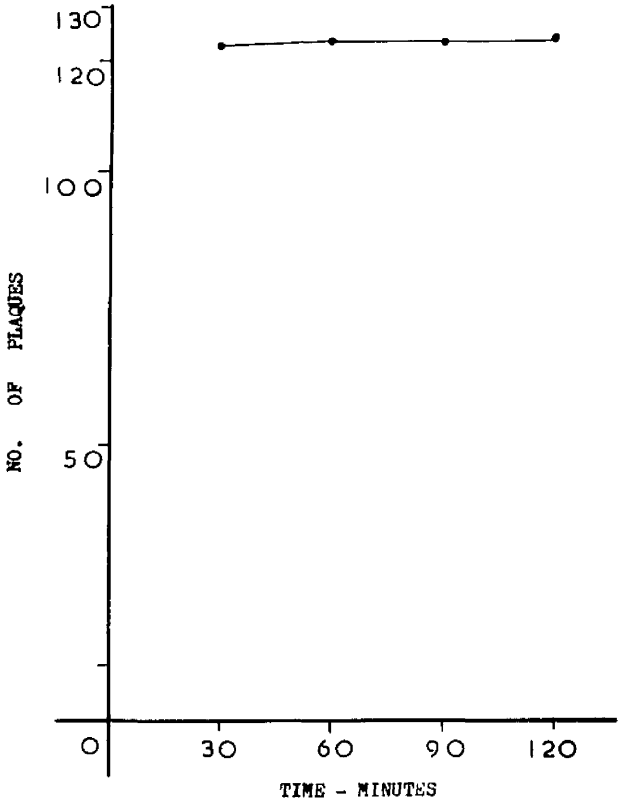

Fig. 7.

Fig. 7 Adsorption of FPV to Cl3 monolayers.

Fig. 8 Adsorption of FPV to Cl3 in suspension.

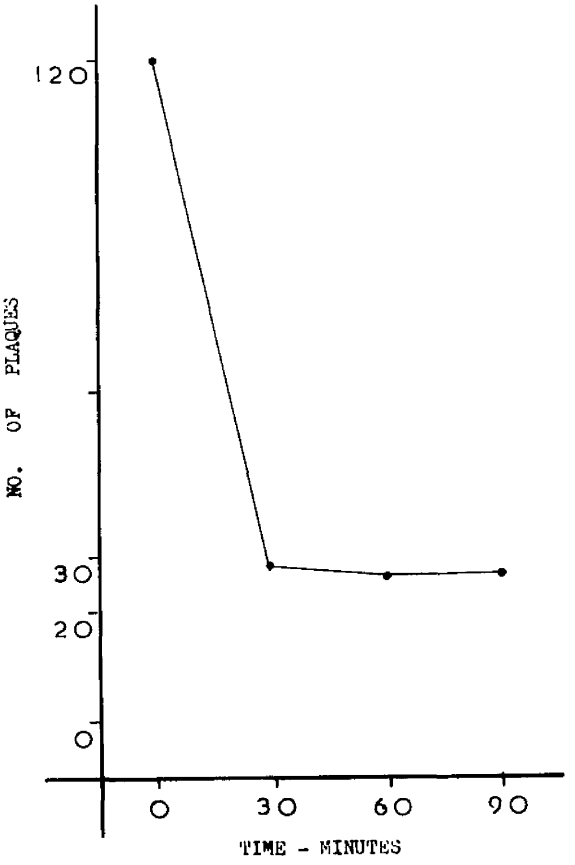

Fig. 8

studied in detail.

The single-step growth curve was carried out at multiplicity of 1 pfu/cell. The results are presented in Fig. 9. After 4 hours latent period the titre of infectious virus gradually rose to reach its final yield by 12 hours, and thereafter remained at the same level. The haemagglutinin appeared by 4 hours and reached its maximum before the production of infectious virus. Only 21% of cells plated for infective centres formed plaques. The yield per infected cell is about 105 pfu/cell.

Newcastle disease virus:

The adsorption time on monolayers and in suspension was determined and is shown in Figs. 10 and 11.

The maximum adsorption of 70% was reached by 90 minutes on monolayers while nearly 85% of total inoculum was adsorbed between 45 and 60 minutes when cells were infected in suspension.

Growth curve:

NDV has a normal growth cycle in BHK-21, C13 cells. The infected cells release new infectious virus and there is no evidence of interference due to incomplete virus.

A single-step growth cycle was performed by infecting C13 cells in suspension at an input ratio of 15 pfu/cell. The procedure followed is described on page 69. The growth cycle is represented in Fig. 12. The latent

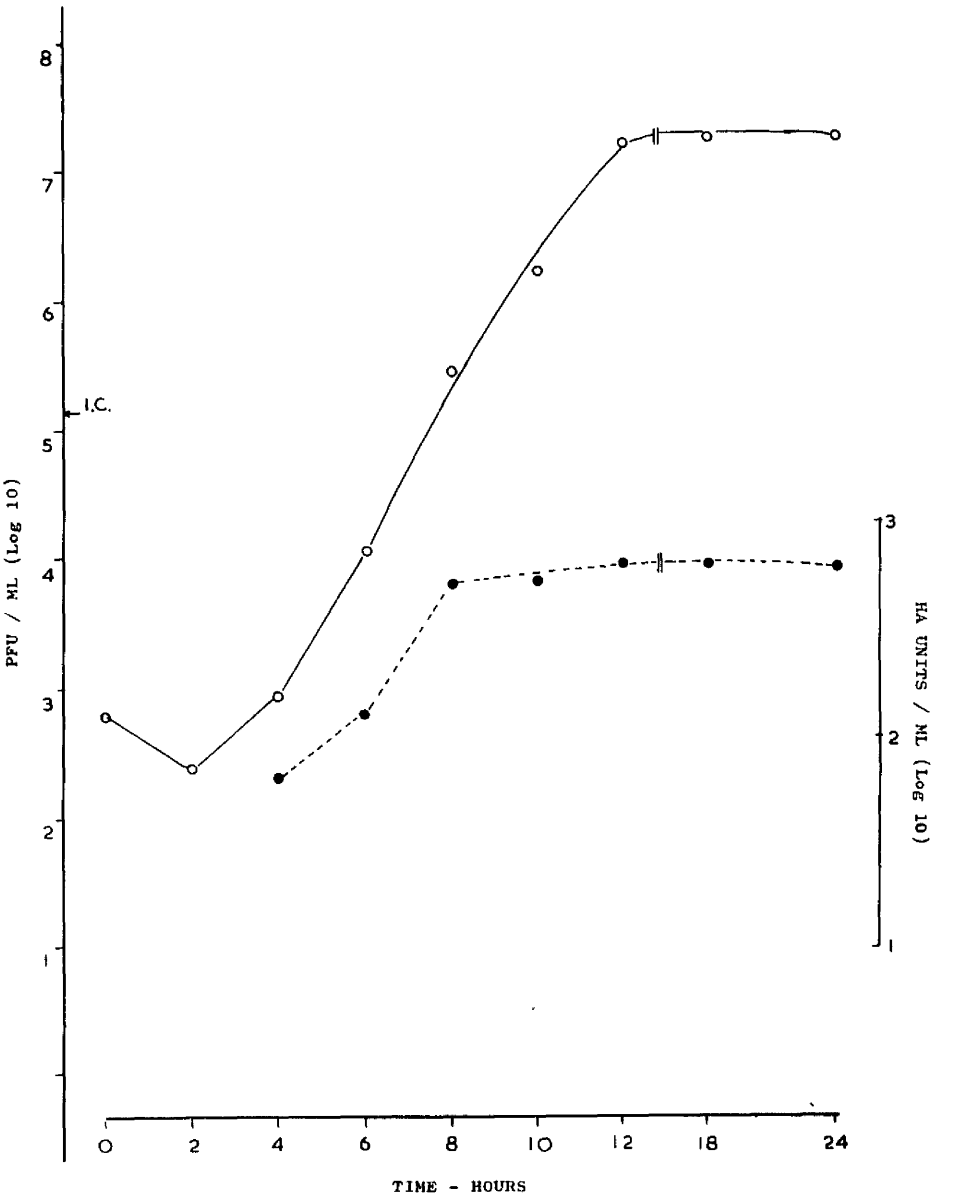

Fig. 9

Fig. 9 FPV : single step growth cycle.

o —— o yield of infectious virus/ml.
o —— o yield of haemagglutinin/ml.

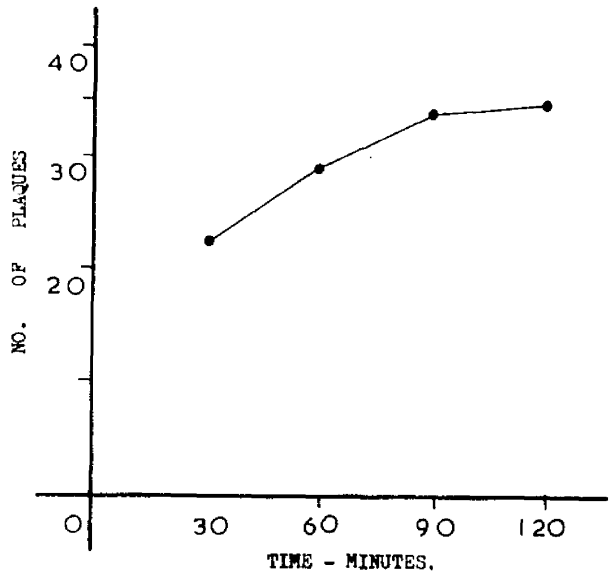

Fig. 10.

Fig. 10 Adsorption of NDV to Cl3 monolayers.

Fig. 11 Adsorption of NDV to Cl3 in suspension.

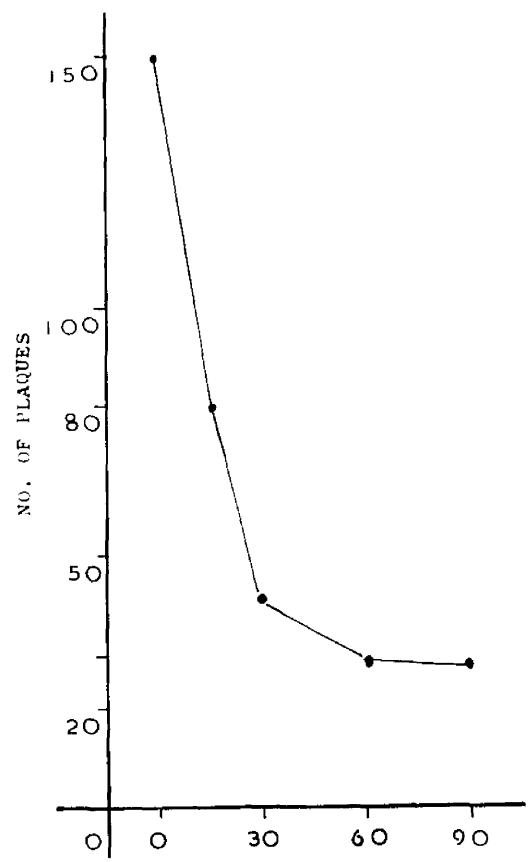

Fig. 11.

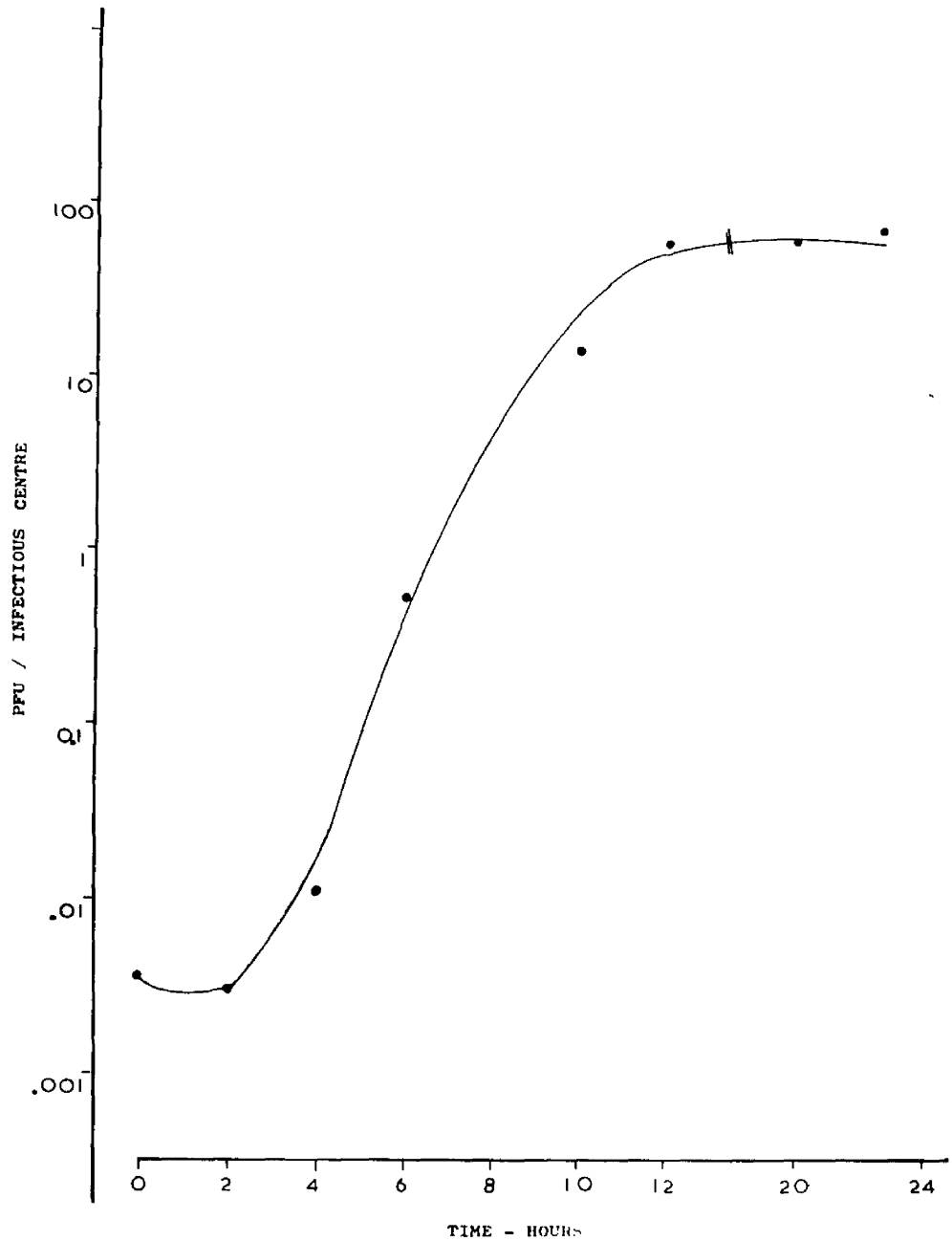

Fig. 12

Fig. 12 NDV : Single step growth cycle in C13 cells.

period was about 2 hours after which virus multiplication increased steadily and was completed by 12 hours. The percentage of cells producing virus was 45%. The yield per infected cell was about 85 pfu/cell.

CHAPTER 7

GROWTH OF RNA VIRUSES IN HEATED CELLS.

Once the multiplication rates of EMC, NWS, FPV, NDV and Reo virus in BHK-21, C13 cells were known, the growth of these viruses in C13 cells exposed to supraoptimal temperatures was considered. The capacity of heated cells to support the growth of the viruses, was tested by the formation of infective centres. It gave a proportionate estimate of the fraction of exposed cells capable of yielding new virus. The results were considered reliable only when reproduced several times, when experiments were repeated under similar conditions.

METHODS:

Infective centre assay:

The procedure for infective centre assay described on page 66 was followed. Infected cells were plated on preformed, confluent, C13 monolayers. After plating infected cells, 0.5 ml of agar medium was added to the plates. This thin layer of agar was allowed to harden and then the remaining 4.5 ml of agar was poured on top. The plates were incubated in CO_2 incubator at 37°C for the required period, depending on the virus under consideration. A minimum number of four plates per sample was used. The

overlay medium was the same as the one used for infectivity assays.

RESULTS:

Encephalomyocarditis:

The number of infectious centres formed was related to the multiplicity of infection. It was observed that when the multiplicities of adsorbed virus pfu per cell was 50 to 1 or more, the plating efficiency of infective centres was nearly 90 to 100%, as seen in Table 1. The frozen and thawed controls were included, to make sure that the plaques formed were not due to excess of unadsorbed virus, but were due to infected cells. The control plates had less than 1% plaques. Hence a multiplicity of 50 to 1 was used for infective centre assays.

Normal C13 cells and the cells exposed to supraoptimal temperatures (method on page 12) were infected with EMC virus. At the end of 30 minutes adsorption, cells were thoroughly washed with ETC to remove unadsorbed virus and about 50 to 100 infected cells in 0.1 ml volume were plated on each monolayer. Four plates were used per sample. The frozen and thawed controls were titrated for free unadsorbed virus.

Fig. 1 represents the result of one experiment, where percentages of infective centres obtained with the cells heated at 41°, 43°, 45°, 46° and 47°C for 15 minutes, are compared with that of normal cells. It indicates that

TABLE 1

Effect of multiplicity of infection on formation of Infectious centres by EMC.

ltiplicity ' infection U per cell	No. of cells plated per monolayer	No. of plaques observed per monolayer	Observed efficiency of plating Infectious centres (%)	Expected percentage of infected cells $P_o = e^{-m}$
2	101	10, 9, 11, 8	9.5	86.5
10	84	34, 37, 31, 36	41.07	100
25	96	42, 43, 46, 41	44.67	100
40	110	66, 62, 59, 68	56.8	100
50	80	70, 74, 78, 68	90.6	100

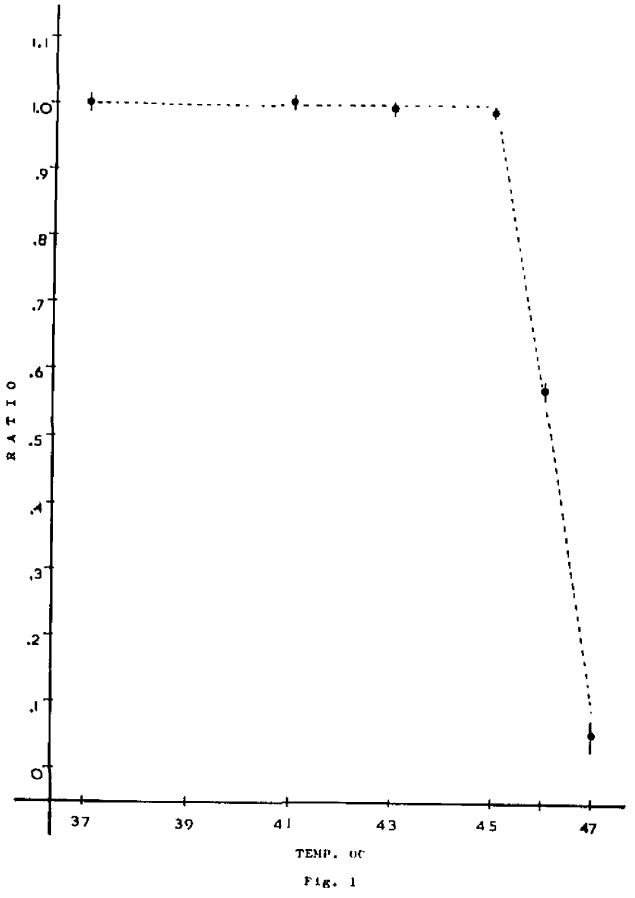

Fig. 1

Fig. 1 EMC : the ratio of infective centres formed by preheated cells to the number formed by normal cells.

temperatures up to 45°C had no adverse effect on the capacity of C13 cells to form infectious centres. The number of cells preheated to 45°C synthesizing EMC virus, was the same as that of normal cells. But the plating efficiency of infective centres of the cells exposed to 46°C was reduced by nearly 50%, and at 47°C, less than 10% of cells were capable of synthesizing new virus. The experiment was repeated several times and the results were similar to the one described here.

The kinetics of the growth cycle of EMC virus in normal cells and in those preheated to 45°C for 15 minutes was compared by following single-step growth curves. The method used is described on page 30. A pool of normal cells and a pool of heated cells were infected with an input multiplicity of 5 pfu to 1 cell. The results of the growth cycles are compared in Fig. 2. The rate of multiplication and the total yield of virus in both the systems was the same. The yield per cell was calculated from the amount of virus present in the final yield and the number of infectious centres formed during latent period. The plating efficiency of infective centres was 45.4% and 44.6% with normal and heated cells respectively. Naturally, the burst size in both the systems was the same, approximately 1000 pfu per infected cell.

It was obvious that C13 cells, when exposed to temperatures up to 45°C, were apparently normal, and continued

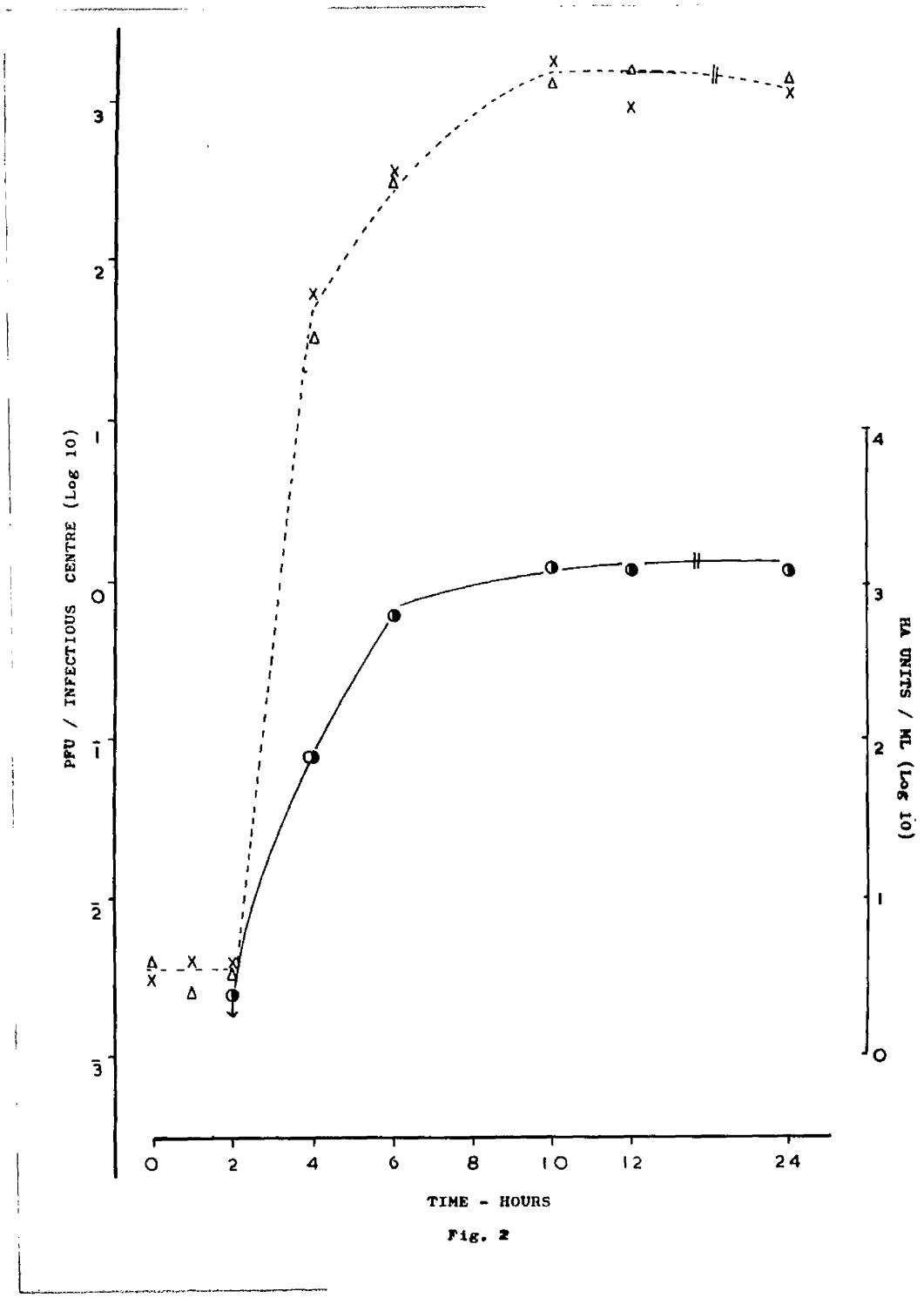

Fig. 2

Fig. 2 The multiplication of EMC in normal cells and preheated cells (45°C for 15 min.).

x —— x yield of infectious virus in normal cells.
o —— o " " haemagglutinin " " "

Δ —— Δ " " infectious virus in preheated cells.
● —— ● " " haemagglutinin in preheated cells.

to synthesize EMC virus with normal efficiency. But at temperatures 46° and 47°, the damaging effect became evident rather suddenly, and within the range of 2°C nearly 90% of cells became ineffective and lost their synthesizing capacity.

The resistance of C13 cells to temperatures up to 45°C was tested, by increasing the duration of exposure. The period of exposure was extended from 15 minutes to 60 minutes. It was difficult to maintain cells in suspension beyond 60 minutes, as they were found to clump inspite of intermittent shaking. Hence the effect of further exposure time was not tested.

Several siliconed tubes containing approximately 1×10^6 cells suspended in 1 ml of ETC, were placed in a water bath maintained at the required temperature. At 15 minute intervals, one tube was removed. These cells were then infected with EMC virus and plated for infective centres. The effect of temperatures 41°, 43° and 45° was considered. The results of three experiments are given in Table 2. The plating efficiency of infective centres of the cells exposed to 41°C for 60 minutes was the same as that of normal cells. Fuerst (1961) found that, if monolayers of L cells, ready for plaque assay of encephalomyocarditis virus, were preheated at 41.5° - 42°C for 60 minutes, they yielded 100% more plaques than unheated monolayers. The

TABLE 2

Effect of time of heating on susceptibility of cells to EMC infection.

No. of experiment	Temperature of incubation	Duration of exposure (minutes)	Percentage of infectious centres
1	Room Temperature		88.02
	41°C	15	86.9
		30	87.2
		45	88.1
		60	88.0
2	Room Temperature		93.1
	43°C	15	92.5
		30	92.04
		45	91.4
		60	80.03
3	Room Temperature		91.9
	45°C	15	92.2
		30	73.4
		45	22.1
		60	5.0

reason for this difference has not been determined. In the experiment discussed here, the total yield of virus was not determined. However, Fuerst's observation supports the finding that the cells preheated to 41°C for 60 minutes can allow virus multiplication. At temperatures 43°C and 45°C the effect of heat on cells became evident after 45 and 15 minutes of exposure respectively. The curve (Fig. 1a) obtained at 45°C resembled a multi-hit curve. It is possible that variation in the sensitivity of cells could be responsible for the nature of the curve obtained here.

Myxoviruses:

Influenza A (NWS):

The growth cycle at different multiplicities discussed on page 74, had indicated that the production of infectious virus, as well as the plating efficiency of infective centres, depended on the input multiplicity of infection. Therefore preliminary experiments were done to find out the multiplicity of infection whereby the maximum number of infectious centres could be obtained.

C13 cells were infected at different input multiplicities of particles per cell. The particle counts of the virus preparation were based on the observation by Milliken and Watson (unpublished) that 10 visible particles by electron microscopy formed one plaque forming unit. The total number of infected cells at the multiplicities of

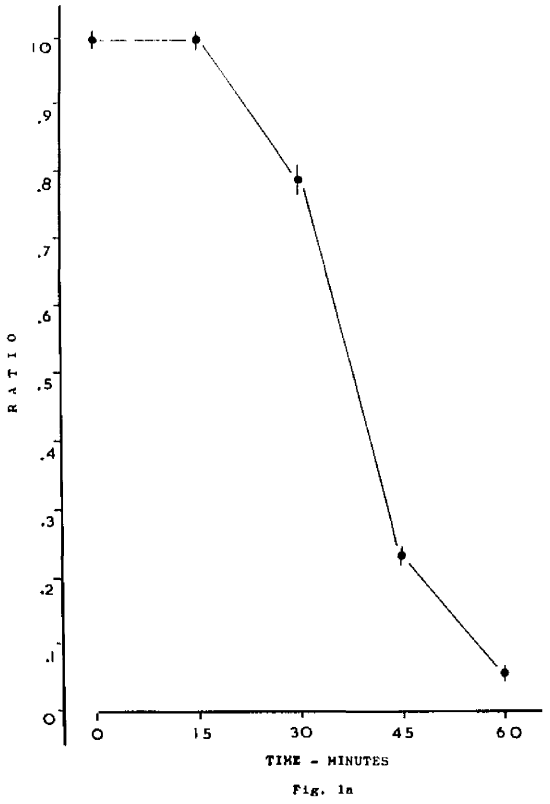

Fig. 1a

Fig. 1a The formation of infective centres by the cells exposed to 45°C for variable length of time.

100 to 1, 10 to 1, 1 to 1 and 1 to 10, was estimated by infective centre assays and a haemadsorption technique. Infective centres were counted at the end of 4 days and haemadsorption counts were done after 20 hours of infection, i.e. at the end of single cycle at low multiplicities. The results are shown in Table 3. The percentage of cells forming infective centres was considerably lower than expected on a Poissonian distribution. But the number of cells haemadsorbing was within statistical expectation. The marked discrepancy observed between the number of infective centres and number of cells giving positive haemadsorption, will be discussed elsewhere.

Since the multiplicity of 10 particles per cell not only insured the infection of all cells in a preparation, but also gave the maximum number of infectious centres, it was used for the following experiments.

Normal and heated cells were infected at an input ratio of 10 particles per cell. The virus-cell mixture was incubated at 37°C for 30 minutes. Cells were thoroughly washed with ETC to remove unadsorbed virus and then plated for infective centres. A minimum number of 500 cells in 0.1 ml volume was added to the monolayer. Four monolayers per sample were used. Frozen and thawed controls were included. In addition to infective centres, cells were plated for haemadsorption. The standard single cell haemadsorption technique described on page 107 was used.

TABLE 3

Effect of multiplicity of infection on formation of Infectious centres by NWS and FPV.

.rus	Multiplicity of infection pfu/cell	Particles/cell	No. of cells monolayer	No. of plaques observed per monolayer	Observed efficiency of plating infectious centres (%)	No. of haem-adsorbing cells (%)	Expected no. of infected cells(%) pfu/cell $P_o = o^{-m}$	Expected no. of infected cells(%) Particles per cell $P_o = e^{-m}$
NWS	10	100	400	15, 9, 9, 4	2.3	100	100	100
	1	10	440	88,76,112,108	22	100	63.31	100
	0.1	1	400	72,84,88,56	9.3	47.5	10.9	63.31
	.01	0.1	460	Nil	Nil	17	2.3	10.9
PV	10		600	11,13, 9,10	1.9	100	100	
	1		400	90,104,98,93	24.2	100	63.31	
	0.1		640	69,65,74,70	11.5	65	10.9	
	.01		620	Nil	Nil	12.1	2.3	

N.B. (1) Infectious centres at absolute and relatively high multiplicity.

(2) A proportion of plaque formation units at low multiplicity fails to form infectious centres but produce haemadsorption.

Haemadsorption counts were done at the end of single cycle. The infective centres were read after 4 days of incubation. The results of one experiment are presented in Fig. 3. In this experiment about 18.2% of normal cells gave infective centres, while 92% of the infected cells were haemadsorbing. Cells exposed to temperatures 41°, 43° and 45°C gave about the same number of infective centres as compared to the normal cells. The percentage of haemadsorbing cells was constant at temperatures 41° and 43°C. At 45°C, the number of cells giving positive haemadsorption had dropped by 8%. At temperatures above 45°C there was a large and abrupt drop in the percentage of infective centres and haemadsorption counts. The experiment was repeated several times and similar results were obtained each time.

The response of heated cells to NWS was, therefore, similar to that of Encephalomyocarditis.

The growth rate of NWS in normal and in cells exposed to 45°C for 15 minutes, was compared by performing a single-step growth cycle. The results are compared in Fig. 4. The rate of synthesis of infectious virus was the same in both systems. The number of infective centres obtained was 20.2% and 21.0% in normal and heated cells respectively. The haemagglutinin was first detected at 4 hours in both systems. The amount of haemagglutinin produced by heated cells at four hours, was a little less when compared with the amount produced by normal cells.

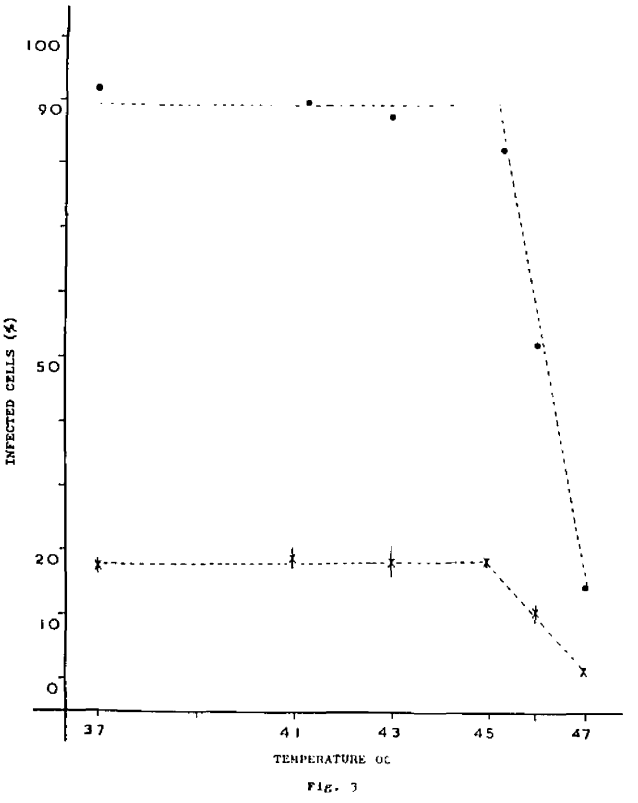

Fig. 3

Fig. 3 NWS : ● --- ● percentage of haemadsorbing cells; x --- x percentage of cells producing infective centres.

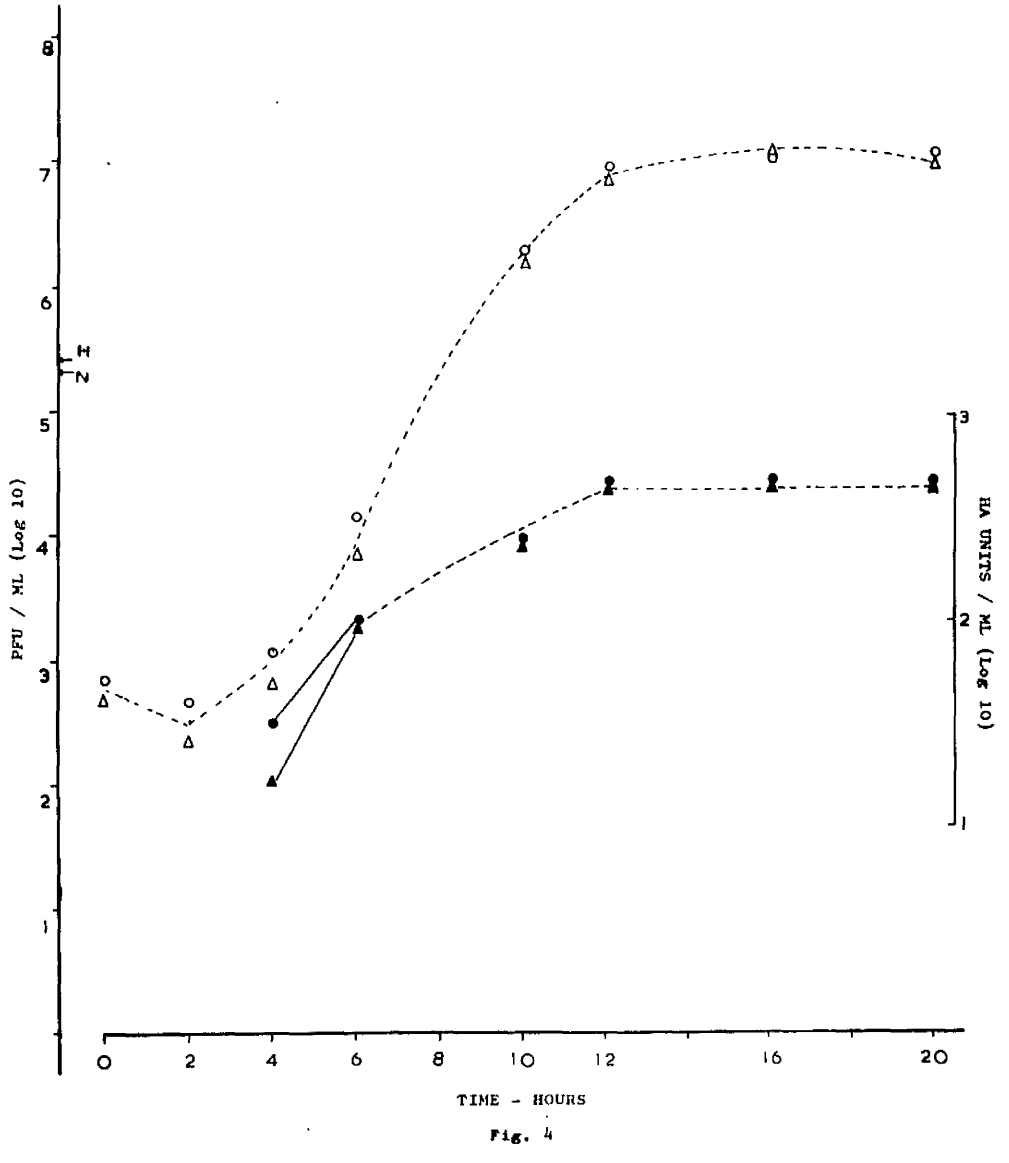

Fig. 4

Fig. 4 The multiplication of NWS in normal cells and preheated cells (45°C for 15 min.).

o --- o yield of infectious virus in normal cells.
o --- o " " haemagglutinin " " "
△ --- △ " " infectious virus in preheated cells.
▲ --- ▲ " " haemagglutinin " " "

But after 4 hours, the rate of synthesis as well as the amount of haemagglutinin produced by heated cells, was the same as normal cells. The pfu/HA ratio was $10^{4.50}$ and $10^{4.40}$ with normal and heated cells respectively. The yield per infected cell for normal and heated cells was 190 pfu and 180 pfu respectively.

Fowl plague virus:

The multiplication of FPV in C13 cells was similar to NWS. The formation of complete and incomplete virus depended upon the multiplicity of infection. Difficulties similar to NWS were encountered in obtaining a reasonable number of infectious centres. Once again, in order to obtain the maximum number of cells producing infectious virus, cells were infected at different multiplicities. The results are shown in Table 3 on page 92. The multiplicity ratio is expressed as the plaque-forming unit per cell. It appears that, at a multiplicity ratio of 1 pfu to 1 cell, almost all the cells are infected and also, a maximum number of cells give infectious centres. As with NWS (see discussion on page 113) non-plaque forming infections must occur. Hence the multiplicity of 1 to 1 was used for the infective centre assays.

The synthesis of FPV in normal and heated cells was compared by infective centre assays. The cells were infected at the input ratio of 1 to 1. The virus-cell

complex was incubated for 30 minutes at 37°C. The infected cells were treated with specific antiserum diluted 1 in 50 in ETC, for 20 minutes at 37°C. This step was necessary as the excess of virus was not removed by several washes. After treatment with antiserum, cells were washed again, twice, to remove antibodies. The cells were then plated for infective centres, a minimum number of 500 cells per monolayer in 0.1 ml volume was plated on the monolayer which was then layered with an overlay medium in two stages as described previously. The results were similar to NWS and are shown in Fig. 5. The plating efficiency was 25.1%.

Newcastle Disease virus:

The growth cycle of NDV in C13 cells was not accompanied by the formation of incomplete virus, hence high multiplicities for infective centre assays were safely used.

The results obtained with normal cells and the cells preheated to temperatures 41°, 43°, 45°, 46° and 47°C are presented in Fig. 6. In this experiment cells were infected at a ratio of 20 pfu per cell, the percentage of infective centre with normal cells was 45.5%. The results were similar to NWS and FPV.

The growth rates of FPV and NDV in normal and heated cells were not compared.

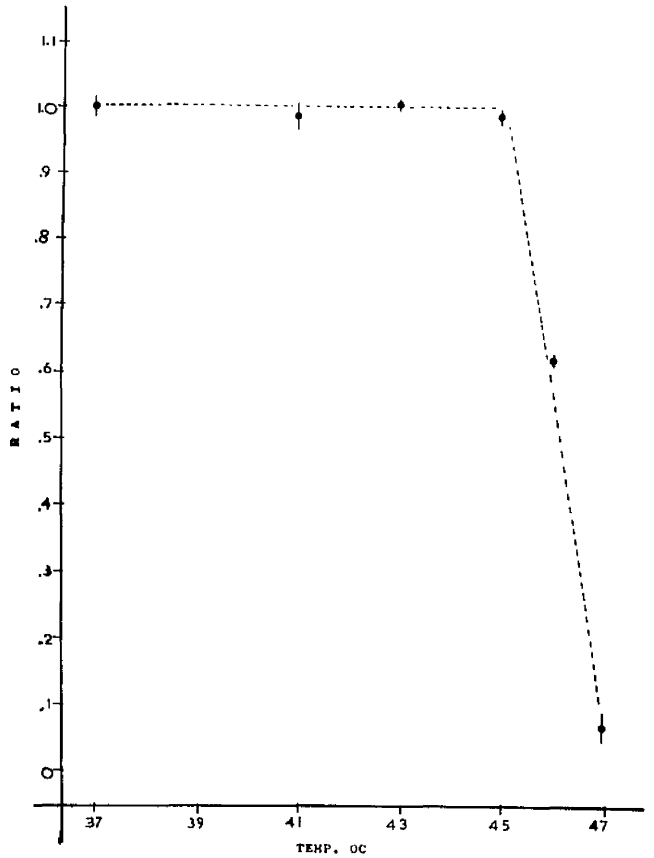

Fig. 5

Fig. 5 FPV : the ratio of infective centres formed by preheated cells to the number formed by normal cells

Fig. 6 NDV : the ratio of infective centres formed by preheated cells to the number formed by normal cells

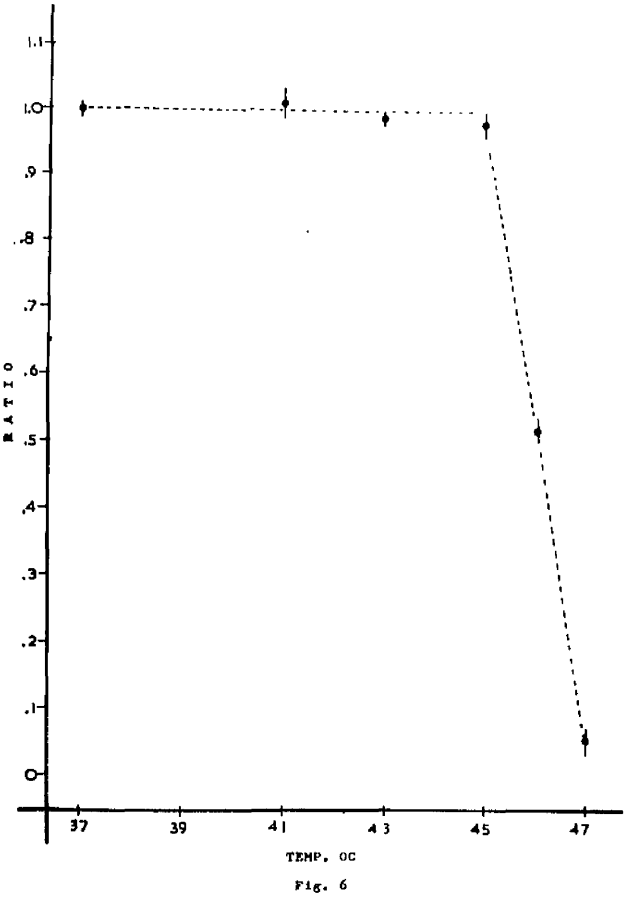

Fig. 6

Reo virus:

The response of heated cells to Reo virus was considered. Normal as well as cells preheated at various temperatures were infected at the multiplicity of 10 to 1. The virus-cell complex was incubated for 60 minutes at 37°C. At the end of adsorption period, cells were washed thrice with ETHr to remove excess of unadsorbed virus and about 100 cells per plate were plated on to the monolayers. The C13 monolayers grown in ETH were washed twice with ETHr before use. Plates were overlaied with agar medium in usual manner. Infective centres appeared at the end of 6 days. From the results presented in Fig. 7 it was evident that the reaction of heated cells to Reo virus was similar to other viruses described above.

The results indicated that, the ability of C13 cells to synthesize RNA viruses, after exposure to supraoptimal temperatures, was not impaired.

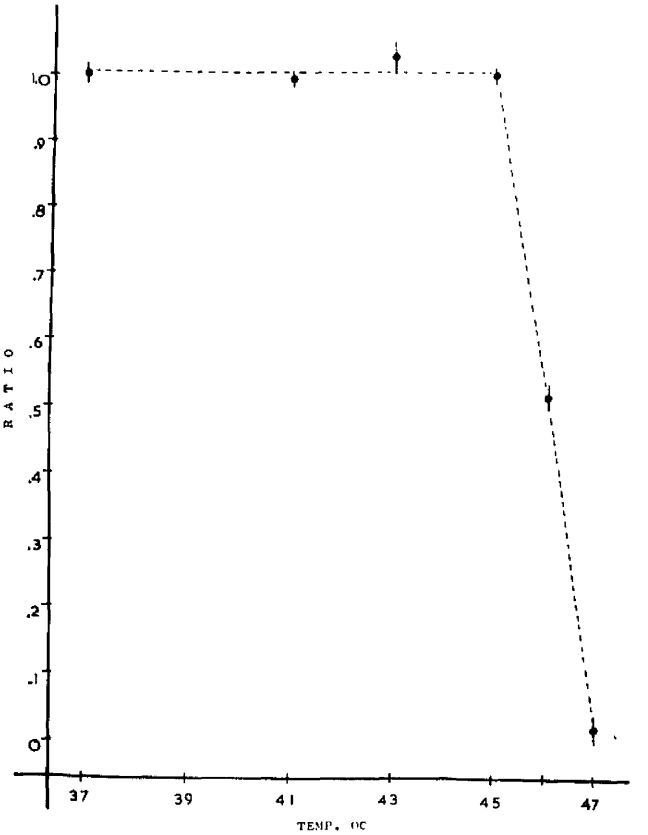

Fig. 7

Fig. 7 Reo : the ratio of infective centres formed by preheated cells to the number formed by normal cells.

CHAPTER 8

EFFECT OF TEMPERATURES ON DIFFERENT CELL LINES.

From the evidence discussed in the previous chapters, it became apparent that the effect of supraoptimal temperatures on BHK-21, C13 cells was characterized by two distinctly different responses of preheated cells to viruses, depending upon the nature of the viruses' nucleic acid. Whether this effect was confined to BHK-21, C13 cells or whether heat had a similar effect on the response of other mammalian cell lines to different viruses, was investigated. Three cell lines originating from different vertebrate species, L cells, secondary chick embryo fibroblastic cells and HeLa cells, were selected. Since the plating efficiency of all three was less than 5%, the effect of supraoptimal temperatures on their survival rates was not considered. The ability of these cells, after exposure to heat, to support virus multiplication was examined, either by infective centre assays or by the single cell haemadsorption technique. Viruses known to grow well and produce a cytopathic effect in the respective tissue cultures were selected from the list given on page 25. Only one virus from each group was used for each cell line. Although care was taken not to use the same virus twice, the use of vaccinia for all three cell lines was inevitable. The HFEM strain of Herpes Simplex, adapted

to BHK-21, C13 cells did not form plaques in L cells and HeLa cells. The susceptibility of secondary chick fibroblasts to the HFEM strain was not tested. Out of the group of viruses containing RNA, Encephalomyocarditis, Fowl plague virus and Newcastle Disease virus were chosen for L cells, secondary chick fibroblasts and HeLa cells respectively.

METHODS AND MATERIALS:

L-cells - Earle's strain L (mouse fibroblasts) cells were grown in baby bottles in Eagle's medium containing 10% calf serum.

Secondary chick embryo fibroblasts - Primary cultures were prepared from decapitated 10 or 11 day old chick embryos by the method of Franklin (1957). Cultures were grown as monolayers in ETC in 60 mm petri dishes and incubated at 37°C in a CO_2 incubator. Once the monolayers were formed, cells were harvested with 0.05% trypsin in versene solution, washed once with ETC and resuspended in ETC. These secondary cultures were used for the experiment.

HeLa cells - HeLa cells, clone S 1, were cultivated in baby bottles in Eagle's medium containing 10% calf serum.

Virus stocks - Preparation of virus stocks has been described in previous chapters.

Infective centre assays - Infective centres with vaccinia were obtained by the suspension method as described on page 42. And for EMC, FPV and NDV infective centres, monolayer method described on page 66 was used.

Haemadsorption technique - A technique, standardised in this laboratory, of single cell haemadsorption was followed. 0.5% of vaccinia sensitive fowl red blood cells suspended in normal saline were used for vaccinia and Newcastle disease viruses.

Exposure of cells to supraoptimal temperatures - Cells were exposed to supraoptimal temperatures for 15 minutes. A detailed description of the method is given on page 12.

RESULTS:

L cells - L cells were exposed to temperatures from above 37°C to 47°C for 15 minutes. The ability of preheated cells to support growth of encephalomyocarditis and vaccinia was tested by infective centre assays.

Encephalomyocarditis:

Normal, as well as heated cells were infected with EMC virus at a multiplicity of 50 to 1. The cells, after 30 minutes' incubation at 37°C, were thoroughly washed to remove unadsorbed virus and then plated for infective centres. The remainder of the cell suspension was frozen and thawed three times during latent period and titrated for unadsorbed virus. The control plates had no plaques. The plating efficiency of infective centres was 77.8%. The Fig. 1 indicates that the normal plating efficiency of infective centres is maintained even after the cells are heated to a temperature of 44°C. But at a temperature of 45°C the number of cells

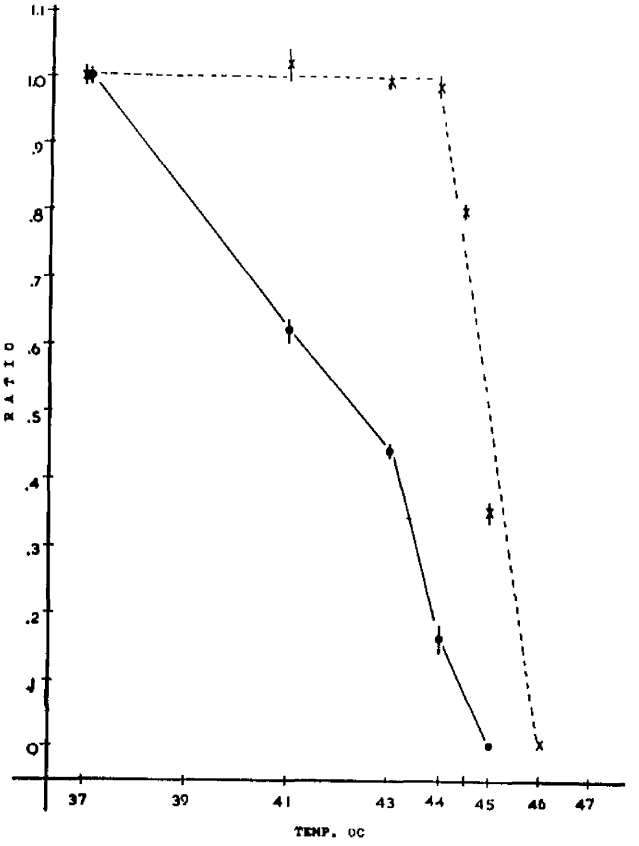

Fig. 1

Fig. 1 EMC (x --- x) and Vaccinia (● --- ●) : each point is a ratio of infective centres formed by preheated cells to the number formed by normal cells.

forming infective centres had dropped by 64%. L cells appeared to be more sensitive to heat than C13 cells where the adverse effect of higher temperatures on the synthesis of EMC virus became evident only above 45°C.

Vaccinia:

Normal and heated L cells were infected with vaccinia at a multiplicity of 5 to 1. The virus-cell mixture was incubated for 60 minutes at 37°C. Infected cells were thoroughly washed free of unadsorbed virus. A minimum number of 100 cells per plate was plated for infective centres. Frozen and thawed controls were included. About 64.5% of normal cells gave infective centres. The results of infective centre assays obtained with normal and heated cells are compared in Fig. 1. The number of infective centres gradually dropped with the rise of temperature, a pattern similar to that obtained with C13 cells.

Similar experiments were performed with secondary chick embryo fibroblasts, where fowl plague virus and vaccinia virus were used.

Fowl plague virus:

Fowl plague virus grows normally in chick fibroblasts (Franklin and Henry, 1960). Cells were infected at an input multiplicity of 10 to 1. About 60.5% of normal cells formed infective centres. The results of infective centre assays obtained with normal and heated cells are compared in Fig. 2. It indicates that the capacity of chick fibroblasts is resistant

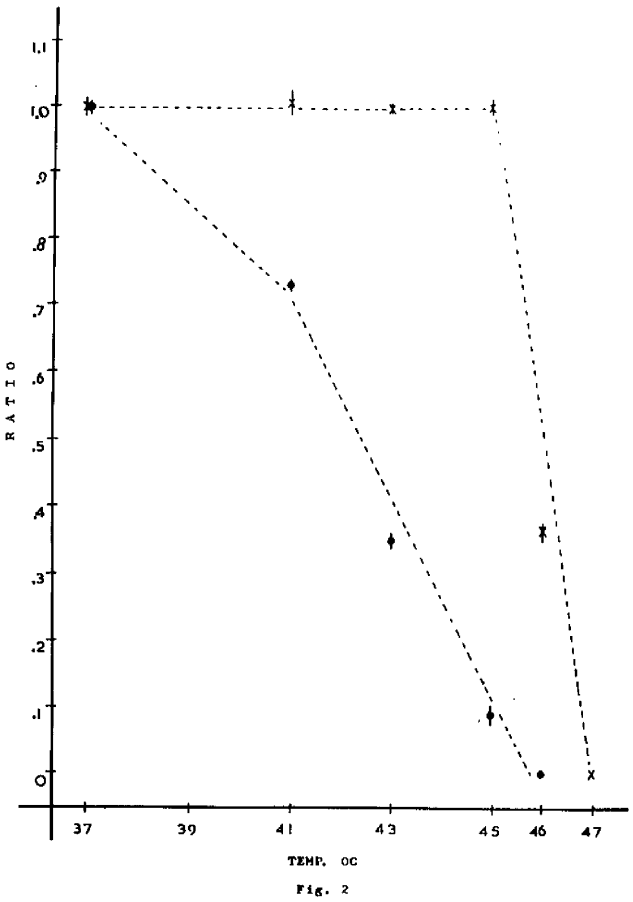

Fig. 2

Fig. 2 FPV (x ---- x) and Vaccinia (● ---- ●) : each point is a ratio of infective centres formed by preheated cells to the number formed by normal cells.

to temperatures up to 45°C, but after 45°C the drop in the number of cells forming infective centres is rather abrupt. The sensitivity of infected chick cells to heat is similar to BHK-21, C13 cells.

Vaccinia:

The results obtained with vaccinia are shown in Fig. 2, on page 105. Chick fibroblasts, like C13, gradually lose the capacity to synthesize vaccinia virus.

HeLa cells:

The synthesis of Newcastle disease virus and vaccinia, in normal and heated cells was examined by the single cell haemadsorption technique. Because of difficulties in obtaining the large number of cells required for infective centre assays, this method was not used.

Since the capacity of preheated C13 cells to support multiplication of vaccinia and NDV was previously measured by infective centres, a comparison of the results obtained with HeLa cells with those of C13 cells was not done simultaneously, using the haemadsorption technique for the estimation of synthesis of virus.

Newcastle disease virus:

Normal and heated cells were infected with NDV at an input multiplicity of 10 to 1. Cells were incubated for 60 minutes at 37°C. After the cells were thoroughly washed and the excess of virus removed, about 1×10^4 cells were

plated in 50 mm petri dishes containing 4 ml of ETC. Plates were then incubated at 37°C in a CO_2 incubator. At the end of 18 hours, that is at the end of a single growth cycle, cells were harvested by using 0.05% trypsin in versene solution. Cells were then spun down and resuspended in 0.5 ml of ETC, to which an equal amount of 0.1% of Fowl red blood cells was added. After a few minutes, a drop of this mixture was allowed to spread on the slides under a coverslip. The cells showing positive haemadsorption were counted under high magnification. A total number of 1000 cells was counted. The results are shown in Table 1.

A similar method was used for vaccinia and the results are shown in Table 1.

The results indicate that the response of heated HeLa cells to NDV and vaccinia is similar to the response, formerly obtained, for C13 cells. Heated cells retained their ability to synthesize NDV even after exposure to temperatures up to 45°C while their sensitivity to vaccinia was gradually lost with the rise of temperature.

Thus, in four separate species of cells the capacity to synthesize certain RNA viruses is always more heat-resistant than the capacity to synthesize certain DNA viruses.

TABLE 1

Susceptibility of preheated BHK-21, C13 and HeLa cells to Vaccinia virus and NDV tested by haemadsorption.

Virus	Cells	Percentage of haemadsorbing cells				
		Room Temperature	41°C	43°C	45°C	46°C
Vaccinia	BHK-21, C13	Occasionally negative	61.9	42.5	8.0	-
	HeLa	100	54.45	40.52	10.6	-
Newcastle disease virus	BHK-21, C13	65.51	69.82	69.34	54.63	19.25
	HeLa	53.6	55.63	60.8	48.61	20.97

CHAPTER 9.

ELECTRON MICROSCOPIC STUDY.

The electron microscopic examination of the normal C13 cells and the cells exposed to 45°C for 15 minutes was undertaken in collaboration with Mrs. E. M. Crawford.

METHOD:

Cells were deposited by centrifugation for 5 minutes at 1000 rpm. The cell pellets were fixed in a cold 1% solution of osmium tetroxide in a Zetterqvist's buffer (pH 7.3) for half an hour. The pellets were then rinsed briefly in distilled water, and dehydrated by rapid passage through an ethanol series. After impregnation in unpolymerized 9 : 1 butyl methyl methacrylate, the cells were embedded in prepolymerized methacrylate syrup (benzoyl peroxide catalyst), stored for 36 hours at 4°, and polymerized at 60° for 12 - 24 hours. Sections were cut on a Cambridge Huxley pattern microtome and mounted on 'Formvar'-covered grids and subsequently stained in lead hydroxide for 10 minutes. The sections were photographed in an Elmiskop I electron microscope.

RESULTS:

Sections of heated cells (Fig. 2) when compared with those of normal cells (Fig. 1) showed no apparent structural abnormality.

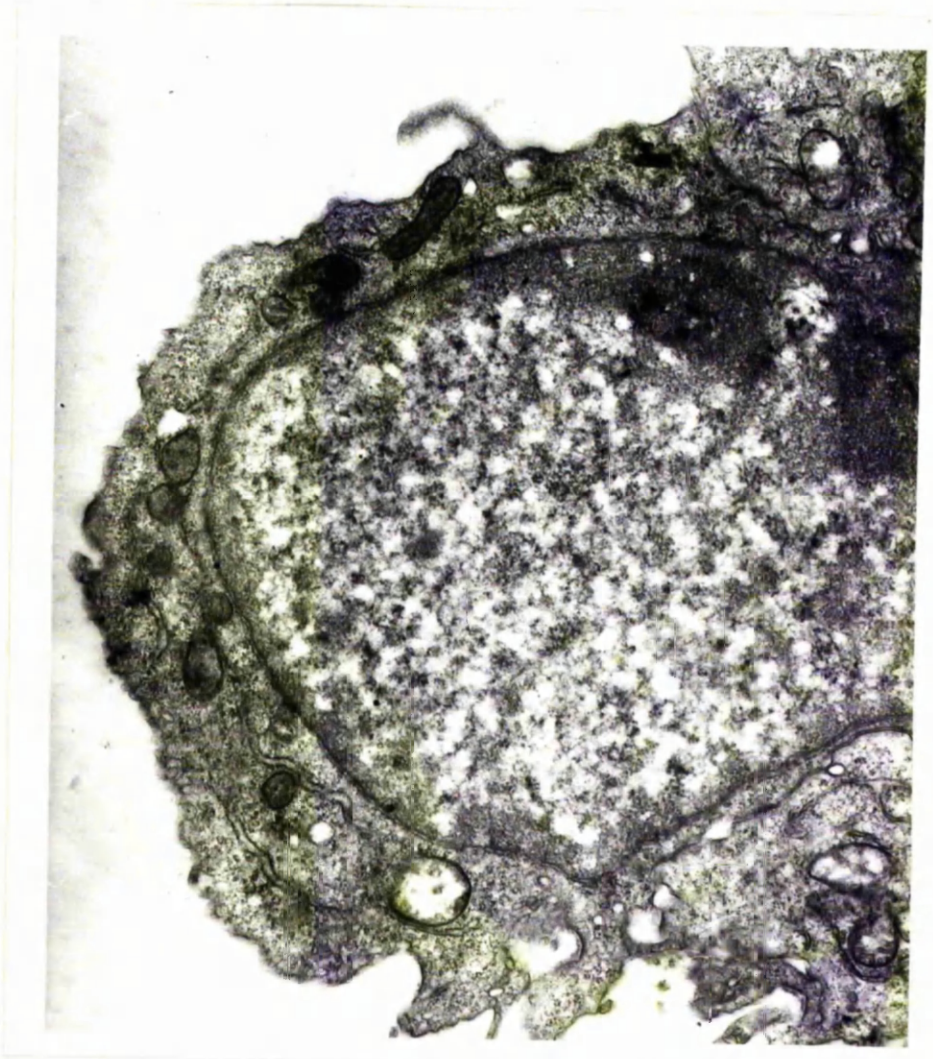

Fig. 1 Normal C13 cells.

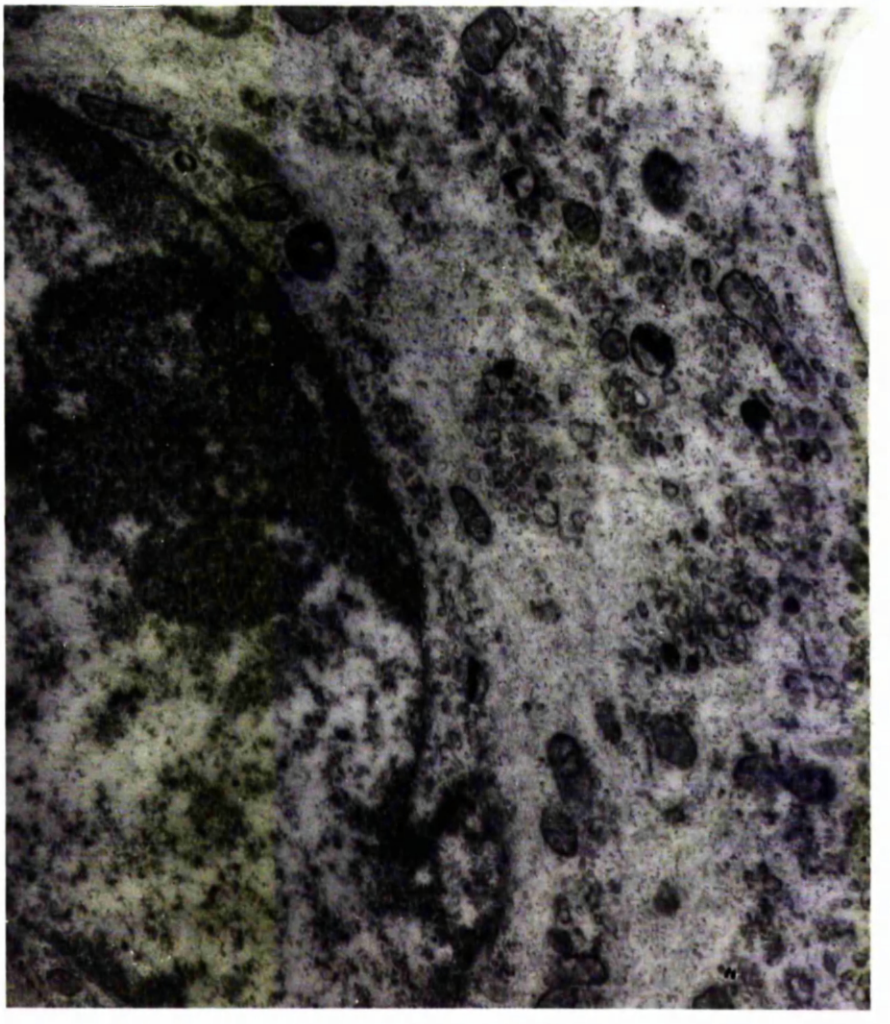

Fig. 2 C13 cells exposed to 45°C for 15 minutes.

CHAPTER 10.

GENERAL DISCUSSION.

The observation reported in the preceding chapters distinguish the heat sensitive and the heat resistant processes in the isolated BHK-21, C13 cells: a) loss of ability of single cells to form macroscopic colonies (Chapter 3), b) an inability of preheated cells to support multiplication of DNA viruses (Chapter 5) and c) the ability of preheated cells to synthesize RNA viruses (Chapter 7).

In preliminary experiments the effect of various supraoptimal temperatures on the survival rate of cells was demonstrated by an increasing loss in the number of cells forming macroscopic colonies, with the rise of temperature. At temperatures 45° and 46°C, 80 to 100% of cells had ceased to divide and were impermeable to trypan blue. The survival curve (Fig. 4, p.20) obtained was a multi-hit curve. The possibility as to whether the effect of heat is a single-hit or a multiple-hit phenomenon has already been discussed on page 22. The variation in the sensitivity of the cells, depending on its physiological state, appears to be the most probable reason for the multi-unit nature of the curve. In that case, action of heat on cells could still be a single-hit phenomenon.

It was difficult to assess the specific action of heat

on the cells as well as the extent of damage inflicted on the cells by heating. Inhibition of cell division could have either been due to structural damage to the reproductive apparatus or due to changes in the biochemical processes involved in the mechanism of cell division. Electron microscopic examination of normal cells and the cells exposed to 45°C for 15 minutes showed no evidence of obvious structural damage.

Prior to the studies on viral synthesis in heated cells, the growth characteristics of all the viruses to be used later were determined.

First, the adsorption rates of each virus were measured, for these had a practical value in the experiments involving plaque titration and quantitative studies. Adsorption of virus particles by suspended cells was more rapid, but also the efficiency of adsorption was better than by monolayers.

The growth cycles of DNA viruses, Herpes Simplex, Pseudorabies and Vaccinia were characteristic and not any different from the ones studied in other cell lines.

But the observation on the growth characteristics of RNA viruses in normal C13 cells made it possible to follow certain features of myxoviruses by taking advantage of the fact that NWS strain of Influenza A forms haemagglutinin as well as infectious virus in C13 cells.

It was noticed that even with the highest yield of

NWS formed during the growth cycle, at a multiplicity of infection of 1 pfu per cell, the ratio of the infectivity titre (4×10^7 pfu/ml) to haemagglutinin titre (5×10^2 HA units/ml) was $10^{4.9}$, approximately 2 log less than would be expected from agglutinin titre (Isaac, 1957). At a multiplicity of 10 pfu per cell, the ratio was even lower, i.e. $10^{3.3}$. These results suggested that multiplicity of infection plays an important role in the formation of incomplete virus in BHK-21, C13 cells as in eggs (Von Magnus, 1955).

Infective centre assays provided further evidence supporting this hypothesis as well as suggesting that at multiplicity lower than 1 pfu per cell, the formation of incomplete virus was due to an excess of incomplete virus in the inoculum. Infectivity ratios were calculated in terms of pfu as well as number of particles per pfu (10 particles/pfu, Milliken and Watson). Infectivity titrations measure the number of infective units in a virus preparation; it does not necessarily follow that, when the particle/infectivity ratio is more than 1, the other particles in the preparation are non-infectious. The method employed for particle counting is incapable of distinguishing between fully infectious particles and non-infectious ones.

The results in Table 3 (p. 92) show that at multiplicity ratio of 10 pfu/cell and 1 pfu/cell, when 100% of cells were infected, as judged by haemadsorption counts, only a few cells

formed infectious centres. The number of infectious centres was higher at 1 pfu/cell than it was at 10 to 1, clearly suggesting that the formation of infectious virus depends on the multiplicity of infection. At a lower multiplicity of 0.1 pfu per cell, when one would expect less interference and a higher fraction of cells producing infectious virus, the proportion of infectious centres to haemadsorbing cells was in fact the same as with the multiplicity of 1 pfu per cell. But at this multiplicity (0.1) the number of infected cells corresponded with the theoretical number calculated from the input of infectious unit per cell, indicating that, at a multiplicity of 0.1 pfu per cell, the plating efficiency of infectious centres was 100%. It must also mean that at all lower multiplicities where there is a less chance of incomplete infection due to multiple infection, every infectious unit adsorbed will give rise to successful infection. It is also true that, at the same multiplicity, the expected number of "infected" cells, when the multiplicity of infection was calculated in terms of particle per cell, was equal to the number of haemadsorbing cells. This implies that all <u>particles</u> were capable of initiating infection, i.e. produce haemagglutinin and, therefore, that about 1 in 10 produce infectious virus. So far as I am aware, this is the first virus-cell system in which one can distinguish between a particle infectivity ratio greater than one being due to an excess of defective virus particles and it is being caused by

a statistical chance, according to the sensitivity of the system, of any one of a population of fully adequate particles initiating infection.

Like NWS, the formation of incomplete and complete virus in FPV infected BHK-21, C13 cells appeared to depend on multiplicity of infection. The results (Table 3, p.92) of infective centre assays with FPV, once again revealed that, at the lower multiplicity of 0.1 pfu/cell, the number of infectious centres produced is equivalent to the theoretical number calculated in terms of infectious units. In this case, the particle infectivity ratio is not measured, hence it is difficult to assess the proportion of non-infectious and infectious particles in the inoculum. All the same, the fact remains that in this cell system, at lower multiplicities, one infectious unit is capable of initiating infection which will always result in the formation of infectious virus.

These were incidental, but important findings observed during the course of investigations and had no bearing on the problem of heat-sensitivity of the cells.

The growth cycles of other RNA viruses used in this study are normal, hence they are not considered here.

All later experiments were designed to answer a question whether the heated cells, after a loss of ability to divide, were still capable of supporting virus growth. The capacity of cells exposed to various temperatures to

grow viruses was tested by infective centre assays because this technique allows one to observe the response of individual cell to virus infection. This is essential in differentiating between a partial loss of capacity by all cells and a complete loss of capacity by some cells in any one population. The multiplication of Herpes Simplex, Pseudorabies, Vaccinia, EMC, Myxoviruses (NWS, FPV and NDV) and Reo virus, in heated cells was compared.

The response of heated cells to various viruses was different for two distinct groups of viruses. One group consisted of Herpes Simplex, Pseudorabies and Vaccinia while in the second group EMC, Myxoviruses and Reo virus were included. This meant that this cell system was capable of distinguishing the DNA-containing viruses from RNA-containing viruses.

The results of infectious centre assays showed that, an increasing number of cells lost their sensitivity to the DNA-containing viruses as the temperature of exposure was raised. At temperatures 45°C and 46°C, 80 to 100% of cells became resistant to the DNA viruses. In contrast, the number of cells synthesizing RNA-containing viruses remained constant up to 45°C and was only reduced by 40 to 50% at 46°C,(Fig. The failure of cells exposed to temperatures above 46°C to synthesize any virus was hardly unexpected considering their physiological state, e.g. change in the permeability (p. 16).

These lines of evidence show that the mechanisms

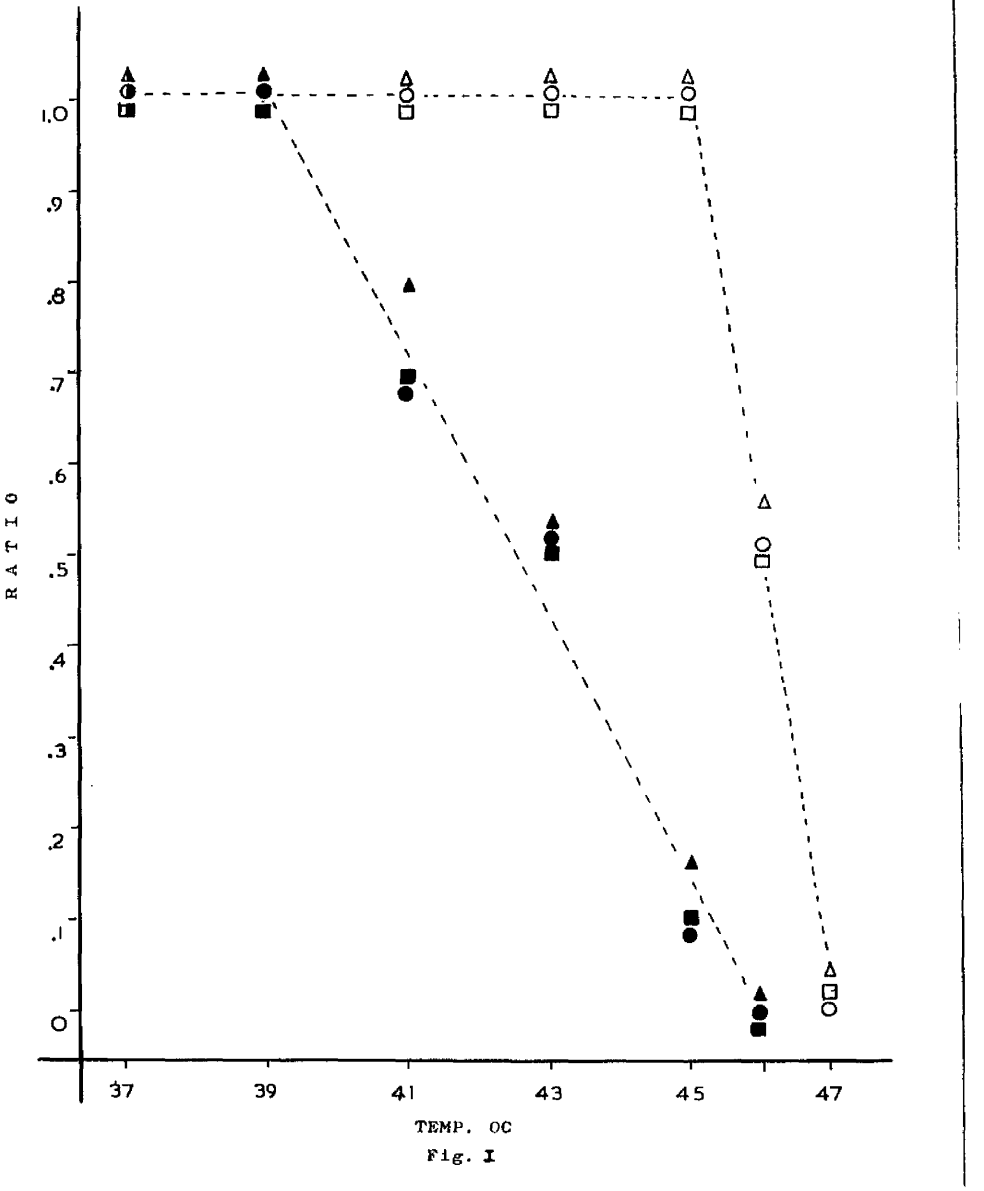

Fig. 1 The results of the individual experiments are presented together in order to indicate the difference between the DNA viruses and RNA viruses tested. The open and closed symbols represent RNA and DNA viruses respectively.

involved in the division of cells and the synthesis of the DNA viruses are heat sensitive; but the replication of RNA viruses is independent of the heat sensitive function(s).

Although the heat sensitive mechanisms need not be identical, it is possible to say from the experimental data at which point in the synthetic cycle of DNA viruses the unknown mechanism operates.

During the cycle of viral replication, viruses pass through an orderly sequence of stages. The known steps are, adsorption, penetration, eclipse, release of viral nucleic acid and the synthesis of viral specific DNA and other components such as protein required for the formation of virus particles. Heat could affect any of these stages which would result in the failure of virus function.

Adsorption:

Damage to the cell surface might prevent adsorption of virus particles on to the cell surface. Measurement of adsorption rates of Herpes Simplex (p. 47) and Vaccinia (p. 57) in heated cells indicated that the rate of adsorption as well as efficiency of adsorption in heated cells was the same as in normal cells. The next stage to adsorption is penetration.

Penetration:

Penetration is an obligatory step in initiating infection of mammalian cells. Direct studies of penetration were not done, but the infecting virus is shown to disappear during latent period. It has been shown that the virus

particles of Herpes Simplex (Holmes and Watson, 1963; Epstein, 1964) and Vaccinia (Dales and Siminovitch, 1961) enter cell by phagocytosis. The phygocytic activity of BHK-21, C13 cells was tested by adding India ink to the suspension of heated cells, the presence of intracellular particles of India ink in majority of cells indicated that the phygocytic activity of cells was not damaged by heat. So it is unlikely that adsorbed virus particles did not penetrate heated cells. The next important steps in the initiation of infection are the eclipse and the uncoating of viral genome.

Eclipse:

The virus from infected heated cells was not released during the latent period by freezing and thawing. Therefore it must be presumed that the heated cells can eclipse added virus at least to the extent of inactivation.

Uncoating of viral genome:

It is presumed that decoating of virus particles is an essential step in the process of virus infection, especially in cases where virus particles are enclosed in membrane(s). Very little is known about this mechanism in the infection of Herpes Simplex. Sufficient information on vaccinia appears to be at hand, to permit the tentative generalization that after phygocytosis, release of the core into the cytoplasmic matrix is effected by enzyme(s). As to the presence of this enzyme(s) in the cells, two controversial views have been expressed. According to Joklik (1964) and Abel (1963),

uncoating enzymes are induced after infection while Dales and Kajioka(1964) believe that these enzymes are present in the cell but are activated when foreign protein enters the cell. Whichever mechanism may be responsible, it is important to keep in mind that heating may either incapacitate the reaction of cells to virus particles or may destroy pre-existing enzymes. In both events, virus particles will fail to initiate infection. Unfortunately it was not possible for me to carry out tests of enzymic activity required for this sort of experiment.

Synthesis of virus components:

Single step growth curves of Herpes Simplex and Vaccinia (p. 49 and p. 57) in heated cells were studied to determine the synthesis of viral components, proteins and infectious virus. The production of antigen in normal as well as heated cells was determined by the complement fixation test. In both cases, results indicate that, the heated cells produced less infectious virus as compared to the normal cells. The ratios of infectivity to antigen suggest that the heated cells produced more antigen than expected for the amount of infectious virus synthesized by these cells. This increase in the synthesis of antigen in heated cells could be due to any of the following reasons:

(1) It is possible that all the heated cells produce damaged components and need to make more antigen before infectious virus is formed.

(2) The yield of mature virus might be limited as a result of the limited quantity of viral DNA but the synthesis of viral proteins continues as normal.

(3) In some cells only, formation of viral DNA is completely inhibited but not the synthesis of protein (antigens).

However, in the absence of definite evidence it can only be guessed as to whether the cells produced excess of antigen or whether some cells which did not score as infectious centres, were synthesizing antigen alone. As stated in Chapter 5, fluorescent antibody studies were too inconclusive in rounded, heated cells.

It therefore appears that one of the heat sensitive steps exists somewhere between eclipse and synthesis of soluble antigen. This leaves certain possibilities. It could be either

(1) Interference with the mechanism of "decoating", or

(2) Interference with the viral DNA synthesis.

A failure in the mechanism of decoating has already been considered.

Several mechanisms appear to have been involved in the replication of DNA viruses. It has been suggested that the viral DNA may be synthesized by DNA enzyme system of the host cell. Increase in the enzyme activity of Herpes Simplex (Keir and Gold, 1963; Russell et al., 1964) and Vaccinia (Joklik, 1962; Green and Pina, 1962a) has been noticed. It is still not known whether the increased enzyme activity is due to

new virus specific enzymes or whether these enzymes are the same as those formed in uninfected cells. However, increased levels of DNA polymerase in Vaccinia (Green, 1962b; Magee, 1962) and DNA-dependent RNA nucleotide transferase (EC 2.7.7.6) in Herpes Simplex infection (Burdon, Keir and Wildy, unpublished) and the current theory of dependence of DNA viruses on DNA dependent RNA polymerase suggest that specific messenger RNA must be involved in DNA virus formation.

Since RNA viruses produce full infectious particles in heated cells, it is clear, therefore, that the synthesis of functional RNA and protein is not impaired. It therefore follows that if messenger RNA is formed by viral DNA, then it would be expected to function normally. This makes it likely that the virus DNA is not "read" into messenger RNA in heated cells. If this is true, then the excess of antigen over infectious virus production in heated cells (p.119) is unlikely to be due to protein synthesis in the absence of DNA replication.

It appears that, in all probability, it is the cellular enzyme system that is affected by heating which in turn prevents the viral DNA synthesis.

In contrast to DNA viruses, the replication of RNA viruses in heated cells was not affected. The failure of RNA viruses to grow in cells exposed to higher temperatures could not be followed because of the physiological state of the cells.

If it is true that the heating of cells inactivates

DNA enzyme system in Cl3 cells then one is justified in concluding that RNA viruses, single stranded as well as double stranded, replicate independently of this system. This supports the conclusion reached by Barry (1964) in case of myxoviruses that although these viruses appear to depend on DNA dependent RNA polymerase as they are Actinomycin D sensitive, they can in fact synthesize independently of the cellular DNA systems. The results with Reo virus are also in accord with the data presented by Gomatos et al. (1962).

The effect of supraoptimal temperatures on three other cell lines was also considered. The sensitivity to viruses of L cells, Chick fibroblastic cells and HeLa cells is similar to that of BHK-21, Cl3 cells. This wide range of cell species indicates that the differential effect of heat on the two main classes of viruses, is likely to be of general application. In this case biochemical investigations should reveal a mechanism which is common to all species of animal cell, which is required for the synthesis of DNA viruses and which is heat-sensitive.

SUMMARY.

The results reported in this dissertation are concerned mainly with the effect of supraoptimal temperatures on BHK-21, C13 cells and on their ability to support the growth of various viruses.

The term "supraoptimal temperature" was defined as any temperature above the optimal temperature of 37°C and below 47°C, above which most cells became stainable by trypan blue.

The effect of supraoptimal temperatures on the multiplication of C13 cells was tested by comparing the plating efficiency of C13 cells, after exposure to higher temperature, with that of normal cells. The plating efficiency of the cells fell gradually with the rise in temperature. Nearly 80 to 100% of cells had ceased to divide between temperatures of 45°C and 46°C.

Before studying heated cells, the characteristics of the growth cycle of a small series of DNA and RNA viruses were established for BHK-21, C13 cells. Some of these had first to be adapted to the cells.

The capacity of heated BHK-21, C13 cells to support synthesis of these DNA and RNA viruses was tested by the formation of infectious centres, by the examination of one-step growth curves, and occasionally by serological test

for synthesis of virus protein and when advantageous, by haemadsorption.

Exposure of the cells to supraoptimal temperatures made the cells insensitive to the DNA viruses. The decline in sensitivity began at about the same temperature and fell at about the same rate as the plating efficiency of BHK-21, C13 cells.

On the other hand, BHK-21, C13 cells, after exposure to supraoptimal temperatures, continued to synthesize the RNA viruses with their normal efficiency.

The findings were not limited to BHK-21, C13 cells but held also for human, chicken and mouse cells tested with one representative virus of each type.

It is suggested that one heat-sensitive biochemical process in all species could be responsible for all these findings.

A P P E N D I X

MEDIA

1.	ETC :	Eagle's medium	80 ml.
		Tryptose phosphate broth	10 ml.
		Calf serum	10 ml.
2.	ETH :	Eagle's medium	80 ml.
		Tryptose phosphate broth	10 ml.
		Human serum	10 ml.
3.	ETHr :	Eagle's medium	80 ml.
		Tryptose phosphate broth	10 ml.
		Horse serum	10 ml.

Eagle's Medium (Glasgow modification).

		To make 1,000 ml.
NaCl		6.4 g.
KCl		0.4 g.
$CaCl_2$		0.2 g.
$MgSO_4.7H_2O$		0.2 g.
$NaH_2PO_4.2H_2O$		0.14 g.
Dextrose		4.5 g.
Ferric nitrate		0.0001 g.
L. Glutamine		0.292 g.
Penicillin		100,000 units
Streptomycin		0.1 g.
Antimycotic	0.02%	1.0 ml.
Phenol Red	1%	1.5 ml.

Dissolve in about 500 ml. of distilled water.

Add:	$NaHCO_3$	2.75 g.
	Amino acids (concentrated)	50 ml.
	Vitamins (concentrated)	4 ml.
	Distilled water to	1,000 ml.

Flush with CO_2 until orange.

Sterilise by Millipore filtration, using G.S. membrane (0.22 μ).

Bottle in 80 ml. amounts and store at $4^{o}C$.

Check for bacterial contamination and plating efficiency.

Tryptose Phosphate Broth

	To make 5,000 ml.
Tryptose Phosphate Broth (Difco Bacto)	147.5 g.
Distilled water to	5,000 ml.

Dissolve with the aid of heat.

Bottle 20 ml. amounts.

Autoclave at 15 lbs. pressure for 15 minutes.

Check sterility by incubating at 37°C for 5 days before issue.

Sera : All sera were sterilized by filtration and stored at -10°C.

Calf serum : Not inactivated.

Human serum : Human serum contained herpes immune antibodies and was not inactivated.

Horse serum : Inactivated at 56°C for 30 minutes.

Plaque assay overlay medium

1.3 Eagle's medium : Eagle's medium with the constituents at 1.3 times the concentration of Eagle's medium except with far less phenol red (1 ml of 1% dye/18 litres)

Agar :

3.6% Noble agar

4.8% Noble agar.

Overlay medium was made by adding 25 ml of melted agar to 75 ml of 1.3 Eagle's medium plus 5 ml of calf serum.

A neutral red containing, staining overlay was made up in an identical manner, except that 3 ml of 0.4% neutral red solution was incorporated.

Preparation of Carboxymethyl cellulose.

5% w/v solution of the sodium salt of carboxymethyl cellulose (L. Light & Co.) in water.

Versene in P.B.S. (Solution A).

	To make 1,000 ml.
NaCl	8 g.
KCl	.2 g.
Na_2HPO_4	1.15 g.
KH_2PO_4	.2 g.

Add:

Diaminoethanetetra - acetic acid, disodium salt (Versene) (BDH quality)	.2 g.
Phenol Red 1%	1.5 ml.

Dissolve in 1,000 ml. of distilled water.

Bottle in 20 ml. amounts.

Autoclave at 15 lbs. pressure for 15 minutes.

Trypsin/Versene:

1 volume Trypsin

4 volumes Versene.

Trypsin 0.25% in Tris Saline.

		To make 1,000 ml.
NaCl		8 g.
KCl	19%	2 ml.
Na_2HPO_4		0.1 g.
Dextrose		1 g.
Tris (hydroxy methyl) amino methane		3 g.

Dissolve in 700 ml. distilled water.

Add N/1 HCl to make pH 7.7 at room temperature.

Add:

Phenol Red	1%	1.5 ml.
Penicillin		100,000 units
Streptomycin		0.1 g.

Make up to 1 litre.

Add:

Trypsin (Difco 1:250)	2.5 g.

Leave at $4^{o}C$ overnight.

Prefilter through 1.2 μ, 0.8 μ, 0.4 μ and 0.22 μ Millipore membranes using a coarse prefilter with each of these.

Sterilise by Millipore filtration, using 0.22 μ Membrane (0.22 μ).

Bottle 100 ml. amounts.

Store at $-20^{o}C$.

Check for bacterial contamination and tryptic efficiency.

The efficiency is checked by testing the digestive activity of varying dilutions of the Trypsin on a piece of X-ray film.

Tris Saline

		To make 50,000 ml.
NaCl		400 g.
KCl	19%	100 ml.
Na_2HPO_4		5 g.
Dextrose		50 g.
Tris (hydroxy methyl) amino methane		150 g.
Phenol Red	1%	75 ml.

Dissolve in about 10 litres distilled water.

Add N/1 HCl to make pH 7.4 at room temperature.

Add:

Penicillin	5,000,000 units
Streptomycin	5 g.
Distilled water to	50,000 ml.

Sterilise by Millipore filtration, using G.S. membrane (0.22 μ).

Bottle 150 ml. amounts.

Store at 4°C.

Check for bacterial contamination.

Tris-calf : Tris saline + 2% calf serum.

Diluent for EMC H.A. titration.

Dissolve 4.5 gm glucose in 100 ml distilled water and mix with 100 ml PBS (complete). Take 95 ml and add 5 ml 1% gelatine solution in PBS.

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