# DNA SYNTHESIS IN CULTURED HAMSTER CELLS AND THE EFFECT OF INFECTION BY HERPESVIRUSES.

by

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TO YVONNE

for her constant patience, humour and support.

CONTENTS.

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## LIST OF ABBREVIATIONS.

Abbreviations and symbols used without definition in this thesis are, in general, those recommended in the Biochemical Journal Instructions to Authors (Biochem J. (1972) 126, 1). In the case of nucleic acids and their constituents, however, the symbols and abbreviations are as set out by the IUPAC-IUB Commission on Biochemical Nomenclature (Eur. J. Biochem. (1970) 15, 203).

Other contractions are as follows:-

| | |
|---|---|
| Acetyl CoA | Acetyle coenzyme |
| BSS | Balanced salt solution |
| C.P.E. | Cytopathic effect |
| CsCl | Caesium chloride |
| $Cs_2SO_4$ | Caesium sulphate |
| DK cells | Dog kidney cells |
| DNA's | Deoxyribonucleic acids |
| DNA polymerase | DNA nucleotidyltransferase (E.C.2.7.7.7) |
| EBV | Epstein-Barr virus |
| EDTA | Ethylenediaminetetra-acetic acid |
| HSV-1 | Herpes simplex virus type 1 |
| HSV-2 | Herpes simplex virus type 2 |
| MDV | Marek's disease virus |
| moi | Multiplicity of infection |
| PHA | Phytohaemagglutinin |
| pfu | Plaque forming units |

List of Abbreviations, contd.

| | |
|---|---|
| pi | Post infection |
| PPLO | Pleuropneumonia-like organisms |
| PPO | 2,5-diphenyloxazole |
| PrV | Pseudorabies virus |
| rRNA | Ribosomal ribonucleic acid species |
| SSC | Standard saline citrate |
| SV40 | Simian virus 40 |
| TCA | Trichloroacetic acid |
| Tris-saline | 0.9% saline containing 20 mM tris-HCl, pH 7.4 at 4° |
| ts | Temperature sensitive |
| u.v. | Ultra-violet |

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# SUMMARY.

## DNA Synthesis in Cultured Hamster Cells and the Effect of Infection by Herpesviruses.

by

David K. Howard

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The purpose of this project was to investigate the control of DNA metabolism in both 'normal' and virus-infected mammalian cells. The investigation adopted three broad approaches: (i) a study of the phenomenon of serum-stimulation of stationary cultures of BHK 21 cells; (ii) a study of the inhibition of cellular DNA synthesis following infection with pseudorabies virus; and (iii) the utilisation of nuclei isolated from both 'normal' and virus-infected cells to study DNA metabolism *in vitro*.

Although the ultimate aim of this project was to gain a deeper understanding of the processes involved in the control of cellular DNA metabolism, considerable effort has been directed towards an investigation of the inhibition of cellular DNA synthesis by infection with pseudorabies virus. In particular, attempts were made to discover whether this inhibition was caused

by the action of a molecule pre-existing in the invading virion or, if not, to identify inhibitory molecules synthesised after infection. Efforts were also made to decide whether initiation and/or propagation of cellular DNA was inhibited.

The investigation into the control of DNA metabolism in uninfected cells concentrated mainly on a study of the stimulation of stationary cultures of BHK cells by the addition of fresh serum including the determination of the levels of a number of key enzymes involved in nucleic acid metabolism and of the rates of synthesis of DNA, RNA and protein. This investigation involved the use of autoradiography to determine the percentage of the stationary cell population stimulated to synthesise DNA and the use of isopycnic centrifugation to identify the nature of the DNA synthesised. Use of actinomycin-D showed the presence of two stages in the pre-replicative phase at which the subsequent synthesis of DNA appeared to depend on RNA synthesis, in good agreement with several other similar systems. The development of this technique provided an alternative to exponentially growing cells in which virus inhibition of cellular DNA synthesis could be studied.

The use of virus which had been inactivated by ultra-violet light and of inhibitors of protein synthesis has shown that the inhibition of cellular DNA synthesis is not brought about by some pre-existing component of the virus particle and that protein synthesis is necessary before this effect can be observed. It is not possible using cycloheximide, however, to inhibit viral

protein synthesis to a sufficient degree to prevent expression of the inhibitory effect on DNA synthesis without at the same time directly inhibiting cellular DNA synthesis. Consequently, it has not been possible to study the proteins produced in normal and in cycloheximide treated virus-infected cells with a view to identifying the inhibitory factor(s) produced after infection, nor has it proved possible to cause an inhibition of cellular DNA synthesis by the addition of crude extracts of infected cells to uninfected cultures.

Some initial screening work on DNA synthesis in cells infected with temperature-sensitive mutants of pseudorabies virus has been carried out and further work with these mutants may prove useful as an approach to an understanding of the control of cellular DNA metabolism.

Alkaline sucrose velocity centrifugation of DNA isolated from virus-infected cells has indicated that the virus inhibits elongation of cellular DNA which had already been initiated at the time of infection, but it has not been possible to establish whether the initiation of new cellular DNA chains is also affected.

The final approach to the problem of control of cellular DNA metabolism has been the development of a system comprising nuclei isolated from control and pseudorabies virus-infected cells and which will incorporate radioactive nucleotides into DNA *in vitro*. Such DNA synthesis mirrors the DNA synthetic capability of the cells from which the nuclei were isolated.

Some preliminary work has been carried out on the effects

of crude 'cytoplasmic' preparations from infected cells on DNA synthesis in the isolated nuclei. A stimulation of DNA synthesis can be observed in infected cell nuclei when these fractions are added to the incubation mixture, but the effect is not so marked in nuclei from uninfected cells. Synthesis of both cellular and viral DNA is stimulated by these fractions, the effect being more marked on cellular DNA synthesis, and there are some indications that this stimulation may be rather non-specific and a result of the action of a cytoplasmic DNA polymerase activity on both cellular and viral DNA.

The in vitro nuclear system seems to hold most promise as a suitable system in which to identify controlling factors produced after virus infection and a study of these factors should lead to a better understanding of the processes involved in the control of DNA metabolism both in infected and in uninfected cells.

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## I. INTRODUCTION.

Genius may anticipate the season of maturity; but in the education of a people, as in that of an individual, memory must be exercised before the powers of reason and fancy can be expanded; nor may the artist hope to equal or surpass till he has learned to imitate the works of his predecessors.

Edward Gibbon, "The Decline and Fall of the Roman Empire."

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### 1. Cell Culture.

Science progresses in five main steps. First there is the observation of more or less evident facts, their codification, and analysis. After that comes the formulation of ideas and general principles based on these facts, and their arrangement into working hypotheses. Third, there is the development of techniques for testing these hypotheses. Fourth is the acquiring, through the use of these techniques, of pertinent but less evident information, and the verification, modification, and refinement of these hypotheses until they themselves become accepted. Finally, there is the integration of these latterly acquired facts with those already known.

The discipline of eukaryotic cell culture beautifully exemplifies these five steps. As a segment of biological thought, the foundations of cell theory are very ancient indeed. In the fourth century before Christ, Theophrastus (320 B.C.) and Aristotle (360 B.C.) described plants and animals as being made up of certain homogeneous elements, sap and blood, fibre and flesh, nerves and veins, wood and bone. But, having no lens for closer examination, they were unable to give more definite descriptions of the structure of these elements. Two millenia later, with the development of the microscope, Hooke (1667) showed that what Theophrastus had called "fibre" and Aristotle "bone" were made up of smaller homogeneous units. These he called "cells". A century and a half later Brown (1833) recognised the ubiquity of "nuclei" in fleshy, as well as in fibrous or bony materials, especially of plants, and Dujardin (1835) noted that the semi-fluid substance which commonly covers the cellular skeleton in the living parts of plants and animals has also an ubiquitous and hence evidently important role. This covering he called the "sarcode". These three concepts, of the ubiquity of Hooke's "cell", of Brown's "nucleus", and of Dujardin's "sarcode", were crystallised in 1838 into the cell theory jointly formulated by Schleiden (1838) and Schwann (1838).

Since the recognition in a formal fashion by these two workers of the ubiquity and fundamental importance of the cell

as the "elementary organism", the importance of studying that elementary unit has been implicit in much of our thinking. Attempts to carry out such studies, however, commonly resulted only in destroying the cells and it was not until 1887 that one of the first successful attempts to duplicate the environment of the eukaryotic cell was made by Arnold, who was able to follow microscopically the migration of frog leucocytes cultivated in the aqueous humour from frogs' eyes, their division into new cells and the processes by which they engulfed bits of debris, etc.

The idea of isolating cells and growing them for the avowed purpose of studying cellular autonomy and interrelations had not been formulated by Arnold as a general objective, and it was Haberlandt (1902) who was the first to express this idea in a clear fashion, and who carried out many experiments with the aim of establishing such cultures. His studies, however, were not successful and like the studies of Arnold they attracted only a passing philosophical interest at the time.

Many of these earlier experiments anticipated by 30 or 40 years techniques which are in general use today. At the time they were difficult to repeat since the media used were generally unsatisfactory or irreproducible and there was some doubt whether they demonstrated genuine survival of healthy tissues or merely somewhat delayed death of the cells.

It was because Harrison's experiment in 1907 demonstrated quite unequivocal continuation of normal function in vitro and

offered a reproducible technique that it has been generally accepted as marking the true beginning of tissue culture. Harrison explanted small pieces of tissue from the medullary tube region of frog embryos into clots of frog lymph. When kept in aseptic conditions the fragments survived for some weeks and axones grew out from the cells. This helped to settle a current controversy about the origin of these structures and illuminated the potentialities of experimental methods using surviving tissues in vitro.

The "traditional" techniques of tissue culture were rapidly established thereafter. Burrows and Carrel made the discovery that embryo extract had a strong growth-promoting effect on certain cells and Carrel's group developed the techniques to allow continuous cultivation of rapidly growing and dividing cells over long periods of time. Success in this latter field suggested the possibility that cells might be grown almost like protozoa or micro-organisms and the perfection of our present methods of cell culture owes a great deal to the group headed by Winton Earle, who were the first to grow cells direct on glass in large numbers, the first to grow cultures from single cells and the first to propagate cells intentionally in suspension.

The implications of the tissue culture method was not lost, even in the very earliest days, and its potential value in such subjects as morphogenesis, cancer research and virology

was immediately recognised. In virology and biochemistry, however, although attempts were made to wrest information from tissue culture material, the technical difficulties proved overwhelming and it was only with the rapid developments of the 1950's that its applications in these fields became widespread.

It was the observations of Enders et al., (1948) that gave the subject its great impetus. They showed conclusively that the poliomyelitis virus could be cultivated in vitro in the absence of nerve tissue. This observation was made at a time when cell culture techniques had undergone some remarkable developments and, with the added practical interest, the number of people in the field increased rapidly and the whole subject evolved with extraordinary speed in the next ten years.

## 2. The Cell Cycle.

### 2.1. General Introduction.

Mammalian cells can be divided into three principal states with respect to their ability to synthesise DNA and divide (Baserga, 1968). Continuously dividing cells that keep moving through the cell cycle from one mitosis to the next constitute the first state. The second consists of cells that have permanently lost their ability to divide, and the third of quiescent cells that ordinarily do not synthesise DNA or divide but can be induced to do so by the application of an appropriate

stimulus, so-called Go cells (Patt and Quastler, 1963). Events occurring in continuously dividing cells can be studied in asynchronous populations or in synchronised populations of cells. The amount of information that can be obtained from temporally heterogeneous populations of cells is necessarily limited, and better results have been obtained by the use of synchronised cell populations. Fortunately, a number of lines of randomly growing cells in culture can be synchronised by chemical or mechanical means, and, although caution must be exercised in interpreting results obtained from cells which have been subjected to such treatment, these methods have allowed biochemical investigations of the various phases of the cell cycle which would be otherwise impossible in asynchronous populations.

## 2.2. Synchronisation of cell cultures.

The utility of synchronised cultures in the study of the biochemistry of several phases of the cellular life cycle of mammalian cells has long been recognised, and numerous methods have been developed to produce such cultures. Most of these methods require that the population be perturbed and it then becomes difficult to prove that the biochemical balance (Anderson *et al.*, 1967) of the culture has not also been altered by the treatment. The ideal synchronisation method would be one which is purely selective and which could be applied to a growing population without affecting the growth rate. The various methods for synchronising cells *in vitro* have been critically

examined by Frindel and Tubiana (1971).

By far the most attractive of the direct methods is that of Terasima and Tolmach (1963) who synchronised HeLa S3 cells by selection of cells in mitotic phase. Further modification of the method by Robbins and Marcus (1964) and by Lindahl and Sorenby (1966) has increased the usefulness of the approach by collecting periodically from the same cultures or by collecting continuously from a single large culture and the difficulty of obtaining large quantitites of mitotic cells has been overcome by using the drug Colcemid. The discovery that colcemid inhibition was reversible in Chinese Hamster Cells (Stubblefield, 1964) led not only to better synchrony techniques (Stubblefield and Klevecz, 1965), but also to a better understanding of the mechanism of Colcemid inhibition.

The use of the drugs Colcemid or colchicine, and other drugs which prevent spindle formation during mitosis, to block the mammalian cell cycle is subject to some criticism, and in some in vivo experiments it has been shown that mitotic figures which have been blocked for four or more hours undergo necrosis and disintegration (Leblond, 1959; Hooper, 1961). These drugs may very well affect cells in nonspecific ways thereby reducing their usefulness, especially in those studies which involve experimental manipulation of the normal cell cycle. Other techniques used to induce synchrony have included temperature changes (Newton and Wildy, 1959), mechanical scraping (Salzman, 1959), trypsinisation (Swaffield and Foley, 1960), but the most

widely used techniques are those which utilise inhibitors of DNA synthesis such as fluorodeoxyuridine (Erikson and Szybalski, 1963; Gold and Helleiner, 1964), amethopterine (Adams et al., 1965), thymidine (Xeros, 1962), deoxyguanosine or deoxyadenosine (Mueller and Kajiwara, 1966a) and 5-amino uracil (Brewen, 1965). Practically every technique so far devised for synchronising cells, however, is subject to criticisms in one way or another. A critical assessment has been made by Feinendegen (1967).

Tests for synchrony include the scoring of mitoses, cell growth, and measurement of DNA synthesis by incorporation of radioactive precursors. They have been reviewed by Sinclair (1969).

## 2.3. The Cell Cycle.

### 2.3.1. General.

Cell division and the events associated with it have been of continuous interest to investigators for over a century, but only during the past 20 years has the cell cycle been studied at a biochemical level. The cell cycle is defined as the interval between the midpoint of mitosis in the parent cell and the midpoint of the subsequent mitosis in one or both daughter cells. Howard and Pelc (1953) have divided the cell cycle into four phases, depicted diagrammatically in Figure 1.1 (1) G1, the period between completion of mitosis and the onset of DNA synthesis; (2) S, the period during which DNA is replicated; (3) G2, the

period between completion of DNA synthesis and the onset of mitosis; and (4) mitosis, the period during which the chromosomes condense, segregate, and are equally distributed between the two daughter cells.

Two important conclusions emerged from the early work on the cell cycle, namely, that mitosis was almost invariably preceded by a duplication of DNA and that the synthesis of DNA was not a continuous process but occurred only during a discrete period of the interphase.

This contrasts with prokaryotes where DNA is not synthesised during a discrete period but as a continuous process throughout the life cycle of the cell (Schaechter et al., 1959; Abbo and Pardee, 1960) and where the pattern of DNA replication may vary according to the rate of growth of the cell (Helmstetter, 1967; Helmstetter and Cooper, 1968; Clarke and Maaløe, 1967).

This simplified description of the mammalian cell cycle outlined here applies only to cells that can be classified as continuously dividing.

### 2.3.2. G1 Phase.

During the G1 interval the differentiated cell expresses its particular phenotype, synthesising substances characteristic of that phenotype.

Since cells vary widely in the length of time they spend in G1, from 5.6 - 30 h (Schultze, 1969), and since they respond individually to extracellular factors which either accelerate or

delay their entrance into the S-phase, it is quite clear that multiple control processes operate to regulate the progression of cells through the G1 interval. An extensive literature already exists on the response of different cell systems to extracellular stimuli which are very diverse and include hormones (Cohen, 1965; Hamilton, 1968; Baserga, 1970), serum proteins (Todaro et al., 1965; Wiebel and Baserga, 1969), vitamins (Taylor et al., 1966), agglutinins (Cooper et al., 1963), amino acids (Brunner, 1973), enzymes (Burger, 1970; Rubin, 1970), lectins (Powell and Leon, 1970), viruses (Kit et al., 1966; Sauer and Defendi, 1966; Dulbecco et al., 1965) and temperature shock (for review see Mitchison, 1971). It is perhaps a bit perplexing to explain how such diverse agents can bring about the ultimate activation of a common set of genes, but, notwithstanding this diversity, the response of the cells usually involves an early change in the pattern of expression of genetic information with rather gross changes in the cytoplasm and membranous structure of the cells. This is followed later by the synthesis of particular species of RNA and protein which are necessary for the cell's entrance into S-phase.

A requirement for protein synthesis to traverse the G1 phase and initiate DNA synthesis has been demonstrated with the use of inhibitors in several mammalian cell types in vivo and in vitro (Black et al., 1967; Mueller and Kajiwara, 1966b; Terasima and Yasukawa, 1966; Terasima et al., 1968). Similar inhibition of DNA synthesis occurs if RNA synthesis is inhibited

in G1 (Baserga et al., 1965a; 1966; Fujiwara, 1967). When protein synthesis is stopped after DNA synthesis is already in progress, DNA synthesis is rapidly depressed (Baserga et al., 1965b; Bennet et al., 1964; Lieberman et al., 1963; Young, 1966). Whether the effect stems from the requirement for newly synthesised proteins to initiate DNA synthesis in segments of chromosomes which initiate late in the S-phase or reflects the need for continuous histone or other protein synthesis during DNA synthesis, has not yet been established.

It appears that during the G1 period there is a series of orderly metabolic events, some of which involve gene expression and are directly related to the onset of DNA synthesis. These events have been discussed in a number of reviews (Baserga, 1968; Baserga and Wiebel, 1969; Cooper, 1971; Epifanova, 1971).

2.3.3. S-Phase.

Throughout the S-phase the activities of enzymes associated with DNA synthesis, such as DNA polymerase, thymidine kinase, thymidylate kinase, thymidylate synthetase, deoxycytidine deaminase, rise or remain high and RNA synthesis continues (for literature see Stein and Baserga, 1972). Protein synthesis also continues at an elevated rate during S-phase and the synthesis of non-histone chromosomal proteins, which was evident during G1 continues (see Stein & Baserga, 1972), although the rate of turnover of these proteins during S-phase appears to be significantly lower than that observed in G1 (Borun and Stein, 1972). The

synthesis of histones has been shown to be tightly coupled to DNA synthesis, both in stimulated Go cells (Takai et al., 1968) and in continuously dividing cells (Spalding et al., 1966; Robbins and Borun, 1967), and what has been provisionally designated as histone messenger RNA is present during S-phase (Borun et al., 1967).

A number of experiments have shown that interactions between the nucleus and the cytoplasm are involved in the regulation of DNA synthesis and it is an old observation that nuclei in the same cytoplasm undergo mitosis in synchrony and, in addition, synchrony of onset of DNA synthesis in nuclei sharing a common cytoplasm has been demonstrated (e.g. Church, 1967; Harris and Watkins, 1965; Kimball and Prescott, 1962; Nygaard et al., 1960).

The study of nucleo-cytoplasmic interaction in the regulation of DNA synthesis has been extended by nuclear transplantation between amoebae in different stages of the cell cycle (Prescott and Goldstein, 1967). These experiments indicate that cytoplasmic rather than nuclear components determine whether or not nuclei synthesise DNA. De Terra (1967) has found a similar situation after cell grafting or nuclear transfer in stentor and the importance of the cytoplasm for DNA synthesis has been demonstrated in a cell-free system (Thomson & McCarthy, 1968).

2.3.4. G2-phase.

During the G2-phase of the cell cycle, RNA and protein synthesis continue, albeit at rates which decrease as the cell approaches mitosis (Taylor, 1960; Robbins and Scharff, 1966; Sisken and Wilkes, 1967). Studies with actinomycin D, puromycin (Tobey et al., 1966b), cycloheximide (Tobey et al., 1966a) and with mengovirus (Tobey et al., 1965) have clearly established that the synthesis of RNA and proteins during the G2 phase of the cell cycle is essential for entry of cells into mitosis.

The synthesis of non-histone chromosomal proteins continues during G2 (Stein and Borun, 1972; Stein et al., 1970; 1971) but their rate of turnover is significantly increased in comparison to that observed during S-phase (Stein et al., 1971; Borun and Stein, 1972). There appear to be qualitative differences in the non-histone chromosomal proteins synthesised in G1, S and G2 (Stein et al., 1970; 1971).

2.3.5. Mitosis.

Mammalian cells synthesise RNA in all phases of the cell cycle except during mitosis when there is a cessation of RNA synthesis (Taylor, 1960) and during which the *in vitro* template activity of the mitotic chromatin is several-fold less than that observed in interphase chromatin (Johnson and Holland, 1965).

A decreased rate of total cellular protein synthesis has

## Fig. 1.1.

### The Cell Cycle.

M = mitosis; from prophase to telophase;

G1 = interval between completion of mitosis and the onset of DNA synthesis;

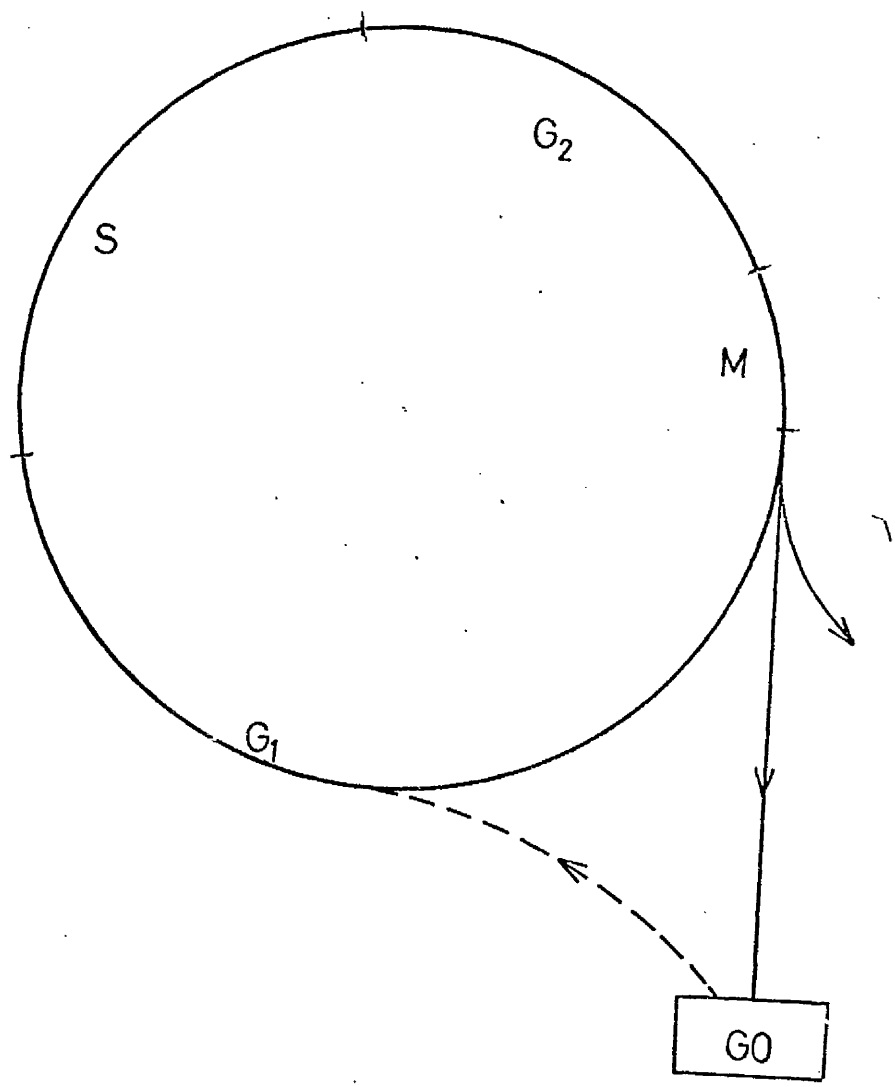
S = period of DNA replication;

G2 = interval between completion of DNA synthesis and mitosis;

$G_0$ = quiescent cells that can be stimulated to synthesise DNA and divide.

from "Biochemistry of Cell Division", ed. R. Baserga, (1969). Charles Thomas, Illinois, U.S.A.

Fig. 1.1.



also been observed during mitosis (Prescott and Bender, 1962; Robbins and Scharff, 1966) and disaggregation of the polysomes occurs (Robbins and Scharff, 1966; Scharff and Robbins, 1966). New RNA synthesis, however, is not necessary for the reformation of the polysomes in G1, the polysome-associated RNA persisting into the subsequent G1 period (Hodge et al., 1969).

Stein and Baserga (1970a) have shown that, unlike total cellular proteins, nonhistone chromosomal proteins are actively synthesised during mitosis and their rate of turnover is greater than that which occurs during S-phase (Stein et al., 1971).

Alterations of the structure of the cell surface also occur during mitosis (Fox et al., 1971).

## 2.4. Characteristics of Cell Culture.

### 2.4.1. Identity of Cell Type.

A number of tissues from an adult (or better, embryonic) organism can be dissociated into their component cells by incubation with proteolytic enzymes. Whenever such a primary tissue explant has been disrupted and the cells allowed to grow in tissue culture media, the cells can often be passaged, or inoculated into a new culture vessel with fresh medium after a considerable length of time. As soon as the cells have been passaged in this way the culture is designated a primary cell line. Frequently, primary cell lines can be passaged repeatedly and, frequently, these lines cease to proliferate and die out

after quite a large number of passages. However, cell lines occasionally can be cultured for such a long time that they apparently have developed the potential to be subcultured indefinitely. Such cell lines are called established cell lines.

The precise physiological environment of the cells in the intact organism is unknown and thus irreproducible and the fact that many primary cultures undergo a "crisis" may merely mean that they have run out of some essential compound of which we have no knowledge. The fact that, nevertheless, cell cultures can be established is thus more a tribute to the adaptability of living cells than to our ingenuity.

An organ situated in the whole animal may consist of many different cell types that intermingle with each other more or less intimately. All of these cell types are capable of growth in cell culture and, with out present non-selective techniques and the loss of morphological characteristics, it is virtually impossible to identify with certainty the type of cell that becomes established as a continuous tissue culture cell.

2.4.2. Dedifferentiation.

Throughout the literature of tissue culture the statement recurs that cells in culture lose their highly specialised functions. When primary cell lines from normal tissue undergo transformation, specialised functions appear usually to be lost but there are some exceptions to this. In

particular fibroblasts usually retain the capacity to secrete collagen (Green & Goldberg, 1963; Davidson, 1963). Moreover, cell lines derived from tumours may exhibit specialised functions and may retain these for a long time in culture. It is not easy to establish a general rule and it may be that there is often some retention of differentiated potentiality in culture which is not realised because of inadequacies of the medium or of inadequate techniques of detection.

2.4.3. Density Dependent Inhibition of Growth.

Primary cell lines do not behave in the same way as established cell lines. When established cell lines have become stationary the medium in which they have grown is usually inadequate for maintenance of a fresh inoculum of cells. However, it can often be observed that cultures of a primary cell line stop growing before the medium is exhausted, a phenomenon known as density dependent inhibition of growth. When certain kinds of 'normal' primary or established cells, mainly fibroblasts, come into contact they immediately become immobilised and cell division, DNA, RNA and protein synthesis are much reduced or eliminated (Eagle, 1965; Stoker, 1967). This phenomenon probably reflects a control mechanism in the intact animal but despite intensive study the mechanism has not been discovered. When primary cell lines become transformed one of the most outstanding features of the transformed cells is the absence or great diminution of density-dependent inhibition of growth. This

is the reason why transformed cells grow to higher densities and pile up on each other.

2.4.4. Chromosome Abnormalities.

In tissue cultures abnormal cells and abnormal cell divisions are frequently encountered. It is common for cytoplasm to fail to divide when the nucleus divides so that multinucleate giant cells occur, and instead of the normal dipolar division of cells, multipolar division may occur, especially in tumour cells. Thus tripolar divisions are not infrequently seen where the cell divides into three.

Other common abnormalities are abnormalities of the population as a whole. In 'normal' populations, all the cells usually have the same number of chromosomes, the diploid number for the species. In established cell lines it is almost the rule to find a chromosome number different from that of the animal of origin. Frequently, the cells have a triploid or tetraploid number of chromosomes or, commonly, a number which is not a simple multiple of the haploid number. This condition is referred to as aneuploidy. In addition, it is common to find great variability in chromosome number from one cell to another in a culture. For example, Hep-2 cells have a modal chromosome frequency of 76 (73-79); HeLa cells, 79 (39-187); KB cells, 77 (68-153); BHK-21(C13) cells 44 (36-45); and WI 38 cells 46 (43-92) (from Registry of Animal cells certified by the Advisory Committee to the animal cell culture collection

of the American Type Culture Collection, 1964).

Even in the whole animal, however, certain chromosome imbalances are compatible with survival into adult life. Thus Down's Syndrome in man is due to trisomy of chromosome 21, so that these individuals have 47 instead of 46 chromosomes. Aneuploid chromosomal complements are probably uncommon as the imbalance produced is, in most cases, incompatible with survival rather than that the mechanism involved is exceptionally rare. In cells in culture chromosomal imbalance may be less of a handicap than in the developing animal since the cells do not form part of a delicately balanced organism.

2.4.5. Effects of Hormones on Tissue Culture Cells.

Studies of the direct effects of hormones on tissue culture metabolism have, on the whole, been disappointing. Attempts to demonstrate a general action of steroid hormones have been unsuccessful except with very high concentrations when an inhibition of growth, probably non-specific, has occurred. This statement applies only to cell culture studies, as some quite dramatic effects have been produced by steroid hormones in organ culture.

Tomkins et al., (1966) have shown that in a rat cell line derived from a hepatoma the synthesis of the enzyme tyrosine transaminase is dependent on the addition and continued presence of corticosteroids which act as "inducers" of the enzyme. They have suggested that the corticosteroids act, not at the

transcriptional level by controlling activity of the structural gene, but at the translational level by controlling the stability of the mRNA.

Very little is known as yet about the action of hormones on cells *in vitro*. It seems quite certain that the presence of hormones is not essential for cell survival but in view of their great importance in the intact animal, it is likely that they are required for the full development and function of differentiated cells.

2.4.6. Interaction among Cells.

Besides density dependent inhibition of growth discussed in Section 2.4.5, other important interactions have been discovered.

Until quite recently it was believed that, with the exception of syncitia such as muscle, animal cells existed as quite separate organisms with no intimate connections. It has now been found, however, that cells within the same organ may have direct electrical continuity, suggesting that ions can flow readily between them. It has also been found that this kind of contact electrical coupling can be established in confluent cells in tissue culture (Potter *et al.*, 1966). Moreover, it has been shown that genetically different cells in close contact with each other can cooperate with each other so that two cells each with a separate genetic defect can complement each other so that both survive. The actual basis for this phenomenon is not understood

but it seems to involve the passage of small molecules between cells through so-called gap junctions (Pitts, 1971).

2.4.7. Genetics of Cultured Cells.

Mutants of animal cells can be isolated by standard selection methods and many have now been described (Taylor et al., 1971). Some of these mutants have been used in studies of genetic transformation and cell hybridisation.

Tissue culture cells do not normally need to possess any system of genetic recombination and so it is unlikely that any mating system will be found among tissue culture cells. This difficulty has been circumvented, however, by the use of u.v.-inactivated Sendai Virus to promote random fusions giving rise to multinucleate cells. In favourable circumstances it is possible to obtain hybrid cell lines from such heterokaryons containing chromosome material from more than one nucleus in the now uninucleate cell. The resulting hybrid cells will be genetically recombinant if they were formed from cells containing different genotypes. In this way it has been possible to localise a number of genes to particular chromosomes, and as techniques improve the precision and scope of these procedures may eventually rival those of bacterial genetics.

2.4.8. Autoradiography.

The dissection by Howard and Pelc (1953) of the cell cycle into four distinct phases was only made possible by the

use of autoradiographic techniques which showed that DNA was replicated in a discrete period of the interphase preceding mitosis.

Autoradiography is one of the most useful techniques for studying biochemical reactions in situ at the level of the individual cell. The developed image on an autoradiogram contains two distinct elements of information: first, the location of the radioactivity relative to the observable structures in the specimen, and second, the intensity of the image which bears a definite but complex relationship to the amount of radioactivity present. The limit of detectability of a given number of molecules is very low and exceeds most of the available assay techniques. Electronic counting on a single sample for more than a few hours is impractical whereas autoradiography permits detection periods of up to at least several months with insignificant increments in background levels. Under certain conditions as few as one disintegration per hour can be detected.

The study of nucleic acid synthesis at the cellular level was drastically changed by the introduction of tritium as a suitable label for autoradiographic purposes and by the availability of tritium-labelled nucleosides with high specific activity. Because of the specificity of the latter as nucleic acid precursors and because of the better resolution of tritium-generated grains tritiated nucleosides are used preferably in studies of DNA and RNA metabolism today.

The discovery by Reichard and Estborn (1951) of thymidine as a specific precursor of DNA with negligible diversion of radioactivity to any other component of DNA and with practically no incorporation into RNA (Friedkin et al., 1956; McQuade et al., 1956) provided a very useful tool for the study of DNA synthesis. Further progress was made in the use of thymidine in autoradiography when thymidine labelled with tritium was produced (Taylor et al., 1957; Verly and Hunebelle, 1957; Verly et al., 1958). Autoradiographic pictures of cells labelled with $^{3}H$-thymidine show a very distinct labelling of the cell nucleus or even of single chromosomes, with almost no scattering of the grains over the border of these cell structures (Plate 2).

### 2.4.9. Nucleotide Metabolism.

The synthesis of polydeoxyribonucleotides can conveniently be considered as proceeding through four main phases:

(1) An initial phase where assembly of the purine- and pyrimidine-nucleoside monophosphates occurs from small molecular precursors;

(2) the conversion of ribonucleotides to deoxyribonucleotides;

(3) the phosphorylation of deoxyribonucleotide monophosphates to the corresponding triphosphates;

(4) polymerisation of deoxyribonucleotides to polynucleotides in the presence of an appropriate polynucleotide template.

Kornberg (1957) pointed out that most cells can obtain the nucleotides necessary for synthesis of polynucleotides by

either a de novo pathway or by utilisation of preformed bases or nucleosides.

The pathway for the de novo production of purines and pyrimidines have been well characterised and are discussed by Davidson (1972).

In addition to the enzymes which catalyse the formation of nucleotides and polynucleotides there appear to exist a large number of catabolic systems operating at all levels of the internucleotide pathways.

The polynucleotides are initially degraded by ribonucleases and deoxyribonucleases, of which there are many types, to nucleoside monophosphates with a large number of oligonucleotide intermediate products. The ultimate products of the nuclease reactions are nucleoside monophosphates with the phosphate group in the 5'-, 2'- or 3'- positions.

The dephosphorylation of nucleoside 5'- or 3'- monophosphates and the nucleoside 5'- polyphosphates is catalysed by a number of phosphatases of low specificity abundant in many tissues and by a number of specific 3'- and 5'- nucleotidases. The nucleosides, the ultimate products of phosphatase action on nucleotides, are split to free bases and pentose moities, presumably by the action of nucleoside phosphorylases.

The cell has the ability to reutilise these breakdown products of nucleotide catabolism in the existence of so-called "salvage" pathways.

Nucleoside phosphorylases catalyse the reaction:

ribonucleoside (deoxyribonucleoside) + Pi $\rightleftharpoons$

base + ribose - 1 - P (deoxyribose - 1 - P).

The equilibrium of the reaction favours nucleoside synthesis from the base (Atkinson and Morton, 1960).

Nucleoside kinases, present in a wide variety of tissues, catalyse the phosphorylation of the nucleoside by ATP to form the monophosphates with the liberation of ADP from ATP. At this point the monophosphates formed from degradation products of nucleotide catabolic reactions can feed back in to the de novo pathway of synthesis of nucleoside di- and tri-phosphates. By utilisation of the same pathways, exogenously supplied, nucleosides can be used by cells to synthesise DNA and RNA.

These "salvage" enzymes have important implications in the study of DNA metabolism in cell cultures since the presence of purine nucleoside kinases and of purine phosphoribosyltransferases permits conversion of antimetabolites that are nucleoside or base analogues respectively into nucleotide analogues that are often powerful regulators of enzyme activity; the presence of the enzyme thymidine kinase in the cells allows $^{3}$H-thymidine to be used as a very suitable label for the study of DNA metabolism by isotope incorporation.

2.4.10. Incorporation of Exogenous $^{3}$H-thymidine into DNA.

Despite the frequent use of $^{3}$H-thymidine for studies of DNA synthesis no definite knowledge was obtained for a long time about how far the exogenous labelled thymidine replaces the

endogenously synthesised thymidine or its phosphorylated derivatives in DNA synthesis. Some references in the literature suggested, however, that the participation of the exogenous thymidine in DNA synthesis is minute. According to Rubini et al., (1962) only about 1% of the endogenous nucleoside or its derivatives is replaced by labelled exogenous thymidine during DNA synthesis. Lang et al., (1966b, 1968), by measuring the radioactivity of $^{3}$H-thymidine incorporated into DNA as a function of different amounts of administered thymidine, reported that the proportion of the entire DNA-thymidylic acid synthesis replaced by the exogenous $^{3}$H-thymidine in the mouse reached only from 0.1% to a few per cent.

2.4.11. Nuclear Pools of Thymidine.

Closely related to the use of $^{3}$H-thymidine as a DNA precursor is the question of the thymidine pool in the cell nucleus. Stone et al., (1965) have studied the relationship between the nuclear $^{3}$H-thymidine pool or its derivatives and DNA synthesis in Tetrahymena pyriformis using an autoradiographic method that makes it possible to demonstrate water-soluble substances within the cell. They found that the uptake of thymidine in the cell is confined to the S-phase, but that a 'soluble' pool of $^{3}$H-thymidine derivatives exists in those cells from the end of one S-phase until the next S-phase and that this pool does not turn over completely during one S-phase. From these findings the authors concluded that the DNA synthesis

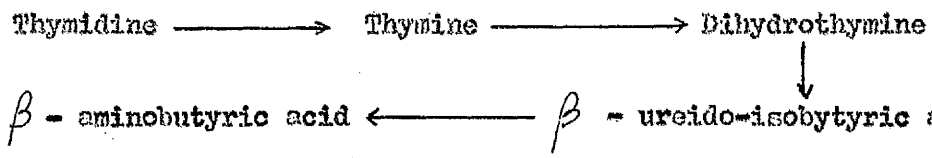
is initiated and maintained for a short time at the expense of this pool, which was formed during the preceding S-phase and that turnover of this pool stimulates the synthesis of additional phosphorylated precursors. Thymidine metabolism and cell kinetics have been reviewed by Cleaver (1967) and Feinendegen (1967).

In BHK 21 cells infected with HSV-1 cellular dTTP pools increase by 35-fold by 6 h post-infection. At 2 h post-infection the pools have already increased 7 - 8 fold, returning to this level by 12 h post-infection. (A. Jamieson, personal communication.) It might be expected that labelling of DNA in infected cells with $^{3}$H-thymidine would be difficult due to dilution of exogenously supplied $^{3}$H-thymidine by endogenous unlabelled thymidine. No such problem is encountered, however, as the viral-induced thymidine kinase appears to be responsible for production of greater than 95 per cent of the cellular dTTP pool, (A. Jamieson, personal communication) and to utilise exogenous thymidine for the eventual production of thymidine triphosphate as expected.

of Thymidine

2.4.12. Catabolism of Thymidine.

Thymidine is rapidly catabolised to thymine in the cell. The cell is able to reduce thymine to dihydrothymine which is further degraded to BUIBA ( $\beta$ - ureido - isobutyric acid). This is depicted schematically below.



That thymidine is catabolised without delay and very rapidly is shown by the appearance of thymidine degradation products in the culture medium as soon as five minutes after the addition of thymidine. This transformation of thymidine to thymine is a much more rapid process than the incorporation into DNA, as was shown in Landschütz ascites cells by Zajicek et al., (1963). By incubation in increasing concentrations of cold thymine the conversion of thymidine to thymine was suppressed so that an increased incorporation of $^{3}$H-thymidine into DNA of the ascites cells was achieved. Lang et al., (1966a) also reported that after incubation of HeLa cells for more than 48 hours the $^{3}$H-thymidine in the incubation medium sharply decreased while there was a correspondingly sharp increase of $^{3}$H-thymine. Only 13-15% of the $^{3}$H-thymidine originally present in the medium was incorporated into the DNA of the cells, indicating that, as in whole animals, only a small part of the applied $^{3}$H-thymidine is incorporated into DNA while most of it is catabolised.

2.4.13. Anomalous Effects Induced by $^{3}$H-thymidine.

Radiation damage to chromosomes resulting from incorporation of $^{3}$H-thymidine has been demonstrated by several investigators (McQuade et al., 1956; Wimber, 1959; Natarajan, 1961; Hsu & Benzes, 1965). Long exposure of HeLa cells to low concentrations of $^{3}$H-thymidine suppresses and can completely inhibit colony growth (Painter et al., 1958). Moreover, Marin and Prescott (1964) have shown that exposure of Chinese Hamster fibro-

blast cells for 1 - 2 h to $^{3}$H-thymidine at concentrations of 1.2 - 2.7 uci/ml in the medium delays division, and significantly decreases the number of third and fourth division labelled metaphases. However, these effects were not seen with smaller concentrations of the labelled nucleoside. Concentrations of 4 uci/ml in the culture medium also increase the duration that cells of Tradescantia labelled with $^{3}$H-thymidine spend in the G2-phase of the cell cycle (Wimber and Quastler, 1963). In mammals it appears that $^{3}$H-thymidine above 1 uci/g body weight causes radiation damage to mouse spermatogonia, and that low concentrations (1 uci or less) are recommended for short-term experiments (Johnson and Cronkite, 1959). However, $^{3}$H-thymidine can be safely used as a tracer both in vivo and in vitro in mammalian cells when pulse labelling and low concentrations of the isotope are utilised.

## 3. Chromosome Structures and Metabolism.

### 3.1. Composition and Structure of Eukaryotic Chromosomes.

#### 3.1.1. Techniques for examining chromosome Structure.

Evidence concerning the structure of chromosome fibres has been obtained by means of electron microscopy, x-ray crystallography, x-ray scattering, hydrodynamic measurements and optical measurements. These approaches have been reviewed by Huberman (1973).

The two most frequent methods of performing electron

microscopy on chromosome fibres are thin sectioning and surface-spreading. The effects of sectioning on fibre structure, however, are essentially unknown and, in addition, the effects of fixing, staining and embedding on the ultrastructure of chromatin can only be guessed at. The surface-spreading method has the advantage over thin sectioning of preserving the overall morphology of whole condensed mitotic chromosomes and any discrepancies between the intracellular chromosome and the chromosome seen by the surface-spreading method, if any, must be confined to the diameter and microscopic arrangement of the fibres, not to their overall disposition. Just as with thin sectioning, however, the effects of cell rupture and surface-spreading on the association or dissociation of proteins and other molecules with or from the fibres and on the state of twisting or coiling of the fibres are unknown. Similar questions can be raised about the validity of measurements using x-rays as these measurements necessitate the use of isolated material.

Measurements using hydrodynamic techniques are open to the usual criticisms about the use of isolated chromatin preparations and the same uncertainties about the purity and structural integrity of the chromatin used for study, compounded by uncertainties about the interpretation of results, have made techniques involving optical measurements inconclusive.

The structure of chromatin can vary greatly depending upon the methods of isolation and analysis. Thus, to relate

a specific property to the intracellular structure of chromatin, that property should be studied under conditions found inside the nucleus. This criterion does not seem to have been met in most studies of chromatin structure.

The roles of various components in the maintenance of chromatin structure and the study of the integrity of the chromatin structure have usually been monitored by hydrodynamic properties or by circular dichroism and occasionally using x-ray diffraction or electron microscopy. The roles of some of the components in the maintenance fibre structure and chromatin functions are discussed in sections 3.2 and 3.3.

### 3.1.2. DNA Structure; repeated sequences and supercoiling of DNA in chromatin.

Measurements of x-ray diffraction from chromosome fibres has led Pardon et al., (1967) and Richards and Pardon (1970) to interpret their results in terms of a supercoil of the DNA duplex with a pitch of 120 Å and diameter of 100 Å. Bram and Ris (1971) measured x-ray scattering from unoriented chromosome fibres and in combination with results from surface-spread electron microscopy these results are interpreted to show an irregularly folded fibre with an average pitch of 45 Å and a diameter of about 60 Å. Viscosity measurements by Sponar et al. (1970) and by others also show that chromosome fibres have a more compact structure than naked DNA but it is not clear that they relate to the supercoil of Pardon et al. (1967). Richards and Pardon

(1970) find that similar x-ray reflections can be produced by a complex of purified DNA with purified histone fractions suggesting that the structure is basically simple; a supercoil is one of the simplest ways to condense a long DNA molecule into a shorter and thicker chromosome fibre.

Highly Repetitive Sequences of DNA.

Generally, about 20-30% of a eukaryotic genome consist of repeated sequences (Hennig and Walker, 1970) and most of these appear to belong to families of related sequences. Despite the fact that these sequences are presumed to be young in evolution (Flamm, 1972) they show signs of having accumulated large numbers of base changes and hence are not stringently conserved. In fact, the degree of nucleotide substitution within the ancestral unit is so extensive, as in the case of guinea pig $\alpha$-satellite, as to argue that few if any base changes have been selected against (Southern, 1970). These sequences are localised in genetically inactive, heterochromatic regions of the chromosome and consist of a short tandemly repeated sequence which is only 6 - 15 nucleotide pairs in length. The idea that these sequences might serve as templates for RNA synthesis appears to be untenable for the above reasons and, in addition, hybridisation experiments have failed to reveal the presence of any RNA sequences complementary to mouse satellite DNA (Flamm et al., 1969). Two of the six possible reading frames yield nonsense codons (Southern, 1970).

The possibility that satellite DNA in mouse might be involved either in the initiation of DNA synthesis (Walker et al., 1969) or in its termination (Flamm et al., 1970) was implied from experiments carried out by Smith et al., (1970) who demonstrated that, following infection of mouse kidney cells with polyoma virus, satellite DNA was replicated 1 or 2 hours before the main band; and from experiments by Flamm et al., (1970) who reported that satellite sequences of synchronised mouse cell cultures were among the last to undergo replication. Flamm et al., (1971), Bostock and Prescott (1971) and Tobia et al., (1971) presented conclusive evidence that mouse satellite sequences are not involved in the initiation of DNA replication. No detectable quantities of satellite are replicated in early S-phase as would be expected of sequences that serve as initiation sites, and it is not until late S-phase that satellite replication becomes apparent.

If it is considered that satellite sequences serve an organisational role within chromosomes, as has been postulated (Walker et al., 1969), and that this function depends upon the sequences serving as recognition sites, then it is necessary that some mechanism exist that can stabilise single-stranded regions. Mouse satellite DNA has this potential in that renaturation of separated light or heavy strands revealed the presence of a short nucleotide sequence which differs entirely from the dominant, basic repeated sequence of the satellite (Flamm et al., 1969). This sequence, which is less than fifty nucleotides long and occurs, on average, once in every one thousand

nucleotides within the satellite duplex, is interspersed along with its complement among the basic repeated sequences on a given heavy or light strand. They have been referred to as "reversed sequences" and it is thought that they can interact in such a way as to stabilise single-stranded regions or loops on opposite strands of the renatured duplex of light and heavy strands. Whether this occurs *in vivo* is not known.

The existence of these regions has led to the theory that satellite blocks may serve as recognition sites for pairing of homologous chromosomes during meiosis, recognition of centromeres of common origin, the process of recombination and determination of, and specification of, the folding patterns of chromosomes (Walker *et al.*, 1969). In support of the pairing hypothesis is the observation that the Y chromosome, which is the only one of the forty mouse chromosomes that does not pair, is also the only chromosome that does not contain localised satellite sequences at its centromere (Flamm, 1972). However, the observations of Hennig *et al.*, (1970) strongly support the idea that satellite DNA plays no role in bringing about somatic pairing in *Drosophila*. They demonstrated that the satellites of different species of *Drosophila* showed marked differences in their nucleotide sequences, in agreement with the findings in mammals. Despite this, there is remarkable accuracy in somatic pairing in hybrids formed from the different species.

One phenomenon in which satellite DNA may play a role is that observed by Michie (1953). He showed that during meiosis

the parental chromosomes segregate non-randomly with those of a given species tending to pass to the same daughter cell. Their ability to do this may be related to the presence of a species-specific satellite at the centromeres (Bostock, 1971). This is compatible with a role of satellite DNA, not in the immediate needs of the cell, but in the evolution and maintenance of new species.

Britten and Davidson (1969) have included all classes of repetitive DNA sequences as likely candidates for a gene regulatory role in their ideas of gene regulation.

### 3.1.3. Chromosomal Proteins.

#### 3.1.3.1. General.

Unlike the single Escherichia coli chromosome which consists of little more than a naked strand of DNA, the mammalian genome is a complex structure consisting of DNA, probably RNA and both acidic and basic chromosomal proteins, the protein component comprising histones and acidic proteins.

The conservation during evolution of the characteristic properties of histones (Delange et al., 1969) tends to indicate that they have a specific function which cannot tolerate major changes in primary structure. However, since they comprise a rather limited group of proteins, it is difficult to ascribe to them any complex role. Hence, their actual function, although specific, may be of a more general nature such as maintaining chromosome structure (see section 3.1.3.3).

Modification of the structure of histones by phosphorylation, acetylation and methylation does introduce the possibility of a more varied role.

Unlike histones, the amount of non-histone chromosomal proteins in chromatin varies with the tissue source and may be correlated with the level of RNA synthesis (Marushige and Dixon, 1969; Marushige and Ozaki, 1967; Dingman and Sporn, 1964). In comparison with histones, the non-histone proteins of chromatin appear to be more heterogeneous and of higher molecular weight. It appears, however, that they show only limited hetrogeneity in various species and tissues (Elgin and Bonner, 1970). This relative lack of heterogeneity need not argue against a specific role as most chromosome preparations are likely to possess similar enzymes, structural proteins and, perhaps, contaminating membrane proteins.

3.1.3.2. Histones.

Histones are the basic proteins bound to the DNA of chromosomes in eukaryotic organisms. Their synthesis, which takes place in the cytoplasm (Robbins and Borun, 1967), is closely related to DNA replication (Prescott, 1966; Robbins and Borun, 1967) and they are sufficiently similar in all organisms studied that they can be recognised by properties such as their amino acid composition, amino acid sequences, or chromatographic and electrophoretic behaviour (for review of role of chromosomal proteins see Huberman, 1973). Some

heterogeneity exists within each class due to minor sequence variations and side-chain modifications, but the five major classes in any given tissue, F1, $F2a_1$, $F2a_2$, F2b and F3, can usually be distinguished (Panyim et al., 1971).

A striking characteristic of the amino acid sequences and partial sequences now available for histones is that the basic amino acids are distributed non-uniformly, nearly all being crowded into one half of the molecule. There is also a striking conservation of sequence during evolution for some of the histones, the conservation being greatest for histone F2a, and least for histone F1 (Delange et al., 1969).

With the exception of the very lysine-rich histone F1, individual histone fractions probably consist of one molecular species (Fambrough and Bonner, 1969; Johns, 1971). The F1 histone fraction has been shown to be heterogeneous in electrophoretic (Panyim and Chalkley, 1969) and ion-exchange chromatographic systems (Kinkade and Cole, 1966; Bustin and Cole, 1969) and the amount of this component appears to be dependent on the physiological state of the animal (Stellwagen and Cole, 1968; Hohmann & Cole, 1969). Johns (1971) has suggested that histone F1 may possibly have a more complex role than the other histone fractions in the control of expression of genetic information.

For reasons outlined in section 3.2.2, however, the concept has now arisen that the histone proteins, rather than functioning as specific gene regulators, a function thought more

likely to be performed by the non-histone chromosomal proteins, probably have an important function in determining and maintaining the chromosome structure *in vivo* in such a way that the normal processes of any particular tissue may be effectively carried out by other specific molecules.

3.1.3.3. Role of Histones in Fibre Structure.

Many attempts have been made to determine which histones are required for fibre structure, but few unambiguous answers have been obtained. For example, many investigators find the properties of isolated chromatin little altered by removal of histone F1, while others find that removal of F1 is deleterious to fibre structure. In addition, those who agree that histone F1 is not necessary for fibre structure disagree among themselves over which of the remaining histones are necessary (see Huberman, 1973, for review).

Henson and Walker (1970) have shown that DNA is more asymmetrical than nucleohistone, histones F2a$_2$, F2b and F3 being involved in this effect and changes in the optical rotatory dispersion spectra of the DNA of chromatin (Tuan and Bonner, 1969) have been ascribed to a slight alteration in the DNA double helix when it is part of the chromatin superstructure (Sponar *et al.*, 1970).

Nuclear Magnetic Resonance studies show that regions of histones F1, F2a and F2b can undergo conformational changes and are responsible for histone-histone interactions. The regions

involved in these interactions appear to be not the basic parts of the histone molecule, which probably bind to DNA, but the remainder of the polypeptide chain (Bradbury and Crane-Robinson, 1971). This technique has further confirmed the impression that the basic region is the only part of histone F2b able to bind to DNA, whereas all of the polypeptide chain of histone F1 is apparently capable of such an interaction (Boublik et al., 1971). It is interesting to note that histone F1 possesses significant amounts of proline and has been reported to have an extended structure with little -helial content (Bradbury et al., 1967; Haydon and Peacocke, 1968). Studies using interphase and metaphase chromosomes indicate that this histone fraction may link DNA-containing fibrils together (Littau et al., 1965; Mirsky et al., 1968; Sluyser and Snellen-Jurgens, 1970).

Olins (1969) has provided evidence that the lysine-rich histones lie in the groove of the DNA helix, and the most tightly bound histones, the arginine-rich histones, have been demonstrated to exist in an extended chain form which appears to lie parallel to the axis of the helix rather than along the grooves themselves (Bradbury and Crane-Robinson, 1971).

3.1.3.4. Non-Histone Chromosomal Proteins.

These proteins are defined as those proteins which remain associated with DNA after the histones have been removed. Unlike histones (section 3.1.3.2), the amount of these proteins

in chromatin varies with the tissue source and may be correlated with the level of RNA synthesis (Marushige and Dixon, 1969; Marushige and Ozaki, 1967; Dingman and Sporn, 1964; Sporn and Dingman, 1966). Wang (1966, 1967) distinguished between chromosomal acidic proteins and the nuclear residual proteins, both of which have been shown to be highly heterogeneous (Wang and Johns, 1968; Patel et al., 1968). There is some evidence to suggest that the residual proteins are nucleolar in origin (Kostraba and Wang, 1970). In comparison with histones, the non-histone proteins of chromatin appear to be more heterogeneous and of higher molecular weight (Bonner et al., 1968a; Elgin and Bonner, 1970; MacGillivray et al., 1971), and it appears that they show limited heterogeneity in various species and tissues (Elgin and Bonner, 1970). Like histones, the non-histone proteins have been shown to be synthesised in the cytoplasm (Stein and Baserga, 1971).

Apart from experiments in which stimuli, such as hormones, induce the synthesis of specific acidic proteins in target tissues (Shelton and Allfrey, 1970; Teng and Hamilton, 1970) only a few studies have been carried out on the turnover of non-histone chromosomal proteins. It appears that these proteins are synthesised during the entire cell cycle at a level higher than that of histones (Stein and Baserga, 1970a)and turn over more rapidly than histones (Holoubek and Crocker, 1968; Hancock, 1969; Stein et al., 1970).

In comparison to histones, which have been reported to be similar in type and amount in both euchromatin and heterochromatin (Frenster et al., 1963; Littau et al., 1964; Frenster, 1965a), non-histone proteins are found to be in two-fold excess in euchromatin.

3.1.4. Chromosomal RNA.

When total chromosomal proteins are isolated, they contain a considerable amount of RNA. Huang and Bonner (1965) first described a special class of RNA containing a high content of dihydrouridylic acid which they considered to be covalently bound to histones and Benjamin et al., (1966) described a similar RNA which was bound to histone and was RNase insensitive. Shih and Bonner (1969) described the properties of a chromosomal RNA from calf thymus and found that it was bound to non-histone protein, and not to histones. A similar RNA from rat ascites cells has been described by Dahmus and McConnell (1969). Bonner et al., (1968b) have made the suggestion that this RNA may provide a simple DNA recognition mechanism, but Heyden and Zachau (1971) have evidence that a large part of the chromosomal RNA from calf thymus chromatin is simply RNA.

Frenster (1965a, b, c) made the suggestion that polyanions might oppose histone binding to DNA and suggested, in particular, that RNA might perform this function. He speculated that the first stage of regulation of transcription might be a loosening of histone binding by polyanions, permitting

local strand separation, which in turn would then permit a "derepressor RNA" to base-pair with one strand and allow transcription from the other, free, strand. Bonner's group has speculated that the special class of RNA, chromosomal RNA, in chromatin may form a stereospecific complex with DNA and hence provide the recognition mechanism for sequences which are to be derepressed. In Frenster's original model, the suggestion was made that the RNA might recognise single-stranded DNA by base pairing, and it was this attractive hypothesis which first drew attention to chromosomal RNA. Bonner's group (Dahmus and Bonner, 1970; Sivolap and Bonner, 1971), however, suggested that this RNA has the unusual property of being able to "hybridise" with double-stranded DNA at low temperatures and in an organ specific manner.

Britten and Davidson (1969) have advanced an elaborate hypothesis to explain genetic regulation in eukaryotic cells, based on the observations with chromosomal RNA and on the general theories of Jacob and Monod (1961). Their proposal has many features in common with the operon model of Jacob and Monod, the two major differences being that the regulator is thought to be RNA and not protein and that an argument is advanced that redundant sequences in DNA might be desirable for the regulator genes.

## 3.2. Gene Expression and its Regulation.

### 3.2.1. Role of Histones.

That histones may serve as gene regulators was originally suggested by Stedman and Stedman (1944) and this concept found support in the demonstrations by Bonner and Huang (1963), Huang and Bonner (1962) and Allfrey et al. (1963) that DNA primed RNA synthesis could be partially or completely suppressed by the addition of histones to the various cell-free system. In studies on the effect of histones on various preparations, however, it is of considerable methodological significance that the components of the medium remain soluble, as Billen and Hnilica (1964) have pointed out. They reported that the inhibition induced by the histones was mainly related to the precipitation of the deoxyribonucleohistone from the preparation.

In an attempt to identify the components of chromatin responsible for the specific restriction of the template activity of chromatin, Paul and Gilmour (1968) investigated the functions of individual components of chromatin by reconstruction of chromatin and nucleoproteins from previously isolated components. They found that to restore the normal transcriptional pattern they had to use total chromosomal proteins and not just histones. Chromatin reconstituted in this way yielded RNA which, by competition experiments, was not distinguishable from RNA transcribed from native chromatin. In a further investigation Gilmour and Paul (1970) suggested that the non-

histone fraction directed the organ specificity of template restriction, and not histones. Similar conclusions have been reached by many workers since then (Spelsberg and Hnilica, 1970; Wang, 1970; Kamiyama and Wang, 1971).

Several findings, however, make it difficult to assign to histones a highly specific role in the fine tuning of gene regulation. In the first place they are present in similar amounts in active and inactive tissue (Dingman and Sporn, 1964) and chromatin (Frenster et al., 1963; Frenster, 1965a; Littau et al., 1964); and second, they do not possess tissue or species specificity (Hnilica and Busch, 1963; Hnilica et al., 1962; Laurence and Butler, 1965; Palau and Butler, 1966; Delange et al., 1969).

Yet, histones may be effective in completely and permanently shutting down the large portion of the mammalian genome that is inactive in differentiated cells of the adult animal. Stellwagen and Cole (1969) have suggested that histones are aspecific inhibitors of DNA transcription and that they simply act by repressing large segments of the genome on a more or less permanent basis. Modification of the structure of histones by phosphorylation, acetylation, methylation, etc., does introduce the possibility of a more varied role;

3.2.2. Modification of Histones.

Acetylation : Acetyl groups are incorporated into N-terminal positions during synthesis of histones (Liew et al., 1970; Marzluff & McCarty, 1970) and also to $\varepsilon$-amino and O-seryl groups of preformed histones (Gershey et al., 1968; Pogo et al., 1968). The donor of these acetyl groups is acetyl CoA (Allfrey, 1970) and a number of enzyme systems have been described which catalyse the reaction (Nohara et al., 1966; Bondy et al., 1970; Gallwitz, 1970). Deacetylase enzymes are also known (Inoue & Fujimoto, 1969; Libby, 1970).

During the cell cycle acetylation of HeLa cell histones F2a, F2b and F3 rises to a maximum coinciding with the end of DNA synthesis. On the other hand, acetylation of histone F1 rises to a maximum at the same time as DNA synthesis. Thereafter the degree of histone acetylation diminishes during the rest of the cell cycle, with the result that histones F1 and F2b lose most of their labelled acetate groups whereas F2 and F3 retain about half of theirs (Shepherd et al., 1971). These findings correlate well with the observation that histone F1 is synthesised before DNA in HeLa cells (Gurley and Hardin, 1970), and that in interphase cells it does not contain internal acetate receptor sites but has an N-terminal acetate group.

Pogo et al. (1966) have shown that in lymphocytes the early induction of RNA synthesis brought about by phytohaemagglutinin

was preceded by acetylation of histones, particularly the arginine-rich fractions, although this may be a non-specific or artifactual effect of the PHA-lymphocyte system (Ono et al., 1968; Monjardino and MacGillivray, 1970). Histone acetylation has been implicated in other systems where gene activation appears to occur (Takaku et al., 1969; Pogo et al., 1967) and controversy exists about yet other systems (Clever and Ellgaard, 1970; compare Allfrey et al., 1968). Thus it would appear that there is no general rule for the association of histone acetylation and the induction of RNA synthesis. Such correspondence is not seen in some systems and is doubted in others.

Phosphorylation : Phosphorylation of histones takes place mainly at serine and threonine residues (Kleinsmith et al., 1966; Benjamin and Goodman, 1969; Langan, 1969; Turkington and Riddle, 1969). A number of histone phosphokinases have been described (Jergil and Dixon, 1970).

Nuclear protein phosphorylation has been found to be dependent on hormone action (Langan, 1968; Turkington and Riddle, 1969) and to decline during cell maturation (Gershey and Kleinsmith, 1969). The phosphate content of histones, particularly F1, varies with the physiological state of the tissue or animal (Stevely and Stocken, 1968; Ord and Stocken, 1969; Fitzgerald et al., 1970) so that there is an increased

level in this fraction and in F3 during DNA synthesis (Ord and Stocken, 1968, 1969; Adams et al., 1970).

In trout testes, protamines are phosphorylated in the cytoplasm immediately after synthesis (Ingles and Dixon, 1967; Marushige et al., 1969) and prior to their replacing histones on DNA. Since protamines of native sperm are not phosphorylated (Ingles and Dixon, 1967), this is taken to indicate that phosphorylation may be associated with the transport of newly synthesised nuclear proteins into the nucleus. Phosphorylation of pre-existing histones, however, does occur in several systems at a time when RNA synthesis is increased (Turkington and Riddle, 1969; Langan, 1969; Ord and Stocken, 1969; Fitzgerald et al., 1970; Cross and Ord, 1970).

In studies with isolated phosphoproteins Teng et al. (1970) have demonstrated tissue-specific patterns of $^{32}P$-labelling and have obtained evidence for species-specific binding of these proteins to DNA, perhaps indicating that phosphorylation of chromatin proteins is involved in allowing the transcription of previously repressed genes. In this respect, Stevely and Stocken (1968) found that phosphorylated F1 histone was less effective as an inhibitor of DNA-dependent RNA synthesis in vitro than nonphosphorylated F1, and Sung and Dixon (1970) have shown that phosphorylation of a serine residue at the probable DNA-binding site of histone F2a1 could lead to detachment of the protein from DNA.

Methylation.

Histones are methylated, after synthesis, at lysine and arginine residues (Allfrey et al., 1964; Paik and Kim, 1968; Burdon and Garven, 1971). A number of methylating enzymes have been described (Comb et al., 1966; Paik and Kim, 1968; Burdon and Garven, 1971). The donor of the methyl group is S-adenosylmethionine which is derived in turn from methionine (Allfrey et al., 1964; Kim and Paik, 1965). Little is known about the biological role of this methylation except that in regenerating liver it occurs after the peak of DNA and histone synthesis (Tidwell et al., 1968).

It is interesting that these changes should take place at a time when chromatin is undergoing conformational changes leading to a compact structure and a curtailment of nucleic acid synthesis

3.2.3. Non-Histone Chromosomal Proteins.

The evidence supporting the role of acidic nuclear proteins in the regulation of gene activity has been reviewed by Stein and Baserga (1972).

Acidic nuclear proteins have been reported to be characteristic of the tissue of origin (Teng et al., 1970; MacGillivray et al., 1971), and an increase in synthesis of non-histone chromosomal proteins has been found in active tissues of chicken (Dingman and Sporn, 1964), in lactating rat mammary glands (Stellwagen and Cole, 1969), in the puffs of

polytene chromosomes (Beermann, 1963; Edstrom & Beermann, 1962), and in the loops of lampbrush chromosomes (Gall and Callan, 1962).

A possible role for acidic nuclear proteins in the control of gene expression is further suggested by various *in vitro* experiments in which these proteins were able to modify the DNA-histone interaction in such a way as to bring about a restoration of histone-inhibited DNA-dependent RNA synthesis (Wang, 1968, 1969a) and to activate repressed gene loci (Wang, 1969b; Kamiyama and Wang, 1971).

A number of findings point to the significance of acidic nuclear proteins in systems where the cells have been stimulated to synthesise DNA (see section 4). Teng and Hamilton (1969) have suggested that non-histone chromosomal proteins may be involved in the oestrogen stimulation of the uterus in the ovarectomised rat and evidence has been presented for an early increase in a specific acidic protein in response to oestradiol in the system (Barker, 1971; Mayol and Thayer, 1970). Stellwagen and Cole (1969) reported a relationship between the synthesis of acidic nuclear proteins in the mammary gland and the stimulation of DNA synthesis and Smith *et al.* (1970) suggest that these proteins are involved in stimulated cellular proliferation in the rat uterus by progesterone. Additional and more direct demonstrations that non-histone chromosomal proteins may be involved in models of stimulated DNA synthesis have come from the investigations of Stein and Baserga (1970b) and Rovera and Baserga (1971) with WI38 cells stimulated to grow and divide by

incubating in fresh medium. In continuously dividing cells there is a significant increase in the specific activity of acidic proteins during G1 (Stein and Borun, 1972), and experiments with inhibitors of DNA synthesis clearly established that, unlike histones whose synthesis is tightly coupled with DNA replication (see section 3.1.3.2), the synthesis of these acidic proteins continues independently of DNA synthesis (Stein et al., 1971; Stein and Borun, 1972). SDS-polyacrylamide gel electrophoretic profiles of acidic nuclear proteins synthesised during G1, S, G2 and mitosis suggest that there are stage-specific differences in the acidic proteins (Stein and Borun, 1972; Stein et al., 1971). In addition, a series of studies not only demonstrated that acidic nuclear proteins have a faster rate of turnover than histones but that the rates of turnover of these proteins vary in different stages of the cell cycle, the highest turnover being observed during mitosis and the lowest during S-phase (Stein et al., 1971; Stein and Borun, 1972).

It is apparent that evidence is accumulating which suggests that acidic nuclear proteins are involved in the control of gene expression and cell proliferation both in continuously dividing cells and in Go cells which are stimulated to proliferate by a variety of stimuli, but the details are not yet clear as to the specific mechanisms by which these proteins interact with the genome and initiate, modify, or augment the transcription of informational macromolecules. It

is known, of course, that a number of acidic proteins can interact specifically with DNA, the best examples being the bacterial and viral repressors studied by Ptashne (1967) and Gilbert and Mueller-Hill (1966). Evidence that eukaryotic non-histone proteins can bind specifically to DNA has been presented by Teng et al. (1970) and Kleinsmith et al. (1970). Given that specific binding to DNA sequences can occur, several modes of action of non-histone proteins can be suggested. On the one hand, there is quite good experimental evidence that some non-histone proteins can neutralise the totally repressive effect of histones in DNA transcription. It has been suggested by Johns (1969) that the inhibition by histones of transcription is due to supercoiling of the DNA (see section 3.1.2) which provides a steric obstruction to movement of RNA polymerase along the DNA strand. It is not difficult to imagine how the binding of a non-histone protein in the groove of DNA not occupied by histones might alter the physical environment in such a way that the molecule could unwind.

On the other hand, non-histone proteins could act in the opposite way, as repressors, by binding rather more tightly to DNA, in the same way as bacterial repressors do; there is indeed evidence that some non-histone proteins may behave in this way. The main physicochemical difference between "derepression" and "repression" might lie merely in the binding energy, and this could conceivably lend itself to allosteric control.

Models of regulation based on non-histone proteins would therefore by somewhat analogous to models of regulation in bacteria in which proteins act as intermediates between regulator genes and structural genes. The main difference in this speculation is that some of the non-histone proteins are seen to alter the configuration of nucleohistone in such a way that it becomes possible for an RNA polymerase to traverse it and transcribe RNA from the DNA.

## 3.3. Chromosomal Replication.

### 3.3.1. General Introduction.

#### Semiconservative Replication and Rates of Growth.

That DNA replication is semiconservative was suggested by the Watson-Crick model and demonstrated in E. coli by the classic work of Meselson and Stahl in 1958. This has since been confirmed in several prokaryotic and eukaryotic systems. The intracellular rate of elongation of nascent DNA chains has been estimated at 30 um/min. for E. coli (Ioannou, 1973) and 1-2 um/min. for BHK cellular DNA (Hayton et al., 1973; Painter and Schaeffer, 1969). In calculating these rates, however, account has to be taken of the bidirectionality of replication which has been shown to occur with many different DNAs.

#### Bidirectional Growth of Nascent DNA Chains.

The original Cairns model of DNA replication in E. coli was put forward in 1963 based on autoradiographs showing two

replicating forks in the DNA (Cairns, 1963). This model assumes that duplication always starts at the same point and advances in the same direction with the aid of a swivel mechanism at the starting or finishing point so that free rotation of the unduplicated part of the DNA may occur. An alternative explanation of the Cairns autoradiograph is of two forks moving in opposite directions from a common origin (Masters and Broda, 1971). Such bidirectional synthesis of DNA has now been demonstrated for many different DNAs including phage (Schnös and Inman, 1970; Tomizawa and Ogawa, 1968; Inman and Schnös, 1971), E. coli (Masters and Broda, 1971), phage T4 (Delius et al., 1971), phage T7 (Wolfson et al., 1972; Wolfson and Dressler, 1972), chinese hamster cells (Huberman and Riggs, 1968), mitochondrial DNA (Kirshner et al., 1968), polyoma virus (Bourgaux and Bourgaux-Ramoisy, 1972, 1971), SV40 virus (Jaenisch et al., 1971) and for mycoplasma hominis H39 (Bode and Morowitz, 1967). Under certain conditions, however, replication may be unidirectional (Lark et al., 1971).

Multiple Initiation Sites in Eukaryotic DNA.

Jacob et al., in 1963 presented their Replicon Model for the regulation of DNA replication in bacteria and in which they defined a replicon to mean a genetic element such as an episome or a chromosome or a section thereof which can only replicate as a whole. They also suggested that the properties of such replicons require that they be capable of controlling their own replication independently of any other controls. In bacterial

cells the whole chromosome constitutes a single replicon. In contrast, however, multiple replication units 10 to 100 um in length are a feature of DNA replication in higher organisms (Cairns, 1966; Painter et al., 1966; Huberman and Riggs, 1968). In addition, the various replication units seem to be synthesised at different times during the S-phase. Remington and Klevecz (1973) have shown that the newly replicated DNA in Don C and diploid Don cell lines can be divided into two and three large temporally distinct fractions respectively. They suggest that there is an increase in the number of temporally distinct fractions of DNA as eukaryotic cells develop from the embryonic stage and age. Mueller and Kajiwara (1966) have shown by exposure of HeLa cells to 5-bromodeoxyuridine that DNA synthesised early in one S-phase in synchronised cultures was again replicated early in a second S-phase, so maintaining its temporal position of synthesis in the S-phase. The DNA synthesised early in the S-phase has a higher GC content than that synthesised late in the S-phase in both HeLa cells and mouse L-cells (Tobia et al., 1970). The significance of these findings is unclear.

### 3.3.2. Membrance Associated DNA Synthesis?

Attachment of chromatin to the nuclear membrane was inferred from studies as early as 1948 (Vanderlyn, 1948), and has been the subject of electronmicroscopic and autoradiographic studies in many eukaryotic cells. A close association of

bacterial DNA with the cell membrane has also been observed and DNA-membrane complexes have been isolated from both bacterial and eukaryotic systems that have allowed biochemical analyses (for literature see Infante et al., 1973).

The involvement of a membrane site in DNA replication was first suggested by Jacob et al. (1963) in their replicon model for the replication of bacterial DNA.

There are several possible relationships between the nuclear membrane of eukaryotic cells and DNA replication, as suggested by Comings and Okada (1973):

(1) DNA synthesis could be initiated at the nuclear membrane;

(2) the replication fork may remain at the nuclear membrane both during initiation and subsequent chain growth;

(3) initiation or continued replication may be membrane-associated only in late replicating heterochromatin;

(4) there may be no association between the nuclear membrane and DNA replication.

Comings and Kakefuda (1968) reported that DNA synthesis was initiated at the nuclear membrane but that the replication point soon left the site of membrane attachment and travelled along the DNA to ther parts of the nucleus. Friedman and Mueller (1969), however, suggested that it was the site of replication rather than the site of initiation that was attached to lipopolysaccharide material in the cell.

Most of these experiments, however, involve isolation of nascent DNA attached to a DNA-protein-lipid complex and such

biochemical studies do not distinguish between a real attachment and a non-specific association of the replication complex with membranes. The results of Comings and Kakefuda (1968), which were based on electron microscopy autoradiography, have been criticised as the cells used appeared to have suffered considerable damage during the process used to synchronise the cell population (Williams & Ockey, 1970; Ockey, 1972).

Results obtained using electron microscopy autoradiography have shown synthesis throughout the entire nucleus but with a peripheral pattern of grains found more frequently at the end of S-phase than at the beginning, suggesting an association of late replicating heterochromatin with the nuclear membrane (Blondel, 1968; Williams and Ockey, 1970; Erlandson and de Harven, 1971).

Several groups of workers, however, have recently shown that nascent DNA is not associated with the nuclear membrane at any stage of the S-phase and that synthesis occurs throughout the entire nucleus (Comings and Okada, 1973; Huberman et al., 1973; Ockey, 1972; Fakan et al., 1972). Comings and Okada (1973) suggest that the peripheral pattern of grains observed in late S-phase is due to synthesis of heterochromatin which is situated mainly at the nuclear membrane and not due to attachment of replicating regions to the membrane. The many results both with prokaryotic and eukaryotic cells, showing an attachment of nascent DNA to isolated membrane-like material,

probably arise as a result of non-specific attachment of the replicating regions to this material during isolation, probably as a result of some peculiar structural arrangement of this region such as single-strandedness of the DNA. Electron microscope autoradiography is the only technique which can give satisfactory answers concerning the attachment of nascent DNA to membranes and since bacteria are too small to allow the use of this technique, such attachment in prokaryotics must remain in doubt.

### 3.3.3. Involvement of RNA in DNA Replication?

In contrast to the DNA-directed RNA polymerases no known DNA polymerase is capable of initiating DNA chains in the absence of a primer 3'-OH group. It has recently been suggested that RNA may supply this primer requirement (Brutlag et al., 1971). It has been shown that rifampicin can cause inhibition of initiation of DNA replication (Bazzicalupco and Tocchini-Valenti, 1972; Staudenbauer and Hofschneider, 1972; Lark, 1972), although there are exceptions (Lark, 1972). Insensitivity of DNA replication to rifampicin, however, does not necessarily mean that an RNA primer is not required for initiation of replication (Schekman et al., 1972).

Conversion of the viral single strand of M13 DNA to its double stranded replicative form requires the synthesis of an RNA primer to initiate DNA synthesis and this RNA synthesis is sensitive to rifampicin (Brutlag et al., 1971;

Wickner *et al.*, 1972). Replication of φX174, however, a virus of base composition similar to that of M13, is not inhibited by rifampicin *in vivo* (Silverstin and Billen, 1971) or *in vitro* (Wickner *et al.*, 1972) although initiation of a DNA strand does require RNA synthesis (Schekman *et al.*, 1972). These latter authors suggest that the absence of rifampicin inhibition is a result of an RNA synthetic system that behaves differently from known RNA polymerases.

The involvement of an RNA primer in DNA replication has been suggested in many cases, including replication of $F^+$, φX174, M13, *E. coli*, chicken embryo cells, fd phage, λ phage (for literature, see Ioannou, 1973), polyoma virus (Magnusson *et al.*, 1973) and of *Physarum polycephalum* (Waqar and Huberman, 1973). Caution should be exercised, however, in the interpretation of some of these results in the light of the demonstration by Umeda *et al.* (1973) that incomplete denaturation of DNA before density gradient analyses, rather than covalent linkage of an RNA primer to DNA, may explain some of the positive results that have been reported.

### 3.3.4. Single-Stranded Regions in the Replicating DNA Duplex.

Studies on DNA replication in *B. subtilis* and phage T4 have revealed the presence of intermediates that display properties consistent with their being wholly or partly single-stranded (Oishi, 1968a, b, c) and similar observations have been made in mammalian systems (Painter and Schaeffer, 1969;

(Hayton et al., 1973). Several models of DNA replication (see section 3.3.7) predict the existence of single-stranded regions in the parental DNA molecule but whether the single-stranded regions identified in B. subtilis and in mammalian cells correspond to those predicted in the models is not clear.

### 3.3.5. Factors that Facilitate Denaturation and Renaturation of DNA Duplexes.

The protein specified by gene 32 of bacteriophate T4 is required in stoichrometric amounts for both replication and recombination (Snustad, 1968). There are about 10,000 molecules per cell and the purified protein consists of a single polypeptide chain with a molecular weight of 35,000 daltons (Alberts and Frey, 1970) although multiple forms are known to occur (Carroll et al., 1972). The protein binds preferentially to single-stranded DNA, the binding being highly cooperative (Alberts and Frey, 1970). Binding to single-stranded DNA under saturating conditions causes the DNA to adopt a highly extended conformation and under non-saturating conditions the protein preferentially binds to AT-rich double-helical regions of phage lambda DNA (Delius and Mantell, 1972).

The strong preferential binding of this protein to single-stranded DNA results in a lowering of the denaturation temperature of double-stranded DNA by about $40^{o}$. The gene

32-protein also promotes renaturation, the rate with 32-protein bound to T4 DNA at 25° being $10^3$ times the rate without bound protein (Alberts and Frey, 1970).

The function of gene 32-protein in DNA replication is unclear but it is possible that this protein participates by promoting unwinding at the replication fork (Alberts and Frey, 1970) and Carroll et al. (1972) have proposed a model of DNA replication incorporating this idea.

The gene 5 protein of phage fd (Oey and Knippers, 1972) and a DNA binding protein isolated from T7-infected cells (Reuben and Gefter, 1973) and from uninfected E. coli (Sigal et al., 1972) have similar properties to that of the T4 gene 32 protein and have been allotted a similar function to that of the latter protein. Herrick and Alberts (cited in Sigal et al., 1972) have isolated from calf thymus a protein which "unwinds" DNA in vitro, but the function of these proteins has still to be conclusively determined.

### 3.3.6. Role of Protein Synthesis in DNA Replication.

Experiments with various organisms have revealed a close dependence of DNA synthesis on synthesis of protein. In prokaryotes it is well documented that protein synthesis is required only for the initiation of DNA synthesis (Lark, 1969) whereas in eukaryotes protein synthesis is required for both the initiation and propagation of DNA synthesis (Cummins and Rusch, 1966; Kim et al., 1968; Littlefield and Jacobs,

1965; Wanka and Moors, 1970).

Cycloheximide and puromycin have been extensively used to study the effects of inhibition of protein synthesis in DNA replication. These antimetabolites inhibit initiation of DNA synthesis in eukaryotes. (Fujiwara, 1972; Hereford and Hartwell, 1973; Highfield and Dewey, 1972), but there is some confusion as to the effects on propagation of chains already initiated (Weintraub and Holtzer, 1972; Hand and Tamm, 1973; Fujiwara, 1972; Hereford and Hartwell, 1973). The conflicting observations may be a result of the use of differing systems by different groups.

The inhibition is not a result of inhibition of uptake of thymidine by the cells nor do these compounds have any effect on repair synthesis (Weintraub and Holtzer, 1972).

Fujiwara (1972) has suggested that the inhibition of initiation of DNA synthesis by cycloheximide may occur at specific sites on the nuclear membrane and a protein(s) synthesised in the middle of the G1 phase of the cell cycle has been shown to be involved in the initiation of DNA replication. It is probable that inhibition of synthesis of this "initiator" protein, or a similar protein, could be responsible for the effect on initiation of DNA synthesis produced by cycloheximide.

### 3.3.7. Models of DNA Replication.

The replication fork in a replicating portion of a

double-stranded DNA molecule is an asymmetrical structure resulting from the different polarity of the two DNA strands. If both strands are polymerised in the direction of fork movement a discontinuous synthesis of at least one strand must be predicted. Otherwise, the postulate of continuous growth of both strands leads to the prediction of a 3' to 5' polymerising activity, which has not been detected in any system despite extensive searching.

Evidence for a discontinuous mechanism was suggested initially by the discoveries that DNA label appears first in short pieces and only later in neo-chromosomal lengths (Okazaki et al., 1968). Further evidence for a discontinuous mechanism for DNA synthesis in animal cells has come from many sources (for review see Goulian, 1970).

Okazaki et al. (1968) presented evidence for a discontinuous model of DNA replication in which short stretches of DNA are synthesised, in either one or both strands, by a 5' to 3' reaction at the replicating point and are subsequently connected to the growing strands (Fig. 1.2a). The fragments observed are about $10^6$ daltons and are synthesised in the 5' to 3' direction and are joined together by polynucleotide ligase (Gellert and Bullock, 1970).

Louarn and Bird (1974), using E. coli in which the genome from phage λ had been incorporated into the host genome, have recently provided evidence that chain growth in E. coli is continuous in the 5' to 3' direction and discontinuous in the

3' to 5' direction. In mutants lacking DNA polymerase I, replication is discontinuous in both directions but the discontinuity in the 5' - 3' strand is probably due to nicks in the DNA being sealed more slowly than in wild type cells.

These authors suggest that the findings of Sugimoto et al., (1969) that DNA replication in E. coli is totally discontinuous is a result of the difficulty of detecting low levels of radioactivity in longer fragments of DNA where the isotope is spread over many fractions. The findings that "Okazaki" fragments hybridise to both strands of the parental duplex probably is due to the bidirectionality of the replication of DNA (see section 3.3.1.). With phage P2 where replication is unidirectional (Schnös and Inman, 1970), the newly synthesised DNA fragments hybridise to only one of the separated strands of the P2 DNA (Okazaki et al., 1974).

"Knife and Fork" Model of DNA Replication.

Guild (1968) suggested a modification of Okazaki's model which suggests that the growing 3'-OH end "turns the corner" and anneals with the complementary parental strand which is then copied in a 5' to 3' direction. Cleavage at the fork by an endonuclease would again leave the original daughter strand with a free 3'-OH to allow synthesis to continue. The short pieces would be joined together by a ligase as is suggested in the discontinuous model. There is no real support, however, for this model which is illustrated in Fig. 1.2b.

"Rolling-Circle" Model of DNA Replication.

A third model of DNA replication was proposed by Gilbert and Dressler (1968) and by Eisen et al. (1968) and is illustrated in Fig. 1.2c. One strand of the progeny DNA is attached covalently to one of the parental strands and is synthesised using the other (circular) parental strand as a template; the other progeny strand is synthesised by an unspecified mechanism.

The synthesis begins by opening one strand of the original circle at a specific point, the newly exposed 5' end is attached to a cellular component (perhaps membranous) and a new copy of this strand synthesised by chain elongation of the 3' end of the old positive strand, using the negative strand which remains closed, as a template. The old positive strand is peeled off as a single strand, but a new negative strand is synthesised upon it in short pieces that are ultimately tied together by a ligase. The synthesis is continuous, one daughter molecule being peeled off endlessly as the growing point continues around the circle. The long strand, which could in principle contain several genome's worth of information, can be used to construct daughter circular molecules by any recombination process between like sequences a genome's length apart.

The prediction by this model of longer-than-unit length strands has been fulfilled in early $\phi$X174 infection during the active replication of duplex rings (Dressler and

Denhardt, 1968; Knippers et al., 1969a; Dressler and Wolfson, 1970). Support has also been provided by the electron micrographic demonstration that intermediates in the replication of ϕX174 single-stranded circles include double-stranded circles with a single-stranded "tail" (Knippers et al., 1969b; Dressler, 1970). Studies on the replication of small DNA viruses at present provide the best support for a rolling circle mechanism and replicating strands which are longer than mature viral DNA have been described for phages T4, λ , P22, T7 and T5 (cited in Goulian, 1970).

"Pre-fork replication" Model of DNA Replication.

As an alternative to multiple initiations on one or both arms of the replicative fork, a pre-fork mechanism for replication, proposed by Haskell and Davern (1969), assumes multiple replication sites at staggered nicks proximal to the replication fork (Fig. 1.2d). These would be separated from the parental primer at their origin by another scission, and exonucleolytic widening of the latter would permit the parental strand to re-establish continuity by reannealing at the gap, followed by closure by ligase. The fragments would ultimately join up to establish continuity of the product strand. Aside from circumventing the problem of initiation of small fragments, this model could account for single-stranded gaps, and also single-stranded nascent fragments if the latter are displaced from their templates by reconstitution

## Fig. 1.2.

### Models of DNA Synthesis.

A. Discontinuous synthesis of 'Okazaki' fragments;

B. Knife and Fork Model;

C. Rolling Circle Model;

D. Pre-fork Replication Model.

o = Primer site;

⟶ = direction of chain growth;

∩ = site of joining;

⟿ = site of endonucleotic cut;

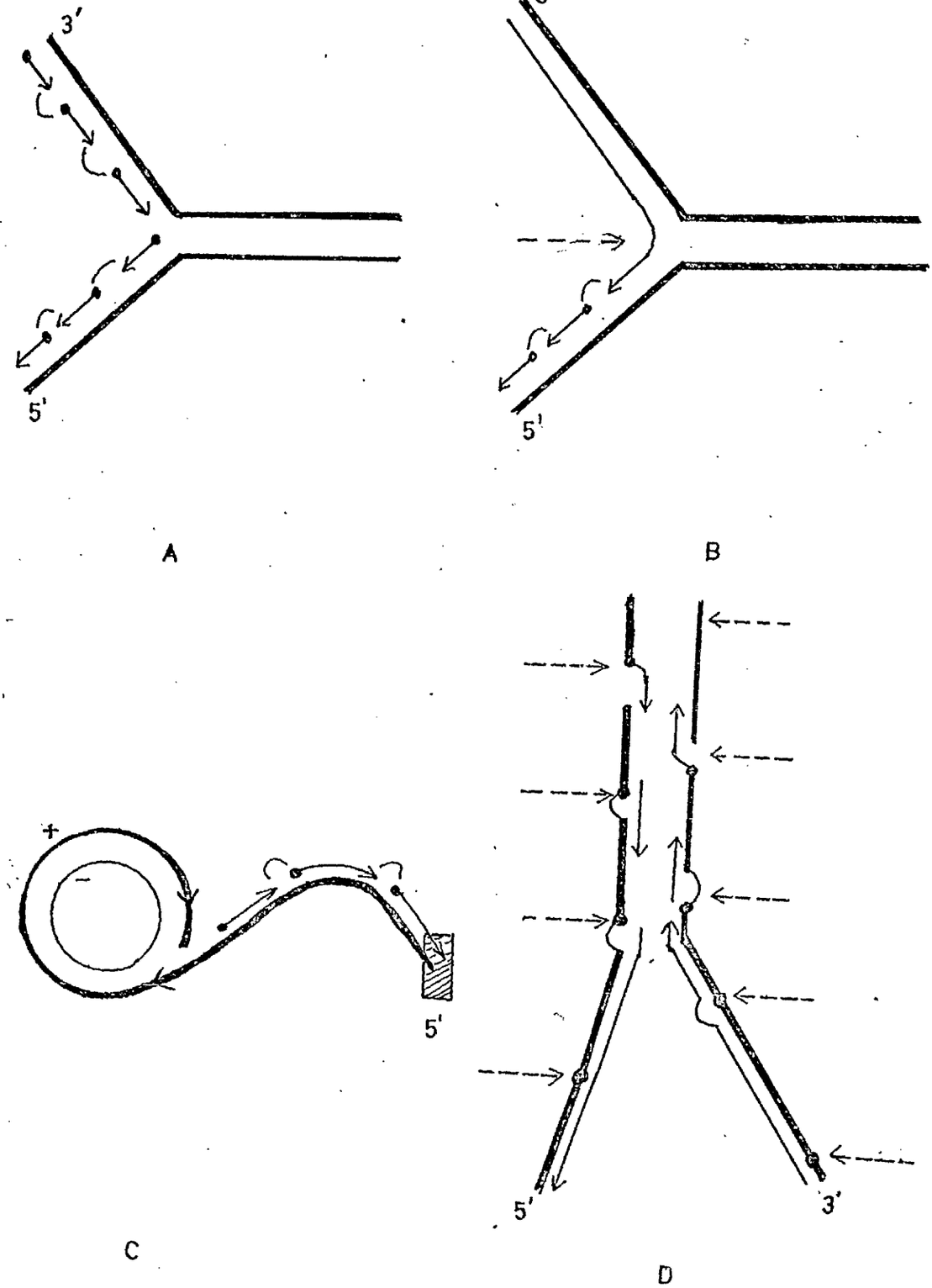
heavy lines = parental material;

light lines = new material.

Figs. 1.2A, B and D from Klein and Bonhoeffer (1972);

Fig. 1.2C from Gilbert and Dressler (1968).

Fig. 1.2.



of the parental duplex.

Summary.

Despite the plethora of information and knowledge on many aspects of DNA synthesis no one, as yet, has managed to combine these facts into a satisfactory explanation of the events occurring during DNA replication, and the overall picture, rather than slowly becoming clear, if anything is even more clouded than it was five years ago. This apparent impasse surely must reflect the true complexity of the events involved in DNA replication and the picture we have of these events probably will be clouded much more before it begins to clear and the facts begin to coalesce into a uniform hypothesis.

## 3.3.8. Special Features of Mammalian DNA Replication.

A sizeable difference in complexity exists between the more complex and diverse classical chromosomes of higher animals and plants and the DNA molecule that must be the ultimate common denominator of all primary genetic systems. It is not only a difference in dimensions, but also one involving chromosome-associated RNA and protein, together with considerable morphological variety and structural complexity.

Unlike prokaryotic systems where there is the comparatively simple problem of replicating a single naked

length of DNA, replication of the eukaryotic chromosome is complicated by the need for duplication of chromosomal proteins and RNA and by the presence of the tight nucleoprotein complex. In addition there is the fact that individual DNA molecules may be 1000 times larger than that in the prokaryote and that the organisation of the genome into a number of chromosomes introduces further complexity. (HeLa cell nuclei containing 187 chromosomes have been demonstrated.)

A clue to the mechanism of replication of these protein-coated chromosomes has been suggested in a study of Euplotes in which Roth (1957) and Prescott (1962) have shown that RNA and protein are partially removed from the area of chromatin immediately ahead of the replication point and that behind this point the DNA content has doubled (Gall, 1959). A mechanism for removal of protein and RNA from the DNA strand prior to replication would perhaps decrease the complexity of the replication process to that in prokaryotes, although there are still the problems of control of removal of chromosomal components and what determines the specificity of reassociation after the DNA has replicated.

Whatever the controlling processes may be, they are extremely efficient, as the temporal relationships of replication of different replicons are maintained from one cell to the next (see section 3.3.1) and areas of heterochromatin remain condensed and genetically inactive throughout all growth and division. The dense heterochromatin becomes diffuse only

during its replication, condensing once again after this process is completed. May and Revel (1963) have shown that $^{3}$H-thymidine is incorporated only into diffuse chromatin which implies that some strict control must be exercised over heterochromatin to cause it to become diffuse at the correct point in the cell cycle for replication and to condense once more afterwards.

Very little is known about the detailed structure of components inside the nucleus, their relationship to each other and of the controlling processes operating on them.

3.3.9. DNA Replication and Cell Division.

For many years it was commonly accepted that the decision to duplicate DNA was intimately connected with cell division. Although these two events are closely associated in normal cell cycles, there is not a universal causal connection between them because there are situations in which one or other of these processes is suppressed.

There are several examples of nonproliferating tissues in which a considerable proportion of the cells have stopped growing in the G2 phase and when provided with an appropriate stimulus will enter mitosis without first synthesising DNA (for review see Tobey et al., 1971). This phenomenon can be observed both in non-proliferating cell populations in the whole animal and in tissue culture populations and in both 'normal' and tumour cell populations.

Cases of the reverse situation - division without DNA synthesis - are much rarer, as might be expected since the biological penalty for this would normally be severe. They do, however, occur in special cases. Polyploid cells in tumours of bean stems can be reduced to the diploid state by successive cell divisions without any DNA synthesis (Rasch et al., 1959) and haploid Amphibian sperm injected into a mitotic egg can be induced to enter mitosis without any prior DNA synthesis (Graham, 1966).

Thus, although DNA synthesis and cell division are normally closely associated, this relationship is not universal. Comparison of the biochemical processes occurring in G2-arrested cells and in 'normal' 'DNA-division' cells may lead to an understanding of the biochemical control processes involved in the regulation of cell growth and division.

### 3.3.10. Use of Isolated Nuclei for the Study of Chromosome Replication.

The application of permeabilised cells and membrane fractions which retain some DNA-synthesising capacity in vitro has stimulated considerably the study of the mechanisms of DNA replication in prokaryotic systems. Similar approaches to the replication of DNA in higher organisms are complicated by nucleocytoplasmic relationships. Since structural inter-relationships between protein complexes, template sites, and substrates may be of critical importance in the synthesis of

DNA, examination of whole isolated nuclei would appear to offer advantages lacking in more refined systems and offer a means of assaying the *in situ* cellular machinery for synthesising DNA.

A number of investigators have examined the incorporation of deoxyribonucleotides into isolated cell nuclei (Friedman and Mueller, 1968; Lynch *et al.*, 1970, 1972; Kaufman *et al.*, 1972; Probst *et al.*, 1972) and in HeLa cell nuclei the synthesis of DNA *in vitro* is replicative rather than repair (Bernard and Erent, 1973). Lynch *et al.* (1970) have shown by autoradiography that only nuclei that were replicating their DNA in the whole animal were capable of incorporating $^{3}$H-thymidine triphosphate *in vitro*, while Lynch *et al.*, (1972) claim that the formation of DNA *in vitro* is by the elongation of DNA chains that were already growing in the whole animal. Kidwell and Mueller (1969), however, using a different cell for the isolation of their nuclei, claim that both elongation of existing nascent DNA and initiation of new DNA chains can occur in the isolated nuclei, although the predominant process is elongation of nascent chains. It remains to be resolved whether initiation of new DNA replication can occur in isolated nuclei.

Systems using isolated nuclei have also been developed for the study of events occurring in the nuclei of cells infected with such viruses as Herpes Simplex (Fine and Ludwig, 1972a, b; Radask, 1973), polyoma (Winnacker *et al.*, 1972;

Magnusson et al., 1972, 1973) and SV40 (Qasba, 1974).

Addition to S-phase nuclei of factors present in S-phase cytoplasm bring about a stimulation of incorporation of precursors into DNA (Kidwell & Mueller, 1969; Kumar and Friedman, 1972; Bernard and Brent, 1973), an effect which is not seen when cytoplasm from G1 or G2 cells is used. Bernard and Brent (1973), however, suggest that this cytosol factor is a DNA polymerase-like protein which is unable to induce incorporation of precursors into DNA in nuclei from G1 cells, suggesting that further control processes are involved inside the nuclei.

The use of isolated nuclei lends itself to an investigation of the role of partially purified cellular components in the processes of DNA replication and should prove a powerful tool in the elucidation of these processes.

## Plate 1.

Electron Micrograph of Pseudorabies Virus.

Magnification x 300,000.

Preparation negatively stained with Phosphotungstic Acid.

Plate 1.

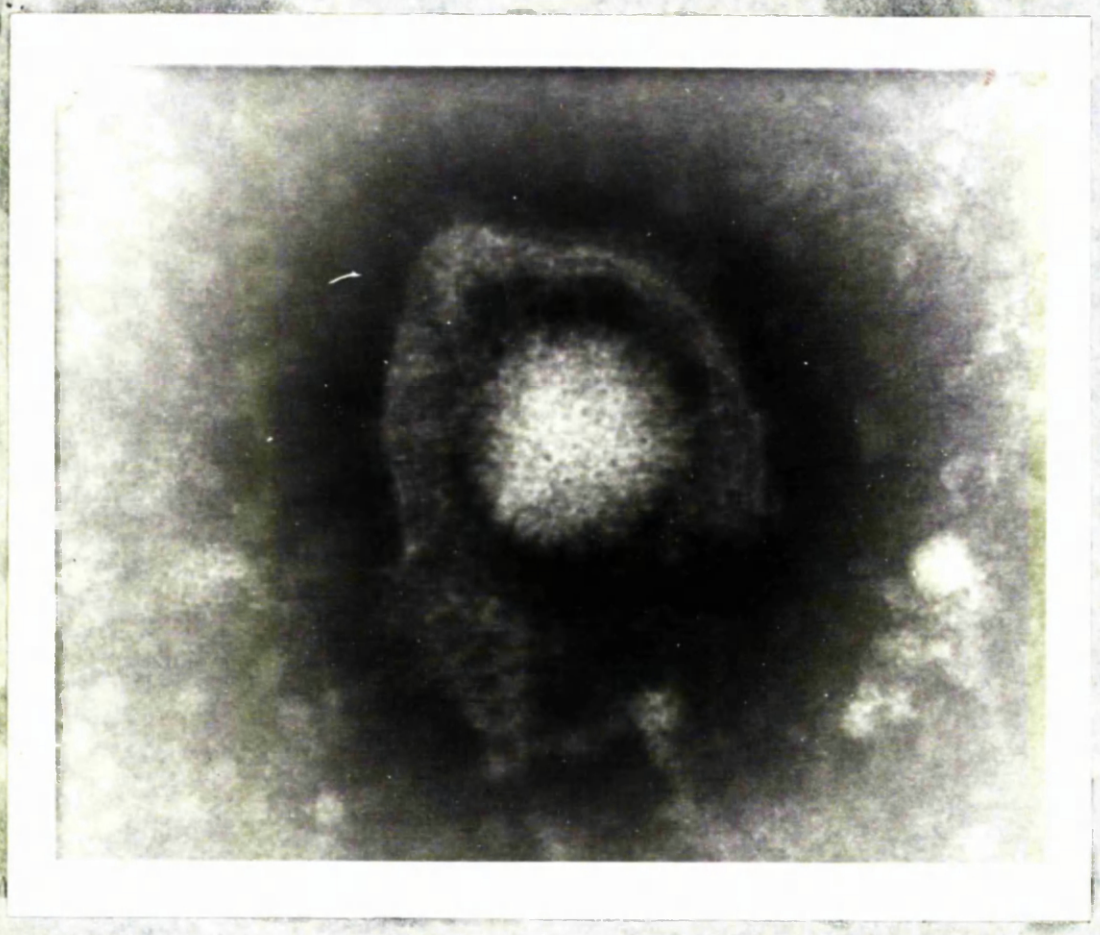


Plate 2.

Autoradiographs of BHK cells at various times following incubation in low serum medium.

A. Exponentially growing cells.

B. 24 h after changing medium to $EC_1$.

C. 48 h after changing medium to $EC_1$.

D. 6 days after changing medium to $EC_1$.

Plate 2.

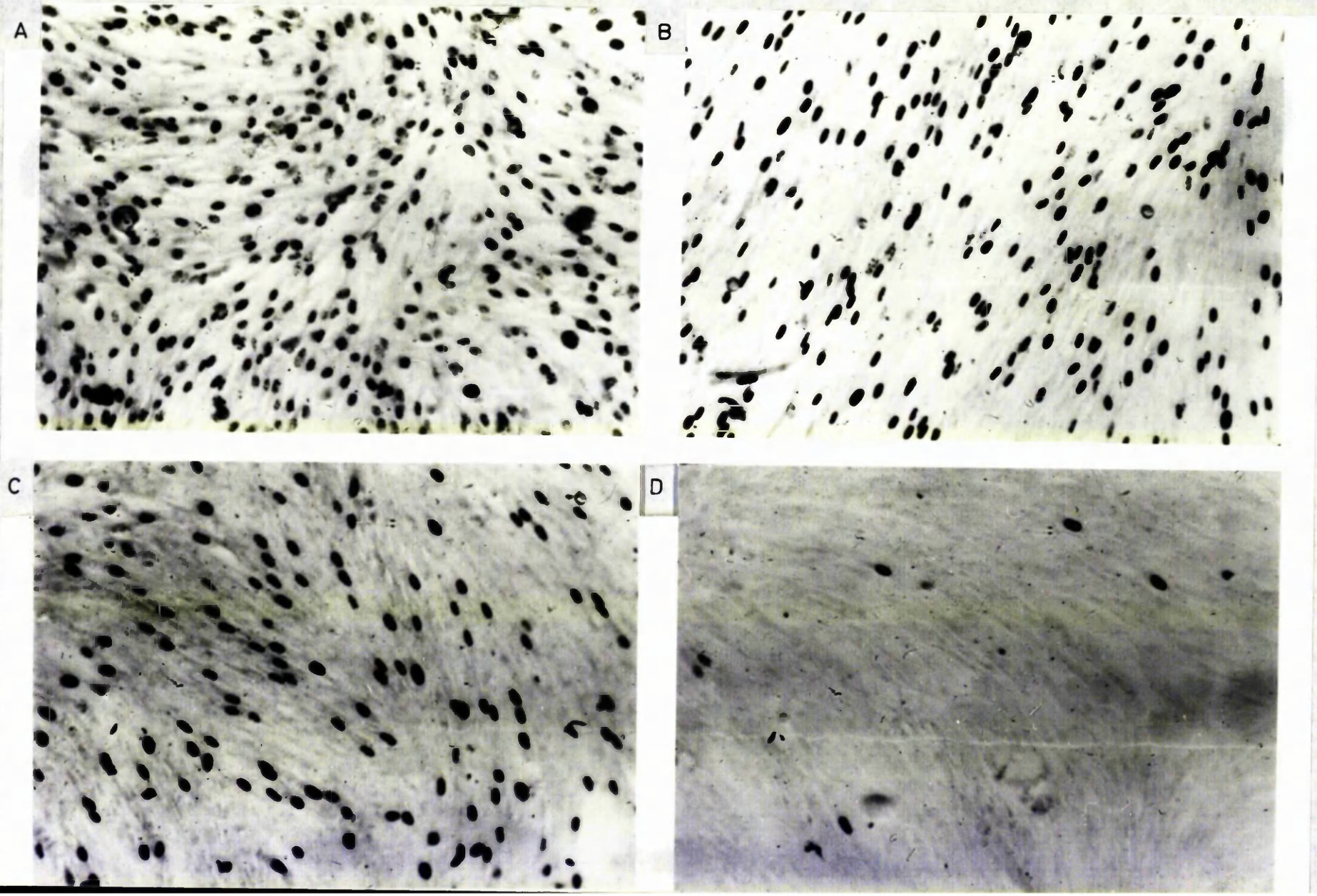


Plate 3.

Autoradiograph of 'Resting' BHK cells infected with Mycoplasma.

Plate 3.

## 4. Model Cell Systems for the Study of Control of Proliferative Growth.

### 4.1. Introduction.

There are several populations of cells which display, in ordinary conditions, a very low level of proliferation, but that can become actively proliferating when exposed to an appropriate stimulus. In these populations of cells, during the quiescent state, DNA synthetic activity is very low and mitoses exceedingly rare. When stimulated, these cells first enter a phase of DNA synthesis followed by a wave of mitosis. The attraction of these systems for the investigator lies in the fact that one can follow the steps, structural or functional, leading from a quiescent state to DNA synthesis and cell division.

These models of stimulated DNA synthesis can be divided into two groups: whole animal studies and studies in tissue culture as tabulated in Table 1.1. Not all of these systems are equally amenable to study. In general, the stimulus of cells in tissue culture can be characterised with greater accuracy, while among whole animal models the best ones are those depending upon a single application of stimulus. Some models have a limited usefulness because of the small size of the response, which reduces the likelihood of identifying the key metabolic events leading to DNA synthesis and cell division.

### 4.2. Pre-replicative phase of stimulated growth.

With one or two exceptions, all those models mentioned

Table 1.1.

Models of Stimulated DNA Synthesis.

| System | Stimulus | |
|---|---|---|
| 1) Tissue culture systems. | | |
| lymphocytes | phytohaemagglutin | For references see Stein and Baserga (1972) |
| density-inhibited cells | change of medium | |
| stationary cell cultures | oncogenic DNA viruses | |
| organ cultures of mammary gland | epidermal growth factor | |
| 2) Whole animal systems. | | |
| liver | partial hepatectomy | Hamilton (1968) |
| uterus | oestrogen | |
| salivary gland | isoproterenol | |
| kidney | folic acid | |

From Stein and Baserga (1972).

above have in common a pre-replicative period, that is an interval of time varying from a minimum of 12 - 15 h to a few days, between the application of the stimulus and the onset of DNA synthesis. Changes in RNA and protein synthesis seem to be the most prominent events in the first few hours of the pre-replicative period, but other changes have been described that may be relevant to the subsequent initiation of DNA synthesis. These have been discussed by Baserga (1968).

## 4.3. Proportion of Cells Stimulated to Synthesise DNA.

It may be useful to comment briefly on the proportion of cells that are stimulated to enter a phase of DNA synthesis in the various models. This is an important point, because it is more likely that one can detect biochemical changes in a population of cells in which a large fraction of the population is stimulated than when a small fraction is involved.

In regenerating liver about 55-60% of the cells are stimulated to enter DNA synthesis in the first 24 h (Bucher and Swaffield, 1964). However, over a span of 3 days after partial hepatectomy, as many as 93% of hepatocytes may enter DNA synthesis (Stöcker, 1966). About 60-80% of the acinar cells in the parotid are stimulated by isoproterenol to synthesise DNA and to divide (Baserga, 1971) and at the peak of synthesis in lymphocytes stimulated with phytohaemagglutinin about 50% of the large mononuclear cells are labelled by tritiated-thymidine

(Cooper et al., 1963). In contact inhibited cells only 6% of the cells are stimulated by dialysed serum (Todaro et al., 1965), although in stationary cultures of BHK cells stimulated by the addition of serum, 80% of the cells incorporated tritiated-thymidine in the first 20 h after stimulation (Clarke et al., 1970). In our hands this same system shows up to 76% of cells incorporating tritiated-thymidine at peak synthesis, with the average being 65-70% (Howard et al., 1974). Thus from the viewpoint of response to the stimulus the serum-stimulated stationary BHK system compares favourably as a model system with the others mentioned.

## 4.4. Factors Present in Serum which Stimulate Growth in BHK Cells.

Cultures of BHK 21 cells continuously release into serum-free medium, active materials which stimulate the incorporation of thymidine in non-confluent, but not in confluent cultures of homotypic cells. The activity is non-dialysable and is retained by membranes with a nominal limitation of molecular weight of 25,000. However, the activity is not removed by centrifugation at 30,000g for four hours. The cells also release a heat labile active material capable of enhancing the effect of added serum. The action of this material is density dependent (Stoker et al., 1971). However, these authors do point out that, since the secreting cells were originally cultured in serum, and since serum also contains enhancing

factors, the activities observed might be the result of a continuous re-secretion of material removed preferentially from serum by the resting cells. It is worth noting however, that since the release of activity from these cells continues at a constant rate for at least six days, the BHK cells may themselves synthesise growth factors which will act like serum factors. Chicken fibroblasts have been observed to synthesise serum-like proteins (Halpern and Rubin, 1970) and since the factors responsible for stimulation of growth of hamster cells are present in hamser serum as well as calf serum it is conceivable that BHK21 cells may express the genetic information necessary for the synthesis of the growth factors in hamster serum. A brief summary of some serum factors which stimulate or inhibit cell growth in culture is given in Table 1.2.

## 4.5. Role of Serum in the Growth of Cultured Cells.

The study of the effects of constant renewal of serum-containing medium by either perfusion (Kruse and Miedena, 1965) or by frequent changes of medium (Temin, 1965) showed that cultures of contact-inhibited fibroblastic cells could grow to much higher population densities than usual if sufficient fresh medium with serum was added. Epithelial cells, however, appear to show less dependence on serum factors for replication, and their growth is limited more by the availability of surface to each cell. Transformed cells, which show very little density dependent inhibition of growth, are little affected by either

Table 1.2.

Factors that stimulate or inhibit growth of cultured cells.

| Source | Characteristics of Factor | References |
|---|---|---|
| Mouse or rat serum | 4 factors: a) promotes growth of 3T3 cells.<br>b) promotes growth of SV40 3T3 cells.<br>c) " " " "<br>d) necessary for cell survival of 3T3 & SV 3T3 cells.<br>Heat stable. | Paul et al., (1971) |
| Human urine | Promotes growth of 3T3 cells - similar to one of above 4 factors. | Holley & Kiernan (1971) |
| Mouse or rat serum | Promotes migration of 3T3 cells, stable to heat, 0.05% SDS, 8M urea; labile to pepsin. | Lipton et al., (1971) |
| Calf serum | Stimulates uptake of uridine and phosphate | Cunningham & Pardee (1969) |
| BHK 21 cells | Stimulates incorporation of thymidine into BHK 21 DNA | Stoker et al., (1971) |
| Calf thymocytes | Stimulates incorporation of thymidine into cell DNA | Sibatini (1973) |
| Calf serum | 3 or 4 factors necessary for growth of 3T3 & SV40 3T3 cells. | Kaplan and Bartholomew (1972) |
| L-cell conditioned medium | Stimulates uptake of thymidine into mouse marrow cells in culture. | Austin et al., (1972) |

Table 1.2 contd.

Factors that stimulate or inhibit growth of cultured cells. (contd.)

| Source | Characteristics of Factor | References |
|---|---|---|
| Calf serum | Prevents cells in G1 phase progressing to S-phase of growth. Heat stable. | Otsuka (1972) |
| Vero M5 cell extracts | Blocks transfer of amino acids from RNA to the growing peptide chain on cell ribosomes. | Englehardt (1971) |
| Serum from partially hepatectomised rats | Stimulates DNA synthesis in liver of normal mice. Heat stable. | Morley and Kingdon (1973) |
| Rat serum | Stimulates incorporation of $^3$H-dTTP into rat liver nuclei *in vitro* | Morioka *et al.*, (1973) |
| L cells | Stimulates DNA synthesis and mitosis in BHK cells | Shodell (1972) |

serum factors or the availability of surface area (Dulbecco and Elkington, 1973).

The experiments with fibroblastic cells, mentioned above, shifted attention to the role of the medium in controlling fibroblastic cell multiplication. Experiments were performed showing that addition of serum in fresh or depleted medium to cultures of stationary cells resulted in stimulation of cell multiplication (Todara et al., 1965; Temin, 1966; Yoshikura and Hirokawa, 1968). Other experiments showed that the final cell population density was directly proportional to the amount of serum in the medium (Temin, 1966, 1968; Todaro et al., 1967). These experiments led to the hypothesis that serum contains some specific factors which are used in cell multiplication, and that it is the availability of these or related factors which usually controls cell multiplication.

Most cells in culture grow attached to a solid substratum, either glass or suitably treated plastic and are unable to multiply in suspension. Attachment of cells can be defined experimentally by a determination of cells remaining on the substratum after inversion of a culture chamber filled with liquid, and provides a good assay for isolation of active components from serum involved in the attachment process. However, the molecular nature of the process and its relation to other aspects of cell growth is unclear at present.

It also appears that serum is needed to prevent cell

detachment in some cases. Temin (1968) noted that chicken fibroblasts detached within a day after removal of serum. With 3T3 cells, Dulbecco (1970a) found that the rate of cell detachment was inversely proportional to the concentration of serum, and directly proportional to temperature. Temin (1970) found that rat embryo fibroblasts did not detach more rapidly in the absence of serum. Interpretation of these studies of cell detachment is complicated by the death of cells in medium without serum which also leads to detachment (Temin, 1968; Dulbecco, 1970b).

4.6. Involvement of Serum in DNA Synthesis.

As discussed earlier, serum appears to contain some specific factors which are necessary for cell multiplication and whose availability controls cell multiplication. The rate of incorporation of thymidine was first used as a quantitative measure of the activity of different fractions of serum by Todaro et al. (1967). Dose-response curves of the amount of serum added against the rate of incorporation of $^{3}$H-thymidine or number of cells synthesising DNA were linear for a variety of normal and transformed fibroblasts from chicken, rat, hamster and mouse (Temin, 1968, 1969, 1970; Clarke et al., 1970; Dulbecco, 1970b ). However, this linearity does not appear to indicate that a single factor in serum is sufficient to stimulate stationary cells to enter into DNA synthesis. Recent work with chicken embryo fibroblasts has indicated that the stimulation of

DNA synthesis by serum is a complex reaction which requires the interaction of serum with cells over a period of a few hours, during which time it was suggested that one or more processes were continuously or successively stimulated by serum before a cell became committed to DNA synthesis (Temin, 1971). Therefore, the approximately linear dose-response curves do not correspond to a "one-hit" process. These curves, however, can still be used as a quantitative measure of the activity of serum or fractions from serum.

Serum need not be present throughout the whole of the pre-replicative period following the initial stimulus to quiescent cells. In the case of chicken fibroblasts about 2-4 hours before a cell started DNA synthesis it was irreversibly committed to start DNA synthesis, even if serum was then removed (Temin, 1971). A few hours of exposure of cells to serum before they started DNA synthesis was sufficient to stimulate cells to start and to complete an entire round of DNA synthesis. In the case of chicken fibroblasts, this time was not decreased by a ten-fold increase in the concentration of serum or by different means of preparing the stationary cells (Temin, 1971). The effects of exposure to serum for periods too short to cause cells to become committed to start DNA synthesis disappeared after overnight incubation in serum-free medium.

## 4.7. Conclusions.

The processes involved in serum stimulation of quiescent

cells are very complex and difficult to study. The diversity of cellular systems studied makes comparisons of different stimulatory factors from serum difficult in the extreme. Such systems, however, if one ignores the initial stimulus and concentrates on the later biochemical changes in the cell population, provide an extemely useful tool for the study of events occurring in cells progressing from a state of non-growth to one of active proliferation. It is prospect hope that such studies may yield valuable insights into events controlling the regular cell cycle which make these studies so attractive.

## 5. Animal Viruses.

### 5.1. RNA containing animal viruses.

#### 5.1.1. Classification.

Fenner (1968) and others have classified the RNA containing animal viruses into seven groups as depicted in Table 1.3. A description of the members of each group is given by Fenner (1968). There are, in addition, many other viruses which have not yet been classified and some of which are discussed by Fenner (1968) and Andrewes (1964).

#### 5.1.2. Morphology and Ultrastructure of the Virion.

Virions belonging to the seven groups catalogued in Table 1.3 all exhibit either icosahedral or tubular symmetry.

The probability that all the non-enveloped spherical

Table 1.3.

Classification of RNA containing Animal Viruses.

| Group | Configuration of RNA | M. wt. of RNA ($\times 10^6$) | Envelope |
|---|---|---|---|
| Picornavirus | SS | about 2 | - |
| Encephalovirus | SS | 2-3 | + |
| Myxovirus | SS | about 3 | + |
| Paramyxovirus | SS | 7.5 | + |
| Rhabdovirus | SS | 6 | + |
| Leukovirus | SS | 10-12 | + |
| Reovirus | DS | 10 | - |

SS = Single-stranded; some viruses contain more than one RNA species.

DS = Double-stranded;

\+ = Envelope present;

\- = No envelope seen.

From Fenner (1968).

animal viruses have icosahedral surface lattices was suggested on theoretical grounds by Caspar and Klug (1962). Capsids are built of repeating subunits called capsomeres and in spherical viruses these are packed to comply with cubic symmetry. The icosahedral form of cubic symmetry allows the use of the greatest possible number of identical asymmetrical subunits and so far all spherical viruses which have been carefully investigated by electron microscopy and x-ray diffraction appear to have icosahedral symmetry.

Although non-enveloped and relatively rigid tubular nucleocapsids are commonly found among the viruses of plants and occasionally in bacteriophages, all the viruses of vertebrates with tubular nucleocapsids are enveloped. Although a great deal is known of the structure of tobacco mosaic virus, very little is known about the structure of the animal viruses which exhibit helical symmetry. The structure of the virion of RNA containing animal viruses is discussed by Fenner (1968).

## 5.2. DNA-containing Animal Viruses.

### 5.2.1. Classification.

The DNA-containing animal viruses have been classified by Fenner (1968) into 5 groups as depicted in Table 1.4.

### 5.2.2. Morphology and Ultrastructure of the Virion.

#### 5.2.2.1. DNA and Information Content.

Herpesvirus DNA, with a molecular weight of $100 \times 10^6$ daltons,

Table 1.4.

Classification of DNA-containing Animal Viruses.

| Group | Configuration of DNA | M. Wt. ($\times 10^6$) | Envelope |
|---|---|---|---|
| Poxvirus | DS | 160-200 | - * |
| Herpesvirus | DS | 100 | + |
| Adenovirus | DS | 20-25 | - |
| Papovavirus | DS | 3.6 | - |
| Parvovirus | SS | 1.8 | - |

DS = Double-stranded;

SS = Single-stranded;

\+ = Envelope present;

\- = Envelope not seen.

From Fenner (1968)

* Poxviruses do contain lipid material but this material is found internally and not as a membrane surrounding the virion (Lenard and Compans, 1974).

carries information sufficient to specify the sequence of more than 100 x $10^3$ amino acids or 200 proteins of 50 x $10^3$ daltons. This amount of information seems very large by comparison with information content of the adenoviruses (specifying up to 50 proteins of 50 x $10^3$ daltons), or the papova viruses (only 4-8 proteins of 50 x $10^3$ daltons). The poxviruses which, unlike the other DNA-containing viruses, replicate in the cytoplasm, are the largest known animal viruses coding for up to 400 proteins of 50 x $10^3$ daltons. The reason for the large amount of information specified by the herpesviruses is unknown but it should be remembered that all of the genetic information carried by the virus may not be expressed in cell cultures or even during a simple lytic cycle in the whole animal. Unlike the small DNA and RNA viruses, with the possible exception of measles virus, herpesviruses have in the course of evolution established a unique relationship with the host they usually infect, the main feature of this relationship being that, following primary infection, and in spite of the appearance of antibody, herpesviruses can survive in some specific tissues for the lifespan of the host without any symptoms of virus infection being apparent for most of the time. This capacity to co-exist is not an indication that the virus is incapable of inflicting injury; herpesviruses frequently cause death or severe illness in species other than their natural host. It seems reasonable to postulate that much of the genetic potential of herpesviruses is required to be expressed

to enable the virus to co-exist with the host in the whole animal but that some of this potential remains suppressed in cell culture or in situations in the whole animal where the host cell is destroyed.

5.2.2.2. The Virion.

All of the known animal DNA-containing viruses, except those of the poxvirus group, have a spherical virion exhibiting icosahedral symmetry. The poxviruses have a "brick-shaped" virion structure exhibiting complex symmetry. The virions are composed of subunits, or capsomeres, ranging in number from 32 in the parvovirus group to 252 in the adenovirus group. Only the herpesviruses are enveloped.

## 5.3. Herpesviruses.

### 5.3.1. General.

The word 'herpes' is derived from Greek and means 'to creep'. It occurs in medical texts dating back at least twenty-five centuries. Herpes catarrhalis, progenitalis, facialis and simplex appear in the 18th and 19th centuries (Beswick, 1962). Gruter (1924) was the first to report the successful transmission of herpesvirus from man to rabbit and the discovery of what appeared to be a single causative agent led to the belief that herpes genitalis, facialis and febrilis are all different clinical manifestations of one disease - herpes

simplex. The virus took on the name of the disease.

Currently, more than fifty herpesviruses are known to infect over thirty different vertebrates, and herpes-like particles have even been observed recently in oysters and fungi (Farley et al., 1972; Kazama and Schornstein, 1972).

Herpesviruses are formally defined as large enveloped virions with an icosahedral capsid consisting of 162 capsomeres and arranged around a DNA core (Lwoff et al., 1962; Lwoff and Tournier, 1966). The DNA is coiled in the form of a doughnut with proteins arranged in the form of a barbell passing through the hole (Furlong, Swift and Roizman, 1972). The capsid containing the core is a uniform, relatively rigid structure, 100 nm in diameter and surrounded by the viral envelope which, like any membrane, is less rigid and consists of lipoproteins probably arranged in an orderly structure (see section 5.3.3.5). It is generally thought that the outer layer of the envelope is derived from the modified lamellae of the nuclear membrane (Morgan et al., 1954; Falke et al., 1959; Siegert and Falke, 1966; Nii et al., 1968a). A more detailed discussion of the structure of the herpesviruses is given in section 5.3.3.

5.3.2. Virus Growth Cycle.

Study of the growth of animal viruses has been greatly influenced by work with bacterial viruses, and many terms commonly used have been derived from bacterial virology, although some, like "burst-size", are inappropriate for most animal viruses.

Ellis and Delbrück (1939) developed the 'one-step growth' experiment as a method of tracing all the complex events that occur during virus replication in individual single cells by manipulating conditions in a population of infected cells so that results at the population level reflected what was happening in any independent virus-cell complex at a given time after infection.

5.3.2.1. Adsorption, Penetration and Incoating.

The mechanisms involved in the initial events in the infection of a cell by a herpesvirus are still unclear. Most of the conclusions are based on somewhat contradictory electron micrographic observations (Morgan et al., 1968; Dales and Silverberg, 1969). The first event, however, seems to be attachment and fusion of the viral envelope with the plasma membrane of the cell. This process is inhibited by strongly polyanionic substances, such as heparin and dextran sulphate (Nahmias et al., 1964), an observation which has led Nahmias and Roizman (1973) to suggest that these polyanionic substances may compete with the herpesviruses, which are strongly negatively charged (Polson and Russel, 1967), for attachment to specific receptors on the cell surface. Cells naturally lacking receptors for HSV have not been described. The rate of adsorption of herpesviruses to susceptible cells is not dependent on the temperature of incubation.

Penetration is dependent upon the temperature of incubation and requires energy expenditure by the cell (Roizman,

1969). Huang and Wagner (1964) found that, once virus had adsorbed to the cell, penetration is relatively rapid.

When the virus envelope has attached to the plasma membrane of the cell there are two ways in which it may enter the cytoplasm. The fused membrane may disaggregate so that the viral capsid enters the cytoplasm directly, as has been suggested by Morgan et al. (1968), or, alternatively, the virus particle with its envelope attached firmly to the cell membrane may be taken into the cell by viropexis (Dales, 1962). This is similar to pinocytosis and involves the formation by invagination of vesicles which enclose the virus and are released into the cytoplasm by pinching-off a piece of membrane from the cell surface. This latter mode of entry is utilised by adenoviruses (Dales, 1962) which, although they do not possess a viral membrane, are taken into cells in pinocytotic vesicles.

Morgan et al. (1959), Epstein et al., 1964 and Siegert and Falke (1966) reported seeing nucleocapsids from HSV free in the cytoplasm of infected cells and in vacuoles and concluded that the virus is taken into the cell by pinocytotic vesicles. However, the significance of these observations is unclear as the ratio of virions and nucleocapsids to infectious units required to produce greater than 90% infection is often greater than 100 : 1 so that conclusions about the role of any particular virion or nucleocapsid becomes extremely difficult to determine.

It has been suggested that lysosomes may be involved in penetration and incoating of herpesviruses (Allison and Sandelin, 1963; Allison and Mallucci, 1965; Welch, 1971), although this has been disputed (Flanagan, 1966).

5.3.2.2. 'Eclipse' Period.

An eclipse period, as is understood by bacterial virologists to mean the period after infection when no infectious virus particles can be detected in infected cells, is not seen with animal viruses as one can always detect some infectious virus throughout the infectious cycle. There is, however, a period shortly after infection during which there is a large decrease in infectious virions present in the infected cells which is similar to the situation seen in virus infected bacteria. This period varies from 2-8 hours for most herpesviruses. It is affected by the temperature of incubation (Farnham and Newton, 1959; Hoggan and Roizman, 1959; Smith, 1963), by the multiplicity of infection (Roizman, 1969) and by prior infection of cells with another mutant (Roizman, 1963).

5.3.2.3. Virus Assembly and Release of Progeny Virions.

Virus assembly begines in the nucleus (Roizman, 1969) where the mature virions first appear (Sydiskis, 1970). Subvirion components are restricted to the nucleus and only the complete virion is released from the cell. Encapsulation of

viral DNA occurs within 2 hours of its synthesis (Olshevsky et al., 1967) and it has been claimed that this process is a random one (Ben-Porat and Kaplan, 1963).

There is generally good agreement that the nucleocapsid is enveloped by the inner lamellae of the nuclear membrane as it exits from the nucleus. Envelopement appears to occur when the nucleocapsid comes into apposition to a thickened virus-modified portion of the inner lamellae of the nuclear membrane (Siegert and Falke, 1966; Shipkey et al., 1967; Darlington and Moss, 1965; Spring et al., 1968) and Ben-Porat and Kaplan (1970) have shown that the virus probably only buds from newly synthesised regions of the nuclear membrane. The proteins in these new regions of nuclear membrane are virus-specified whereas the phospholipids are derived in most part from other pre-existing cellular membranes. The appearance in the cell of the first enveloped mature progeny virion signals the end of the eclipse phase of the virus growth cycle.

The release of the virus from the infected cell is temperature dependent (Hoggan and Roizman, 1959). A network of branched tubules 65 nm in diameter become apparent in HEp-2 cells 8 hours p.i. with HSV (Schwartz and Roizman, 1969). The membranes limiting the tubules are contiguous at one end with the outer lamellae of the nuclear membrane and at the other with the cytoplasmic membrane. Virions fill the tubules and appear outside the cell 16 hours p.i.. The network of tubules is not present in uninfected cells or in DK cells abortively infected

with HSV. The source of genetic information for the tubules is unknown but it is conceivable, since nuclear and cytoplasmic membrances become altered after infection, that these tubules may arise by modification of pre-existing structures by the virus. Similar tubules have been described in cells infected with Simian Virus 40 (Mayor et al., 1962; Grandboulan, 1963; Oshiro et al., 1967) and with adenoviruses (Dales, 1962).

5.3.2.4. Virus Yield.

Virus yield increases exponentially from the end of the eclipse phase until almost the end of the reproductive cycle. The best virus preparations of Watson et al. (1964) contained about 10 virions per infectious unit, although routine preparations of virus contain 10-100 virions per infectious unit (Smith, 1963). Under optimal conditions, the yield of HSV is 10,000 to 100,000 virions per cell (Roizman, 1969).

5.3.2.5. Role of Arginine in the Virus Growth Cycle.

Herpes Simplex Virus type 1 does not multiply in the absence of arginine in cells in continuous culture (Tankersley, 1964; Roizman et al., 1965, 1967; Becker et al., 1967; Inglis, 1968). Adsorption, penetration and uncoating are not prevented by the lack of arginine (Inglis, 1968), nor are the reproductive events occurring during the first 4 hours of infection (Roizman et al., 1967) nor viral DNA synthesis (Becker et al., 1967; Mark & Kaplan, 1971). Courtney et al. (1971)

and Mark and Kaplan (1971) reported that the major virus proteins are synthesised in the cytoplasm of these cells, although at a reduced rate. These proteins, however, do not migrate to the nucleus and do not assemble into particles as they do normally. They will, however, migrate to the nucleus once arginine is supplied to the cells (Courtney et al., 1971).

Viral membrane glycoproteins are also synthesised in arginine-deprived cells but are not integrated into the nuclear membrane as they are normally in infected cells (Mark and Kaplan, 1972).

The lack of migration of viral proteins to the nucleus may result from the fact that a protein, the synthesis of which is more affected by arginine deprivation than that of other proteins, plays a central role in the assembly of viral particles. It is also possible, however, that the lack of assembly of viral particles in the nucleus is directly related to the lack of characteristic virus-induced alterations of the nuclear membrane, since the nuclear membrane has been shown to play a role in the assembly of adenovirus (Winters and Russell, 1971).

A similar effect of arginine deprivation is seen in adenovirus-infected cells in continuous culture. In KB cell monolayers maintained without arginine for 30 hours virus maturation begins immediately upon restoration of arginine to the culture fluid (Rouse and Schlesinger, 1967). Addition of

FdUrd simultaneously with restoration of the arginine fails to inhibit virus maturation; $^{3}$H-dThd present only during arginine deprivation is incorporated into the DNA of virions matured after restoration of arginine; immunofluorescence and haemagglutination tests show that functional capsid subunits are also produced prior to the addition of arginine; addition of radioactively labelled arginine after a 30 hour starvation period, and purification of the virus by equilibrium density gradient centrifugation in caesium chloride, indicates that the arginine label is incorporated in significant amounts in the final viral product at the density characteristic of infectious virions. Thus it appears that the assembly of adenovirus virions requires the synthesis and incorporation of an arginine-rich component. There appears to be a very similar function of arginine in the maturation of herpesviruses (Becker et al., 1967). The nature of the postulated arginine-rich proteins has not been established, although Maizel et al. (1968a, 1968b) suggest that polypeptides associated with the DNA-containing core of the virus are relatively rich in arginine. HSV virions may also contain an arginine-rich "internal" peptide (Becker and Olshevsky, 1972).

### 5.3.3. Virion Ultrastructure and Morphology.

#### 5.3.3.1. Naked and Enveloped Particles.

Wildy et al. (1960) described two basic types of particles

distinguished by the presence or absence of an outer coat or envelope. The naked particle or capsid has an overall diameter of 100 nm and is composed of 162 hexagonal capsomeres each of which is about 10 nm in diameter and have axial holes about 4 nm in diameter.

Some of the particles are enveloped by a membrane varying in diameter from 150-200 nm and occasionally two or more capsids may be enveloped by one envelope.

Epstein (1962a) has described the envelope as a triple-layered membranous structure indistinguishable from host cell membrane. The virus envelope is discussed further in section 5.3.3.5.

5.3.3.2. The Core.

In thin sections of enveloped particles the core appears as a densely stained region of about 30 nm diameter. Epstein (1962b) showed by enzyme digestion that the dense core region contained DNA. Roizman et al., (1969) described a larger body of about 45 nm diameter consisting of beadlike projections surrounding a central core 25 nm in diameter. They regard this structure as an inner capsid surrounding a central core and has been correlated by them with structures appearing in thin sections of nuclei of infected cells. They have also postulated the existence of a middle capsid having a diamter of 70-80 nm and to which the capsomeres of the outer capsid adhere. The existence of these additional components, however,

still remains speculative.

5.3.3.3. Viral DNA.

Russell (1962) and Ben-Porat and Kaplan (1962) were, independently, the first to show by direct methods that herpesviruses contain DNA. The DNA of all herpesviruses, while having a GC content greater than that of animal cells, has been found to vary considerably in GC content, from 55% GC for equine abortion virus to 74% GC for pseudorabies virus, and to have a molecular weight of about $10^8$ daltons (for review, see Roizman, 1969).

The DNAs of herpes simplex viruses type 1 and type 2 are linear double-stranded molecules (Becker et al., 1968; Kieff et al., 1971) and studies of the kinetics of reassociation of HSV type 1-DNA indicate that each gene probably occurs only once, i.e. there are no repetitive sequences (Frenkel and Roizman, 1971).

5.3.3.4. Viral Proteins.

As will be discussed in section 5.3.4.2. 50 percent of the HSV-type 1 genome is transcribed during productive infection. Assuming that all RNA transcripts specify proteins, it can be calculated that Viral DNA codes for the sequence of nearly 55,000 amino acids. To date 49 proteins, accounting for 75% of the total genetic information of the virus, have been identified (Honess and Roizman, 1973). Of this number, at

least 27 are structural proteins of the virus.

Gibson and Roizman (1972) have reported that the newly-assembled DNA-containing capsids which accumulate in the nucleus consist of six proteins ranging in molecular weight from 25,000 to 155,000 daltons.

Olshevsky and Becker (1970) and Becker and Olshevsky (1972) have shown the presence of nine polypeptides in the mature virion of HSV-type 1. The viral capsid is composed mainly of one peptide of molecular weight about 110,000 daltons of which they suggest there are nine molecules in each of the 162 capsomeres. Shimono et al. (1969) have shown that the main capsid polypeptide in Pseudorabies Virions has a molecular weight of 120,000 daltons. Olshevsky and Becker (1970) have found that one of the virion peptides of molecular weight 40,000 daltons and designated peptide VII, is enriched in arginine content and have suggested that it is one of the internal proteins bound in close proximity to the DNA core. Peptide V, of molecular weight 69,000 daltons, is a virus-specific glycoprotein and is probably the first to attach to the nucleocapsid during envelopment. Two other glycosylated peptides, peptide III (106,000 daltons) and IV (83,000 daltons), are added to the virion during envelopment. Becker and Olshevsky (1972) have summarised the known structure of the virion as shown in Figure 1.3.

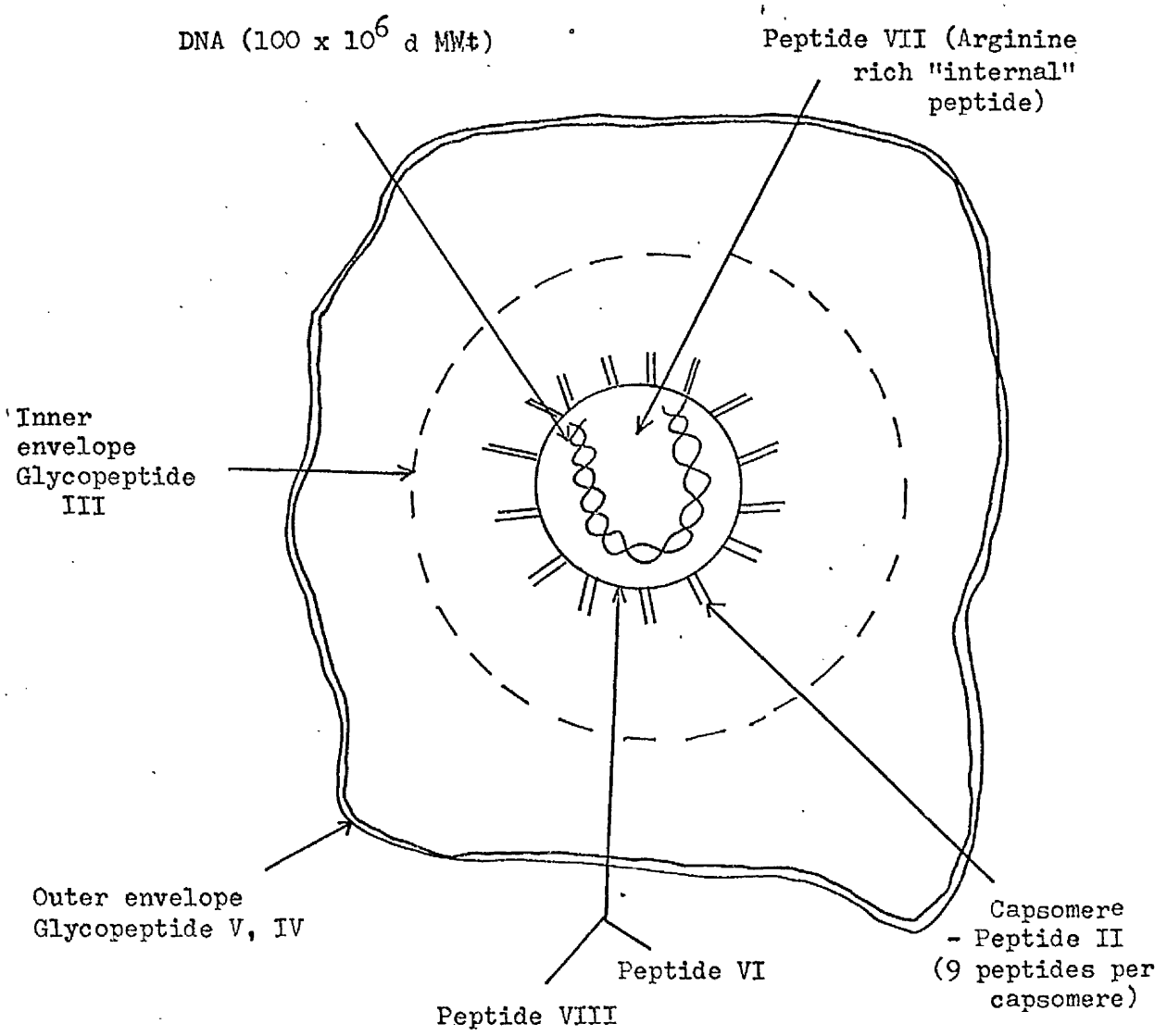
Stevens et al. (1969) showed the appearance of five virus-induced acid-extractable proteins in the nuclei of pseudo-

Fig. 1.3.

Schematic diagram suggesting the localisation of the seven major viral peptides in the HSV Virion.

from Becker and Olshevesky (1972).

Fig. 1.3.


DNA (100 x $10^6$ d MWt)
Peptide VII (Arginine rich "internal" peptide)
Inner envelope Glycopeptide III
Outer envelope Glycopeptide V, IV
Peptide VIII
Peptide VI
Capsomere - Peptide II (9 peptides per capsomere)

rabies virus infected cells 4 hours after infection. Four of these were virus structural proteins; one was not. All of the proteins contained tryptophan and, therefore, were not 'classic' histones. Chantler and Stevely (1973) have identified three basic proteins in chromatin preparations from pseudorabies virus-infected cells. They appear to be virus-specified and are similar in size and charge to host histones. Unlike histones, however, they contain tryptophan. They also find three similar proteins in HSV-type 1 infected cells and two of these proteins have a similar electrophoretic mobility to two of the pseudorabies-induced proteins. Partially purified preparations of pseudorabies virus contain low amounts of all three proteins.

5.3.3.5. Virus Membrane.

The envelope surrounding the herpesviruses forms a loose, but rather impermeable, coat around the virus particle and morphologically resembles other cellular membranes. It contains lipids, polyamines and at least 12 glycoproteins (Heine et al., 1972; Gibson and Roizman, 1971).

Analyses of the infectivity of the virion and of subviral particles indicate that the enveloped particle is most probably the epidemiologically important infectious unit (for review see Roizman, 1969). Although an intact envelope is not required for a particle to be infectious (Spring and Roizman, 1968), study of capsids obtained by stripping the

envelope with detergents show that the preponderance of infectivity is associated with enveloped or partially enveloped particles. Even though the function of the envelope in conferring infectivity on the capsid is still unclear, it is likely that the envelope allows the virus to withstand physical stress and to adsorb to cells with a higher efficiency than particles that are not enveloped.

Although most herpesvirus strains that have been studied acquire their envelopes from the inner nuclear membrane (Ben-Porat and Kaplan, 1971; 1972), this process is not universal. Herpes saimiri, for example, acquires its membrane component while still within the nucleus (Heine et al., 1971).

Preparations of enveloped virions contain little, if any, of the proteins present in the cells at the time of infection (Ben-Porat and Kaplan, 1970; Spear and Roizman, 1972), whereas most of the phospholipids of the viral membrane pre-existed in the cells at the time of infection (Asher et al., 1969; Ben-Porat and Kaplan, 1971).

The convolutions of the nuclear membrane observed by electron microscopy in herpesvirus-infected cells suggests that infection may stimulate the synthesis of new nuclear membrane and evidence of this has been presented by Ben-Porat and Kaplan (1972).

Only nucleocapsids acquire an outer envelope; envelopement of empty capsids is a rare event. This has been

demonstrated mainly by electron microscopy (Nii et al., 1968; Spring et al., 1968) but is also supported by biochemical analyses of the different types of viral particles produced by the cells. The mechanism which ensures that only nucleocapsids are enveloped is not clearly understood, but it seems likely that the empty and full capsids differ in some respects in their surface structure.

5.3.4. Metabolism of Infected Cells.

5.3.4.1. Inhibition of host Macromolecular Synthesis.

During the first hours of infection profound alterations in host cell macromolecular syntheses are brought about by the virus with a concomitant induction of synthesis of viral macromolecular species and their precursors.

Host RNA, DNA and protein syntheses are inhibited during the first 3-5 hours p.i. (Roizman and Roane, 1964; Roizman et al., 1965; Sydiskis and Roizman, 1966, 1967; Aurelian and Roizman, 1965; Hay et al., 1966; Ben-Porat and and Kaplan, 1965). The inhibition of the syntheses of host macromolecules is accompanied by disaggregation of the nucleolus, disaggregation of the polyribosomes and aggregation of chromosomes at the nuclear membrane (Sydiskis and Roizman, 1966; 1967).

The efficiency of the inhibitory process appears to be determined by the host cell. The process is very efficient in HEp-2 cells infected with HSV at relatively low m.o.i. but much higher m.o.i. are required to produce equivalent inhibition

of the macromolecular syntheses of dog kidney cells using the same virus (Aurelian and Roizman, 1965).

DNA Synthesis.

According to Ben-Porat and Kaplan (1965) the inhibition of cellular DNA synthesis by pseudorabies virus is not due to accumulation of viral DNA and competition of viral DNA with cellular DNA to act as a template for replication. They used FdUrd to reduce viral DNA synthesis to 20% of the normal amount and demonstrated that cellular DNA synthesis was still inhibited at the same rate.

Fewer than 20% of the viral DNA molecules, however, are probably actively involved in DNA replication (Kaplan and Ben-Porat, 1966; Kaplan, 1964) and the conclusions reached by Ben-Porat and Kaplan (1965) based on the use of FdUrd must be suspect.

Protein synthesis is required before the virus can inhibit host cell DNA synthesis (Ben-Porat and Kaplan, 1965) and the inhibition is not due to extensive degradation of host cell DNA (Kaplan and Ben-Porat, 1963), although in some cases the DNA may be cleaved to relatively small molecules (Nonoyama and Pagano, 1972).

It has been shown by Ensminger and Tamm (1969, 1970) that Newcastle Disease Virus, Mengovirus and Reovirus all block host cell DNA synthesis at the level of initiation of synthesis upon new regions of chromosomal DNA and that Newcastle Disease Virus and Mengovirus inhibit cellular DNA synthesis indirectly

through a primary viral inhibition of cellular protein synthesis (see section 3.3.6). It is not clear in herpesvirus infected cells whether the inhibition of protein synthesis is the cause or consequence of the inhibition of host cell nucleic acid synthesis.

Protein Synthesis.

Infection of cells with most of the herpesviruses which have been studied results in the inhibition of cell-specific protein synthesis and in the induction of virus-specific protein synthesis.

In HEp-2 cells infected with HSV, there is a decrease during the early stages of infection in the rate of incorporation of amino acids into proteins, followed by a recovery in the rate that is due to the increasing rate of synthesis of viral proteins (Roizman et al., 1965; Sydiskis and Roizman, 1966). The changes in the rates of incorporation of amino acids in PrV infected RK cells are not due to changes in the intracellular pools but reflect a decrease in the rate of synthesis of cellular proteins and a concomitant increase in the synthesis of viral proteins (Kaplan et al., 1970; Sutton and Stevens, 1972). The shift in synthesis of proteins from cellular to viral is reflected in the profiles of polyribosomes in sucrose density gradients and is discussed in section 5.3.4.2.

RNA Synthesis.

Whereas the inhibition of host DNA and protein synthesis appears to be total in HSV-infected cells, there is

some suggestion that the inhibition of host RNA synthesis is selective. This suggestion is based on the observation that the synthesis of ribosomal RNA declines more quickly than that of other species RNA (Wagner and Roizman, 1969; Rakusanova and Kaplan, 1970).

The modification of host RNA synthesis occurs at several levels (Roizman et al., 1970). Firstly there is a selective reduction in host RNA synthesis. Ribosomal precursor RNA is reduced by as much as 70% of that found in uninfected cells, and secondly, the host RNA that continues to be synthesised is not properly processed. Normally the 45s ribosomal RNA is methylated and then cleaved in a series of steps yielding 18s and 28s ribosomal RNA. In infected cells, a small proportion of the 45s RNA continues to be made and to be methylated. The residual RNA, however, is degraded rather than cleaved into the 18s and 28s segments. Concurrently, the nucleolus disaggregates into its components. Thirdly, the small amount of RNA that is processed is transported to the cytoplasm in a different way from viral RNA and does not enter the polyribosomal pool to direct host protein synthesis.

5.3.4.2. Replication, Transcription and Translation of the Virus Genome.

Replication of Virus DNA.

Herpesvirus DNA is synthesised within the nucleus of

the infected cell, and specifically within the intranuclear inclusion body (Munk & Sauer, 1963). Cohen et al., (1971) have shown that replication of HSV DNA is independent of the growth cycle of the cell whereas equine abortion virus can initiate replication of its DNA only in cells which are in the S-phase of growth (Lawrence, 1971).

Protein synthesis is required for the initiation of HSV DNA synthesis (Roizman and Roane, 1964) and for the continuation of the normal rate of synthesis already initiated in PrV-infected RK cells (Kaplan and Ben-Porat, 1966). Replication is semiconservative (Kaplan and Ben-Porat, 1964) and, although few DNA molecules are in the process of being replicated at any given time, the process is rapid (Kaplan and Ben-Porat, 1966). Immediately after its synthesis, viral DNA seems to consist of nicked strands which are later joined together by a ligase. Not all of these nicks, however, are repaired, and they appear in the DNA of mature virions at sites which may be specific (Kieff et al., 1971; Frenkel and Roizman, 1972; Wilkie, 1973). Frenkel and Roizman (1972) compared their results to those obtained with the bacteriophage T5 in which one strand of the DNA duplex is always found intact, while the other always has alkali-labile interruptions (Bujard, 1969; Jacquemin-Sablon and Richardson, 1970; Hayward & Smith, 1972). Wilkie (1973), however, finds that both strands can be found intact and that breaks can also be found in both strands, similar to the situation reported for bacteriophage

SP50 (Reznikoff and Thomas, 1969).

Thoren et al., (1972) have shown that SV40 DNA replication is not initiated at random, but rather that there is a single specific initiation site for DNA replication. In contrast, on the basis of their results with 'nicked' strands, Frenkel and Roizman (1972a) have suggested that replication of the larger HSV genome begins at several initiation points along the molecule and that the short regions synthesised are joined together by a ligase later in the synthetic process and Wilkie (1973) has suggested that the mature DNA molecule may arise as a result of processing of a high molecular weight precursor.

Virus DNA is incorporated into mature virions within 2 hours of synthesis (Olshevsky et al., 1967; Pringle et al., 1973).

Viral RNA.

Viral DNA is transcribed in the nucleus of the infected cell. At least a portion of the viral RNA is made as a high molecular weight molecule that is cleaved into smaller molecules (Roizman et al., 1970), and about 50 per cent of the viral DNA is transcribed (Roizman and Frenkel, 1973; Frenkel and Roizman, 1972b). A temporal control of transcription emerges from the observation that 44 per cent of HSV type-1 DNA is transcribed before the onset of DNA synthesis; 48-50 per cent is transcribed late in infection. A second control, regulating abundance of RNA, specifies that the transcripts of

some DNA sequences are present much more abundantly than those of others (Roizman and Frenkel, 1973; Frenkel and Roizman, 1972). Early in infection, the abundant and scarce transcripts are derived from 14 and 30 per cent respectively of the HSV-type-1-DNA. An additional 4 to 5 per cent of the HSV-type 1-DNA is transcribed and accumulates in relatively abundant amounts late in infection. Silverstein et al., (1973) and Bachenheimer and Roizman (1972) have shown that the abundant species are adenylated, whereas the scarce species are not. These studies have also revealed that adenylation takes place in the nucleus and that the polyadenylic acid chains linked to the 3' end of the RNA contains as many as 160 adenosine residues.

In the cytoplasm, virus-specific RNA enters into free and membrane-bound polyribosomes and directs the synthesis of structural and nonstructural proteins of the virus (Roizman, 1969) and there is evidence to indicate the existence of controls that regulate the time of synthesis and the abundance of both structural and nonstructural proteins (Honess and Roizman, 1973).

Synthesis of Virus Proteins.

The shift in synthesis of proteins from cellular to viral is reflected in the profiles of polyribosomes in sucrose density gradients (Sydiskis and Roizman, 1966, 1967, 1968). The polyribosomes of uninfected HEp-2 cells are polydisperse, but with a peak fraction sedimenting at 170s. These polysomes

disappear between one and two hours after productive infection with HSV and are replaced by polydisperse polyribosomes with a sedimentation coefficient of the peak fraction of about 270s.

Viral proteins first appear in the cytoplasm (Fujiwara and Kaplan, 1967) and then move into the nucleus slowly and selectively (Spear and Roizman, 1968). Less than half of the protein synthesised during a short pulse with radioactive amino acids appeared in the nucleus after a 3 hour chase and 3 proteins appeared to be restricted to the cytoplasm.

5.3.4.3. Virus-induced Enzymes.

The finding that there is an increase in activity of many enzymes already present in the host cell when they are infected with a herpesvirus is at first glance, surprising. Although the normal mammalian cell is equipped with these enzymes in some measure or another, there is no guarantee that the levels of activity of the enzymes and their specificities are suitable for efficient and accurate production of new virus particles after initiation of infection. Another possibility, as suggested by Newton (1970), is that the activity of the enzyme in the host cell may vary with the physiological state of the cell and the ability of a virus to induce these particular enzymes may allow it a degree of independence of the division status of the cell which would

ensure a larger population of cells giving productive infections. In this respect it is interesting to note that the normal site of infection by herpesviruses in the whole animal is in nervous tissue in which very few, if any, of the neuronal cells are dividing and in which enzyme levels are probably greatly reduced. A third possibility is that the virus-induced enzymes may be less sensitive to feedback inhibition than the host enzymes and allowing virus enzyme to function under conditions in which the corresponding host enzyme is inhibited.

DNA polymerase.

Infection of susceptible cells by HSV leads to the induction of a new DNA polymerase (Keir and Gold, 1963). This enzyme differs in several respects from that of the host cell. The virus-induced enzyme has a much higher salt optimum than the cellular enzyme, is more heat stable and less sensitive to iodoacetimide. The two enzymes can also be distinguished immunologically (Keir et al., 1966; Watson et al., 1966), and an enzyme with the same immunological specificities is induced by HSV in several different cell lines (for discussion, see Keir, 1968).

Weissbach et al., (1973) have partially purified and characterised the HSV-induced polymerase in HeLa cells. The molecular weight was estimated to be $1.8 \times 10^5$ daltons and appears to prefer poly dG.dC templates to activated DNA which the authors suggest may be a reflection of the high GC

content of HSV DNA. The other properties of the enzyme are similar to those described previously by Keir (1968).

Deoxyribonuclease.

A DNase activity has been shown to appear after infection of cells with HSV (Keir and Gold, 1963; Russell et al., 1964). This activity is not detectable in uninfected cells (Morrison and Keir, 1967) and differs in its requirements and heat stability from that of the uninfected cell enzyme (for summary of differences, see Keir, 1968). The virus-induced enzyme is not induced in the presence of actinomycin D (Keir, 1968) or puromycin (Morrison, 1967) and acts by producing 5'-nucleoside monophosphates sequentially from the 3'-end of a suitable DNA substrate (Keir, 1968).

Thymidine kinase.

The enzyme is present in both "normal" and "neoplastic" cells grown in vitro. In BHK 21 cells the activity of the enzyme increases as much as 20-fold between 2 and 8 hours after infection with HSV-type 1 (Klemperer et al., 1967).

The evidence that thymidine kinase in HSV-infected cells is specified by the virus is particularly strong, in that there are differences in electrophoretic, immunological and physical properties, between the host enzyme and the HSV-specified enzyme (for discussion, see Keir, 1968).

J. P. Durham (personal communication) using the enzyme isolated from sucrose velocity gradients has evidence to show that the HSV-specified enzyme in BHK21 cells is either a

completely virus-coded protein or a virus-modified cellular enzyme with a molecular weight of greater than 200,000 daltons as opposed to a molecular weight of 70,000 daltons for the BHK enzyme. In contrast, in PrV-infected BHK cells, only increased levels of the 70,000 daltons species is observed, suggesting that this virus does not induce its own thymidine kinase.

deoxythymidylate, deoxycytidylate, deoxadenylate and deoxyguanylate kinases.

Hamada et al., (1966) reported that deoxythymidylate kinase activity increased in Rabbit Kidney cells infected with pseudorabies virus whereas the activity of the other three nucleoside monophosphate kinases remained unaltered. Prusoff et al., (1965) observed a similar increase in deoxythymidylate kinase in African Green Monkey kidney cells infected with HSV, but they were unable to differentiate between the properties of the enzyme in extracts of infected and uninfected cells. Deoxyadenylate kinase from infected and uninfected cells could not be differentiated by antisera prepared against uninfected cell extracts (Hamada et al., 1966).

deoxynucleoside kinases.

Pereira and Morrison (1970) have reported an increase in deoxycytidine kinase activity in HSV infected BHK cells and J. P. Durham (personal communication) has confirmed this observation but has shown that the levels of deoxyadenosine kinase and deoxyguanosine kinase remained unaltered after infection.

## 5.4. Viruses and Cancer.

### 5.4.1. General.

There is still no consensus about the cause or causes of cancer; indeed, it is often difficult to find a consensus about any aspect of cancer research. The sharply conflicting views of investigators in different subdisciplines has been most aptly summarised by one scientist, Charles Heidelberger who argues that "the mechanism of cancer is a mirror into which each man looks and sees himself".

The concept that viruses can cause cancer is attractive for two reasons. Introduction of viral genetic material into cells can account for their permanent transformation to the malignant state and, second, identification of a viral cause for a cancer may permit the development of vaccines or drugs to prevent and ultimately erradicate the disease.

#### RNA Tumour Viruses.

The first tumour virus to be studied seriously was discovered by Rous (1910). He had been studying a spontaneous chicken sarcoma and showed that a cell-free filtrate could induce a tumour to grow at the inoculation site, establishing a virus as the aetiological cause of the tumour (Rous, 1911). The name Rous Sarcoma Virus is now given to a large number of independently isolated chicken viruses that induce similar sarcomas; the general name

avian sarcoma virus is also used.

Bittner (1936) obtained evidence that a mouse mammary gland carcinoma is transmitted from mother to offspring by a factor in milk, and subsequently showed that the factor which causes the disease is a filterable virus (Bittner, 1942).

In 1951, Gross successfully transmitted leukaemia by inoculating mice with extracts of leukaemic cells and since then several strains of murine leukaemia virus, including the Friend, Moloney, Rauscher and Graffi strains, have been isolated. Murine sarcoma viruses have also been identified (Harvey, 1964; Moloney, 1966; Kirsten and Mayer, 1967). Leukaemia and/or sarcoma viruses have also been isolated from cats, rats, hamsters, monkeys and there is evidence for guinea pig and bovine leukaemia viruses and for leukaemia and sarcoma viruses of gibbons and one species of snake.

RNA tumour viruses are generally divided into three main classes, labelled A, B and C. Type C RNA viruses have been shown to infect a large number of animal species and most of these viruses cause mainly leukaemias, lymphomas and sarcomas. Type B RNA viruses have been associated primarily with certain carcinomas of the breast and type A RNA viruses, which are found intracellularly, appear to be precursors to type B or type C particles (Sarkar et al., 1972).

To date, no one has isolated C-type particles directly from biopsies of human tumours and unequivocal biochemical

evidence of the presence of components of C-type viruses in human cells has yet to be obtained.

Reverse transcriptase appears to be a universal component of type C RNA tumour viruses and several enzyme activities capable of using synthetic RNA and perhaps natural RNA templates for the synthesis of complementary DNA have been found in normal, as well as tumour, cells of various species, including man. These findings have been reviewed by Temin and Baltimore (1972). Whether any of these enzymes are related to the reverse transcriptases of tumour virus particles remains to be proven.

5.4.2. Herpesviruses.

Whether the small DNA tumour viruses and the adenoviruses ever cause tumours outside the laboratory is open to doubt, but at least one herpesvirus, Marek's disease virus, indisputably is a natural carcinogen. That fact alone forces us to consider seriously certain human herpesviruses as potential carcinogens.

Several herpesviruses which have been associated with the aetiology of tumours in various species, including man, are enumerated in Table 1.5.

Evidence is accumulating for a causal role of herpesviruses in Marek's lymphoma of chickens, in Lucke renal adenocarcinoma of frogs, and in lymphomas and leukaemias of non-human primates, rabbits, and guinea pigs. In man, Epstein-Barr virus has

been consistently linked with Burkitt's lymphoma and with nasopharyngeal carcinoma, whereas HSV-1 and HSV-2 have been associated for some years with squamous cell carcinoma of the lip and cancer of the cervix respectively. Recently, Sabin and Tarro (1973) carried out an immunological survey on a large number of patients suffering from various forms of cancer and on a control population exhibiting no symptoms of cancer. Of 29 different areas of the body exhibiting cancerous growth, only 9 from specific areas reacted with antisera to non-virion components of HSV-1 and HSV-2. None of the control patients displayed any reaction with the antisera and, in addition, several advanced cancers of the lip or oropharynx reacted with HSV-1 antisera only, and not with antisera to HSV-2. Thirteen advanced cancers of the cervix and one of the vulva reacted with antisera to both HSV-1 and HSV-2. They suggest that their data taken together indicate that HSV-1 and HSV-2 play an aetiological role in certain human tumours. Most of the studies on the relation between HSV and human cancer have dealt with HSV-2 and cervical carcinoma, but in spite of supporting epidemiological data and *in vitro* and *in vivo* studies on the potential carcinogenesis of HSV-2, the causal role of the virus in cervical carcinogenesis remains to be established.

It is, of course, very difficult to prove conclusively that a suspect agent is the cause of any human cancer, but using criteria similar to those employed to support a causal

Table 1.5.

Association of Herpesviruses with Tumours.

| Virus | Natural Host | Clinical Disease in Natural Host | | References |
|---|---|---|---|---|
| | | Nonmalignant | Malignant | |
| HSV1 | Man | Several | Squamous cell carcinoma of the lip | Wyburn-Mason, 1957; Kvasnicka, 1963, 64, 65. |
| HSV2 | Man | Several | Cervical and possibly other genital tumours | Nahmias et al., 1970; Rawls et al., 1969; Aurelian et al., 1970; Duff and Rapp, 1971. |
| EBV | Man | Infectious mononucleosis | Burkitt's lymphoma | Klein, 1971; Henle et al., 1968; Niederman et al., 1968; de-The et al., 1969a, b; Levy et al., 1971. |
| MDV | Chickens | Degenerative lesions in feather follicle epithelium, bursa of fabricius, thymus | Neurolymphomatosis, lymphoid tumours of gonads, kidney, lung, heart, spleen, liver, skin. | Biggs et al., 1970, 1972. |
| Guinea pig herpes virus | Guinea pig | - | Lymphocytic leukaemia | Hsiung and Kaplow, 1969. |
| Cottontail rabbit herpes virus | Cottontail rabbit | Transient lymphocytic infiltration of liver & kidney | Abnormal lymphocytes in peripheral blood, liver, kidney; occasional death. | Hinze, 1971a, 1971b. |

role of cigarette smoking in lung cancer, the association between HSV-2 and cervical cancer stands up well (Nahmias et al., 1972).

We know very little about the biology at the cellular and molecular levels of the interactions between herpesviruses and their non-permissive hosts. It has been suggested, however, that all or part of the genomes of these viruses may establish stable associations with host cell genomes in the same way that SV40 and polyoma virus DNAs stably integrate with cell genomes. One biopsy of cervical carcinoma tissue has shown that cells of that tumour contained one copy of about 40 percent of the HSV-2 genome and RNA transcripts corresponding to about 5 percent of the sequences in the viral DNA (Frenkel et al., 1972). This result is consistent with the above suggestion of integration of the viral genome into the host cell genome.

Whatever the molecular mechanisms involved, however, at least some herpesviruses are oncogenic either for animals of their natural host species or for animals of species maintained in the laboratory.

## II. MATERIALS AND METHODS.

Science is nothing but trained and organised common sense.

T. H. Huxley.

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### Materials.

#### Virus.

The strain of pseudorabies virus used in these experiments was originally obtained from Dr. A. S. Kaplan, Albert Einstein Medical Centre, Philadelphia. It was cloned by isolation from a single plaque at limiting dilution. Cloning was repeated three times before the first wild type stock was established (Kaplan and Vatter, 1959). Wild type and mutant stocks were stored at -70°C.

Temperature-sensitive mutants of pseudorabies virus were isolated following exposure to 5 ug/ml 5-bromodeoxyuridine as described by Pringle et al., (1973).

#### Cells.

BHK 21 clone 13 cells were a continuous line of hamster fibroblasts described by Macpherson and Stoker (1962).

#### Radiochemicals.

All isotopically-labelled compounds were obtained from

the Radiochemical Centre, Amersham, Bucks., England.

Media.

The Glasgow modification of Eagle's medium (Busby, House and McDonald, 1964) containing 100 units Penicillin, 100 ug/ml Streptomycin (Glaxo Laboratories) and 0.002% (W/v) phenol red was routinely used. The vitamins and amino acids were obtained from Flow Laboratories, Irvine, Ayrshire.

Tryptose Phosphate Broth consisting of a 2.95% (W/v) solution of tryptose phosphate broth in distilled water was purchased from Difco Bacto Laboratories, East Molesey, Surrey, England.

ETC 10. 80% (V/v) Eagle's medium,
10% (V/v) tryptose phosphate broth,
10% calf serum (Flow Laboratories).

EC 1. 99% (V/v) Eagle's medium,
1% (V/v) calf serum.

Trypsin-Versene Solution.

1 volume 0.25% (W/v) trypsin solution in 4 volumes 0.6 mM EDTA.

Heparin: Sigma Chemical Company London.

A stock solution of 10 mg/ml in distilled water was sterilised by filtration.

SSC : (Standard Saline Citrate).

0.15M sodium chloride, 0.015M trisodium citrate.

PBS(a) : 0.17M NaCl, 3.4 mM KCl, 10mM $Na_2HPO_4$, 2mM $KH_2PO_4$, pH 7.4 (Dulbecco and Vogt, 1954).

SDS : ethanol-recrystallised sodium dodecyl (lauryl) sulphate in distilled water.

Stock solution of 20% ($^{W}/v$) stored at 37°C.

Formol saline : 4% ($^{V}/v$) formaldehyde in 85 mM NaCl, 0.1M $Na_2SO_4$.

Gelatine-chrome alum :

5g gelatine, 0.5g chrome alum ($CrK(SO4)_2.12H_2O$) 5 ml formaldehyde (40% $^{V}/v$ solution) and 1 ml Photo-Flo (Kodak Ltd., London) in a total volume of 1 litre.

D19b developer :

72g $Na_2SO_3$, 48g $Na_2CO_3$, 4g KBr, 8.8g hydroquinone, 2.2g Metol (Kodak Ltd., London) to a total volume of 1 litre.

Amfix : 20% ($^{V}/v$) concentrated Amfix (May and Baker Ltd., Dagenham, England) in distilled water.

Giemsa stain :

A 1.5% ($^{w}/_{v}$) suspension of Giemsa (George T. Gurr Ltd., London) in glycerol, heated to 56° for 90-120 minutes and diluted with an equal volume of methanol was used as stock (Dacie, 1956).

All other chemicals used were of Analar grade or equivalent where possible and were purchased from BDH Ltd., BDH Laboratory Chemicals Division, Poole, England, or from Hopkin and Williams Ltd., Chadwell Heath, Essex, England. HHepes buffer, N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid was purchased from Calbiochem., San Diego, California, U.S.A.

Deoxyribonucleoside-5'-triphosphates; were supplied by P - L Biochemicals Inc., Milwaukee, Wis., 53205.

Uridine-5'-triphosphate: was supplied by Boehringer Mannheim GmbH, Germany.

Pronase : was supplied by BDH Ltd., Poole, England and Sigma Chemical Company, P.O.Box 14508, St. Louis, Mo., 63178 supplied the poly-L-$\alpha$-ornithine.

Dithiothreitol, actinomycin D and cycloheximide were supplied by Calbiochem., Los Angeles, California 90054.

Glass coverslips : 13mm diameter glass coverslips (Chance) size

No.1 were purchased from McFarlane Robson Ltd., Burnfield Avenue, Thornliebank, Glasgow.

## Methods.

### 1. Tissue Culture Techniques.

#### 1.1. Propagation of Cells.

BHK 21 clone 13 cells were propagated at 37° in burrlers containing 180 ml EC10 in an atmosphere consisting of 95% air and 5% $CO_2$. Bottles were routinely seeded with 20 x $10^6$ cells and cells harvested after three days incubation.

#### 1.2. Harvesting Cells.

The medium was poured off and the cell monolayer washed once with 20 ml trypsin-versene at 37°, followed by another wash with 20 ml trypsin-versene solution also at 37°. The cells were washed out of the bottles using a small volume of ETC and centrifuged at 500g for 5 mins. The cell pellet was washed once with ETC and the cells resuspended in fresh ETC for dispersal into fresh bottles.

All cell stock preparations were routinely checked for bacterial and PPLO contamination. Contaminated stocks were discarded.

#### 1.3. Cell Systems for the study of Virus and Host Cell Metabolism.

##### 1.3.1. Exponentially growing cells.

Cells were suspended in warm ETC and dispersed into

burrlers, 40 oz. Roux bottles or 50 mm plastic petri dishes, at a density of 0.5 - 2.0 x $10^5$ cells/ml. The cells were allowed to grow for 16 - 24 hours before infection with virus.

1.3.2. Low Serum or 'Resting' Cells.

Cells grown to two generations short of confluence were treated as described by Howard et al., (1974). Briefly, the medium was removed from cells growing exponentially in ETC, and the cell sheet washed twice with warm Eagles medium containing neither tryptose phosphate broth nor calf serum. 100 ml of ECl was added to each burrler (50 ml/roux and 5 ml/50 mm Petri dish) and incubation continued at 37° for 5½ days. At this point the cells were either infected with pseudorabies virus or stimulated to recommence growth and division by the addition of calf serum to 10% (v/v).

1.4. Procedure for Infecting Cells.

1.4.1. Exponentially Growing Cells.

The culture fluid was decanted aseptically from burrlers and replaced with either 20 ml conditioned ETC (i.e. medium which had been previously used to cultivate cells) containing an inoculum of pseudorabies virus at the required input multiplicity, or 20 ml conditioned ETC. The corresponding volumes of conditioned ETC for Roux bottles and 50 mm Petri dishes were 10 ml and 0.2 ml respectively. After an adsorption

period of 45 - 60 minutes at 37° the medium containing unadsorbed virus was removed and replaced with 100 ml conditioned ETC (50 ml and 5 ml in a Roux and 50 mm Petri dish respectively) and the incubation continued for the required length of time at 37°.

1.4.2. Low Serum or 'Resting' Cells.

Cells were infected as above except that conditioned ECl was used in place of ETC.

1.5. Production of Virus Stocks.

After decanting the medium, semi-confluent cultures of cells growing in ETC in roller bottles were infected with a low multiplicity of 1 p.f.u. per 300 cells with the virus in 20 ml fresh ETC. The virus was allowed to adsorb for 60 minutes at 37° after which time a further 50 ml of fresh ETC was added to each bottle. Incubation was continued for 27 - 30 hours by which time the cells exhibited extensive C.P.E. The cells at this point were dislodged by shaking them off the glass and the suspension centrifuged at 900 g for 10 mins. at 4°.

The supernatant was decanted and the cell pellet suspended in a suitable volume of ice-cold BSS-bicarbonate. This suspension was disrupted by three cycles of rapid freezing and thawing in a solid $CO_2$-methanol mixture, and the debris removed by centrifugation. This supernatant, containing cell-

associated virus and the original supernatant containing cell-released virus were centrifuged separately at 9,000g for 90 minutes at 4°. The pellet containing pseudorabies virus was suspended in PBS(a) or BSS-bicarbonate. All virus stock preparations were routinely checked for contamination by PPLO. During all manipulations the temperature was kept at 4° and the virus suspension was stored at -70° in 1 ml aliquots.

1.6. Isolation and Propagation of Temperature Sensitive Mutants of Pseudorabies Virus.

Temperature-sensitive mutants of PrV were isolated and propagated as described by Pringle et al., (1973).

1.7. Procedures for Infecting Cells with Temperature Sensitive Mutants of Pseudorabies Virus.

The culture fluid was decanted aseptically from BHK cells grown at 37° in screw-capped 30 ml bottles and the cells infected with PrV at 1 pfu/cell in 0.2 ml conditioned medium. After virus adsorption for 60 mins. at room temperature incubation was resumed in conditioned medium at 37° or 41°.

1.8. Titration of Virus.

Virus infectivity was assayed by inoculating confluent

monolayers of BHK cells in 50 mm plastic Petri dishes with 0.2 ml of ten-fold serial dilutions of virus in ETC or in PBS(a) supplemented with 10% (v/v) calf serum. Virus was allowed to adsorb to the cells for 45 - 60 minutes at 37° after which time 4 ml of fresh ETC was added to the infected cells and incubation continued at 37°. After a further two hours a further 1 ml of ETC containing 250 ug heparin to prevent vertical transmission of virus was added. The medium was removed 28 - 30 hours later, the cell sheet washed with PBS(a) and fixed in 10% formol saline. When fixed, the cell sheet was stained with Giemsa, dried and plaques counted at low magnification under the microscope.

## 2. Biochemical Techniques.

### 2.1. General.

#### 2.1.1. Preparation of Coverslips for Autoradiography.

13 mm diameter glass coverslips were boiled for 10 minutes in 0.1N NaOH, washed for 3 hours in tap water, twice for three hours each in distilled water, one hour in ethanol and air dried. Sterilisation was carried out at 120° for two hours.

#### 2.1.2. Preparation of Buffers.

Buffers were prepared by weighing out the required amount of solid and dissolving it in less than the required amount of distilled water. The pH was adjusted with hydrochloric acid

or sodium hydroxide and the volume adjusted with distilled water.

2.2 Labelling of cells and Autoradiography.

Cells were labelled for 1 hour periods unless otherwise stated. DNA was routinely labelled with 8 uci/ml 6-$^{3}$H-thymidine at a final concentration of $4 \times 10^{-6}$M deoxythymidine for exponentially growing cells and $4 \times 10^{-7}$M for 'resting' cells. Protein was labelled with 0.2 - 1.0 uci/ml of a mixture of individually purified $^{14}$C-amino acids.

Autoradiography was performed on cells grown on 13 mm glass coverslips or in 50 mm Petri dishes. The coverslips were removed after the labelling period and, after fixing the cells in 10% formol saline, they were placed in toluene-PPO scintillation fluid, and the TCA insoluble material determined in a liquid scintillation counter. Four coverslips were routinely placed in each of two duplicate 50 mm Petri dishes and the average radioactivity content determined. Each coverslip was removed after determining the radioactive content, washed twice in PPO-free toluene, once in ethanol and air dried. They were mounted, cells uppermost, on 0.8 - 1 mm glass microscope slides previously degreased in ethanol and coated with gelatine-chrome-alum. The coverslips were overlaid with Kodak A.R.10 stripping film and exposed in the dark in a moisture-free atmosphere. The films were developed in D19b developer for 5 mins., fixed for 4 mins. in Amfix, washed for 10 mins. in distilled water

and immediately stained for 90-120 secs. with a freshly prepared 1:20 dilution in water of Giemsa stain. After air drying, the slides were examined microscopically. Occasionally, the alternative method of overlaying a monolayer culture of labelled cells with Ilford Nuclear Research Photographic emulsion, type L.4, was used. This emulsion was developed as for Kodak A.R.10 stripping film.

Cells containing radioactive precursors in their DNA were seen to have silver grains over their nuclei.

2.3. Determination of Mitotic Index.

Cells grown in 50 mm plastic Petri dishes were maintained in mitotic arrest by adding colcemid at 20 ug/ml for one hour periods. After fixing the cell sheet in 10% formol saline and washing in tap water, the cells were stained for 5 - 10 min. in Harris's Haemotoxylin and then washed extensively in tap water. Triplicate cultures were used for each time point and 500 cells in each dish were scored.

2.4. Preparation of Cell Extracts.

Cells growing in burrlers were infected in the normal fashion and at a suitable time later the cells were scraped off in tris-saline pH 7.4 and centrifuged at 500 g for 5 mins. The cell pellet was resuspended in a small volume of 20 mM Hepes -NaOH buffer pH 7.0, sonicated at 1.5 amps for 10 seconds in an M.S.E. ultrasonic power unit and the degree of cell disruption

checked microscopically. Half of the sonicate was centrifuged at 308,000 g for 30 mins. in a Spinco SW56 rotor. The top portion of the supernatant was removed. A mock-infected control culture was similarly treated. At later stages extracts were prepared as by Suarez et al., (1972). Samples were removed at all stages and checked for sterility and for the presence of infectious virus.

0.2 or 0.4 ml of centrifuged or uncentrifuged extracts were added to monolayer cultures of cells in 50 mm Petri dishes containing 5 ml medium, and the cultures labelled with 8 uci/ml $^{3}$H-dThd at a final concentration of $4 \times 10^{-6}$M or with 0.2 uci/ml of a mixture of individually purified $^{14}$C-amino acids at various times afterwards. At the end of each labelling period the cells were scraped off in a small volume of ice-cold BSS, centrifuged and the pellet washed twice with 10 ml ice-cold 10% TCA. The resulting pellet was left to drain overnight and the next morning dissolved in 0.5 ml hyamine hydroxide at 60° for 10 minutes and the radioactivity determined in 10 ml toluene-PPO scintillation fluid as described in section 2.4.1.

## 2.5. Preparation of Nuclei and *in vitro* DNA Synthesis.

The cells were harvested as described in section 1.2 except that BSS-bicarbonate was used to wash the cells out of the bottles, and were suspended in 3-4 volumes of ice-cold 20 mM Hepes, pH 8.0 containing 1mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1mM

dithiothreitol. Following homogenisation with ten strokes of a loose fitting homogeniser, sucrose was added to 400 mM and the nuclei pelleted by centrifugation at 1,000 g for 4 min. The nuclear pellet was resuspended in 2 volumes 50 mM Hepes, pH 8.0 containing 1 mM $MgCl_2$ 0.5 mM $CaCl_2$ and 1 mM dithiothreitol and 0.1 ml aliquots incubated with 0.1 ml of a mixture containing 96 mM Hepes, pH 8.0, 100 mM NaCl, 12 mM $MgCl_2$, 4 mM ATP, 2 mM dithiothreitol, 400 mM sucrose, 1.6 mM each dATP, dCTP, dGTP, 0.04 mM dTTP and 1 uci $^3$H-dTTP. The incubation was terminated by the addition of NaOH to a final concentration of 1 M, the suspension incubated at 37° for 45 mins., and the nuclei pelleted by centrifugation at 1,000 g for 5 min. after the addition of ice-cold trichloracetic acid containing 50 mM sodium pyrophosphate to a final concentration of 5%.

The nuclei were taken through 5 cycles of solution in NaOH and precipitation in TCA and the final acid-insoluble radioactivity determined in 0.5 ml hyamine hydroxide and 10 ml toluene containing 0.5% PPO.

DNA polymerase activity in 'cytoplasmic' preparations was determined in a reaction mixture containing 0.1 M tris-HCl, pH 7.5 at 37°, 10 mM $MgCl_2$, 10 mM mercaptoethanol, 50 mM KCl, 0.4 mM each dATP, dGTP, and dCTP, 0.04 mM dTTP, 2 uci $^3$H-dTTP, 150 ug native or denatured DNA and 40 ul of the cytoplasmic preparation in a total volume of 100 ul. The reaction was stopped after 30 min. by the addition of 500 ug

denatured salmon testes DNA in ice-cold TCA containing 20 mM disodium tetraphosphate. Acid-insoluble material was retained on a Whatman's No. 1 filter paper disc and the radioactive content determined by liquid scintillation counting.

2.5.1. Preparation of Nuclear Supernatant Fractions.

Nuclei were prepared as described in the previous section (2.5) and the supernatant fractions centrifuged at 165,000 g for 30 mins. at $4^{\circ}$. The supernatants from these centrifuged samples were dialysed overnight at $4^{\circ}$ against 50 mM Hepes-NaOH, pH 8.0 at $4^{\circ}$, containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1 mM dithiothreitol. Fractions were stored at $-20^{\circ}$ until ready for use.

2.6. U. V. Inactivation of Virus.

$3 \times 10^9$ pfu/ml pseudorabies virus in complete PBS plus 1% (v/v) calf serum was irradiated in 0.5 ml batches in 50 mm Petri dishes with no lid. They were irradiated at 16 ergs/$mm^2$/sec for 30 minutes at $4^{\circ}$. The lamp was calibrated as described by Hatchard and Parker, (1956).

2.7. Gradient Centrifugation.

2.7.1. Caesium Chloride Equilibrium Density Gradient Centrifugation.

Cells in 50 mm Petri dishes were dissolved in 3 ml SSC

containing 2% SDS. When using temperature sensitive mutants of PrV and infecting cells for 24 hour periods, mature virus and cellular products released into the medium were collected by centrifugation at 165,000 g for 30 min. and pooled with the material released from the cells by 2% SDS before overnight incubation with pronase. Pronase (250 ug/ml) was added and the suspension incubated at 37° for 12 - 16 hours, when a further 125 ug/ml pronase was added for a further 3 hours. Caesium chloride was added to give a density of 1.70 $g/cm^3$ and the suspension cleared by centrifugation at 1,000 g for 4 mins. The supernatant solution was then centrifuged at 59,000 g for 60 - 72 hours at 18°. Fractions were collected by puncturing the bottom of the tube.

Figures 3.17 and 3.18 are typical examples of the distribution of radioactivity throughout the isopycnic gradients. The radioactivity in the fractions under each peak was summed to give an estimate of the amounts of cellular and viral DNA present at various times after infection. For example, in figure 3.18a fractions 30-42 were taken to represent viral DNA, and fractions 46-60 to represent cellular DNA. In all cases the median buoyant densities of the two peaks was determined to ensure that it corresponded to the known values for viral and cellular DNAs {1.73 and 1.70 g/ml respectively (Pringle et al., 1973)}.

suspension, a further 0.6 ml of 0.5 M NaOH-0.1 M EDTA was layered over the cell suspension. Loaded gradients were held

at $4^{o}$ for 12 - 16 hours and were then centrifuged in the SW27 rotor at 82,500 g for 4 hours at $4^{o}$. Gradients were harvested from the bottom using a peristaltic pump and 50 ul or 100 ul fractions spotted on Whatman No. 1 filter paper discs. Radioactivity was determined as in section 2.8.3.

## 2.8. Determination of Radioactivity.

Three basic techniques were used.

### 2.8.1. Use of Hyamine Hydroxide to Dissolve Radioactive Material.

Cells were scraped off in PBS(a) and washed three times in ice-cold 5% TCA with low speed centrifugation between each wash. The pellet was dissolved in 0.5 ml hyamine hydroxide (1 M in methanol) at $60^{o}$ for 10 - 15 minutes and radioactivity determined in 10 ml toluene/PPO scintillation fluid (0.5% ($^{w}/_{v}$) PPO).

### 2.8.2. Determination of Radioactivity in Cells grown on Coverslips.

Coverslips were washed three times in ice-cold 5% TCA after fixing the cells in 10% formol saline. They were then rinsed once in distilled water, once in alcohol, air dried and the acid-insoluble radioactivity determined in 5 or 10 ml toluene/PPO scintillation fluid.

2.8.3. Determination of Radioactivity in Samples from Caesium chloride and alkaline Sucrose Density Gradients.

50 ul samples from each fraction of the gradients were spotted on Whatman No. 1 2.5 cm filter paper discs, washed twice in ice-cold 5% TCA, once in water, once in alcohol, once in ether and air dried. The TCA-insoluble material was eluted from the discs with 0.5 ml 0.2 M NaOH overnight at 37° and, after neutralisation with 0.5 ml 0.25 M HCl, radioactivity determined in 10 ml triton-toluene liquid scintillation fluid (Patterson and Greene, 1965).

2.9. Use of Actinomycin-D and Cycloheximide.

When using cycloheximide to study the effects of inhibition of protein synthesis on the inhibition of host macromolecular synthesis by pseudorabies virus, the drug was added in sterile distilled water to the medium immediately after the 45 min. period of adsorption of virus to the cells and remained in the medium throughout the experiment.

Both actinomycin-D and cycloheximide in sterile distilled water were added to the culture medium at various times after addition of serum to stationary serum deprived BHK cells and remained in the medium throughout the remainder of the experiment.

In all cases the actinomycin-D and cycloheximide solutions were made up fresh immediately before use.

## 2.10. Preparation of Rabbit Antiserum to Pseudorabies Virion Antigens.

### 2.10.1. Inactivation of Virus.

Formalin was added to the virus preparation to a final concentration of 1:400 formalin and incubated at $37^{\circ}$ overnight. After titration the inactivated virus preparation was stored at $4^{\circ}C$. The formaldehyde was not neutralised before use of the inactivated virus.

### 2.10.2. Preparation of Antiserum.

Equal volumes of inactivated virus and Freund's incomplete adjuvant were emulsified together by pumping in and out of a syringe until the emulsion was stable. Live virus was diluted as necessary in PBS(a) to the required concentration for the later inoculations.

Rabbits were given two inoculations intramuscularly of inactivated virus originally containing $10^8$ - $10^9$ pfu in Freund's incomplete adjuvant, with 14 days between inoculations. A fortnight after the second inoculation of inactivated virus, 225 pfu of live virus was administered intravenously. The dose was increased 100-fold at weekly intervals for three weeks, the last dose containing $2 \times 10^8$ pfu of live virus.

### 2.10.3. Titration of Antiserum.

About 10 - 20 ml serum was drawn off from the ear vein before administration of any virus and immediately before each

subsequent inoculation. Serum was inactivated at 56° for 30 mins. as a 1:10 dilution in PBS(a). It was then diluted as necessary in PBS(a), usually in 5-fold steps and mixed with an equal volume of virus diluted to give about 150 pfu in 0.1 ml of the final mixture. The mixtures were incubated at 37° for 60 mins. before being plated out on preformed monolayers of BHK21 cells in 50 mm Petri dishes. 0.1 ml of each mixture was put on each of 4 replicate cultures, adsorbed at 37° for 30 mins. and then overlaid with EC10. The cultures were incubated at 37° in a 5% $CO_2$ atmosphere for 24 h before staining and counting of plaques. Heparin was not found to be necessary to prevent vertical transmission of virus. The titre of the serum is that dilution giving 50% reduction in plaque count, obtained by plotting a plaque count vs log dilution graph and inspecting it.

## III. RESULTS.

"Whether or not it is clear to you, no doubt the Universe is unfolding as it should."

Pierre Trudeau.

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### 1. Serum-stimulated "Resting" BHK Cells.

#### 1.1. Optimal conditions for uptake of radioactive thymidine.

Cells were grown on glass coverslips in plastic Petri dishes and were labelled during the logarithmic phase of growth for a one hour period with $^{3}$H-thymidine of constant specific activity and at varying concentrations of unlabelled thymidine. It is clear from the results shown in Figure 3.1 that a thymidine concentration of $4 \times 10^{-6}$ M allowed maximal incorporation of radioactivity and this concentration was used in all experiments with exponentially growing cells unless otherwise stated. A concentration of $4 \times 10^{-7}$ M thymidine was chosen for labelling stationary cell cultures as nuclear pools of nucleotides are decreased in such cultures and in the G1 phase of the cell cycle (Nordenskjöld et al., 1970).

At this concentration of thymidine exponentially growing cells exhibit linear incorporation of $^{3}$H-thymidine into acid insoluble material for three hours, after which time no further increase in incorporation is observed (Figure 3.2).

Fig. 3.1.

The Effect of thymidine on the incorporation of $^{3}$H-dThd into acid-insoluble material in exponentially growing BHK cells.

Cells were grown on glass coverslips in 50 mm Petri dishes and incubated for one hour with 10 uCi/mM $^{3}$H-dThd at varying concentrations of thymidine as shown. The medium was then removed, the cell sheet washed once in ice-cold PBS(a) and fixed in 10% formol saline. Acid-insoluble radioactivity was determined by liquid scintillation counting.

Fig. 3.1

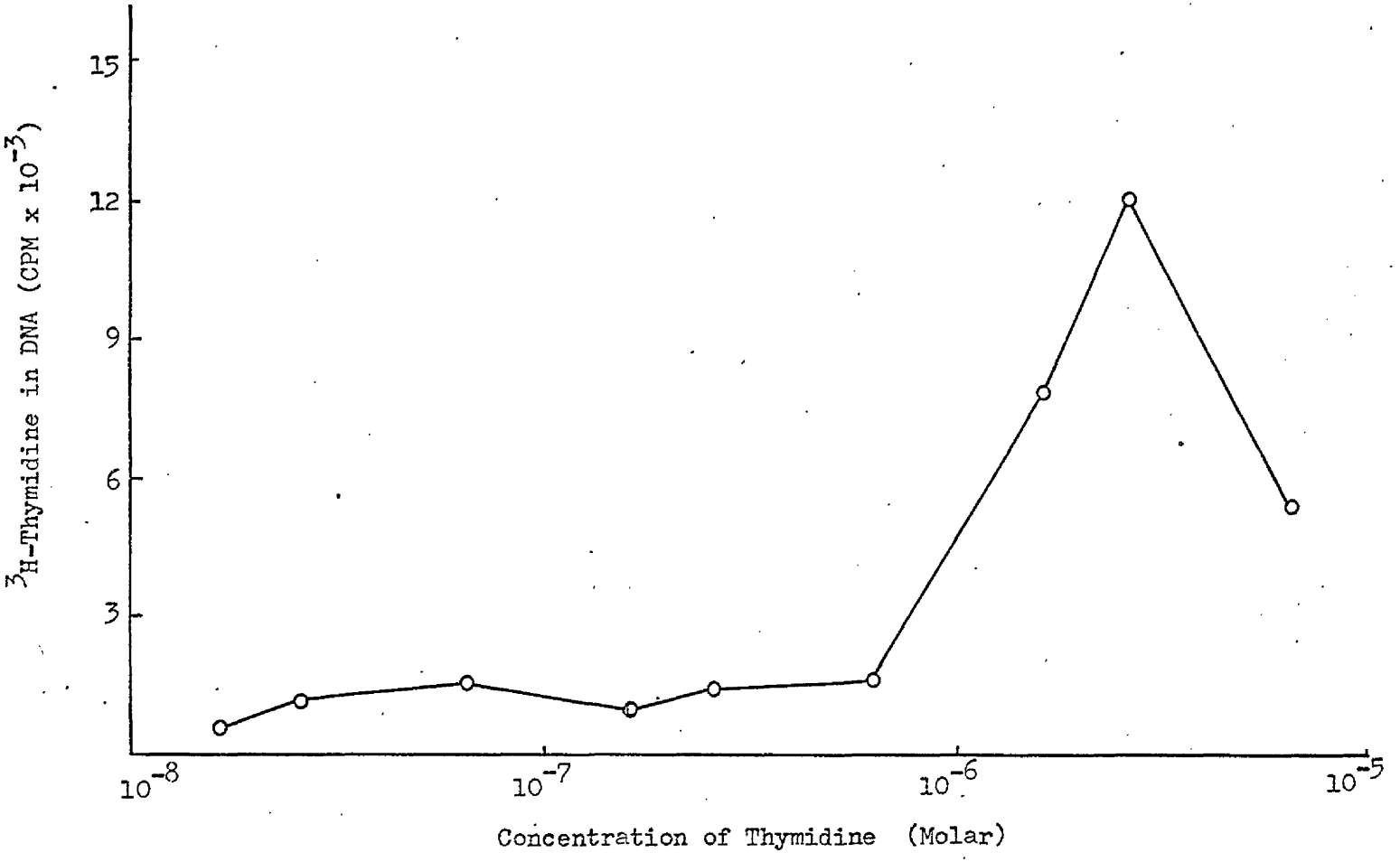
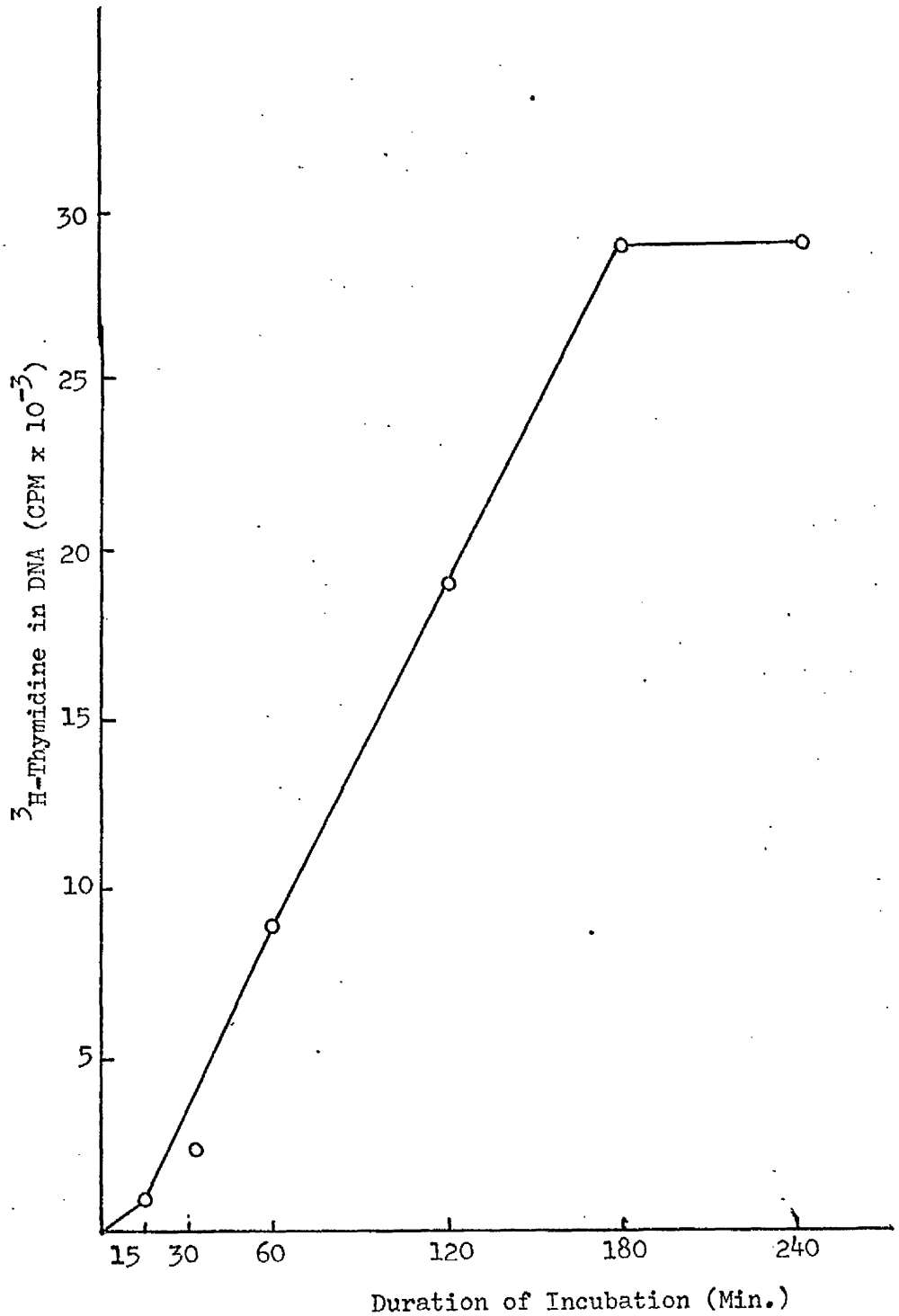

15
12
9
6
3
$^{3}$H-Thymidine in DNA (CPM x $10^{-3}$)
$10^{-8}$
$10^{-7}$
$10^{-6}$
$10^{-5}$
Concentration of Thymidine (Molar)

Fig. 3.2.

**Incorporation of $^{3}$H-dThd into acid-insoluble material in exponentially growing BHK cells as a function of the labelling period.**

Cells were grown on glass coverslips in ETC. While still in the exponential phase of growth duplicate cultures were incubated with 8 uCi/ml $^{3}$H-dTHd at $4 \times 10^{-6}$ M for periods ranging from 15 min. to 4 h. Cells were fixed in 10% formol saline, acid washed and radioactivity determined by liquid scintillation counting.

Fig. 3.2


30
25
20
15
10
5
$^3$H-Thymidine in DNA (CPM x $10^{-3}$)
15
30
60
120
180
240
Duration of Incubation (Min.)

1.2. Time Course of DNA Synthesis after Deprivation of Serum and its Readdition Five Days Later.

When C13 cells growing exponentially in ETC are transferred to Eagle's medium containing only 1% serum there is a rapid decrease in the percentage of cells incorporating thymidine (Figure 3.3a). The time course of this decrease is dependent upon the density of the cells, at higher densities the incorporation of thymidine falling faster (Figure 3.4). In all cases, however, not more than 0.5% of the cells are incorporating label by 6 days and there is no increase in cell number. In contrast to the curve of the percentage of cells incorporating thymidine, the total amount of $^{3}$H-thymidine incorporated actually rises until 48 h before beginning to fall (Figure 3.3a). The reason for this apparent discrepancy appears to be that after transfer to EC1, although fewer cells are incorporating label, those that do so take up a greater amount of label. Thus, autoradiograms of exponentially growing cells and cells 24 and 48 h after transfer to EC1 show that the latter cells contain far more radioactivity (Plate 1).

Upon the addition of serum to resting cells which had been incubated in EC1 for 5½ days, the incorporation of $^{3}$H-thymidine increases after 10 - 11 h, reaching a peak at 12 - 13 h, falling again and rising to a second, larger peak at 15 - 17 h. This is followed by a doubling in cell number (Figure 3.3b). This pattern of DNA synthesis has been

Fig. 3.3.

Effect of lowering and raising the serum concentration on DNA Synthesis in BHK cells.

Sub-confluent cultures of BHK cells grown on glass coverslips were incubated in $EC_1$, as described in the Methods section (Methods section 1.3.2.). Labelling of cells and autoradiography were performed as described in Methods section 2.2. and cell numbers were determined by triplicate haemocytometer counts being made on each culture, with duplicate cultures for each time point.

A. cells placed in EC1 at zero time;

B. serum added to 10% at zero time to cells incubated for 5.5 days in EC1.

o——o $^{3}$H-thymidine incorporation;

●——● % of cells incorporating $^{3}$H-thymidine;

□——□ cell number.

Fig. 3.3

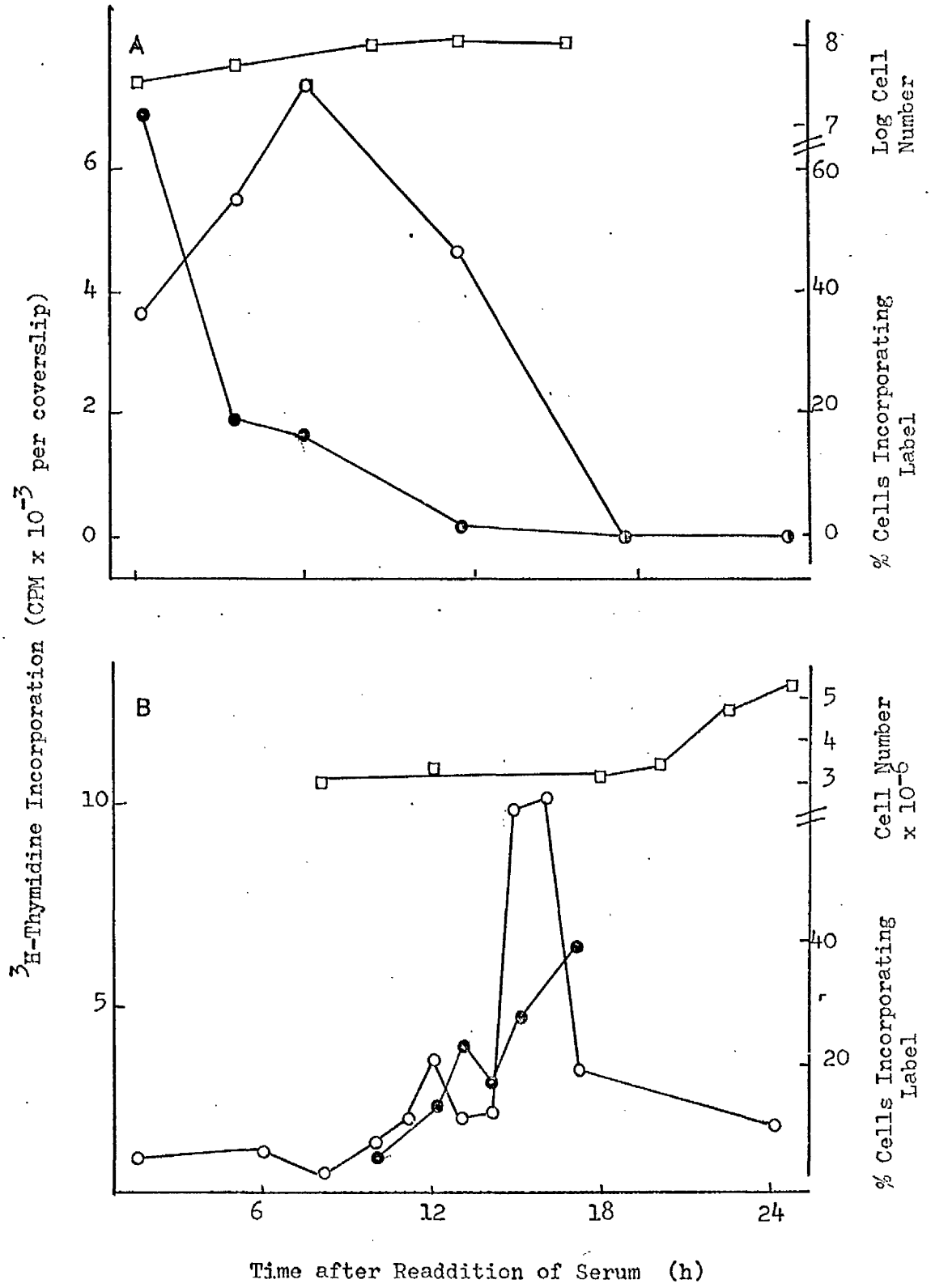

A
B
$^3$H-Thymidine Incorporation (CPM x $10^{-3}$ per coverslip)
% Cells Incorporating Label
Log Cell Number
Cell Number x $10^{-6}$
Time after Readdition of Serum (h)
0
2
4
6
8
10
20
40
60
3
5
12
18
24

Fig. 3.4.

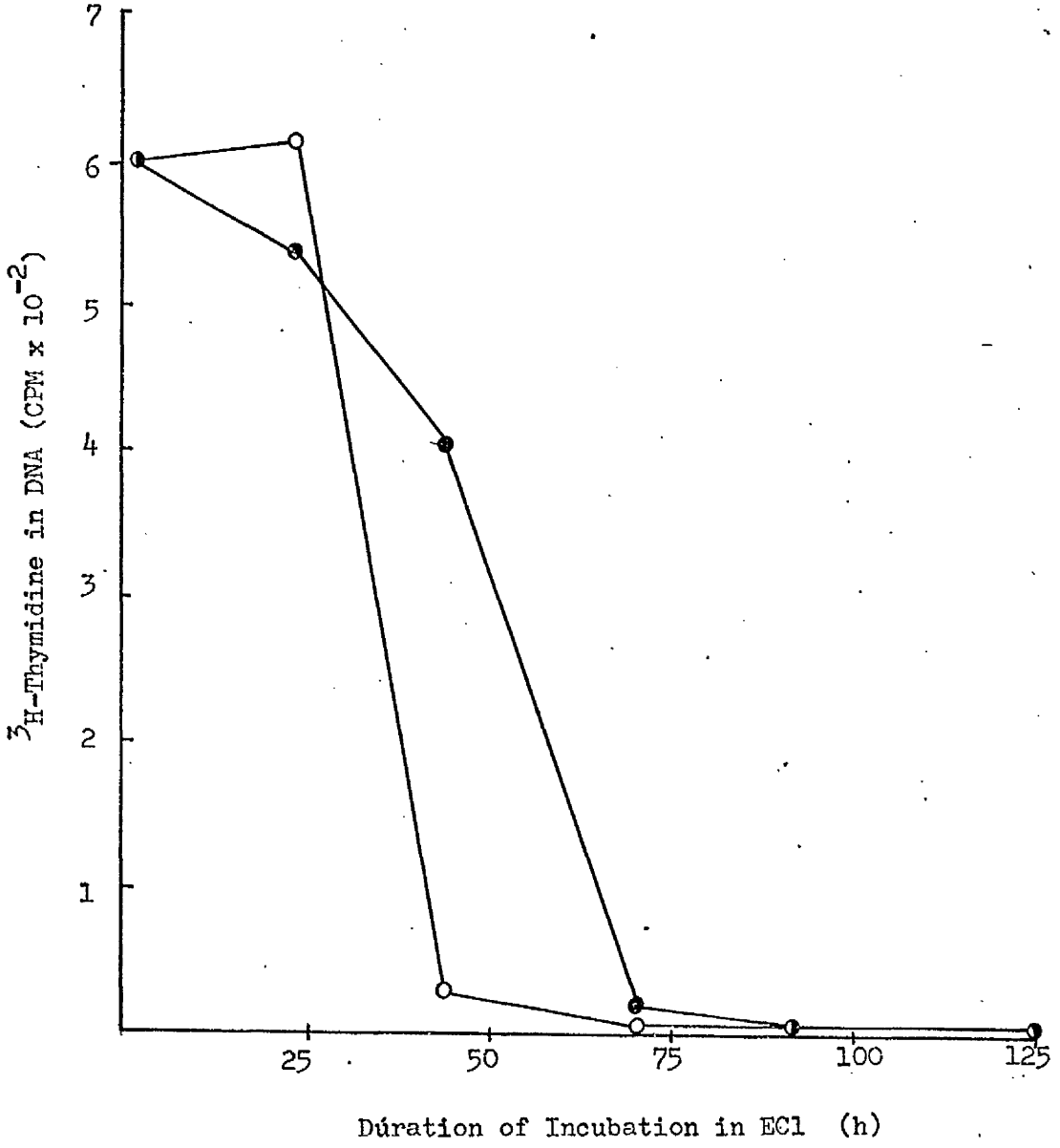
Effect of density of cell sheet on the decrease in DNA synthesis in BHK cells incubated in low serum medium.

BHK cells were seeded in ETC at $5 \times 10^5$ or $2 \times 10^6$ cells per 50 mm Petri dish and the experiment performed as in the legend to Figure 3.3. Autoradiography and cell counts were not performed on these cultures.

o——o seeded at $2 \times 10^6$ cells per dish;

●——● seeded at $5 \times 10^5$ cells per dish.

Fig. 3.4


7
6
5
4
3
2
1
$^3$H-Thymidine in DNA (CPM x $10^{-2}$)
25
50
75
100
125
Duration of Incubation in ECl (h)

constantly observed in experiments carried out over a period of about eighteen months. Autoradiography shows that both peaks of DNA synthesis involve incorporation of $^{3}$H-thymidine into nuclear material and CsCl gradient equilibrium centrifugation confirmed that the incorporation was into material with the same density as the chromosomal DNA of exponentially growing cells (Figure 3.5). A smaller percentage of cells, however, incorporated $^{3}$H-thymidine at 12 - 13 h than did at 15 - 17 h (Figure 3.3b) suggesting the possibility that the two peaks might be due to the presence of different sub-populations of cells, perhaps resulting from not all the cells being arrested at the same stage of the cell cycle when they are transferred to EC1. This is unlikely though, as the time covered by the individual peaks of $^{3}$H-thymidine incorporation is far shorter than the normal S-phase of BHK cells.

To rule out this possibility, however, the course of mitosis after these periods of DNA synthesis was determined and only a single peak of mitotic figures was observed (Figure 3.6). In addition, autoradiography of cells labelled from 11 - 12 h, 15 - 16 h and 11 - 16 h showed that the number of cells labelled in the 11 - 16 h period was no greater than the number labelled in the 15 - 16 h pulse (Table 3.1). This is the result expected if both peaks represent the same population of cells.

ERRATUM.

Between pages 141 and 142 insert

"Remington and Klevecz (1973) have observed that there is turn-over of DNA and loss of $^{3}$H-thymidine from DNA in Don C and Don cell lines in culture. Pulse-chase experiments with BHK cells revealed no discernible loss of $^{3}$H-thymidine from the DNA, so that turn-over, if it occurs, must either be on a very small scale or very rapid so that the pulse used measures predominantly stable DNA (Figure 3.7)."

Table 3.1.

'Resting' BHK cells were stimulated by the readdition of serum to 10% and labelled for the periods indicated with 8 uci/ml $^{3}$H-dThd. At the end of these periods the cells were fixed in formol saline acid-washed and autoradiographic determinations made as described in the Methods section 2.2.

Table 3.1.

Determination of the percentage of cells incorporating $^{3}$H-thymidine during various labelling periods after stimulation of 'resting' BHK cells by the readdition of serum to 10%.

| Labelling period | % of cells incorporating $^{3}$H-dThd into nuclei |
|---|---|
| 11 - 12 h | 30.1 |
| 15 - 16 h | 75.8 |
| 11 - 16 h | 68.7 |

Fig. 3.5.

Buoyant density in caesium chloride of DNA isolated from BHK cells 12 and 16 h after the readdition of serum to 'resting' cells.

'Resting' BHK cells were stimulated by adding serum to 10% and labelled for 1 h periods with 8 uCi/ml $^{3}$H-thymidine at 11 and 15 h after serum addition. Isopycnic centrifugation was performed as described in the Methods section 2.7.1.

●——● 11-12 h;

Δ——Δ 15-16 h after serum addition;

□——□ exponentially growing cells.

Fig. 3.5

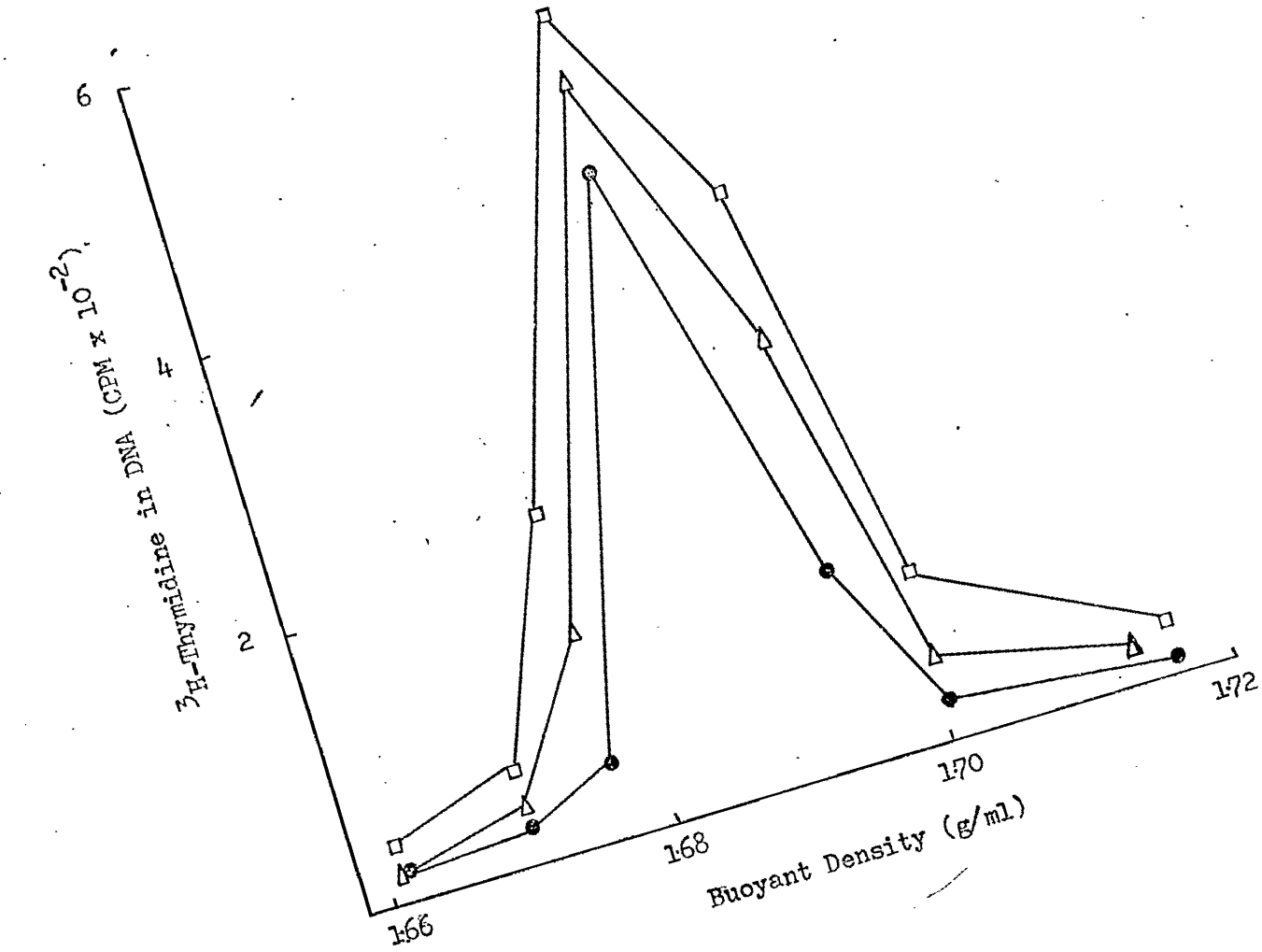

6
4
2
$^3$H-Thymidine in DNA (CPM x $10^{-2}$)
166
168
170
172
Buoyant Density (g/ml)

Fig. 3.6.

Time course of Mitosis in 'resting' BHK cells which have been stimulated to grow and divide by the addition of serum.

Conditions were as described in Methods section 2.3.

Fig. 3.6

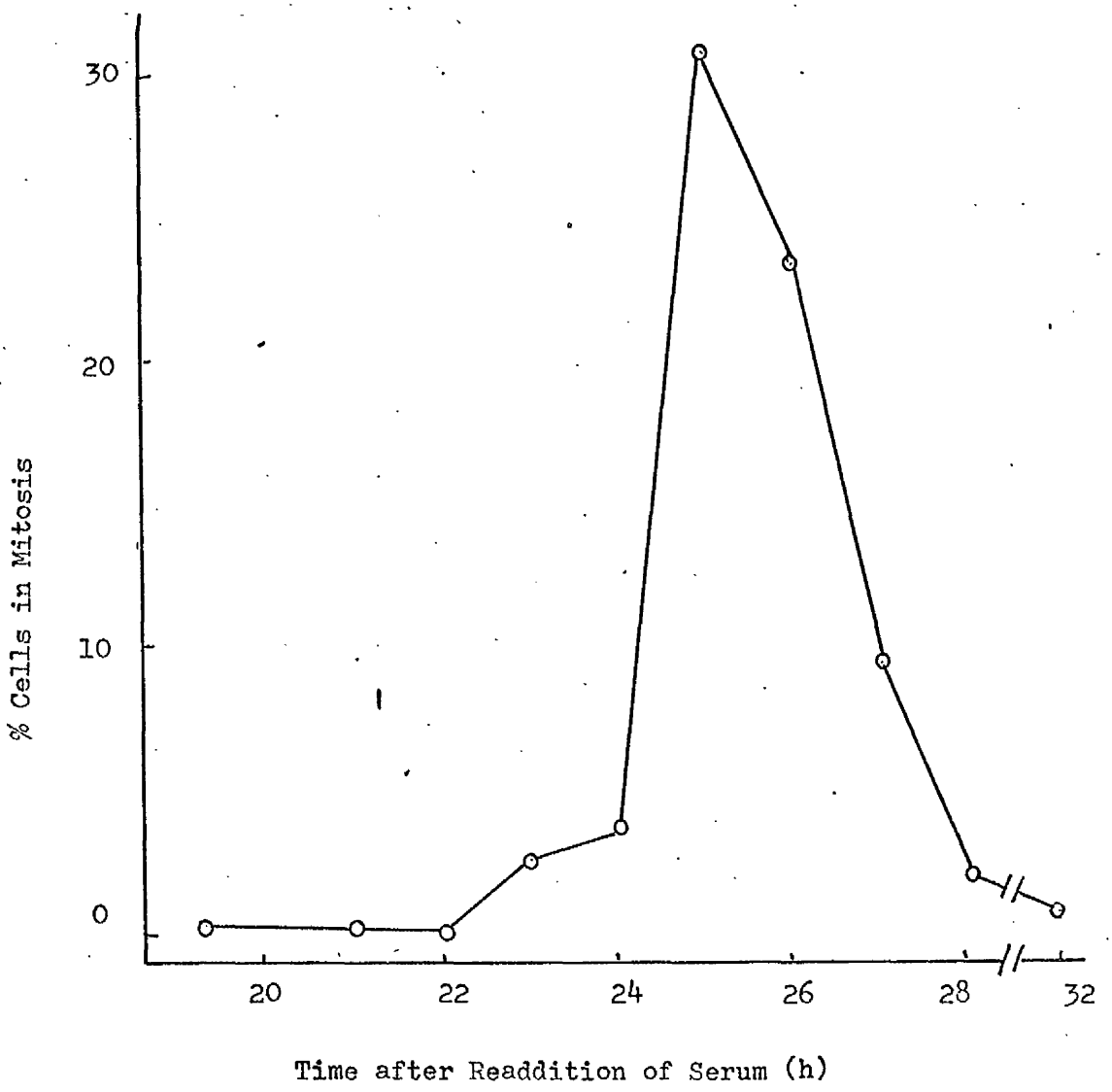

30
20
10
0
% Cells in Mitosis
20
22
24
26
28
32
Time after Readdition of Serum (h)

Fig. 3.7.

Stability of DNA Synthesised after readdition of serum to 'resting' BHK cells.

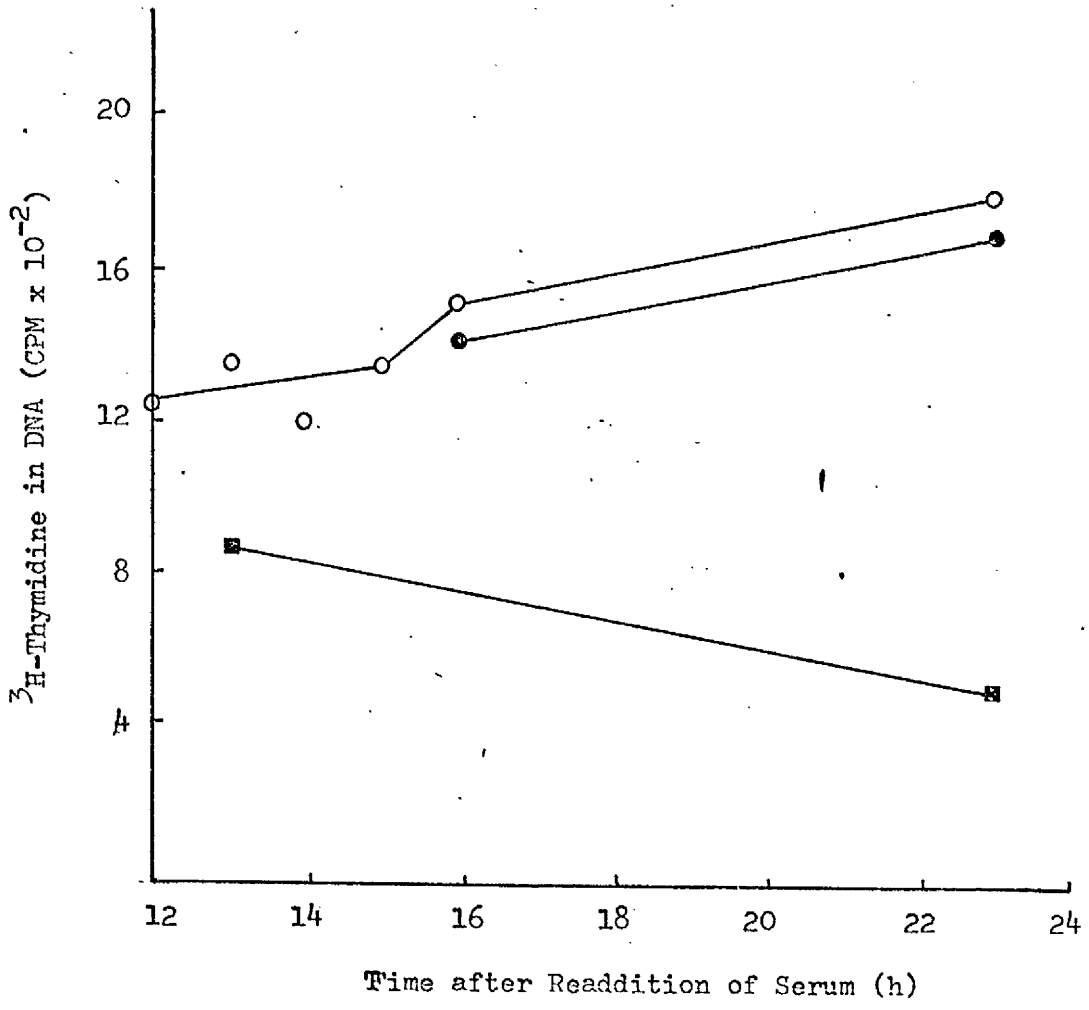
'Resting' BHK cells grown on glass coverslips were stimulated by adding serum to 10% and the cells labelled for one hour periods with 8 uCi/ml $^{3}$H-thymidine at 11, 12 and 15 h after serum addition. At the end of the labelling period the cells were washed in 5 ml fresh $EC_{10}$ containing a 2.5-fold excess of cold thymidine and incubation continued in 5 ml of the same medium. At the times indicated the cell sheet was fixed in formol saline and the acid-insoluble radioactivity determined by liquid scintillation counting.

o—o labelled 11-12 h;

■—■ labelled 12-13 h;

●—● labelled 15-16 h.

Fig. 3.7


20
16
12
8
4
$^3$H-Thymidine in DNA (CPM x $10^{-2}$)
12
14
16
18
20
22
24
Time after Readdition of Serum (h)

Ellem and Mironescu (1972) have shown in a similar serum-stimulated cell system that between the fifth and tenth hour after serum addition there is an RNA species synthesised in the cells, and when this point is reached the cells are committed to DNA synthesis. The situation in serum-stimulated BHK cells seems to be more complex, with the synthesis of perhaps two RNA species being necessary to allow DNA synthesis to proceed (Figure 3.8). If actinomycin D is added up to two hours after serum addition no DNA synthesis takes place between 15 and 16 h after the serum stimulation. If this inhibitor of RNA synthesis is added between three and seven hours after the addition of serum some incorporation of $^{3}$H-thymidine into acid-insoluble material occurs (although it does not approach the level obtained in untreated cells), suggesting that some step occurring at about three hours after serum addition, and sensitive to this drug, is necessary to allow DNA synthesis to occur. Addition of the drug eight hours after the serum stimulus again brings about an inhibition of DNA synthesis at 16 h but after this time the drug has a progressively smaller inhibitory effect on DNA synthesis. Cyclohexamide, as expected, severely restricts DNA synthesis.

## 2.2 Herpesvirus Infection.

Having characterised the resting cell system, it was thought that the study of the biology of pseudorabies virus growth could be greatly simplified by using this cell system

Fig. 3.8.

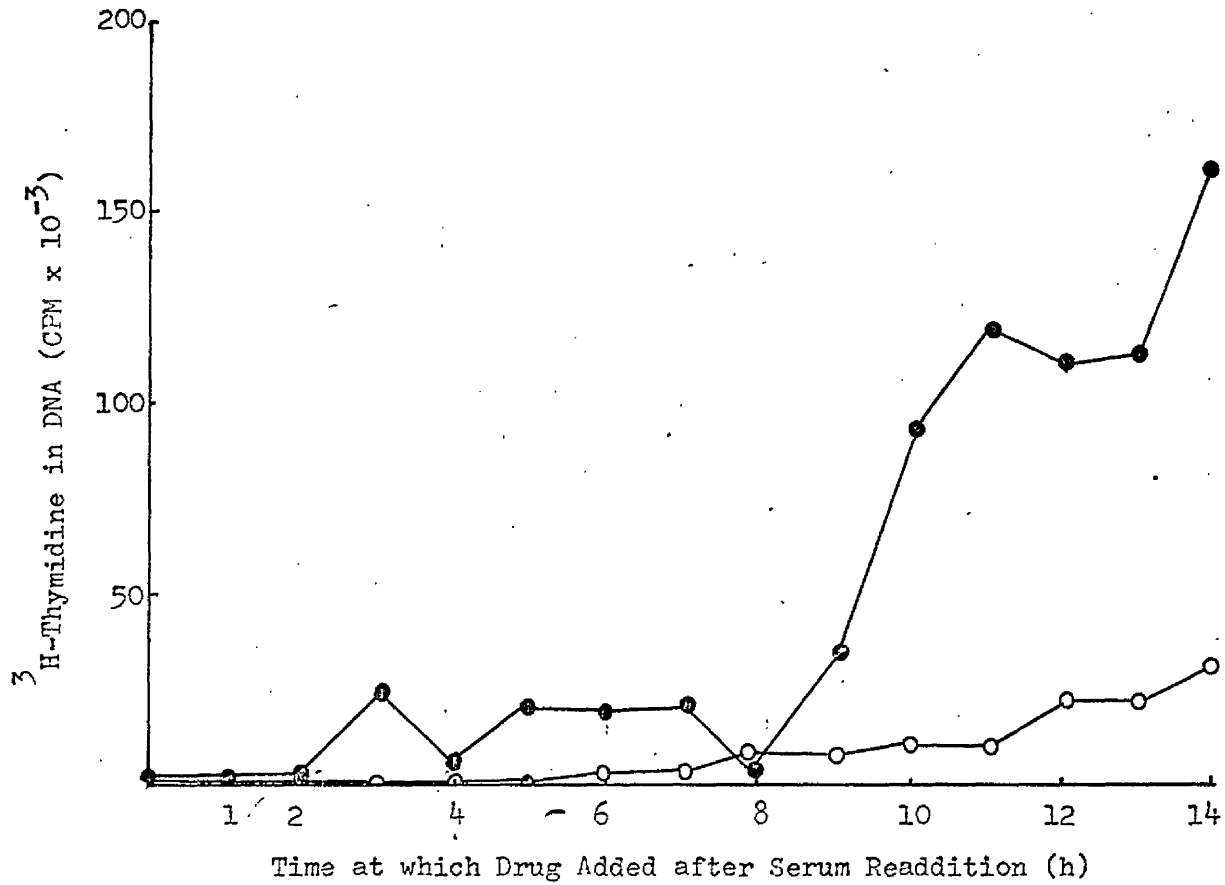
DNA synthesis in serum-stimulated 'resting' BHK cells in the presence of actinomycin-D or cycloheximide.

'Resting' cultures of BHK cells were stimulated by the addition of serum to 10% and at the times indicated actinomycin-D or cycloheximide were added to the medium of duplicate cultures to a final concentration of 0.1 ug/ml and 2 ug/ml respectively. All cultures were labelled with 8 uCi/ml $^{3}$H-thymidine at 4 x $10^{-6}$M from 15-16 h after serum addition, and the cells harvested by scraping into ice-cold tris-saline pH 7.5. The cell pellet was then dissolved in hyamine hydroxide and the acid-insoluble radioactivity subsequently determined by liquid scintillation counting.

●——● actinomycin-D treated;

o——o cycloheximide treated.

Fig. 3.8


200
150
100
50
$^3$H-Thymidine in DNA (CPM x $10^{-3}$)
1
2
4
6
8
10
12
14
Time at which Drug Added after Serum Readdition (h)

in which viral metabolites could be studied with the minimum of interference by host macromolecules. In addition, study of the inhibition of host macromolecular synthesis could be facilitated by use of this synchronous cell system.

2.1. Percentage of Cells Infected with Various Amounts of Virus.

To ensure that when studying inhibition of host cell macromolecular synthesis there were no uninfected cells to complicate interpretation of results, the amount of virus required to productively infect all the cells in the culture was determined. The results are shown in Figure 3.9. It can be seen that 20 pfu/cell was sufficient to cause production of viral DNA in 90-100% of the cells by 4 h p.i. which is the earliest time at which one can detect viral DNA synthesis. This level of virus was used in all subsequent experiments unless otherwise stated.

2.2. Antiserum to Pseudorabies Virion Antigens.

When BHK cells are induced to enter the "resting" state, those cells which are still incorporating label after 6 days in ECl appear to take up a greater amount than normally expected (see Plate 1 D). These cells are still observed after infection of the culture by pseudorabies virus. To determine whether they are susceptible to infection by pseudorabies virus or whether they are resistant, which seems possible, attempts were made to obtain fluorescent antibody to pseudorabies virion components. Pseudorabies virus, however, is extremely pathogenic

Fig. 3.9.

Percentage of cell population infected with pseudorabies virus added at various multiplicities of infection.

Virus was adsorbed in conditioned medium for 60 min. at 37° to 'resting' cells grown on glass coverslips. Unadsorbed virus was then removed and the incubation continued in conditioned medium. At various times after infection the cells were labelled for one hour with 0.2 uCi/ml $^{3}$H-thymidine at $4 \times 10^{-7}$M, fixed in formol saline, acid washed and autoradiographic determinations carried out as described in the Methods section 2.2.

o—o uninfected cells;

●—● infected with 1 pfu/cell;

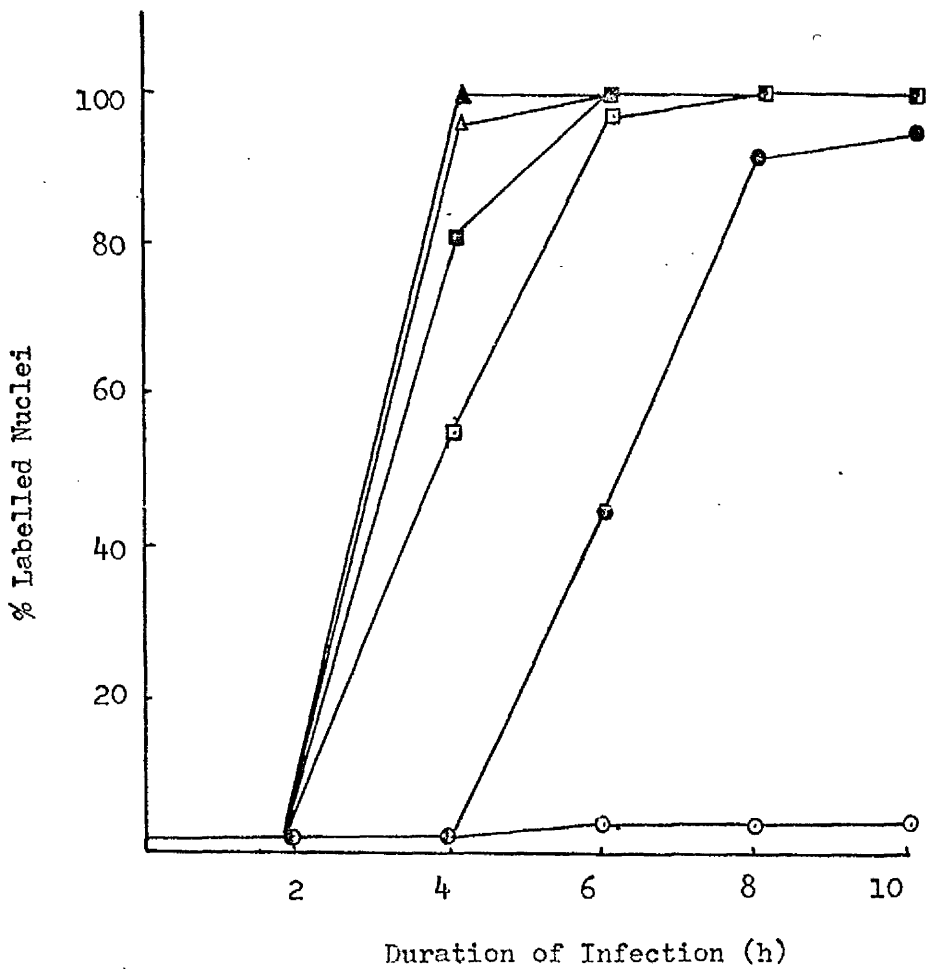
□—□ infected with 10 pfu/cell;

■—■ infected with 20 pfu/cell;

Δ—Δ infected with 50 pfu/cell;

▲—▲ infected with 100 pfu/cell.

Fig. 3.9


100
80
60
40
20
% Labelled Nuclei
2
4
6
8
10
Duration of Infection (h)

to rabbits (the animals used to produce antiserum) causing painful death in a matter of hours. In addition, the virus seems to be only weakly antigenic in these and other animals. Many difficulties, both practical and administrative, were encountered with this part of the project and no active antibody against pseudorabies virion antigens was obtained, despite strenuous effort. So far, the above question remains unanswered.

2.3. Appearance of Mature Progeny Virus.

Production of mature infectious virus particles follows the appearance of viral DNA in the cell, both in 'resting' and in exponentially growing cells (Figure 3.10). Both appearance of viral DNA and production of mature progeny virus proceed more slowly in resting cells than in exponentially growing cells and this is probably a reflection of the depleted pools of metabolic intermediates found in resting cells.

2.4. Inhibition of Cellular DNA Synthesis.

2.4.1. Time Course of Inhibition of Cellular DNA Synthesis and Synthesis of Viral DNA after Infection of BHK Cells with Pseudorabies Virus.

Kaplan and Ben-Porat (1963) reported the inhibition of host cell DNA synthesis by pseudorabies virus in rabbit kidney cells. A similar inhibition of host cell DNA synthesis is produced in BHK cells by this virus. The time course of this inhibition in exponentially growing BHK cells is shown in Figure 3.11. The synthesis of host DNA is inhibited between

Fig. 3.10.

Production of infectious progeny virus after infection of 'resting' and exponentially growing BHK cells.

Cells grown in 50 mm Petri dishes were infected with pseudorabies virus at 10 pfu/cell. Virus was adsorbed to the cells at 37° for 45 mins., excess unadsorbed virus then removed and the incubation continued in conditioned medium. At the times indicated the cell sheet was scraped off into the culture medium and cell associated virus released by three cycles of rapid freezing and thawing in a methanol-$CO_2$ freezing mixture. Cell debris was removed by centrifugation at 1000 g for 5 min. and virus determined by the plaque assay technique as described in the Methods section 1.8.

Viral DNA synthesis was determined by labelling with 8 uCi/ml $^3$H-thymidine for one hour periods. Isopycnic caesium chloride centrifugation was carried out as described in the Methods section 2.7.1.

A. cells incubated for 6 days in ECl;

B. exponentially growing cells.

●—● viral DNA;

o—o infectious virus.

Fig. 3.10

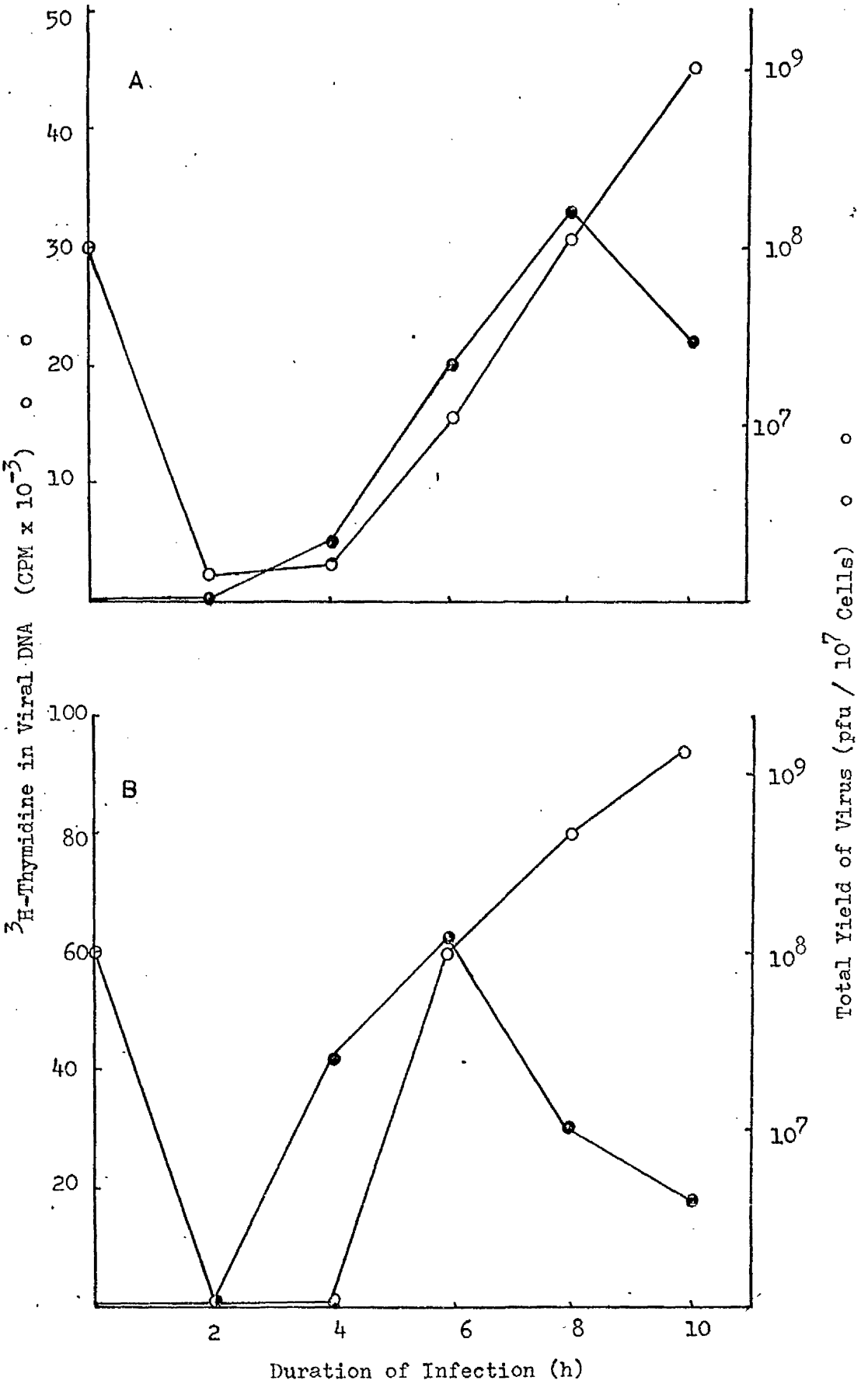

A
B
50
40
30
20
10
100
80
60
40
20
$10^9$
$10^8$
$10^7$
2
4
6
8
10
$^3$H-Thymidine in Viral DNA (CPM x $10^{-3}$)
Total Yield of Virus (pfu / $10^7$ Cells)
Duration of Infection (h)

Fig. 3.11.

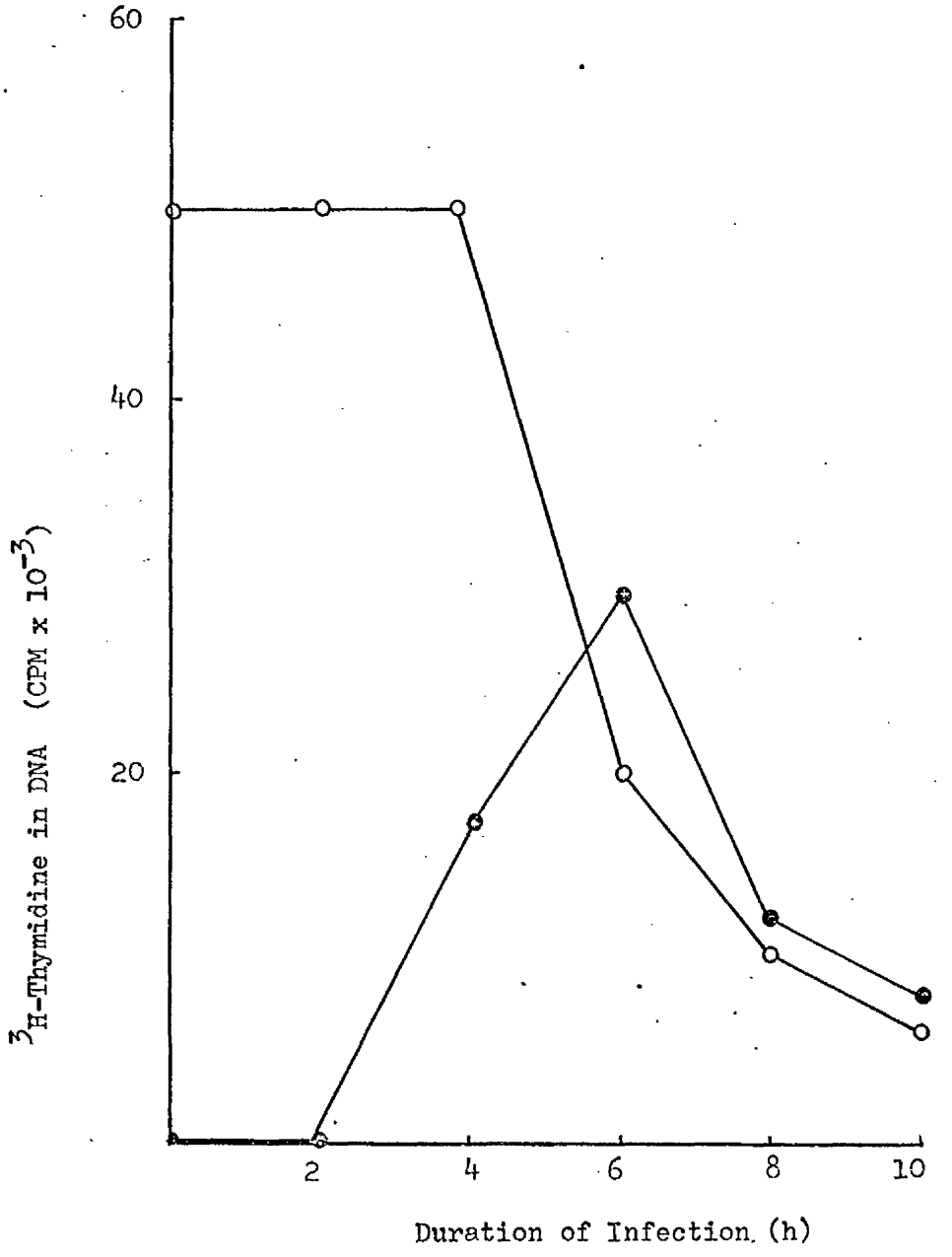
Time course of cellular and viral DNA synthesis in exponentially growing BHK cells infected with pseudorabies virus.

Duplicate cultures of exponentially growing BHK cells were infected with pseudorabies virus as described in the Methods section 1.4.1., and cells labelled at the times indicated with 8 uCi/ml $^{3}$H-thymidine at $4 \times 10^{-6}$ M for one h periods. Isopycnic caesium chloride centrifugation was carried out as described in the Methods section 2.7.1.

o—o cellular DNA;

●—● viral DNA.

Fig. 3.11


60
40
20
2
4
6
8
10
$^{3}$H-Thymidine in DNA (CPM x $10^{-3}$)
Duration of Infection (h)

4 and 6 hours after infection and rapidly falls to a very low level. Viral DNA synthesis is first detected between 4 and 6 hours. It is not clear whether the host cell DNA synthesis remaining at 10 h post infection is due to a fraction of host DNA whose synthesis is resistant to inhibition by pseudorabies virus or due simply to some cells not being infected as early in the virus growth cycle as the bulk of the population. Occasionally, the inhibition of host cell DNA synthesis and the appearance of viral DNA occur as early as 3 h post infection. Pseudorabies virus also inhibits the synthesis of host cell DNA in 'resting' cells stimulated by the addition of serum. This effect is seen by 3 h post infection at which time viral DNA also first appears (Figure 3.12). The level of host DNA synthesis plateaus from 3 to 5 h post infection but by 6 h the inhibition is complete.

2.4.2. Effect of Multiplicity of Infection on Inhibition of Cellular DNA Synthesis by Pseudorabies Virus.

It was thought that this biphase effect on host cell DNA synthesis may be due either to a differential effect of virus on initiation and elongation of host DNA synthesis or more simply due to some cells not being infected until 6 hours post infection. In an attempt to differentiate between these two possibilities the same experiment was performed with varying multiplicities of virus to bring about infection of the whole cell population at earlier times (Figure 3.13). It appears that increasing the multiplicity of infection of virus from

Fig. 3.12.

Time course of cellular and viral DNA synthesis in serum-stimulated 'resting' BHK cells infected with pseudorabies virus.

'Resting' BHK cells were stimulated by addition of serum to 10% at zero time and virus adsorbed at various times later for 60 min. periods at 37°. After removal of unadsorbed virus, incubation was continued in the same medium and all cultures labelled from 15-16 h after serum addition with 0.8 uCi/ml $^{3}$H-thymidine at $4 \times 10^{-7}$M. Total DNA was extracted and analysed by isopycnic caesium chloride centrifugation as described in the Methods section 2.7.1.

o——o cellular DNA;

●——● viral DNA.

Fig. 3.12

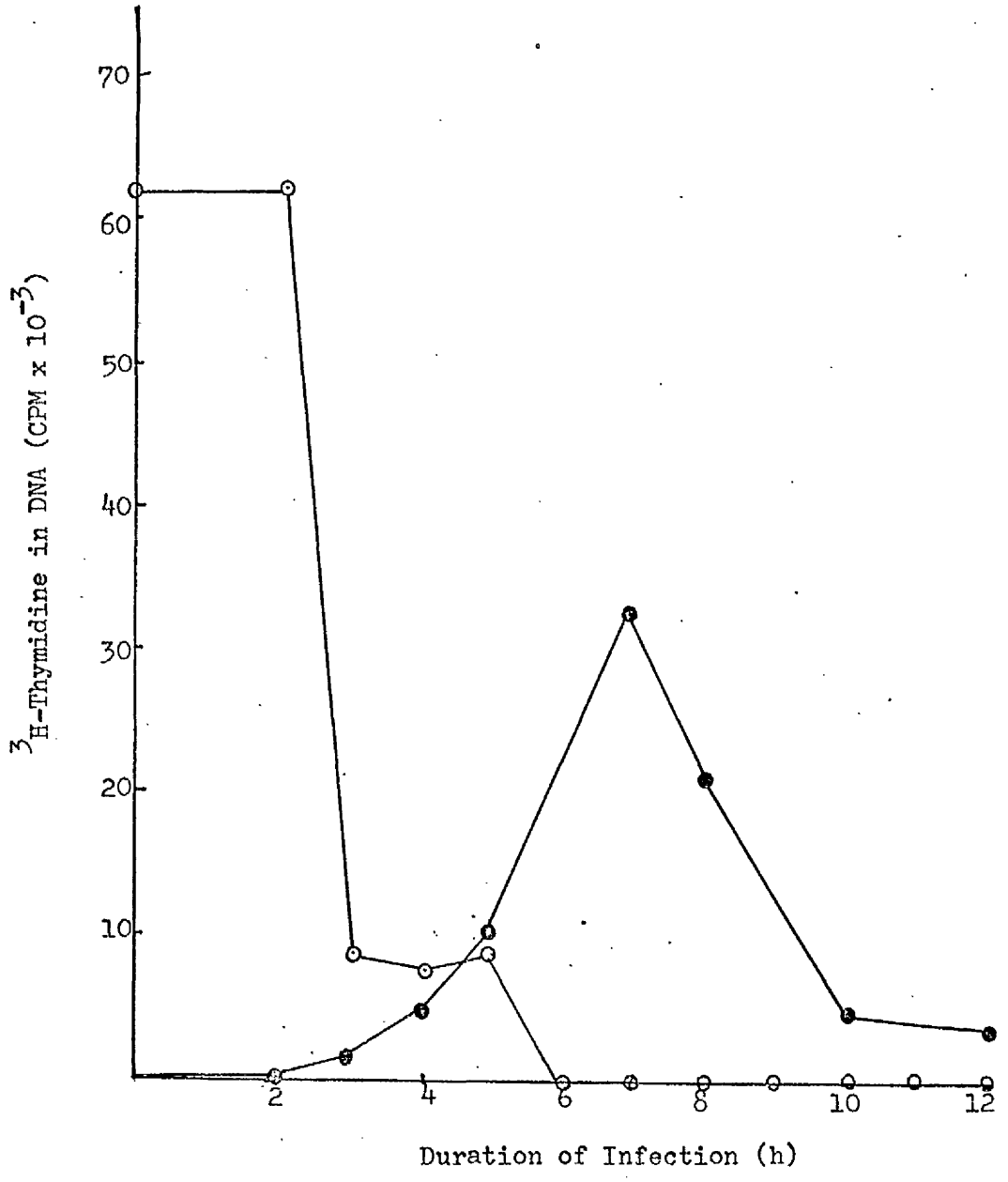

70
60
50
40
30
20
10
$^3$H-Thymidine in DNA (CPM x $10^{-3}$)
2
4
6
8
10
12
Duration of Infection (h)

Fig. 3.13.

**Effect on inhibition of cellular DNA synthesis in serum-stimulated 'resting' BHK cells of varying the input multiplicity of virus.**

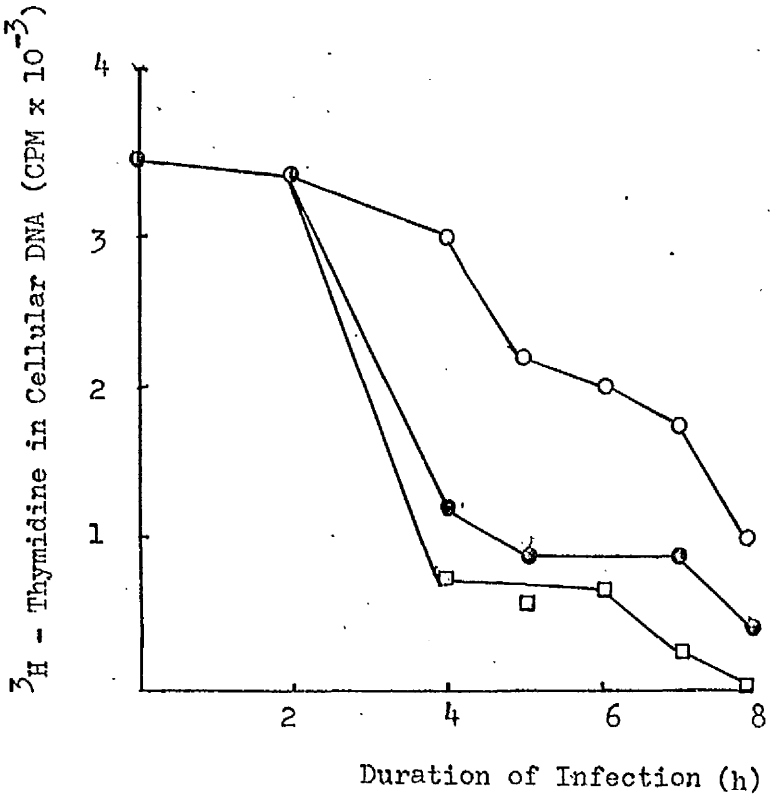
Cells were infected at various multiplicities of infection and labelled with $^{3}$H-thymidine as described in the legend to Figure 3.12. Cellular DNA was determined by isopycnic caesium chloride centrifugation as described in the Methods section 2.7.1.

o——o infected with 1 pfu/cell;

●——● infected with 20 pfu/cell;

□——□ infected with 100 pfu/cell.

Fig. 3.13


$^3_H$ - Thymidine in Cellular DNA (CPM x $10^{-3}$)
4
3
2
1
2
4
6
8
Duration of Infection (h)

1 pfu/cell to 100 pfu/cell has little effect on the profile of inhibition of cell DNA synthesis by the virus. Using 1 pfu/cell the inhibition is much slower, as one might expect since at this m.o.i. only 45% of the cells are infected by 6 h (see Figure 3.9). At a m.o.i. of 100 pfu/cell all cells are infected by between 4 and 5 hours p.i., making it unlikely that the residual host DNA synthesis from 3 to 6 h p.i. is due to DNA synthesis in uninfected cells.

2.4.3. Inhibition of Elongation of Cellular DNA by Pseudorabies Virus.

Kaplan and Ben-Porat (1963) claimed that the decrease in the rate of cellular DNA synthesis after infection with pseudorabies virus was not due to the inability of the infected rabbit kidney cells to enter into the S-phase of the growth cycle. Ensminger and Tamm (1970) using sedimentation analyses in alkaline sucrose gradients showed that Newcastle Disease Virus and Mengovirus inhibit the initiation of cellular DNA synthesis but that propagation of chains already initiated proceeds with normal kinetics. Applying this technique to the study of inhibition of cellular DNA synthesis by pseudorabies virus is complicated by the fact that pseudorabies virus is a DNA-containing virus whereas the viruses used by Ensminger and Tamm had an RNA genome. Sedimentation profiles of total DNA from pseudorabies infected cells are complicated by the presence of viral DNA at later times, but by a suitable choice of time at which to study these profiles the interference by

viral DNA can be minimised. Figure 3.14 depicts the results of such an experiment. As expected, DNA from uninfected cells subjected to velocity sedimentation on alkaline sucrose gradients after a 3 h 'chase' with 100-fold excess unlabelled thymidine moves further into the gradient than that from 'unchased' cells (Figures 3.14a, b). 5 ug/ml cycloheximide does not prevent this migration (Figure 3.14c). In comparison, the profiles obtained with DNA from 'unchased' infected cells 4-5 h post infection, is similar to that of 'unchased' uninfected cells while a 3 h chase has not resulted inaa progress of the DNA further into the gradient (Figures 3.14c, d). This would indicate that pseudorabies virus infection leads to an inhibition of propagation of host cell DNA synthesis. Whether there is also an effect on initiation of host cell DNA synthesis is not clear as the radioactivity in these sucrose gradients is qualitative but not quantitative.

2.4.4. Inhibition of Cellular DNA synthesis by Virion Components.

It was considered a possibility that the factor(s) responsible for the inhibition of host DNA synthesis by pseudorabies virus may be a component of the mature infectious virions. It is well known that virion proteins in many viruses have enzyme activity. For example, it has been reported that vaccinia virus has a virion-associated DNA-dependent RNA polymerase (Kates and McAuslan, 1967; Munyon et al., 1967); that reovirus has a virion-associated double-stranded RNA-

Fig. 3.14.

Inhibition of propagation of cellular DNA synthesis by infection of BHK cells with pseudorabies virus.

Exponentially growing cells were infected as described in the Methods section 1.4.1. Labelling was carried out for 10 min. at 4.5 h p.i. with 8 uCi/ml $^{3}$H-thymidine at 4 x $10^{-6}$M. The cell sheet was then quickly washed in ice-cold PBS(a), scraped off in ice-cold tris-saline, pH 7.5, and the cell pellet collected by centrifugation at 1000 g for 2.5 min. A chase was carried out by washing the cell sheet in warm conditioned medium after the labelling period and continuing the incubation in the presence of 100-fold excess unlabelled thymidine for 3 h. Cycloheximide, when used, was added to uninfected cells to a final concentration of 5 ug/ml for 4 h before labelling. Velocity sedimentation in alkaline sucrose gradients was performed as described in the Methods section 2.7.2.

A. uninfected cells labelled for 10 min;

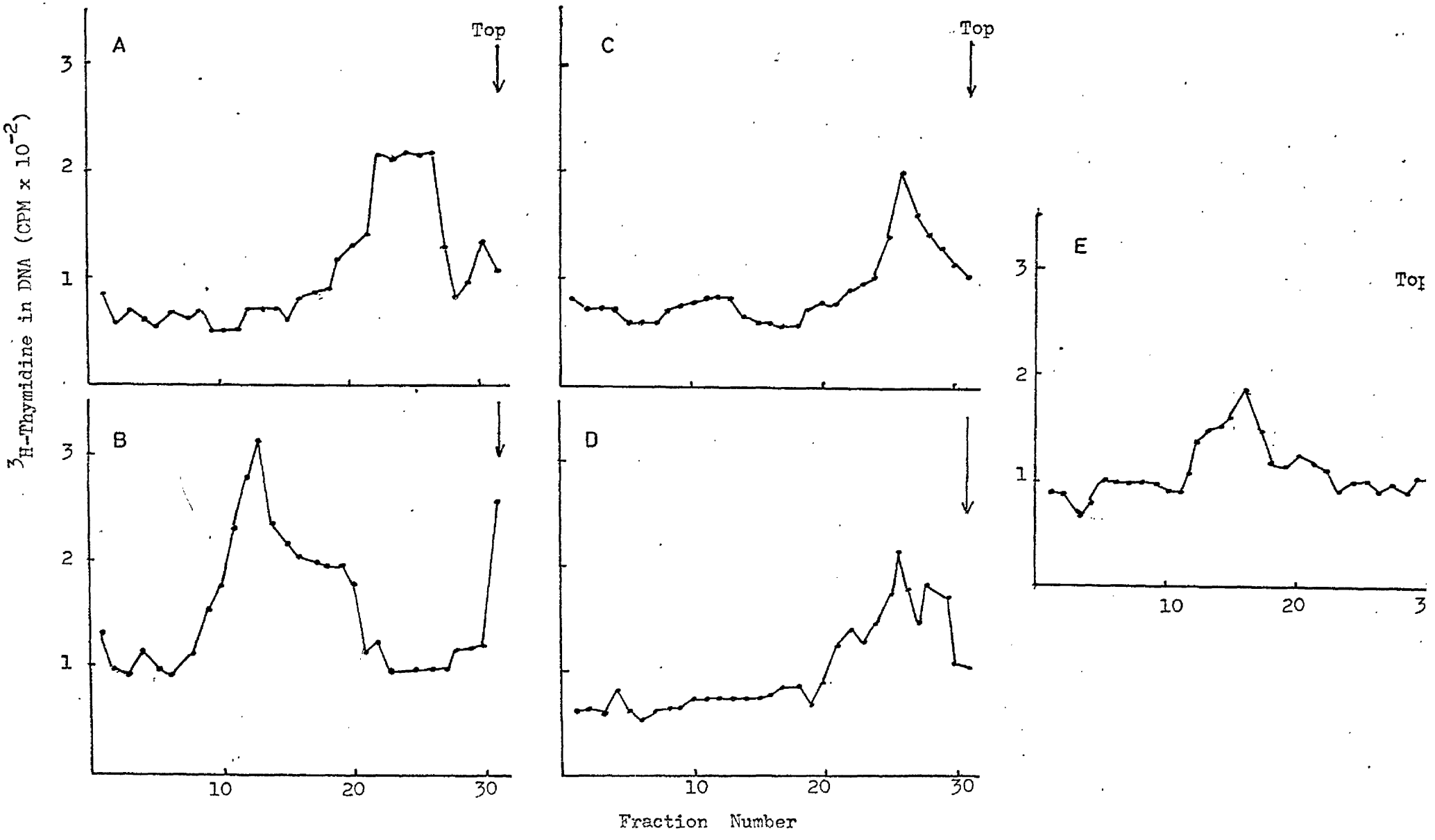
B. uninfected cells labelled for 10 min. and chased for 3 h;

C. uninfected cells in presence of 5 ug/ml cycloheximide, labelled for 10 min. and chased for 3 h;

D. infected cells labelled for 10 min;

E. infected cells labelled for 10 min. and chased for 3 h.

Fig. 3.14


A
B
C
D
E
Top
Top
Top
3
2
1
10
20
30
$^3$H-Thymidine in DNA (CPM x $10^{-2}$)
Fraction Number

dependent RNA polymerase (Borsa and Graham, 1968; Shatkin and Sipe, 1968); that adenovirus types 2 and 12 have an endonuclease activity associated with the penton peptides (Burlingham et al., 1971); and that the herpes simplex virion contains a protein kinase (Rubenstein et al., 1972).

In an attempt to determine whether the effect on cellular DNA synthesis was caused by a virion-associated factor ultra-violet light was used to inactivate the virus genome and so prevent production of virus-coded factors after infection. Yamashita et al., (1971) reported that u.v.-irradiated adenovirus types 2 and 12 suppressed the initiation of cellular DNA synthesis in HEK cells but that chain elongation of nascent DNA progressed normally. This suppression was dependent on the multiplicity of infection and the activity responsible was found in the u.v.-irradiated virion fractions, isolated in isopycnic CsCl gradients. Yaoi and Amano (1970) have reported a similar effect of u.v.-irradiated vesicular Stomatitis Virus on the initiation of DNA synthesis in cultured chick embryo cells. Once again no effect on propagation of nascent DNA chains was observed. Our results are shown in Figure 3.15. As can be seen from Figure 3.15a, u.v.-inactivated virus does not synthesise any appreciable amounts of viral DNA in 'resting' cells. No mature progeny virus could be detected by the plaque assay technique. It is also clear that, while the wild-type virus produces the typical rapid inhibition of cellular DNA synthesis in exponentially

Fig. 3.15.

Effect on cellular DNA synthesis of infection of BHK cells with u.v.-inactivated pseudorabies virus.

BHK cells grown on coverslips were either incubated for 4 days in EC1 before use or were used when growing exponentially. Virus was adsorbed to the cells for 45 min. at 37° and after removal of unadsorbed virus the incubation was continued in conditioned medium. Cells were labelled for one hour periods at the times indicated with 8 uCi/ml $^{3}$H-thymidine at a final concentration of $4 \times 10^{-6}$M. At the end of the labelling period the cells were fixed in 10% formol saline and acid-insoluble radioactivity determined by liquid scintillation counting.

A. cells incubated for 4 days in EC1;

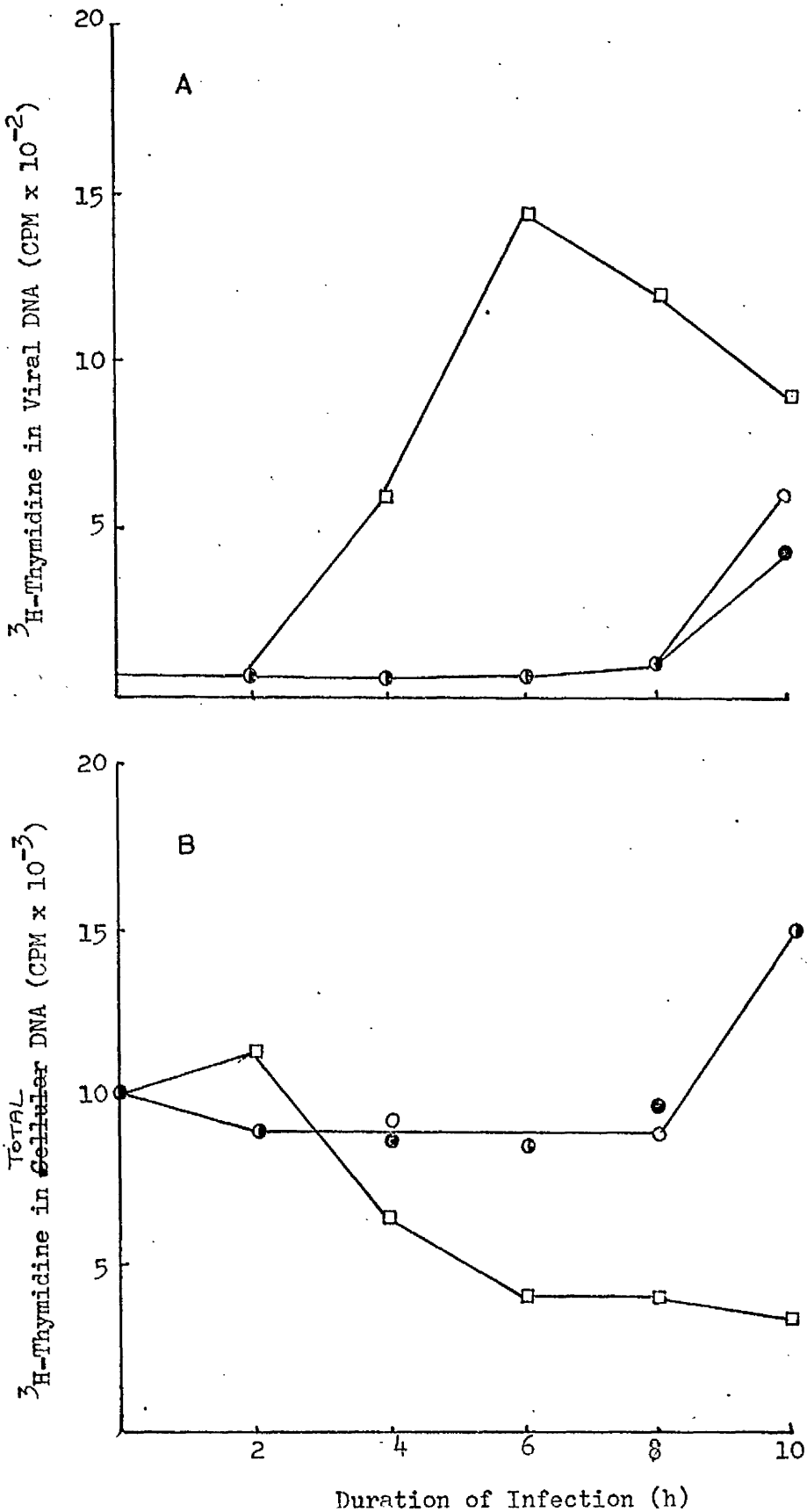
B. exponentially growing cells.

o—o uninfected cells;

infected with wild-type virus;

infected with u.v.-inactivated virus.

Fig. 3.15


A
B
20
15
10
5
$^3$H-Thymidine in Viral DNA (CPM x $10^{-2}$)
$^3$H-Thymidine in Total DNA (CPM x $10^{-3}$)
2
4
6
8
10
Duration of Infection (h)

growing cells, the u.v.-irradiated virus has little or no effect on cellular DNA synthesis (Figure 3.15b). The rise in DNA synthesis between 8 and 10 h p.i. in the uninfected and u.v.-irradiated virus infected cultures is probably due to the stimulation of cellular DNA synthesis caused by the addition of fresh medium to the cells after adsorption of virus. These results seem to indicate that the factor(s) responsible for the inhibition of cellular DNA synthesis are produced after infection by a process which requires a functional virus genome, and which are not components of the infecting virion.

It is conceivable, however, that u.v.-irradiation may have altered the virion structure so that inhibitory factor(s) present therein were no longer able to function. An attempt to clarify this point was made by irradiating the virus with x-rays, but due to technical and other difficulties with the nuclear reactor this series of experiments had to be curtailed without having obtained a suitably inactivated virus preparation.

2.4.5. Effect of Inhibitors of Protein Synthesis on the Inhibitory Process.

Assuming that inhibition of cellular DNA synthesis is due to synthesis of a specific factor(s) by the virus early in infection, one might expect that inhibitors of protein synthesis might prevent the production of this factor and so prevent the virus from displaying the inhibitory effect on cellular DNA

synthesis. It is clear from Figures 3.16a and 3.16b that, while protein synthesis in uninfected BHK cells is dramatically reduced by low levels of cycloheximide, DNA synthesis is little affected at levels of 0.5 ug/ml or less. It is important to establish that the drug has little direct effect on DNA synthesis as this may make interpretation of later results confusing. In virus-infected 'resting' cells the inhibition of protein synthesis is slightly greater than in similar uninfected cells (Figure 3.16c). This is probably due to the inhibitory effect of the virus itself on cellular protein synthesis (zero cycloheximide curve in Figure 3.16c). Viral DNA synthesis can proceed in the presence of low levels of cycloheximide but appears to be delayed by several hours (Figure 3.16d). The DNA synthesised in these cells is typical virus DNA as determined by isopycnic centrifugation in caesium chloride. Cellular DNA synthesis is still inhibited by pseudorabies virus in the presence of 0.2 ug/ml cycloheximide (Figure 3.16e) although this effect is less pronounced at later times than in the cells which have not been treated with the drug. It appears that these low levels of drug are insufficient to prevent production of the viral product(s) necessary for the inhibition of cellular DNA synthesis. Higher levels of the drug, however, have a significant effect on cellular DNA synthesis in the absence of added virus and were not used in these experiments.

Fig. 3.16.

**Effect of cycloheximide on the inhibition of cellular DNA synthesis by pseudorabies virus in BHK cells.**

Cells were infected with virus as described in the Methods section 1.4. and treated with cycloheximide as described in the Methods section 2.9. Cultures were labelled for one hour periods with either 8 uCi/ml $^{3}$H-thymidine at a final concentration of $4 \times 10^{-6}$ or $4 \times 10^{-7}$M or with 0.2 uCi/ml of a $^{14}$C-amino acid mixture.

Radioactivity in DNA was estimated as described in the Methods section 2.8.2. or extracted for analysis on isopycnic caesium chloride density gradients as described in the Methods section 2.7.1. Radioactivity in protein was estimated by dissolving the acid-washed cell pellet in hyamine hydroxide as described in the Methods section 2.8.1.

A. protein synthesis in exponentially growing cells treated with cycloheximide;

B. DNA synthesis in exponentially growing cells treated with cycloheximide.

●—● untreated cultures; △—△ 0.5 ug/ml cycloheximide;

o—o 0.1 ug/ml cycloheximide; □—□ 1.0 ug/ml cycloheximide;

▲—▲ 0.2 ug/ml cycloheximide; ■—■ 2.0 ug/ml cycloheximide;

o—o 5.0 ug/ml cycloheximide.

C. protein synthesis in pseudorabies virus infected 'resting' cells treated with cycloheximide;

continued.....

(2)

Fig. 3.16 contd.

B. Viral DNA synthesis in pseudorabies virus infected 'resting' cells treated with cycloheximide;

E. cellular DNA synthesis in exponentially growing cells infected with pseudorabies virus and treated with cycloheximide.

o——o untreated, uninfected cultures;

●——● untreated, infected cultures;

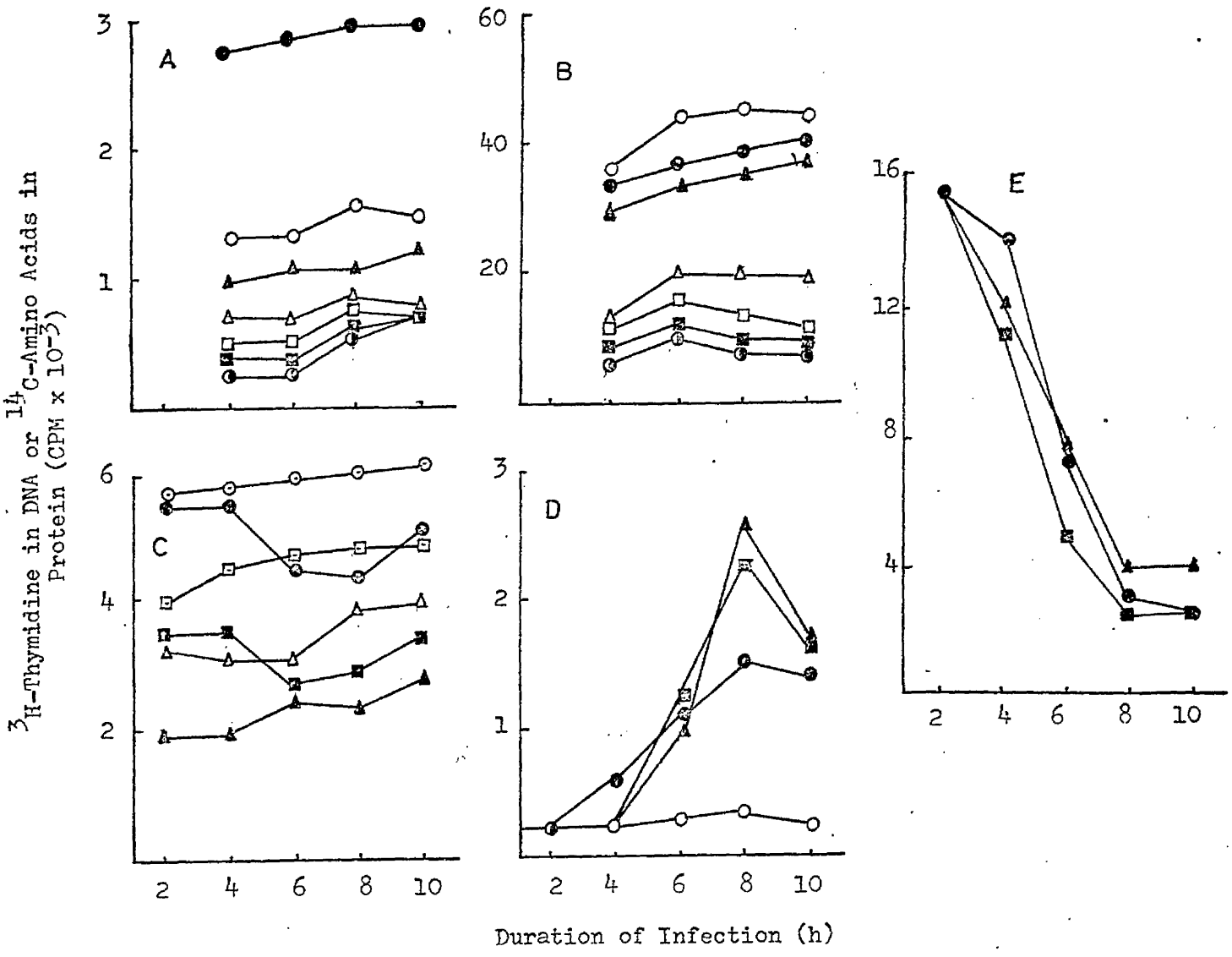
□——□ uninfected, 0.1 ug/ml cycloheximide;

■——■ infected, 0.1 ug/ml cycloheximide;

Δ——Δ uninfected, 0.2 ug/ml cycloheximide;

▲——▲ infected, 0.2 ug/ml cycloheximide.

Fig. 3.16


A
B
C
D
E
$^{3}$H-Thymidine in DNA or $^{14}$C-Amino Acids in Protein (CPM x $10^{-3}$)
Duration of Infection (h)

2.4.6. Addition of Extracts of Pseudorabies Virus-Infected Cells to Uninfected Cell Cultures.

In an attempt to identify the factor(s) in infected cells responsible for bringing about the inhibition of cellular DNA synthesis, simple extracts of infected cells were prepared and added back to uninfected cells on the assumption that uptake of these factor(s) by these cells would bring about an inhibition of cellular DNA synthesis. Care was taken to ensure that the extracts contained no infectious virus. Elimination of infectious virus was achieved either by ultracentrifugation to pellet the virus and thence using only the top regions of the supernatant, or by u.v.-irradiation of the extract. All extracts were examined for the presence of virus by the plaque assay technique.

Poly-L-$\alpha$-ornithine was used at 10 ug/ml to assist the uptake by the cells of material from the extracts. These experiments, however, did not reveal the presence of any factors taken up by cells and causing inhibition of cellular DNA. This may simply reflect that the rather vigorous techniques used to prepare these extracts have destroyed the inhibitory factors or that these factors are not taken up by the cells in an active form. Alternatively, they may conceivably be part of a large DNA replication complex, the destruction of which in the isolation of the factor(s) destroys their inhibitory activity.

2.4.7. Temperature Sensitive Mutants of Pseudorabies Virus.

The processes involved in the replication of herpesviruses have been rather poorly defined, part of the reason being the complexity arising from a genome whose coding potential may encompass 150 - 200 polypeptides. Some resolution of the complexity should be achieved by the isolation and study of temperature sensitive (ts) virus mutants since any indispensable function of the viral genome will potentially be affected by mutation of this type.

2.4.7.1. Isolation.

The isolation and characterisation of the ten ts mutants of pseudorabies virus described here has been reported by Pringle et al., (1973). These mutants were isolated after exposure to 5 ug/ml BrdUrd and, unlike the wild type virus, are unable to multiply in BHK cells at 41°.

2.4.7.2. Synthesis of Viral DNA and Inhibition of Cellular DNA Synthesis.

All of the mutants synthesise viral DNA and inhibit host cell DNA synthesis at the permissive temperature (37°). No mutant, however, incorporates radioactivity into viral DNA as effectively as wild type virus (Table 3.2). On the other hand, nine of the ten mutants inhibit cellular DNA synthesis at 37° to approximately the same extent as wild type virus. The exception is ts 6 which restricts cellular DNA synthesis to about 5% of the mock-infected cell level.

## Table 3.2

The data are taken from CsCl gradient fractionations of cellular and PrV DNA in several different experiments; examples of these are given in Figs. 3.17 and 3.18. Total radioactivity in peaks has been expressed as a percentage either of mock-infected cell DNA synthesis (for host DNA) or of $ts^+$ viral DNA synthesis (for viral DNA). Labelling time was from 4 to 24 hr.

Table 3.2

DNA Synthesis as a Percentage of Mock-infected or Wild-type PrV-infected Value

| In cells infected with | Permissive temperature (37°) | | Nonpermissive temperature (41°) | |
|---|---|---|---|---|
| | Host DNA | Viral DNA | Host DNA | Viral DNA |
| $ts^+$ | 18 | 100 | 15 | 100 |
| ts 1 | 20 | 32 | 58 | 4 |
| ts 2 | 21 | 60 | 7 | 25 |
| ts 3 | 19 | 50 | 31 | 38 |
| ts 4 | 21 | 71 | 29 | 0 |
| ts 5 | 15 | 26 | 66 | 0 |
| ts 6 | 5 | 32 | 11 | 17 |
| ts 7 | 21 | 32 | 37 | 0 |
| ts 8 | 16 | 35 | 19 | 19 |
| ts 9 | 18 | 36 | 37 | 28 |
| ts 10 | 21 | 30 | 44 | 1 |
| Mock-infected cells | 100 | - | 100 | - |

At the restrictive temperature (41°) three of the mutants fail to synthesise detectable amounts of viral DNA and two others exhibit only very low levels of incorporation of $^{3}$H-thymidine. None of the mutants synthesise viral DNA to the same extent as wild type virus. Cellular DNA synthesis is inhibited by the mutants to a variable extent (Table 3.2) and there is some correlation between the ability to inhibit this host function and the ability to synthesise viral DNA. The mutants which are defective for DNA synthesis reduce host DNA synthesis by 29 - 66% of normal whereas the other five mutants inhibit cellular DNA synthesis to 7 - 31% of the mock-infected value. Similar results were obtained using ($^{32}$P)-orthophosphate, thus ruling out the possibility that the apparent lack of viral DNA synthesis was due to a defect in thymidine uptake. No differences could be detected in the density in caesium chloride of mutant virus DNA as compared to that of the wild-type virus. Figures 3.17 and 3.18 are illustrative of the profiles obtained in caesium chloride isopycnic gradients.

3. DNA Synthesis in vitro in Nuclei Isolated from Pseudorabies Virus-Infected and Uninfected BHK Cells.

It has been shown by several groups of workers that isolated nuclei from virus-infected cells can support the continued maturation of virus for some time after isolation. For example,

Fine and Ludwig (1970a, b) and Radask (1973) have shown that isolated nuclei from herpesvirus infected cells can incorporate radioactive precursors of DNA into acid-insoluble material and Sussenbach and van der Vliet (1973) have shown that nuclei from adenovirus infected KB cells synthesise viral DNA and have interpreted their results to show that the mechanism of viral DNA synthesis in the isolated nuclei reflects the intracellular situation.

A system was developed using isolated nuclei from pseudorabies infected BHK cells in which the processes involved in virus DNA synthesis and the inhibition of cellular DNA synthesis could conceivably be studied and which allowed a study of the roles of cytoplasmic and nuclear components in these processes. The system used was based on that developed by Winnacker et al., (1972) and was modified to our own requirements.

3.1. General requirements for optimal incorporation of $^{3}$H-dTTP and the procedure for isolation of acid-insoluble material.

The incubation was stopped after 5 min. at 37° with 1 M alkali and the nuclei washed 6 times for 45' at 37° in alkali. Figure 3.19 indicates the amount of acid-insoluble radioactivity left in the nuclei after each wash. On the basis of these results nuclei were routinely washed 6 times in alkali before determining the radioactivity remaining.

The incubation mixture contained all four deoxyribonucleoside

Fig. 3.17.

Isopycnic caesium chloride analysis of DNA isolated from BHK cells infected with temperature-sensitive mutants of pseudorabies virus.

Cultures of BHK cells at 37° or 41° were infected with pseudorabies virus at 1 pfu/cell, labelled with 8 uCi/ml $^{3}$H-thymidine at a final concentration of $4 \times 10^{-6}$ M from 4 to 24 h after infection and the DNA analysed on isopycnic caesium chloride gradients as described in the Methods section 2.7.1.

A. mock-infected cells grown at 37°;

B. mock-infected cells grown at 41°;

C. wild-type PrV grown at 37°;

D. wild-type PrV grown at 41°.

The fractions are numbered from the bottom of the tube.

Fig. 3.17

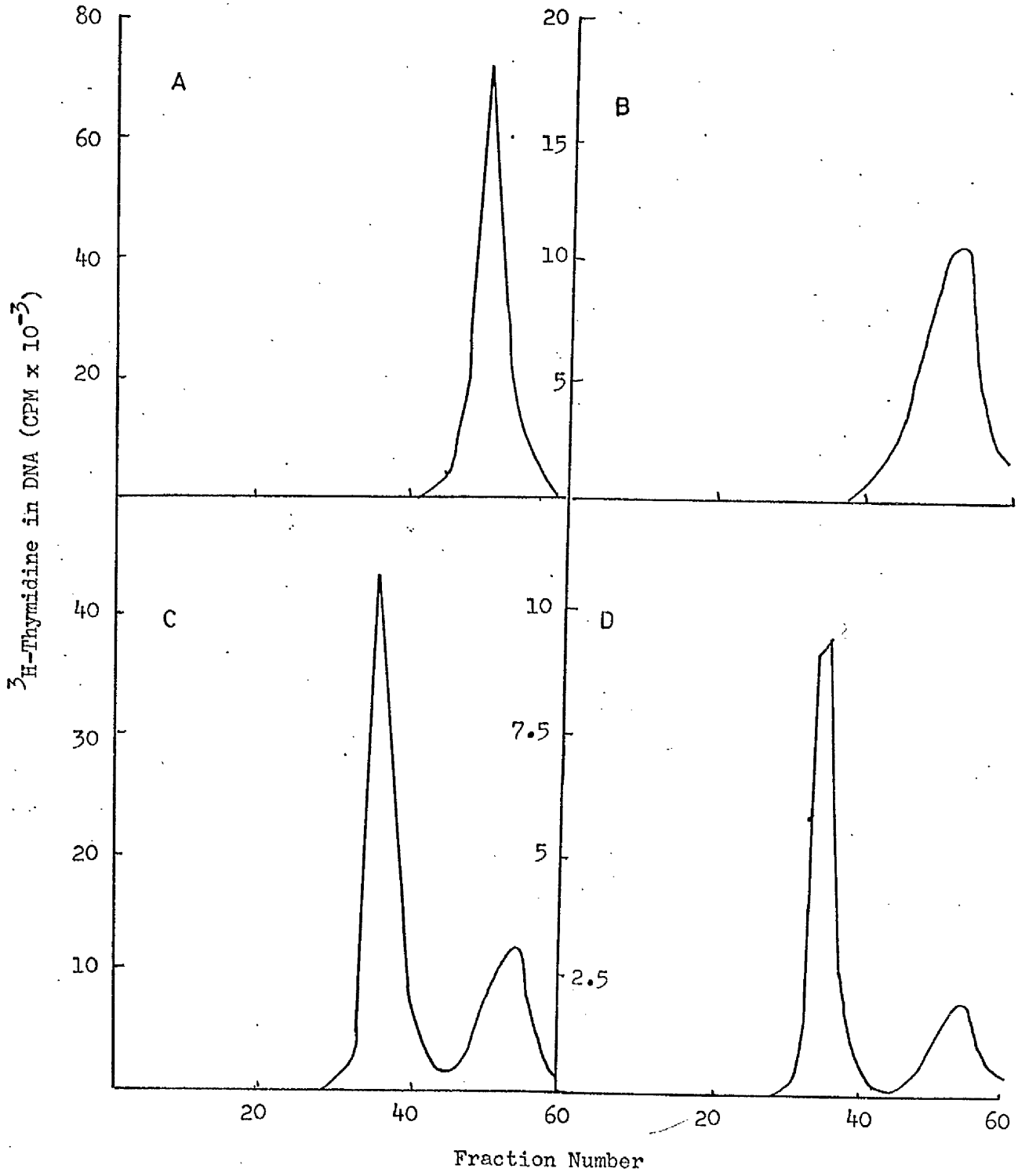

A
B
C
D
80
60
40
20
20
15
10
5
40
30
20
10
10
7.5
5
2.5
20
40
60
20
40
60
$^3$H-Thymidine in DNA (CPM x $10^{-3}$)
Fraction Number

Fig. 3.18.

Caesium chloride analysis of DNA isolated from BHK cells infected with temperature-sensitive mutants of pseudorabies virus.

The methods were as described in the legend to Figure 3.17.

A. ts 2;

B. ts 9;

C. ts 10.

Fig. 3.18

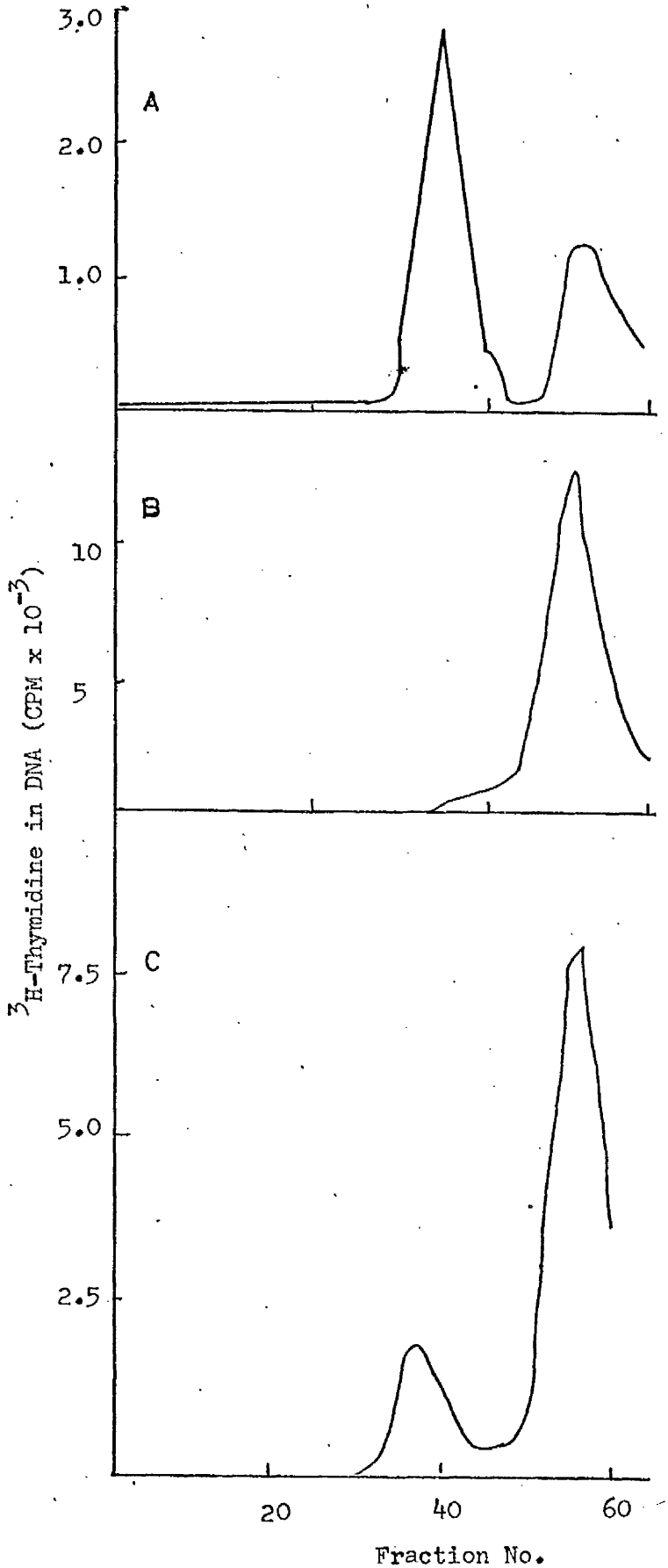

3.0
2.0
1.0
A
10
5
B
7.5
5.0
2.5
C
20
40
60
Fraction No.
$^3$H-Thymidine in DNA (CPM x $10^{-3}$)

Fig. 3.19.

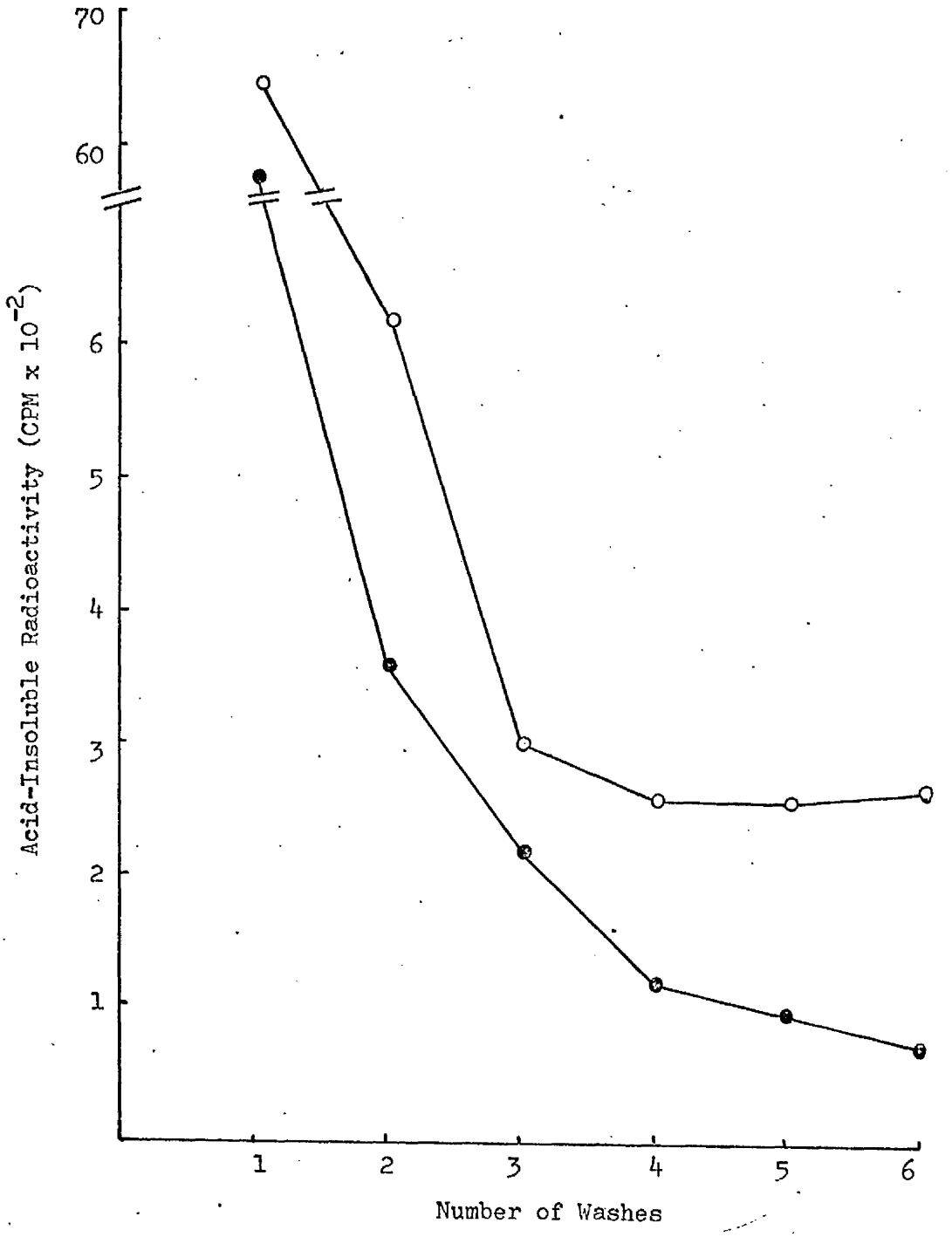
Acid-insoluble radioactivity remaining in isolated nuclei after washing in alkali.

Cells were harvested and nuclei prepared as described in the Methods section 2.5. After incubation for 5 min. at 37° as described in the Methods section 2.5 the nuclei were given successive washes in 1 M NaOH. After each wash acid-insoluble radioactivity was determined by dissolving the nuclear pellet in hyamine hydroxide and counting as described in the Methods section 2.8.1. All determinations were made in duplicate.

o——o incubated at 37°;

●——● incubated at 4°.

Fig. 3.19


70
60
6
5
4
3
2
1
Acid-Insoluble Radioactivity (CPM x $10^{-2}$)
1
2
3
4
5
6
Number of Washes

triphosphates, all except deoxythymidine triphosphate being present at 0.4 mM. Winnacker et al., (1972) find no inhibition of incorporation of $^{3}$H-dTTP with concentrations of deoxyribonucleoside triphosphates of up to 1 mM.

The ATP requirement is shown in Figure 3.20(a). Optimal synthesis in uninfected nuclei was found at 2 mM ATP; higher concentrations resulted in a slight inhibition. In infected nuclei ATP had a pronounced inhibitory effect and 2 mM was chosen to be used routinely in assays.

Figure 3.20(b) and (c) shows the stimulation of the reaction by $Na^{+}$ and $Mg^{2+}$ ions. Addition of $Na^{+}$ ions had a maximal effect up to 50 mM in uninfected nuclei, higher concentrations causing a pronounced inhibition. In infected nuclei, $Na^{+}$ ions continued to cause a stimulation in incorporation up to 80 mM, the highest concentration looked at. Addition of $Mg^{2+}$ ions gave a maximal stimulation of incorporation at around 6.5 mM in both the uninfected and infected nuclei. A slight inhibition was seen on either side of this maximum, being greater in uninfected nuclei.

$Ca^{2+}$ ions had little effect on incorporation in uninfected nuclei; a pronounced inhibition of incorporation of $^{3}$H-dTTP in infected cell nuclei, however, was seen in the presence of $Ca^{2+}$ ions (Figure 3.20(d)). The presence of dithiothreitol had little effect on incorporation in either uninfected or infected nuclei (Figure 3.20(e)).

Addition of deoxythymidine triphosphate caused a pronounced

inhibition of incorporation $^{3}$H-dTTP in both uninfected and infected nuclei as would be expected (Figure 3.20(f)). A concentration of 0.01 mM deoxythymidine triphosphate was used routinely when assaying both uninfected and infected nuclei. Greater incorporation was found with infected cells than with uninfected cells (approx. x 5-10) which probably reflects the differences in deoxythymidine triphosphate pool sizes in the two types of nuclei. The pool size in uninfected nuclei was estimated by isotope dilution to be 1.5 p moles per $10^{7}$ nuclei whilst that in infected nuclei was estimated to be 0.15 p moles per $10^{7}$ nuclei.

## 3.2. Time Course of Incorporation of $^{3}$H-dTTP in Uninfected Cell Nuclei.

The time course of incorporation of $^{3}$H-dTTP into acid-insoluble material is shown in Figure 3.21. Maximum incorporation is obtained between 2 and 5 min. of incubation and 5 min. was chosen as the incubation time of choice in all subsequent experiments.

The density of the cell sheet at time of harvesting has a pronounced effect on the incorporation of $^{3}$H-dTTP into nuclei prepared from these cells. Nuclei prepared from a confluent monolayer incorporated isotope to only 5% of the level obtained with nuclei from a sparse, exponentially growing monolayer (Table 3.3). Thus it appears that density dependent inhibition of growth, which is exhibited only to a slight extent

Table 3.3.

Cells were harvested at different stages of growth and nuclei prepared and incubated as described in the Methods section 2.5.

Table 3.3.

Effect of density of cell sheet when harvested on the subsequent incorporation of $^{3}$H-dTTP into nuclei *in vitro*.

| | cpm | % of non-confluent |
|---|---|---|
| Non-confluent exponentially growing cells | 597 | 100 |
| Confluent monolayer | 30 | 5 |
| "Resting" cells | 35 | 6 |

Fig. 3.20.

Requirements for optimal incorporation of $^{3}$H-dTTP into isolated nuclei.

Nuclei were incubated at 37° for 5 min. with reaction mixtures in which the concentration of one constituent was varied.

A. ATP varied;

B. NaCl varied;

C. $MgCl_2$ varied;

D. $CaCl_2$ varied;

E. dithiothreitol varied;

F. dTTP varied.

o——o uninfected cell nuclei;

●——● infected cell nuclei.

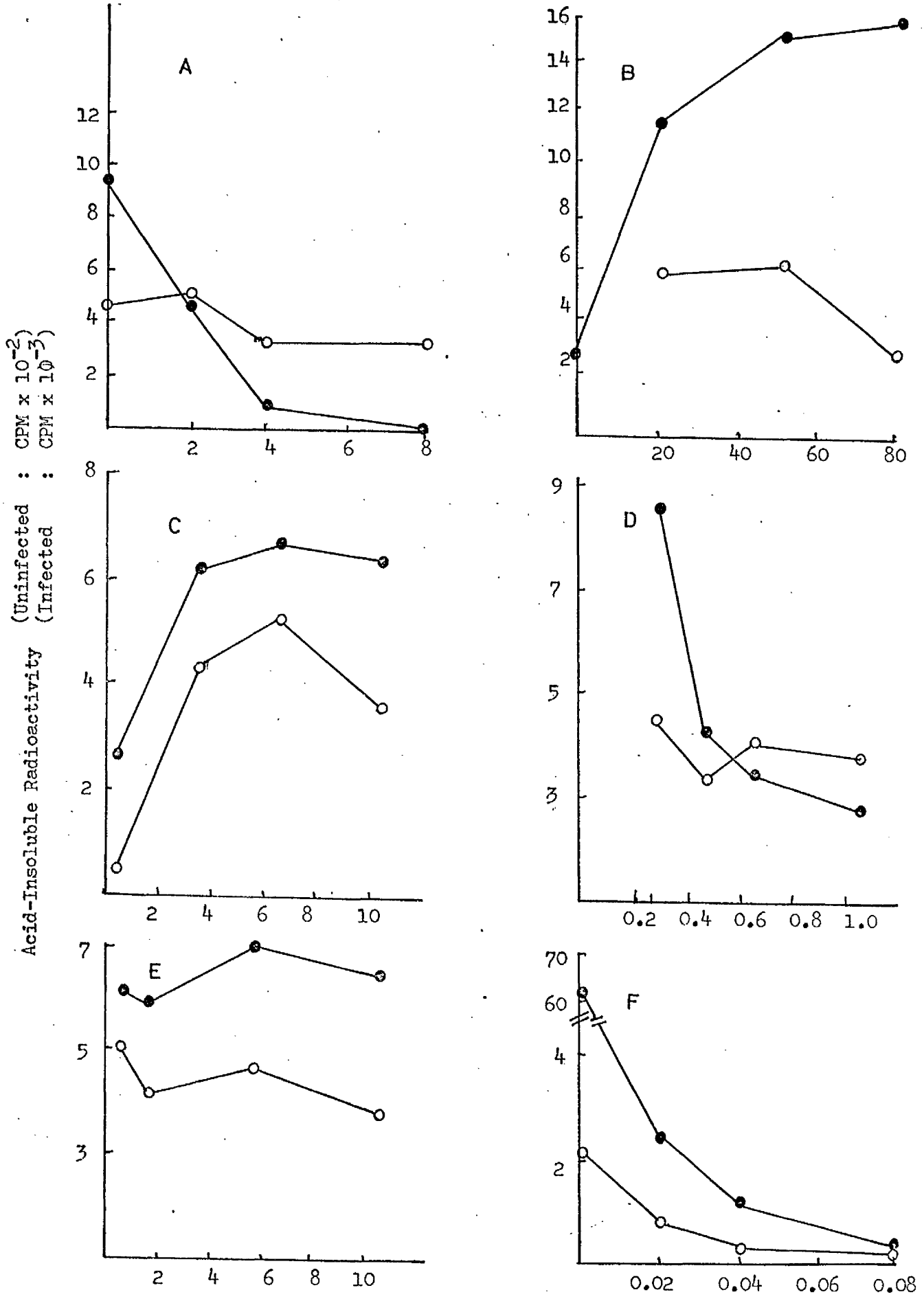

A
B
C
D
E
F
Acid-Insoluble Radioactivity (Uninfected : CPM x $10^{-2}$) (Infected : CPM x $10^{-3}$)
Concentration (mM)

Fig. 3.21.

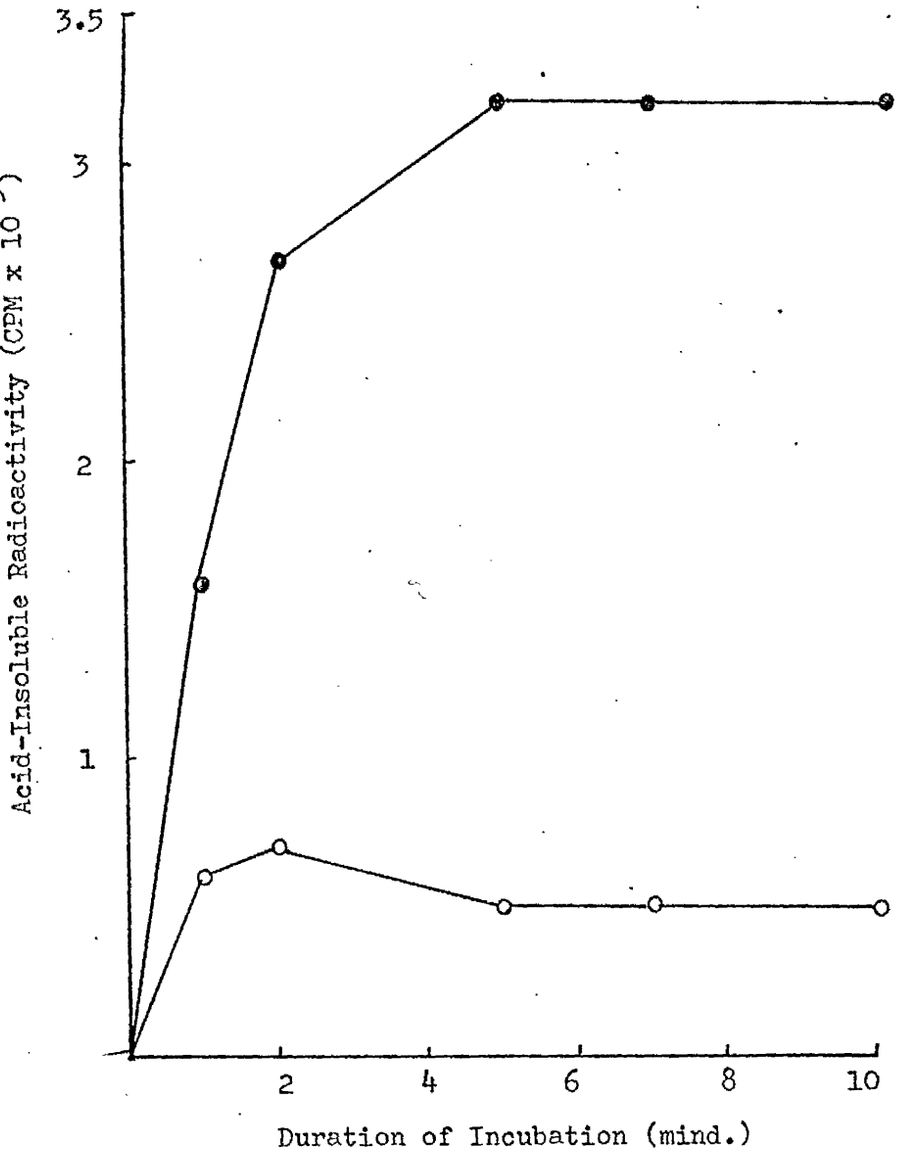
Time course of incorporation of $^{3}$H-dTTP in uninfected cell nuclei in vitro.

Nuclei were incubated for varying lengths of time at 37° as described in the Methods section 2.5.

o——o DNA synthesised in the absence of exogenous dTTP;

●——● DNA synthesised in presence of 0.02 mM dTTP.

Fig. 3.21


3.5
3
2
1
Acid-Insoluble Radioactivity (CPM x $10^{-3}$)
2
4
6
8
10
Duration of Incubation (mind.)

by BHK cells, is reflected in DNA synthesis in isolated nuclei *in vitro*.

### 3.3. Effect of ATP on Incorporation of $^{3}$H-dTTP.

In prokaryotic systems, in the presence of ATP and with suitable preparations, incorporation of $^{3}$H-dTTP can be shown to be due to semiconservative replication, whereas in the absence of ATP synthesis is of the repair type.

In mammalian systems using isolated nuclei it has been shown that ATP is required for maximal incorporation of $^{3}$H-dTTP into the nuclei (Lynch *et al.*, 1970). Additional evidence has been provided that in the presence of ATP these nuclei elongate *in vitro* DNA chains which were growing in the whole animal and it was suggested that the process *in vitro* is replicative in nature and is carried out in the same way as *in vivo*.

Figure 3.22 illustrates the effects produced by ATP in uninfected and infected BHK nuclei incubated *in vitro*. The presence of ATP causes a marked stimulation in incorporation of $^{3}$H-dTTP into uninfected nuclei over that in the absence of ATP (Figure 3.22(a)). This is in agreement with results published for other similar systems. In the virus-infected system, however, the presence of ATP does not cause any appreciable stimulation of incorporation over that in the absence of ATP (Figure 3.22(b)). On the basis of the result shown in Figure 3.20(a) one might expect to see an inhibition of

Fig. 3.22.

Effect of ATP on incorporation of $^{3}$H-dTTP into acid-insoluble material in isolated nuclei in vitro.

Nuclei were incubated for 5 min. at 37° as described in the Methods section 2.5. in the presence or absence of 2 mM ATP.

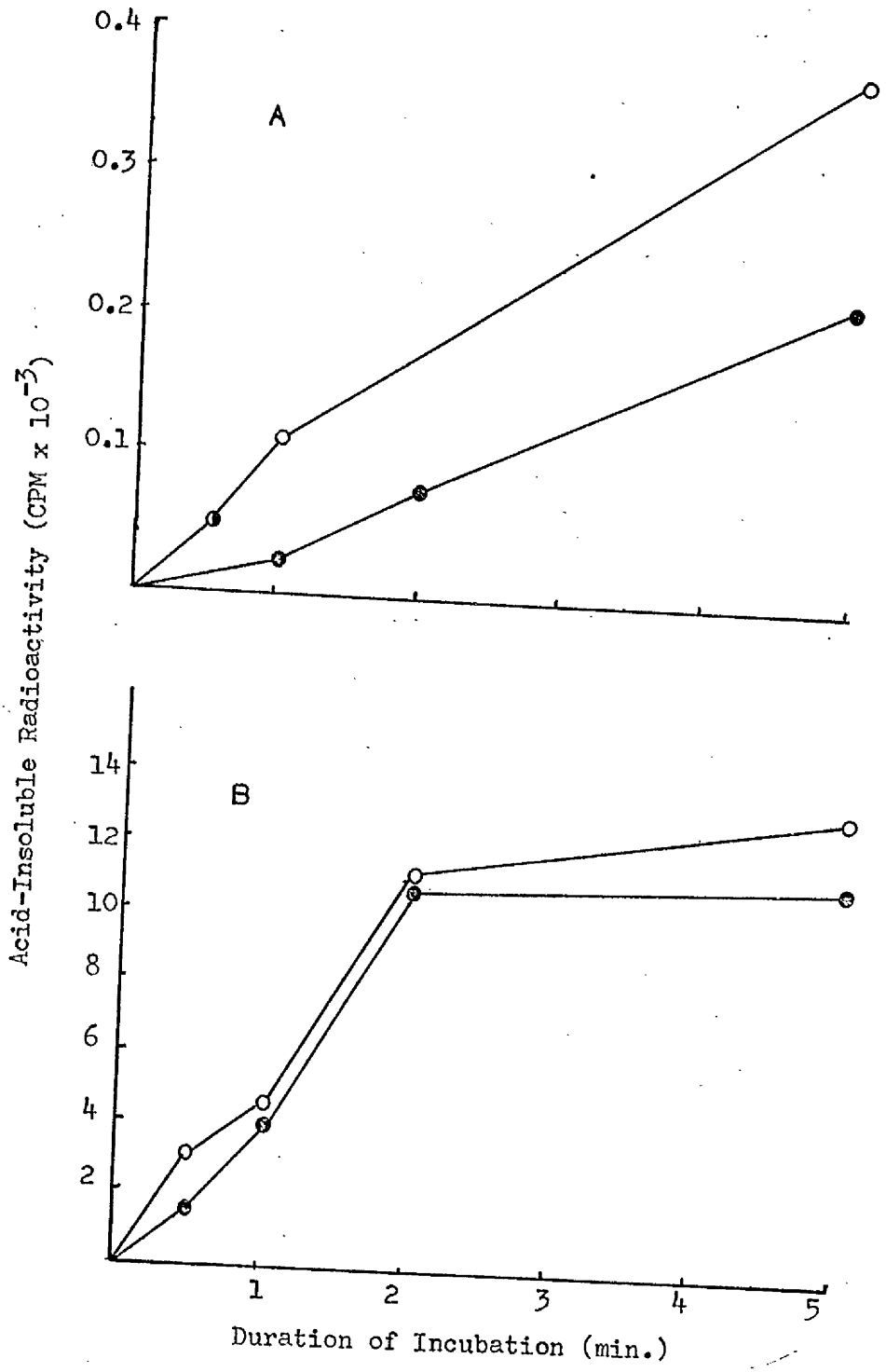
A. uninfected cell nuclei;

B. infected cell nuclei.

o——o 2 mM ATP present;

●——● absence of ATP.

Fig. 3.22


A
B
0.4
0.3
0.2
0.1
14
12
10
8
6
4
2
1
2
3
4
5
Acid-Insoluble Radioactivity (CPM x $10^{-3}$)
Duration of Incubation (min.)

incorporation here and the difference cannot be explained. The results do agree, however, in that the marked stimulation seen with the uninfected cell nuclei is not seen in the infected cell nuclei. This may indicate that the virus system uses a different mechanism from the uninfected cell in the replication of its DNA, and if this is the case it may provide a basis for simple chemotherapy of herpesvirus infections.

## 3.4. Effect of Omission of Single Deoxyribonucleoside Triphosphates.

Figures 3.23 and 3.24 illustrate the effects of omitting single deoxyribonucleotides on the incorporation of $^{3}$H-dTTP into uninfected and infected cell nuclei respectively. It is clear that omission of any of the three triphosphates, dATP, dCTP or dGTP causes a stimulation of incorporation of $^{3}$H-dTTP into DNA, contrary to what might be expected. In infected cell nuclei the incorporation of $^{3}$H-dTTP into DNA is not, or only slightly, inhibited by omission of all three deoxynucleoside triphosphates. The presence of dTTP in both systems seems to alter the profile of incorporation of $^{3}$H-dTTP into DNA, although why this should be so is unclear.

## 3.5. Nuclei from Serum-Stimulated 'Resting' Cells.

Figure 3.25 represents the incorporation of $^{3}$H-dTTP into nuclei from cells incubated for various lengths of time in ECl. As can be seen, the incorporation into the nuclei declines with

Fig. 3.23.

**Effect of omission of single deoxyribonucleoside triphosphates on the incorporation of $^{3}$H-dTTP into acid-insoluble material in uninfected BHK cell nuclei *in vitro*.**

Nuclei were incubated for varying lengths of time at 37° with incubation mixes in which one deoxyribonucleoside triphosphate had been omitted and the acid-insoluble radioactivity determined as described in the Methods section 2.5.

A. in the absence of exogenous dTTP;

B. in the presence of 0.02 mM exogenous dTTP.

o——o omitting dGTP;

●——● omitting dATP;

□——□ omitting dCTP;

■——■ dGTP, dATP and dCTP all present.

Fig. 3.23

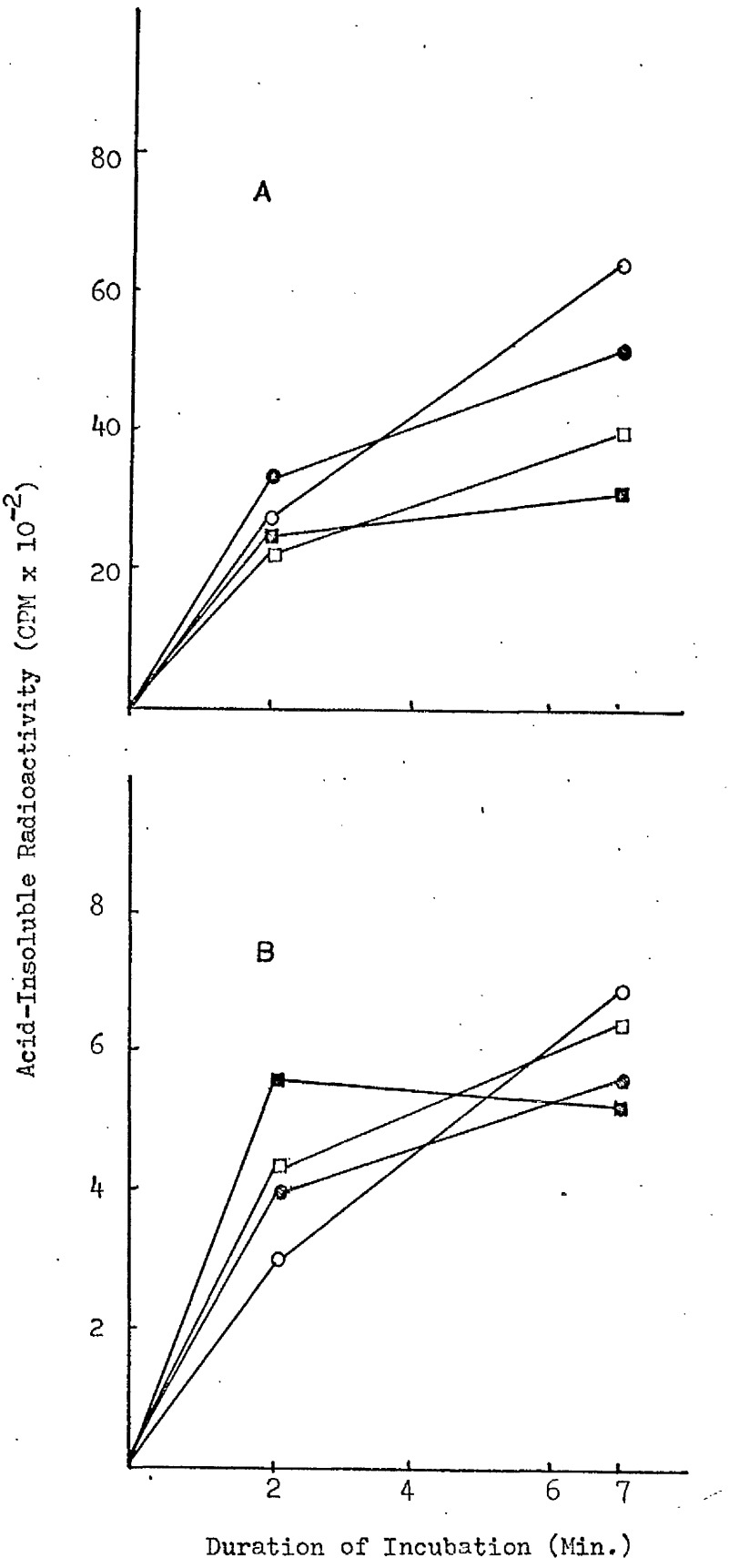

A
B
80
60
40
20
8
6
4
2
2
4
6
7
Acid-Insoluble Radioactivity (CPM x $10^{-2}$)
Duration of Incubation (Min.)

Fig. 3.24.

Effect of omission of single deoxyribonucleoside triphosphates on the incorporation in vitro of $^{3}$H-dTTP into acid-insoluble material in nuclei isolated from pseudorabies virus-infected BHK cells.

Methods were as described in the legend to Figure 3.23.

A. in the absence of exogenous dTTP;

B. in the presence of 0.02 mM exogenous dTTP.

o——o omitting dGTP;

●——● omitting dATP;

□——□ omitting dCTP;

■——■ dGTP, dATP and dCTP all present;

Δ——Δ omitting dGTP, dATP and dCTP.

Fig. 3.24

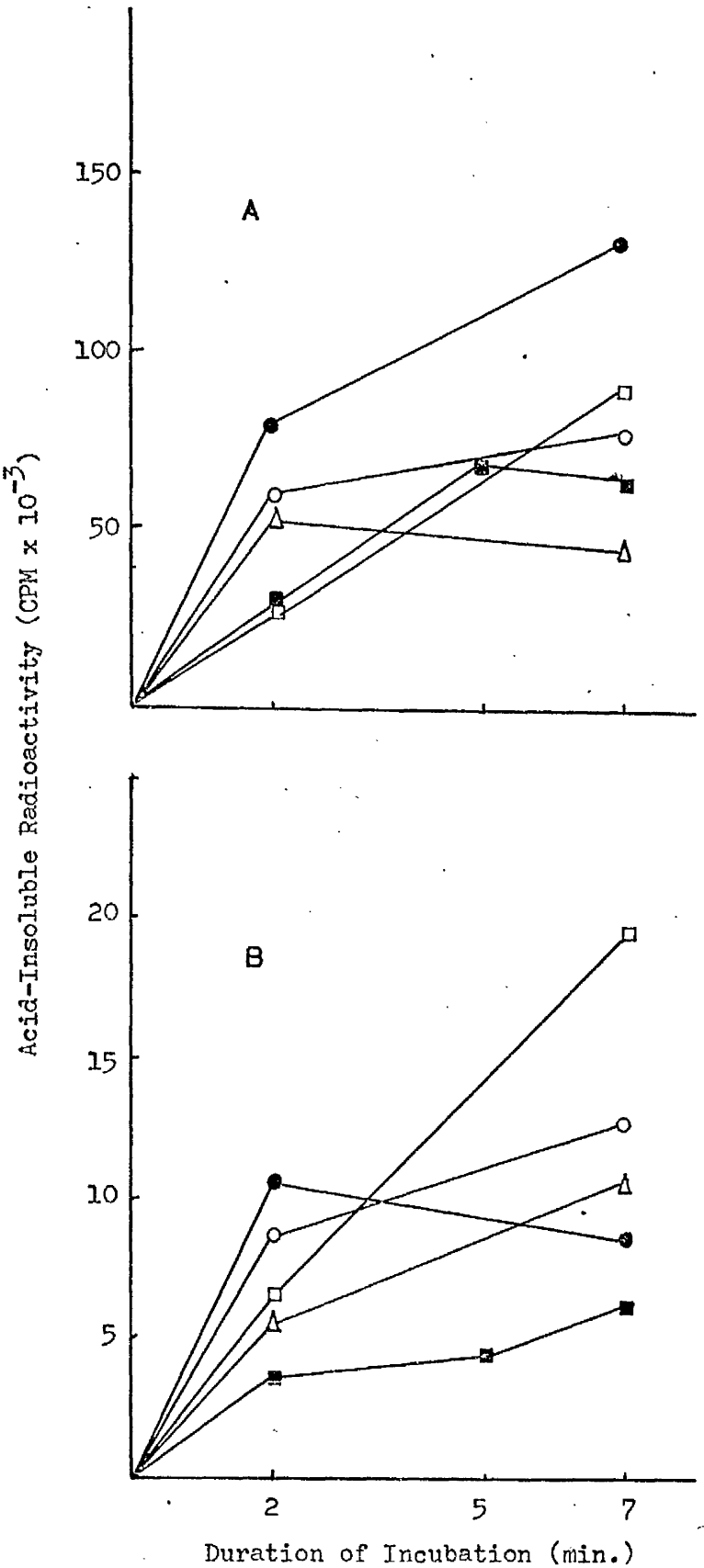
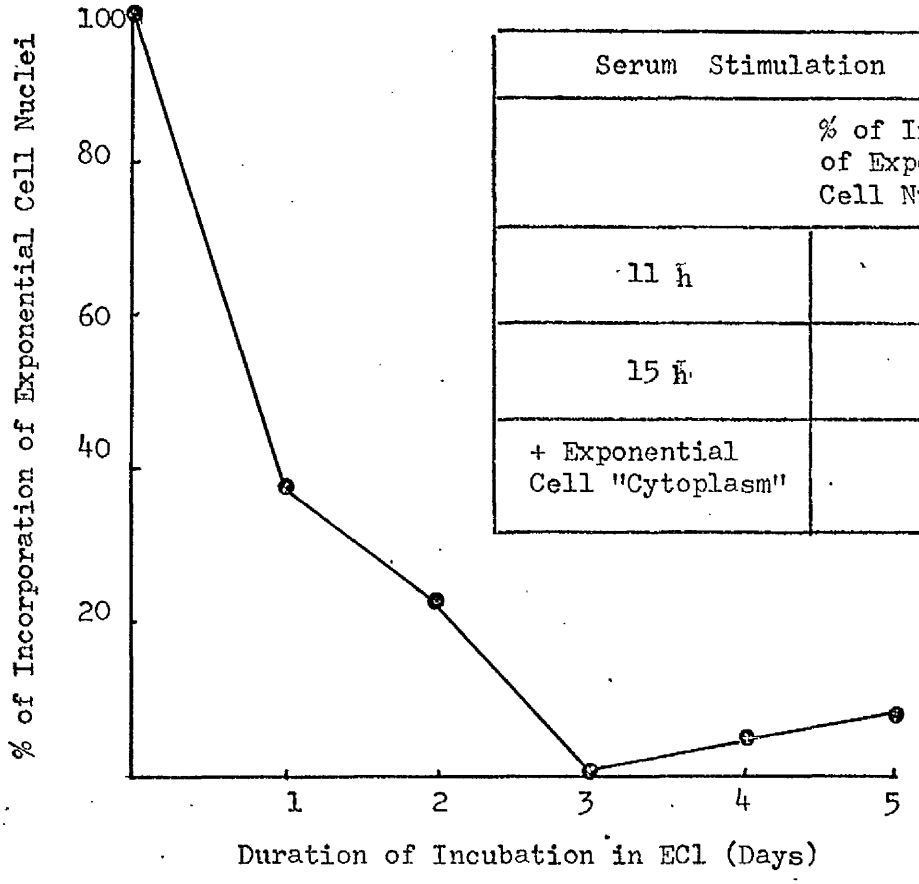

150
100
50
A
20
15
10
5
B
2
5
7
Acid-Insoluble Radioactivity (CPM x $10^{-3}$)
Duration of Incubation (min.)

Fig. 3.25.

**Incorporation of $^{3}$H-dTTP into acid-insoluble material in nuclei from 'resting' and serum-stimulated 'resting' BHK cells.**

Nuclei were prepared as described in the Methods section 2.5 from cells which had been incubated for various periods of time in PCl or which had been stimulated to grow by the addition of serum to 10%. Incubations were carried out as described in the Methods section 2.5.

Fig. 3.25




| Serum Stimulation | |
|---|---|
| | % of Incorporation of Exponential Cell Nuclei |
| 11 h | 96% |
| 15 h | 11% |
| + Exponential Cell "Cytoplasm" | 65% |

time in a similar fashion to the decrease in DNA synthesis obtained with whole cells incubated in ECl. Nuclei isolated 11 h after stimulation of 'resting' cells by serum incorporate isotope to the same extent as nuclei from an exponentially growing population of cells. This corresponds to the time of the first peak of DNA synthesis seen in whole cells after stimulation of resting cells. At the time of the second peak, however, there is little stimulation of incorporation into nuclei isolated at this time. Addition to nuclei from resting cells of a crude cytoplasmic preparation from exponentially growing cells stimulates incorporation of isotope to 60% of the exponential cell level.

Thus, in some measure, the isolated nuclei do mimic the effects seen with whole cells in the serum-stimulated resting cell system and should prove useful in gaining further understanding of the processes involved in DNA replication in this system.

## 3.6. Distribution of Acid-Insoluble $^{3}$H-dTTP between Viral and Cellular DNA.

Nuclei prepared from BHK cells 5.5 h after infection with virus incorporate isotope into both viral and cellular DNA as determined by caesium chloride isopycnic centrifugation. Figure 3.26 illustrates the profile obtained with DNA from nuclei incubated at 37° for 5 min. After incubation for only 2 min. the DNA on caesium chloride isopycnic gradients cannot

be identified as either cellular or viral.

One difference between the viral DNA synthesised in isolated nuclei and that in the whole cell is that the viral DNA made in isolated nuclei has a much higher buoyant density in caesium chloride than that from whole cells. The buoyant density of viral DNA from whole cells is 1.73 g/ml, corresponding to a GC content of 72%, while that of viral DNA from isolated nuclei is 1.83 g/ml.

## 3.7. Viral DNA Synthesis in Nuclei Isolated at Various Times after Infection.

Figure 3.27 illustrates the type of DNA synthesised in virus-infected cell nuclei isolated at various times after infection. The appearance of virus DNA mirrors its appearance in whole cells (compare Figure 3.11), and an inhibition of cellular DNA synthesis can be observed, although at later times than is found in the whole cell.

Figure 3.28 represents the results of a similar experiment in which total DNA synthesis in infected nuclei was measured and, in addition, the activity of DNA polymerase in the supernatant from the nuclear preparations was determined. Initially, while incorporation of isotope into the nucleus is high, the activity of supernatant DNA polymerase is low with both denatured and native DNA templates. As incorporation into the nuclei decreases, however, the activity of the supernatant DNA polymerase is seen to increase when assayed with a

Fig. 3.26.

**Distribution of acid-insoluble $^{3}$H-dTTP between viral and cellular DNA isolated from pseudorabies virus-infected BHK cell nuclei.**

Nuclei were prepared 5 h after infection as described in the Methods section 2.5. They were incubated for 5 min. at 37° in a reaction mixture containing 4 uCi $^{3}$H-dTTP and the reaction stopped by the addition of ice-cold SSC containing 2% SDS and leaving on ice for 5 min. The samples were then subjected to analysis on isopycnic caesium chloride density gradients as described in the Methods section 2.7.1.

Fig. 3.26

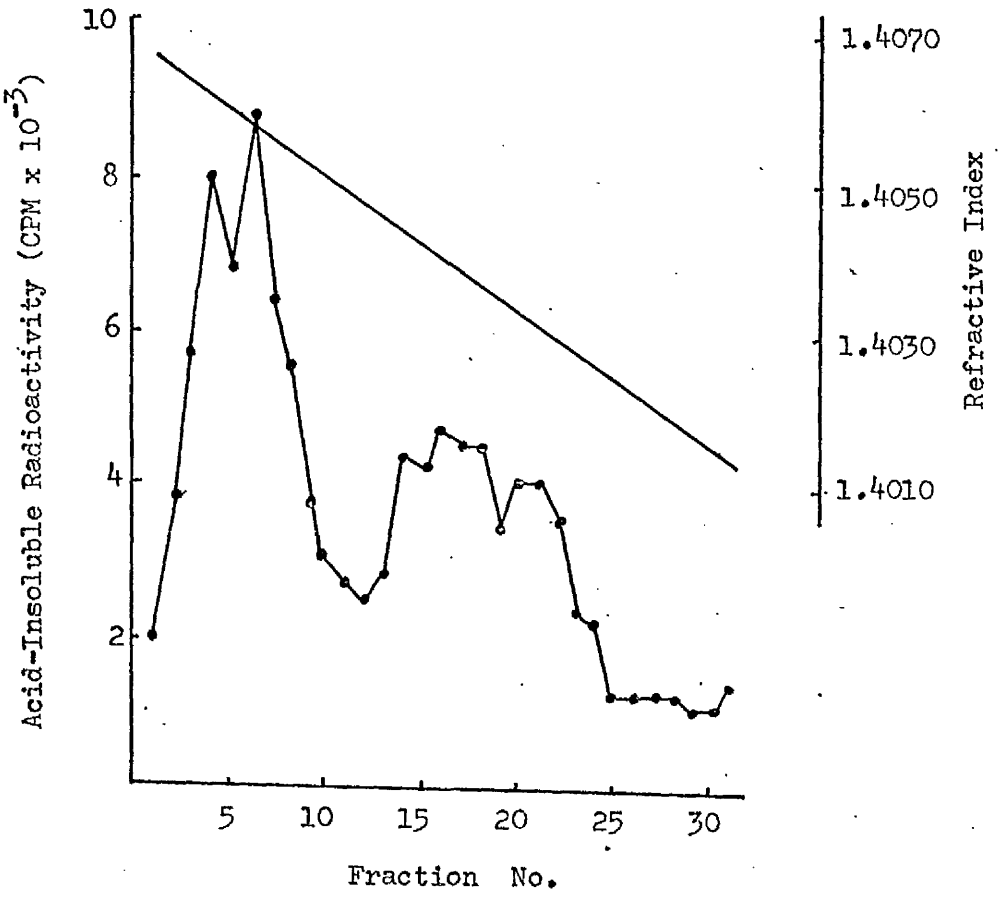

Acid-Insoluble Radioactivity (CPM x $10^{-3}$)
10
8
6
4
2
5
10
15
20
25
30
Fraction No.
1.4070
1.4050
1.4030
1.4010
Refractive Index

Fig. 3.27.

Viral DNA synthesis in nuclei isolated from BHK cells at various times after infection with pseudorabies virus.

Nuclei were prepared at various times after infection as described in the Methods section 2.5. Incubations were stopped by the addition of ice-cold SSC containing 2% SDS and leaving on ice for 5 min. The samples were subjected to analysis on isopycnic caesium chloride density gradients as described in the Methods section 2.7.1.

o—o cellular DNA;

●—● viral DNA.

Fig. 3.27

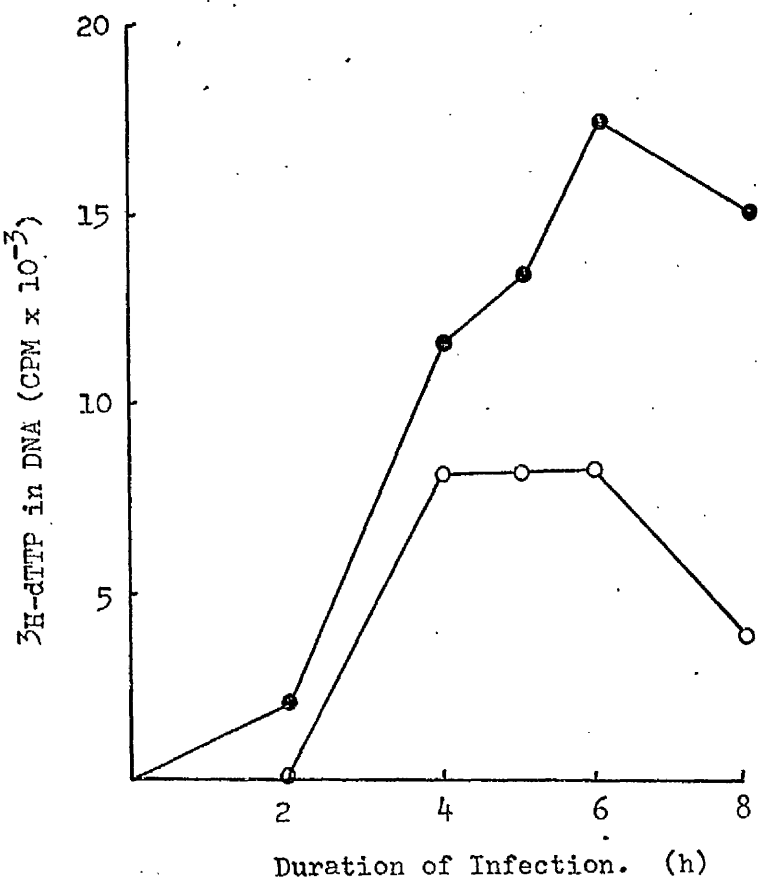


Fig. 3.28.

'Cytoplasmic' DNA polymerase assayed at various times after infection of BHK cells with pseudorabies virus.

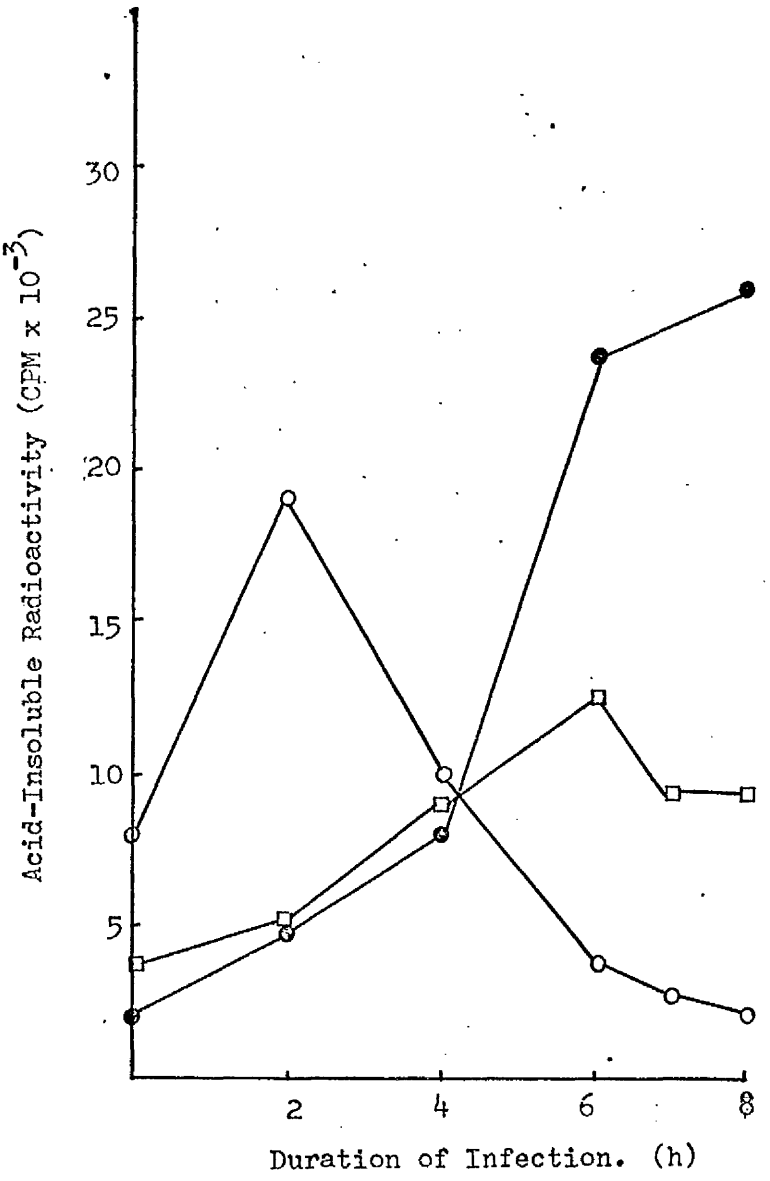
Infected cell nuclei were prepared and incubated as described in the Methods section 2.5. The procedure for the determination of DNA polymerase activity in the nuclear supernatant fractions is also described in the Methods section 2.5.

o——o total nuclear DNA;

●——● DNA polymerase activity assayed with denatured DNA as template;

□——□ DNA polymerase activity assayed with Native DNA as template.

Fig. 3.28


Acid-Insoluble Radioactivity (CPM x $10^{-3}$)
30
25
20
15
10
5
2
4
6
8
Duration of Infection. (h)

denatured DNA as template but not with a native DNA template. This may suggest that the DNA polymerase activity being measured is responsible for virus DNA synthesis and, as virus DNA synthesis ceases, the polymerase is transported from the nucleus to the cytoplasm. Howard et al., (1974) reported a similar apparent migration of DNA polymerase activity preferring a denatured DNA as template in their serum-stimulated 'resting' cell system after the peak period of DNA synthesis.

## 3.8. Addition of crude preparations of infected cell "cytoplasm" to nuclei isolated from uninfected and infected cells.

Further endeavours to identify a factor(s) in infected cells which brings about an inhibition of cellular DNA synthesis were attempted by adding to isolated nuclei crude preparations of supernatant obtained after disruption of infected cells by homogenisation and removal by centrifugation of nuclei.

No such inhibitory effect could be detected but instead, a stimulation of incorporation of isotope into infected cell nuclei was observed (Figure 3.29). If nuclei were prepared by homogenisation in isotonic buffer instead of hypotonic buffer, no stimulation could be detected. This probably reflects an inability of the stimulatory factor(s) to enter these nuclei which are probably not so damaged as those prepared by homogenisation in hypotonic buffer. The identity of this

factor is unknown, but may simply be a virus-coded DNA polymerase as such an activity does appear in these "cytoplasmic" preparations after infection (Figure 3.28).

A similar stimulatory effect is observed with uninfected cell nuclei, although the effect is not as marked as seen in the virus-infected cell nuclei. Again, no stimulation of incorporation could be detected in nuclei prepared in isotonic homogenisation buffer (Figure 3.30).

## 3.9. Distribution of Acid-Insoluble $^{3}$H-dTTP between Viral and Cellular DNA in Nuclei Stimulated by the Addition of Crude Preparations of "Cytoplasm" from Infected Cells.

The "cytoplasmic" preparations stimulate incorporation of $^{3}$H-dTTP into both cellular and viral DNA although there is a greater effect on cellular DNA synthesis (Figure 3.31). The stimulation produced increases with increasing amounts of cytoplasm until a plateau is reach between 150 and 200 ul of cytoplasm per 200 ul of nuclear suspension. Caesium chloride density gradient centrifugation reveals that both viral and cellular DNA synthesised after the addition of cytoplasm to the nuclei are very heterodisperse, suggesting that the stimulatory effect may be non-specific (Figure 3.32).

Fig. 3.29.

Effect of crude preparations of infected BHK cell nuclear supernatant fractions on the incorporation of $^{3}$H-dTTP into acid-insoluble material in infected BHK cell nuclei.

Nuclear supernatant fractions were prepared by isolating nuclei at various times after infection by homoganisation of cells in either hypotonic buffer or isotonic buffer (Methods section 2.5.1.). 50 ul fractions of supernatant were incubated with 100 ul of a suspension of nuclei isolated 5 h p.i. for 1 min. at 37° before assaying for ability of the nuclei to incorporate $^{3}$H-dTTP as described in the Methods section 2.5.

o——o incorporation of $^{3}$H-dTTP by nuclei isolated in hypotonic buffer;

●——● incorporation of $^{3}$H-dTTP by nuclei isolated in isotonic buffer.

Fig. 3.29

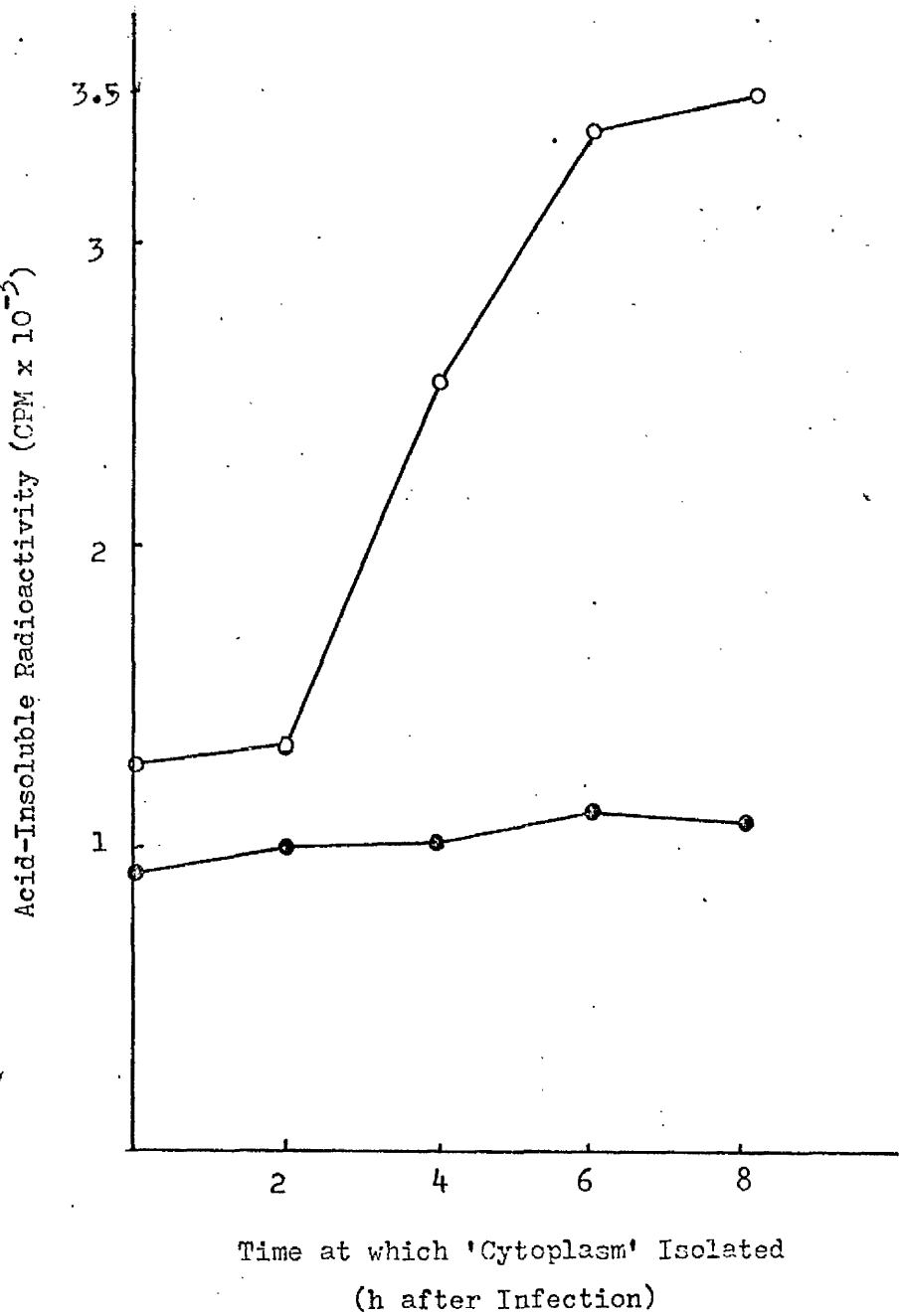

Acid-Insoluble Radioactivity (CPM x $10^{-3}$)
3.5
3
2
1
2
4
6
8
Time at which 'Cytoplasm' Isolated
(h after Infection)

Fig. 3.30.

**Effect of crude preparations of infected BHK cell nuclear supernatant fractions on the incorporation of $^{3}$H-dTTP into material in uninfected BHK cell nuclei.**

Methods are as described in the legend to Figure 3.29 except that uninfected cell nuclei were incubated with the supernatant fractions from infected cell nuclei.

o—o incorporation of $^{3}$H-dTTP into nuclei prepared in hypotonic buffer;

●—● incorporation of $^{3}$H-dTTP into nuclei prepared in isotonic buffer.

Fig. 3.30

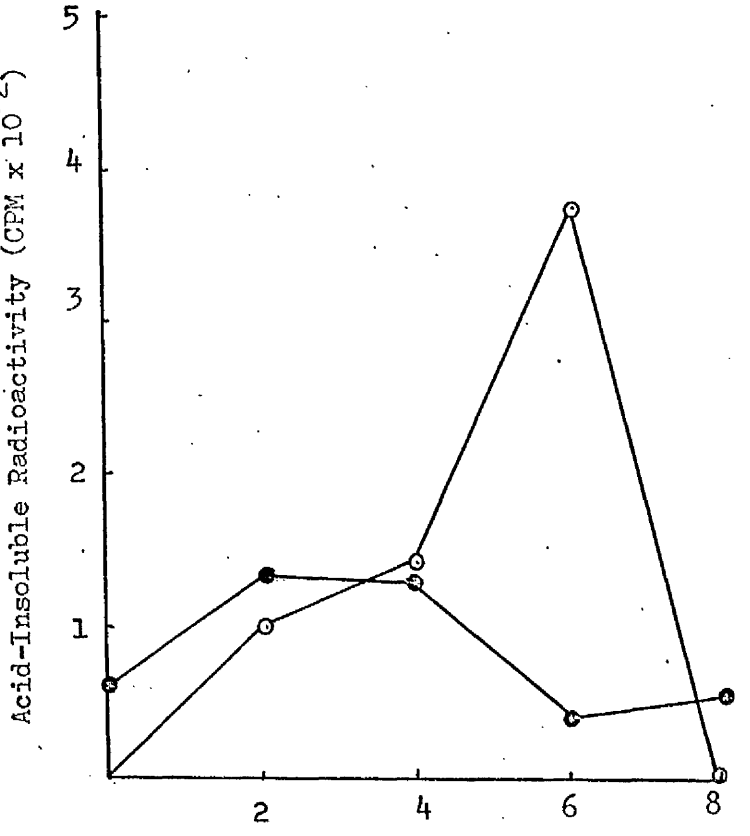

Acid-Insoluble Radioactivity (CPM x $10^{-2}$)
5
4
3
2
1
2
4
6
8
Time at which 'Cytoplasm' Isolated
(H after Infection)

Fig. 3.31.

**Distribution of acid-insoluble $^{3}$H-dTTP between viral and cellular DNA isolated from nuclei from pseudorabies virus-infected BHK cells which had been stimulated in vitro by the addition of crude preparations of nuclear supernatant from pseudorabies virus-infected BHK cells.**

Nuclear supernatant fractions were prepared by homogenisation of infected BHK cells in hypotonic buffer 5 h p.i. as described in the Methods section 2.5.1. 250 ul of supernatant was incubated with 200 ul of a suspension of nuclei isolated from infected cells 5 h p.i. in a reaction mixture containing 5 uCi $^{3}$H-dTTP. The reaction was stopped by the addition of ice-cold SSC containing 2% SDS and leaving on ice for 5 min. Samples were subjected to isopycnic caesium chloride density gradient analysis as described in the Methods section 2.7.1.

Fig. 3.31

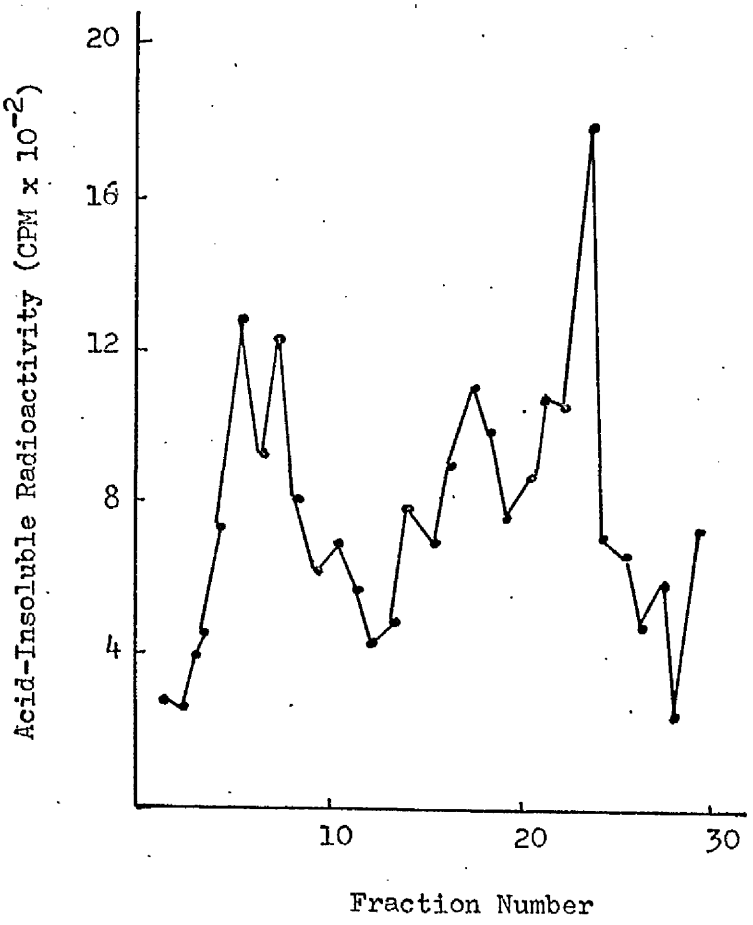

Acid-Insoluble Radioactivity (CPM x $10^{-2}$)
20
16
12
8
4
10
20
30
Fraction Number

Fig. 3.32.

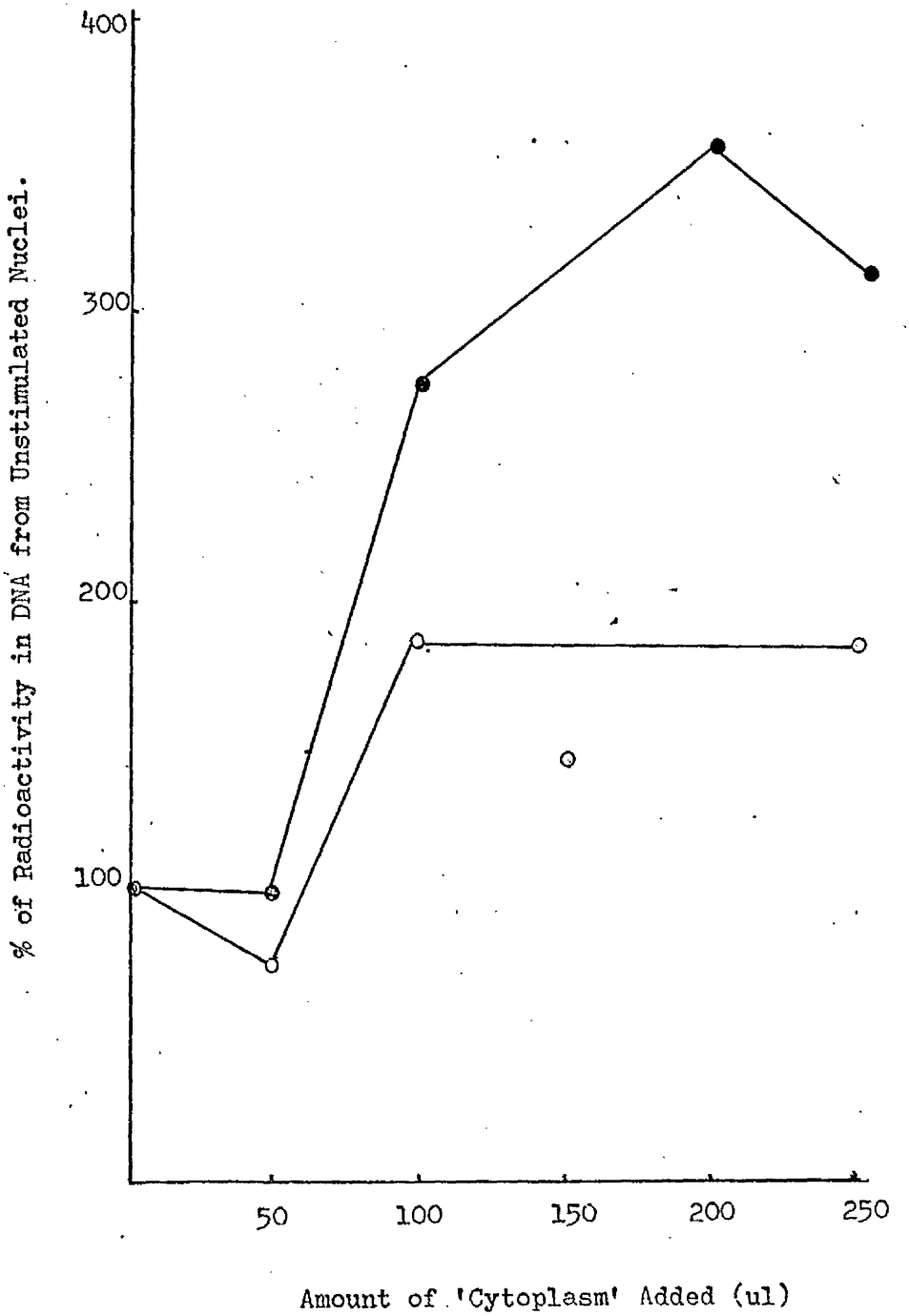
**Stimulation of incorporation of $^{3}$H-dTTP into cellular and viral DNA in nuclei from pseudorabies virus-infected BHK cells by the addition of crude nuclear supernatant fractions from pseudorabies virus-infected BHK cells.**

Methods are as described in the legend to Figure 3.31 except that varying amounts of supernatant material was added to nuclei isolated 5 h after infection of BHK cells with pseudorabies virus.

●——● incorporation of $^{3}$H-dTTP into cellular DNA;

o——o incorporation of $^{3}$H-dTTP into viral DNA.

Fig. 3.32


400
300
200
100
% of Radioactivity in DNA from Unstimulated Nuclei.
50
100
150
200
250
Amount of 'Cytoplasm' Added (ul)

# IV. DISCUSSION.

"This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

Winston Churchill.

---

## 1. Effects of Levels of Serum on BHK Cells.

Although density-dependent inhibition of growth of cells is currently being extensively used to study stationary cells and their stimulation to growth, there are doubts as to the real nature of this phenomenon and it may not be as "physiological" as is sometimes claimed (Dulbecco and Elkington, 1973).

BHK cells do not exhibit strong density-dependent inhibition of growth, forming multilayers as thick as transformed cells (House and Stoker, 1966). We thought it useful to compare the results of serum deprivation and subsequent stimulation of the stationary cells with those obtained using other techniques, basing our system on the original observations of Bürk (1967) that when non-confluent monolayer cultures of BHK cells are maintained for 2 - 3 days in medium containing 0.5% serum they cease growing with the cells in the G1 phase of the cell cycle.

### 1.1. Effects of Serum Depreivation on DNA Synthesis and Cell Division.

The more rapid decrease in incorporation of thymidine in

dense cultures than in sparse cultures (Figure 3.4) is not surprising, as one might expect the increased competition in dense cultures for serum growth factors to lead to a more rapid exhaustion of these factors and a consequently more rapid decrease in cell growth. In both cases, however, by 6 days not more than 0.5% of the cells are incorporating label and there is no increase in cell number. Beyond 7 days the cells begin to die and the percentage stimulated into DNA synthesis by adding serum falls. Cell death after 7 days appears to proceed more rapidly in dense cultures than in sparse cultures.

As mentioned in the Results section 3.1.2., it appears that after transfer of cells to ECl, although fewer cells are incorporating label, those that do so take up a greater amount of label (Plate 1). A similar phenomenon has been observed with mouse fibroblast cells which cease growth in medium containing low levels of serum. This increased labelling could be explained on the basis of transfer of $^{3}$H-thymidine to those cells able to synthesise DNA from those in contact with it which themselves do not synthesise DNA. Such a transfer of labelled nucleotides between cells has been shown by Pitts (1971). This explanation, however, seems unlikely as cells can take up exogenous thymidine only during the S-phase (Stone et al., 1965; M. E. Finbow, personal communication). Those cells not actively synthesising DNA do contain a soluble pool of thymidine nucleotides (Stone et al.,

1965) which exists in those cells from the end of one S-phase until the next S-phase, but these thymidine derivatives should not be labelled under the conditions used by us in this experiment. The true reason for the increased incorporation of $^{3}$H-thymidine into acid-insoluble material is not clear.

1.2. Effects of Re-addition of Serum to Resting Cultures on DNA Synthesis and Cell Division.

Figure 3.3(b) depicts the pattern of DNA synthesis and cell division seen upon stimulation of resting cell cultures by the addition of fresh serum. Autoradiography has confirmed that both peaks of DNA synthesis represent incorporation of $^{3}$H-thymidine into nuclear material and the incorporation is into material with the same buoyant density in neutral caesium chloride as the chromosomal DNA of exponentially growing cells (Figure 3.5). On the basis of several criteria detailed in Results section 3.1.2., the two peaks of DNA synthesis represent incorporation of thymidine into the same population of cells.

The double peak is most unlikely to be due to contaminatinn of the cultures with mycoplasma as, in addition to the negative routine PPLO checks, autoradiography after $^{3}$H-thymidine labelling did not show grains in the cytoplasm where most PPLO reproduce (Stanbridge, 1971), no RNA with characteristics similar to the usual 16 and 23 s ribosomal RNA of all PPLO (Stanbridge, 1971) could be detected by polyacrylamide gel electrophoresis and no

DNA which may have originated from mycoplasm could be seen in caesium chloride gradients.

It is also possible that $^{3}$H-thymidine labelling does not provide an accurate measure of DNA synthesis. In hamster fibroblasts, however, which show multiple peaks of isotope incorporation, the incorporation of thymidine does parallel DNA synthesis as measured directly by DNA fluorescence (Remington and Klevecz, 1973). In stimulated cells the size of the thymidine triphosphate pool does change (Nordenskjold et al., 1970) but there is a corresponding increase in the uptake of thymidine from the medium (Cunningham and Remo, 1973) so that the specific activity of the pool remains fairly constant.

Fluctuations in the rate of DNA replication during the S-phase have been reported in a wide variety of experimental systems (see Remington and Klevecz, 1973, for survey). Bimodal incorporation of $^{3}$H-thymidine is usually observed in diploid cell types derived from embryonic or very young organisms. The rate of overall DNA synthesis in heteroploid cell lines is generally thought to be continuous and unimodal during the S-phase (Remington and Klevecz, 1973). Nonetheless, biphasic incorporation of $^{3}$H-thymidine has been reported in some heteroploid cell lines (Painter and Schaefer, 1971), although the two maxima are far less distinct than with diploid cell lines.

BHK cells, which are aneuploid, exhibit a clear biphasic incorporation of $^{3}$H-thymidine and thus appear to resemble more

closely normal diploid cells, especially since their original derivation from a young organism might lead one to expect a bimodal S-period.

In contrast to the findings of Remington and Klevecz (1973) there is no discernible loss of $^{3}$H-thymidine from the DNA of BHK cells, so that turnover of the DNA, if it occurs, must either be on a small scale or very rapid so that the 1 hour pulse used measures predominantly stable DNA.

DNA polymerase activity in resting cells is 20% of that in exponentially growing cells and after stimulation of the cells by the addition of fresh serum both the particulate and supernatant activities rise to a maximum during S-phase, but at different periods during this phase (Howard et al., 1974).

1.3. Effects of Inhibitors of RNA and Protein Synthesis on DNA Synthesis following Stimulation of Resting Cells by the Addition of Serum.

An early increase in RNA synthesis has been observed in most models of stimulated DNA synthesis, this increase being sensitive to low levels of actinomycin D which do not affect DNA synthesis per se (For survey, see Stein and Baserga, 1972).

There is evidence in many systems for a very early increase in RNA synthesis, reaching a maximum a few hours after the stimulus has been applied to the cells. In some systems, but not others, there is a second increase in RNA synthesis occurring late in the pre-replicative period, and even more

pronounced than that occurring in the early pre-replicative period. This second increase is due to an increase in synthesis of both ribosomal and messenger RNA (For survey, see Baserga, 1968).

Stationary cultures of BHK cells stimulated to grow and divide by the addition of serum exhibit two stages of the pre-replicative phase during which they are particularly sensitive to low levels of actinomycin-D. The first occurs during the first two hours after addition of serum, the presence of actinomycin-D during this period completely preventing DNA synthesis between 15 and 16 hours after the stimulus. The presence of actinomycin-D after this initial two hour period does not prevent some DNA synthesis occurring between 15 and 16 hours but the inhibitory effect of the drug on subsequent DNA synthesis is much less when it is added at times later than 9 or 10 hours after the stimulus, indicating that RNA synthesis necessary for DNA synthesis occurs at about 8 hours. Ellem and Mironescu (1972) have shown that RNA synthesis at this time in a similar system is mainly of rRNA. The identity of the RNA species synthesised in the first two hours after the stimulus and at 8 or 9 hours later has not been determined but this system does resemble most others studied in exhibiting two actinomycin-D sensitive periods after stimulation to grow and divide.

## 2. Infection of BHK Cells with Pseudorabies Virus.

### 2.1. Inhibition of Cellular DNA Synthesis.

#### 2.1.1. Possible Virus-induced Mechanisms of Inhibition of Cellular DNA Synthesis.

In pseudorabies virus-infected rabbit kidney cells cellular DNA synthesis is completely inhibited by 7.5 h p.i. in the same nuclei in which viral DNA synthesis is proceeding at a rapid rate (Kaplan and Ben-Porat, 1963). The virus brings about a similar cessation of cellular DNA synthesis in BHK cells (Figs. 3.11 and 3.12). There are several possible ways in which this inhibition could be brought about by the virus and these are illustrated in Figure 4.1.

After infection the inhibition of cellular DNA synthesis may be caused by the action of a component present in the invading virion and which would not require transcription and translation in order for the inhibition to occur. There is a precedent for this in the case of the adenovirus fiber and hexon proteins (Levine and Ginsberg, 1968; Yamashita et al., 1971) and in the case of vesicular stomatitis virus (Yaoi and Amano, 1970). The alternative mechanism would involve transcription of the viral genome and translation of the RNA to produce virus-coded proteins which would then act to produce inhibition of cellular DNA synthesis, as occurs with bacteriophage Pe (Marcus and Newton, 1971; Lavi and Marcus, 1972).

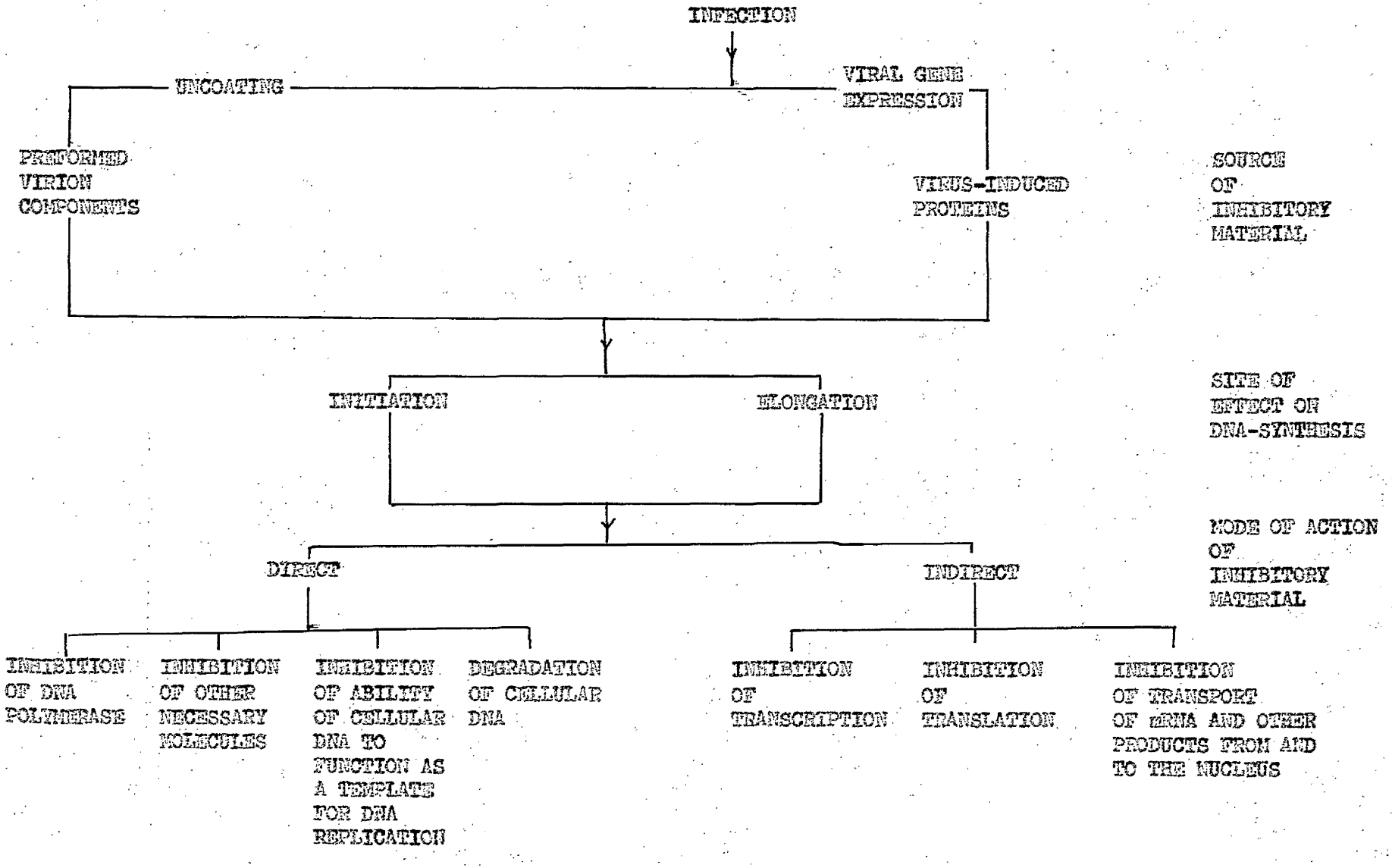
Whatever the component which actually brings about the cessation of DNA synthesis, it can act either at the level of

Fig. 4.1.

Inhibition of cellular DNA synthesis by Herpesviruses.

Possible modes of action of virus-induced inhibitory material.

Fig. 4.1.


INFECTION
UNCOATING
VIRAL GENE EXPRESSION
PREFORMED VIRION COMPONENTS
VIRUS-INDUCED PROTEINS
SOURCE OF INHIBITORY MATERIAL
INITIATION
ELONGATION
SITE OF EFFECT ON DNA-SYNTHESIS
DIRECT
INDIRECT
MODE OF ACTION OF INHIBITORY MATERIAL
INHIBITION OF DNA POLYMERASE
INHIBITION OF OTHER NECESSARY MOLECULES
INHIBITION OF ABILITY OF CELLULAR DNA TO FUNCTION AS A TEMPLATE FOR DNA REPLICATION
DEGRADATION OF CELLULAR DNA
INHIBITION OF TRANSCRIPTION
INHIBITION OF TRANSLATION
INHIBITION OF TRANSPORT OF mRNA AND OTHER PRODUCTS FROM AND TO THE NUCLEUS

initiation of DNA synthesis, at the level of propagation of DNA chains whose replication has already been initiated or at both levels.

The inhibition could be caused by a direct action on the cellular DNA replication complex, for example, by causing an inhibition of the cellular polymerase or other molecules involved in the replication process, by binding to the DNA template thus preventing replication or by causing a depolymerisation of cellular DNA. An indirect inhibition could conceivably occur by means of an inhibition of transcription or translation of host macromolecules coding for proteins necessary for cellular DNA replication or by inhibiting the transfer of host RNA and proteins from and to the nucleus.

2.1.2. Involvement of a Virion Component.

The inhibition of DNA synthesis brought about by pseudorabies virus would appear not to be caused by the action of some component in the invading virion itself as protein synthesis is necessary before the expression of this inhibitory function (Ben-Porat and Kaplan, 1965) and since u.v.-inactivated pseudorabies virus (Figure 3.15) and u.v.-inactivated HSV-1 (I. W. Halliburton, personal communication) do not exhibit this function.

2.1.3. Is Cellular DNA Synthesis Inhibited at the Level of Initiation and/or Propagation?

Cohen et al., (1971) have claimed that HSV-1 inhibits

both initiation and propagation of cellular DNA synthesis and in our system it is clear that inhibition of DNA synthesis occurs at the level of propagation of DNA chains already initiated (Figure 3.14). Whether initiation is also affected is unknown.

2.1.4. Does Degradation of Cellular DNA take place?

Herpesvirus infection does not lead to extensive degradation of host cell DNA, but in some cases it may be cleaved to relatively small molecules (Nonoyama and Pagano, 1972). Due to the rapidity of the inhibition of cellular DNA synthesis, however, it is unlikely that this degradation of cellular DNA plays an important role in the inhibitory process, although endonucleolytic cleavage, if unrepaired, could bring about a cessation of DNA synthesis.

2.1.5. Inhibition of Cellular DNA Polymerase.

Host cell DNA polymerase activity can be separated from the virus-induced DNA polymerase activity in extracts of HSV-infected cells (J. M. Morrison, personal communication). Whether this activity is active in the whole cell, however, is not clear as it is quite possible that inhibitory factors produced by the virus have been removed from the polymerase molecule during its isolation from the cell.

2.1.6. Inhibition of Transcription, Translation and Transport of RNA from the nucleus to the cytoplasm.

As discussed in section 5.3.4. of the Introduction, infection of cells with herpesviruses leads to a drastic inhibition

of cellular RNA and protein synthesis, the cellular RNA which does continue to be synthesised is improperly processed and the process of transfer to the cytoplasm is also altered. In the light of these findings it is entirely possible that inhibition of cellular DNA synthesis may occur as a secondary effect to the inhibition of cellular RNA and protein synthesis.

2.1.7. Conclusions.

The only one of the above possible mechanisms of inhibition of cellular DNA synthesis that can be discounted is an effect produced by a virion component without the necessity for transcription and translation of the virus genome. It is not possible to decide whether the process acts directly on the cellular DNA replication complex or whether it occurs indirectly as a result of inhibition of cellular RNA and protein synthesis or of inhibition of transport of macromolecules from the nucleus.

## 2.2. Attempts to Identify the Products Responsible for Inhibition of Cellular DNA Synthesis.

### 2.2.1. Use of Cycloheximide to Inhibit Viral Protein Synthesis.

Ben-Porat and Kaplan (1965) have reported that puromycin prevents pseudorabies virus from expressing the ability to inhibit host-cell DNA synthesis. Inhibition of protein synthesis, however, can itself bring about a cessation of DNA synthesis by preventing synthesis of "initiation proteins" (Fujiwara, 1972) and of histones and other chromosomal proteins necessary to produce a complete daughter chromatid.

Using low levels of cycloheximide which still cause a partial cessation of protein synthesis but do not have adverse effects on cellular DNA synthesis, it is reasonable to expect that a comparison could be made of the proteins synthesised in the presence and absence of the drug and perhaps an identification of proteins involved in inhibition of DNA synthesis. In our hands, however, it has not been possible to inhibit viral protein synthesis without at the same time directly inhibiting cellular DNA synthesis.

2.2.2. Addition of extracts of infected cells to uninfected cultures.

As an alternative to the above approach we reasoned that if the virus is coding for an inhibitory molecule, the addition of extracts of infected cells to uninfected cultures might be expected to lead to inhibition of cellular DNA synthesis in these cultures, provided that this inhibitor could enter the cell and still maintain its activity. Suarez et al., (1972) have shown that SV40 transformed nonpermissive cells, in which neither infectious viral DNA nor virions had been previously detected, reproducibly yielded infectious SV40 DNA after treatment of the cells with extracts of normal permissive cells. They showed that the activating factor(s) was insensitive to DNase and RNase but was sensitive to heat and to proteolytic enzymes. Using similar techniques, however, and poly-L-$\alpha$-ornithine to increase the cellular uptake of proteins (Ryser, 1967), we could observe no reproducible

effects on cellular DNA synthesis as a result of the addition of these extracts to the cells, and consequently have been unable to identify inhibitory factors in virus-infected cells.

## 2.3. Inhibition of Cellular DNA Synthesis in Cells Infected with Temperature-Sensitive Mutants of Pseudorabies Virus.

Five of the ten temperature-sensitive mutants of pseudorabies are defective in the synthesis of viral DNA at the non-permissive temperature. The possibility that the lack of viral DNA synthesis was due to a defect in thymidine uptake was ruled out by an identical pattern of orthophosphate incorporation. Characterisation of herpes simplex virus 2 (Halliburton and Timbury, 1973), by similar methods to those used by us, indicated that herpes viral genes responsible for viral DNA synthesis are particularly sensitive to mutation. This may result from sensitivity of a small part of the viral genome to BrdUrd or, alternatively, a large part of the herpesvirus genome may be involved in the synthesis of viral DNA. In contrast, only a small percentage of adenovirus temperature-sensitive mutants are defective in viral DNA synthesis (Wilkie et al., 1973) and this difference between adenoviruses and herpesviruses in their response to mutagens may mean that a considerably larger number of viral genes is necessary for viral DNA synthesis in herpesviruses than in adenoviruses (Subak-Sharpe, 1973).

The finding that the viral DNA-positive mutants are those which most effectively inhibit host cell DNA synthesis suggests that these functions are probably temporally related, but the evidence is not sufficiently firm to allow a decision to be made on whether or not those two functions are closely correlated.

It seems clear from the complementation data (Pringle et al., 1973) that at least four separate functions are necessary for viral DNA synthesis, but what these functions are, or whether they are also involved in the control of the inhibition of cellular DNA synthesis, is not known.

## 3. DNA Synthesis in BHK Cell Nuclei in vitro.

### 3.1. Kinetics of Incorporation of $^{3}$H-dTTP.

Uninfected BHK cell nuclei can incorporate labelled substrate linearly for only 2 - 5 min. As discussed by Bernard and Brent (1973) there are several explanations which may be offered for the cessation of DNA synthesis in isolated nuclei. This phenomenon may be due to depletion or destruction of precursors or cofactors, to inactivation of cytoplasmic factors or to complete utilisation of available template in the absence of initiation of new template sites. They showed with nuclei from HeLa cells, however, that the first two explanations did not apply and that the third was the most likely to be correct. In the BHK system, however, although the

third explanation may apply, the fact that addition of crude cytoplasmic preparations to the nuclei caused a stimulation in DNA synthesis may imply that inactivation of cytoplasmic factors may play an important role in the cessation of DNA synthesis. Winnacker et al., (1972) have reported a similar rapid cessation of DNA synthesis in nuclei from both BHK and 3T6 cells which had been infected with polyoma virus and suggested that this effect is due to a very limited capacity of the nuclei to initiate new rounds of replication.

The virus infected cell nuclei consistently incorporate 5 - 10 times as much radioactivity as do the uninfected cell nuclei. This may merely reflect the ten-fold difference in thymidine triphosphate pools in the two systems or it may reflect the presence of increased numbers of breaks in the DNA which may act as points of initiation for DNA synthesis. Lynch et al., (1970) have shown that the addition of pancreatic DNase to rat liver nuclei produces a large increase in the incorporation of $^{3}$H-dTTP, and the presence in pseudorabies-infected cell nuclei of a virus-induced endonuclease may cause a similar effect to that observed by Lynch et al., (1970).

3.2. Requirements for DNA Synthesis.

The requirements of these nuclei to synthesise DNA are very similar to those reported by Winnacker et al., (1972), who used the same cell line. They reported that their system

required ATP at 2 to 4 mM to produce optimal DNA synthesis, a concentration which we have confirmed as being optimal. In pseudorabies virus infected cell nuclei, however, ATP has a pronounced inhibitory effect on incorporation of $^{3}$H-dTTP into DNA. It is not known whether this reflects a difference in the ATP pools sizes in infected and uninfected cell nuclei or whether this is a reflection of different DNA synthetic machinery in the infected cell nuclei. If the latter alternative could be confirmed, this could have far-reaching implications in chemotherapy of herpesvirus infections.

It appears that the role of ATP in mammalian cell nuclear systems is not simply one of phosphorylation of deoxynucleotides to maintain them in their triphosphate form but that it is more directly involved with the DNA replication reaction (Bernard and Brent, 1973).

The presence of $Ca^{2+}$ ions had little effect on incorporation into uninfected cell nuclei but did cause an inhibition in infected cell nuclei. Winnacker et al., (1973) have reported that the presence of $Ca^{2+}$ ions accelerates deterioration of the nuclear system with time and it may be that this effect is more pronounced in the inherently less stable infected-cell nuclei.

3.3. Biological Significance of the DNA Synthesised by Nuclei Isolated from Virus-Infected and Uninfected Cells.

Three pieces of evidence point to the biological significance of the DNA synthesised by BHK cell nuclei in vitro. Firstly,

the DNA synthetic capability of nuclei isolated from cells incubated for varying lengths of time in medium containing low levels of serum reflects the synthetic capability shown by the whole cell. When stationary cultures of BHK cells are stimulated to grow and divide by the addition of serum, nuclei isolated 11 h after the addition of serum regain the ability to incorporate $^{3}$H-dTTP as well as nuclei from exponentially growing cells. Nuclei isolated 15 h after the addition of serum, however, regain only 11% of the ability of nuclei from exponentially growing cells to incorporate $^{3}$H-dTTP whereas it would be expected that these nuclei should incorporate to the same extent as those isolated at 11 h as the cells are still synthesising DNA at this point. The reason for this discrepancy is not clear. Secondly, nuclei isolated from virus-infected cells synthesise both viral and cellular DNA and the distribution of radioactivity between the two DNAs as a function of time is similar to the distribution observed in whole cells. There is one obvious difference, however, between the viral DNA made in isolated nuclei and that made in whole cells. The buoyant density of viral DNA made in whole cells is 1.73 g/ml, corresponding to a GC content of 72%, whereas that of viral DNA from isolated nuclei is 1.83 g/ml. The cause of this discrepancy is not known, but it seems likely that the increased density will be due to the presence of single-stranded regions in the replicating viral DNA molecules or to the presence of RNA attached to the viral DNA

molecules. The attachment of RNA to the viral DNA can be shown by denaturation of the duplex in 90% formamide and centrifugation in sucrose gradients containing 99% formamide. Treatment of fractions of this gradient with RNase or alkali and a second cycle of centrifugation in isopycnic $CS_2SO_4$ gradients should abolish the density difference due to attachment of RNA to the viral DNA (Waqar and Huberman, 1973).

Finally, incorporation of $^3$H-dTTP into cellular DNA progressively decreases with time in virus-infected nuclei, probably reflecting the inhibition of cellular DNA synthesis observed in whole cells, although the effect in isolated nuclei is not observed until later times. Several groups of workers have shown that DNA synthesis in isolated nuclei occurs by elongation of DNA chains which were initiated *in vivo* and that little or no new initiation occurs in the isolated nuclei (Lynch *et al.*, 1970; 1972; Magnusson *et al.*, 1972). If this is also the case in pseudorabies virus-infected BHK cell nuclei, then it is surprising that any host DNA is synthesised at all as the virus causes inhibition of elongation of DNA chains *in vivo*. Assuming that cellular DNA synthesis in these nuclei is a replicative process the failure to inhibit elongation of cellular DNA probably reflects the loss of inhibitory macromolecules from the nuclei during isolation. This in turn may suggest that the inhibition of synthesis observed in these nuclei reflects an inhibition of initiation of cellular DNA synthesis *in vivo* in addition to the inhibition of elongation of DNA chains *in vivo*.

A much less attractive explanation is that DNA synthesis

in these nuclei is not replicative in nature but rather unscheduled repair replication. Kaufman et al., (1972) have shown that, in rat liver nuclei which were exhibiting unscheduled incorporation of $^{3}$H-dTTP into DNA, the DNA synthesis was independent of the ATP concentration and was only slightly inhibited by the omission of single or multiple deoxyribonucleotides, if at all. On the basis of this and additional evidence, they suggest that these nuclei incorporate $^{3}$H-dTTP into DNA in a repair process rather than a replicative process. In virus-infected BHK cell nuclei the incorporation of $^{3}$H-dTTP is independent of the presence of ATP at the chosen concentration of 2 mM, and the omission of single deoxyribonucleoside triphosphates in both uninfected and infected cell nuclei causes a stimulation of incorporation of $^{3}$H-dTTP rather than an inhibition. These observations in conjunction with the dramatic increase in incorporation of $^{3}$H-dTTP into cellular DNA in nuclei isolated 5 - 6 h after infection, a time when viral nuclease activity is present in the cell, may suggest that the incorporation of $^{3}$H-dTTP by the nuclei is a repair rather than a replicative process. The time course of the rise and fall in viral DNA synthesis in these nuclei, however, argues for a replicative rather than a repair process unless the sensitivity of viral DNA to nuclease action also follows this pattern. A more thorough investigation of the synthetic process in these nuclei is necessary to distinguish between a repair and a replicative process.

3.4. Effects of Omitting Deoxyribonucleotides from the Incubation Mixtures.

The results of omitting single deoxynucleotides are difficult to interpret and no adequate explanation of these results can be offered.

In HSV infected BHK cells the deoxyadenosine triphosphate pool size decreases almost to zero within two hours of infection and remains at that level until as late as 12 h after infection (A. Jamieson, personal communication). If this effect can also be shown in pseudorabies virus-infected cells and if this low level of nucleotide is necessary to allow viral DNA replication to proceed this would explain why the presence of 400 uM exogenous dATP causes a dramatic decrease in the incorporation of $^{3}$H-dTTP into DNA. Thymidine triphosphate has been reported to inhibit the reduction of both adenosine- and guanosine-diphosphates to the deoxynucleotide form (for discussion, see Davidson, 1972). Cohen (1972), however, has reported that the HSV-induced ribonucleotide reductase in KB cells is refractory to inhibition by dTTP. Results presented here, however, suggest that in pseudorabies virus-infected cell nuclei in the presence of dTTP and absence of dATP there is less dATP available than when dTTP is omitted, suggesting that any ribonucleotide reductase present is inhibited by 10 uM dTTP. A stimulation in the incorporation of $^{3}$H-dTTP caused by the omission of dATP is still observed but is much less than that seen in the absence of dTTP. The results obtained by omitting dCTP in the

presence and absence of dTTP, however, are not in agreement with this theory and the true explanation of the results remains unclear.

## 3.5. Effects of Crude Cytoplasmic Preparations on Incorporation of $^{3}$H-dTTP into DNA.

In the absence of success in identifying virus-coded molecules involved in the inhibition of cellular DNA synthesis attempts were made to use the isolated nuclear system in such a study. Bernard and Brent (1973) described some properties of a cytoplasmic factor from HeLa cells which stimulates incorporation of $^{3}$H-dTTP into DNA in isolated nuclei and which is found only in cytoplasm from S-phase cells. The cytoplasmic preparations used by us stimulate incorporation of $^{3}$H-dTTP into DNA in virus-infected cell nuclei rather than, as we had hoped, produce an inhibition of incorporation. The factor(s) present in those preparations stimulate incorporation of $^{3}$H-dTTP into both cellular and viral DNA and seem to have a greater effect on cellular DNA. The stimulation produced increases initially with increasing amounts of cytoplasm but finally plateaus at around 150-200 ul cytoplasm per 200 ul nuclear suspension.

Examination of the profiles in neutral caesium chloride of DNA from nuclei stimulated by the addition of cytoplasm preparations reveals that both the viral and cellular DNA are very heterogeneous. In conjunction with the appearance of a DNA polymerase activity in the cytoplasm after infection, this

suggests that the stimulation may be rather non-specific, perhaps nothing more than a random synthesis of DNA at sites of strand breaks by the cytoplasmic polymerase. Nevertheless, this may be a more specific effect and a more detailed investigation of the effects of these preparations, and of purified viral DNA polymerases, on incorporation of $^{3}$H-dTTP into DNA in these nuclei is planned.

It is clear, however, despite the drawbacks to the system and the need for further characterisation of the DNA synthetic processes occurring in the nuclei, that this system holds promise as a suitable system in which to identify macromolecules produced as a consequence of virus infection and which play a role in the control of the alterations to host cell metabolism after infection. An understanding of these processes should, in turn, lead to a better understanding of the control processes operating in the normal uninfected cell.

## 4. Future Approaches.

The large size of the herpesvirus genome and the considerable complexity of the cell make the simplified system using isolated nuclei an attractive one and perhaps offers the best approach to a further understanding of the events occurring in herpesvirus-infected cell cultures.

Future approaches to the synthesis of DNA in nuclei isolated from uninfected and infected cells *in vitro* must include

a better characterisation of the events occurring within the nuclei. In particular, it is necessary to determine whether DNA synthesis in these nuclei is a repair or replicative process and a conclusive answer to the question of whether or not RNA is involved in viral DNA synthesis would help in our understanding of how this process occurs.

The basis has also been laid for an identification of virus-induced factors which play a role in the alteration of DNA metabolism in the infected cell nuclei and a study of identifiable virus-induced products, such as the DNA polymerase and endonuclease, can also now be made.

A further study of the role of ATP in DNA synthesis in isolated nuclei to confirm and further characterise the inhibition caused by this substance will need to be performed as this effect could conceivably have some clinical importance.

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