The Behaviour of Polyoma Viral DNA in Mammalian Cells

by

Gordon A. Nicholas, B.Sc.

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The University of Glasgow

Department of Biochemistry,
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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TO MY MOTHER AND FATHER

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Abbreviations

These are laid down in the Biochemical Journal Instructions to Authors (revised, 1971) with the following additions:-

BHK21/13 cells	Baby hamster kidney cells, clone 13.
BSA	Bovine serum albumin.
DNase I	Pancreatic deoxyribonuclease (E.C.3.1.4.5), deoxyribonucleate oligonucleotidohydrolase.
Pancreatic RNase	Pancreatic ribonuclease (E.C.2.7.7.16), polyribonucleotide 2-oligonucleotidotransferase.
PFU	Plaque-forming units.
RDE	Receptor-destroying enzyme.
SDS	Sodium dodecyl sulphate.
TCA	Trichloroacetic acid.

CONTENTS

I. INTRODUCTION

Polyoma virus - introduction

In 1953 a filterable agent was recovered from leukaemic mice which, on inoculation into newborn mice, gave rise to tumours of the parotid gland (Gross, 1953; Stewart, 1953). Later, it was discovered that the virus responsible for the parotid gland tumours could be propagated in mouse embryo tissue cultures (Stewart, Eddy & Borgese, 1958) and because this virus was able to produce tumours in many organs, apart from the parotid gland, Stewart and Eddy named it polyoma virus. Tumours may also be induced by polyoma virus in hamsters, rats, guinea pigs and rabbits.

Polyoma virus and the closely related Simian Virus 40 are members of the Papovavirus Group. The name Papova, an abbreviation for papilloma, polyoma and vacuolating agent, was suggested by Melnick (1962). Where appropriate, polyoma virus and SV40 will be described together.

Polyoma virus - physical and chemical properties

The polyoma virus particle or virion does not possess an outer lipid-containing membrane but consists only of DNA (Smith, Freeman, Vogt & Dulbecco, 1960) contained within a protein shell or capsid. The capsid has icosahedral symmetry and is made up from 72 sub-units or capsomeres (Klug, 1965), which are, in turn, assembled from proteins. Sixty of the capsomeres should contain 6 sub-units while the remaining twelve should contain only 5 sub-units, amounting to 420 sub-units for the complete capsid.

Table 1

Properties of polyoma virus and SV40 (Green, 1970)

	Polyoma virus	SV40
Particle weight (daltons)	25×10^6	17×10^6
Diameter of virion (nm)	40-45	40
Molecular weight of DNA	$2.9\text{-}3.4 \times 10^6$	$2.3\text{-}2.5 \times 10^6$
Mole % G+C of DNA	48	41

Purified virus preparations contain varying amounts of "empty" particles which do not contain DNA (Crawford, Crawford & Watson, 1962). These may be distinguished from "full" particles by electron microscopy using negative staining and by their lower buoyant density (Crawford et al. 1962).

Some of the properties of the virion and DNA for polyoma virus and SV40 are shown in Table 1.

(a) Proteins of the virion

The analysis by polyacrylamide gel electrophoresis of sodium dodecyl sulphate-disrupted full or empty virus particles indicated the presence of a major protein component (Thorne & Warden, 1967; Fine, Mass & Murakami, 1968). Fine et al. (1968) estimated that this protein had a molecular weight of about 50,000. The total protein content of a polyoma virion is approximately 2.2×10^7 daltons, consistent with the particle being composed of 420 such protein molecules as predicted by Caspar & Klug (1962).

In addition, comparison of the amino acid composition of protein from full and empty particle preparations indicated the presence of a minor basic protein component in full but not empty virus particles (Murakami, Fine, Harrington & Ben-Sassan, 1968). This was borne out by polyacrylamide gel electrophoresis of proteins from full polyoma virus particles, disrupted by treatment with urea, at pH4.5 in the presence of 10M-urea (Fine et al. 1968). It was estimated

that this basic protein comprised about 10% of total virus protein. The basic nature of this protein and the fact that it was only found in virus particles which contained DNA, suggested that perhaps it was an internal protein involved in the packaging of viral DNA during virus maturation.

Further studies, however, have indicated that not one but possibly four such internal proteins may be present in the polyoma virion (Crawford & Murakami, 1970). Analysis of dissociated polyoma virions by SDS-polyacrylamide gel electrophoresis showed that the capsid protein and four internal proteins were present in the ratio 80:5:5:5:5 respectively in the virion. Molecular weights of 38,000-44,000 for the capsid protein and 30,000, 23,000, 20,000 and 17,000 for the internal proteins were reported: the total molecular weight of the 5 proteins is 130,000. Polyoma viral DNA, with a molecular weight of approximately 3×10^6, consists of about 5,000 base pairs which contain enough information for 1,700 amino acids or, in effect, 200,000 daltons of protein. 65% of the total coding potential would be required, therefore, to specify all of the above proteins. This figure seems rather high and would suggest that some of the components were not virus-coded. They may be basic proteins of the host cell, either having a prescribed function in the virion or remaining as firmly-associated impurities after the virus purification procedure.

SV40 virions are reported to contain three proteins (Anderer, Schlumberger, Koch, Frank & Eggers, 1967). Degradation of SV40 virions at pH10.5 and low ionic strength strips off the two major capsid proteins to leave a stable complex containing viral DNA and an internal protein (Anderer, Koch & Schlumberger, 1968). The failure to reconstitute the complex from its component parts under the conditions of isolation was good evidence that the complex was not an artifact of the extraction procedure. The protein contained in the complex was found in empty as well as full particles, indicating that it has an affinity both for viral DNA and the two capsid proteins. Anderer et al. (1967) estimated that an SV40 virion contained about 80 molecules of the internal protein. It was also shown that antigenic sites of the internal protein were partially exposed over the entire surface of the virion (Koch, Becht & Anderer, 1971). It is possible that there are only 72 internal protein molecules, one for each of the capsomeres, and that this protein has an important rôle in virus assembly. The exact protein constitution of SV40 virions, however, is still not known. Recently, other workers have claimed that SV40 virions may contain as many as six proteins (Girard, Marty & Suarez, 1970; Estes, Huang & Pagano, 1971).

(b) Viral DNA

Polyoma viral DNA, extracted from purified virus, is double-stranded (Crawford, 1963) and can be separated into three components, I, II and III, by sedimentation in the ultracentrifuge (Dulbecco & Vogt, 1963; Weil & Vinograd, 1963).

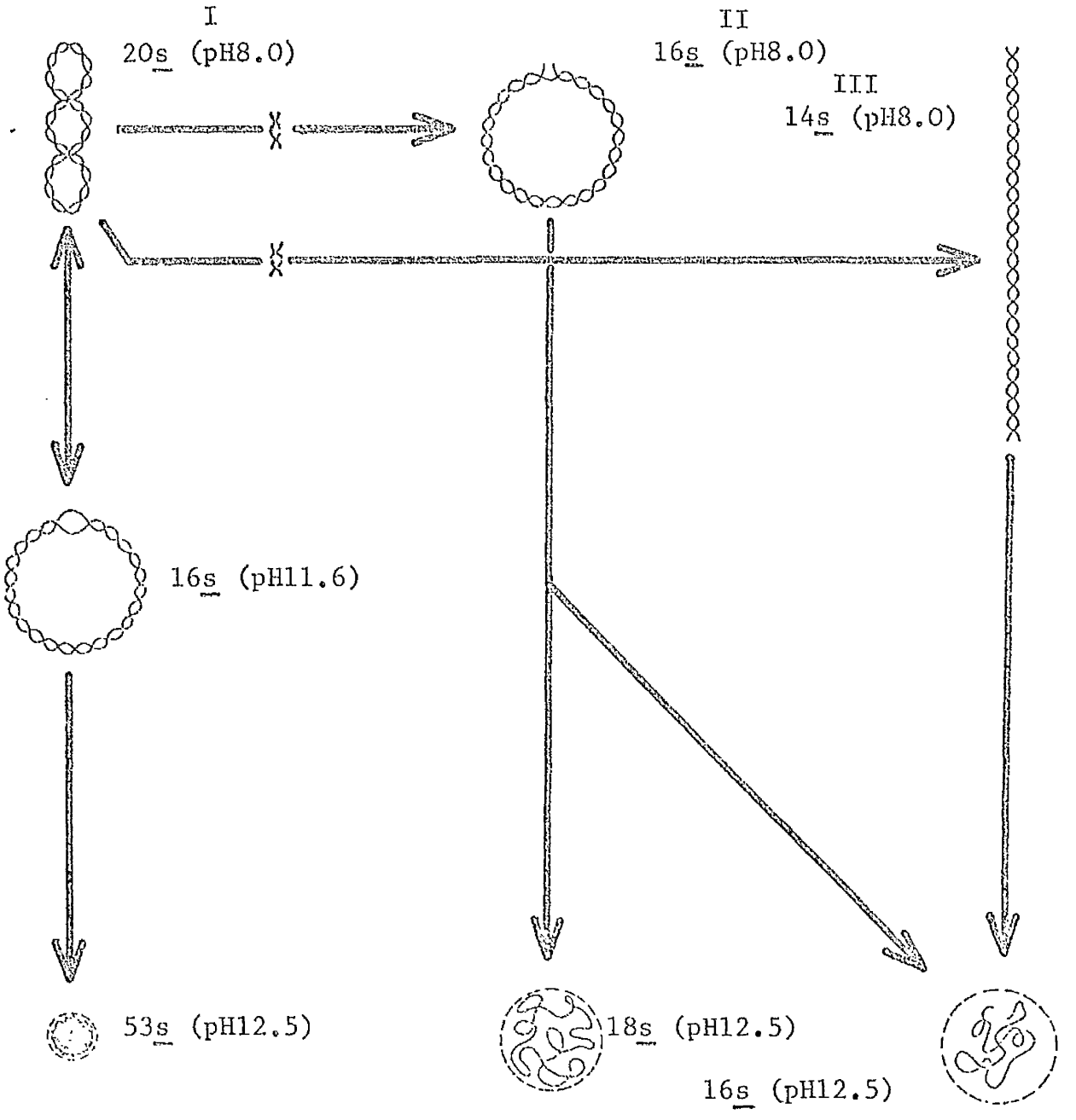
Component I consists of covalently closed cyclic duplex molecules. Each strand of this base-paired molecule is separately continuous (Dulbecco & Vogt, 1963; Weil & Vinograd, 1963). This structure explains the unusually fast renaturation kinetics of component I molecules (Weil, 1963). It was later shown that these molecules are supercoiled or twisted upon themselves (Vinograd, Lebowitz, Radloff, Watson & Laipis, 1965).

Supercoiling is due to a deficiency, rather than an excess, of turns in the double helix as shown by experiments with ethidium bromide (Crawford & Waring, 1967). Ethidium bromide is a phenanthridine dye which forms a reversible complex with double-stranded DNA. Dye molecules intercalate between the base pairs of the DNA causing a local unwinding of the double helix. Addition of increasing amounts of ethidium bromide to supercoiled polyoma viral DNA results in a gradual removal of supercoiling until a non-supercoiled conformation is obtained. This would only be possible if supercoiling was due to a deficiency of turns in the double helix. This deficiency of right-handed turns in the double helix can be made up for by the molecule adopting a conformation with right-handed twists or supercoils. Further addition of ethidium bromide to polyoma viral DNA results in the reappearance of supercoiling, this time in a left-handed sense.

Supercoiling may also be removed by partial denaturation either by heating to 40°C in the presence of formaldehyde (Crawford & Black, 1964) or at pH11.6 (Vinograd et al. 1965). As shown in Fig. 1, further

Fig. 1

Structure and properties of polyoma viral DNA



The dashed circles around the alkali-denatured forms indicate the relative hydrodynamic diameters.

denaturation of component I at pH12.5 leads to the formation of a compact structure with a high sedimentation coefficient.

Component II consists of open cyclic duplex molecules having single strand scissions (Fig. 1). Component II can be formed from component I in vitro by scission of one phosphodiester bond of one of the DNA strands by DNase I (Vinograd et al. 1965). Further treatment with DNase I does not affect the circular integrity of the molecule until single strand nicks in opposite strands occur close to each other. When this occurs the ring opens into a linear form (Fig. 1). The occurence of component II in viral DNA preparations may be an artifact of the extraction technique.

Component III is mainly degraded cellular DNA, which has been packaged instead of viral DNA in virus particles, and has a linear double-stranded structure (Michel, Hirt & Weil, 1967; Winocour, 1967, 1968). Virions containing such cellular DNA are known as pseudovirions and, in the case of SV40, appear to occur to a greater extent when the virus is grown in primary culture cells rather than in cell lines (Levine & Teresky, 1970). Pseudovirions may contain cellular histones associated with the encapsidated cellular DNA and this may account for some of the minor proteins reported to be present in normal virions (see p. 5). The properties of SV40 DNA are similar to those of polyoma viral DNA.

Polyoma virus - consequences of viral infection of cells

The technique of growing animal cells in culture, developed since the late 1940's, has provided an invaluable tool, not only for the propagation of viruses, but also for studying virus-cell interactions in general.

Cells in culture, which take up polyoma virus or SV40, can be classified as either permissive or non-permissive, according to whether or not they support viral replication. Permissive cells support viral DNA replication and virus production and are finally killed; this is known as a productive or lytic infection. Non-permissive cells, however, do not support viral replication.

(a) Infection of permissive cells

In general, mouse cells are permissive for the replication of polyoma virus. The first cell systems used were primary or secondary mouse embryo or baby mouse kidney cells prepared from mouse tissue by trypsinisation. More recently 3T3 cells have been used extensively. 3T3 is an established line of mouse fibroblasts, originally derived from mouse embryo cells (Todaro & Green, 1963).

The lytic response has been used to establish a plaque assay for polyoma virus (Dulbecco & Freeman, 1959; Sachs, Fogel & Winocour, 1959). The virus has a high particle to infectivity ratio, approximately 100 particles, as seen in the electron microscope, corresponding to one plaque-forming unit (PFU) (Crawford et al. 1962).

Monkey cells are permissive for SV40 replication. Studies on this interaction have been carried out with primary African green monkey kidney (AGMK) cells and the established monkey cell lines, BSC-1 and CV-1.

The following sections deal with the events which occur after the infection of permissive cells with polyoma virus. Although the general sequence of events is known the time scale may vary with the virus strain, the host cell and the physiological conditions of the host cell. The events which follow are termed "early" or "late" depending on whether or not they precede the onset of the polyoma viral DNA replication.

(i) Adsorbtion, penetration and uncoating of virus

The processes by which the viral DNA is introduced to the cell are only poorly understood. One approach to this problem is to infect cells with virus and to follow loss of viral infectivity in the medium or loss of accessibility of virus to anti-viral antibodies at the cell surface. The success of such experiments depends very much on the accuracy and sensitivity of the virus assay method. Electron microscopy has also been used to study the fate of ingested virus particles but, because only a small number of particles are ever visualised, the usefulness of this technique is doubtful. In addition, it must be remembered that, out of possibly 10,000 virus particles entering the cell, only one is required to initiate the infective process and the fate of each virus particle cannot be followed.

What has been discovered about adsorption, penetration and uncoating concerns, then, the major population of virus particles which enter a cell.

Initially, the polyoma virus particle is thought to adsorb to the cell surface, held by weak physical forces. Adsorption is reversible and virus particles detach from the cell surface at high pH and increased temperature (Crawford, 1962). Adsorption may be localised at specific receptor sites on the cell surface because adsorbed virus is released when cells are treated with receptor-destroying enzyme (RDE) which is a neuraminidase or sialidase (Hartley & Rowe, 1959). Many animal cells and some erythrocytes possess similar receptors and the haemagglutination of guinea pig red blood cells by polyoma virus is the basis of a convenient assay for the virus.

It seems likely that polyoma virus enters the cell by phagocytosis. This process takes place within minutes after adsorption and virus particles contained with cytoplasmic vacuoles may be seen by electron microscopy (Mattern, Takemoto & Wendell, 1966).

The ultimate destination of the viral DNA is the nucleus since it is here that virus replication occurs (Henle, Deinhardt & Rodriguez, 1959; Sachs & Fogel, 1960), but the site of virus uncoating remains unresolved. Experiments with radioactively-labelled virus led Khare & Consigli (1965) to suggest that the uncoating of polyoma virus took place in the cytoplasm, adjacent to the nucleus, at about 6h after infection and that viral DNA, alone, entered the nucleus. Similar

experiments with SV40, however, have indicated that virus particles may enter the nucleus (Barbanti—Brodano, Swetly & Koprowski, 1970), a finding corroborated by electron microscopy (Hummeler, Tomassini & Sokol, 1970). Mattern et al. (1966) detected polyoma virus particles in the nuclear membrane of infected cells.

The mechanism of uncoating is also obscure. It might be the result of attack on the virus particle by cellular enzymes or, alternatively, physical stress applied to a virion, firmly held in a membrane, might cause an alteration in capsid structure and allow viral DNA to be released. It seems likely that, as a result of adsorbtion, penetration and uncoating, the viral DNA is introduced into the nucleus of the infected cell.

(ii) Synthesis of early viral mRNA

The extent of transcription prior to viral DNA replication has been studied in the case of SV40. DNA-RNA hybridisation experiments have shown that early viral-specific messenger RNA (viral mRNA) synthesised in SV40-infected monkey cells corresponds to transcription of between 28 and 40% of the viral genome (Aloni, Winocour & Sachs, 1968; Oda & Dulbecco, 1968; Sauer & Kidwai, 1968). By combining the hybridisation technique with sedimentation velocity analysis and polyacrylamide gel electrophoresis, Tonegawa, Walter, Bernardini & Dulbecco (1970) have estimated that early viral mRNA has a molecular weight of 8×10^5. This corresponds to about half of a single strand of SV40 DNA. Martin (1970) obtained a similar result.

Transcription of early viral mRNA is probably mediated by the DNA-dependent RNA polymerase of the host cell but the mechanism, which restricts transcription to only a section of the viral genome, is obscure. The possibility of complete transcription followed by selective degradation cannot be ruled out.

(iii) Synthesis of viral-specific antigens

Early viral mRNA is responsible for the synthesis of viral-specific proteins prior to viral DNA replication. It seems likely that one of these proteins is an antigen, which is detected in the nucleus as early as 12h after infection with polyoma virus (Weil & Kára, 1970). This antigen, which is immunologically distinct from capsid protein, reacts with serum from animals bearing polyoma virus-induced tumours (Habel, 1965) and is known as the tumour antigen or T-antigen. The T-antigen may be detected by complement fixation or immunofluorescence. A similar, but immunologically distinct antigen is found in SV40-infected permissive cells (Black, Rowe, Turner & Huebner, 1963; Hatanaka & Dulbecco, 1966).

The presence of another viral-specific antigen was suspected when it was discovered that hamsters and mice, after immunisation with polyoma virus, rejected the transplantation of polyoma virus-induced tumour cells from syngeneic animals (Habel, 1961; Sjögren, Hellström & Klein, 1961). This antigen (the transplantation antigen) is thought to reside at the surface of the tumour cells. The transplantation antigen is also found on the surface of mouse cells

infected with polyoma virus (Hellström & Sjögren, 1966; Irlin, 1967) and there is some evidence that it appears prior to the onset of viral DNA synthesis (Irlin, 1967; Jarrett & Pitts, unpublished). A similar SV40-specific transplantation antigen has also been described (Defendi, 1963; Habel & Eddy, 1963; Koch & Sabin, 1963).

The T-antigen and transplantation antigens are viral-specific in that the same antigens are induced by virus in cells of different species. This is strong evidence that these antigens are coded for by the viral DNA.

(iv) Induced activity of enzymes

Increases in the activities of a number of enzymes, associated with DNA synthesis, have been reported in polyoma viral-infected mouse cells. These enzymes are DNA polymerase (DNA nucleotidyltransferase, E.C.2.7.7.7), thymidine kinase (ATP: thymidine 5'-phosphotransferase, E.C.2.7.1.21), polynucleotide ligase, deoxycytidine kinase, TMP kinase, dCMP kinase (ATP: deoxyCMP phosphotransferase, E.C.2.7.4.5), CDP reductase, dihydrofolate reductase, dCMP deaminase and TMP synthetase (Dulbecco, Hartwell & Vogt, 1965; Frearson, Kit & Dubbs, 1965; Kit, Dubbs & Frearson, 1966a; Sheinin, 1966a; Kára & Weil, 1967; Beard & Pitts, 1968; Sambrook & Shatkin, 1969; Beard, 1971).

The increase in activities of some of these enzymes has been shown to be prevented by puromycin, an inhibitor of protein synthesis,

but not by inhibitors of DNA synthesis (Kit & Dubbs, 1969). Possible activation or stabilisation of pre-existing enzyme molecules, as a result of viral infection, has been ruled out in some cases by mixing extracts of infected and uninfected cells and assaying the enzyme activity of the mixture (Hartwell, Vogt & Dulbecco, 1965). Though unproven, it seems likely that a product of the parental viral DNA is responsible for initiating the de novo synthesis of enzyme molecules.

The properties of viral-induced enzymes and the enzymes of uninfected cells have been compared in an attempt to determine whether or not the viral-induced enzymes are viral-coded (Kit & Dubbs, 1969; Smart & Pitts, 1971). Polyoma viral DNA could not code for all of the above enzymes and there is no significant evidence to suggest that any are viral-coded.

Good evidence, that the induced thymidine kinase is not viral-coded, was obtained by Basilico, Matsuya & Green (1969) who showed that thymidine kinase was not induced when 3T3 cells lacking thymidine kinase activity (TK-3T3) were infected with polyoma virus.

(v) Induced synthesis of cellular DNA

Polyoma virus infection of confluent cultures of mouse kidney cells, which have a low level of DNA synthesis, results in a large increase in cellular DNA synthesis (Dulbecco et al. 1965; Weil, Michel & Ruschmann, 1965; Winocour, Kaye & Stollar, 1965). Similarly, muscle cells, which are in their terminal state of differentiation

and do not synthesise DNA or undergo mitosis, resume the synthesis of DNA after polyoma viral infection (Fogel & Defendi, 1967; Yaffe & Gershon, 1967).

Moreover, polyoma viral infection has been shown to induce DNA synthesis in mouse embryo cells, in which DNA synthesis was first reduced to a low level by X-irradiation (Gershon, Hausen, Sachs & Winocour, 1965), by the incorporation of iododeoxyuridine into the cellular DNA (Kasamaki, Ben-Porat & Kaplan, 1968) or by maintaining the cells in Eagle's medium containing a limiting amount of calf serum (Fried & Pitts, 1968).

The induction of cellular DNA synthesis appears to parallel the increase in enzyme activities described above and it has been shown, by autoradiography and immunofluorescence, that the cells in which induction occurs go on to synthesise progeny virus (Basilico, Marin & di Mayorca, 1966; Vogt, Dulbecco & Smith, 1966). Studies on the nitrous acid inactivation of polyoma viral DNA have shown that induction of cellular DNA synthesis is one-fifth as susceptible as viral infectivity to inactivation, suggesting that only one-fifth of the viral genome is required for the induction of cellular DNA synthesis (Gershon et al. 1965).

The induction of cellular DNA synthesis is not the result of repair but appears to represent genuine DNA replication. A doubling of the DNA content of infected cells was reported by Gershon et al. (1965) and further evidence for replication was the finding that

60-80% of the total DNA in an infected mouse cell culture contained one strand which had been synthesised prior to viral infection (Weil et al. 1965; Ben-Porat, Kaplan & Tennant, 1967). Furthermore, induced cellular DNA has the same buoyant density as that of uninfected cells (Winocour et al. 1965). Recently, however, Cheevers, Branton & Sheinin (1970) have shown that some of the DNA, which has replicated, contains discontinuities in the newly synthesised strand.

An inhibition of cellular DNA synthesis has been reported for exponentially-growing mouse embryo cells infected with polyoma virus at high multiplicity (Sheinin & Quinn, 1965; Sheinin 1966b). This result, which contrasts with the work described above, may be due to the physiological condition of the cells in which DNA is being synthesised actively prior to infection. Alternatively, it may be a consequence of the high multiplicities of virus employed in these experiments.

(vi) Viral DNA replication

Synthesis of viral DNA can first be detected about the time of the induction of enzyme activities and cellular DNA synthesis. The onset of viral DNA synthesis occurs at 12-24h after infection depending on the cell system used.

Hirt (1966) showed that polyoma viral DNA replicates in a semiconservative manner, which requires that the two strands of a replicating molecule separate after replication. A separation of

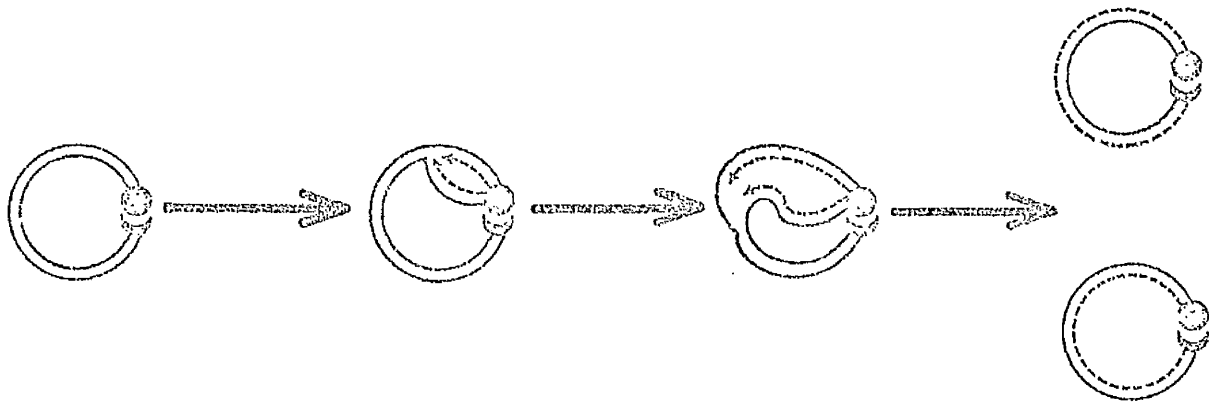
the strands of polyoma viral DNA, a closed circular duplex structure, is not possible without first introducing at least one single-strand scission. It was shown (Hirt, 1969; Bourgaux, Bourgaux-Ramoisy & Dulbecco, 1969) that replication of the ring proceeded in a manner compatible with the mechanism proposed by Cairns (1963, 1966), which is shown in Fig. 2. Hirt (1969) detected such replicating molecules, which have a characteristic triple-branched structure, by electron microscopy. Later, Bourgaux et al. (1969) detected replicative intermediates by labelling infected cultures for short periods with [^{3}H] thymidine. After alkaline denaturation, these replicative intermediates yielded single-stranded rings and a heterogeneous population of single-stranded linear molecules, not longer than the strands of mature viral DNA. These date excluded the rolling circle model (Gilbert & Dressler, 1968) as a possible mode of replication, because a feature of this mechanism (Fig. 3) is the formation of strands longer than unit length.

A simple model for the replication of polyoma viral DNA is shown in Fig. 4. Three steps are envisaged: a ring-opening step involving an endonuclease, a polymerisation step mediated by a polymerase or polymerising complex and a ring-closure step involving a ligase.

Although viral DNA replication occurs in the nucleus of the infected cell it is not known whether replication takes place at

Fig. 2

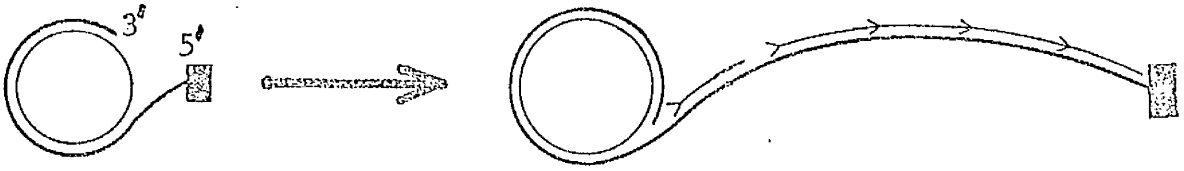
Replication of cyclic DNA molecules: The Cairns Mechanism



The two DNA chains are represented by concentric circles linked at a ball and socket joint which represents the hypothetical "swivel". Duplication begins at the swivel and proceeds in an anti-clockwise manner. Newly synthesised DNA chains are represented by dashed lines and the arrowheads mark the replicating fork.

Fig. 3

Replication of cyclic DNA molecules: The Rolling Circle Model

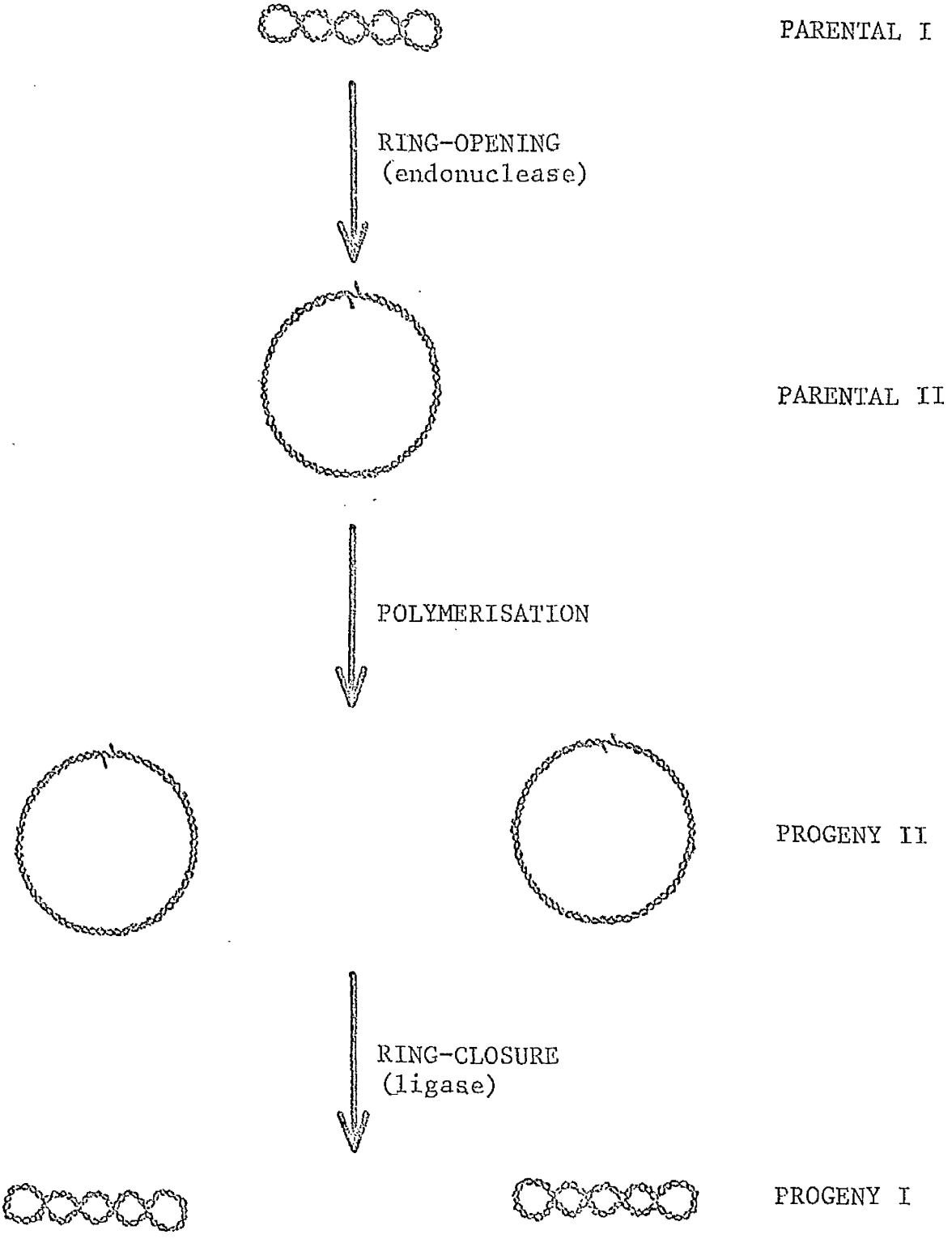


(a) (b)

One of the parental DNA strands is opened by an endonuclease and the newly-formed 5' end is transferred to a membrane site as shown in (a). A new copy of this strand is synthesised by 5' to 3' chain elongation at a 3' end using the other strand, which always remains cyclic, as a template. Such synthesis results in the continuous displacement of the other end of the same strand to form a tail as shown in (b). At the same time the displaced strand acts as a template for the synthesis of the other strand formed by 5' to 3' synthesis in short pieces which are subsequently joined by a ligase. The resultant double-stranded tail may then be used for the formation of cyclic progeny DNA molecules by a recombination process between sequences the length of a genome apart.

Fig. 4

Replication of polyoma viral DNA



specific sites, for example on a membrane. Equally obscure is the specificity of the enzymes involved in replication. There may be a unique phosphodiester bond in one of the strands that is cleaved by the endonuclease and from which point polymerisation is initiated. The same bond will then be re-formed by the ligase when polymerisation is complete.

The phenomenon of supercoiling of polyoma viral DNA is associated with the ring-closure step. Wang, Baumgarten & Olivera (1967) suggested three possible causes:-

(1) A portion of the molecule is unwound at the time of ring-closure to accommodate the ligase.

(2) The number of base pairs per helical turn is higher in the environment of ring-closure than in vitro.

(3) The DNA is associated with a protein core at the time of ring-closure and extraction of the DNA results in the loss of this protein core.

The mode of replication of SV40 DNA has been shown to be similar to that of polyoma viral DNA (Levine, Kang & Billheimer, 1970). An interesting finding of Eason & Vinograd (1971) is that unencapsidated progeny SV40 DNA is heterogeneous with respect to the number of supercoils whereas SV40 DNA found in virions is homogeneous. It is not known if the heterogeneous material, after nicking and re-ligasing is ever found in mature virions.

(vii) Synthesis of late viral mRNA

After the onset of polyoma viral DNA replication there is an increase in the amount of viral-specific mRNA synthesised (Benjamin, 1966; Weil, Pétursson, Kára & Diggelman, 1967; Cheevers & Sheinin, 1970; Hudson, Goldstein & Weil, 1970).

In SV40-infected monkey cells the viral-specific mRNA synthesised at this time (late SV40 mRNA) contains not only early viral mRNA but also other viral-specific mRNA sequences (Aloni et al. (1968); Oda & Dulbecco, 1968; Sauer & Kidwai, 1968). Since it has been demonstrated (Martin & Axelrod, 1969a) that all SV40 genes are transcribed in SV40-infected monkey cells, it must be concluded that the entire SV40 genome is transcribed during the late period.

Late SV40 mRNA may consist of one polycistronic messenger RNA, equivalent in size to a strand of SV40 DNA (Martin, 1970). However RNA capable of hybridising to viral DNA is heterogeneous with respect to molecular weight and material of greater and smaller molecular size is detected. Tonegawa et al. (1970) have claimed that late SV40 mRNA consists of two main components of molecular weight 7.9×10^5 and 5.6×10^5.

Studies on the synthesis of viral mRNA in polyoma viral-infected cells have not been as detailed (Martin & Axelrod, 1969b; Hudson et al. 1970), but it seems likely that late viral mRNA represents the transcription product of the complete polyoma viral genome.

(viii) Modification of the cell surface

Certain glycoproteins of plant origin, such as wheat germ agglutinin and concanavalin A, are able to bind to sites on the surface of animal cells causing the cells to agglutinate. Agglutination is specific for a carbohydrate-containing determinant on the cell surface.

Normally, the agglutinin-binding sites are thought to be inaccessible since agglutination is only achieved at relatively high concentrations of the plant agglutinin. However these sites become exposed after the cells have been treated briefly with trypsin (E.C.3.4.4.4) and the cells are agglutinated at lower agglutinin concentrations (Burger, 1969). Using a synchronised population of 3T3 cells, Fox, Sheppard & Burger (1971) showed that the agglutinin-binding sites become exposed during mitosis.

Polyoma viral infection of 3T3 cells results in the exposure of plant agglutinin-binding sites (Benjamin & Burger, 1970; Eckhart, Dulbecco & Burger, 1971). Inhibitors of DNA synthesis prevent the surface modification taking place (Benjamin & Burger, 1970; Eckhart et al. 1971). The surface modification appears to be closely related to the induction of DNA synthesis; it occurs in SV40-infected CV-1 cells in which there is an induction of DNA synthesis but does not take place in one line of BSC-1 cells infected with SV40 in which there is no induction of DNA synthesis (Ritzi & Levine, 1970); there

is an induction of DNA synthesis and a surface modification, however, when the same BSC-1 cells are infected with adenovirus 5, which shows that these cells are potentially capable of undergoing a surface modification (Sheppard, Levine & Burger, 1971); an induction of DNA synthesis after SV40 infection has been reported with a different BSC-1 cell line and, in this case, the surface modification takes place as well (Sheppard et al. 1971). The fact that a surface modification takes place in SV40-infected 3T3 cells (Ben-Bassatt, Inbar & Sachs, 1970), although these cells do not support viral DNA replication, would indicate that the apparent requirement for DNA synthesis pertains to cellular and not viral DNA synthesis.

Infection of 3T3 cells with the temperature-sensitive ts-3 mutant of polyoma virus at the non-permissive temperature, at which there is no induction of DNA synthesis, does not lead to a surface modification. Although, clearly, closely related, cellular DNA synthesis and the surface modification are not inseparable. Infection of 3T3 cells with NG-18, a host range mutant (Benjamin, 1970) of polyoma virus, resulted in induction of cellular DNA synthesis but not in the exposure of concanavalin A-binding sites (Benjamin & Burger, 1970). This suggests that cellular DNA synthesis, although necessary, is not a sufficient pre-requisite for the surface modification to occur.

(ix) Capsid protein synthesis, virus maturation and cell death

Late viral mRNA almost certainly carries the information necessary for polyoma viral capsid protein synthesis, which is first detected several hours after the onset of viral DNA synthesis (Weil et al. 1967).

Presumably, capsid protein is transported from the ribosomes into the nucleus where it can be detected by immunofluorescence.

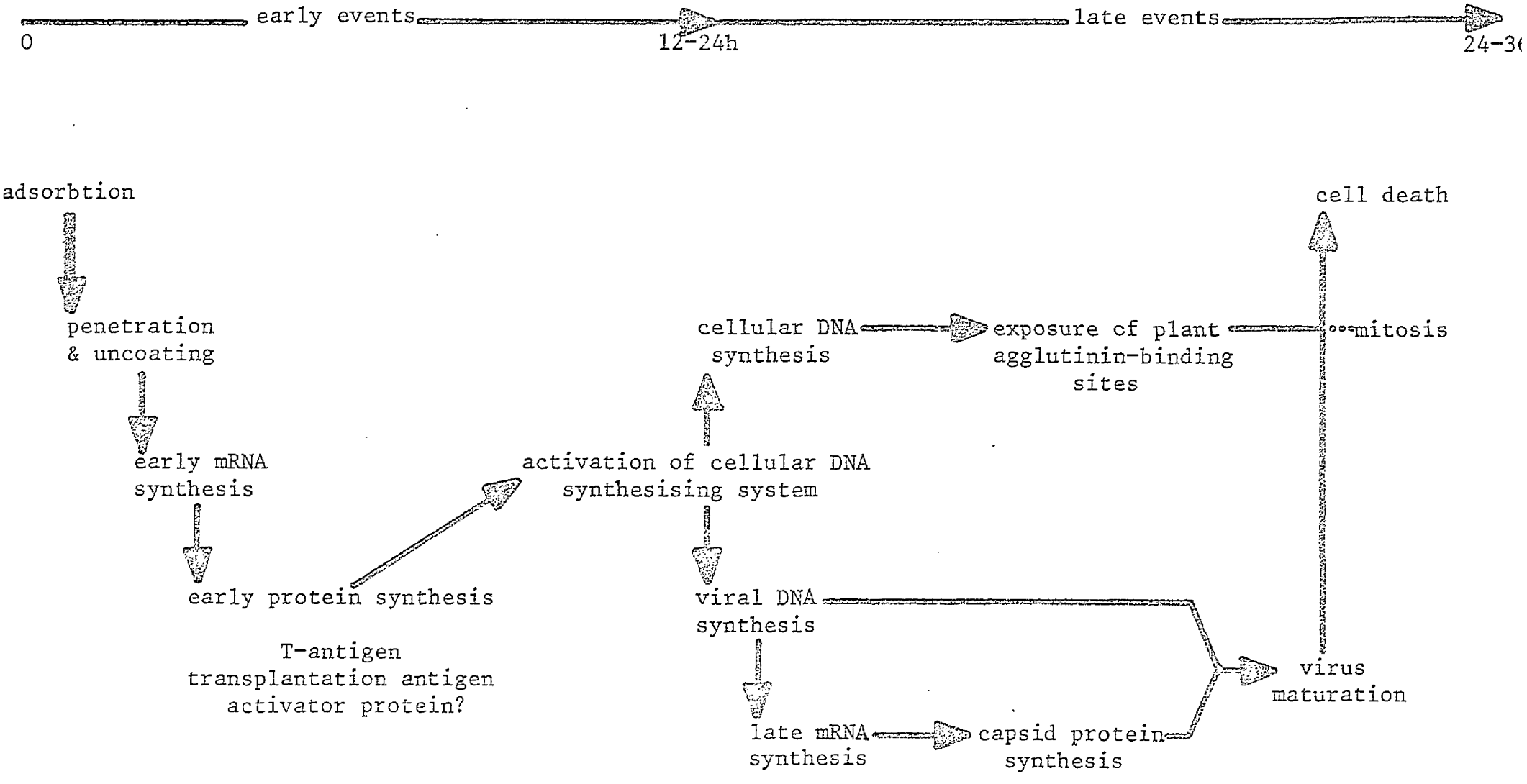
Little is known about the formation of intact polyoma virus particles from component parts except that the process takes place in the nucleus of the infected cell (Henle et al. 1959; Sachs & Fogel, 1960). The abundance of empty virus particles in virus preparations (Crawford et al. 1962) suggests that possibly virion proteins are capable of self-assembly in the absence of viral DNA; however, empty particles may be formed by loss of viral DNA subsequent to the assembly of full particles.

Green, Miller & Hendler (1971) have shown that replicating and newly-replicated polyoma viral DNA can be extracted from infected cells in the form of a nucleoprotein complex. This complex has recently been found to contain capsid protein (Green, 1971), but is distinct from mature virus particles. The formation of the nucleoprotein complex may be a stage in virus maturation.

Electron microscopy of polyoma viral-infected and SV40-infected cells (Bernard, Fèbvre & Cramer, 1959; Granboulan, Tournier, Wicker & Bernard, 1963; Mattern et al. 1966) has shown the development of densely

Fig. 5

Polyoma viral infection of permissive cells



staining filaments in the nuclei of such cells. Although the nature of the filaments is unknown it has been suggested that they may be a developmental form of the virus. Later, virus particles, sometimes organised in crystalline arrays, are observed in the nuclei. Eventually, when degeneration of the cell nucleus has become apparent, virus particles are observed in the cytoplasm, where they adsorb in monolayers to various cell membranes.

(x) Summary

The events taking place following the infection of permissive cells with polyoma virus or SV40 are summarised in Fig. 5. The early events terminate in the activation of the cellular DNA synthesising system, probably by one of the protein products of early viral mRNA. Weil & Kára (1970) have suggested that the T-antigen has the rôle of activator, but there is no evidence to support this.

Late events include the replication of viral DNA and the synthesis of viral capsid protein, culminating in the assembly of progeny virus. These processes appear to interfere with normal DNA synthesis and cell division and the cell is eventually killed.

(b) Infection of non-permissive cells

Hamster and rat cells are non-permissive for polyoma viral replication. Mouse cells are non-permissive for SV40 replication. Rat and hamster embryo cells have been used to study this virus-cell

interaction in the case of polyoma virus but much work has centred around the BHK21/13 cell line isolated from baby Syrian hamster kidney tissue by Macpherson & Stoker (1962).

A small proportion of hamster cells infected with polyoma virus and a substantial (up to 50%) proportion of mouse (3T3) cells infected with SV40 become transformed (Vogt & Dulbecco, 1960; Stoker & Macpherson, 1961; Macpherson & Stoker, 1962; Todaro & Green, 1966a). The following sections deal with the phenomenon of cell transformation.

(i) Properties of transformed cells

Cells transformed by polyoma virus or SV40 can be cloned and shown to be free of virus particles. Such cells acquire several new properties which are retained permanently.

Transformed cells produce tumours when inoculated into the syngeneic host animal. In tissue culture, unlike normal cells, they no longer require surface attachment for growth and will grow in suspension culture. Growth of polyoma viral-transformed cells in agar forms the basis of a convenient transformation assay (Macpherson & Montagnier, 1964).

Transformed cells are not subject to all the controls regulating the growth of normal cells. Contact inhibition of movement (Stoker & Rubin, 1967) is no longer apparent and, whereas normal cells tend to form parallel arrangements in a monolayer, transformed cells pile up in random array (Macpherson & Stoker, 1962) and reach higher saturation densities. Transformed cells also have a higher rate of

glycolysis than normal cells.

The characteristic loss of growth control of transformed cells is related possibly to the modification of their cell surface. In contrast to normal cells, the sites on the cell surface, which bind various plant agglutinins (see p.25), are exposed in transformed cells throughout the cell cycle (Aub, Tieslau & Lankester, 1963; Burger & Goldberg, 1967; Burger, 1968; Inbar & Sachs, 1969; Pollack & Burger, 1969). Gross differences in the chemical composition of cell surface constituents between normal and transformed cells have also been reported. Hakomori & Murakami (1968) found that polyoma viral-transformed BHK21/13 cells have a decreased content of the glycolipid hematoside N-acetyl-neuraminyllactosylceramide and a corresponding increase in lactosylceramide, compared to normal BHK21/13 cells. Cytoplasmic membranes isolated from SV40-transformed 3T3 cells contain lower sialic acid and galactosamine but increased glucosamine levels, compared to normal 3T3 cells (Wu, Meezan, Black & Robbins, 1969). Recently, Buck, Glick & Warren (1971) have detected molecular weight differences in glycopeptides isolated from normal and polyoma viral-transformed cells.

Finally, cells transformed by polyoma virus contain the T-antigen in the nucleus (Habel, 1965) and the transplantation antigen at the cell surface (Habel, 1961; Sjögren et al. 1961). These viral-specific antigens are described more fully on p. 14 . The corresponding SV40-specific antigens are found in SV40-transformed cells. At the present

time, the contribution, if any, of these antigens to the transformed state is not known.

(ii) Evidence for the persistence of viral genes in transformed cells

DNA-RNA hybridisation experiments have demonstrated the presence of viral-specific mRNA in polyoma virus and SV40-transformed cells (Benjamin, 1966; Aloni et al. 1968; Oda & Dulbecco, 1968; Sauer & Kidwai, 1968; Martin & Axelrod, 1969a,b).

Studies on the viral mRNA synthesised in SV40-transformed mouse cells indicate that such RNA lacks most late mRNA sequences (see p. 24) and is contained in RNA molecules which are longer than a strand of SV40 DNA (Lindberg & Darnell, 1970; Martin, 1970; Tonegawa et al. 1970). SV40 and polyoma viral mRNA is transcribed from viral DNA in the nuclei of transformed cells (Westphal & Dulbecco, 1968). Sambrook, Westphal, Srinivasan & Dulbecco (1968) demonstrated that, in SV40-transformed 3T3 cells, SV40 DNA is associated with the cellular DNA by alkali-stable linkages.

When SV40-transformed cells are fused with permissive cells, in the presence of inactivated sendai virus, infectious SV40 virus is produced (Gerber, 1966; Koprowski, Jensen & Steplewski, 1967; Watkins & Dulbecco, 1967). This process, which must result from the full expression of the complete SV40 genome, contained in transformed cells, after fusion with permissive cells, is known as virus rescue. Attempts to rescue polyoma virus from polyoma viral-transformed cells have, so far, been unsuccessful. This indicates that perhaps only a

segment of the polyoma viral genome is present in cells transformed by the virus.

Early estimates showed that as many as sixty viral DNA equivalents per cell are present in some SV40-transformed cells but fewer than five in polyoma viral-transformed cells (Westphal & Dulbecco, 1968). More recently, however, it has been shown that SV40-transformed cells may contain only one viral DNA equivalent per cell (Gelb, Kohne & Martin, 1971).

In order to decide whether the presence of viral DNA is necessary for the maintenance of the polyoma viral-transformed cell phenotype, Marin & Littlefield (1968) studied the loss of the transformed phenotype by cells which lose DNA. They selected a tetraploid BHK21/13 cell line from two genetically different diploid BHK21/13 cell lines. One parental line lacked inosinic pyrophosphorylase, the other thymidine kinase. The tetraploid line was then transformed with polyoma virus and loss of DNA from these cells was promoted by selecting for cells which had lost the ability to make inosinic pyrophosphorylase and were therefore resistant to 6-thioguanine. All of the surviving clones had a reduced chromosome complement and some had lost the transformed phenotype (Marin & Littlefield, 1968; Marin & Macpherson, 1969). In addition the hybrid clones which had lost the transformed phenotype could be retransformed with polyoma virus. This indicated that the loss of viral genes but not cellular genes, perhaps necessary for the expression of the transformed phenotype, is associated with

reversion to the normal phenotype (Marin & Macpherson, 1969). A similar study has been made in the case of SV40 (Weiss, Ephrussi & Scaletta, 1968). These workers studied the loss of SV40 T-antigen synthesising ability by hybrid cells, formed from SV40-transformed human cells and normal mouse cells. These hybrid cells lose human chromosomes spontaneously and their loss was associated with the loss of the ability of the cells to synthesise T-antigen. Furthermore, these cells regained the ability to synthesise T-antigen after re-transformation by SV40 (Weiss, 1970).

These findings provide strong evidence that the continued presence of at least part of the viral genome is necessary for the maintenance of the transformed state.

(iii) The mechanism of transformation

Transformation by polyoma virus is a rare event, about 10^4 PFU being required to initiate one transforming event in BHK21/13 cells (Stoker & Macpherson, 1964). The dose-response curve of transformation is linear up to a maximum at which only about 5% of the cells are transformed (Macpherson & Montagnier, 1964). This low frequency of transformation is not due to any genetic difference in the cells that become transformed because isolated clones of a given population show the same low frequency of transformation (Black, 1964). Cells show only small differences in transformation efficiency at different stages in the cell cycle (Basilico & Marin, 1966), which tends to rule out the possibility that only cells in a particular physiological

condition may be transformed. The transformation of 3T3 cells by SV40 is a less inefficient process and, under optimal conditions, 50% of the clones that arise after the plating of infected cells are transformed (Todaro & Green, 1966a).

The linearity of the dose-response curve suggests that a single infectious virus particle is sufficient to cause one transformation event (Macpherson & Montagnier, 1964; Black, 1966). However the inefficiency of the transformation process, particularly in the polyoma virus-BHK21/13 system, has made difficult the biochemical study of events leading up to transformation. The permanent expression of the transformed cell phenotype appears to result from the integration of viral DNA in the host cell chromosomes. Todaro & Green (1966b) showed that SV40-infected 3T3 cells must undergo one cell division in order to fix the transformed state and several further divisions to allow the full expression of the transformed phenotype. It seems likely that the insertion of SV40 DNA into 3T3 cellular DNA occurs during the period of DNA synthesis in the cell cycle (Todaro & Green, 1967).

It is generally assumed that all of the viral genetic information is required for the productive infection of permissive cells by polyoma virus or SV40. Parts of the genome, for example that specifying the coat protein, may not be necessary, however, for transformation. The inactivating effects of various radiations on the infectivity and transforming ability of polyoma virus have been compared, in order to

obtain an estimate of the fraction of the genome required for transformation. Infectivity was approximately five times more sensitive than transformation to UV-irradiation and to the indirect effect of X-rays (Latarjet, Cramer & Montagnier, 1967). Similar results were obtained by Gershon et al. (1965) in studies on the inactivation, by nitrous acid, of the plaque-forming and transforming activities of polyoma virus. It was concluded, therefore, that transformation requires only one-fifth of the polyoma viral genome.

Stoker (1968) showed that exposure of BHK21/13 cells to high multiplicities of polyoma virus resulted, not only in the permanent transformation of the usual small fraction of the cell population, but also in a high proportion of the cells acquiring the ability to grow in suspension for several cell divisions. This phenomenon, whereby polyoma viral-infected BHK21/13 cells transiently acquired at least one of the properties of transformed cells, was termed abortive transformation (Stoker, 1968).

Abortive transformation of BHK21/13 cells by polyoma virus may be related to the induction of cellular DNA synthesis that occurs in non-permissive cells infected with SV40 at high multiplicities (Gershon, Sachs & Winocour, 1966; Henry, Black, Oxman & Weissman, 1966; Sauer & Defendi, 1966; Kit, DeTorres, Dubbs & Salvi, 1967).

Black (1966) has shown that at high multiplicities of infection 100% of 3T3 cells exposed to SV40 synthesise T-antigen, although only 20 to 30% of the cells become transformed. Only transformed cells continue to synthesise T-antigen and the remainder lose it during subsequent growth (Oxman & Black, 1966). Synthesis of T-antigen has also been reported after polyoma viral-infection of rat and hamster embryo cells (Fogel, Gilden & Defendi, 1967).

The events occuring after the infection of non-permissive cells with polyoma virus or SV40, at high multiplicities, appear to resemble the early events in the infection of permissive cells. In SV40-infected 3T3 cells, however, there is no detectable synthesis of viral DNA (Henry et al. 1966) or viral capsid protein and infectious virus (Black, 1966). It is possible that limited synthesis of viral capsid protein (Fraser & Crawford, 1965) and virus (Bourgaux, 1964) may occur after polyoma viral infection of BHK21/13 cells but this remains to be substantiated.

Polyoma virus - known gene functions of viral DNA

Polyoma viral DNA has a molecular weight of 3×10^6, sufficient to code for 5-10 proteins. From the preceding sections it is clear that polyoma viral DNA codes at least for the capsid protein, internal protein(s), the viral-specific T-antigen and transplantation antigen, and functions responsible for transformation and the induction of cellular DNA synthesis.

Identification of some of the polyoma viral gene functions has been facilitated by the isolation of temperature-sensitive mutants of polyoma virus (Fried, 1965, 1970; di Mayorca, Callender, Marin & Giordano, 1969; Eckhart, 1969). Wild-type polyoma virus is treated with a chemical mutagen, either nitrous acid or hydroxylamine, and plaques of surviving virus isolated on monolayers of permissive cells. From the surviving virus ts mutants may be isolated, which show markedly reduced growth at 38.5°C (the non-permissive temperature) compared to 31.5°C (the permissive temperature). By observing whether a given biochemical change occurs at the non-permissive temperature after infection with a ts mutant, it can be established whether that mutated gene product is required for that biochemical change to occur. ts mutants of polyoma virus may be arranged into five complementation groups on the basis of their complementation properties as shown in Table 2. Each complementation group can be identified with a viral gene.

Groups I & IV

ts mutants in these groups appear to be defective in the genes which specify two viral structural proteins. This is consistent with a major capsid protein and at least one internal protein being coded for by the viral DNA.

Group II

This group of ts mutants is defective in a gene involved with viral DNA synthesis and in the initiation of transformation. BHK21/13

Table 2

Temperature-sensitive mutants of polyoma virus

	I ts10	II ts-a	III P155	IV 1260	V ts-3
Virus particles thermostable	-	+	+	-	
Infectious viral DNA synthesis	+	-	-	+	-
Induction of cellular DNA synthesis	+	+	+	+	-
Initiation of transformation	+	-	+	+	+
Maintenance of transformation	+	+	+	+	-
T-antigen synthesis	+	-			
Exposure of plant agglutinin-binding sites		+			-

cells are not transformed by the ts-a mutant at the non-permissive temperature, but cells transformed with this mutant at the permissive temperature remain transformed at the non-permissive temperature (Fried, 1965). This indicates that the ts-a function is involved in the initiation but not the maintenance of the transformed state. It has been suggested that the ts-a function may be an endonuclease, which introduces nicks in viral DNA, required both for replication and for integration into host cell DNA in transformation. Further support for this idea has been provided by Cuzin, Vogt, Dieckmann & Berg (1970), who showed that closed circular monomers, dimers and trimers of viral DNA were produced when 3T3 cells transformed by the ts-a mutant and stable at the non-permissive temperature, were incubated at the permissive temperature. A possible explanation is that viral DNA molecules, integrated in tandem in the cell chromosomes, are excised at the permissive temperature due to the reactivation of the ts-a function.

Group III

The P155 mutant is defective in the synthesis of viral DNA but not in transformation (Eckhart, 1969). This indicates that at least one viral function is involved in viral DNA synthesis but not in transformation.

Group V

The ts-3 mutant is defective in a function required for viral DNA synthesis, the induction of cellular DNA synthesis and the maintenance

of the transformed state. Using BHK21/13 cells transformed by this mutant, Dulbecco & Eckhart (1970) were able to demonstrate that the maintenance of the transformed state was under the direct control of a viral gene function.

Evidence that the viral gene function required for the induction of cellular DNA synthesis might also be involved in transformation had already been obtained by Gershon et al. (1965). They showed that the transforming and DNA inducing capacity of the virus were inactivated by nitrous acid at the same rates, which indicated that the same fraction of the genome was involved.

The existence of the ts-a and ts-3 mutants is further evidence for the distinction between the initiation and maintenance of transformation. The abortive transformation of BHK21/13 cells by the ts-a mutant at the non-permissive temperature (Stoker & Dulbecco, 1969) is therefore the result of the expression of the ts-3 gene function.

In conclusion, from the known consequences of viral infection and from the study of temperature-sensitive mutants, as many as seven gene functions can be associated with polyoma viral DNA. These are:

(1) Capsid protein

(2) Internal protein(s)

(3) Tumour antigen

(4) Transplantation antigen

(5) ts-a Function - endonuclease? - viral DNA replication
initiation of transformation

(6) ts-3 Function - induction of DNA synthesis
maintenance of transformation
viral DNA synthesis

(7) Group III Function - viral DNA synthesis

Aim of present work

Mouse embryo cells are permissive to polyoma virus replication. In contrast, BHK21/13 cells are apparently non-permissive to virus replication but undergo transformation and abortive transformation.

This thesis describes the study of the infection of BHK21/13 cells by polyoma virus in an effort to establish the basis of the non-permissive state of these cells.

II. MATERIALS

1. Chemicals

Amfix was obtained from May and Baker, Ltd., Dagenham, England.

Acrylamide, amino acids, 2,5-diphenyloxazole (PPO), sodium dodecyl sulphate (SDS) and vitamins were obtained from Koch-Light Laboratories, Colnbrook, Bucks., England.

ATP, dATP, dCTP, dGTP, dTTP, thymidine, tris and amino acids were obtained from Sigma Chemical Co., St. Louis, Missouri, USA.

CsCl (AnalaR grade) was obtained from Hopkin & Williams Ltd., Chadwell Heath, Essex, England.

DePeX and Giemsa stain were obtained from George T. Gurr Ltd., London.

Ethidium bromide was obtained from Boots Pure Drug Co., Ltd., Nottingham, England.

Hyamine Hydroxide (1M-solution in methanol) was obtained from Nuclear Enterprises Ltd., Edinburgh.

Metol and Photo-Flo were obtained from Kodak Ltd., London.

N',N'-Methylenebisacrylamide was obtained from British Drug Houses Ltd., Poole, England.

Phenol (AnalaR grade) was obtained from British Drug Houses Ltd., and redistilled.

Sephadex was obtained from Pharmacia AB, Uppsala, Sweden.

Other chemicals, mostly of AnalaR grade, were obtained from British Drug Houses Ltd.

2. Radiochemicals

[8-^{3}H]dATP of specific activity 5.66Ci/mmole was obtained from Schwarz Bioresearch Inc., Orangeburg, New York, USA.

Other radiochemicals, obtained from The Radiochemical Centre, Amersham, Bucks., England, were:-

L-[4,5-^{3}H]leucine of specific activity > 10,000mCi/mmole.

L-[^{35}S]methionine of specific activity 5,000-20,000mCi/mmole.

[^{32}P]orthophosphate of high specific activity.

[Me-^{3}H]thymidine of specific activity >10,000mCi/mmole.

3. Biological materials

Tissue culture cells

Secondary cultures of mouse embryo cells in ETC were provided by the Cytology Department of the Institute of Virology, University of Glasgow.

BHK21/13 cells, an established line of hamster fibroblasts (Stoker & Macpherson, 1964) and PyY cells, a line of polyoma viral-transformed hamster fibroblasts (Macpherson & Stoker, 1962) were supplied by the Wellcome Tissue Culture Unit, Department of Biochemistry, University of Glasgow.

Mutant L cells, which lacked inosinic pyrophosphorylase, and hybrid BHK-L cells were given by Dr. J. W. Littlefield, Massachusetts General Hospital, Boston, Mass., U.S.A.

Polyoma virus

The polyoma virus used was a small plaque strain, described by

Diamond & Crawford (1964) and was originally obtained from the Institute of Virology, University of Glasgow.

Cell culture

Amino acids, vitamins, calf serum and foetal calf serum were obtained from Flow Laboratories Ltd., Irvine, Ayrshire, Scotland.

Hepes buffer was obtained from Bio-cult Laboratories, Glasgow.

Penicillin and streptomycin were obtained from Glaxo Laboratories Ltd., Greenford, Middlesex, England.

Tryptose phosphate broth (dehydrated) was obtained from Difco Laboratories, East Molesey, Surrey, England.

Enzymes

DNase I was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.

Pronase (B grade) was obtained from Calbiochem, Los Angeles, California, U.S.A.

Receptor-destroying enzyme (RDE) (neuraminidase) was obtained from Behringwerke AG, Marburg-Lahn, Germany.

Pancreatic RNase-A was obtained from Sigma Chemical Co. Before use, a solution of the enzyme (1mg/ml) was heated at 90-95^{o}C for 10min to destroy DNase activity.

Trypsin (E.C.3.4.4.4) was obtained from Difco Laboratories.

Bovine serum albumin (BSA) was obtained from Sigma Chemical Co.

Gelatine was obtained from Hopkin & Williams Ltd.

4. Miscellaneous

AR.10 fine grain autoradiographic stripping plates were obtained from Kodak Ltd.

Chance No. 1 glass coverslips (13mm diameter) were obtained from Macfarlane Robson Ltd., Glasgow.

Porcelain coverslip racks were obtained from Arthur H. Thomas Company, Philadelphia, U.S.A.

Sartorious nitrocellulose filter discs were obtained from V. A. Howe & Co., Ltd., London.

Swinnex filter holders were obtained from Millipore (UK) Ltd., London.

Solutions

Amfix	solution contained 20% (v/v) conc. Amfix in water.
Counting Fluid	had the composition shown in Table 10.
D19b	developer had the composition shown in Table 9.
Eagle's medium	had the composition shown in Table 3.
Formol saline	solution contained 4% (v/v) formaldehyde in 0.08M-NaCl, 0.1M-Na_2SO_4.
Gel buffer	had the composition shown in Table 11.
Gelatine-chrome alum	solution had the composition shown in Table 8.
Giemsa	stain contained 0.75% Giemsa in glycerol-methanol (1:1, v/v).
PBS(a)	was similar to the phosphate-buffered saline of Dulbecco & Vogt (1954), and had the composition shown in Table 6.

RDE — solution was tris saline containing 25units/ml of RDE.

Scintillation fluid — contained 0.5% 2,5-diphenyloxazole (PPO) in toluene.

SSC — was 0.15M-NaCl, 0.015M-sodium citrate adjusted to pH7.0.

TEM — buffer was 0.02M-tris-HCl, 0.001M-EDTA, 0.005M-2-mercaptoethanol, pH7.5.

TEMK — buffer was TEM buffer containing 0.15M-KCl.

Tris saline — had the composition shown in Table 7.

Trypsin solution — had the composition shown in Table 4.

Trypsin-versene — solution contained 20% (v/v) trypsin solution in versene solution.

Tryptose phosphate broth — contained 2.95% (w/v) dehydrated tryptose phosphate broth in water.

Versene — solution had the composition shown in Table 5.

Cell culture media

Cell culture media had the compositions by volume given below:-

ETC:- Eagle's medium-tryptose phosphate broth-calf serum (8:1:1).

EC 10%:- Eagle's medium-calf serum (9:1).

EC 1%:- Eagle's medium-calf serum (99:1).

EC 0.5%:- Eagle's medium-calf serum (199:1).

Hepes-buffered Eagle's medium, pH7.0, contained 0.7% 1M-Hepes buffer instead of $NaHCO_3$.

Table 3

Eagle's medium

NaCl	6.8g
KCl	0.4g
$CaCl_2.6H_2O$	0.393g
$MgSO_4.7H_2O$	0.2g
$NaH_2PO_4.2H_2O$	0.14g
Glucose	4.5g
$NaHCO_3$	2.4g
L-arginine.HCl	0.0421g
L-cystine	0.0240g
L-glutamine	0.2920g
L-histidine.HCl	0.0192g
L-isoleucine	0.0525g
L-leucine	0.0525g
L-lysine.HCl	0.0731g
L-methionine	0.0149g
L-phenylalanine	0.0330g
L-threonine	0.0476g
L-tryptophan	0.0082g
L-tyrosine	0.0362g
L-valine	0.0469g
D-calcium pantothenate	0.002g
Choline chloride	0.002g
Folic acid	0.002g
i-inositol	0.004g
Nicotinamide	0.002g
Pyridoxal.HCl	0.002g
Riboflavin	0.0002g
Thiamine.HCl	0.002g
Penicillin	100,000units
Streptomycin	0.1g
Phenol red	0.015g
Total volume	1litre

Solution flushed with CO_2 until pH7.0

Table 4

Trypsin solution	
Trypsin	2.5g
NaCl	6.15g
Sodium citrate	2.96g
Phenol red	0.015g
Solution adjusted to pH7.8	
Total volume	1litre

Table 5

Versene solution	
EDTA	0.2g
NaCl	8g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g
Phenol red	0.015g
Total volume	1litre

Table 6

PBS(a)

NaCl	10g
KCl	0.25g
Na_2HPO_4	1.44g
KH_2PO_4	0.25g
Total volume	1litre

Table 7

Tris saline

NaCl	8g
KCl	0.38g
Na_2HPO_4	0.1g
Glucose	1g
Tris	3g
Penicillin	100,000units
Streptomycin	0.1g
Phenol red	0.015g
Solution adjusted to pH7.4	
Total volume	1litre

Table 8

Gelatine-chrome alum

Gelatine	5g
Chrome alum ($CrK(SO_4)_2.12H_2O$	0.5g
Formaldehyde (40% (v/v) solution)	5ml
Photo-Flo	1ml
Total volume	1litre

Table 9

D19b

Na_2SO_3	72g
Na_2CO_3	48g
KBr	4g
Hydroquinone	8.8g
Metol	2.2g
Total volume	1litre

Table 10

Counting fluid

NaCl	70g
Citric acid	105g
Mercuric chloride	10g
Total volume	10litres

Table 11

Gel buffer

$NaH_2PO_4.2H_2O$	8.8g
$Na_2HPO_4.12H_2O$	51.5g
Sodium dodecyl sulphate (SDS)	2g
Total volume	1litre

III. METHODS

Unless otherwise stated, high speed centrifugation was performed using a Spinco Model L or L2 ultracentrifuge.

Radioactivity was determined using a Nuclear-Chicago 720 Series, Packard Tri-carb or Phillips liquid scintillation spectrometer.

1. Cell culture

All cultures were maintained at 37°C in an atmosphere containing 5% CO_2 in air. Cultures in plastic Petri dishes were kept in a humidified incubator, continually flushed with air containing 5% CO_2. Cultures in 80oz Winchester bottles were rotated continuously on a culture rack of the type described by House & Wildy (1965) (roller bottle culture).

Centrifugation of cells was performed using an MSE Major centrifuge.

(a) Mouse embryo cells

Exponentially growing cultures

(i) Primary cultures of mouse embryo cells were produced by seeding 2×10^8 cells from minced, trypsinised mouse embryos in ETC (200ml) in 80oz Winchester bottles.

(ii) Secondary cultures. After incubation for 5 days primary cultures were washed with versene (20ml) and treated with trypsin-versene (25ml) at 20°C until the cells detached on shaking. The cells were dispersed by pipetting and 5×10^7 cells plated in 80oz bottles containing ETC (200ml).

(iii) Tertiary cultures. Confluent secondary cultures were removed from the glass by trypsinisation and plated in ETC or EC 10% to give tertiary cultures.

Stationary cultures

Confluent secondary cell cultures were removed from the roller bottles by trypsinisation after 3 days. After dispersion the cells were chilled by addition to 5-10vol. of Eagle's medium in centrifuge bottles at 0°C. Following centrifugation at 900g at 0°C for 10min, the cells were resuspended in ice-cold EC 0.5% at 5×10^5 cells/ml and plated in 90mm plastic Petri dishes (7.5×10^6 cells) or 50mm plastic Petri dishes (2.5×10^6 cells). Cells were maintained in EC 0.5% for 4 days prior to use. Plastic Petri dishes sometimes contained clean, sterile, 13mm diameter glass coverslips.

(b) BHK21/13 cells

Exponentially growing cultures

This established cell line was propagated continuously in ETC by trypsinising cell monolayers every 3 or 4 days and initiating a sub-culture with one-tenth of the cell suspension.

Stationary cultures

Confluent monolayers in ETC were trypsinised, resuspended in EC 10% at 2×10^5 cells/ml and plated in 90mm plastic Petri dishes (2×10^6 cells) or 50mm plastic Petri dishes (0.75×10^6 cells). After incubation for 14-18h the medium was removed and the cell sheet washed with Eagle's medium (5ml) at 20°C and then overlaid with EC 1% (12.5ml and 4ml respectively). Cells were maintained in EC 1% for 5 days prior to use. Plastic Petri dishes sometimes contained clean, sterile, 13mm diameter glass coverslips.

(c) PyY cells were propagated continuously in ETC.

(d) Mutant L and hybrid BHK-L cells were propagated continuously in Eagle's medium-foetal calf serum (9:1 v/v).

Cell counting

Cells were counted using an "Improved Neubeuer" haemocytometer or a Coulter Counter (Model B).

To determine the number of cells on plastic Petri dishes, the medium was removed and the cell sheet washed once with versene solution and drained. 4ml of trypsin solution was added, followed by 1ml of calf serum when the cells had detached. The cells were dispersed by pipetting and counted. When the Coulter Counter was used the cell suspension was diluted 1:50 (v/v) in Counting Fluid (Table 10) before counting.

2. Polyoma virus

(a) Production

Polyoma virus was grown in mouse embryo cells by a modification of the method of Crawford (1962). Secondary cultures grown in ETC were trypsinised and the cell suspension used to seed two 80oz bottles containing ETC (200ml). After incubation for 4 days the cells were infected with polyoma virus at a multiplicity of approximately 10 PFU/cell.

The medium was poured off and virus, contained in Eagle's medium (10ml), was allowed to adsorb to the cells for 2h at 37°C. ETC (200ml)

was then added to each bottle and the cultures were incubated at 37°C. After 2 days the medium was replaced.

5-7 days after virus infection the bottles were shaken to dislodge the cell sheets into the medium, any remaining fragments being removed by shaking with versene. The suspension was, if necessary, made acid by gassing with CO_2 and cooled to 4°C to promote virus adsorption to cell debris. It was then centrifuged at 900g for 10min using an MSE Major centrifuge. The pellet from four roller bottle cultures was suspended in tris saline (10ml) and the cells disrupted by freezing and thawing three times. After centrifugation at 900g for 10min, the supernatant was removed and the pellet resuspended in 10ml of tris saline. RDE (2.5ml) was added and the suspension incubated at 37°C for 24h. After centrifugation, the supernatant (RDE extract) was removed and the pellet resuspended in 10ml of tris saline. After incubation at 37°C for 24h the material was again centrifuged and the supernatant (tris saline wash) removed.

Crude virus stocks, prepared in this way, and containing about 10^{10} PFU/ml were stored at -10°C.

(b) Purification

Virus was purified from crude stocks by a modification of the method of Crawford et al. (1962). RDE extracts and tris saline washes were centrifuged at 2000g for 15min using an MSE Major centrifuge, to sediment large particles. The supernatants were then centrifuged at 80,000g for 2h to pellet the virus. The pellets were resuspended in

0.2M-tris-HCl buffer, pH7.5 and 1ml volumes layered on 3ml of a solution of CsCl in the same buffer (sp.gr.=1.4g/ml) to give a final overall density of 1.3g/ml. After centrifugation at 100,000g for 18-24h, virus material was present in two main bands, the lower denser band (sp.gr.=1.32g/ml consisting of "full" polyoma virus particles and the upper, lighter band (sp.gr.=1.29g/ml) of "empty" particles containing no DNA (Crawford et al. 1962). Fractions were collected drop-wise from the bottom of the tube. Those containing the lower band were pooled, diluted at least five times in PBS(a) and centrifuged at 130,000g for 4h. The sedimented virus was resuspended in 0.1 x PBS(a) and stored at -70°C.

(c) Assay by haemagglutination (HA)

Two-fold serial dilutions were made in 0.2ml volumes of PBS(a) in perspex haemagglutination trays. An equal volume of a 1% suspension of guinea pig red blood cells in PBS(a) was added to each dilution, and the tray was shaken to mix the virus and red cells.

After at least 3h at 4°C, the trays were examined and the end points recorded as the dilution of virus at which the red cells were only partially agglutinated.

1 HA unit = 10^5 PFU/ml.

3. Infection of cells

Exponentially growing cells were infected 24h after plating. Stationary BHK21/13 and mouse embryo cells were infected 5 and 4 days respectively after plating, by which time DNA synthesis and enzyme activities had fallen to low levels (see Results section).

In all cases the appropriate amount of virus was contained in Eagle's medium which had been gassed with CO_2 to about pH6.0. 3ml and 1ml respectively of virus suspension was added to cultures in 90mm and 50mm plastic Petri dishes after the medium had been removed. Virus was allowed to adsorb to the cells for 1h at 37°C, during which time the plates were occasionally tipped to redistribute the liquid and the pH was maintained at 6.0 by increasing the CO_2 content in the atmosphere surrounding the plates.

The virus inoculum was removed and the cells overlaid with the original medium for stationary cultures or fresh EC 10% for exponentially growing cultures. (10ml for 90mm plates and 4ml for 50mm plates).

Mock-infected cultures were treated in an identical manner except that the adsorption inoculum did not contain polyoma virus.

These operations were performed as quickly as possible to avoid exposing the cells to alkaline pH but carefully to ensure that pipetting did not result in mechanical damage to the cell sheets.

4. Glass coverslips

Glass coverslips, on which cells were to be grown, were immersed in boiling dilute NaOH for 20min. NaOH was removed under running tap water and the coverslips were thoroughly washed several times with distilled water followed by ethanol. Finally, the coverslips were sterilised in an oven at 120°C.

Transfer of coverslips to plastic Petri dishes was carried out under aseptic conditions.

5. [^{3}H]Thymidine incorporation into cells

Cell cultures in plastic Petri dishes containing glass coverslips were pulsed with [^{3}H]thymidine (2.5×10^{-6}M; 1μCi/ml). At the end of the pulse the coverslips were placed in a porcelain rack and fixed in formol-saline overnight. After fixing they were washed for 10min periods with 10% (w/v) TCA (twice) at 0°C and with distilled water at 0°C. Finally they were rinsed with ethanol at 20°C and dried in air.

Such coverslips were used for the measurement of total [^{3}H]thymidine incorporation and the percentage of cells incorporating [^{3}H]thymidine.

(a) Total [^{3}H]thymidine incorporation

Coverslips bearing an intact cell sheet were assayed for total [^{3}H]thymidine incorporation into acid-insoluble material by direct counting in toluene-based scintillation fluid (Fried & Pitts, 1968) using a liquid scintillation spectrometer.

(b) Autoradiographic determination of the percentage of cells incorporating [^{3}H]thymidine

Coverslips were mounted with DePeX, cells uppermost, on 0.8-1mm glass microscope slides which had been degreased in ethanol and coated with a film of gelatine-chrome alum. The slides were covered with AR.10 stripping film and exposed at 4°C for several weeks, before being processed at 20°C, all these steps being carried out by safelight. The autoradiographs were developed in D19b for 5min, rinsed in water, fixed with Amfix (no hardener) for 4min and rinsed again with water. They were immediately stained with Giemsa (freshly diluted 1:20 v/v in water) for 5min, rinsed with water and finally dried in air.

Microscopic examination revealed that cells, which had incorporated [^{3}H]thymidine into acid-insoluble material, had silver grains coincident with their nuclei (labelled cells).

6. Preparation of cell extracts for assay of DNA polymerase and thymidine kinase activities

(a) Harvesting of cells

For the preparation of small quantities of extract, the cell sheets on two 90mm plastic Petri dishes were washed twice with PBS(a) (5ml) and once with TEM (5ml), both at 0°C. The cells were then scraped into a total of 0.5ml of TEM and stored at -70°C until used for the preparation of extracts.

Large quantities of extract were prepared.from roller bottle cell cultures. The medium was poured off and the cell sheet first washed with PBS(a) (20ml) and then scraped off into a small volume of the same buffer at 20°C. The cell suspension was immediately added to 10-20vol. of PBS(a) at 0°C. and centrifuged at 900g for 10min. The pellet was washed twice more with PBS(a) by resuspension and centrifugation, all at 0°C. It was finally washed briefly with TEM at 0°C and the cells resuspended in this buffer (1ml per roller bottle culture). The cell suspension was stored at -70°C until further use.

(b) Preparation of extracts

The thawed cell suspension was homogenised at 0°C with 12 strokes in a Potter Elvehjem homogeniser. KCl was added to give a final concn. of 0.15M (TEMK buffer) and the suspension was centrifuged at 6,000g for 10min using an MSE Major centrifuge. The supernatant was removed and was used for the determination of the activities of DNA polymerase and thymidine kinase. Enzyme assays were usually performed immediately after the preparation of the extract but, if not, the extracts were stored at -70°C. The protein concn. of extracts was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

7. Preparation of DNA

(a) Mouse Embryo DNA

The method was based on that of Marmur (1961), the starting material being the residue of minced, trypsinised mouse embryos remaining after the preparation of primary cultures (Methods, section 1 (a) (i)).

1vol. of packed tissue was suspended in 1vol. of Eagle's medium and 2vol. of 0.05M-tris-HCl, 0.1M-EDTA, pH8.5 were added. After the addition of sodium dodecyl sulphate (SDS) to a final concentration of 0.2%, the suspension was incubated at 37°C for 30min with 50μg/ml of pronase (Reich, Baum, Rose, Rowe & Weissman, 1966). The concn. of SDS was then increased to 1%, and the viscous suspension heated at 50°C for 30min. After cooling to 20°C, $NaClO_4$ was added to a concentration of approximately 1M. An equal volume of chloroform-iso-amyl alcohol (24:1, v/v) was added and the mixture shaken mechanically for 30min at 20°C. After centrifugation at 2,000g for 20min, using an MSE Major centrifuge, the upper aqueous phase was removed. 2vol. of ethanol were layered on top and the mixture stirred gently with a glass rod. The crude DNA, wound on the rod, was redissolved by gentle agitation in the minimum volume of 0.1 x SSC. The salt concn. was increased ten-fold by the addition of 10 x SSC and the solution re-extracted with chloroform-iso-amyl alcohol as before. After the DNA had been redissolved in 0.1 x SSC, it was incubated for 30min at 25°C with RNase (50μg/ml).

Several more cycles of deproteinisation were carried out until little insoluble material could be seen at the interface between the aqueous and organic layers.

The DNA was then dissolved in the minimum volume of 0.1 x SSC and 0.1vol. of 3M-sodium acetate, 0.001M-EDTA, pH7.0, added. The solution was cooled to 4°C, the fluffy precipitate of DNA was washed successively in 70%, 85% and 95% ethanol, and dried, first in air, and then in a vacuum desiccator. The final DNA preparation was dissolved in 0.01M-KCl, at 4mg/ml, and stored at -20°C.

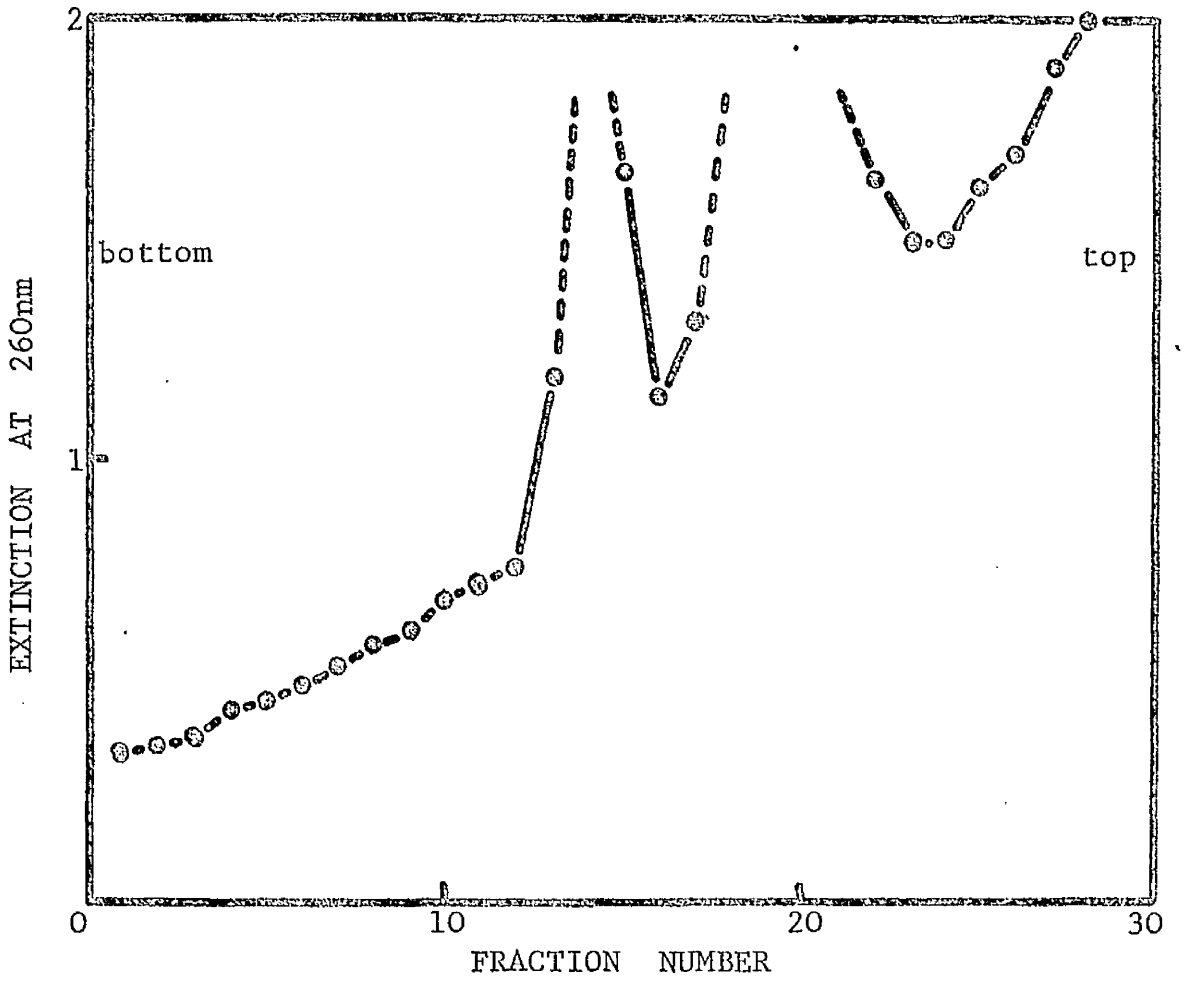
The DNA, diluted to 2mg/ml, was denatured immediately before use by boiling for 10min and then cooling quickly in ice. Hyperchromic shifts of 25-30% were obtained with different preparations.

(b) Polyoma viral DNA

Purified "full" particles of polyma virus (Methods, section 2 (b)) suspended in 0.1 x PBS(a) containing 0.001M-EDTA, were treated with 1% SDS (final concn.) at 50°C for 30min. The solution was cooled and made 1M with respect to NaCl by adding 5M-NaCl. After standing overnight at 4°C, the precipitated SDS was removed by centrifugation at 18,000g for 30min. The supernatant was layered on to an equal vol. of a solution of CsCl in 0.02M-tris-HCl, pH7.5, 0.001M-EDTA (sp.gr.=1.5g/ml) and centrifuged at 100,000g for 24h. The supernatant was decanted carefully, and the pellet of DNA redissolved in 0.02M-tris-HCl, pH7.5, 0.001M-EDTA.

The DNA was fractionated by equilibrium centrifugation in CsCl containing 100µg/ml of ethidium bromide (Radloff, Bauer & Vinograd, 1967)

Purification of polyoma viral DNA



DNA extracted from purified polyoma virus was mixed in 3ml of CsCl solution (sp.gr.=1.57g/ml; pH7.5) containing 100µg/ml of ethidium bromide and centrifuged at 100,000g for 72h in the SW50 rotor of the ultracentrifuge. Fractions were collected from the bottom of the tube and diluted with 0.3ml of PBS(a). The extinctions at 260nm were determined using a Unicam SP500 spectrophotometer.

to separate supercoiled component I (sp.gr.=1.587g/ml) from components II and III (sp.gr.=1.550g/ml). DNA was contained in a solution of CsCl in 0.02M-tris-HCl, pH7.5, 0.001M-EDTA containing 100µg/ml of ethidium bromide (total volume 3ml; sp.gr.=1.56-1.57g/ml) and centrifuged at 100,000g for 72h. Fractions were collected from the bottom of the tube and the positions of the DNA components estimated by reading the extinction at 260nm as shown in Fig. 6. The fractions containing component I were pooled and the ethidium bromide removed by three extractions with propan-2-ol in the presence of CsCl (Cuzin et al. 1970) . CsCl was removed from the DNA preparation by dialysis against 0.02M-tris-HCl, pH7.5, 0.001M-EDTA.

The concentrations of all DNA solutions were determined from their extinctions at 260nm. (E 1.0=50µg/ml).

8. DNA polymerase assay

This assay has been described by Fried & Pitts, (1968).

(a) Assay mixture

The standard assay mixture contained in 0.25ml: tris-HCl buffer pH7.7, 5µmoles; KCl, 15µmoles; EDTA, 0.1µmole; dATP, dGTP, dCTP, and dTTP, 50nmoles each; $MgSO_4$, 2µmoles; 2-mercaptoethanol, 1µmole; BSA, 50µg; heat-denatured mouse embryo DNA, 75µg; enzyme extract containing 100-150µg of protein. dATP was labelled with $[^3H]$ (1µCi per assay mixture). 50µl samples were removed in duplicate at zero time as controls.

After incubation at 37°C for 1h, the reaction in the assay mixture was terminated by cooling to 0°C and two further 50µl samples were removed.

(b) Detection of acid-insoluble radioactivity

Each 50µl aliquot was added to 0.5ml of 10% (w/v) $Na_4P_2O_7$ (sodium pyrophosphate) at 0°C. 50µg of BSA was added as carrier prior to precipitation, followed by 4ml of 5% (w/v) TCA containing 1% pyrophosphate. The tubes were left at 0°C for 10-30min and the precipitates collected by filtration through nitrocellulose filter discs (25mm diameter; 0.45µm pore diameter). The filter discs were washed with 30ml of 5% TCA containing 1% pyrophosphate, and dried. The pyrophosphate reduced the non-specific adsorption of [^{3}H]dATP to acid-insoluble material and to the filter discs.

The filter discs were immersed in toluene-based scintillation fluid and the acid-insoluble radioactivity determined using a liquid scintillation spectrometer. The radioactivities of zero time samples were subtracted from all results.

The specific activity of DNA polymerase was expressed in nmoles of dATP incorporated into acid-insoluble material/h/mg enzyme protein.

9. Thymidine kinase assay

(a) Assay mixture

The standard assay mixture contained in 0.25ml: tris-HCl buffer pH7.9, 12.5μmoles; $MgCl_2$, 1μmole; 2-mercaptoethanol, 1μmole; ATP, 1μmole; thymidine, 50nmoles; enzyme extract containing 100-150μg of protein. Thymidine was labelled with $[^3H]$ (1μCi per assay mixture). Assays were performed in duplicate. After incubation at 37°C for 15min, the reaction was terminated by the addition of 20μl of ice-cold 50% (w/v) TCA.

(b) Estimation of phosphorylated derivatives of thymidine

20μl of a solution of 5mM-thymidine, 5mM-dTMP were added and the mixture was centrifuged at 2,500g at 4°C for 10min, using an MSE Major centrifuge, to remove acid-insoluble material. 40μl of the supernatant was spotted on Whatman DE-81 DEAE-cellulose paper and the phosphorylated derivatives of thymidine separated from thymidine by ascending chromatography using 4M-formic acid, 0.1M-ammonium formate as solvent. The location of the phosphorylated derivatives was determined from the position of reference markers (thymidine, dTMP, dTDP and dTTP) as revealed by their fluorescence under a UV-lamp. The chromatogram was cut into half inch strips from the solvent-front and the strips incubated in 0.5ml of hyamine hydroxide at 60°C for 1h. 10ml of toluene-based scintillation fluid was added and the radioactivity determined using a liquid scintillation spectrometer. From the radioactivity distribution obtained the radioactivity of phosphorylated

derivatives of thymidine was determined.

The specific activity of thymidine kinase was expressed in nmoles of thymidine phosphorylated/15min/mg enzyme protein.

10. Estimation of [^{3}H]polyoma DNA in viral-infected cells

Viral-infected cells on 50 or 90mm plastic Petri dishes, were pulsed with [^{3}H]thymidine (2.5×10^{-7}M; 1μCi/ml) to label viral DNA synthesised during the period of the pulse. At the end of the pulse the medium was removed and the cell sheet washed twice with PBS(a). The cells were lysed with 1ml of 0.6% SDS solution in 0.01M-tris-HCl, pH7.5, 0.01M-EDTA. [^{3}H]viral DNA was assayed by equilibrium centrifugation or by sedimentation velocity centrifugation.

(a) Equilibrium centrifugation

After 10-20min at 20°C the viscous SDS-lysate was scraped into a centrifuge tube, and if necessary stored at -10°C. The SDS-lysate was warmed to redissolve the precipitated SDS, and was contained in a solution of CsCl in 0.1M-tris-HCl, pH7.5, 0.01M-EDTA containing 100μg/ml of ethidium bromide (total volume 3ml; sp.gr.=1.56-1.57g/ml). This mixture was covered with paraffin oil and centrifuged at 100,000g for 72h in the SW50 rotor of the ultracentrifuge. The SDS formed a lamella on top of the CsCl solution. Fractions were collected from the bottom of the tube in vials containing 50μg of BSA as carrier. 4ml of 5% (w/v) TCA

at 0°C was added to each vial and, after 10min at 0°C, the precipitated material was collected by filtration through nitrocellulose filter discs (25mm diameter; 0.45μm pore diameter). The discs were washed with 20ml of TCA and dried.

The filter discs were immersed in toluene-based scintillation fluid and the radioactivity determined using a liquid scintillation spectrometer.

This method (Radloff et al. 1967) effects a separation of supercoiled component I (sp.gr.=1.587g/ml) from components II and III and cellular DNA (sp.gr.=1.550g/ml) and the amount of [^{3}H]polyoma viral DNA (component I) was estimated from the radioactivity distribution obtained.

(b) Sedimentation velocity centrifugation

After 10-20min at 20°C the viscous SDS-lysate was carefully scraped, with the minimum of shearing, into a centrifuge tube. Polyoma viral DNA was selectively extracted from the lysate by the method of Hirt (1967). 5M-NaCl was added to give a final concn. of 1M and the sample was mixed by slowly inverting the tube 10 times. The sample was stored at 4°C for at least 8h and centrifuged at 17,000g for 30min at 4°C in the SW50 rotor of the ultracentrifuge to precipitate the cellular DNA. The supernatant, containing [^{3}H]polyoma viral DNA, was carefully removed by pouring and was stored at 4°C. After warming to redissolve precipitated SDS, the supernatant was analysed by sedimentation velocity centrifugation through neutral or alkaline CsCl according to the method of Dulbecco & Vogt (1963).

(i) Sedimentation through neutral CsCl

0.2ml of the Hirt supernatant was carefully layered on 3ml of CsCl contained in 0.05M-tris-HCl, pH7.5, 0.001M-EDTA (sp.gr.=1.5g/ml), covered with paraffin oil, and centrifuged at 100,000g for 3.5h in the SW50 rotor of the ultracentrifuge. Fractions were collected from the bottom of the tube and the distribution of acid-insoluble radioactivity determined as before (Methods, section 10 (a)). The amount of [^{3}H]polyoma viral DNA was estimated from the radioactivity profile obtained.

(ii) Sedimentation through alkaline CsCl

0.2ml of the Hirt supernatant was carefully layered on 3ml of CsCl contained in 0.01M-phosphate buffer, pH12.5, 0.01M-EDTA (sp.gr.=1.5g/ml), covered with paraffin oil, and centrifuged at 100,000g for 2h in the SW50 rotor of the ultracentrifuge. Fractions were collected from the bottom of the tube and the distribution of acid-insoluble radioactivity determined as before (Methods, section 10 (a)). This method effects a good separation of component I from components II and III and the radioactivities of these components was estimated from the radioactivity profile obtained.

11. Preparation of RNA

(a) Source of RNA

(i) [^{32}P]phosphate-labelled polyoma viral-infected stationary mouse embryo cells

3 days after plating the cells (Methods, section 1 (a)) in 90mm plastic Petri dishes, the medium was replaced with orthophosphate-free EC 0.5%*. (EC 0.5%* contained 0.5% dialysed serum and had been pre-incubated at 37°C for 3-4 days to minimise stimulation of the cells (Smart, 1968)). 24h later the cells were infected with polyoma virus (50 PFU/cell) (Methods, section 3) and the cells overlaid with 5ml of EC 0.5%* containing 10% of the normal phosphate concn. (Results, section B 4 (a)) and [^{32}P]orthophosphate (50μCi/ml). At 8,16,24,32 and 40h after infection, plates were removed for extraction of RNA.

(ii) Polyoma viral-infected stationary mouse embryo and BHK21/13 cells

Stationary mouse embryo and BHK21/13 cells (Methods, section 1 (a) (b)) were infected with polyoma virus (50 PFU/cell) (Methods, section 3). At 8,16,24,32 and 40h after infection, plates were removed for extraction of RNA.

(iii) Exponentially-growing mouse embryo, BHK21/13 and PyY cells

Tertiary cultures of mouse embryo cells (Methods, section 1 (a) (iii)), BHK21/13 cells and PyY cells were grown in ETC in roller bottles. When confluent they were removed for extraction of RNA.

(b) Extraction of RNA

The method of extraction was based on that of Martin & Axelrod (1969a).

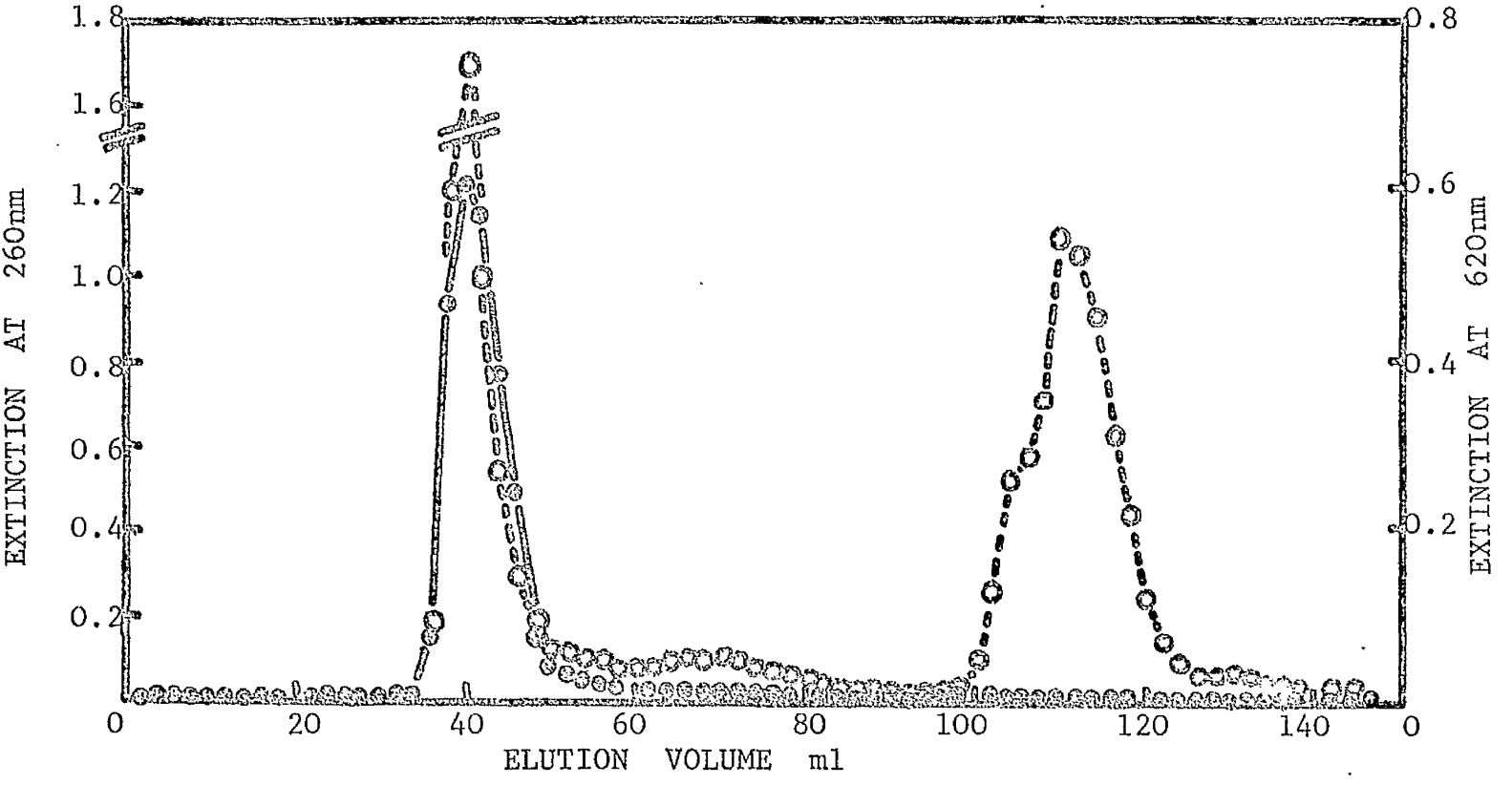
All solutions were sterilised by autoclaving and glassware flamed prior to use to minimise degradation of RNA by ribonuclease.

For cultures in roller bottles, the cell sheet was washed twice with ice-cold PBS(a) (20ml) and the cells were scraped from the glass, dispersed and pipetted into centrifuge bottles containing ice-cold PBS(a). After centrifugation at 900g at 4°C for 10min using an MSE Major centrifuge, the cell pellet was lysed with 20ml of 0.35% SDS contained in 0.1M-NaCl, 0.01M-sodium acetate, pH5.3 at 20°C. The SDS-lysate was stored at -70°C.

For cultures on 90mm plastic Petri dishes, the cell sheet was washed twice with ice-cold PBS(a) (5ml) and the cells lysed with 1ml of 0.35% SDS contained in 0.1M-NaCl, 0.01M-sodium acetate, pH5.3 at 20°C. SDS-lysates from similar plates were pooled and stored at -70°C.

After thawing, the SDS-lysates were extracted with an equal volume of phenol at 60°C. The mixture was shaken vigourously for 3-5min and centrifuged at 4,000g for 10min using an MSE Major centrifuge. The lower phenol layer was discarded and the aqueous layer re-extracted with an equal volume of phenol at 60°C as before. The upper aqueous layer was carefully removed and precipitated with 2vol. of 95% ethanol at -10°C overnight. After centrifugation at 4,000g at 4°C for 10min, the precipitate was resuspended in 5ml of 0.1M-NaCl, 0.002M-$MgCl_2$, 0.01M-sodium acetate, pH5.3. This solution was treated with DNase I (50µg/ml) at 20°C for 3h and then exposed to pronase (50µg/ml), previously self-digested for 2h at 37°C, at 20°C for 2h. The RNA was extracted twice

Purification of RNA by gel filtration using Sephadex G-100



A Sephadex G-100 column (25 x 2.5cm) was eluted at 4°C with buffer at a flow rate of 20ml/h using a peristaltic pump. The void volume (V_o) of the column was determined by applying 1ml of a dilute solution of blue dextran to the column and eluting the material with buffer. 2ml fractions were collected and the extinctions at 620nm determined (—o—). V_o was 40ml.

1ml of RNA solution (approximately 1mg/ml) was applied to the column and eluted with buffer at 4°C. 2ml fractions were collected and the extinctions at 260nm determined (--o--). Material appearing in the void volume was pooled.

Extinctions at 260nm and 620nm were determined using a Unicam SP500 spectrophotometer.

with an equal volume of 88% (v/v) phenol at 20°C, as before, and the aqueous layer precipitated with 95% ethanol at -10°C. After centrifugation at 4,000g at 4°C for 10min the precipitate was resuspended in the minimum volume of 0.1M-NaCl, 0.01M-sodium acetate, pH5.3 containing 0.02% sodium azide. The RNA was separated from DNA fragments by fractionation at 4°C on a Sephadex G-100 column (25 x 2.5cm). 1ml samples were applied to the column and eluted with 0.1M-NaCl, 0.01M-sodium acetate, pH5.3 containing 0.02% sodium azide. RNA appearing in the void volume (Fig. 7) was collected in 2vol. of 95% ethanol and precipitated at -10°C. After centrifugation at 4,000g for 10min the precipitated RNA was resuspended at 1mg/ml in 4 x SSC containing 0.1% SDS and stored at -70°C.

The concentrations of all RNA solutions were determined from their extinctions at 260nm. (E 1.0=43.5µg/ml).

[^{32}P]phosphate-labelled RNA extracted, using this technique, from polyoma viral-infected stationary mouse embryo cells was 99.93% resistant to further DNase I treatment and was 99.85% degraded by alkali as shown in Table 12.

Table 12

$[^{32}P]$RNA: Resistance to DNase I and sensitivity to alkali

Treatment	Acid-insoluble material d.p.m.	% of untreated sample
-	131,371	100
DNase (50ug/ml) 4h at 30°C	131,285	99.93
0.3M NaOH (final concn.) 30h at 30°C	197	0.15

2.5µg of $[^{32}P]$RNA, from polyoma viral-infected mouse embryo cells, contained in 0.5ml of 0.1M-NaCl, 0.002M-MgCl , 0.01M-sodium acetate, pH5.3 was subjected to the above treatments. 50µg of BSA was added and the acid-insoluble radioactivity determined after precipitation with 5% (w/v) TCA at 0°C and filtration through nitrocellulose filter discs. Residual acid-insoluble radioactivity was expressed as % of untreated sample. The average of duplicate determinations is shown.

12. DNA-RNA hybridisation

(a) Preparation of polyoma viral DNA (Component II)

Purified polyoma viral DNA (component I) (Methods, section 7 (b)) was purified further by equilibrium centrifugation in CsCl containing ethidium bromide (Methods, section 7 (b)). This material was converted to component II by limited attack by DNase I (Westphal & Dulbecco, 1968). The reaction mixture contained in 0.5ml of 0.04M-NaCl, 0.02M-$MgCl_2$, 0.01M-tris-HCl, pH8.0 : polyoma viral DNA, 10µg; BSA, 5µg; DNase I, 0.1ng. The reaction mixture was incubated at 30°C and at various times 10µl aliquots were removed for analysis by sedimentation through alkaline CsCl using a Spinco Model E analytical ultracentrifuge. During analysis the main reaction mixture was frozen at -10°C. The reaction mixture was incubated at 37°C for 2h for 100% conversion of component I to component II. The preparation was extracted with 88% (v/v) phenol at 20°C and dialysed against 0.1 x SSC.

(b) Binding of component II DNA to nitrocellulose filter discs

The following operations were carried out at 20°C. Nitrocellulose filters (13mm diameter; 0.45µm pore diameter) were immersed in 4 x SSC (5ml/filter) for at least 1h and loaded in Millipore Swinnex filter holders attached to a 10ml plastic disposable syringe with the plunger removed. The filter holder assemblies were arranged in a group of six, connected to a water pump. 10ml of 4 x SSC was drawn through each filter

at a flow rate of 2ml/min. 5ml of 4 x SSC containing 10ng of DNA, previously denatured by heating at 100°C for 10min in 0.1 x SSC, was drawn through each filter, followed by 10ml of 4 x SSC, all at a flow rate of 2ml/min. The filters were inverted in the filter holders and a further 10ml of 4 x SSC drawn through. After removing the filters, they were pressed between two Whatman No. 1 filter papers and left overnight. They were finally baked in a vacuum oven at 80°C for 3h and stored in a vacuum desiccator at 20°C.

(c) DNA-RNA hybridisation

The hybridisation reactions were performed in cylindrical plastic vials (4 x 1.5cm) with tight-fitting caps. The reaction mixture contained RNA in 0.25ml of 4 x SSC containing 0.1% SDS. One DNA-bearing filter and one blank filter were placed in the reaction mixture with the blank filter uppermost. Air bubbles were removed by directing a slow stream of air onto the surface of the reaction mixture. The vials were capped and incubated at 67°C for 16h.

In competition experiments, when the filters were incubated first with unlabelled RNA and then with [^{32}P]RNA, the filters were removed from the first reaction mixture, carefully blotted between Whatman No. 1 filter papers and transferred to the second reaction mixture.

After the incubation at 67°C, the reaction mixtures were allowed to cool to 20°C and the remaining operations performed at 20°C. The filters were removed and washed 4 times by immersing for 5min in 4 x SSC (2.5ml/filter). They were then immersed in 2 x SSC (2.5ml/filter) and

treated with RNase (20μg/ml) for 1h. After RNase treatment the filters were further washed 3 times in 4 x SSC (2.5ml/filter). The filters were then loaded in Millipore Swinnex filter holders and each side of every filter washed by drawing through 20ml of 4 x SSC at a flow-rate of 2ml/min, as before.

Finally the filters were dried, immersed in 10ml of toluene-based scintillation fluid and the radioactivity determined using a liquid scintillation spectrometer.

13. Fractionation of proteins by SDS-polyacrylamide gel electrophoresis

(a) Extraction of proteins

Cells on 90mm plastic Petri dishes were pulsed with [^{3}H]leucine or [^{35}S]methionine. At the end of the pulse, the cell sheet was washed twice with 5ml of ice-cold PBS(a) and the cells lysed with 1ml of 0.01M-sodium phosphate, pH7.0, containing 1% SDS and 1% 2-mercaptoethanol. The SDS-lysate was heated at 100°C for 10min and then incubated at 37°C for 2h. The proteins were then examined by SDS-polyacrylamide gel electrophoresis using the method of Weber & Osborn (1969).

(b) Preparation of 10% (w/v) polyacrylamide gels

22.2g of acrylamide and 0.6g of methylenebisacrylamide were dissolved in water to give 100ml of solution. Insoluble material was removed by filtration through Whatman No. 1 filter paper and the solution

was stored in a dark bottle at 4°C. For a typical run of 10 gels, 15ml of gel buffer (Table 11) were deaerated, using a water pump, and mixed with 13.5ml of acrylamide solution. After further deaeration, 1.5ml of freshly made ammonium persulphate solution (5mg/ml) and 0.05ml of N,N,N',N'-tetramethylethylenediamine were added. After mixing, 2.5ml of this solution were added to perspex gel tubes, 10cm long with an internal diameter of 6mm, temporarily sealed at the bottom. Before the gel hardened a few drops of water were layered on top of the gel solution. After 10 to 20min an interface could be seen, indicating that the gel had solidified. Just before use the water layer was drawn off and the gel tubes were positioned in the electrophoresis apparatus.

(c) Electrophoresis of proteins

For each gel, 5µg of marker dye (0.05% bromophenol blue), 1 drop of glycerol, 5µl of 2-mercaptoethanol and 100µl of protein solution were mixed in a small test tube. After mixing, the solutions were applied to the top of the gels and gel buffer (Table 11), diluted 1:1 with water, was carefully layered over each sample to fill the tubes. The two compartments of the electrophoresis apparatus were filled with gel buffer, diluted 1:1 with water. Air bubbles, collecting around the bottom of the gel tubes, were drawn off using a bent Pasteur pipette. Electrophoresis was performed at 20°C at a constant current of 8mA per gel with the anode in the lower chamber and was continued for 6h by which time the bromophenol blue marker had moved through 80% of the gel.

After electrophoresis, the gels were removed from the tubes by directing a stream of water between the gel and the wall of the tube using a syringe. The gel was frozen using powdered "Drikold" and sliced into 1mm cylindrical sections using a Mickle gel-slicer. The gel sections were put in scintillation vials (2 sections/vial) and treated with 0.3ml of hydrogen peroxide (100 volumes) at 60°C for 6h followed by 0.5ml of hyamine hydroxide at 60°C for 15min. 4ml of 2-methoxyethanol and 6ml of toluene-based scintillation fluid were added and the radioactivity determined using a liquid scintillation spectrometer. In this way the distribution in the gel of radioactive-labelled proteins was determined.

IV. RESULTS

The Results section consists of two parts:-

Section A

Development of a stationary BHK cell system.

Section B

Infection of stationary BHK cells with polyoma virus.

Section A

1. Advantages of stationary cell systems in the study of polyoma virus-cell interactions

In biochemical studies on the infection of cells by viruses it is desirable that a large proportion of the cell population is infected synchronously so that measurements on the population of cells infected represent the events occuring in individual infected cells. Some studies on the productive infection of permissive cells by polyoma virus have been carried out with mouse embryo cells. It requires high multiplicities of virus, however, to infect the majority of growing mouse embryo cells (Sheinin & Quinn, 1965).

Fried & Pitts (1968) developed a stationary mouse embryo cell system in which the cells were maintained in Eagle's medium containing only 0.5% calf serum. After infection with polyoma virus at a multiplicity of 20 PFU/cell, 90% of such cells were infected and synthesised viral capsid antigen whereas only 5% of exponentially growing mouse embryo cells were infected under the same conditions. This stationary cell system which allows the majority of cells to show a viral-specific response after infection at a moderate viral multiplicity is ideally suited for biochemical studies. Moreover, stationary mouse embryo cells, obtained by this method, had low levels of DNA synthesis and DNA polymerase activity and the system was shown

to be an excellent one for studying the stimulation, by polyoma virus, of these cellular functions.

In the study described here the applicability of the low serum method of Fried & Pitts (1968) for obtaining stationary cultures of BHK cells has been investigated. In view of the advantages of the stationary mouse embryo system mentioned above, it was thought that a stationary BHK cell system would facilitate a study of the non-permissive BHK cell-polyoma virus interaction and enable the block in viral replication to be characterised.

2. Stationary mouse embryo cells

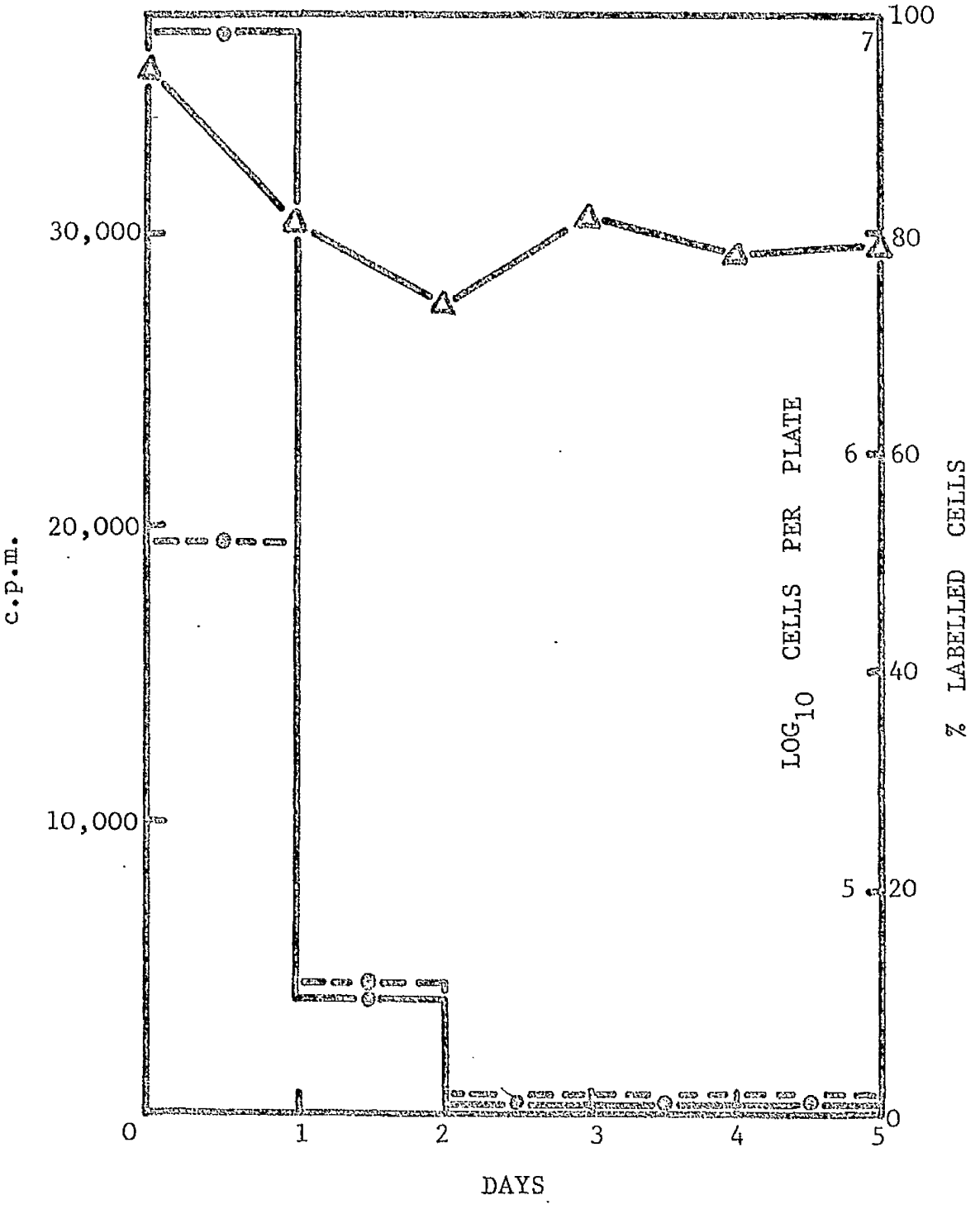
For comparison purposes, stationary mouse embryo cells were prepared by the low serum method of Fried & Pitts (1968). Secondary cultures of mouse embryo cells were removed from the glass by trypsinisation and seeded on plastic Petri dishes in Eagle's medium containing 0.5% calf serum (Methods, section 1 (a)). Some plates contained coverslips to facilitate measurements of DNA synthesis. After 4 days in low serum medium, the cells had a low level of DNA synthesis as measured by total incorporation of [^{3}H]thymidine into acid-insoluble material (Fig. 8). Autoradiography showed that, by this time, only 1% of the cells were incorporating [^{3}H]thymidine. The cell number fell by about 50% during the first day after plating but remained approximately constant (3.0-3.5 x 10^6 cells/90mm plate) thereafter.

Fig. 8

Stationary mouse embryo cells

7.5×10^6 cells were plated in EC 0.5% (15ml) on 90mm plates. Cells on plates containing coverslips were pulsed with [^{3}H]thymidine (2.5×10^{-6}M; 1μCi/ml) for 24h periods. At the end of the pulse three coverslips from each of duplicate plates were used to determine the total incorporation of [^{3}H]thymidine (Methods, section 5 (a)) and the average incorporation/coverslip is shown (—○—). Two of these coverslips were used for the autoradiographic determination (Methods, section 5 (b)) of the % of cells incorporating [^{3}H]thymidine (- -○- -). At least 500 cells on each coverslip were examined for every point.

On successive days after plating the average cell number on duplicate plates was determined (—△—). Two separate cell counts (200-300 cells) were made for each plate using a haemocytometer (Methods, section 1) and the average count is shown.


30,000
20,000
10,000
c.p.m.
0
1
2
3
4
5
DAYS
7
6
5
LOG_{10} CELLS PER PLATE
100
80
60
40
20
0
% LABELLED CELLS

These data were in good agreement with the original work.

Microscopic examination revealed that stationary mouse embryo cells had a fibroblast-like morphology and did not adopt a particularly ordered arrangement in the monolayer. Initially internal features such as nuclei and nucleoli were clearly visible but after 4 or 5 days in low serum medium these features and the boundaries of the cells were only visualised with difficulty. These observations were probably related to the reduced activity of stationary cells as compared to growing cells.

The usefulness of stationary mouse embryo cells is indicated by Table 13. Polyoma viral infection of mouse embryo cells after 4 days in low serum medium resulted in a 9-fold stimulation of DNA synthesis as compared to control mock-infected cultures. The percentage of cells incorporating [^{3}H]thymidine increased to 68% in viral-infected cultures while only 3% of mock-infected cells were labelled. DNA synthesis was also stimulated by overlaying the cells with EC 10%; total incorporation of [^{3}H]thymidine was 44-fold greater than in mock-infected cultures and 82% of the cells had incorporated [^{3}H]thymidine.

The procedure of overlaying the cells with EC 10%, and observing the resultant burst of DNA synthesis, is a good test for establishing the viability of stationary cells.

Table 13

Stationary mouse embryo cells: stimulation of DNA synthesis by polyoma viral infection or serum

Treatment	DNA synthesis c.p.m.	% labelled cells
Mock-infected	349	3
Viral-infected	3,076	68
EC 10%	15,441	82

Stationary mouse embryo cells in 90mm plates containing coverslips were infected with polyoma virus (50 PFU/cell), mock-infected or overlaid with EC 10%. The cells were pulsed with [^{3}H]thymidine (2.5×10^{-6}M; 1μCi/ml) for 24h between 20 and 44h after treatment. At the end of the pulse three coverslips from each of duplicate plates were used to measure total incorporation of [^{3}H]thymidine. The average incorporation/coverslip was taken as a measure of DNA synthesis. Two of these coverslips were used for the autoradiographic determination of the % of cells incorporating [^{3}H]thymidine. At least 500 cells on each coverslip were examined.

3. Application of low serum method to BHK cells

When the low serum method was used for BHK cells it was noticed that, after trypsinisation and resuspension in low serum medium, such cells aggregated in large clumps which were not easily broken up. Even when clumps were dispersed by much pipetting they quickly re-formed. This made the determination of cell number difficult. When such cells were plated and examined the following day it was found that although some cells had attached to the surface of the plate, many remained in clumps in suspension.

The degree of clumping appeared to be inversely related to the amount of serum protein in the medium because a suspension of single cells was easily obtained in medium containing 10% serum.

After 4 days in EC 0.5% BHK cells still had a high level of DNA synthesis, which only increased 3-fold after overlaying the cells with EC 10% (Table 14). In addition, 30% of the cells were incorporating [^{3}H]thymidine. A small stimulation of DNA synthesis after viral-infection occurred in this experiment but, because of the high background level of DNA synthesis, little significance can be placed on these data.

An attempt was made to reduce the level of DNA synthesis by maintaining the BHK cells in medium containing lower amounts of serum. BHK cells were plated and maintained for 5 days in medium containing 0.25% and 0.1% serum. Table 15 shows that reducing the serum concentration to 0.25% had little effect on the level of DNA synthesis in BHK cells after 5 days. Further reducing the serum concentration to 0.1% resulted

Table 14

BHK cells: use of the low serum method for obtaining stationary cultures

Treatment	DNA synthesis c.p.m.	% labelled cells
Mock-infected	7,783	30
Viral-infected	8,977	36
EC 10%	19,953	88

7.5×10^6 cells were plated in EC 0.5% (15ml) in 90mm plates containing coverslips. 4 days after plating the cells were infected with polyoma virus (50 PFU/cell), mock-infected or overlaid with EC 10%. The cells were pulsed with [^{3}H]thymidine (2.5×10^{-6}M; 1μCi/ml) for 24h between 20 and 44h after treatment. At the end of the pulse three coverslips from each of duplicate plates were used to measure total incorporation of [^{3}H]thymidine. The average incorporation/coverslip was taken as a measure of DNA synthesis. Two of these coverslips were used for the autoradiographic determination of the % of cells incorporating [^{3}H]thymidine. At least 500 cells on each coverslip were examined.

Table 15

BHK cells: Effect of reduced serum concentrations on DNA synthesis

Serum concn. in medium %	DNA synthesis c.p.m.	% labelled cells
0.5	4,236	26
0.25	4,475	24
0.1	753	21

7.5×10^6 cells were plated in Eagle's medium containing 0.5%, 0.25% or 0.1% serum in 90mm plates containing coverslips. 5 days after plating the cells were pulsed with [^{3}H]thymidine (2.5×10^{-6}M; 1μCi/ml) for 24h. At the end of the pulse three coverslips from each of duplicate plates were used to measure total incorporation of [^{3}H]thymidine The average incorporation/coverslip was taken as measure of DNA synthesis. Two of these coverslips were used for the autoradiographic determination of the % of cells incorporating [^{3}H]thymidine. At least 500 cells on each coverslip were examined.

in only a small decrease in the percentage of cells incorporating [^{3}H]thymidine but gave rise to large decrease in the total incorporation of [^{3}H]thymidine. However this was possibly the result of a decrease in cell number because in EC 0.1% the cells rapidly degenerated and many cells which had originally plated began to detach.

Clumped cells were present in suspension in all these experiments, which may account for the high residual levels of DNA synthesis as compared with stationary mouse embryo cells prepared by the low serum method. The results obtained from these studies suggested that the direct application of the low serum method of Fried & Pitts (1968) was unsuitable for the preparation of stationary cultures of BHK cells.

4. Function of tryptose phosphate

BHK cells are normally grown in medium which consists of Eagle's medium-calf serum-tryptose phosphate in the proportions 9:1:1 by volume. Although the exact function of tryptose phosphate is not known, BHK cells apparently grow faster and to higher densities in its presence. The studies on the applicability of the low serum method for BHK cells, described in the previous section were performed with cells which had been passaged several times in the absence of tryptose phosphate. This was an attempt to condition the cells to medium lacking tryptose phosphate as it was reasoned that, without tryptose phosphate, maintenance of BHK cells in a stationary state would be facilitated. It was apparent,

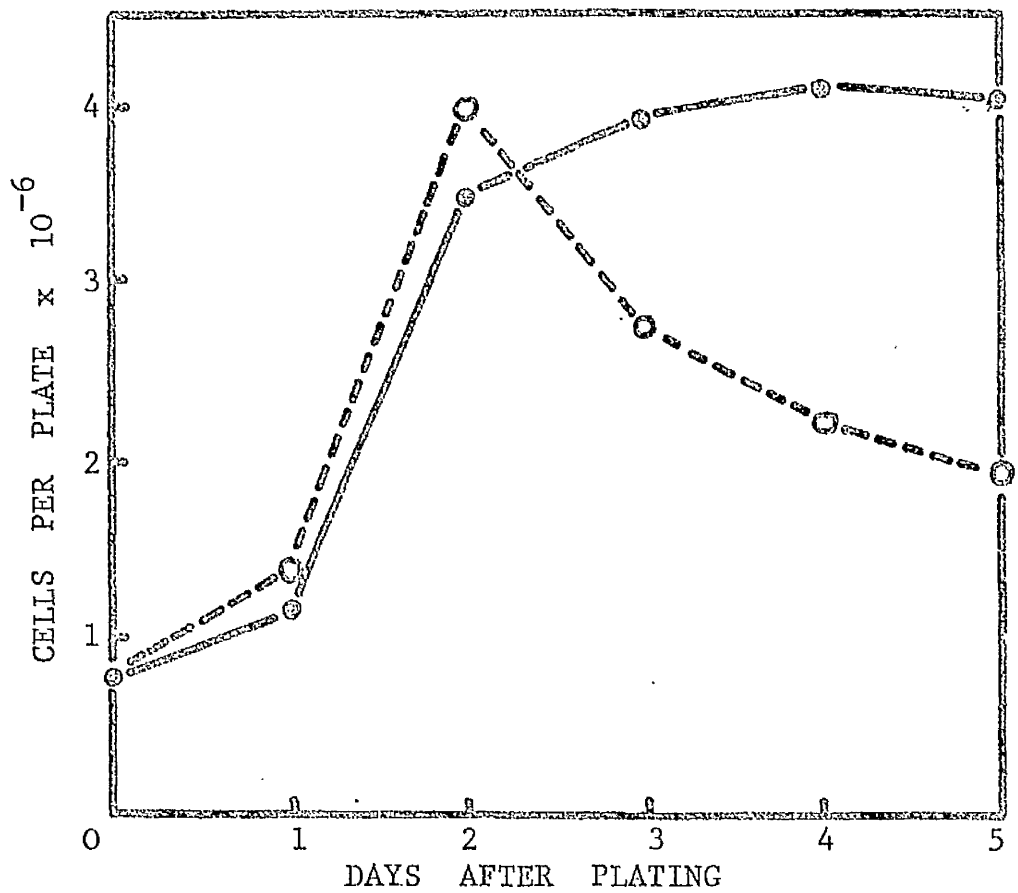
however, that several passages in EC 10% only resulted in degeneration of BHK cultures and the condition of the cells was then dependent on the number of such passages. As this added another variable to an already complex problem, BHK cells continuously propagated in ETC were used in all further investigations.

The effect of tryptose phosphate on the growth of BHK cells is shown in Fig. 9. There was little difference in the rate of growth of cells grown in the presence or absence of tryptose phosphate up to 2 days after plating. However by this time the cultures had become acid and, whereas the cells grown on tryptose phosphate continued to grow to saturation density, those without tryptose phosphate began to detach from the cell monolayer. By 5 days after plating, cultures without tryptose phosphate contained 52% of the cells in suspension but cultures grown on tryptose phosphate consisted of a stable monolayer of cells.

Under these conditions tryptose phosphate appeared to have little effect on the growth rate of BHK cells but, when the cells became acid after two days growth, tryptose phosphate prevented the cells from detaching. It is possible that a component on the cell surface, sensitive to acid conditions, was protected by a constituent of tryptose phosphate. It seems unlikely that tryptose phosphate was acting as a pH-buffer because after 5 days cultures with and without tryptose phosphate appeared to be equally acid.

Fig. 9

Effect of tryptose phosphate on the growth of BHK cells



0.75×10^6 cells in ETC or EC 10% were plated in 50mm plates. On successive days after plating the number of cells/plate in the monolayer was determined using a Coulter Counter (Methods, section 1). Five separate counts of at least 2,000 cells were made for each of duplicate plates and the average cell number is shown.

—●— cells grown in ETC; - - ○ - - cells grown in EC 10%

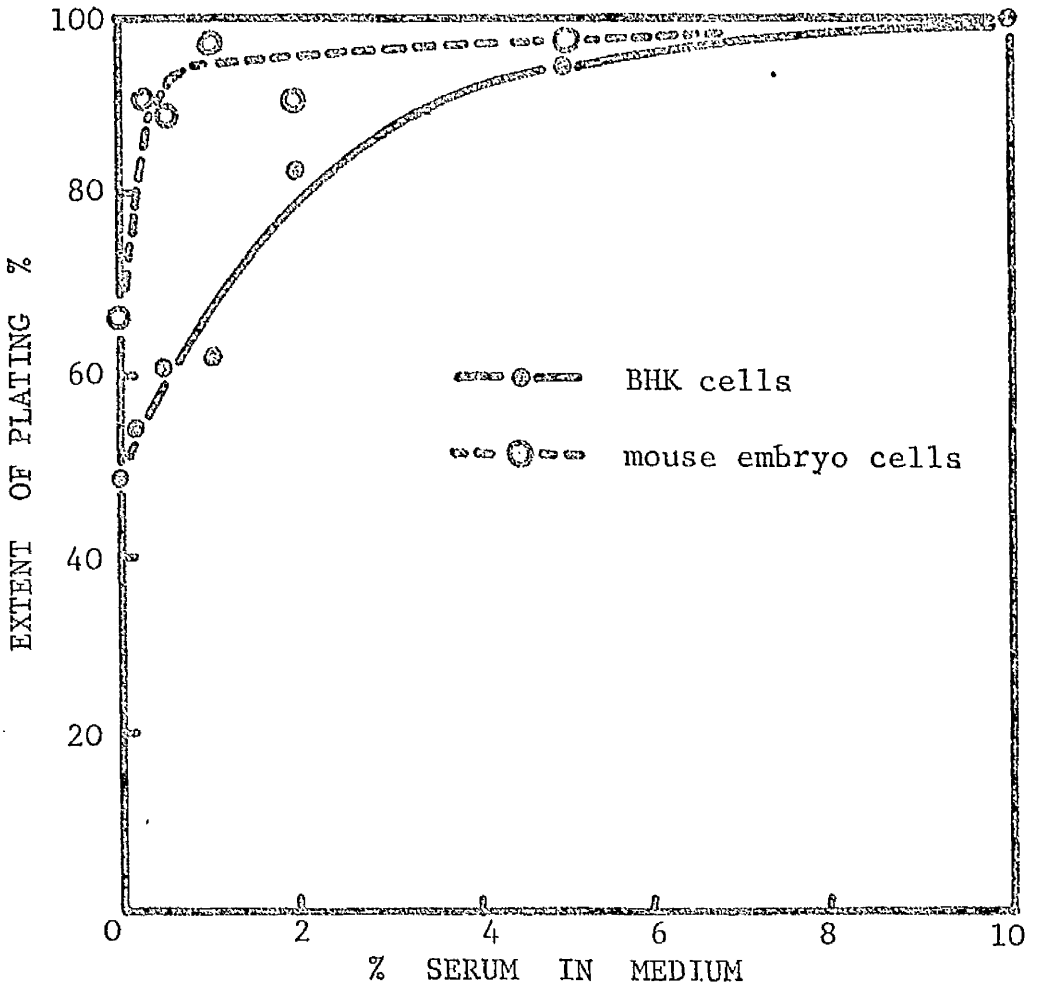
5. Effect of serum concentration on the plating of BHK and mouse embryo cells

It was shown earlier (Results, section A 3) that in medium containing 0.5% serum BHK cells, unlike mouse embryo cells, plated inefficiently and clumps of cells accumulated in suspension. The plating behaviour of BHK and mouse embryo cells at different serum concentrations was studied to establish the difference between BHK and mouse embryo cells and to find the optimal conditions for plating BHK cells. In these studies cells which were not removed from the plates by washing with versene but were removed by trypsin were said to have plated successfully.

BHK and mouse embryo cells were plated in medium containing various concentrations of serum and 16h later the number of cells plated was determined as shown in Fig. 10. The number of cells plated in 10% serum was called 100% and the number of cells plated at other serum concentrations was expressed as a percentage of this (the extent of plating). In 0.5% serum almost 95% of mouse embryo cells plated whereas 5% serum in the medium was required before BHK cells plated to the same extent.

To show that Fig. 10 represents the extent of plating of cells and not cell growth, the growth of BHK cells, already plated, in 24h as a function of serum concentration in the medium is shown in Fig. 11. BHK cells grow much faster in 10% serum than in 5% serum (Fig. 11)

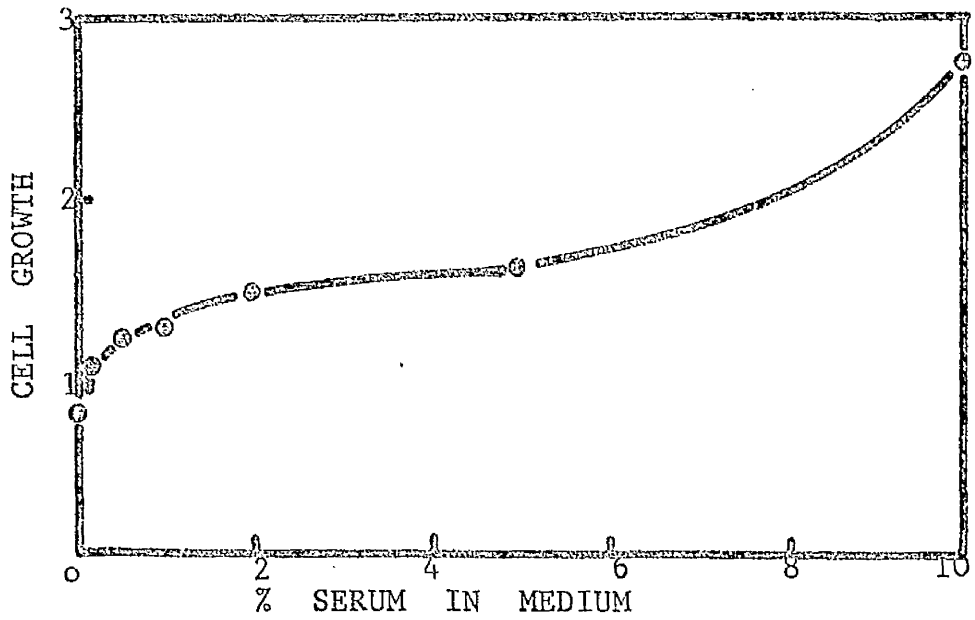
Effect of serum concentration on the plating of BHK and mouse embryo cells



2×10^6 cells in medium (without tryptose phosphate) containing various amounts of serum were seeded in 50mm plates. After 16h the number of cells/plate in the monolayer was determined using a Coulter Counter (Methods, section 1). Five separate counts of at least 2,000 cells were made for each of duplicate plates and the average cell number (N) obtained.

Extent of plating in x% of serum $= \frac{N_{x\%}}{N_{10\%}} \times 100\%$

Effect of serum concentration on the growth of BHK cells



0.75×10^6 cells in EC 10% were plated in 50mm plates. After 18h the medium was removed and the cell monolayer first washed with Eagle's medium at 20°C and then overlaid with medium (4ml) containing various amounts of serum. The number of cells/plate in the monolayer was determined (Methods, section 1) at this time (N_o) and again after 24h (N_{24}). Five separate counts of at least 1,800 cells were made for each of duplicate plates using a Coulter Counter and the average cell number obtained.

$$\text{Cell growth in 24h} = \frac{N_{24}}{N_o}$$

whereas there was little difference in the extent of plating of cells at these serum concentrations (Fig. 10).

The different plating behaviour of BHK and mouse embryo cells (Fig. 10) may be due to different cell surface properties. It is possible that the high serum requirement for efficient plating of BHK cells may be related to the clumping of the cells which occurred in low serum medium (Results, section A 3). It follows from Fig. 10 that seeding of BHK cells in less than 5% serum would result in less than 95% of the cells becoming plated. Therefore, in all further studies BHK cells were plated in medium containing 10% serum which was subsequently replaced with low serum medium.

6. A modified low serum technique

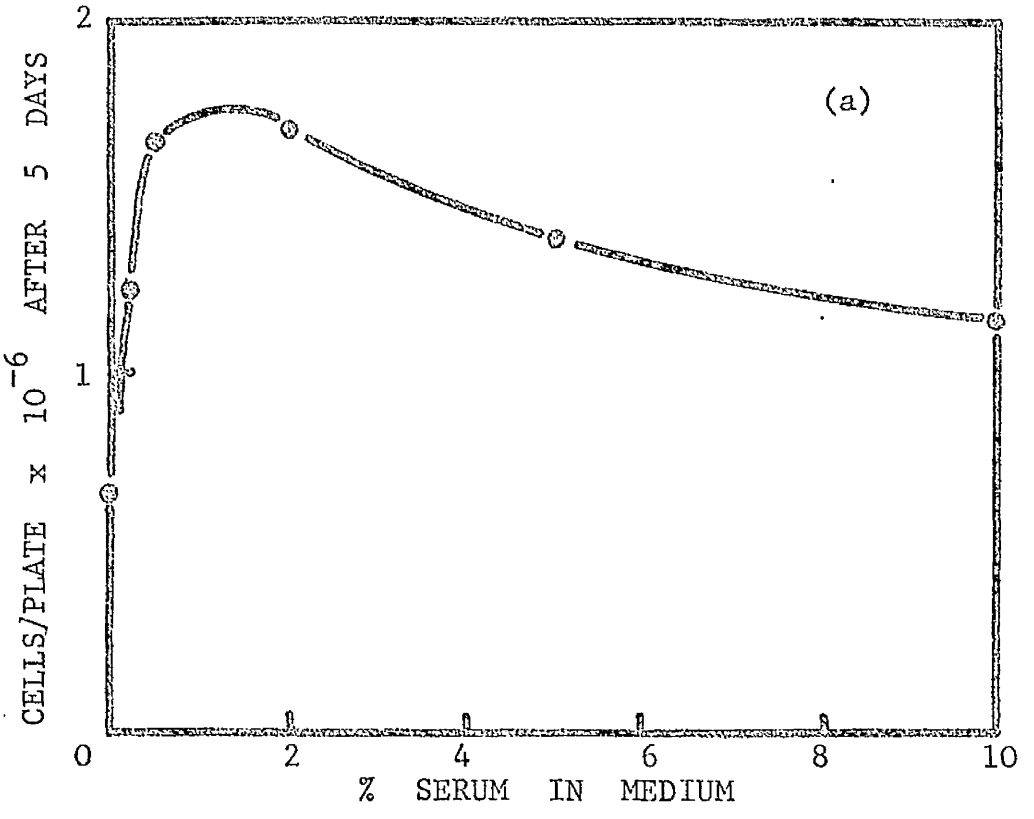
(a) The effect of the final serum concentration

BHK cells which had been passaged in ETC were plated in EC 10% and 16h later the medium was replaced with medium containing various serum concentrations up to 10%. The number of cells in the monolayer was determined on subsequent days for the next 5 days. The cell number after 5 days as a function of serum concentration in the medium is shown in Fig. 12(a). The cell number was a maximum at a serum concentration between 0.5 and 2%.

To account for the maximum in Fig. 12(a) the cell counts on subsequent days in 0,2 and 10% serum in the same experiment are shown

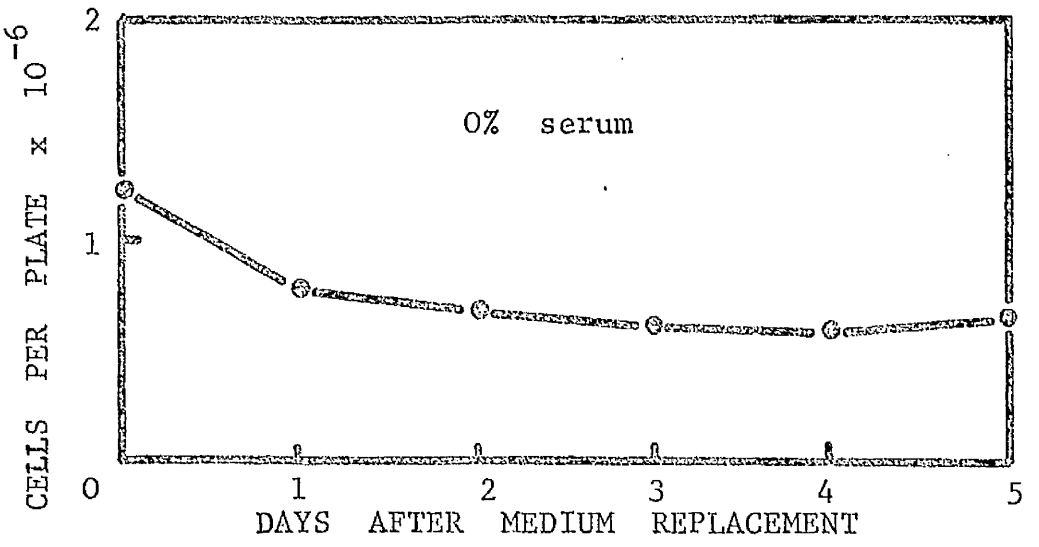
Fig. 12

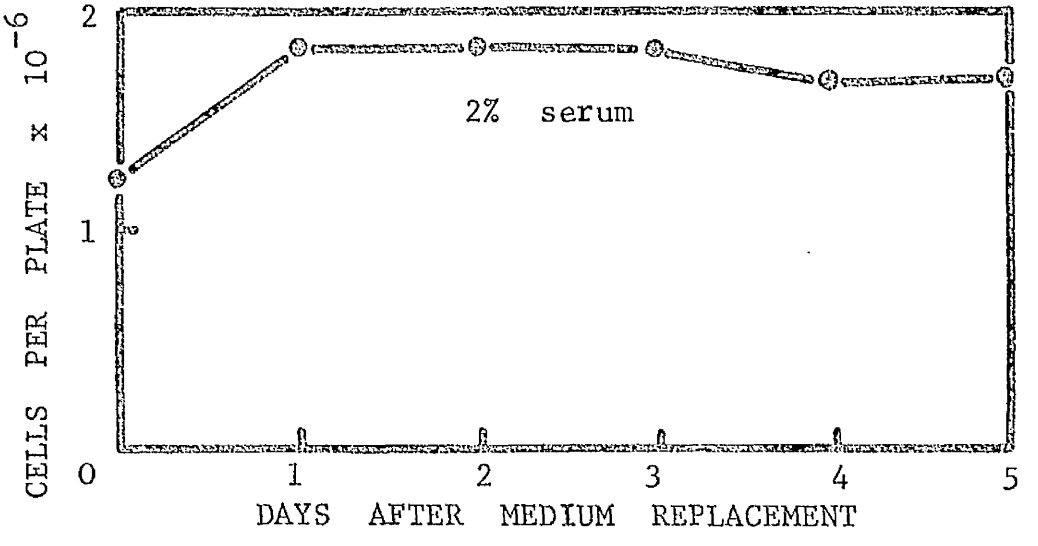
Effect of the final serum concentration

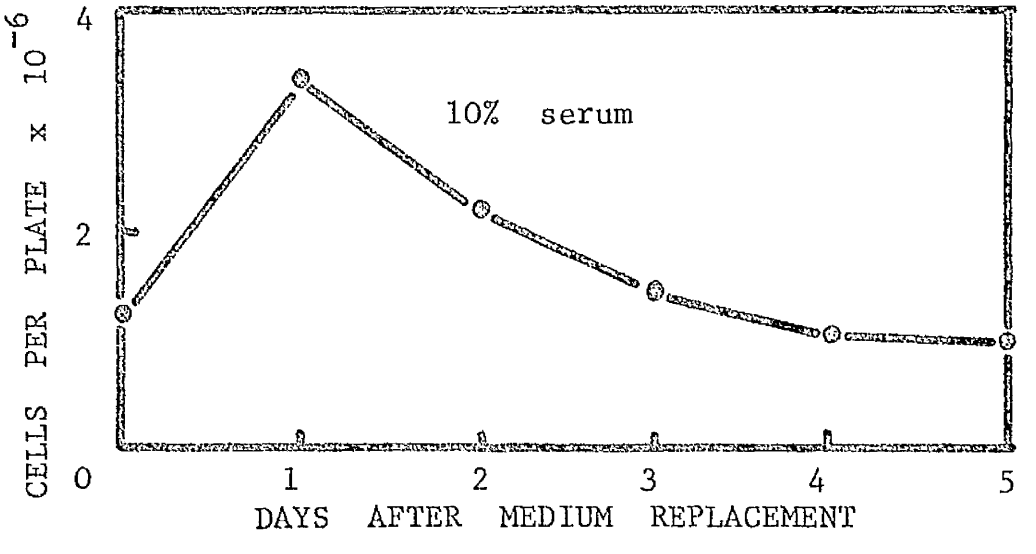


0.75×10^6 BHK cells in EC 10% were plated in 50mm plates. After 16h the medium was removed and the cell monolayer first washed with Eagle's medium at 20°C and then overlaid with medium (4ml) containing various amounts of serum. The number of cells/plate in the monolayer was determined (Methods, section 1) at this time and on each subsequent day for 5 days. Five separate counts of at least 1,100 cells were made for each of duplicate plates using a Coulter Counter and the average cell number obtained.

Fig. 12(b)


0% serum
CELLS PER PLATE x 10^{-6}
2
1
0
1
2
3
4
5
DAYS AFTER MEDIUM REPLACEMENT


2% serum
CELLS PER PLATE x 10^{-6}
2
1
0
1
2
3
4
5
DAYS AFTER MEDIUM REPLACEMENT


10% serum
CELLS PER PLATE x 10^{-6}
4
2
0
1
2
3
4
5
DAYS AFTER MEDIUM REPLACEMENT

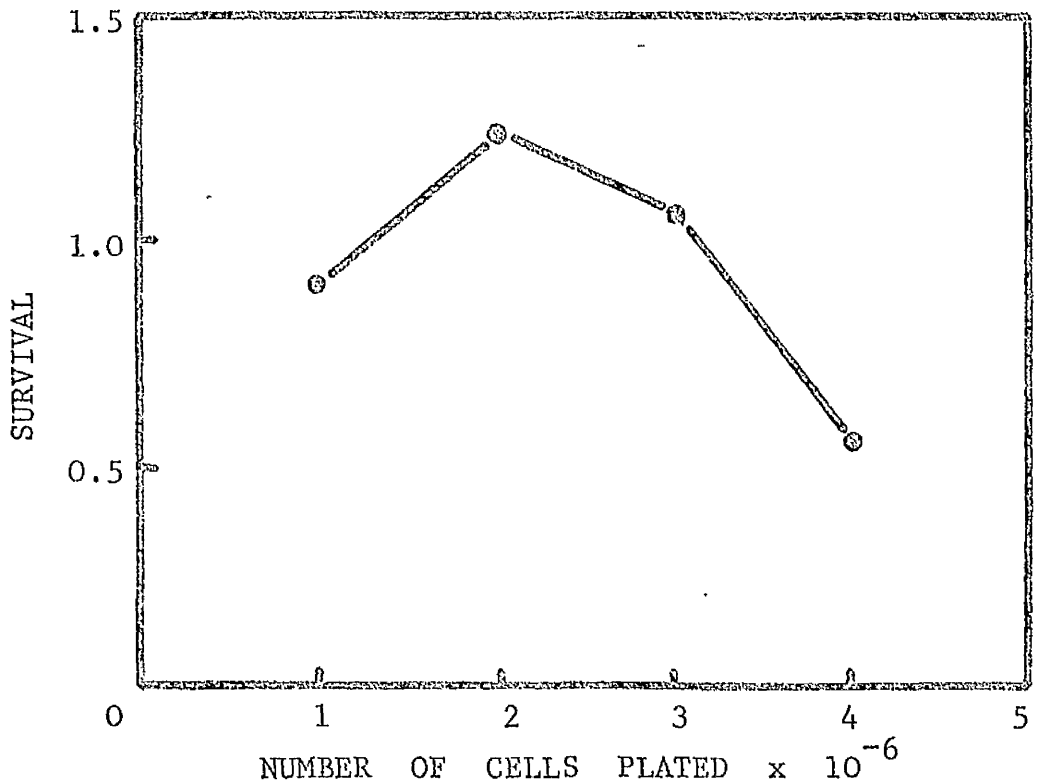
in Fig. 12(b). Microscopic examination of cells in 0% serum revealed that they rapidly degenerated and no growth was detectable by cell counting. Cells in 10% serum grew during the first day but later the cell number fell as cells detached concomitant with increasing acidity of the medium. (See Results, section A 4). At serum concentrations close to the maximum in Fig. 12(a), for example 2%, the cells grew more slowly than in 10% serum and then apparently stopped dividing. The pH of these latter cultures remained unchanged and few detached cells could be detected.

It was concluded from these data that serum concentrations less than 0.5% were not sufficient to maintain the viability of BHK cells. Possibly serum protein is required for stabilisation of a cell surface component. This has been suggested for 3T3 cells by Dulbecco (1970a). Conversely at serum concentrations above 2% the cells deteriorated after growth which resulted in an increase of the acidity of medium. Serum concentrations between 0.5 and 2% appeared to be optimal for maintaining the cells in a non-dividing condition. In subsequent experiments BHK cells were maintained in EC 1% for 5 days after the medium change.

(b) The effect of cell density

BHK cells were plated in EC 10% at various cell densities and the medium replaced with EC 1% after 16h. The cells were maintained in this medium for 5 days and the number of cells in the monolayer was determined. The survival of cells was the ratio of the number of

Effect of cell density on the survival of BHK cells



1,2,3 and 4 x 10^6 BHK cells in EC 10% were plated in 90mm plates. After 16h the medium was removed and the cell monolayer first washed with Eagle's medium at 20°C and then overlaid with EC 1% (10ml). After 5 days the number of cells/plate in the monolayer was determined using a haemocytometer. Four separate cell counts (50-150 cells) were made for each of duplicate plates and the average cell number obtained.

$$\text{Survival} = \frac{\text{Cell number/plate after 5 days in EC 1\%}}{\text{Number of cells plated}}$$

cells remaining after 5 days to the number plated. As shown in Fig. 13 survival was greatest when 2 x 10^6 cells/90mm plate were plated. At higher cell densities (3 and 4 x 10^6 cells/plate) the medium became acid and the cells began to detach after 3 or 4 days in EC 1%. The pH of cultures obtained from the plating of 2 x 10^6 cells was unchanged and few cells could be detected in suspension. In all further studies cells were plated at 2 x 10^6 cells/90mm plate and 0.75 x 10^6 cells/50mm plate.

(c) A stationary BHK cell system

BHK cells, passaged in ETC, were removed from the glass by trypsinisation and plated in EC 10% at 2 x 10^6 cells/90mm plate. 14-18h later the medium was replaced with EC 1%. DNA synthesis and cell number were monitored on subsequent days for 5 days (Fig. 14(a)).

The cell number was approximately constant (3.0-3.5 x 10^6 cells) at 3,4 and 5 days after the medium change. DNA synthesis, as measured by total incorporation of [^{3}H]thymidine, decreased on each successive day in EC 1% and by the fifth day had reached a very low level. Similarly, autoradiography showed that the percentage of labelled cells also decreased and by the fourth and fifth days less than 1% cells were incorporating [^{3}H]thymidine. These results were comparable to those obtained with stationary mouse embryo cells (Results, section A 2).

After 5 days in EC 1%, BHK cells were still viable for there was a large increase in DNA synthesis when the cells were overlaid with EC 10% (Fig. 14(b)). Between 12 and 24h after serum-stimulation 75%

Fig. 14(a)

Stationary BHK cells

2×10^6 cells in EC 10% were plated in 90mm plates. After 16h the medium was removed and the cell monolayer first washed with Eagle's medium at $20^{\circ}C$ and then overlaid with EC 1% (10ml).

Cells on plates containing coverslips were pulsed with [^{3}H]thymidine (2.5×10^{-6}M; 1μCi/ml) for 24h periods. At the end of the pulse three coverslips from each of duplicate plates were used to determine the total incorporation of [^{3}H]thymidine (Methods, section 5 (a)) and the average incorporation/coverslip is shown (—●—). Two of these coverslips were used for the autoradiographic determination (Methods, section 5 (b)) of the % of cells incorporating [^{3}H]thymidine (- -○- -). At least 500 cells on each coverslip were examined for every point.

On successive days after plating the average cell number on duplicate plates was determined (—▲—). Five separate counts of at least 4,000 cells were made for each of duplicate plates using a Coulter Counter and the average cell number obtained.

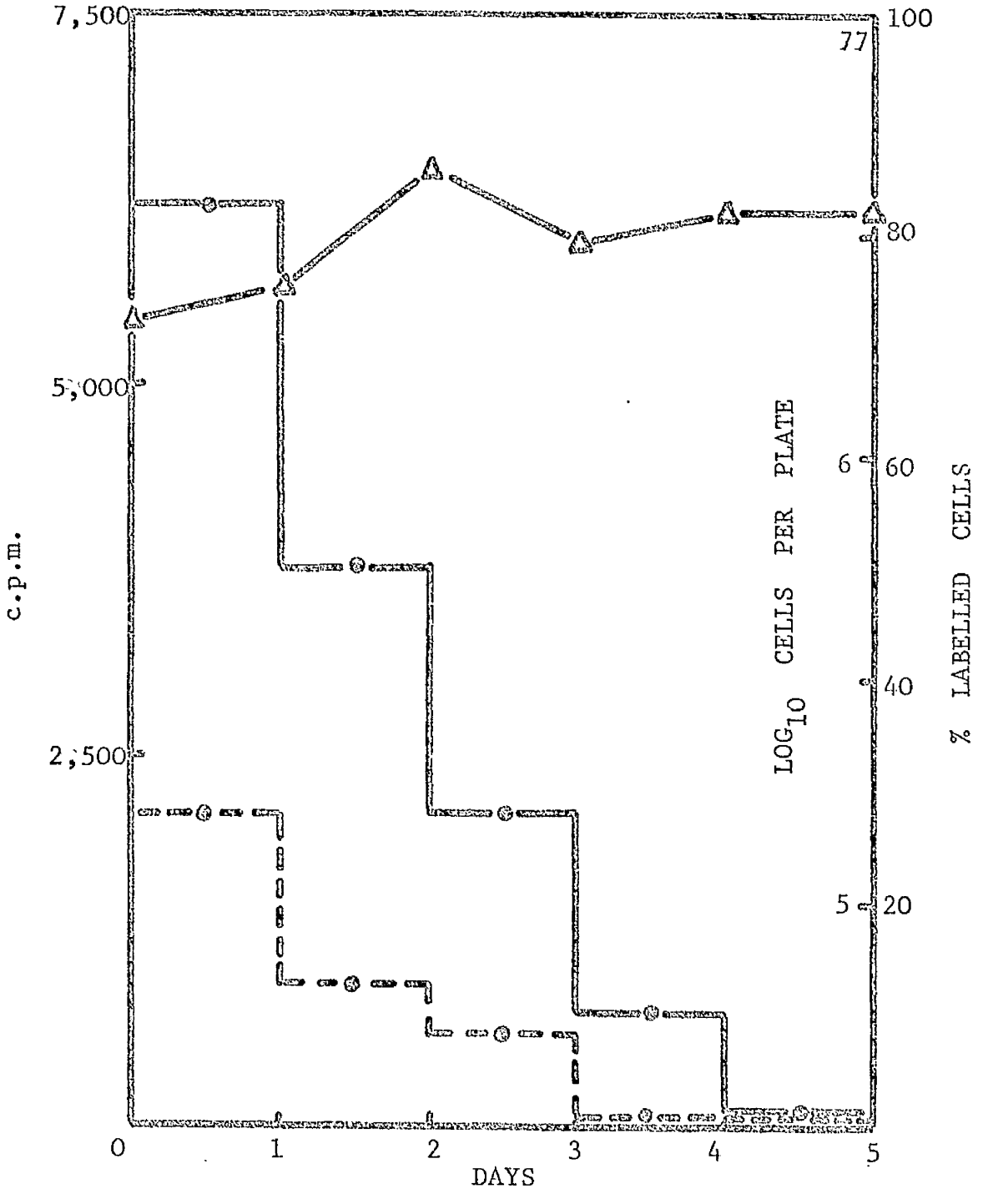
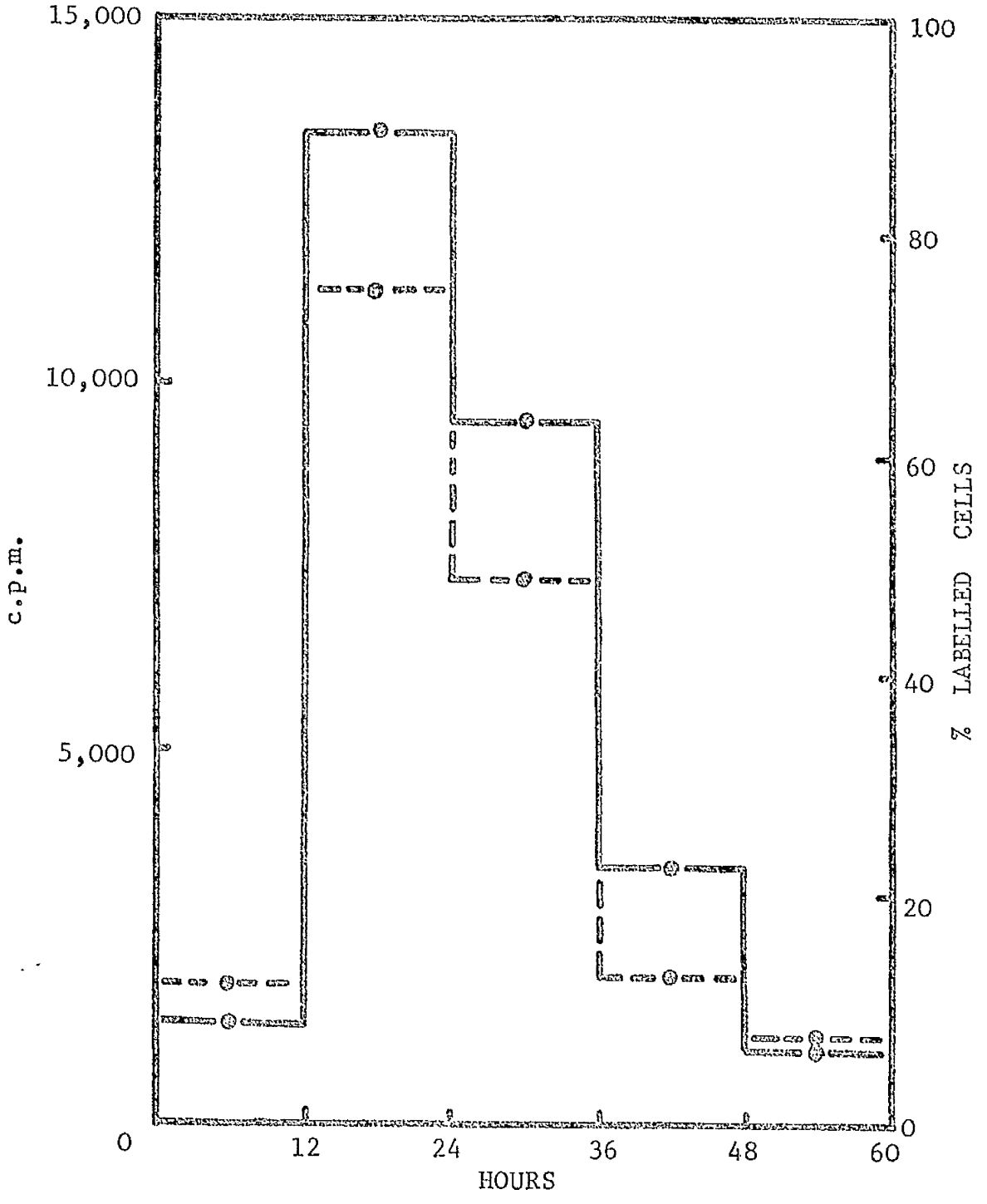

7,500
5,000
2,500
c.p.m.
100
80
60
40
20
% LABELLED CELLS
77
6
5
LOG10 CELLS PER PLATE
0
1
2
3
4
5
DAYS

Fig. 14(b)

Serum-stimulation of stationary BHK cells

After 5 days in EC 1% the medium was replaced with 10ml of EC 10%. Cells on plates containing coverslips were pulsed with [^{3}H] thymidine (2.5×10^{-6}M; 1μCi/ml) for 12h periods. At the end of the pulse three coverslips from each of duplicate plates were used for the determination of total incorporation of [^{3}H] thymidine/coverslip (⊶⊷) and the autoradiographic determination of the % of cells incorporating [^{3}H] thymidine (⊶⊷), as in Fig. 14(a).


15,000
10,000
5,000
0
c.p.m.
100
80
60
40
20
0
% LABELLED CELLS
12
24
36
48
60
HOURS

cells were incorporating [^{3}H]thymidine. That the remaining 25% cells were not labelled is probably not due to their lack of viability but to the asynchrony of the onset of DNA synthesis.

BHK cells, maintained for 5 days in EC 1% had fibroblast-like morphology and were organised in parallel arrays in the monolayer. Such cells were just confluent and the crowding observed in growing cultures, wherever two parallel arrays, perpendicular to one another, intersected, was much less prevalent. As in the case of stationary mouse embryo cells the boundaries and internal structures of stationary BHK cells were less distinct than with growing cells.

This method of preparing stationary BHK cell cultures circumvents the problem of clumping or adhesiveness of BHK cells under low serum conditions encountered here (Results, section A 3) and elsewhere (Edwards, Campbell & Williams, 1971). The method is very reproducible and has been successfully adopted by other workers (Abrahams, Low & Hay, 1971) for studying the infection of stationary BHK cells by herpes and pseudorabies viruses.

Section B

To characterise the block in polyoma virus replication in viral-infected non-permissive cells, the process of infection in stationary non-permissive BHK cells was compared with that in stationary permissive mouse embryo cells.

1. The effect of viral infection on cellular DNA synthesis

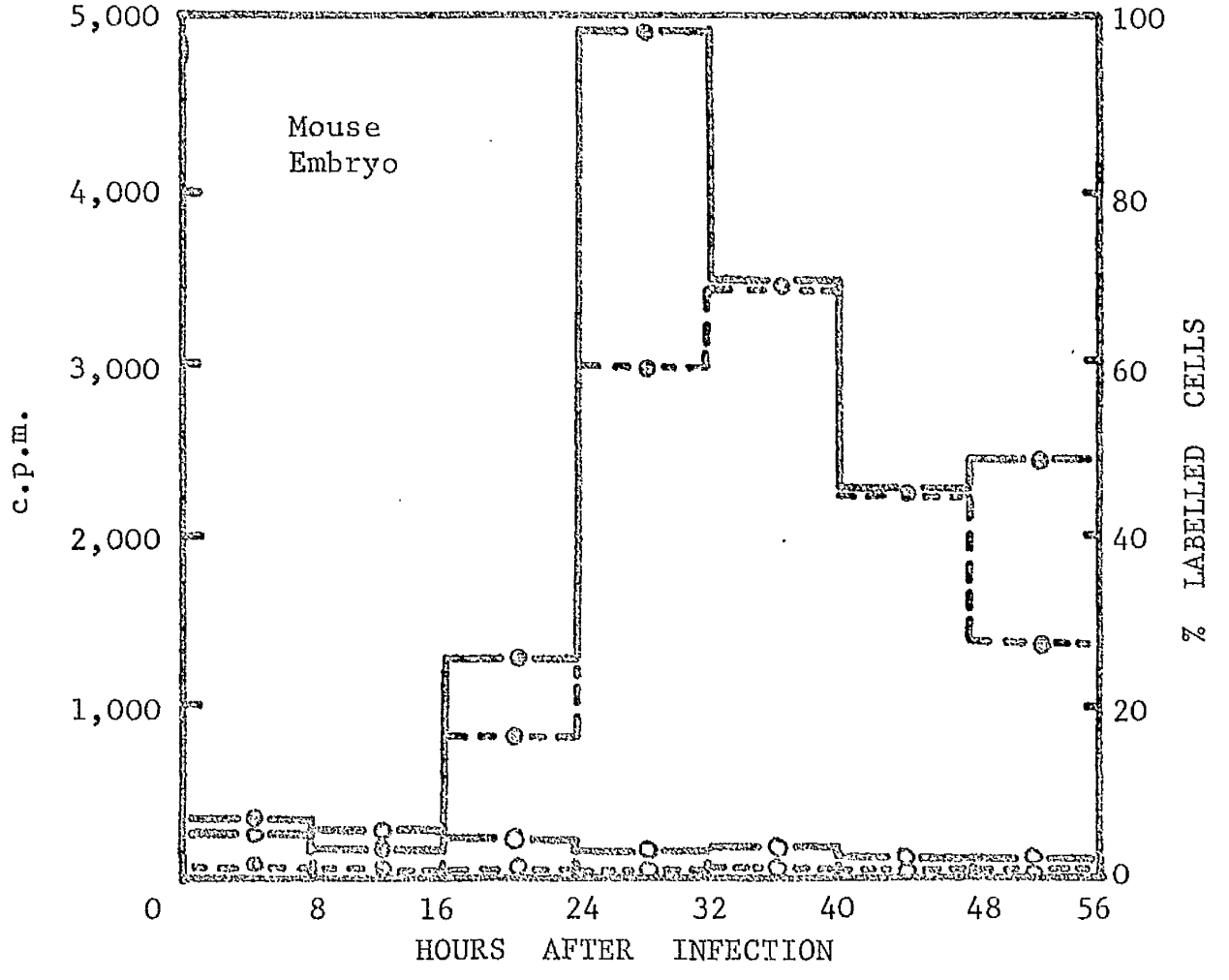
Stationary BHK and mouse embryo cells were infected with polyoma virus at 50 PFU/cell. DNA synthesis was monitored by measuring the incorporation of [^{3}H]thymidine into acid-insoluble material resulting from 8 or 12h pulses with the isotope. Total incorporation of [^{3}H]thymidine increased after viral infection of mouse embryo cells and was maximal between 24 and 32h after infection (Fig. 15). In this period [^{3}H]thymidine incorporation into viral-infected cells was 38-fold greater than that into mock-infected cells in which there was no increase in [^{3}H]thymidine incorporation. Moreover in viral-infected mouse embryo cells the percentage of cells incorporating [^{3}H]thymidine increased from 1% and was maximal at 70% between 32 and 40h after infection. In mock-infected cultures only 1 to 2% of cells incorporated [^{3}H]thymidine throughout.

When the [^{3}H]thymidine-labelled DNA synthesised in viral-infected mouse embryo cells between 0 and 72h after infection was analysed by equilibrium centrifugation in CsCl containing ethidium bromide (Fig. 16) it was found that only 11% of the total acid-insoluble radioactivity

Fig. 15

Effect of polyoma viral infection on DNA synthesis in stationary cells

Stationary BHK and mouse embryo cells (Methods, section 1) in 90mm plates containing coverslips were infected with polyoma virus (50 PFU/cell) or mock-infected and pulsed with [^{3}H] thymidine (2.5×10^{-6}M; 1μCi/ml) for 8 or 12h periods. At the end of the pulse three coverslips from each of duplicate plates were used to determine the total incorporation of [^{3}H] thymidine in viral-infected (⊖) or mock-infected (○) cells and the average incorporation/coverslip is shown. Two of these coverslips were used for the autoradiographic determination of the % of cells incorporating [^{3}H]thymidine in viral-infected (⊖) or mock-infected (○) cultures. At least 500 cells on each coverslip were examined for every point.


Mouse
Embryo
c.p.m.
% LABELLED CELLS
5,000
4,000
3,000
2,000
1,000
0
100
80
60
40
20
0
8
16
24
32
40
48
56
HOURS AFTER INFECTION

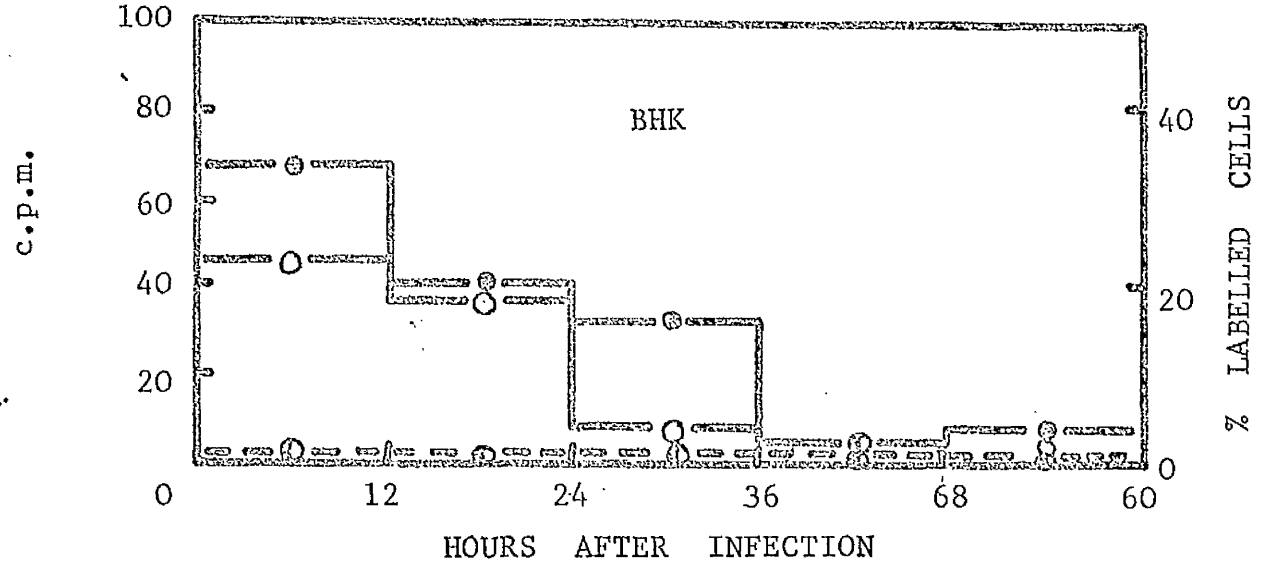
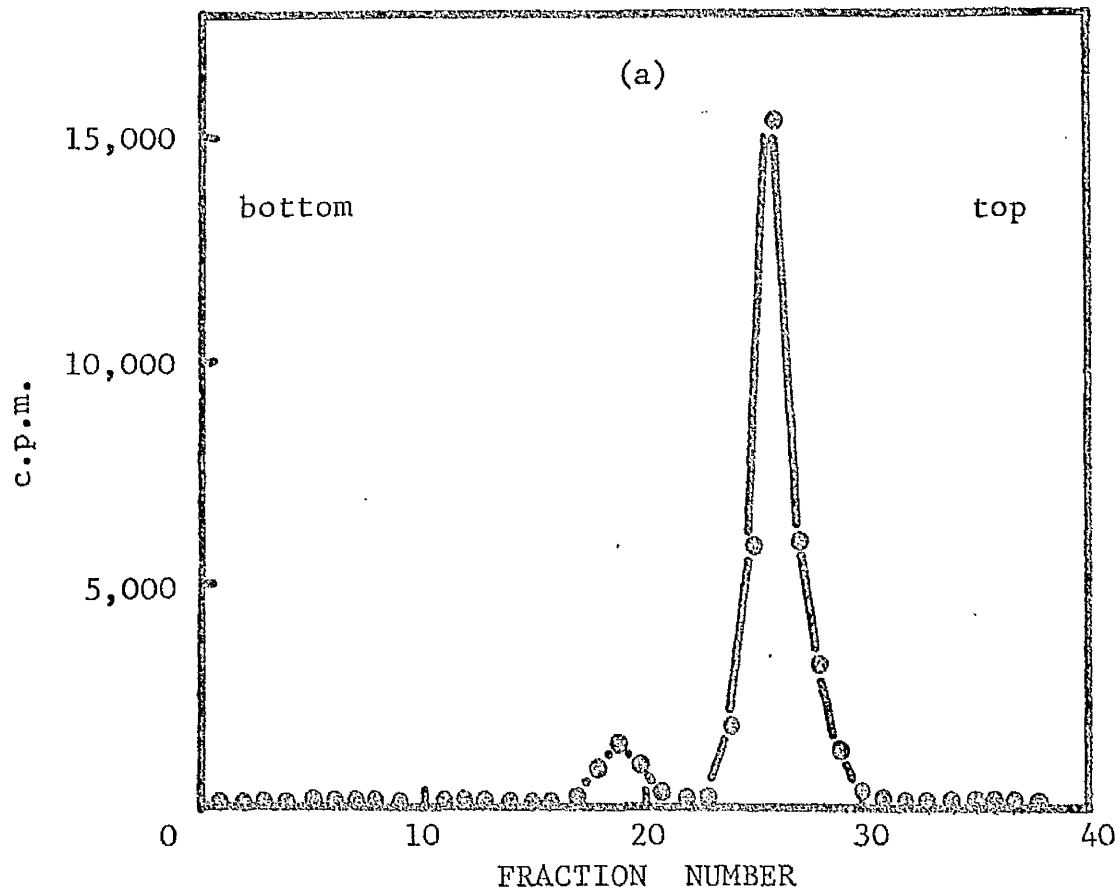

BHK
c.p.m.
% LABELLED CELLS
100
80
60
40
20
0
40
20
0
12
24
36
68
60
HOURS AFTER INFECTION

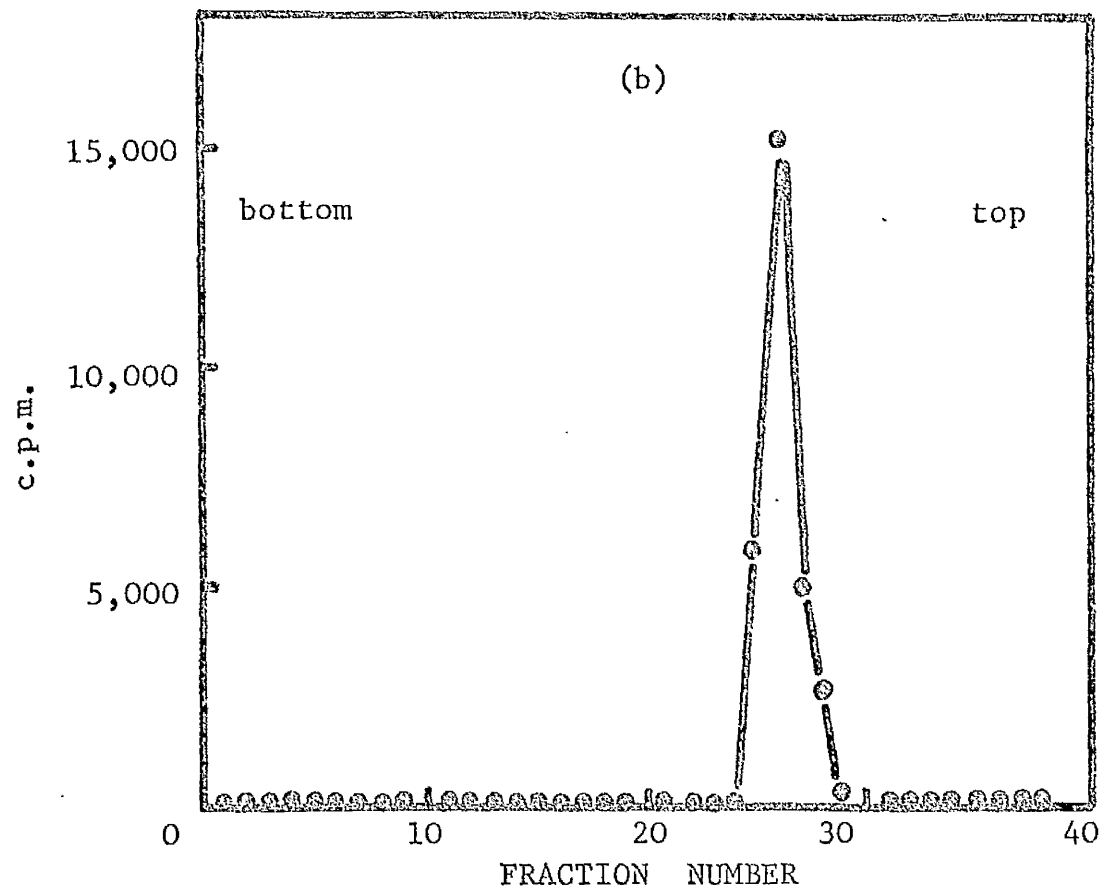
Fig. 16

Analysis of DNA synthesised in viral-infected mouse embryo cells

(a) Stationary cells in 90mm plates were infected with polyoma virus (50 PFU/cell) and pulsed with [^{3}H]thymidine (2.5×10^{-6}M; 1μCi/ml) between 0 and 72h after infection. At the end of the pulse, the cells were lysed with SDS and the [^{3}H]DNA analysed by equilibrium centrifugation at 100,000g for 72h in CsCl (sp.gr.=1.57g/ml) containing 100μg/ml of ethidium bromide. Fractions were collected from the bottom of the tube and the distribution of acid-insoluble radioactivity determined (Methods, section 10 (a)).

(b) Analysis of [^{3}H]DNA synthesised in mouse embryo cells after replacing the low serum medium over stationary cells with EC 10%. Otherwise, treated as in (a).


(a)
15,000
10,000
5,000
c.p.m.
bottom
top
0
10
20
30
40
FRACTION NUMBER


(b)
15,000
10,000
5,000
c.p.m.
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top
0
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30
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FRACTION NUMBER

banded in the position characteristic of supercoiled polyoma viral DNA. Therefore, the increase in DNA synthesis in viral-infected stationary mouse embryo cells (Fig. 15) represented mainly a stimulation of the synthesis of non-supercoiled DNA (presumably cellular DNA). By 24h the cell boundaries, nuclei and nucleoli of viral-infected cells were much more distinct than in the case of stationary or mock-infected cells.

In contrast, after infection of stationary BHK cells with polyoma virus at the same multiplicity there was no increase either in total incorporation of [^{3}H]thymidine or in the percentage of cells incorporating [^{3}H]thymidine (Fig. 15). However when these stationary BHK cells were overlaid with medium containing 10% serum there was a large increase in DNA synthesis. Between 12 and 14h after treatment with 10% serum there was a 580-fold increase in total incorporation of [^{3}H]thymidine and 75% of the cells were incorporating [^{3}H]thymidine during this period. Microscopic examination of these cells showed that the cell boundaries and internal details became much more distinct. By 24h, the cell density of the cultures had increased and much of the ordered arrangement, characteristic of the stationary cells, had been lost due to overcrowding presumably after growth.

These data and observations indicated that the inability of polyoma viral infection to induce DNA synthesis in stationary BHK cells was not due to a loss of viability of the cells.

2. The effect of viral infection on the activities of DNA polymerase and thymidine kinase

For these studies stationary BHK and mouse embryo cells in 90mm plastic Petri dishes were infected with polyoma virus at 50 PFU/cell, mock-infected or overlaid with medium containing 10% serum. At various times after these treatments the cells from two similar plates were collected (Methods, section 6 (a)) for the determination of the activities of DNA polymerase and thymidine kinase. The enzyme assays were carried out on homogenised extracts of these cells (Methods, section 6 (b)) and are described in Methods, sections 8 and 9.

(a) DNA polymerase

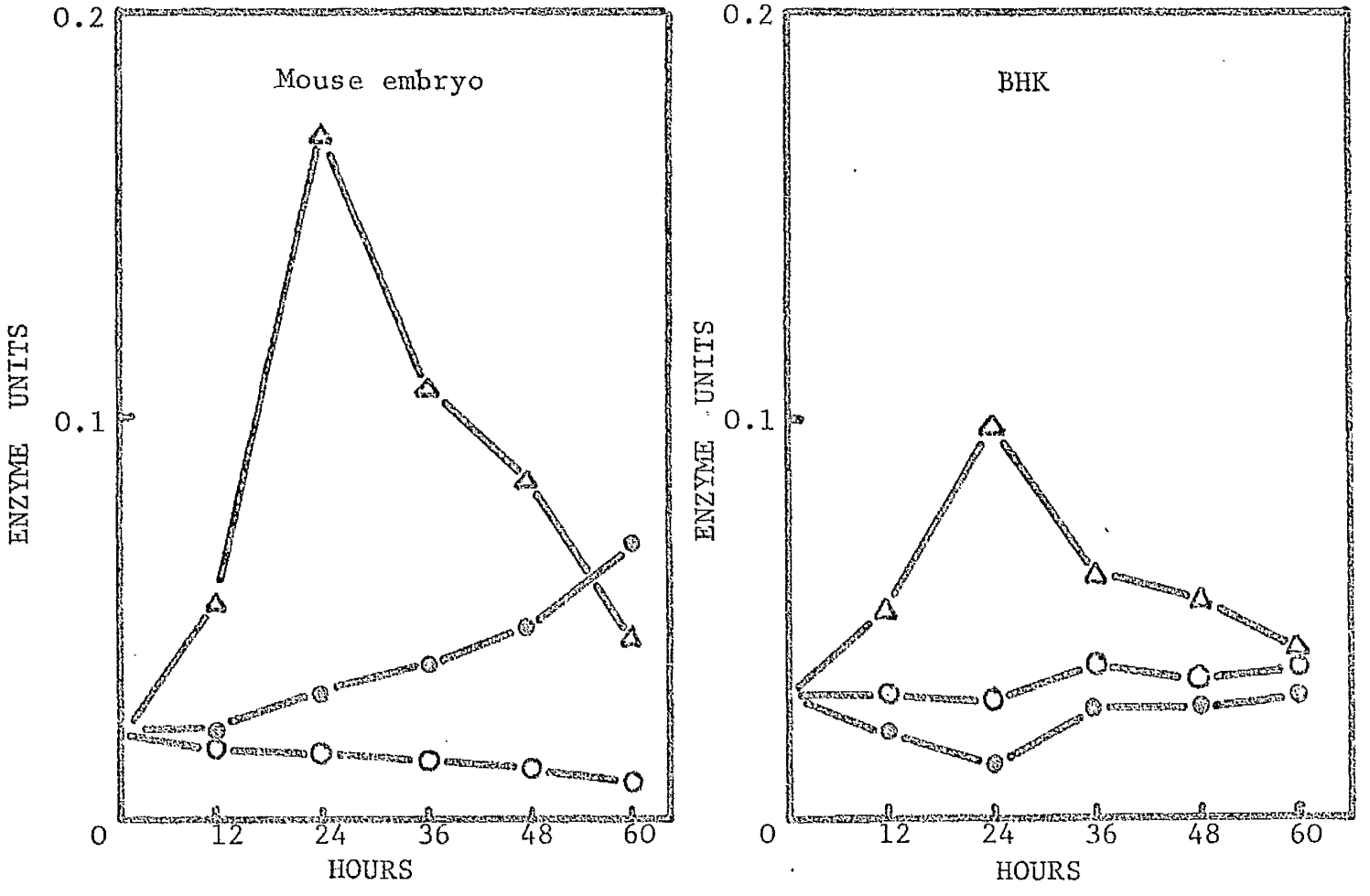
Polyoma viral-infection of stationary mouse embryo cells resulted in a steady increase in DNA polymerase activity (Fig. 17). In mock-infected cultures there was a steady decline in activity. At 60h after infection the DNA polymerase activity of viral-infected cells was 7-fold greater than that of mock-infected cells. In contrast, viral-infection of stationary BHK cells did not result in an increase in the activity of the enzyme. The activity of viral-infected cells was at all times less than that of mock-infected BHK cells.

However when the stationary cells were overlaid with medium containing 10% serum there was an increase in DNA polymerase activity maximal after 24h, in stationary BHK cells as well as in stationary mouse embryo cells. At the maximum levels, 24h after serum-stimulation,

Fig. 17

Effect of viral infection and serum on

DNA polymerase activity of stationary cells



the DNA polymerase activity of BHK and mouse embryo cells was 3.5 and 10 times, respectively, greater than the zero time activity.

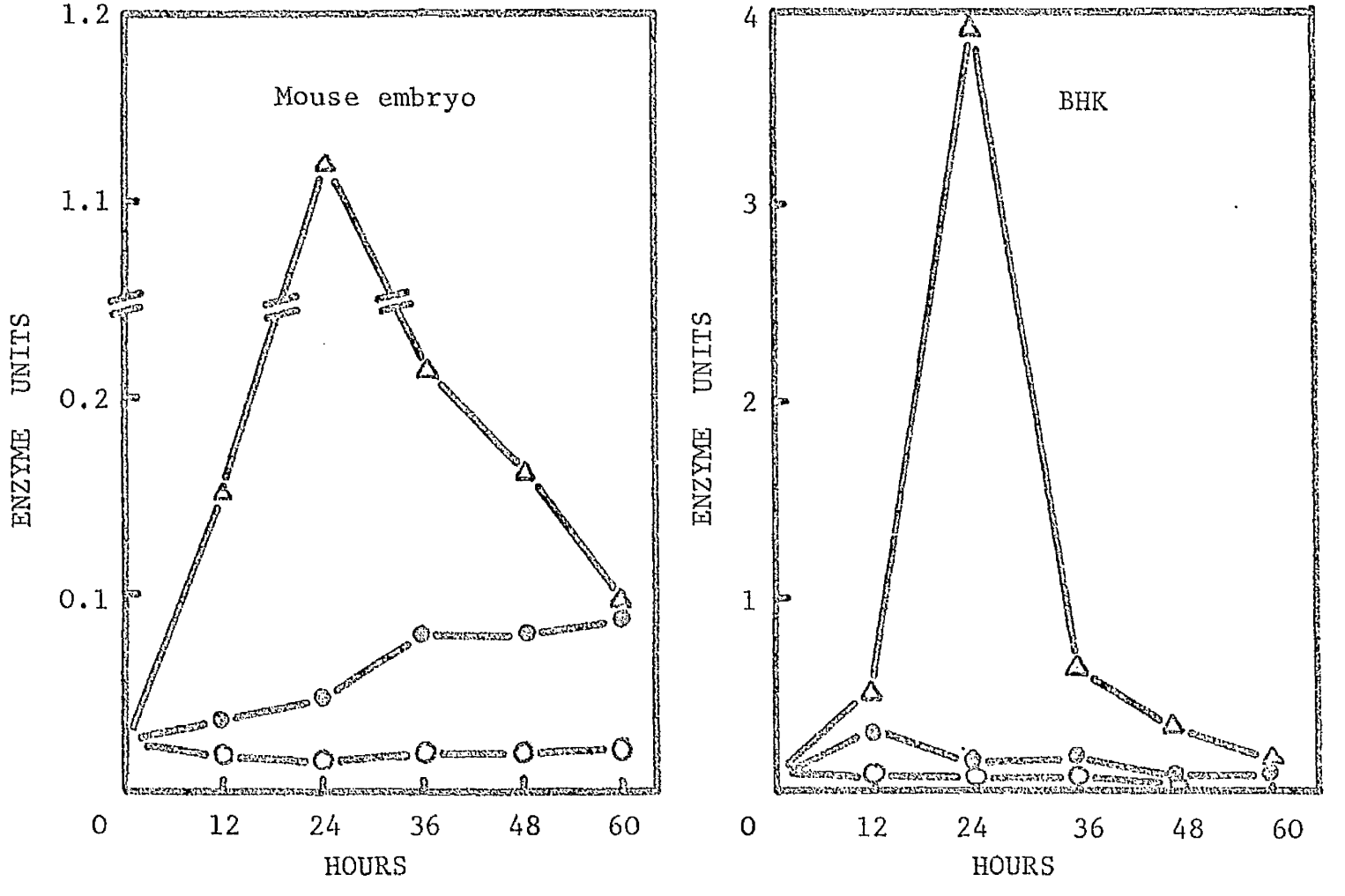
(b) Thymidine kinase

Thymidine kinase activity of stationary mouse embryo cells increased after infection with polyoma virus (Fig. 18). The activity approximately levelled off at 36h after viral infection and at this time was 3.5-fold greater than the activity of mock-infected cultures. The thymidine kinase activity of mock-infected cultures remained approximately constant throughout the time-course studied. 12h after viral infection of stationary BHK cells there was a small increase in thymidine kinase activity but at all other times there was no significant increase in activity compared to that at zero time. This early stimulation of activity, quite different from the pattern of stimulation in viral-infected mouse embryo cells, was not reproducible, however, and it was concluded that polyoma viral infection of stationary BHK cells did not result in a significant increase in thymidine kinase activity.

However, when the stationary cells were overlaid with medium containing 10% serum, there was an increase in thymidine kinase activity, maximal after 24h, in stationary BHK cells as well as in stationary mouse embryo cells. At the maximum levels, 24h after serum-stimulation, the thymidine kinase activity of BHK and mouse embryo cells was 33 and 13 times, respectively, greater than the zero time activity.

Fig. 18

Effect of viral infection and serum on thymidine kinase activity of stationary cells



3. Synthesis of polyoma viral DNA

Hirt (1967) described a technique for the selective extraction of polyoma viral DNA from infected mouse cell cultures in which the undegraded cellular DNA is precipitated in the presence of 0.6% SDS and 1M-NaCl. Labelled viral DNA in the supernatant can then be assayed by sedimentation velocity or ethidium bromide-CsCl equilibrium centrifugation.

(a) Viral-infected stationary mouse embryo cells

The Hirt extraction method is illustrated in Fig. 19. Stationary mouse embryo cells were infected with polyoma virus and labelled with [^{3}H]thymidine. The cells were subjected to Hirt extraction and the supernatant material centrifuged through neutral CsCl. As a control, stationary mouse embryo cells which had been overlaid with EC 10% were treated identically. Most of the radioactivity in the infected cell supernatant sedimented as a single band. No such band was obtained in the control experiment. When the infected cell supernatant material was centrifuged through alkaline CsCl (Fig. 20) the presence of a fast-moving band of radioactivity was indicative of polyoma viral DNA (component I). A slow moving minor band, component II and/or degraded cellular DNA, was also obtained.

The time course of polyoma viral DNA synthesis in infected mouse embryo cells was determined by using the Hirt extraction, followed by sedimentation through neutral CsCl to estimate [^{3}H]polyoma viral DNA

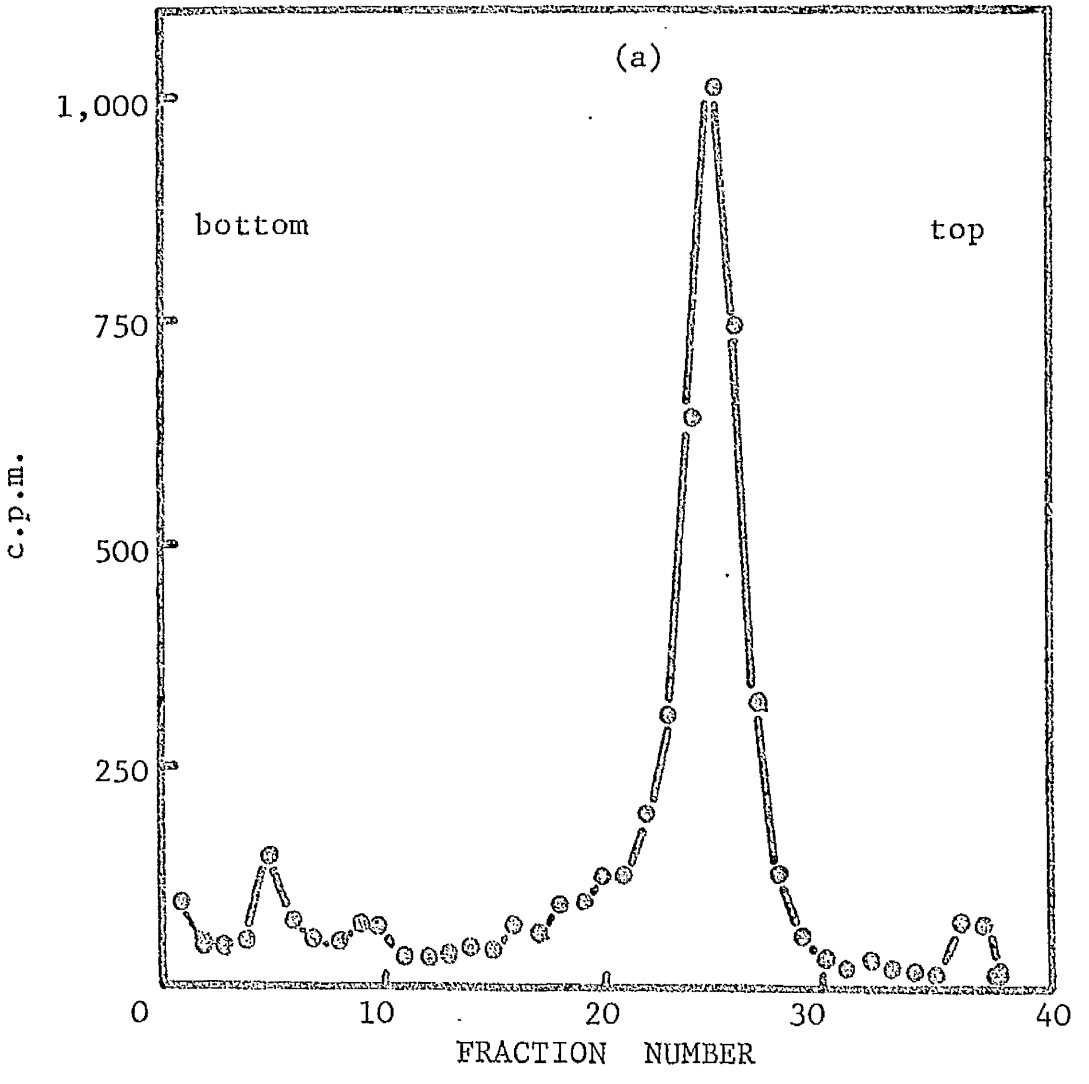
Fig. 19

Hirt extraction of polyoma viral DNA:

sedimentation through neutral CsCl

(a) Stationary mouse embryo cells in 90mm plates were infected with polyoma virus (50 PFU/cell) and pulsed with [^{3}H]thymidine (2.5×10^{-7}M; 1μCi/ml) between 20 and 44h after infection. At the end of the pulse the cells were lysed with 1ml of 0.6% SDS in 0.01M-tris-HCl, pH7.5, 0.01M-EDTA. The lysate was extracted by the Hirt method (Methods, section 10 (b)) and a 0.2ml aliquot of the supernatant centrifuged through 3ml of neutral CsCl (sp.gr.=1.5g/ml; pH7.5) at 100,000g for 3.5h. Fractions were collected from the bottom of the tubes and the distribution of acid-insoluble radioactivity determined (Methods, section 10 (a)).

(b) Stationary mouse embryo cells were overlaid with EC 10% and pulsed with [^{3}H]thymidine (2.5×10^{-7}M; 1μCi/ml) between 20 and 44h after the medium replacement. Otherwise treated as in (a).


(a)
1,000
750
500
250
0
c.p.m.
bottom
top
10
20
30
40
FRACTION NUMBER

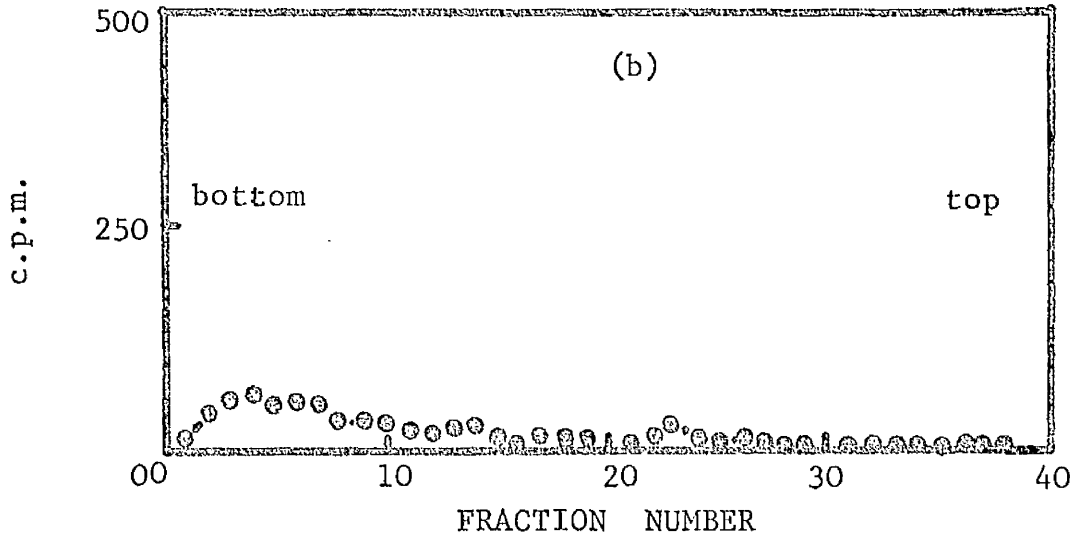
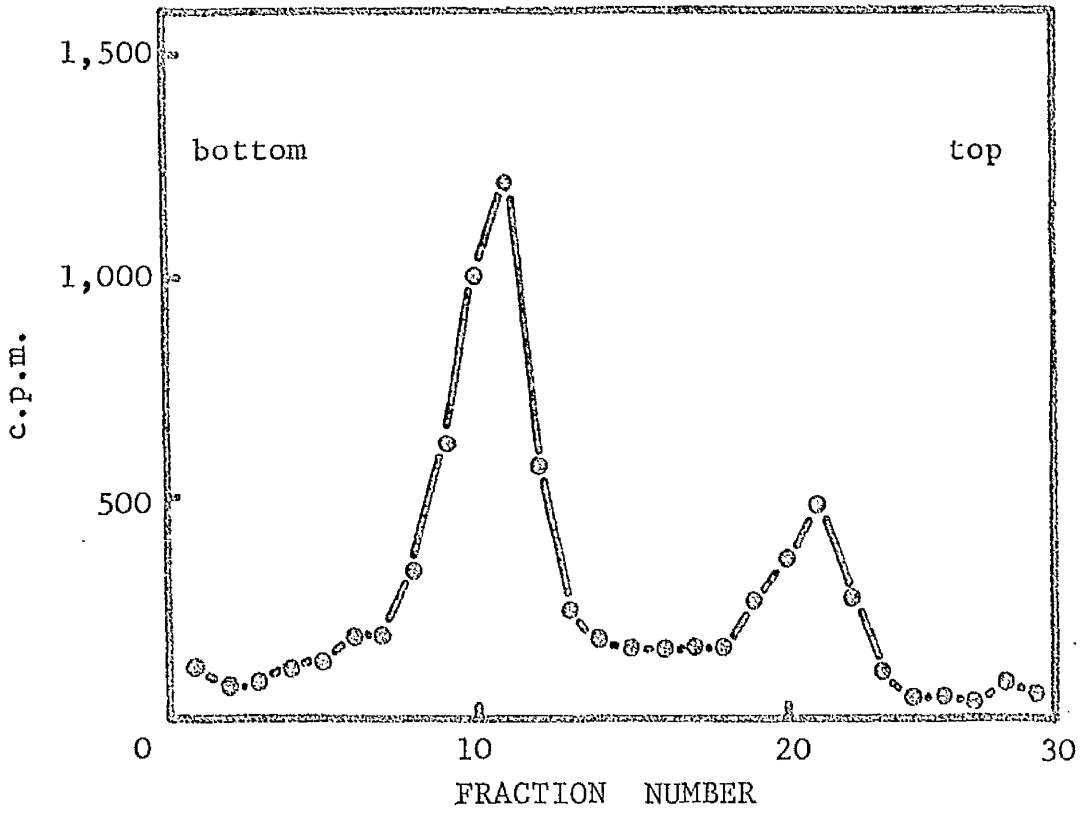

(b)
500
250
00
c.p.m.
bottom
top
10
20
30
40
FRACTION NUMBER

Fig. 20

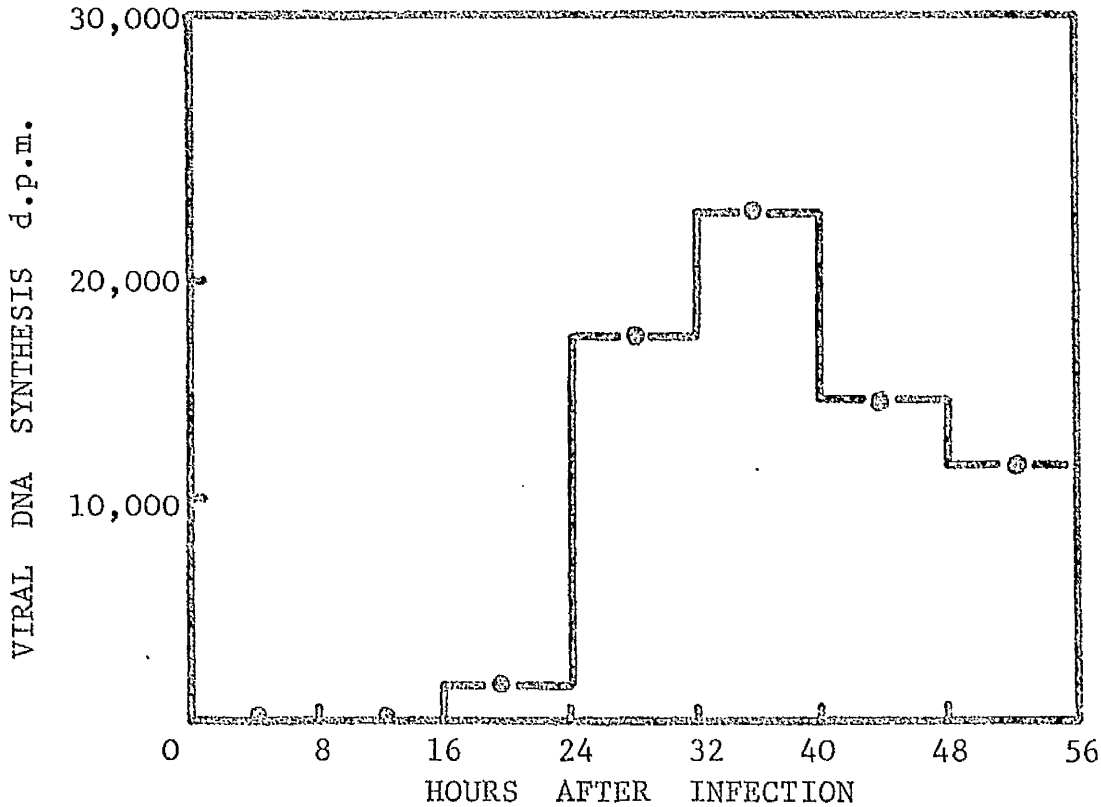
Hirt extraction of polyoma viral DNA: sedimentation through alkaline CsCl



A 0.2ml aliquot of the Hirt supernatant described in Fig. 19(a) was centrifuged through 3ml of alkaline CsCl (sp.gr.=1.5g/ml; pH12.5) at 100,000g for 2h (Methods, section 10 (b)). Fractions were collected from the bottom of the tube and the distribution of acid-insoluble radioactivity determined (Methods, section 10 (a)).

Fig. 21

Time course of polyoma viral DNA synthesis in infected stationary mouse embryo cells



Stationary mouse embryo cells in 50mm plates were infected with polyoma virus (50 PFU/cell) and pulsed with [^{3}H]thymidine (2.5×10^{-7}M; 1μCi/ml) for 8h periods after infection. The [^{3}H]viral DNA synthesised during each period was estimated by centrifugation of a 0.2ml aliquot of the supernatant, obtained from a Hirt extraction, through neutral CsCl as before.

synthesised during 8h pulses with [^{3}H]thymidine (Fig 21). Viral DNA synthesis was first detected between 16 and 24h and the rate of synthesis was maximal between 32 and 40h after infection.

(b) Viral-infected stationary BHK cells

The above method was used in an attempt to detect polyoma viral DNA synthesis in infected stationary BHK cells. The infected cells were pulsed with [^{3}H]thymidine for 8h periods up to 48h after infection. At the end of the pulse the cells were lysed with SDS, the lysate extracted by the Hirt method, and the supernatants centrifuged through neutral CsCl. Fig 22(a) shows the distribution of radioactivity obtained from infected BHK cells pulsed with [^{3}H]thymidine between 32 and 40h after infection, the period when viral DNA is synthesised at maximal rate in infected stationary mouse embryo cells. For comparison, the corresponding data for infected stationary mouse embryo cells is shown in Fig. 22(b). No synthesis of viral DNA could be detected in infected BHK cells during the period 32-40h after infection. Similarly, no viral DNA synthesis could be detected in these cells during the other five 8h periods up to 48h after infection.

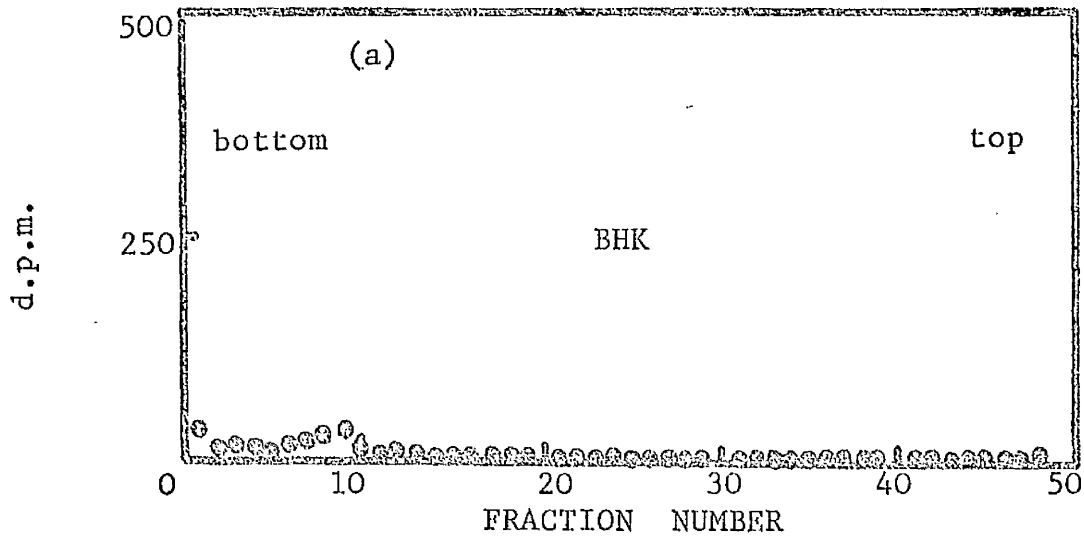
If synthesis of [^{3}H]viral DNA had taken place in infected BHK cells to an extent of 1% of that in infected mouse embryo cells it would have been detectable. As no synthesis of component II viral DNA either could be detected, these data suggest that in infected stationary BHK cells the polymerisation step in viral DNA replication

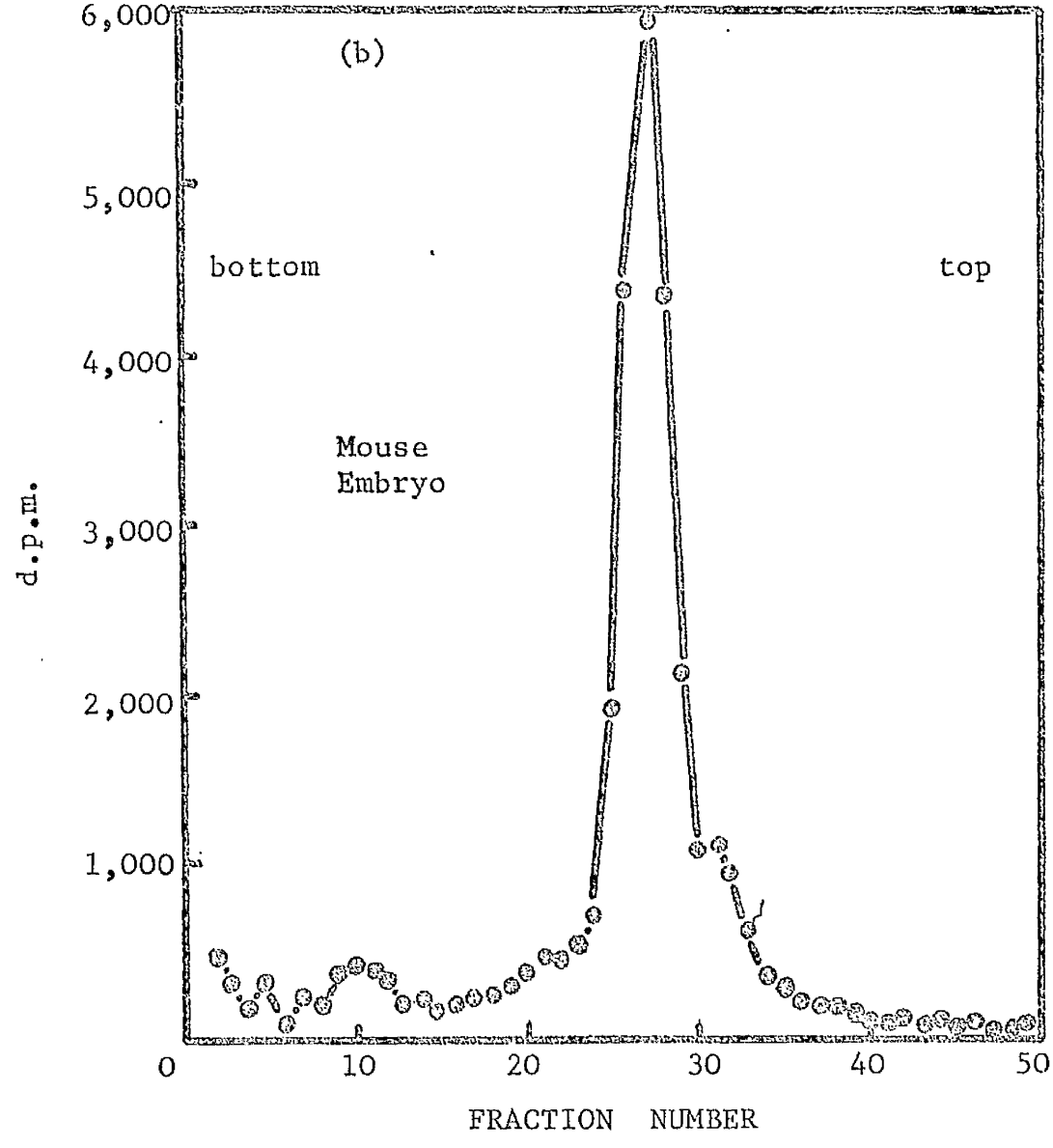
Fig. 22

Viral DNA synthesis in infected cells:

comparison between infected BHK and mouse embryo cells

Stationary cells in 50mm plates were infected with polyoma virus (50 PFU/cell) and pulsed with [^{3}H]thymidine (2.5×10^{-7}M; 1μCi/ml) between 32 and 40h after infection. At the end of the pulse the cells were lysed with SDS and the lysate extracted by the Hirt method (Methods, section 10 (b)). A 0.2ml aliquot of the Hirt supernatant was centrifuged through 3ml of CsCl (sp.gr.=1.5g/ml; pH7.5) at 100,000g for 3.5h. Fractions were collected from the bottom of the tube and the distribution of acid-insoluble radioactivity determined (Methods, section 10 (a)).


(a)
500
250
0
d.p.m.
bottom
top
BHK
10
20
30
40
50
FRACTION NUMBER


(b)
6,000
5,000
4,000
3,000
2,000
1,000
0
d.p.m.
bottom
top
Mouse
Embryo
10
20
30
40
50
FRACTION NUMBER

(see p. 19) takes place either to a very limited extent or not at all. This could be due to a partial or complete block in the polymerisation step itself or in the nicking step required to convert parental component I to component II prior to polymerisation.

The process of viral DNA replication in infected cells may require viral-coded proteins as well as cellular functions. It is possible that in infected stationary BHK cells there is a failure, partial or complete, to synthesise necessary viral proteins. Since the expression of the polyoma viral genome in infected cells could be regulated at the levels of transcription and translation, it was of interest, first of all, to examine the transcription of polyoma viral DNA in infected stationary BHK cells.

4. Synthesis of viral-specific RNA in viral-infected cells

Using the DNA-RNA hybridisation technique, Martin & Axelrod (1969a,b) were able to show that the complete polyoma viral genome was transcribed in viral-infected mouse embryo cells. They also showed by a novel competition method, that the viral-specific RNA made in certain polyoma viral-transformed cell lines corresponded to transcription of only 40% of the viral genome.

It should be possible, therefore, to use this method to compare the transcription of polyoma viral DNA in viral-infected stationary BHK and mouse embryo cells.

In outline, [^{32}P]-labelled RNA, prepared from viral-infected mouse embryo cells, was used as marker viral-specific RNA. Unlabelled RNA from viral-infected BHK cells (and other sources, as controls) was incubated with polyoma viral DNA under hybridisation conditions. Hybridisation of homologous viral RNA was detected by a further incubation of the viral DNA with [^{32}P]-labelled marker RNA. A decrease in [^{32}P]RNA hybridising with viral DNA was indicative of the presence of viral RNA in the unlabelled RNA preparation.

(a) Preparation of RNA from viral-infected cells

The preparation of [^{32}P]RNA from viral-infected stationary mouse embryo cells is described in detail in Methods, section 11 (a) (i). Immediately after virus adsorption the cells were labelled with [^{32}P]orthophosphate and to ensure that [^{32}P]RNA of high specific

activity was obtained the medium contained a reduced amount of orthophosphate. It was necessary, however, to ensure that medium orthophosphate was not significantly depleted during the labelling period otherwise [^{32}P]RNA synthesised at the beginning and end of the period would have very different specific activities.

Viral-infected mouse embryo cells were maintained in medium containing various amounts of orthophosphate (expressed as a percentage of the normal orthophosphate concn. of medium) and labelled with [^{32}P]orthophosphate. At a series of times after infection the incorporation of [^{32}P]orthophosphate into acid-insoluble material was determined and expressed as a percentage of the input radioactivity. The time course of incorporation up to 40h after infection at different phosphate concentrations is shown in Fig. 23(a). Fig. 23(b) shows the incorporation between 0 and 40h as a function of phosphate concentration. After 40h, only 16.7% of orthophosphate in medium containing 10% of the normal orthophosphate concentration was incorporated into acid-insoluble material. As this did not represent an excessive depletion of orthophosphate, 10% normal phosphate medium was used prior to the preparation of [^{32}P]RNA.

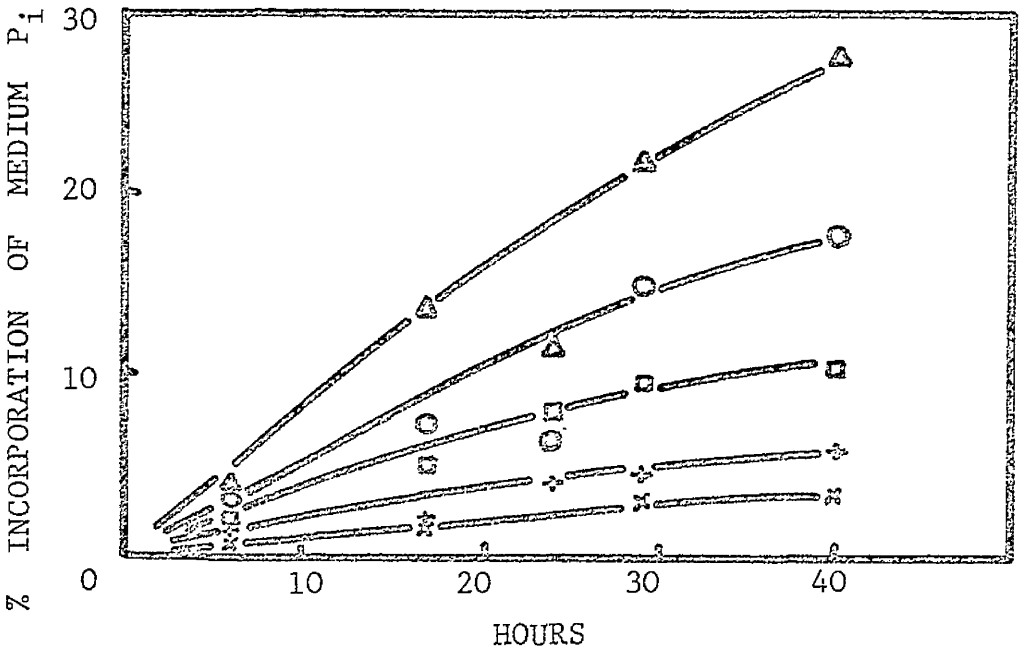
RNA was extracted from the pooled SDS-lysates of viral-infected cells obtained at 8h intervals up to 40h after infection. This ensured that all classes of viral-specific RNA, made at different times after infection, were present in the preparation.

Fig. 23

Incorporation of orthophosphate by viral-infected stationary mouse embryo cells

The medium on stationary mouse embryo cells in 90mm plates was replaced 24h prior to viral infection with 10ml of orthophosphate-free EC 0.5%*. (EC 0.5%* contained 0.5% of dialysed serum and had been pre-incubated at 37°C for 3-4 days). After infection with polyoma virus (50 PFU/cell) the cells were overlaid with 10ml of EC 0.5%* containing various concentrations of orthophosphate. The cells were labelled with [^{32}P] orthophosphate (1μCi/plate) and incubated at 37°C. At various times after infection the cells were collected. The medium was removed and the cells washed twice with ice-cold PBS(a) and then scraped from the plates. The cells were collected on Whatman GF/A glass fibre filters and washed with 30ml of ice-cold 5% (w/v) TCA. The filters were dried and counted in toluene-based scintillation fluid using a liquid scintillation spectrometer. Incorporated radioactivity was expressed as a percentage of the input radioactivity.

(a)



The medium contained the following concentrations of P_i expressed as the % of the P_i concentration of normal medium:-

5%

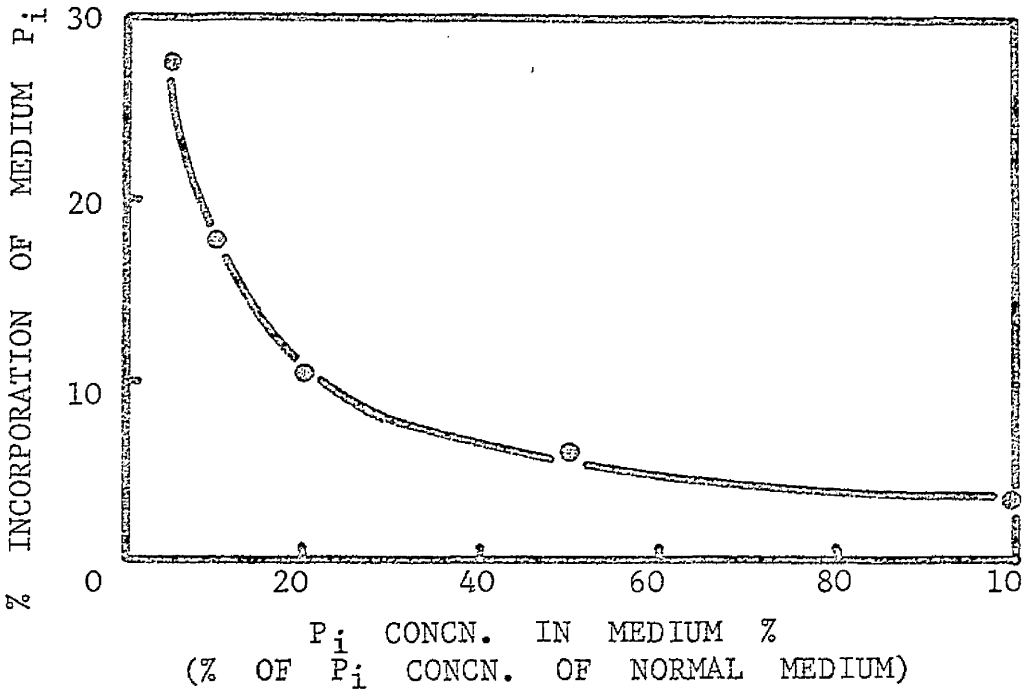
10%

20%

50%

100%

(b)



The % incorporation of medium P_i in 40h was taken from (a).

(b) DNA-RNA hybridisation

10ng lots of heat-denatured polyoma viral DNA (component II, prepared in vitro from purified component I: see Methods, section 12 (a)) were immobilised on nitrocellulose filter discs. The amount of [^{32}P]RNA required to saturate the viral DNA on a filter was found by incubating the filter at 67°C for 16h with increasing amounts of this RNA in 4 x SSC containing 1% SDS and determining the amount of hybridised [^{32}P]RNA. Fig. 24 shows that approximately 100µg of [^{32}P]RNA was required to saturate the viral DNA sites.

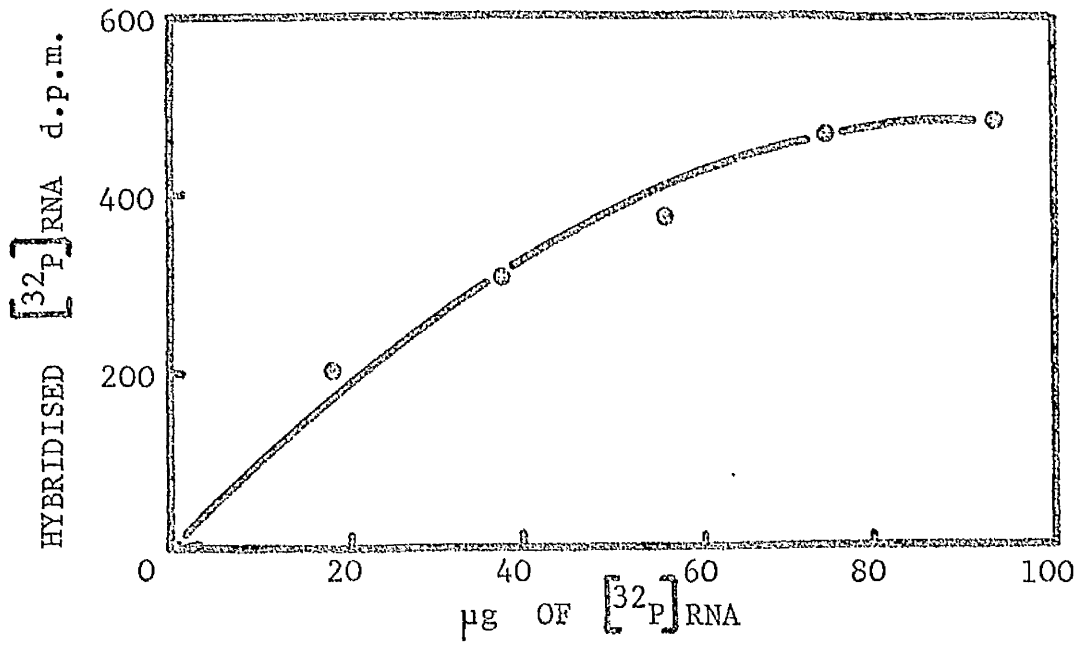
The ability of various types of unlabelled RNA to hybridise with viral DNA was then tested. Filters bearing viral DNA were incubated with unlabelled RNA at 67°C for 16h and then transferred into another hybridisation reaction mixture containing saturating amounts of [^{32}P]RNA. After incubation at 67°C for a further 16h the amount of hybridised [^{32}P]RNA was determined and expressed as the percentage of the amount of hybridised [^{32}P]RNA in the absence of unlabelled RNA.

Table 16 shows that RNA from uninfected BHK and mouse embryo cells (Methods, section 11) did not compete with the [^{32}P]RNA for viral DNA sites. This indicated that viral DNA hybridised only with viral-specific RNA and not with cellular RNA from either BHK or mouse embryo cells.

The procedure was repeated with unlabelled RNA obtained from viral-infected stationary BHK and mouse embryo cells and from PyY cells, a line of polyoma viral-transformed BHK cells. Each of these types of RNA competed effectively with [^{32}P]RNA for viral DNA sites (Fig. 25), indicating that viral—specific RNA was present in all of these cells.

Fig. 24

Saturation of polyoma viral DNA with $[^{32}P]$RNA from infected stationary mouse embryo cells



10ng amounts of viral DNA immobilised on nitrocellulose filters were incubated with $[^{32}P]$RNA in 0.25ml of 4 x SSC containing 0.1% SDS at 67°C for 16h. The filters were treated with pancreatic RNase, washed and dried. The hybridised $[^{32}P]$RNA was determined by counting the filters in toluene-based scintillation fluid, using a liquid scintillation spectrometer. The radioactivity of a blank filter, similarly incubated, was subtracted in each case. Each point represents the average of duplicate incubations.

Table 16

Specificity of the hybridisation of viral DNA with viral RNA

FIRST INCUBATION	SECOND INCUBATION	HYBRIDISED $[^{32}P]$RNA d.p.m.	% HYBRIDISED $[^{32}P]$RNA
Buffer	$[^{32}P]$RNA 100µg	328	100
BHK RNA 176µg		366	111
ME RNA 157µg		332	101

10ng amounts of polyoma viral DNA, immobilised on nitrocellulose filters, were subjected to two consecutive incubations at 67°C for 16h. The first incubation was carried out in 0.25ml of buffer (4 x SSC containing 0.1% SDS) either alone or in the presence of unlabelled RNA from uninfected BHK cells (BHK RNA) or uninfected mouse embryo cells (ME RNA). The filters were each transferred to 0.25ml of buffer containing 100µg of $[^{32}P]$RNA and incubated a second time. The amount of hybridised $[^{32}P]$RNA was determined as before and corrected by subtracting the radioactivity of a blank filter, similarly incubated. Each value represents the average of duplicate incubations.

To obtain an estimate of the percentage of the viral genome transcribed in viral-infected BHK cells it is necessary to extrapolate the data of Fig. 25 to infinite concentration of RNA. In theory, this can be done by means of a double-reciprocal plot as shown in Fig. 26. The reciprocal of the intercept on the vertical axis for each type of RNA is a measure of the percentage of the viral genome transcribed.

The entire genome is known to be transcribed in viral-infected mouse embryo cells and the data obtained in these competition studies is consistent with this. The data obtained for PyY RNA is not linear but it is apparent that this RNA contained viral-specific RNA which was the transcription product of only a fraction of the viral genome. This is in agreement with the observations of Martin & Axelrod (1969b), who reported that only 40% of the viral genome was transcribed in two polyoma viral-transformed cell lines tested.

The double-reciprocal plot of the data for RNA from viral-infected BHK cells is linear and the intercept on the vertical axis is 1×10^{-2} indicating that the complete viral genome was transcribed in these cells. The initial slopes of the curves in Fig. 25 indicate that lesser amounts of viral-specific RNA were produced in these cells than in viral-infected mouse embryo cells.

It was concluded that a possible failure to synthesise polyoma viral proteins, necessary for viral DNA replication, in infected stationary BHK cells is apparently not due to a failure at the level of transcription. However, there may be a failure at the level of translation and the following section describes studies on viral proteins synthesised in infected stationary BHK and mouse embryo cells.

Fig. 25

Detection of polyoma viral-specific RNA in viral-infected and viral-transformed cells by competitive hybridisation

100ng amounts of polyoma viral DNA, immobilised on nitrocellulose filters, were subjected to two consecutive incubations at 67°C for 16h. The first incubation was carried out in 0.25ml of buffer (4 x SSC containing 0.1% SDS) either alone or in the presence of various amounts of unlabelled RNA from viral-infected stationary mouse embryo cells (Py-ME RNA), viral-infected stationary BHK cells (Py-BHK RNA) or PyY cells (PyY RNA). The filters were each transferred to 0.25ml of buffer containing 100µg of [^{32}P]RNA and incubated a second time. The amount of hybridised [^{32}P]RNA was determined as before and corrected by subtracting the radioactivity of a blank filter, similarly incubated. Each point represents the average of duplicate incubations.

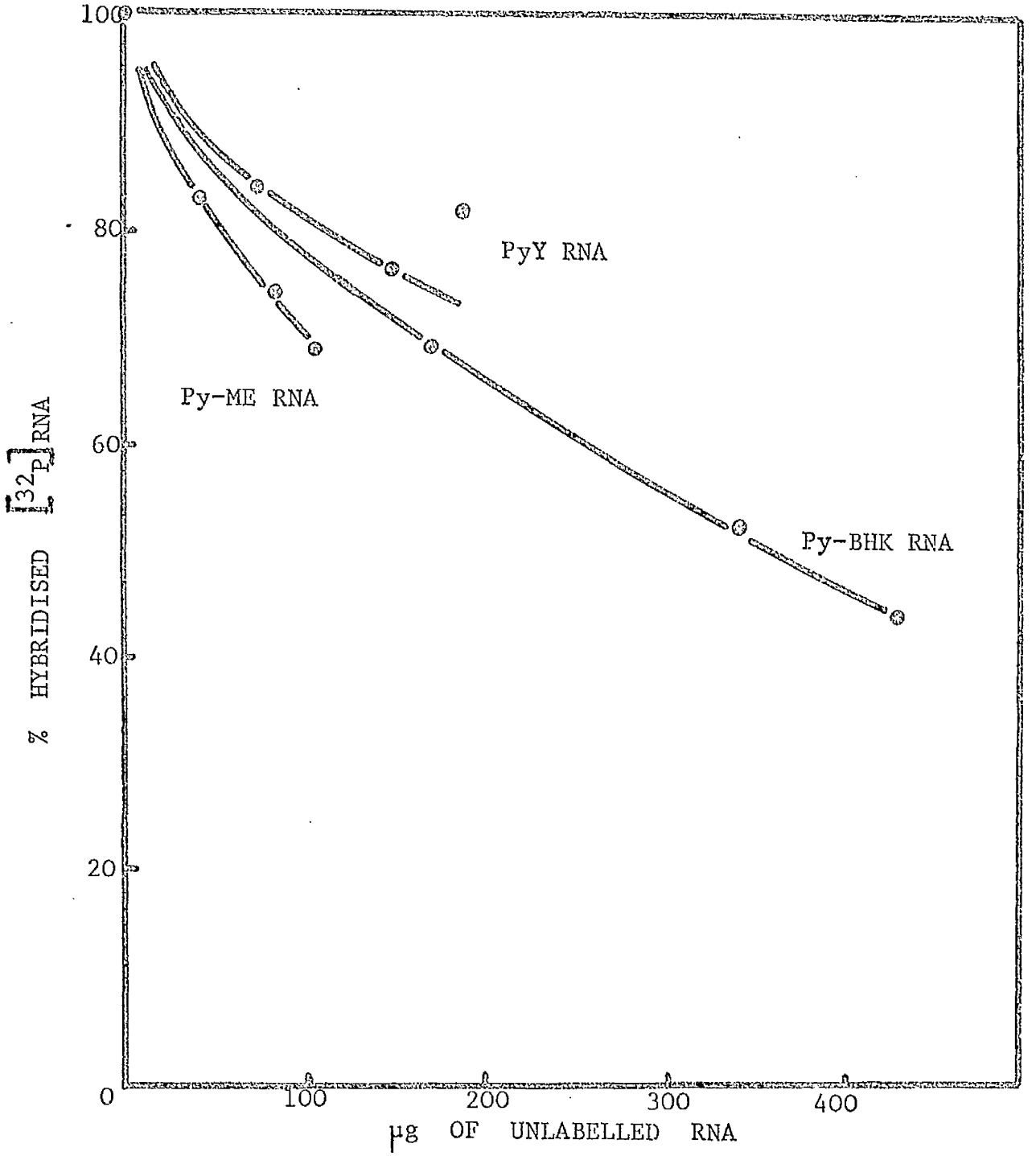
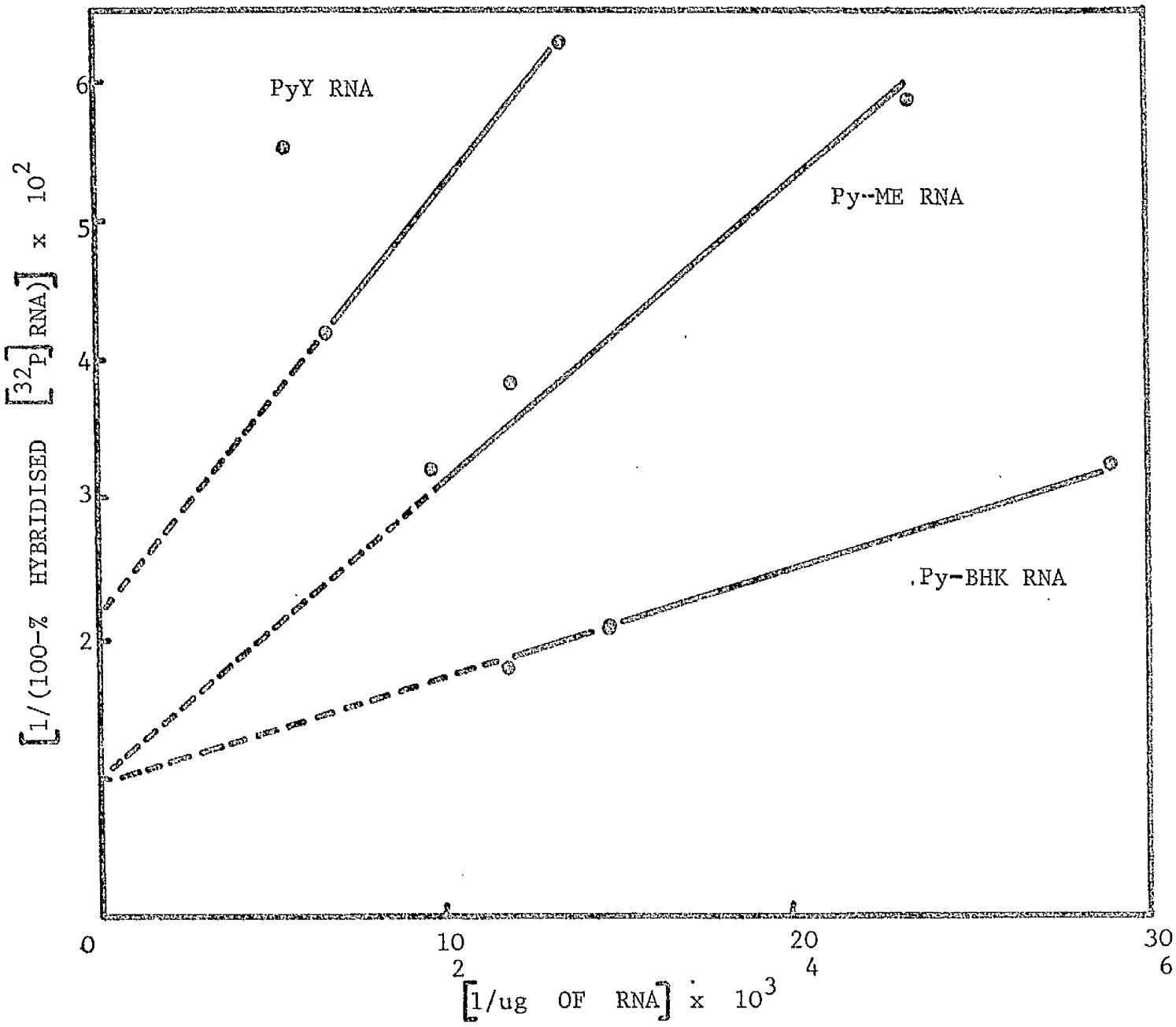

100
80
60
40
20
0
% HYBRIDISED [32P] RNA
PyY RNA
Py-ME RNA
Py-BHK RNA
100
200
300
400
µg OF UNLABELLED RNA

Fig. 26

Double-reciprocal plot of the data of Fig. 25



5. Synthesis of viral-specific proteins in viral-infected cells

It seems likely that the full complement of viral-specific proteins is made during the productive infection of mouse embryo cells but it is possible that some viral proteins may not be made in viral-infected BHK cells.

As a first approach to this problem, studies were made on a method of specifically labelling viral proteins, synthesised in infected cells. Sodium fluoride reversibly inhibits protein synthesis and causes the dissociation of polyribosomes. It is thought to inhibit some stage of the initiation of protein synthesis but not affect chain-elongation (Marks, Burka, Canconi, Perl & Rifkind, 1965; Ravel, Mosteller & Hardesty, 1966). When Krebs ascites tumour cells, infected with encephalomyocarditis virus (EMC), were treated with NaF and pulsed with [^{35}S]methionine after release from the NaF block, mainly viral-specific proteins were labelled (Smith, Marcker & Mathews, 1970).

The applicability of this method for polyoma viral-infected cells was investigated. Stationary mouse embryo cells were infected with polyoma virus or mock-infected. Protein synthesis in viral-infected and mock-infected cells after release from NaF inhibition was compared by pulsing the cells with [^{3}H]leucine for 5min periods and determining the incorporation of [^{3}H]leucine into acid-insoluble material (Fig. 27). In viral-infected cells there was a large burst of protein synthesis

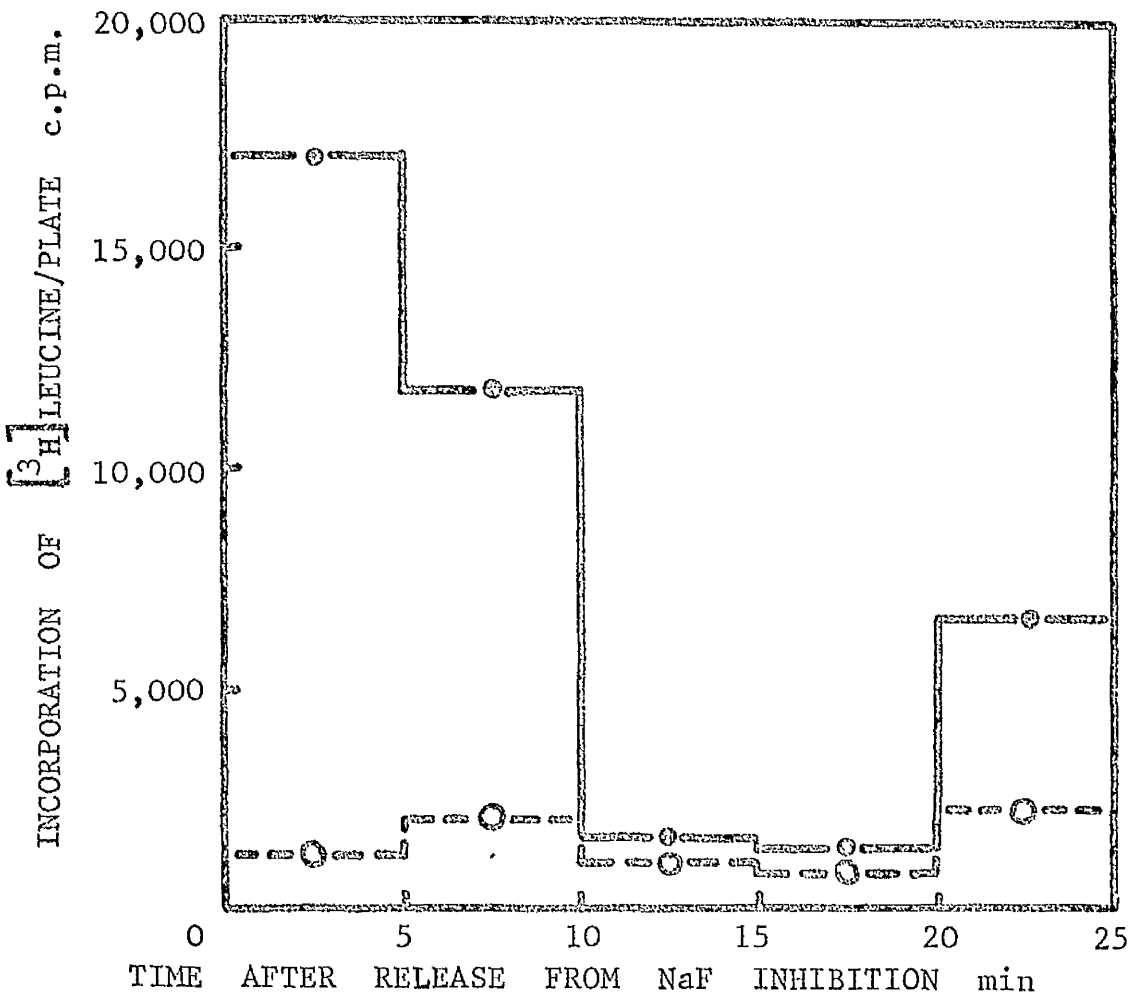
Fig. 27

Protein synthesis in viral-infected mouse embryo cells after release from sodium fluoride inhibition

Stationary mouse embryo cells in 90mm plates were infected with polyoma virus (50 PFU/cell) or mock-infected. 30h after infection NaF was added to a final concn. of 10mM and the cultures incubated at 37°C for a further 30min. The following operations were performed in a 37°C hot room: the medium was removed and the cell monolayers washed three times with 5ml of PBS(a) at 4°C; 3ml of hepes-buffered Eagle's medium (containing 5% of the normal leucine concn.) at 37°C was added to each plate and the cells pulsed with [^{3}H]leucine (10μCi/plate) for 5min periods; at the end of a pulse the medium was removed and the cells overlaid with ice-cold 5% (w/v) TCA. The cells were scraped from the plates and the acid-insoluble material collected on Whatman GF/A glass fibre filters. After washing with 30ml of ice-cold 5% TCA and drying, the filters were counted in toluene-based scintillation fluid using a liquid scintillation spectrometer. Each point represents the average incorporation of radioactivity by cells on duplicate plates.

—⊙— Infected cells

- -⊙- - Mock-infected cells


INCORPORATION OF [3H]LEUCINE/PLATE c.p.m.
20,000
15,000
10,000
5,000
0
5
10
15
20
25
TIME AFTER RELEASE FROM NaF INHIBITION min

occurring between 0 and 10min after release from NaF inhibition. This was followed by a lag and then a second burst of protein synthesis starting between 20 and 25min. In mock-infected cultures there was a poorly-defined initial burst of protein synthesis, a lag and then a second burst. By analogy with the EMC system the initial burst of protein synthesis in viral-infected cells possibly represented the synchronised initiation of viral-specific protein synthesis, before cellular proteins began to be made in the second burst.

Provided that this interpretation was correct, this system afforded an excellent means of specifically labelling viral proteins. [^{35}S]methionine-labelled proteins synthesised in the first 10min after release from NaF inhibition in viral-infected stationary mouse embryo cells were fractionated by electrophoresis through 10% polyacrylamide gels in the presence of 0.1% SDS. The distribution of radioactivity in the gels after electrophoresis is shown in Fig. 28. [^{35}S]methionine-labelled material was distributed randomly throughout the gels and there was little difference in the profiles obtained using infected or mock-infected cells whether or not they had been treated with NaF. It appeared that after release from NaF inhibition a heterogeneous collection of proteins and not just viral proteins were synthesised in viral-infected mouse embryo cells. If viral proteins alone had been synthesised, as in EMC-infected cells, a small number of bands of radioactivity might have been expected after electrophoresis.

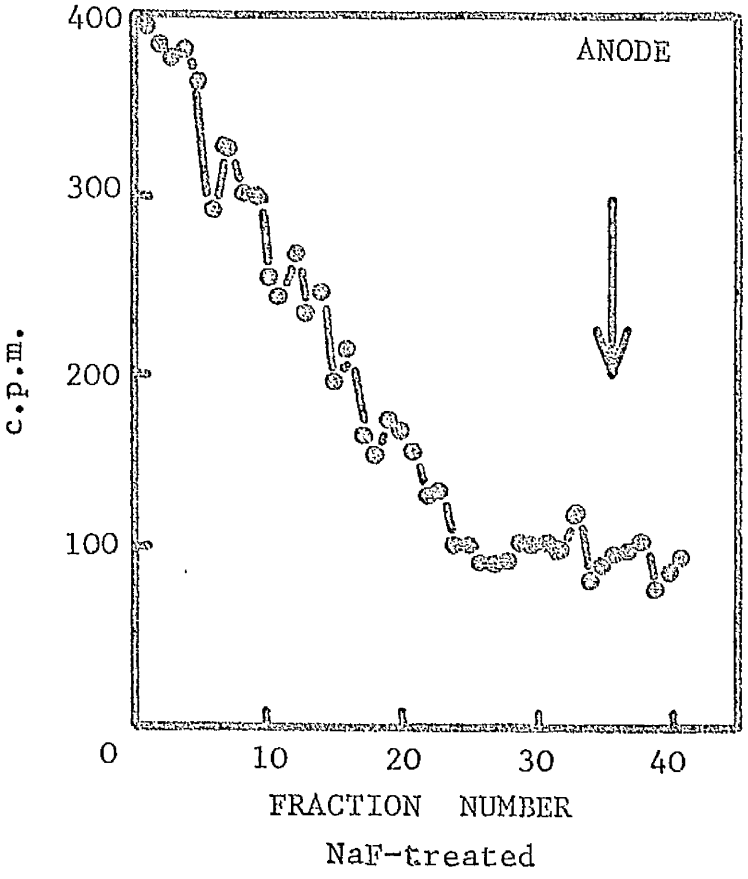
Attempts to separate and characterise the viral proteins synthesised

Fig. 28

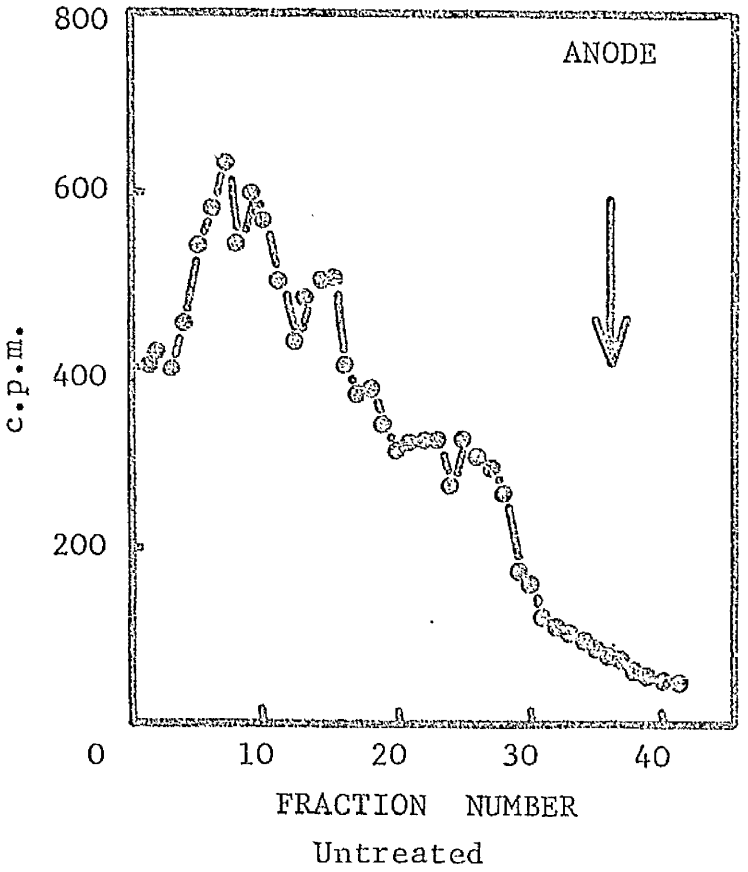
SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labelled proteins in infected mouse embryo cells after release from NaF inhibition

Infected and mock-infected cells were treated with NaF and released from NaF inhibition as described in the legend to Fig. 27. Untreated cultures were subjected to the same washing procedure as NaF-treated cultures. All cultures were overlaid with 2ml of hepes-buffered Eagle's medium (containing 5% of the normal methionine concn.) and incubated at 37°C in the presence of [^{35}S]methionine (10μCi/plate). for 10min. The medium was then removed and the cells first washed twice with 5ml of ice-cold PBS(a) and lysed with 1ml of 1% SDS in 0.01M-sodium phosphate, pH7.0, containing 1% 2-mercaptoethanol [^{35}S]methionine-labelled proteins were fractionated by SDS-polyacrylamide gel electrophoresis (Methods, section 13). The arrow indicates the position of the bromophenol blue marker.

(a) Viral-infected cells

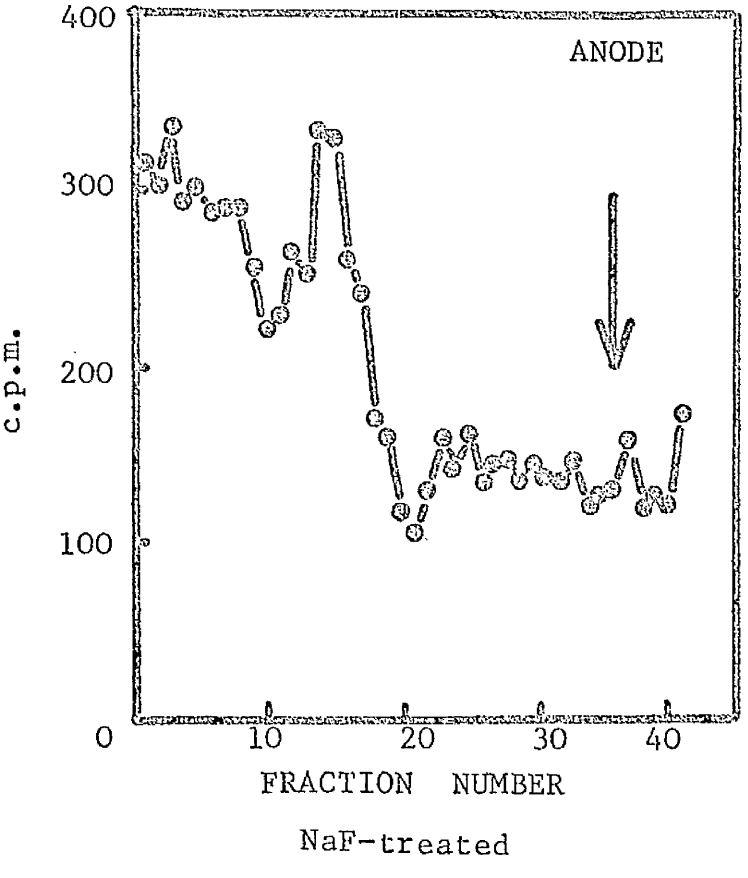


NaF-treated

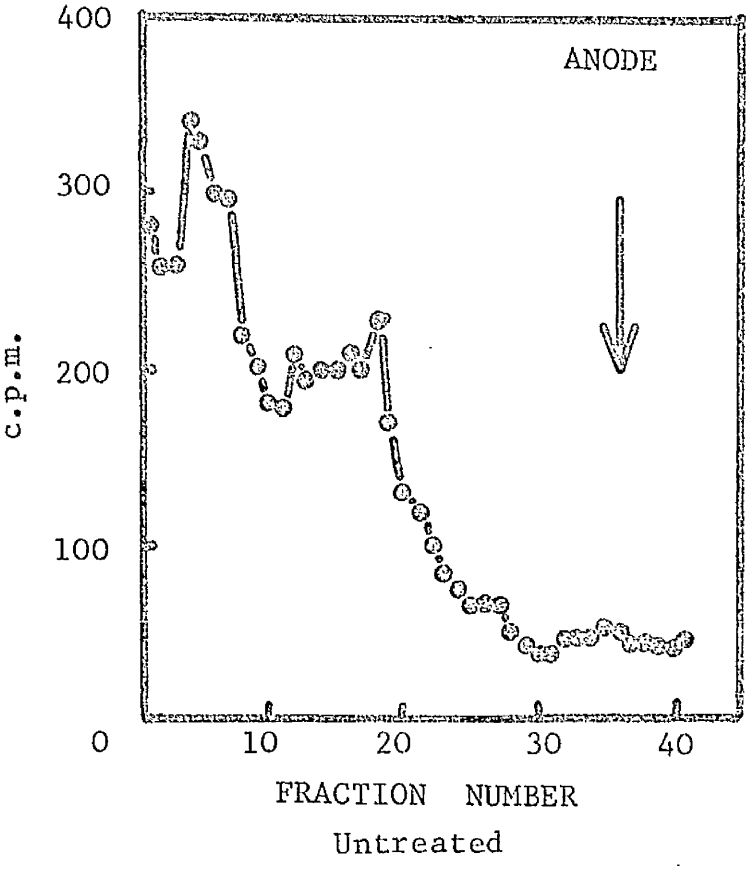


Untreated

(b) Mock-infected cells



NaF-treated



Untreated

in infected BHK and mouse embryo cells were not continued: instead, the possibility that BHK cells may lack cellular functions required for polyoma viral DNA replication was investigated.

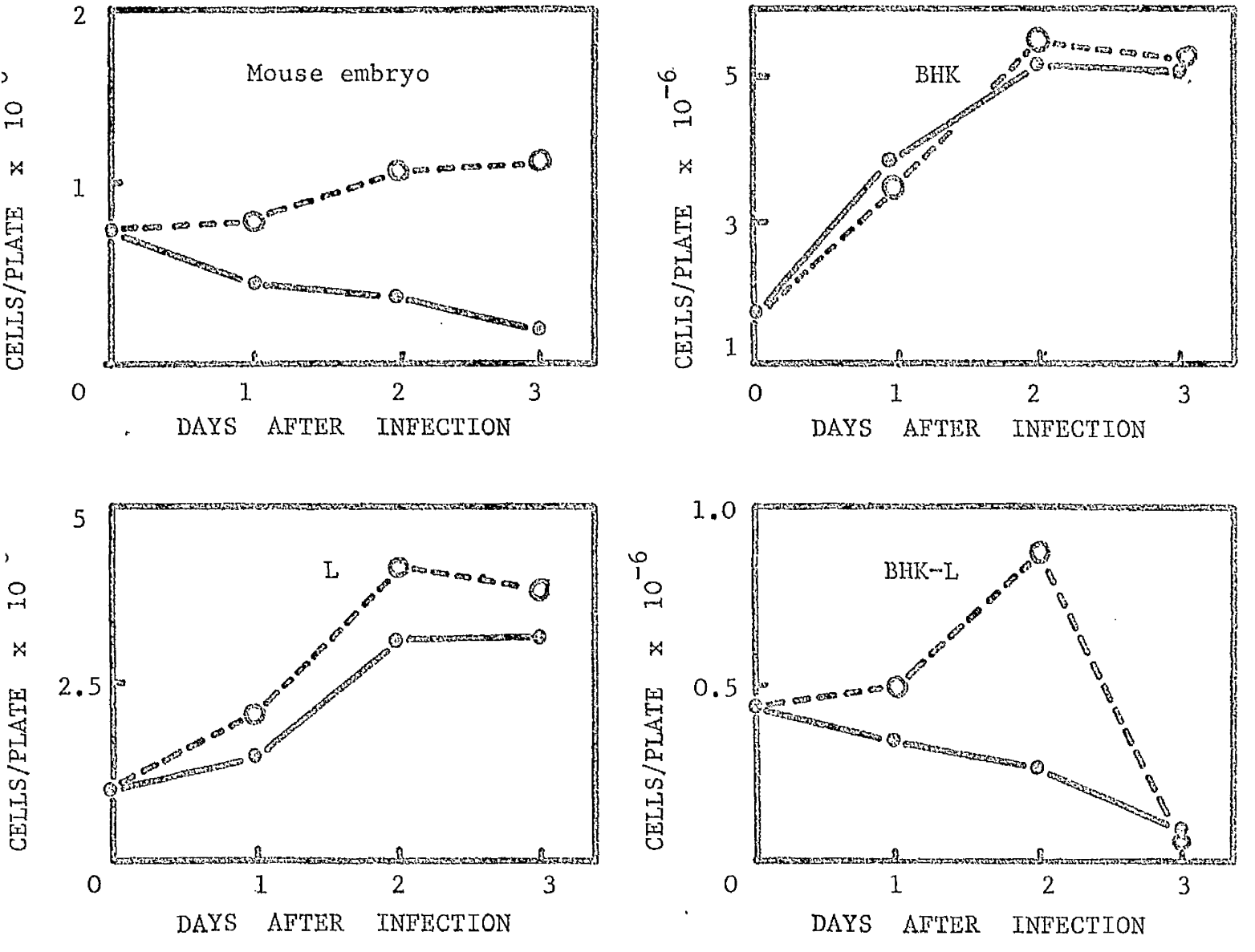
6. The possible requirement of cellular functions for viral DNA replication

Polyoma viral DNA replication could not be detected in viral-infected stationary BHK cells (Results, section B 3). A block in viral DNA replication in these cells may be due to a deficiency or complete absence of some necessary cellular functions which are present in mouse embryo cells. Viral DNA replication might be possible in BHK cells if these necessary mouse cell functions were made available. Hybrid BHK-mouse cells, formed by fusion in the presence of inactivated sendai virus, provided a system for examining this possibility.

A BHK-L hybrid cell line was obtained by the fusion of BHK cells and L cells, a line derived from mouse fibroblasts. The hybrid cells had fibroblast-like morphology similar to that of BHK cells but quite distinct from the more epithelial-like morphology of L cells.

The effect of polyoma viral infection on the growth of BHK-L, BHK, L and mouse embryo cells is shown in Fig. 29. These cells were grown in EC 10% and infected at a viral multiplicity of 100 PFU/cell or mock-infected. Mock-infected BHK, L and mouse embryo cells grew to confluence by 2 days. Mock infected BHK-L cells grew with considerable acid

Effect of polyoma viral infection on cell growth



0.75×10^6 cells in EC 10% were plated in 50mm plates. After 24h the cells were infected with polyoma virus (100 PFU/cell) or mock-infected. On subsequent days the cell number of infected cells (⊙) and mock-infected cells (○) was determined using a Coulter Counter. Five separate counts of at least 200 cells were made for each of duplicate plates and the average obtained.

production during the first and second days and during the third day about 90% of cells detached. In this tendancy to detach under acid conditions, BHK-L cells resembled BHK cells (Results, section A 4) but detachment was much more pronounced in the case of BHK-L cells, presumably because the acid production was greater.

Polyoma viral infection of mouse embryo cells resulted in a cytopathic effect as early as 24h after infection. No cell growth could be detected and by 3 days after infection the cell sheet was beginning to detach. Viral infection had little effect on the growth of BHK and L cells, but had a dramatic effect on the growth of BHK-L cells. There was little acid production in infected BHK-L cells, in contrast to mock-infected cells, and an acute cytopathic effect was observed in accord with the observed decrease in cell number.

These observations indicated that hybrid BHK-L cells, differed from both parental cell strains, BHK and L, in showing a response to polyoma viral infection that was characteristic of the permissive mouse embryo cells.

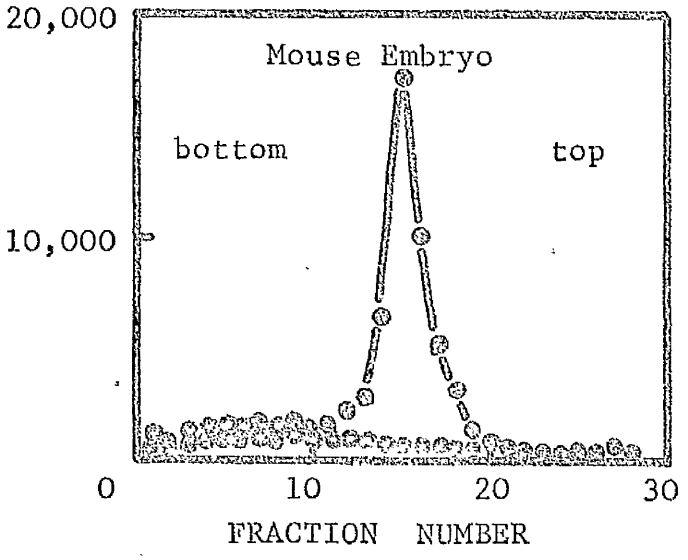
Mouse embryo, BHK, L and BHK-L cells, grown in EC 10% were viral-infected and labelled with [^{3}H]thymidine. After Hirt extraction [^{3}H]viral DNA was assayed by sedimentation velocity centrifugation through neutral CsCl (Fig. 30). The Hirt extraction method was found to be much less effective in quantitatively removing BHK and BHK-L cellular DNA. Therefore, although it is apparent that viral DNA was synthesised in infected mouse embryo cells, the interpretation of the other radioactivity

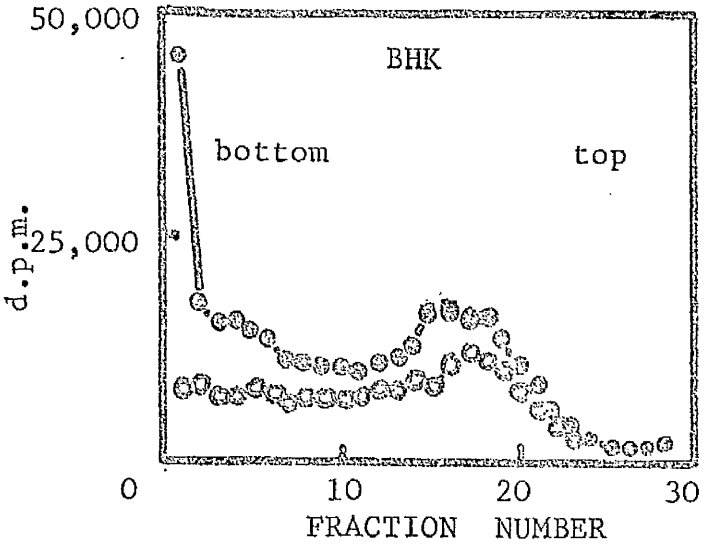
Fig. 30

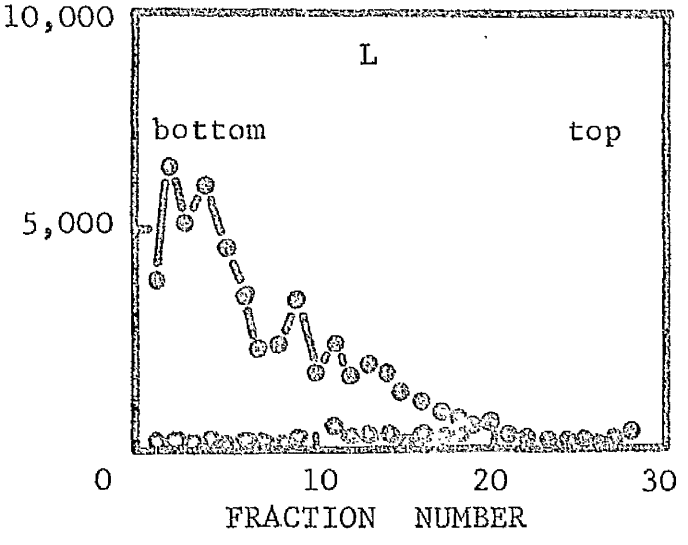
Polyoma viral infection of mouse embryo, BHK, L and BHK-L cells: viral DNA synthesis

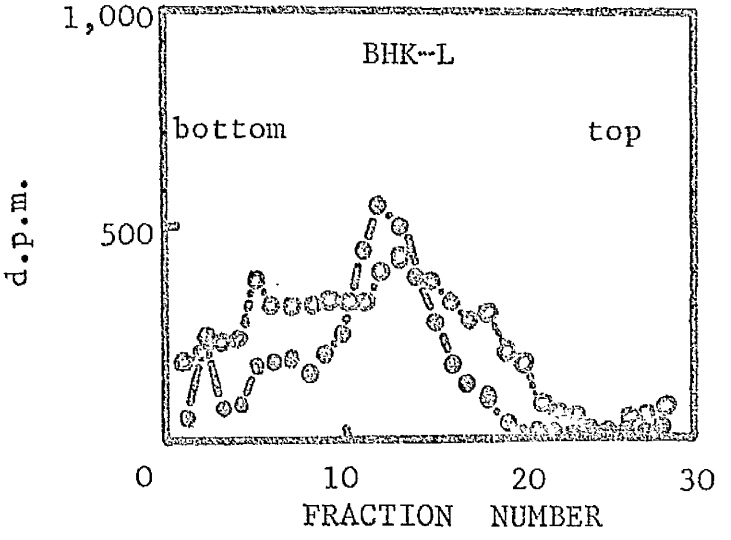
2×10^6 cells in EC 10% were plated in 90mm plates. 24h later the cells were infected with polyoma virus (100 PFU/cell) or mock-infected and pulsed with [^{3}H]thymidine (2.5×10^{-7}M; 1μCi/ml) between 24 and 48h after infection. At the end of the pulse the cells were lysed with SDS and Hirt-extracted (Methods, section 10 (b)). A 0.2ml aliquot of the Hirt supernatant was centrifuged through 3ml of CsCl (sp.gr.=1.5; pH7.5) at 100,000g for 3.5h. Fractions were collected from the bottom of the tubes and the distribution of acid-insoluble radioactivity determined (Methods, section 10 (a)).

—O— Infected cells —○— Mock-infected cells


20,000
10,000
0
Mouse Embryo
bottom
top
10
20
30
FRACTION NUMBER


50,000
25,000
0
d.p.m.
BHK
bottom
top
10
20
30
FRACTION NUMBER

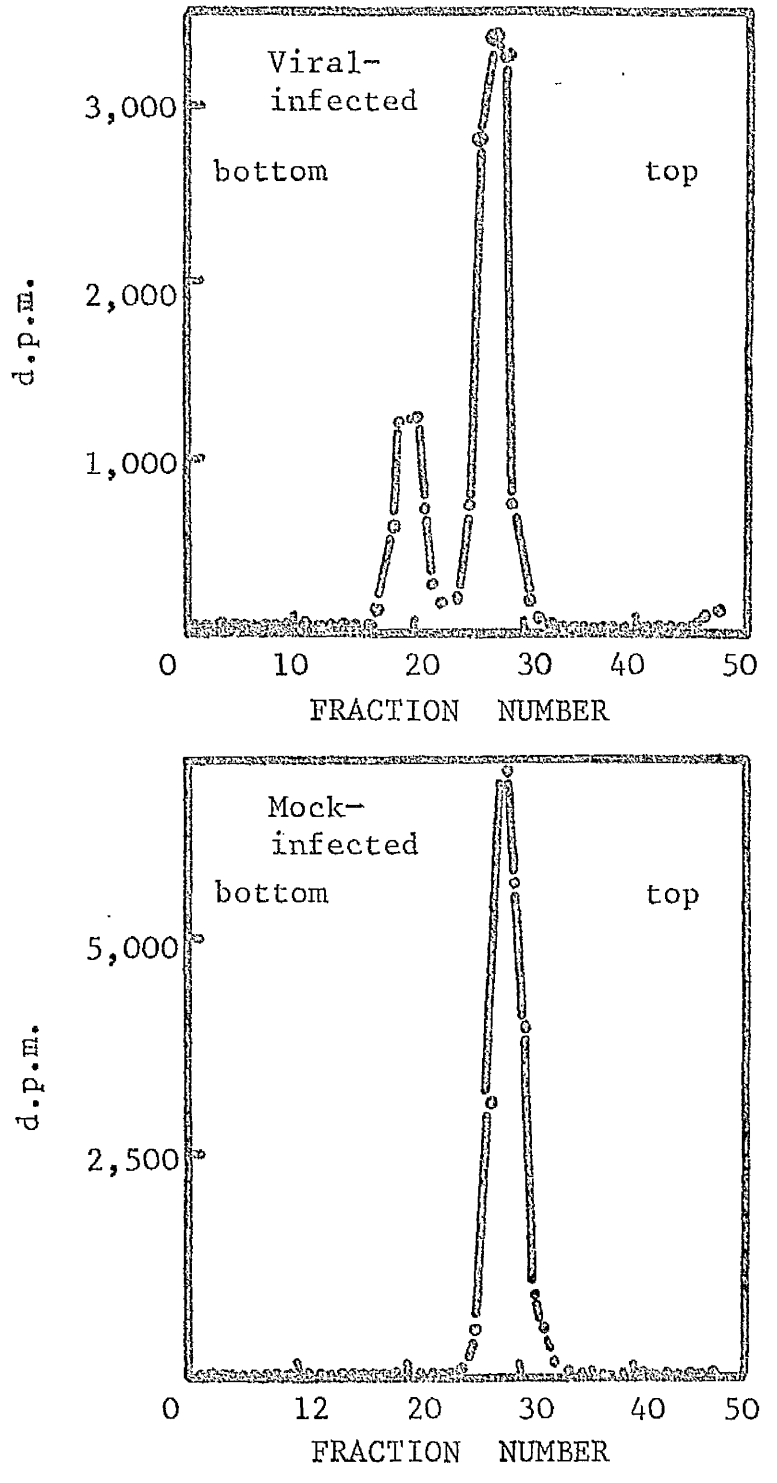

10,000
5,000
0
L
bottom
top
10
20
30
FRACTION NUMBER


1,000
500
0
d.p.m.
BHK--L
bottom
top
10
20
30
FRACTION NUMBER

profiles is more difficult, due to high background radioactivity. In the case of BHK-L cells, however, there was a well-defined peak of radioactivity in the position of viral DNA. Viral DNA synthesis could not be detected in either infected BHK or infected L cells.

The Hirt supernatants from infected and mock-infected BHK-L cells were analysed by equilibrium centrifugation in CsCl containing ethidium bromide (Fig. 31). The material from mock-infected cells gave one band of radioactivity due to cellular DNA while that from infected cells gave two bands, the minor heavy band being characteristic of component I polyoma viral DNA. [^{3}H]viral DNA synthesis in BHK-L cells represented 3% of that in mouse embryo cells.

Briefly summarising these studies, viral DNA synthesis could not be detected in BHK or L cells but was detected in hybrid cells, obtained by the fusion of these cells. The hybrid cell may be thought of as a BHK cell which has acquired mouse cellular functions; these functions then allow the BHK cell to synthesise viral DNA after infection.



0.5ml of the Hirt supernatants from the infected or mock-infected BHK-L cells, described in the legend for Fig. 30, were assayed for component I viral DNA by equilibrium centrifugation in ethidium bromide-CsCl (Methods, section 10 (a)).

V. DISCUSSION

The effect of polyoma viral infection on cellular functions

As described in the Introduction the infection of permissive cells with polyoma virus results in the activation of the cellular DNA synthesising system prior to viral DNA replication. In the case of stationary mouse embryo cells there was an induction of cellular DNA synthesis and also an increase in the activities of DNA polymerase and thymidine kinase after infection with polyoma virus (Results, section B 1 & 2).

The induction of cellular DNA synthesis in polyoma virus or SV40 infected non-permissive cells has been studied less throoughly. Infection of 3T3 cells with SV40 at a multiplicity of 30 PFU/cell did not result in stimulation of DNA synthesis (Todaro & Green, 1966b). However a stimulation was reported in SV40-infected 3T3 cells at high virus multiplicities (Gershon et al. 1966; Henry et al. 1966; Kit et al. 1967). Polyoma viral-infection of rat kidney cells (Winocour et al. 1965), rat embryo cells (Gershon et al. 1965; Sheinin, 1966c) and hamster embryo cells (Weil et al. 1965) gave only poor stimulation of cellular DNA synthesis at high virus multiplicities.

The development of a stationary BHK cell system, described here (Results, Section A), provided an excellent system for investigating the effect of polyoma viral infection on cellular DNA synthesis and enzyme activities in the non-permissive BHK cells. There was no detectable induction of cellular DNA synthesis (Results, Section B 1) and no significant increase in the activities of DNA polymerase and thymidine

kinase (Results, section B 2) after infection of stationary BHK cells at a viral multiplicity of 50 PFU/cell. Under these conditions the majority of viral-infected stationary mouse embryo cells were stimulated to synthesise DNA.

Induction of cellular functions in permissive cells may be necessary for viral replication because of the small number of functions coded for by SV40 and polyoma viral DNA. It is perhaps significant that the larger DNA viruses, such as herpesviruses and poxviruses, shut off rather than stimulate cellular DNA synthesis (Kaplan & Ben-Porat, 1968). Presumably these viruses bring many more functions into the cell than do the smaller DNA viruses, and therefore do not rely on cellular functions, for example cellular enzymes, to the same extent.

In non-permissive cells infected with polyoma virus or SV40 an induction of cellular DNA synthesis may facilitate the transformation process. As has been shown for the SV40-3T3 system (Todaro & Green, 1966b, 1967), cellular DNA synthesis is probably necessary for the insertion of the viral genome into cellular DNA. Recently it has been demonstrated that in SV40-infected 3T3 cells the transformation rate is greater for cells in which there is an induction of cellular DNA synthesis than for cells in which cellular DNA synthesis is not induced (Fox & Levine, 1971).

Two of the factors which can affect DNA synthesis and cell growth in monolayer culture are cell density and serum in the medium (Clarke, Stoker, Ludlow & Thornton, 1970; Dulbecco, 1970b; Dulbecco & Stoker, 1970).

Dulbecco (1970b) devised an elegant method for determining the separate contribution of each of these factors to DNA synthesis and derived the parameters Topoinhibition (TI) and Wound Serum Requirement (WSR). TI is a measure of the inhibition of DNA synthesis, due to interaction between cells at high cell densities. WSR, on the other hand, is a measure of the true serum requirement of individual cells for DNA synthesis in the absence of topoinhibition (i.e. at low cell densities).

Dulbecco (1970b) has suggested that serum has at least three functions apart from having a direct role in DNA synthesis as measured by WSR; these are for cell survival, for counteracting topoinhibition in certain systems and for mitosis. Different factors in serum may be responsible for these different functions (Bürk, 1966; Jainchill & Todaro, 1970; Holley & Kiernan, 1971; Paul, Lipton & Klinger, 1971).

Cells transformed by polyoma virus or SV40 have a reduced WSR and TI compared to normal cells (Dulbecco, 1970b) consistent with the observation that at a given serum concentration transformed cells grow more rapidly and to higher cell densities than normal cells. BHK cells transformed by the temperature-sensitive ts-3 mutant of polyoma virus become subject to topoinhibition when the cells are transferred to the non-permissive temperature (Dulbecco & Eckhart, 1970). These cells show enhanced agglutination by plant agglutinins at the permissive temperature but not at the non-permissive temperature (Eckhart et al. 1971). These results indicate that in transformed cells the release from topoinhibition and the surface alteration, measured by enhanced agglutination properties are connected and mediated by a viral gene.

The mechanism of topoinhibition is not understood (Dulbecco & Stoker, 1970) but conceivably it is the result of interaction between the surfaces of neighbouring cells. Even a minor modification of the cell surface might be expected to have a considerable effect on this control mechanism.

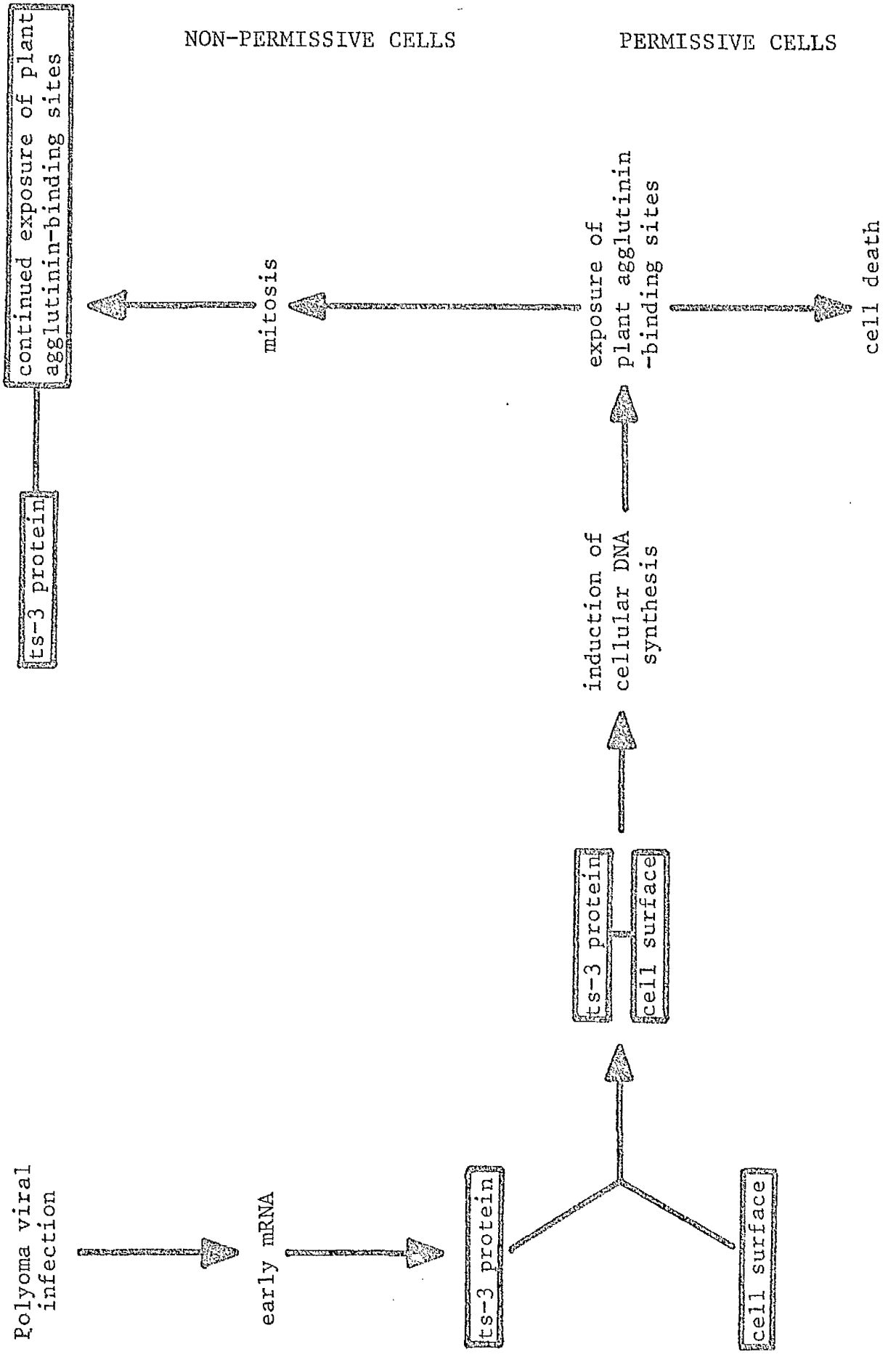
Infection of 3T3 cells with polyoma virus results in a surface modification characterised by the exposure of sites which will bind plant agglutinins (Benjamin & Burger, 1970; Eckhart et al. 1971). However the surface modification, associated with enhanced agglutinability, appears to require, rather than cause, the induction of cellular DNA synthesis in this system (see Introduction). Exposure of plant agglutinin sites only occurs during mitosis in normal cells (Fox et al. 1971) and the exposure of these sites in viral-infected permissive cells may thus be characteristic of the potentially mitotic state of those cells after cellular DNA synthesis.

The ts-3 protein appears to have two functions; one is the induction of cellular DNA synthesis and the other is maintaining the exposure of plant agglutinin-binding sites. A simple model which attempts to explain the mechanism of cellular DNA induction and the exposure of plant agglutinin-binding sites is shown in Fig. 32.

According to this model the ts-3 protein is expressed early in infection and interacts with the cell surface causing a minor rearrangement sufficient to release the cell from topoinhibition and possibly reduce the dependence of the cell on serum. The net effect of this surface

Fig. 32

The induction of cellular DNA synthesis and the exposure of plant agglutinin-binding sites after infection of cells with polyoma virus: a proposed model



rearrangement is to induce the cell to synthesise DNA. Exposure of plant agglutinin-binding sites then follows as the normal consequence of DNA synthesis as in normal growing cells. In normal cells the plant agglutinin-binding sites are no longer exposed after mitosis. However in abortively transformed and transformed cells these sites remain exposed and it is suggested that the cell surface is prevented from returning to the normal conformation by the ts-3 protein. Different sites on the ts-3 protein may be responsible for the initial interaction with the cell surface and for the locking of the exposure of plant agglutinin-binding sites. This may explain the uncoupling of the induction of cellular DNA synthesis and plant agglutinin-binding site exposure which occurs after infection with the NG-18 mutant of polyoma virus (see p. 26).

The model is able to account for the observation that the continuous functioning of the ts-3 protein is required for the expression of the transformed cell phenotype (Dulbecco & Eckhart, 1970; Eckhart et al. 1971). In permanently transformed cells it seems likely that integration of viral genes in the cellular DNA ensures the continued synthesis of this protein.

The temperature-sensitive ts-a mutant of polyoma virus is unable to initiate stable transformation of BHK cells at the non-permissive temperature (Fried, 1965) presumably because the ts-a function is required for the integration of the viral genome into cellular DNA. This mutant, however, is able to cause abortive transformation at the

non-permissive temperature (Stoker & Dulbecco, 1969). This illustrates that the initial expression of the transformed cell phenotype does not require stable insertion of the viral DNA in the host cell chromosomes. This, also, is in agreement with the model described above.

The failure of polyoma viral infection to induce cellular DNA synthesis in stationary BHK cells maintained in EC 1% for five days (Results, Section B 1) may be due to the exhaustion of a serum factor necessary for the induction of DNA synthesis to occur. The requirement of a serum factor for the abortive transformation of BHK cells would explain the difference between these results and those of Stoker (1968), where BHK cells were maintained in a stationary state in suspension culture in medium containing 10% serum. BHK cells require to be attached to a surface for growth to occur. After polyoma viral infection the ts-3 protein may interact with the cell surface according to the model described earlier. This interaction may abolish the requirement of the cell for surface attachment and in medium containing 10% serum DNA synthesis would be initiated and the infected cells would undergo the few cell divisions characteristic of abortive transformation.

The more recent work of Taylor-Papadimitriou, Stoker & Riddle (1971) shows that abortive transformation of serum-depleted BHK cells takes place at high virus multiplicities. This suggests that, if the serum factors were depleted to the same extent in both systems, the

induction of DNA synthesis in polyoma viral-infected stationary BHK cells is a much less efficient process than in infected stationary mouse embryo cells. At a virus multiplicity of 50 PFU/cell the majority of infected stationary mouse embryo cells are induced to synthesise DNA (Results, Section B 1). In contrast, even at a virus multiplicity of 1,000 PFU/cell less than 40% of infected BHK cells exhibited an induction of DNA synthesis (Taylor-Papadimitriou et al. 1971). Similarly the induction of cellular DNA synthesis after SV40 infection is much less efficient in non-permissive cells than in permissive cells. This may be a consequence of restricted expression of the viral genome in non-permissive cells prior to integration of viral DNA.

Transcription and Translation of polyoma viral DNA in infected and transformed cells

In permissive cells infected with polyoma virus the entire genome is transcribed (Martin & Axelrod, 1969b). It seems likely that only a fraction of the genome is transcribed early after infection and that at late times transcription is complete as has been shown for SV40-infected permissive cells (see Introduction). Early viral mRNA is responsible presumably for the synthesis of early viral proteins such as the T-antigen and the protein involved in the induction of the cellular DNA synthesising system.

The synthesis of late viral proteins appears to have a requirement for DNA synthesis. Inhibitors of DNA synthesis do not interfere with the synthesis of the T-antigen but block the synthesis of viral capsid protein and mature virions in permissive cells infected with polyoma virus (Consigli, Minocha & Aboahmed, 1968) or SV40- (Butel & Rapp, 1965; Kit, Dubbs, Frearson & Melnick, 1966b). Recently it has been shown that arabinofuranosyl-cytosine (ara-c), an inhibitor of DNA synthesis, prevents the synthesis of late viral mRNA sequences in SV40-infected permissive cells (Sauer, 1971).

It is apparent therefore that the expression of the viral genome in polyoma virus or SV40-infected permissive cells is controlled at the transcriptional level. However the mechanism which restricts transcription prior to viral DNA replication and subsequently allows complete transcription is not understood. Viral DNA is probably transcribed by the DNA-dependent RNA polymerase of the host cell. It is possible that late viral mRNA sequences are synthesised only after the specificity of this enzyme has been altered, for example by a σ factor (Travers, 1970) which may be either viral-coded or viral-induced. Alternatively, integration of viral DNA in the host cell DNA may be necessary before late viral mRNA sequences can be synthesised.

Viral DNA synthesis may be a requirement for transcription of late viral mRNA because mouse embryo cells infected with the ts-a mutant of polyoma virus are unable to synthesise capsid protein at the non-permissive temperature (di Mayorca et al. 1969). Under these conditions

viral DNA is not synthesised but cellular DNA synthesis is induced (Fried, 1970). In this connection Sauer (1971) has suggested that only replicative intermediates of viral DNA may serve as templates for the transcription of late viral mRNA, the rationale being that certain initiation sites for the DNA-dependent RNA polymerase might only become available during viral DNA replication.

The viral mRNA synthesised in some SV40-transformed cell lines contains early and some late viral mRNA sequences (Aloni et al. 1968; Oda & Dulbecco, 1968; Sauer & Kidwai, 1968; Martin & Axelrod, 1969a). Sauer (1971) found that ara-c did not inhibit the synthesis of late viral mRNA sequences in SV40-transformed cells. This contrasts with the requirement of DNA synthesis for synthesis of late viral mRNA in SV40-infected permissive cells (Sauer, 1971). The control of transcription of viral DNA therefore appears to be different in transformed cells and viral-infected permissive cells. This may be due to the integrated state of viral DNA in transformed cells.

Some SV40-transformed cell lines yield infectious virus when fused with permissive cells (Gerber, 1966; Koprowski et al. 1967; Watkins & Dulbecco, 1967) indicating that these transformed cells contain the complete viral genome. Late functions are apparently not expressed in such SV40-transformed cells, however, and this is apparently due to either transcriptional or translational control or both (Martin & Axelrod, 1969a). In contrast in polyoma viral-infected or SV40-infected permissive cells, the expression of the viral genome appears to be

controlled mainly at the level of transcription.

Little is known about the expression of viral DNA in polyoma viral-infected or SV40-infected non-permissive cells. The best evidence that polyoma viral DNA is at least partly expressed in infected BHK cells is the phenomenon of abortive transformation (Stoker, 1968). This process certainly requires the synthesis of at least one polyoma viral protein. The study of the transcription and translation of polyoma viral DNA in infected stationary BHK cells described here is therefore of interest.

Using the DNA-RNA hybridisation technique it was found that the complete viral genome is apparently transcribed in polyoma viral-infected BHK cells (Results, Section B 4). This would indicate that the block to polyoma virus replication in BHK cells is not the result of a failure to synthesis viral mRNA. It should be remembered however that while hybridisation techniques permit a comparison of polynucleotide sequences, minor differences might not be detectable. Therefore, although the polyoma viral mRNA sequences synthesised in infected BHK and mouse embryo cells were identical by this technique, there remains the possibility that the viral mRNA made in infected BHK cells may lack a few RNA sequences, essential for the initiation of viral protein synthesis.

This problem would be resolved by comparing the viral proteins synthesised in polyoma viral-infected BHK and mouse embryo cells (Results, Section B 5). It seems likely that the full complement of

polyoma viral-coded proteins is synthesised during the productive infection of mouse embryo cells. Comparison of these proteins with the viral proteins synthesised in infected BHK cells would indicate whether there is a translational block in the expression of the polyoma viral genome in infected BHK cells. Smith et al. (1970) used a method employing release from NaF inhibition of protein synthesis to specifically label viral proteins synthesised in encephalomyocarditis viral-infected Krebs ascites tumour cells. After release from the NaF block, synthesis of EMC viral proteins began prior to cellular protein synthesis and, therefore, could be specifically labelled with [^{35}S]methionine This technique offered the possibility of solving the problem of viral protein synthesis in polyoma viral-infected BHK cells and was therefore investigated.

Initial experiments showed that a burst of protein synthesis occurred in viral-infected but not in mock-infected mouse embryo cells after release from NaF block (Fig. 27) as in the EMC virus system. However, analysis by SDS-polyacrylamide gel electrophoresis of the proteins synthesised during this burst period indicated that they contained a heterogeneous collection of proteins (Fig. 28). If viral proteins alone had been synthesised a small number of distinct bands of radioactivity might have been expected. The results obtained suggested that cellular proteins were being synthesised during the period immediately after release from the NaF block, in contrast to the results obtained with the EMC virus system.

The extent of synthesis of polyoma viral proteins in infected BHK cells remains unresolved. However, the expression of the viral genome in these cells does not appear to be impaired at the level of transcription. The detection of capsid antigen synthesis by immunofluorescence in a small proportion of BHK cells after infection with polyoma virus at high input multiplicities (Fraser & Crawford, 1965) indicated that synthesis of late viral proteins is possible in this system. That synthesis of capsid antigen be confined to only a small proportion of the infected cell population, however, suggests that perhaps the translation of polyoma viral mRNA sequences on BHK cell ribosomes is very restricted. In polyoma viral-transformed BHK cells, where at least a section of the viral DNA is probably integrated into the cellular DNA, viral mRNA sequences may be associated with cellular mRNA sequences which then may facilitate efficient translation of viral proteins on BHK cell ribosomes.

Polyoma viral DNA replication in infected cells

Synthesis of polyoma viral DNA could not be detected in viral-infected stationary BHK cells by assaying for [^{3}H]thymidine-labelled progeny viral DNA (Results, section B 3). The sensitivity of the method of assay was such that if 1% of the [^{3}H]viral DNA synthesised in infected mouse embryo cells had been synthesised in infected BHK cells it would have been detected. Similarly, viral DNA replication

could not be detected in growing cultures of BHK cells infected with polyoma virus (Fig. 30). The failure of viral-infected BHK cells to synthesise detectable amounts of progeny open or closed cyclic duplex viral DNA suggests that viral DNA replication takes place only to a very limited extent or not at all in these cells.

A possibility that cannot be overlooked is that polyoma viral DNA may replicate in BHK cells by a different mechanism from that in mouse cells. If, for example, viral DNA replicated by a rolling circle-type mechanism (Fig. 3) and the maturation of unit-size molecules was blocked, then the methods employed would not have allowed detection of such multimeric, non-supercoiled viral DNA molecules. This unlikely possibility could be tested by DNA-DNA or DNA-RNA hybridisation methods as could the possibility, mentioned earlier, that replication by the usual mechanism takes place only to a limited extent in infected BHK cells. It is now possible to detect less than one molecule of viral DNA per cell by the DNA-DNA hybridisation technique (Gelb et al. 1971).

Replication of viral DNA in infected BHK cells, even to a limited extent, might be expected to facilitate the transformation process in that a greater number of viral DNA molecules would be made available for integration into the cellular DNA. However as was discussed in the last section, viral DNA replication in permissive cells may be responsible for the initiation of the late events culminating in cell death. A block to viral DNA replication in

infected BHK cells may ensure their survival. From the work described here it would appear that the position of such a block is either at the conversion of parental closed cyclic duplex viral DNA to give open cyclic duplexes or at the polymerisation step which should yield progeny open cyclic duplexes (see Fig. 4). No attempt was made to distinguish between these two possibilities because of the difficulty in interpreting the results of experiments designed to assay the ring-opening of parental viral DNA molecules. For example, Barbanti-Brodano et al. (1970) reported that ring-opening of parental viral DNA took place in the nucleus of permissive cells infected with SV40, the DNA of which was radioactively-labelled. However, this may represent non-specific endonuclease action in the nucleus of infected cells unrelated to viral DNA replication. Moreover, perhaps much less than 1% of the viral DNA molecules entering a nucleus may take part in viral DNA replication and the fate of these few molecules could not be followed unambiguously using such a method.

Having found an apparent block to viral DNA replication in viral-infected BHK cells it was of interest to investigate the cause of the block. The most likely mechanism of polyoma viral DNA replication (Fig. 4) would indicate a requirement for at least three enzymes: an endonuclease, a polymerisation enzyme and a ligase. In addition non-enzymic functions, possibly proteins, may have a structural rôle in viral DNA replication. Some of these functions, including the enzymes may be viral-coded and the others of cellular origin.

Three possible reasons why polyoma viral DNA replication may be blocked in viral-infected BHK cells are:-

(a) Failure to synthesise a necessary viral-coded protein.

(b) Deficiency or absence of a necessary cellular function.

(c) Presence of a cellular inhibitor.

Viral-infected BHK cells may fail to synthesise a viral-coded protein necessary for viral DNA replication. The DNA-RNA hybridisation experiments discussed in the last section suggested that such a failure does not arise from restricted transcription. However the poor induction of cellular DNA synthesis in this system (Results, section B 1) and the absence of detectable capsid antigen synthesis in most infected BHK cells may be an indication of the inefficient translation of viral-coded proteins.

The presence or absence of certain cellular functions may ultimately be responsible for the non-permissive state of BHK cells. Polyoma viral infection of L cells appears to give rise to a latent interaction in which there is little or no cytopathic effect and only occasional synthesis of virus after persistent infection (Hare, Balduzzi & Morgan, 1963; Henle, Hinze & Henle, 1963). Viral DNA synthesis could not be detected after polyoma viral infection of growing cultures of either BHK or L cells. A limited amount of viral DNA synthesis was detected, however, after viral infection of BHK-L hybrid cells, formed by fusion of BHK and L cells in the presence of inactivated sendai virus. (Results, section B 6).

This indicates that there is complementation of BHK and L cellular functions in the hybrid BHK-L cell which then acquires the ability to synthesise viral DNA. This would tend to rule out the possibility that BHK cells contain an inhibitor of viral DNA replication, but would support the idea that BHK cells lack a cellular function essential for viral DNA replication. A similar conclusion followed studies on the permissiveness for polyoma viral DNA replication of 3T3-BHK hybrid cells containing various mouse: hamster chromosome ratios (Basilico, Matsuya & Green, 1970).

The BHK-L hybrid cell may be regarded as a BHK cell with acquired L cell functions which confer on the BHK cell the ability to support viral DNA replication. The nature of these functions is not known and there are many possibilities. It was mentioned earlier that polyoma viral mRNA may not be utilised on BHK cell ribosomes. The presence of L cell ribosomes in the hybrid may permit the synthesis of an essential viral protein required for viral DNA replication which could then proceed normally. Alternatively viral DNA replication may require specific sites only present perhaps in the nuclear membrane of mouse cells and these might be present in L cells and in BHK-L hybrid cells. It is possible that BHK cells may only lack a single essential function such as a structural protein or an enzyme involved in viral DNA replication.

Conclusion

The findings reported here support the idea that BHK cells are non-permissive for polyoma virus replication.

Infection of stationary BHK cells with polyoma virus did not result in a stimulation of the cellular DNA synthesising system; this contrasted with the stimulation that followed infection of stationary mouse embryo cells.

Replication of viral DNA could not be detected in stationary or growing BHK cells after infection with polyoma virus. It is concluded that the block in replication is either at the ring-opening or polymerisation steps (p. 19).

The non-permissive nature of BHK cells is probably due to a deficiency of some cellular function(s), present in mouse cells, which is necessary for the replication of polyoma virus.

VI. SUMMARY

1. The physical and chemical properties of polyoma virus are described. The literature concerning the interaction of the virus with cells, permissive and non-permissive to virus replication, is reviewed.

2. A method has been developed for maintaining BHK21/13 cells (BHK cells) in a stationary condition; this involves the use of medium containing 1% calf serum. Stationary BHK cells obtained by this method had low levels of DNA synthesis, DNA polymerase activity and thymidine kinase activity.

3. No significant increase in DNA synthesis, or the activities of DNA polymerase and thymidine kinase followed the infection of stationary BHK cells with polyoma virus at an input multiplicity of 50 PFU/cell. In contrast after viral infection of stationary mouse embryo cells under the same conditions there was a 38-fold stimulation of DNA synthesis and 7 and 3.5-fold increases in the activities of DNA polymerase and thymidine kinase respectively.

4. Replication of polyoma viral DNA could not be detected in infected stationary BHK cells. It is concluded that the position of the block in replication is either at the endonuclease-mediated conversion of parental closed cyclic duplex DNA to open cyclic duplex DNA or at the subsequent polymerisation step which should yield progeny open cyclic duplex molecules.

5. DNA-RNA hybridisation studies provided evidence that viral-specific RNA sequences were synthesised in stationary BHK cells infected with polyoma virus. Extrapolation of this data by means of a double-reciprocal plot indicated that the complete viral genome was transcribed.

6. An unsuccessful attempt was made to characterise the viral proteins synthesised in stationary BHK cells infected with polyoma virus.

7. Limited replication of polyoma viral DNA was detected after infection of growing cultures of hybrid cells formed by the fusion of BHK and L cells, although it was not detected after infection of either of the parental cell strains. Similarly, a cytopathic effect following infection with polyoma virus was observed in the case of the hybrid cells but not in the case of either of the parental cell strains.

8. The implications of these results with regard to the non-permissive nature of BHK cells is discussed. It is concluded that BHK cells are deficient in some cellular function(s) necessary for the replication of polyoma virus.

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