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INDUCTION OF ENZYMES IN MAMMALIAN CELLS INFECTED WITH HERPES SIMPLEX VIRUS

Ponnamperuma A. J. Perera, B. Sc.

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ABBREVIATIONS

The abbreviations used in this thesis are those recommended by the Editorial Board of the Biochemical Journal, published in Biochem. J. (1969) 112, 1. Given below is a list of abbreviations not included in the recommended list.

DNA polymerase	DNA nucleotidyl transferase (E. C. 2. 7. 7. 7)
DNase	deoxyribonuclease
dCyd kinase	ATP: deoxycytine 5'- phosphotransferase
d Thd kinase	ATP: thymidine 5'-phosphotransferase (E. C. 2. 7. 1. 21)
dAdo kinase	ATP: deoxyadenosine 5'-phosphotransferase
dGuo kinase	ATP: deoxyguanosine 5'-phosphotransferase
dTMP kinase	ATP: deoxy-thymidinemonophosphate phosphotransferase
dCMP kinase	ATP: deoxy-CMP phosphotransferase (E. C. 2. 7. 4. 5)
dCMP deaminase	deoxy-CMP aminohydrolase
FdUrd, BrdUrd	fluoro deoxyuridine, bromo deoxyuridine
BES	NN-Bis-(2-hydroxyethyl)-2-amino ethane sulphonic acid
PCA	perchloric acid
TCA	trichloroacetic acid

SDS	sodium dodecyl sulphate
BHK-21/C-13 cells	a strain of cultured cells derived from baby hamster kidney (MacPherson and Stoker, 1962)
HEp-2 cells	human epithelioid carcinoma, No. 2
P. F. U. / cell	plaque forming units/cell, a unit of infectivity

CONTENTS

SUMMARY

Herpes simplex virus and BHK cells provided an excellent system for the study of virus-host cell interactions. The main emphasis of this work was laid on the induction of new enzymes after virus infection. During this investigation, evidence for the induction of a new deoxycytidine kinase was obtained, while there were indications of an isoenzyme. This is the first instance of a virus-induced deocycytidine kinase that has been reported.

The principal evidence which led to the above conclusion came from studies on inhibition and heat stability of host and virus-induced deoxycytidine kinases. Deoxycytidine triphosphate at 2×10^{-5}M strongly inhibited host cell deoxycytidine kinase but had very little effect on the virus-induced dCyd kinase. At a ten-fold higher concentration deoxycytidine triphosphate inhibited both host and virus-induced kinases. In contrast, deoxycytidine triphosphate at this concentration activated virus-induced deoxythymidine kinase whilst inhibiting the host deoxythymidine kinase. Deoxythymidine triphosphate was a potent inhibitor of both dCyd and deoxythymidine kinases. At 2×10^{-4}M, deoxythymidine triphosphate inhibited host deoxythymidine kinase and deoxycytidine kinases of control and infected cells but had only a small effect on the virus-induced dThd kinase. This suggested the induction of a new deoxycytidine kinase in BHK cells following

herpes infection, which was distinct from host and virus-induced deoxythymidine kinases and host deoxycytidine kinase.

Additional evidence for the induction of a new deoxycytidine kinase came from the heat sensitivity studies of the pyrimidine deoxyribonucleoside kinases. The substrate-stabilised profiles of these enzymes against heat inactivation were clearly different. Furthermore, mixing studies with control and virus-infected cell extracts proved the absence of a possible activator or an inhibitor in this system which increased the activity of host deoxycytidine kinase following herpes infection. The requirement for the synthesis of protein for the occurrence of this increase in activity came from studies with inhibitors of macromolecular synthesis. The virus-induced deoxycytidine kinase was resolved into two components during fractionation on DEAE-cellulose.

In order to understand the functions of the virus-induced pyrimidine deoxyribonucleoside kinases an investigation was carried out into the variation of acid-soluble pool size within the host cell following virus-infection. It was found that the uptake of pyrimidine deoxyribonucleoside precursors into the acid-soluble pool increased in a manner which paralleled the increase in activity of pyrimidine deoxyribonucleoside kinases. The discovery of an increased pool size within the infected cell suggested an alteration of host cell's

mechanism of controlling nucleotide synthesis and may explain the requirement for new virus-coded enzymes which are less susceptible to feedback inhibition.

The high levels of activity of purine deoxyribonucleoside kinases in the uninfected cell relative to the activity of the pyrimidine kinases and the strong inhibitory effect of deoxyadenosine triphosphate on host DNA synthesis perhaps provide satisfactory reasons for the absence of induction of purine deoxyribonucleoside kinases after herpes infection. The discovery of two possibly virus-coded DNA exonucleases and the high levels of deoxyribonucleoside kinases within the infected cell strongly suggested the control of host DNA synthesis and the regulation of the production of viral progeny DNA through the co-ordination of virus-induced enzymes.

There was a progressive incorporation of labelled host deoxyribonucleotides into four buoyant density bands of DNA in addition to double-stranded herpes DNA, following infection of BHK cells by herpes virus. These appeared simultaneously with the production of progeny virus. One has been tentatively identified as being single-stranded viral DNA. The others have very high buoyant densities and may represent DNA-RNA hybrids. These putative hybrids may represent transcription of late functions of herpes virus. It is likely that cut-off of early virus-induced enzymes which is recognised to be one of the late viral functions may be mediated by the synthesis of such late viral RNA.

INTRODUCTION

1. EVOLUTION

1.1. The Origin of Life

The theory of evolution by natural selection was proposed by Charles Darwin in his book entitled "The Origin of Species", in 1859. Since then, the concept of evolutionary change has been substantially verified by studies on fossils and the inherited characteristics of organisms. In recent years, there has been increasing interest in the problems of the origin of life. With the advent of the idea of a reducing primordial atmosphere (Oparin, 1938) containing ammonia, water vapour and methane and the experimental findings of Miller (1953) and Ponnamperuma et al. (1963), the feasibility of the synthesis of polypeptides and polynucleotides under pre-biological conditions became apparent. Oparin pointed out that colloidal protein molecules tend to form aggregates called coacervate droplets. These droplets have a marked tendency to adsorb and incorporate various substances from the surrounding solution. If we accept the coacervate droplet as a model for the primitive cell, then its formation may be envisaged through a process of molecular aggregation of polypeptides, polynucleotides and carbohydrates followed by encapsulation of these within a lipid membrane (Bernal, 1967). Reproduction in this primitive system could have taken place

by the physical fragmentation of large droplets formed by harmonious interactions within the cell. As the available nutrient material was used up only cells possessing efficient machinery which could utilise modified substrates survived. This process of evolution of synthetic ability may have continued until cells could make all essential components.

1.2. Pro- and Eukaryotic Cells

Organisms have been divided into prokaryotes and eukaryotes according to their intra-cellular structures (Bernal, 1967). The former type has a single chromosome, without the distinct nucleus, and elaborate mitotic mechanism which characterizes the other, more complex type of cell, the eukaryotes. In the eukaryotes, the DNA molecules are associated with basic proteins to form several highly organised chromosomes.

A detailed analysis of the DNA from different organisms indicates that the variation in base composition and the nearest neighbour base frequency is relatively small in eukaryotes and very large in prokaryotes (McCarthy, 1965). The evolution of DNA is most noticeable in micro-organisms, since they can rapidly multiply to build up large populations. Bacteria, for example, exhibit a wide range of base compositions, although within a given species the base composition is more homogeneous (Marmur, Falkow and Mandel, 1963).

On comparison of different bacterial species one can arrive at the following generalisations.

1. The composition of different bacteria is similar: ribosomes, t-RNA and membranes are similar in properties; cell walls vary but have many common features.

2. The basic metabolic pathways in different bacteria are essentially similar.

3. The deviation from randomness of the dinucleotide frequencies in DNA varies systematically with the base ratio (Freese and Strack, 1962; Kaiser and Baldwin, 1962).

From these observations a common ancestral origin for bacteria seems probable.

During the process of evolution the size of genome has expanded very markedly. As a result DNA content per cell has increased from 1×10^{-14} g for Escherichia coli to 6×10^{-12} g for a mammalian cell. The increase in genome size could have occurred by gene duplication and translocation mechanisms (Nei, 1969). Indirect evidence for this hypothesis comes from the existence of isoenzymes and the multiplicity of genes for ribosomal RNA and transfer RNA.

Unlike bacteria, higher organisms possess specialized machinery for differentiation. An analysis of the compositions of cytochrome C (Margoliash and Smith, 1965), haemoglobin (Jukes, 1966)

and eukaryotic transfer (Brown, 1963) and ribosomal RNA (Pinder, Gould and Smith, 1969) reveals the conservative nature of the corresponding genes during the course of evolution. The close homology of base sequences of RNA during early embryogenesis of echinoderms as compared with adult RNA (Whitley, Whitley and McCarthy, 1967) points towards a common ancestral origin for different sea urchin species. This also suggests that stable RNA molecules from unfertilized eggs are derived from genetic loci common to all echinoderms, whereas those from larvae are produced by genes which have diverged appreciably during evolution of the various species.

1.3. Evolution of DNA Viruses

Modern viruses are obligatory intracellular parasites and their propagation depends on the suitability of the host cell. This has led some to suggest that viruses may be of recent origin and that the host-virus relationship may be evolutionary in nature. Since viral DNA is present as a unique single - or double-stranded DNA it could have either come from a small fragment of bacterial or mammalian DNA. Evidence for the latter source comes from the similarity of the nearest neighbour frequency patterns of DNA of the papovaviruses and their host (Subak-Sharpe et al., 1966b). But however this same argument cannot be used for the herpes viruses as their nearest neighbour frequency patterns differ from those of their hosts.

Other biologists, however, consider the evolution of first cells

as having occurred by self-replication of molecules such as nucleic acids followed by acquisition of a primitive cytoplasm. According to this hypothesis, the first cells arose from a virus-like precursor. It is suggested that viruses could have undergone reproduction by making use of free nutrients of the "primordial soup". It is difficult at this stage to decide between the two alternative models available.

1.3.1. Bacteriophages

There is no homology in the base sequences between viral and host genomes in virulent bacteriophages as determined by hybridisation studies (McCarthy, 1965). Since homology is not known to be required for successful virus-host relationship of virulent bacteriophages, only a small part of the viral genome with host range properties need to be considered. A high degree of homology exists between the T even phages T_2, T_4 and T_6 and T_3 and T_7 respectively. But no two members of the two groups show any homology. The presence of hydroxy-methylcytosine in place of cytosine in the even series explains part of this discrepancy.

Lysogenic phages λ_L and P22 show a certain degree of homology towards phages T_3 and hence to T_7 (McCarthy, 1965). It was reported that all four were similar in morphology and had similar content of DNA of the same base composition. From this evidence it appears that this group of viruses have been able to evolve varying degrees of virulence

towards a range of hosts. The base composition of lysogenic bacteriophage DNA is similar to its host DNA. Further, phages which transduce bacterial genes are bacterial in origin (Hayes, 1968). This has prompted some to postulate that most of the viral genetic material is bacterial in origin.

1.3.2. Animal Viruses

Successful infection of any cell depends on the ability of the invading virus to take over the translation apparatus of the cell. Modification of any part of this apparatus by the virus needs information for the synthesis of new components and also perhaps for the inhibition of a host function. But this requires a relatively large viral genome. The papova viruses on the other hand possess a coding potential of 5 to 10 polypeptides of average size. The presence of seven viral gene functions have been recognised for the expression of polyoma and SV40, without making any allowances for the modification of host translational apparatus. Of these, 4 viral genes have been identified in polyoma (Eckhart, 1969). Therefore, this does not leave much scope for the coding of putative components required for the modification of translational apparatus.

The continued presence of viral DNA in SV40 transformed cells has been shown by reactivation of SV40 by fusion with a permissive strain of cells (Watkins and Dulbecco, 1967) and also by DNA

hybridisation studies (Aloni et al., 1969). These findings strongly favour the hypothesis that the virus genome originated from cellular DNA.

Viruses of herpes and pox groups have relatively large genomes. The doublet frequency patterns and base compositions of the DNAs differ from those of the host cell (Subak-Sharpe et al., 1966b). This demands the synthesis of new components for the subversion of cellular machinery and the synthesis of viral components. Accordingly on infection, a number of new virus specific enzymes are produced (Keir, 1968), and additionally, the population of transfer RNA species is altered (Hay et al., 1966). Therefore it seems very unlikely that such viruses could have evolved from the host DNA.

2. VIRUSES

2.1. Definition

Viruses are organisms whose genome comprises either DNA or RNA and which reproduce exclusively inside living cells, using the synthetic machinery of the latter to direct the synthesis of viral proteins and nucleic acids (Lwoff, 1957; Luria, 1959). This definition accommodates animal, plant and bacterial viruses but excludes the rickettsia and psittacosis groups which are considered to be bacteria (Lwoff and Tournier, 1966).

2.2. Structure of Viruses

Viruses are usually comprised of protein and nucleic acid, these being organized in a specific manner to give the virus particle or virion. The viral nucleic acid is enclosed in a serologically specific protein assembly referred to as a capsid. The capsid is composed of capsomeres which are symmetric clusters of structural units (Crick and Watson, 1956). The number of capsomeres for a given virus is a constant and the symmetry of this capsid can be helical, cubical or binal. The capsid with the enclosed nucleic acid is the nucleocapsid. The nucleocapsid can exist in a naked or an enveloped form. The envelope consists of material which may be viral and/or host in origin.

2.3. Replication of DNA Viruses

The successful production of mature virions depends on the ability of the invading viral genome to subvert the host cellular machinery for the synthesis and assembly of viral components. To understand the extent of cellular metabolic modifications in such systems, it is necessary to analyze the virus-host cell interaction throughout the virus cell cycle. Virulent T-even bacteriophages of E. coli. have provided the most complete information regarding the virus-host cell interaction and have been used as models to facilitate understanding of the more complex, animal cell-virus interactions.

2.3.1. Attachment and Penetration

The bacterial cell wall consists of a complex network of mucopolysaccharides. The precise nature of interaction between bacterial receptors and phage attachment proteins remains obscure. T-even bacteriophages possess an elaborate injection mechanism whereby DNA and some protein pass through the bacterial cell wall leaving behind a phage "ghost" (Hershey and Chase, 1952). During the penetration of T_2 phage DNA it is believed that viral lysozyme weakens the cell wall and the nucleoside triphosphates within the virus particle provide energy for contraction of the tail sheath which ejects the viral genome into the cytoplasm.

The initial attachment of animal viruses to host cells is

probably an electrostatic attraction between the virus and charged groups on the cell surface (Puck and Sagik, 1953). Unlike bacteriophages, animal DNA viruses do not appear to have such sophisticated mechanisms for the introduction of viral DNA into the cytoplasm. Electron microscopic studies on the entry of vaccinia (Dales and Kajioka, 1964) and herpes simplex (Holms and Watson, 1963) virions into cells reveal the introduction of these particles by invagination of cellular membrane followed by pinocytosis. But recent investigations (Morgan et al., 1968) indicate that most of the herpes virions enter mammalian cells by partial digestion of the cell wall. The viral particles thus introduced into the cytoplasm are intact and have to be "uncoated" before any transcription could take place.

2.3.2. Eclipse Phase

This is the period when the infectivity of the input virus is at a very low level. The uncoating of virions begins immediately after penetration and results in the production of naked DNA or viral cores. A host cell enzyme is responsible for this first stage of intra cellular uncoating in the case of pox virus cores. For the release of naked pox virus DNA the synthesis of a new protein has been found to be essential (Joklik, 1964). It is postulated that it is a new protein which is produced by derepression of host cell DNA by a viral protein released in the first stage of viral uncoating (Joklik, 1966).

The lag before uncoating is shortened by increasing the multiplicity of infection. In contrast to vaccinia, the time course of uncoating of herpes viral DNA and its transfer from the cytoplasm to the nucleus is not effected by inhibition of nucleic acid and protein synthesis (Hochberg and Becker, 1968).

2.3.3. Effect on Host Cell Metabolism

The introduction of phage genetic material into a bacterium leads to changes in cellular function and structure. This ranges from reversible alterations in the pattern of synthesis in the case of the temperate phages (Smith and Levine, 1965), to complete disruption as observed after infection with T-even phages or T_5. The mechanism for the rapid cessation of bacterial DNA, RNA and protein synthesis as compared with the slow breakdown of bacterial DNA is not clear. The breakdown of bacterial DNA is caused by phage directed nucleases (Wiberg, 1966). An alteration in one fraction of leucine t-RNA has been found in T_2 phage infected bacterium (Sueoka and Kano-Sueoka, 1964). This could selectively prevent the translation of host m-RNA and indirectly its production. But however, this does not explain the abrupt cessation of host DNA synthesis.

Infection of mammalian cells with animal viruses can cause either stimulation or inhibition of host cell macromolecular synthesis, depending on the particular virus and the state of the cell at infection

(Kaplan and Ben-Porat, 1968). Herpes virus causes an inhibition of host macromolecular synthesis; this could be effected by

(i) a virus structural protein,

or (ii) a virus-specific protein made soon after infection.

Newton (1967) has claimed that the inhibitory effect caused by herpes on host DNA synthesis is due to a non-DNA component of the virion.

Effects such as inhibition of mitosis (Stoker and Newton, 1959) and aberration of chromosomes of the host cell (Stich et al., 1964) has been observed after herpes infection. Furthermore, several DNase activities with different specificities have been reported in herpes infected BHK-21 and HEp-2 cells (Morrison and Keir, 1968). Destruction of a small proportion of histone-unprotected DNA by a virus-induced DNase might be involved in arresting DNA synthesis.

Papovaviruses appear to stimulate host DNA synthesis (Hatanaka and Dulbecco, 1966). This appears to be a pre-requisite for the satisfactory replication of these viruses. Since the viral genome is incapable of coding for more than 5-10 proteins, the synthesis of viral components is largely dependent on functions transcribed from the host's genome. Cellular DNA made after polyoma infection is labile and is degraded into fragments of molecular weight 3×10^6 daltons. These are subsequently encapsulated into non infective virions during maturation (Ben-Porat and Kaplan, 1967).

The significance of this phenomenon is not clear.

Inhibition of protein synthesis after herpes infection and disaggregation of host-cell polysomes take place simultaneously. With the synthesis of viral protein a new population of larger polysomes appeared (Sydiskis and Roizman, 1967)with an increased number of ribosomes per m RNA (Penman et al., 1963). The rate of inhibition of protein synthesis increased with the multiplicity of infection (Roizman and Borman, 1965).

Following herpes infection new species of arginyl-and seryl-t RNA appear to be synthesised (Subak-Sharpe, Shepherd and Hay, 1966a). Becker et al (1967) demonstrated that arginine starvation did not effect the synthesis of viral DNA but prevented the formation of mature virions. This may explain the specific requirement for arginine and hence arginyl-t RNAs in herpes-infected cells.

2.3.4. Synthesis and Control of Early Virus Specified Proteins

The first step in T_4 phage infection is the formation of a transcription complex containing phage DNA and E. coli RNA polymerase (Oleson et al., 1969). From this complex a class of RNA is synthesised which hybridises with phage DNA (Green, 1964) and shows a high rate of turnover (Volkin and Astrachan, 1956). This virus-specified m RNA is then translated on existing host ribosomes (Brenner et al., 1961) producing virus-specific early proteins.

In phage-infected bacteria three classes of new proteins can be recognised. Of these, class II proteins represent early enzymes and class III constitute late proteins. Class I proteins represent the earliest proteins synthesised and are found only in T_5 infected cells. There is no corresponding protein of this class available in other phage-cell systems. Class I proteins are synthesised as a result of the first step transfer of DNA from phage T_5. Among these proteins is a component that is responsible for the shut off host protein synthesis and also proteins of Class I. (McCorquodale et al., 1967).

Many of the enzymes involved in the synthesis of viral DNA are virus specified. These belong to the Class II type of proteins. Of these, deoxycytidylate hydroxymethylase, α and β glucosyl transferase, deoxycytidine triphosphatase and DNA methylase are of utmost significance for the synthesis of T-even bacteriophages (Stent, 1963). See section 3.4.

Rapidly labelled DNA-like RNA has been identified following infection with herpes (Hay et al., 1966) vaccinia and adenoviruses (Green, 1966). This species of RNA has been found to be virus specific by hybridisation studies and represent the early m RNA.

For the transcription of viral DNA a DNA dependent RNA polymerase is required. Since the host RNA polymerase is firmly

bound to cellular nucleoprotein it is difficult to visualize the transcription of the virus message. It has been shown that transcription starts before uncoating of vaccinia is complete (Kates and McAuslan, 1967a). This suggests that vaccinia may possess DNA-dependent RNA polymerase activity in the virus capsid. Indeed, Kates and McAuslan (1967b) have demonstrated the presence of this activity in the cores of vaccinia virus. However, the work of Gharpure (1965) indicates the possibility that at least some viral functions may be transcribed by the host cell RNA polymerase.

The normal cell is equipped with the enzymes required for the synthesis of DNA. But there is no guarantee that their activity and specificity will be adequate for the replication of viral DNA. Accordingly, on infection of BHK cells virus specific enzymes are produced (Keir, 1968). See section 4.

2.3.5. Synthesis of Viral DNA

Bacterial enzymes continue to function in phage-infected cells to provide energy and some of the DNA precursors for the synthesis of viral DNA. The virus-induced enzymes ensure the supply of correct DNA precursors both from de novo synthesis and the degraded host DNA (Cohen, 1961, 1963). Thus, for example,

dCTP is converted to dCMP to act as a substrate for dCMP hydroxymethylase and also to prevent dCTP being utilized for phage DNA synthesis.

With T-even phage, DNA from a parent molecule appears in the progeny dispersed among many particles (Kozinski, 1961). This is due to the high frequency of recombination between semi-conservatively replicated molecules in the phage DNA pool. Phages with less DNA, such as λ, undergo less breakage, so that entire strands from infecting particles are found in progeny particles paired with new strands (Meselson and Weigle, 1961). This shows that λ phage DNA undergoes semiconservative replication during virus multiplication.

Bacterial mutants exist which are temperature sensitive with respect to DNA (Bonhoeffer and Schaller, 1965). The sensitive step in such strains is not at the level of triphosphate or of DNA polymerase. The ability or inability of viruses to grow in such strains at the restrictive temperature is an indication of the autonomy of host DNA synthetic machinery in the viral system. DNA synthesis of autonomous viruses such as T_1, T_4, T_7 or P_1 is unimpeded in temperature sensitive host mutants. But replication of less autonomous viruses such as λ or ØX 174 is blocked.

Large concatemers of DNA have been isolated as intermediates

in viral replication of T_4 (Frankel, 1966a, b, c), λ (Smith and Skalka, 1966) and several other bacteriophages. These concatemers have been shown to be shear sensitive. It is probably due to single stranded regions on them (Frankel, 1968).

Replication of animal virus DNA proceeds in a semi-conservative fashion (Kaplan and Ben-Porat, 1964). The larger animal viruses probably make use of virus-specified DNA polymerase in preference to host DNA polymerase for their replication (Keir et al., 1966b). See section 4.7. The non-autonomous small viruses like SV40 and polyoma depend on the activation of host DNA synthesising apparatus for their replication (Dulbecco et al., 1965). The ability of the papovaviruses to transform cells may also be related to this function (Eckhart, 1969).

Cytosine arabinoside (Levitt and Becker, 1967) and 5-fluoro deoxyuridine (Newton and Tamm, 1959) inhibit both host and viral DNA synthesis. But their action can be reversed by the addition of the appropriate deoxyribonucleosides. This suggests that most, if not all of the DNA precursors for virus replication come via the de novo pathway. This hypothesis will be dealt with in greater detail in the discussion section.

2.3.6. Synthesis and Control of Late Virus Specified Proteins

There is an absolute requirement for DNA synthesis prior to the synthesis of late m-RNA in T_4-infected bacteria. DNA replication, however, is not a sufficient requirement for late viral m-RNA synthesis, the function of at least several "maturation gene" products is also required (Bolle, Epstein and Salser, 1968).

Structural proteins, maturation factors and late enzymes (like lysozyme) are produced late in infection following the cut-off of early protein synthesis. On infection of bacteria with U.V. - irradiated virus particles, only the early functions of the virus genome are transcribed (Sekiguchi and Cohen, 1964). The failure of the late proteins to appear and inability of the viral DNA to replicate under these conditions (Dirksen et al., 1960) suggest that transcription of m-RNA for late proteins may well come from the progeny virus DNA. Using actinomycin it has been shown that T_4 lysozyme m-RNA is produced late in the phage cycle (Protass and Korn, 1966).

Viral genome is transcribed sequentially in adenovirus-2 infected KB cells. About $^1/5$ of the genome is transcribed prior to viral DNA synthesis. Early cytoplasmic RNA has a lower G + C content than early nuclear virus-specific RNA. But at later times after infection cytoplasmic and nuclear RNA possess the same base composition (Thomas and Green, 1969). These findings suggest that

regulation of early viral gene expression may involve control of both transcription and translation. Synthesis of early and late m RNA species have also been demonstrated in vaccinia - (Oda and Joklik, 1967) and polyoma - (Benjamin, 1966) infected cells.

Structural proteins of animal DNA viruses are synthesised in the cytoplasm following the shut-off of the virus-specified early proteins (Thomas and Green, 1966). Viral proteins are transported across the nuclear membrane into the nucleus (Olshevsky et al., 1967). The viral proteins first appear in the soluble fraction and later in the viral capsids. The proteins synthesised in the cytoplasm takes about 2 hours to reach the nucleus during active virus synthesis. The migration of protein to nucleus occur less efficiently when viral DNA synthesis is inhibited. But however, in cytosine arabinoside-treated cells where host and viral DNA is inhibited the viral protein still migrates to the nucleus (Ben-Porat et al., 1969).

2.3.7. Maturation and Release of Progeny Virus

The assembly of subvirions and the maturation of phage particles take place in an orderly manner. Wood and Edgar (1967) have shown that about 45 viral genes are involved in the assembly of phage T_4. Many steps have been characterised by in vitro complementation using incomplete proteins obtained from cells infected with different mutants under non-permissive conditions.

The release of mature virus particles from the infected bacterium marks the end of the viral replication cycle. The release is preceded by the lysis of bacterial cell wall by phage-induced lysozyme. Mutants defective in lysozyme function are unable to lyse the cells.

Electron microscopy reveals that maturation of animal DNA viruses proceeds in a stepwise manner (Nii et al., 1968). The efficiency of encapsulation of DNA of large animal DNA viruses is relatively small when compared with bacteriophages. The efficiency of encapsulation decreases with the size of the viral genome. The overproduction of viral components and maturation of only a small proportion of progeny DNA may well be a general feature of animal virus infection (Green, 1962; Russell et al., 1964).

The process of envelopment for equine abortion, pseudorabies and herpes viruses are similar (Darlington et al., 1968). After assembly in the nucleus, the nucleocapsid acquires an envelope by budding from the inner nuclear membrane. Once enveloped the virus particles appear in vesicles and vacuoles in the cell cytoplasm. Herpes virus particles have been shown to be capable of being transferred from one cell to another through the site of cellular contact (Hoggan et al., 1961). Mature virus is released not only at the time of cell lysis, but also progressively before this event (Scott and Tokumaru, 1964).

3. REGULATION OF PROTEIN SYNTHESIS

3.1. Gene Regulation at the Level of Transcription

The synthesis of enzymes in bacteria is under the control of structural, regulator and operator genes. The structural genes determine the primary structure of the proteins while regulator and operator genes control the rate of protein synthesis by means of protein repressors. A repressor can be either inactivated or activated by certain metabolites giving rise to induction or repression of enzyme synthesis. This system of regulation is taken in general to explain the effects of external agents on the synthesis of individual proteins (Jacob and Monod, 1961), assuming that the information contained in the structural gene is both necessary and sufficient to define the structure of a protein.

In eukaryotic cells a simple theory like that of Jacob and Monod's is unable to explain the phenomena of differentiation and redundant nucleotide sequences of the genome. Britten and Davidson (1969) have proposed a model in which early synthesis of activator RNA initiated by an inducer, causes the transcription of a producer gene by activating a DNA sequence linked to it (receptor gene). The striking difference from the bacterial model is the existence of sensor receptor genes and a multiplicity of receptor genes and activator genes. But there is no decisive evidence to clarify the precise role of "activator RNA" in eukaryotic cells.

3.2. Gene Expression

Bell (1969) has isolated a special class of cytoplasmic particles (I-somes) which contain a special type of DNA (I-DNA) in eukaryotic cells. These particles have been found to be associated with rapidly-labelled RNA. I-DNA has been shown to be associated with polyribosomes by sedimentation analysis. During translation a ternary structure containing I-DNA, polyribosomes and protein is envisaged. Earlier Georgiev (1967) and Spirin (1969) reported the identification of monocistronic m-RNA molecules associated with protein in the cytoplasm of embryonic cells.

From the above observations, it may be that "messenger" DNAs (I-DNA) each representing a discrete segment of genome pass into the cytoplasm where they associate with proteins to form I-somes. It may be this DNA which acts as a template for RNA synthesis.

3.3. Enzyme Induction

Wild type E. coli grown in the absence of β-galactoside has about 1 to 10 units of β-galactosidase per mg. dry weight. But, in the presence of a suitable inducer, an average of 10,000 units per mg. dry weight is obtained. The increase in the specific activity of β-galactosidase has been shown to be due to the synthesis of entirely new enzyme molecules rather than the activation or convertion of pre-existing precursors, by the following experimental results.

(i) The induced protein is antigenically distinct from all the proteins present in uninduced cells (Cohn and Torriani, 1952).

(ii) It does not derive any significant fraction of its sulphur (Monod and Cohn, 1953), or carbon (Rotman and Spiegelman, 1954) from pre-existing proteins.

While such dramatic changes cannot be obtained in animal cells, certain related phenomena have been observed.

Alkaline phosphatase present in early stages of differentiation of chick embryo cells, disappears on maturation (Moog, 1944). By addition of phenylphosphate to long established strains of human fibroblasts, Cox and Pontecorvo (1961) succeeded in raising the enzyme to a high level of activity. Administration of adrenal steroids into synchronized rat hepatoma cells produced large amounts of tyrosine aminotransferase (Martin, Tomkins and Bresler, 1969). Cells pre—exposed to Dexamathasone sulphate have maximal TAT activity in mitotic and early G_1 phase in the absence of the inducer. After the third hour of G_1 phase, TAT synthesis is inhibited by a specific post-transcriptional repressor.

3.4. Enzyme Repression

The term "repression" (Vogel, 1957) may be defined as the inhibition of the rate of enzyme synthesis by an end product of a

sequence of reactions or a close derivative thereof in which the enzyme is involved. The repressor probably acts by controlling either directly or indirectly the structural gene. Repressors have been shown to be protein in nature (Bretscher, 1968).

Wild type E. coli grown in the presence of arginine show only traces of ornithine-carbamyl transferase. As soon as arginine is removed from the growth medium the rate of enzyme synthesis increases about 1,000 fold and remains constant. The repressing metabolite acts here as would a dissociable inhibitor in an enzyme system (Jacob and Monod, 1961). A possibly analogous effect in animal cells comes from the work of Walker and Walker (1962) who claim that arginine - glycine transamidinase is reduced to 8% of its normal 11-day value in chick embryo liver on administration of creatine on the 6th day.

Negative Feedback Inhibition

This control mechanism involves the inhibition of activity of an enzyme in an anabolic sequence by the ultimate product of the sequence. Repression and negative feedback inhibition need not be mutually exclusive. In fact, they are found simultaneously in certain organisms (Yates and Pardee, 1956). Negative feedback inhibition is a common feature encountered in the regulation of enzymes involved in the biosynthesis of deoxyribonucleoside triphosphates in both mammalian and bacterial systems (Davidson, 1969).

3.5. Induction of Host Enzymes by Viruses

The term "induction" as applied to the enzymes of virus-infected cells is generally taken to mean an increase in the level of a particular enzyme activity following virus - cell interaction. In this sense, it is not necessarily synonymous with Jacob and Monod's concept of enzyme induction. The induction may be due to:

(a) synthesis of host cell enzymes brought about by the invading virus.

(b) some direct effect not necessarily involving new protein synthesis, such as an increase in the stability of an existing enzyme or

(c) synthesis of new, virus-specified enzymes (see Section 4).

Oncogenic viruses are known to stimulate the DNA-synthesising apparatus of the host cell. Infection of contact-inhibited mouse kidney cells with polyoma virus does not cause synthesis of virus-specific capsid proteins if DNA replication is blocked. On the other hand, the enzymatic pathways involved in biosynthesis of deoxyribonucleotides and of DNA are activated (Kara and Weil, 1967). These results imply that the observed increase in enzymatic activities does not require the prior synthesis of either host or viral DNA.

In mammalian cells, the pool of dTTP is small and is accurately regulated by intricate feedback controls (Cleaver and Holford, 1965).

After infection, the pool sizes are increased. This may indicate that the synthesis of deoxyribonucleotides is no longer closely controlled. Furthermore, virus infection does not trigger off the mitotic apparatus of differentiated cells as does partial hepatectomy (Lieberman and Kane, 1965). Therefore it would appear that after polyoma infection, an "activator" is produced which interacts with a specific cellular regulatory element like the "receptor gene" of Britten and Davidson's model. This de-repression of DNA synthesising apparatus is irreversible. (See section 2.3.4. for further details on control of early virus specified proteins).

Direct evidence for the existence of viral regulator genes comes from temperate phages (Jacob and Monod, 1961). When bacteria are infected with a temperate phage some of the cells are transformed into lysogenic bacteria. These lysogenic bacteria are not susceptible to lytic infection by the same phage. This immunity is a physiological property of the lysogenic cell rather than some structural effect of the attachment of the prophage to the chromosome (Campbell, 1967). Whatever the basis of the immunity the mechanism which prevents super-infecting phage from functioning in a lysogenic cell may be sufficient to inhibit the prophage itself from expressing its viral function. With certain types of prophages transition from prophage state to the vegetative state can be induced by exposure of the culture to heat, U. V. or

chemical compounds (Lwoff, 1953). Early functions appear as a pre-requisite for the vegative multiplication of the phage genome.

The problem of regulation of protein synthesis in cells infected with autonomous viruses is much more complex. There is competition between early and late genes of the virus and those of the host genome for expression. After infection by a phage such as T_4 transcription of the host genome is shut off at an early stage, and the mechanism by which this is brought about may be similar to that by which late phage functions are also repressed at early times.

4. VIRUS-SPECIFIED ENZYME INDUCTION

Following virus infection large increases in enzyme activities have been observed in both bacterial and mammalian systems. Many of the enzymes are essential for the synthesis of mature virus progeny, but the significance of others is less obvious. A survey of enzymes induced by animal DNA viruses will be made in this section.

4.1. Deoxythymidine Kinase

The activity of dThd kinase increases in mammalian cells infected with herpes simplex, pseudorabies, vaccinia, polyoma and SV40 viruses (Keir, 1968). Immunological experiments carried out on the enzyme obtained from non-infected cells and cells infected with herpes simplex or pseudorabies virus, indicate that antigenically distinct enzymes are present in the infected cell (Klemperer et al., 1967; Hamada et al., 1966). A strain of mouse fibroblast cells lacking deoxythymidine kinase acquires the activity after infection with herpes simplex virus (Kit and Dubbs, 1963). Mutants of herpes and vaccinia which have lost the ability to induce dThd kinase grow normally (Kit and Dubbs, 1963a) suggesting that the enzyme is not essential for virus multiplication. Although virus-induced dThd kinases differ in thermal stability, K_m values and enzymatic properties from those of host enzymes, the observed differences may be the result of physical or chemical alterations of the normal enzyme or de-repression of an isoenzyme of dThd kinase. It is interesting to note that bacteriophages in general do not

induce increase in levels of activity of dThd kinase but instead induce increases in dTMP synthetase.

4.2. Deoxycytidine Kinase

Information regarding deoxycytidine kinase in the literature is very scanty. Kara and Weil (1967) have reported an increase in activity of dCyd kinase on infection of contact-inhibited mouse kidney cells with polyoma virus. It has been shown to be distinct from dThd kinase. High levels of dCyd kinase have been reported for lymphoid tissues and tumors derived from them (Durham and Ives, 1969).

4.3. dTMP Kinase

The levels of activity of dTMP kinase increases on infection of mammalian cells with herpes simplex (Newton, 1964), pseudorabies (Nohara and Kaplan 1963), polyoma and SV40 (Black, 1968). It has been reported that infection of monkey kidney cells with herpes virus does not give a detectable difference in the level of dTMP kinase (Prusoff et al., 1965). The increase in dTMP kinase induced by pseudorabies virus has been shown to be due to stabilization of the enzyme present in the cell at the time of infection (Kaplan and Ben-Porat, 1968). dTMP kinase activity of neoplastic human tissues has been reported to be higher than corresponding activities in normal tissues (Gordon et al., 1968).

4.4. dCMP Deaminase

The activity of this enzyme increases following infection of

cells with polyoma (Hartwell et al., 1965), herpes (McGeoch and Keir, unpublished results) and adenoviruses (Ledinko, 1967). The dramatic decline in enzymatic activity observed with herpes simplex, beyond 6 hr. p.i. is not clear. This may be attributable to activation and subsequent inhibition of the host cell enzyme as a consequence of response to fluctuations of the intracellular concentrations of a effectors and repressors (Malley and Malley, 1962).

4.5. dTMP Synthetase

Infection of cells with herpes virus does not change the levels of activity of dTMP synthetase or dihydrofolic acid reductase (Freason et al., 1966). Papova viruses on the other hand, do induce increases in the levels of activity of dTMP synthetase (Black, 1968). It is of interest to note that dTMP synthetase level of bacteria increase after phage infection, but that phage mutants lacking thymidylate synthetase multiply suboptimally, on thymineless bacteria in the absence of exagenous thymine. Thus the enzyme would seem to be useful but not essential for phage replication.

4.6. DNase

DNase activity increases after infection with herpes (Keir and Gold, 1963), pseudorabies (Morrison and Keir, unpublished results) cowpox and vaccinia viruses (McAuslan, 1965; Jungwirth and Joklik, 1965). The DNase induced by herpes simplex has been shown to be enzymically and immunologically different from the DNase of non-infected

cells (Morrison and Keir, 1968). Virus-induced alkaline DNase has been partially purified and characterised as an exonuclease. The activities of acid DNase and alkaline phosphomonoesterase have not been found to be significantly altered after herpes infection (Morrison and Keir, 1968). Pox virus has been claimed to induce three DNases different from those of the host cell (McAuslan and Kates, 1966). T-even bacteriophages have been reported to induce three DNases after infection of E. coli (Sadowski and Hurwitz, 1969), which itself contains at least seven DNases.

4.7. DNA Polymerase

After infection with DNA viruses DNA polymerase activities of both bacterial and mammalian cells have been found to be increased. Of the animal viruses, enzymes induced by herpes simplex (Keir, et al., 1966a) and vaccinia viruses (Jungwirth and Joklik, 1965) have both chemical and immunological properties distinct from host cell enzymes (Keir et al., 1966b; Magee and Miller, 1967).

4.8. Other Enzymes

Infection of mammalian cells with polyoma and SV40 viruses gave increased activities of dihydrofolic acid reductase and cytidine diphosphate reductase (Kaplan and Ben-Porat, 1968), whereas herpes simplex virus did not induce increases in the levels of dihydrofolic acid reductase (Frearson et al., 1966) or of hydrolytic enzymes contained in lysosomes (Flanagan, 1966). Adenovirus 5 has been shown to induce a

2-3 fold increase in the activity of aspartate transcarbamylase (Consigli and Ginsberg, 1964). This has been identified as a virus-induced activation of a host enzyme.

5. STRUCTURE AND METABOLISM OF DNA

5.1. Structure

5.1.1. Composition

Deoxyribonucleic acids contain the purine bases, adenine and guanine, and the pyrimidine bases, thymine and cytosine. In addition to these, several other bases replacing one of the normal bases in whole or in part have been detected in certain DNAs:-

(i) 5' - methylcytosine occurs in small amounts in most species, partially replacing cytosine.

(ii) 5' - hydroxymethylcytosine wholly replaces cytosine in the DNA of T-even bacteriophages (Wyatt and Cohen, 1950).

(iii) 6' - methylaminopurine replaces adenine to a small extent in bacterial and bacteriophage DNAs (Dunn and Smith, 1958).

(iv) Uracil wholly replaces thymine in the DNA of bacteriophage PBS8 of Bacillus Subtilis (Takahashi and Marmur, 1963).

(v) 5' - hydroxymethyluracil wholly replaces thymine in the DNA of bacteriophage SP8 of Bacillus Subtilis (Kallen et al., 1962).

The bases are attached to deoxyribose of the phosphodiester back bone by a β - N - glycosidic type of linkage. In addition, glucose residues are known to be present in DNA of bacteriophages carrying unusual bases. For example, in the T-even phages hydroxymethylcytosine residues are usually glucosylated in a manner characteristic of each phage

Thus, T2 phage has 75% of its hydroxymethylcytosine groups glucosylated, with 69% α-glucosyl and 6% diglucosyl substitutions, whereas T4 has 70% α-and 30% β-glucosyl substitutions. A mutant of phage SP8 has D - mannose instead of glucose (Rosenberg, 1965).

5.1.2. Primary Structure

DNA is a linear heteropolymer of purine and pyrimidine deoxyribonucleotides. The N - glycosidic bond of the purine deoxyribonucleotides is sensitive to acid and treatment of DNA at p^H 1.6 gives rise to "apurinic acid". The internucleotide bonds of apurinic acid are intact. Further acid hydrolysis of this product gives rise to deoxyribonucleosides and deoxyribonucleoside - 3', 5' diphosphates showing that both the 3' - and 5' - OH groups are esterified by phosphoric acid in the intact DNA. This has also been demonstrated by degradation of DNA to 3' - or 5' - monophosphates by means of specific nucleases and diesterases followed by chromatographic analysis. The absence of a 2' - OH gp on the sugar moiety of deoxyribonucleotides is explicitly indicated by the resistance of DNA towards alkali treatment.

Chemical or enzymatic hydrolysis of a DNA sample followed by separation and analysis of the products gives the base composition of the DNA . Chargaff (1955) observed certain regularities in the base composition of DNAs from different sources and demonstrated that molar proportions of adenine to thymine and guanine to cytosine were equal (A = T, G = C). It was also observed that there were wide

variations in base composition between the DNAs of different species, although, DNAs from different organs and tissues of any one species were essentially the same. DNAs are commonly characterised by their percentage content of guanine and cytosine (G + C content). Thus BHK-21 cell DNA and herpes simplex virus DNA have G + C contents of 42% and 68% respectively (Russell and Crawford, 1963).

The number of nucleotide pairs per eukaryotic cell is of the order of 10^9. The determination of the nucleotide sequence in such a genome is therefore a formidable task. The current techniques available are not capable of handling such problems (Burton, 1965). Although, the nucleotide sequences of some t-RNAs and (Holley et al., 1965) and of 5S-RNA (Brownlee et al., 1967) have been determined, these are several orders smaller and have involved the use of techniques not applicable to the DNA situation.

5.1.3. Secondary Structure

X-ray analysis contributed considerably towards the elucidation of the secondary structure of DNA. Astbury and Bell were the first to obtain useful X-ray diffraction patterns of DNA fibres. These indicated a regular "repeating unit" with a periodicity of 3.34 Å perpendicular to the fibre axis. The authors incorrectly concluded that stacking was at the level of individual nucleotides.

Watson and Crick (1953) proposed that DNA was organized as a double helix. They based their proposal on the X-ray diffraction results

of Wilkins (1953) and Franklin and Gosling (1963), and upon the base analyses of Chargaff (1951) and Wyatt (1952). The model embodied the following basic features:-

(i) DNA consists of two right-handed, polydeoxyribonucleotide strands wound around the same axis;

(ii) the polynucleotide strands are antiparallel; i.e. the internucleoside phosphodiester bonds run from 3' - to 5' - carbon atoms on one strand, and from 5' - to 3' - carbon atoms on the other;

(iii) the strands are held together by interchain H-bonding between the base pairs (adenine - thymine and guanine - cytosine);

(iv) these base pairs lie normal to the long axis. The planes containing the successive base pairs are parallel and 3.4 Å apart.

Since its enunciation, the basic features of the model have remained unchanged, although, certain refinements with regard to the configuration of DNA has been made (Wilkins, 1963). It is now accepted that 3 configurations with different number of nucleotides per helical turn are possible for DNA. In addition, it is now recognised that the stability of the DNA helix in solid state and in solution is chiefly due to the base stacking forces (Josse and Eigner, 1966). The double helical

model of DNA has been a most important stimulus to biologists, especially since it immediately implied a plausible mechanism for DNA replication (Watson and Crick, 1953).

5.1.4. DNA Replication

The postulated mechanism involves the unfolding, sequentially or otherwise, of the helix to produce single-stranded regions which can act as templates for the synthesis of new strands. The complete unfolding of the whole molecule in this manner, however, seems energetically unlikely (Davidson, 1969). Furthermore, it is difficult to explain the replication of circular DNA by the above model. Cairns (1966) has suggested a "swivel" type of mechanism for replication of circular DNA assuming that duplication always starts at the same point and advances in the same direction.

5.2. Synthesis of DNA

5.2.1. In vivo Synthesis

Good evidence for semi-conservative replication of DNA was obtained from the classic experiment of Meselson and Stahl (1958), in which DNA from E. coli grown in a N^{15} medium and subsequently transferred to a normal N^{14} medium was found to pass through a (N^{15}/N^{14}) hybrid state. These results show that DNA molecules of E. coli are composed of two strands, and that, on replication, each daughter molecule receives one parental strand. The strands do not undergo detectable fragmentation but remain intact for generations.

Labelling of E. coli DNA with ^{3}H followed by autoradiography indicates that the amount of ^{3}H incorporated per unit length of newly-synthesized DNA is consistent with the presence of only one newly-synthesized DNA strand in each daughter double helix (Cairns, 1966). This work also showed that E. coli DNA was circular and replicated unidirectionally at a Y-shaped replicating point.

5.2.2. In vitro Synthesis

In 1956, Kornberg and his collaborators demonstrated the uptake of ^{14}C-thymidine into acid-insoluble DNA using a cell-free extract of E. coli in the presence of Mg^{2+} and ATP. It became evident that dThd was converted to dTTP by a kinase system prior to its incorporation into DNA, and thus that deoxynucleoside triphosphates were the probable immediate precursors of DNA. Using partially purified DNA polymerase from E. coli, Kornberg (1961) demonstrated the synthesis of DNA in vitro by polymerisation of the four deoxy-nucleoside 5' -triphosphates in the presence of a DNA template and Mg^{2+}. The polymerization involved the nucleophilic attack of a 3' - hydroxyl on the α-phosphate of a triphosphate residue producing a 3', 5' phosphodiester bond with concomittant release of pyrophosphate. The enzyme copied the DNA template in a precise manner. DNA polymerases with broadly similar properties have now been isolated from a variety of cell types and in cells infected with DNA viruses.

The DNA polymerase of E. coli has been purified to homogeneity and catalyses the following reactions (Kornberg, 1969).

(i) 5' ⟶ 3' growth of a DNA chain by polymerisation of the deoxynucleoside triphosphates;

(ii) exonucleolytic hydrolysis of a DNA chain from the 3' -OH end (3' ⟶ 5' direction);

(iii) exonucleolytic hydrolysis of a DNA from the 5' -p end (5' ⟶ 3' direction).

(iv) pyrophosphorylysis of a DNA chain from the 3' end and

(v) exchange of inorganic pyrophosphate with the terminal pyrophosphate group of a deoxynucleoside triphosphate.

The different template-primer requirements of the polymerase of E. coli and of the polymerase induced by T_4 phage are of considerable interest. Both bacterial and phage polymerases can use a double-stranded DNA template, the strands of which have been partially degraded from 3' -OH end by exonuclease III. The helix is restored to its original state by extension from the 3' -termini. On the other hand, T_4-induced polymerase cannot exploit nicked regions in the DNA as can the E. coli enzyme. For example, single strand breaks in a DNA duplex caused by pancreatic DNase treatment increase the rate of polymerisation by E. coli enzyme 10 to 20 fold. Whereas no such effect is observed with the phage-induced enzyme.

Kornberg (1969) has postulated a model for helix replication in vivo involving the action of DNA polymerase in conjunction with DNA endonuclease and ligase activities. Recently, a thymine-requiring mutant of E. coli was found to produce single-stranded breaks in DNA after thymine starvation (Freifelder, 1969). dATP concentrations such as are found in thymine starved cells are known to inhibit E. coli DNA ligase. If strand breaks are the result of some normal process, then the loss of circular DNA after thymine starvation could be attributed to the inhibition of ligase by dATP. This provides evidence for the coordinated action of enzymes as suggested in Kornberg's model.

De Lucia and Cairns (1969) have reported the isolation of a mutant of E. coli, extracts of which show greatly reduced DNA polymerase activity. The mutant cells show increased sensitivity towards U. V. irradiation and methylmethane sulphonate treatment. Since this strain grows normally and is not defective in genetic recombination (Gross and Gross, 1969) it has been suggested that the DNA polymerase isolated from E. coli is involved in repair rather than replication of DNA.

With bacterial DNA polymerase the final product of in vitro synthesis consists of up to twenty times more DNA than was supplied as template, while in the case of mammalian DNA polymerases the reaction does not proceed beyond the limit of one-fold replication

(Bollum, 1963). This may be a property inherent in mammalian DNA polymerases.

5.3. DNA Modification

The structure of DNA is known to be modified by both chemical and enzymatic agents. The modifications caused by mutagenic chemicals and their mode of action have been extensively studies in bacterial systems by genetic recombination tests. Only enzyme-mediated alterations will be dealt with here.

5.3.1. Host-controlled Methylation

Viruses have been reported to undergo non-heritable changes upon passage through certain host strains. This may be interpreted as serving as a defence mechanism against invading foreign agents. DNA methylating enzymes isolated from E. coli and other bacterial strains (Gold and Hurwitz, 1963; Fujimoto et al., 1965) have the capacity of methylating heterologous but not homologous DNAs (Arber, 1968). Host controlled modifications of phage fd and its correlation with specific methylation of deoxyribonucleotides have been reported by Arber and Smith (1966).

5.3.2. Virus-induced Methylation

Methylation of T-even phages does not appear to be essential for any function of T-phage DNA. This is seen from studies on mixed infection of E. coli with phages T_2 and T_3, in which progeny phages are produced which lack methyl groups. This is due to the presence of a

T_3-induced enzyme which cleaves S-adenosyl methionine (Gefter et al., 1966). T_2-phage produced in this fashion grows quite normally on a variety of bacterial strains and shows normal recombination. It has been reported that polyoma virus infection produced methylation of host DNA (Kaye and Winacour, 1967) whilst viral DNA was unmethylated. The DNA of herpes virus has similarly been shown to be unmethylated (Low, Hay and Keir).

5.3.3. Virus-induced Degradation of Host DNA

Extensive breakdown of E. coli DNA was observed after T_4-phage infection. The first product obtained was a high molecular weight material of 50 to 70 S. Further degradation appeared to occur in discrete steps. But infection with U. V. irradiated phage T_4 did not give rise to this further degradation (Warren and Bose, 1968). These results suggest that observed degradation of host DNA begins early and requires sequential action of several phage induced endo - as well as exo-deoxyribonucleases.

Extensive breakdown of cytosine-containing T_4 DNA is observed on infection with a T_4-phage mutant lacking dCMP hydroxymethylase. When a mutation in gene 46 is also present in the same phage, neither E. coli nor T_4 DNA is degraded. This implies that genes 46 and 47 control one or more DNases which preferentially attack DNA containing cytosine rather than hydroxymethylcytosine, thus imparting protection on the viral genome.

5.4. Nucleotide Metabolism

Cells possess pools of nucleic acid precursors distributed in various compartments of the cell. During active DNA synthesis the appropriate precursors are withdrawn from the pool. This depletion of the deoxyribonucleotide pool probably triggers off the nucleotide - synthesising machinery. The levels of dATP and dTTP have been implicated in the regulation of deoxyribonucleotide synthesis (Grav, 1967).

The cellular deoxyribonucleotide pool is generally of the order of 0.1% of the total nucleotide content of DNA (Grav, 1967). This is relatively small compared with the amounts required for DNA synthesis. Therefore, any attempt at inhibition of DNA synthesis should directly involve blocking of a precursor or precursors *via* the ribonucleotide reductase pathway (*de novo* synthesis)

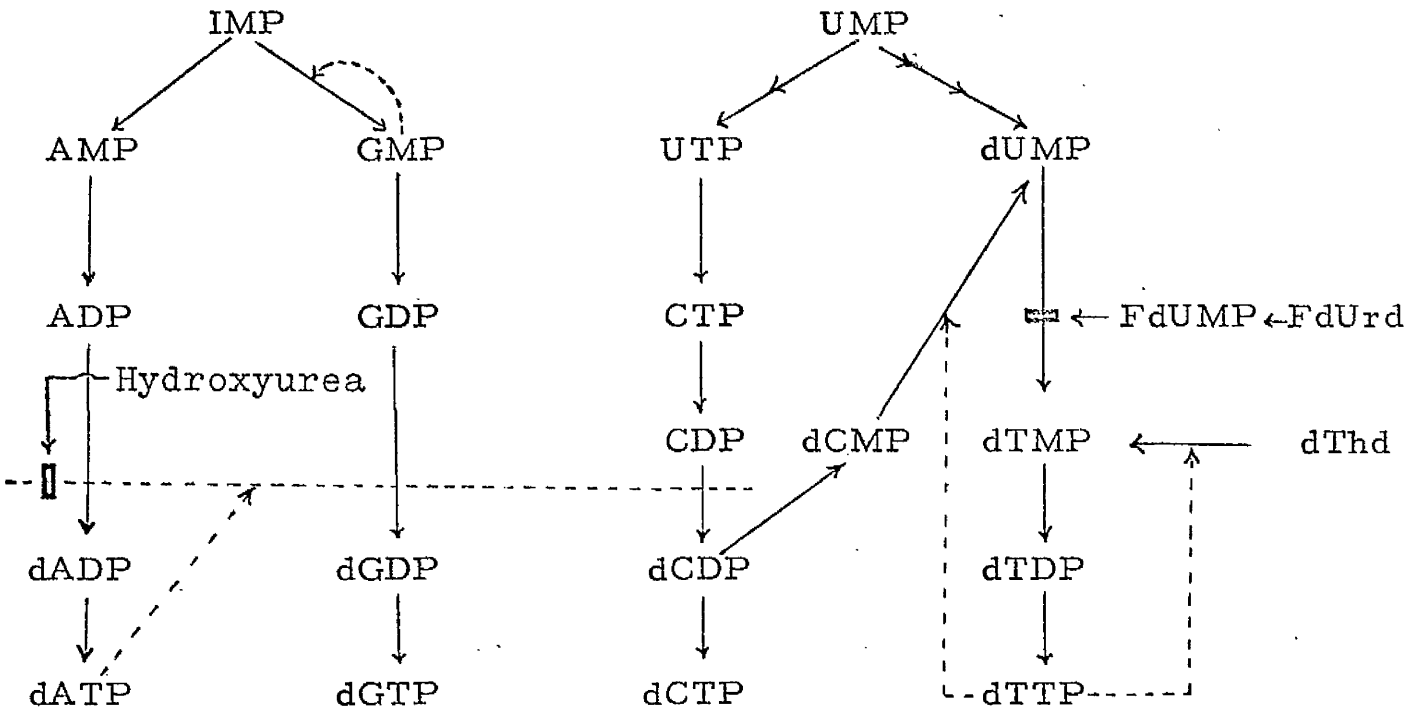
Biosynthesis of deoxyribo-mononucleotides

5.4.1. Ribonucleotide Synthesis

IMP and UMP are the respective end products of the pathways of purine and pyrimidine biosynthesis. IMP is aminated to AMP *via* adenylosuccinic acid. There is a requirement for GTP as a co-enzyme inthe reaction forming adenylosuccinic acid. The formation of GMP from IMP proceeds *via* Xanthosine 5' -monophosphate. XMP is aminated to GMP in the presence of glutamine. AMP, GMP and UMP are phosphorylated to their respective diphosphates prior to being transferred

Figure I.

Pathways Involved in the Biosynthesis of Deoxyribonucleoside Triphosphates



Feedback mechanisms are indicated by dotted lines

from the large ribonucleotide pool to the specifically controlled deoxyribonucleotide pool.

UMP is converted by kinases through UDP to UTP. UTP is then aminated to give CTP. CTP has to be dephosphorylated to CDP before undergoing reduction.

5.4.2. Ribonucleotide Reduction

The reduction of ADP, GDP, UDP and CDP to deoxyribose derivatives take place with the glycosidic linkage between sugar and base intact (Reichard, 1964). Very low levels of dATP are known to inhibit this reductive process and hence the DNA synthesis (Morris and Fischer, 1960). In the micro-organism Lactobacillus leichmannii, however, the reduction takes place at the triphosphate level and requires a cobamide enzyme.

The partially purified ribonucleotide reductase system from Novikoff hepatoma reduces all 4 ribonucleotides (Moore and Hurlbert, 1966) and in addition responds to the addition of certain nucleoside triphosphate allosteric effectors in a manner closely resembling that of the E. coli system. Reduction of both CDP and UDP were stimulated by ATP and inhibited by dATP. dGTP and dTTP inhibited the reductase reaction even in the presence of ATP. The reduction of purine ribonucleotides was very little affected by ATP but was strongly inhibited by dATP.

Brown and Reichard (1969) have separated ribonucleoside diphosphate reductase from E. coli into two inactive sub-units, B_1 and B_2. When they were mixed together, enzyme activity was restored. On sucrose gradients, the active complex had a sedimentation value of 9.75, whereas, in the presence of the negative effector dATP, the S value increased to 15.5S. ATP and dTTP did not alter the conformation appreciably.

Allosteric control of ribonucleoside diphosphate reduction

Sub-unit B_2 does not have any nucleotide binding site, while B_1, either alone or in combination with B_2 possesses 4 nucleotide binding sites. These fall into two classes.

Class h - site has a high affinity for dATP and binds ATP, dATP, dGTP and dTTP.

Class l - site has a lower affinity for dATP and binds only to ATP and dATP.

It is envisaged (Brown et al., 1969) that l-site probably regulates overall activity of enzyme whilst h-site influences substrate specificity.

5.4.3. Thymidylate Synthesis and Phosphorylation

dUMP is a key precursor of thymidylate and can arise by reduction of uridine nucleotides or deamination of dCMP. dUMP is rapidly methylated by the enzyme thymidylate synthetase in a reaction involving N^5, N^{10} - methylene tetrahydrofolic acid. 5-fluorodeoxyuridylic acid is a potent inhibitor of this enzyme; this explains the

potent inhibition of thymidylate and hence DNA synthesis by FdUrd. It has been suggested that dUMP may activate or induce the formation of thymidylate synthetase (Maley and Maley, 1960).

Phosphorylation

Specific kinases phosphorylate deoxyribonucleoside mono - and diphosphates to their respective triphosphates, the immediate precursors of DNA synthesis. One of the important kinases frequently induced or activated to make use of exogenous thymidine in the cellular environment is the thymidine kinase. Its activity is greatly increased in regenerating rat liver (Weissman et al., 1960), rapidly proliferating tissues and mammalian cells infected with DNA viruses (Keir, 1968). Since dTTP is known to inhibit thymidine kinase (Potter, 1964) and dCMP deaminase by negative feedback inhibition, large accumulation of dTTP either from thymidine or dCMP deaminase pathway is prevented. The effect of feedback control mechanism in herpes infected cells will be dealt in a more rigorous manner in the discussion section.

Regulation of Deoxythymidine Kinase

The molecular weight of the monomeric enzyme from E. coli is approximately 42,000 daltons as estimated by gel filtration. There is a good correlation between the extent of activation or inhibition of dThd kinase and its sedimentation coefficient. The sedimentation coefficient increases from 3.4 - 3.5S to 5.3 - 5.5S on addition of the activator dCDP, while addition of the inhibitor dTTP increases it

further to 5.9 - 6.0S. This small difference may reflect a difference in the conformation of the dimer formed from monomers in the presence of activators and inhibitors (Iwatsuki and Okazaki, 1967).

6. OBJECTIVES OF THE PROJECT

The aim of this project was to study the deoxyribonucleoside kinases of mammalian cells before and after infection with herpes virus, and to characterise any virus induced activities. Since the discovery of the virus-induced deoxycytidine kinase further investigations have been carried out to establish the significance of virus induced enzymes in the synthesis of viral DNA. The effects of multiplicity of infection and the nutritional state of the cells before infection on the induction and control of virus-induced enzymes have been carefully examined.

II. MATERIALS

1. Standard Materials

1.1. Chemicals

Where possible, all chemicals used were "Analar" grade or its equivalent. Deoxyribonucleosides and deoxyribonucleotides were purchased from Calbiochem, Los Angeles, California and P-L Biochemicals, Milwaukee, Wisconsin. Deoxyribonucleoside 5'- triphosphates were purified on DEAE sephadex columns. Salmon testes DNA was purchased from Worthington Biochemicals Corp., Freehold, New Jersey. BES and imidazole were purchased from BDH Biochemicals, Poole, Dorset. Actinomycin D was purchased from Calbiochem, Los Angeles, California. Puromycin hydrochloride and hydroxyurea were purchased from Nutritional Biochemical Corporation Cleveland, Ohio. Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne. CsCl was purchased from Harshaw Chemical Co., Cleveland, Ohio. Kieselguhr (Hyflo Supercel) was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

1.2. Radiochemicals

^{32}P-labelled inorganic phosphate and ^{3}H- and ^{14}C- labelled deoxynucleosides were purchased from the Radiochemical Centre, Amersham, Bucks. α^{32}-P labelled dATP and dTTP were purchased from International Chemical and Nuclear Corporation, City of Industry,

California. ^{3}H- dATP and ^{3}H- ATP were purchased from Schwarz Bioresearch Inc., Orangeburg, New York. Where necessary, radiochemicals were purified by paper chromatography using isobutyric/ ammonia/water as solvent.

1.3. Liquid Scintillation Material

1,4 di-2 (5-phenyl oxazolyl)-benzene, (POPOP) and 2, 5-diphenyl oxazole (PPO) were purchased from Koch Light Laboratories Ltd., Colnbrook, Bucks. Hyamine hydroxide, 1M in methanol, napthalene and dioxan were purchased from Nuclear Enterprises (G.B.) Ltd., Edinburgh.

1.4. Media for Chromatography and Electrophoresis

Whatman Nos. 1 and 3 MM filter paper and ion-exchange celluloses, DE52 and DE81 were purchased from H. Reeve Angel & Co. Ltd., London. Sephadex G-25, G-50 and G-100 were purchased from Pharmacia (G.B.) Ltd., London. Hydroxyapatite was purchased from Bio. Rad Laboratories, Richmond, California.

1.5. Biological Materials

Tissue Culture Cells

BHK-21/C-13 cells were a continuous line of hamster fibroblasts (Macpherson and Stoker, 1962).

Virus

The particular strain of Herpes simplex virus used was a

specifically purified sub strain of HFEM referred to as the α-strain (Russell et al., 1964).

1.6. Enzymes

Pronase was purchased from Calbiochem, Los Angeles, California and snake venom 5'-nucleotidase from Sigma Chemical Co., St. Louis, Mo.

1.7. Miscellaneous

Eagle's medium and calf serum were purchased from the Institute of Virology, University of Glasgow. Oxoid Agar No. 3 was purchased from the Oxoid division of Oxo Ltd., London. Difco bacto peptone was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. T_2 phage infected E. coli R2 and λ phage-infected E. coli W 3110 were generous gifts of Dr. J. Pitts of this department.

2. Composition of Media and Standard Solutions

2.1. Media for Tissue Culture Work

Eagle's medium A modification of Eagle's medium (Busby, House and MacDonald, 1964) containing 100 µg./ml. streptomycin, 100 units/ml. of penicillin, 0.002% (w/v) phenol red and 0.2 µg./ml. of antimycotic agent n-butyl, p-hydroxy benzoate was used.

EC_{10} medium Consisted of Eagle's medium supplemented with 10% calf serum.

ET_5C_5 medium Consisted of Eagle's medium supplemented with 5% tryptose and 5% calf serum.

Tryptose phosphate broth It consisted of a 2.95% (w/v) solution of tryptose phosphate broth (Difco Bacto) in water.

Buffered saline solution (BSS) Consisted of 0.116M-NaCl, 5.4mM-KCl, 1mM-$MgSO_4$, 1mM-NaH_2PO_4, 1.8mM-$CaCl_2$ and 0.002% (w/v) phenol red made neutral with 8.4% (w/v) $NaHCO_3$.

Phosphate buffered saline A (PBS Sol. A). Consisted of 0.17M-NaCl, 3.4mM-KCl, 10mM-Na_2HPO_4 and 2mM-KH_2PO_4 at pH 7.4 (Dulbecco and Vogt, 1954).

Tris-saline Consisted of 25mM-Tris/HCl buffer (pH 7.4), 0.14M-NaCl, 5mM-KCl, 0.7mM-Na_2HPO_4, 5mM-dextrose containing 0.002% (w/v) phenol red, 100 μg./ml. of streptomycin and 100 units/ml. of penicillin.

Versene Consisted of 0.6mM-EDTA in PBS solution A to which 0.002% (w/v) phenol red was added.

Trypsin-Versene This was a 0.05% (w/v) solution of trypsin in versene.

Formal saline Contained 4% (v/v) formaldehyde in 85mM-NaCl and 0.1M-Na_2SO_4.

Giemsa stain This was a suspension of 1.5% (w/v) Giemsa in glycerol heated at 56°C for 90 min. and diluted with an equal volume of methanol (Dacie, 1956).

2.2. Media for Phage Production

Nutrient agar Contained 1% (w/v) Difco bacto peptone, 1%(w/v) Oxoid

agar No. 3, 0.8% NaCl and 0.1% glucose.

Soft agar Contained 0.6% Oxoid agar No. 3 dissolved in water.

HB broth Contained a solution of 0.5% Difco bacto peptone, 0.1% glucose, 0.5% NaCl and 0.8% Difco nutrient broth whose pH was adjusted to 7.2 with 10N-NaOH.

2.3. Standard Solutions

Standard Citrate solution (SSC). This was a solution containing 0.15M NaCl and 15mM trisodium citrate at pH 7.0.

Stock Imidazole buffer (IB) This was a 1.0M solution of imidazole, whose pH had been adjusted to 7.0 with HCl acid.

Stock solution of BES buffer This was a 1.0M solution of BES whose pH had been adjusted to 7.0 with 10N-NaOH.

2.4. Liquid Scintillators

Dioxan based scintillator consisted of 10% napthalene, and 0.7% PPO dissolved in scintillation grade dioxan.

Toluene based scintillator consisted of 0.5% POP in "Analar" toluene. In earlier measurements 0.003% POPOP was included in the solution, its omission made no significant difference.

III. METHODS

1. Tissue Culture Techniques

1.1. Propagation of Cells

BHK-21/C-13 cells were propagated in 180 ml. of modified Eagle's medium supplemented with 10% calf serum (EC_{10}) in 80 ounce roller bottles gassed with 95% air + 5% CO_2 and incubated at 37°C (House and Wildy, 1965). Under these conditions the cell population doubled every 20 - 22 hr. Cells were routinely harvested after three days and each bottle seeded with 20×10^6 cells. All incubations were at 37°C, unless otherwise indicated.

1.2. Harvesting Cells

The medium was decanted and the monolayer of cells washed once with 20 ml. of warm versene. The cells were then trypsinised using 25 ml. of 0.05% trypsin in versene. The cells were dispersed, poured into 5 ml. of EC_{10} and centrifuged at 500 g for 10 min. The cell pellet was washed once with Eagle's medium and the cells suspended in EC_{10} or $EC_{0.5}$ when the cells were being set to resting (see below).

1.3. Cell Systems For The Study of Virus-Host Cell Interactions

1.3.1. High Serum Cells

Cells were suspended in EC_{10} at a cell density of 0.5×10^6 cells/ml. and then dispensed into 50 mm. Petri dishes at 4 ml./dish.

After 40 hours the cells were ready to be harvested for the determination of enzyme activity. When dialysable amounts of enzyme were required the cells were either grown in 90 mm. Petri dishes to a final concentration of 8×10^6 cells/dish or in roller bottles to a concentration 75×10^6 cells/bottle.

1.3.2. Resting Cells

Cells suspended in $EC_{0.5}$ were dispensed into 50 mm. Petri dishes at a cell density of 1.6×10^6 cells/ml. using 5 ml. of medium. In the case of 90 mm. Petri dishes 15×10^6 cells were seeded using 15 ml. of $EC_{0.5}$ and in the case of roller bottles 150×10^6 cells were seeded using 100 ml. of $EC_{0.5}$. After three days the cells were ready to be infected. A modified method (Fried and Pitts, 1968) was also adopted during latter part of the research project. Confluent roller bottles were washed once with 20 ml. of versene/bottle and then treated with 25 ml. of 0.05% trypsin/versene on the bench. The cells were shaken off the glass and dispersed gently into single cells. The cells were then immediately transferred into 80 ml. of ice-cold Eagle's medium per bottle and centrifuged at 500 g for 10 min. The cell pellet was resuspended in $EC_{0.5}$ and seeded into 50 mm. dishes at 8×10^6 cells/dish or 150×10^6 cells/roller bottle.

1.4. Procedure for Infecting Cells

1.4.1. High Serum Cells

Growth medium was removed from roller bottle cultures

containing approximately 150 x 10^6 cells/bottle and replaced with either 20 ml. inoculum of herpes virus in EC_2 (3 x 10^9 P. F. U. /bottle) or 20 ml. of EC_2. After an adsorption period of 1 hr. the medium was removed and the cell sheet washed with 2 x 25 ml. warm BSS. 100 ml. EC_{10} was added and the bottles incubated at 37° C till the time of harvesting. Infected cells were normally harvested between 10-12 hr. after infection.

1.4.2. Low Serum Cells

After three days the low serum medium was removed from the monolayers and centrifuged at 500 g for 10 min. to remove any cell debris. The medium was then gassed with 5% CO_2 and incubated at 37° C. The monolayers (75 x 10^6 cells/bottle) were mock-infected with 20 ml. of the above used medium or inoculated with herpes virus suspended in 20 ml. of used medium at a multiplicity 20 P. F. U. /cell. After 1 hour for adsorption of virus, the medium was removed and the cell sheet washed with 25 ml. warm BSS. 100 ml. of used $EC_{0.5}$ was then added and cells incubated for 10 - 12 hr. before being harvested.

1.5. Production of Virus Stocks

Cells were grown in roller bottles to populations of 200 - 400 x 10^6 cells per bottle within 3 days, using $EC_{10}T_{10}$. The medium was decanted and the cells infected at the low multiplicity of 1 P. F. U. per 300 to 1,000 cells with the virus in 20 ml. EC_5T_5. After an hour,

a further 30 ml. of EC_5T_5 was added and the cells incubated for 2 to 3 days at 37°C until the cells exhibited complete cytopathic effect. The pH of the medium was maintained neutral by addition of 8.4% (w/v) $NaHCO_3$ during the course of virus multiplication. This enhanced the growth and stability of the virus.

Harvesting of Virus

Virus-infected cells were dislodged by shaking the cells off the glass. The cell suspension was centrifuged at 900 g for 10 mins. The supernatant was decanted and the cell pellet suspended in 10 - 15 ml. of supernatant. The cell suspension was disrupted with ultrasonic vibration and the resulting sonicate was centrifuged at 900 g for 10 min. The opalescent supernatant was pooled with the earlier supernatant fraction and centrifuged at 30,000 g for 30 min. The pellet thus obtained was resuspended by sonication in $EC_{0.5}$ and subjected to centrifugation at 900 g for 10 min. The supernate thus prepared contained a fairly clean preparation of high titre virus (10^{10} P.F.U./ roller bottle) as indicated by electron microscopic observations. During all manipulations the temperature was maintained below 5°C. The virus suspension was stored at -70°C in 2 ml. portions.

1.6. Titration of Virus

Stock virus solutions were stored at -70°C for over a fortnight before any estimations of infectivity were made. This was to allow for any possible inactivation of virus to take place during

storage at -70°C. A specimen of the virus preparation was thawed and serially diluted in EC_2. The infectivity of diluted virus suspensions was estimated using a sensitive plaque technique (Russell, 1962). When plaque formation was complete, the cells were fixed with formal saline and stained with Giemsa stain. Excess dye was washed off with PBS solution A and the plaques counted. If it was desired to keep the virus viable in the plaques, neutral red was added directly without fixing the cells. Plaques were counted after overnight incubation at 37°C.

2. Biochemical Techniques

2.1 Preparation of Crude Cell Extracts

Cell monolayers were washed once with ice cold BSS and scraped into conical tubes using BSS solution. Cells were centrifuged at 500g for 10 min, the medium decanted and the cells suspended in 2 to 5 volumes of 10mM-BES buffer pH 7.0 containing 5mM-mercapto-ethanol.

Cells were disrupted by ultrasonic vibrations (4 x 30 sec. with MSE probe type ultrasonic unit) and the homogeneous suspension centrifuged at 105,000g for 90 min. The supernatant fluid was removed and dialysed overnight against 100 volumes of buffer. All operations were carried out at 0 - 4°.

The labile kinases were stabilised during enzyme extraction and dialysis by the presence of 10^{-6}M deoxyribonucleoside. The dialysed supernatant was stored at -70°C in sealed containers. The pellet contained negligible amounts of deoxypyrimidine kinases and DNase activity.

2.2. Preparation of Phagicin

2.2.1. Small Scale Preparation of Phage Lysate Using the Agar Overlay Method

25 ml. amounts of nutrient agar were poured into 90 mm. Petri dishes and allowed to set. 4 ml. amounts of soft agar at 42°C were mixed with 0.5 ml. of E. coli W3110 and 0.2 ml. of crude

phage λ lysate and poured uniformly over the hard agar base. Plates were incubated overnight at 37°C in a humidified incubator until the lysis of bacteria was complete.

The soft agar containing the phage lysate was extracted with 1 to 2 ml. of PBS solution A and centrifuged at 1,000g for 15 min. The supernatant was treated with a Dawe Soniprobe for 3 min. at 2.5 amps. The sonicate was centrifuged at 105,000g for 30 min. and the supernate stored at -20°C.

2.2.2. Large Scale Preparation of Phage Lysate by Infecting Cells in Suspension

0.5 ml. of E. coli W3110 was added to 50 ml. of HB broth at 37°C and incubated until the turbidity at 450 nm. was about 0.8 extinction units. The cell suspension was then infected at a multiplicity of 2 P.F.U./cell and incubated at 42°C for 15 min. The temperature was then lowered to 37°C and incubation continued until lysis was complete.

The crude cell lysate was clarified by centrifugation at 15,000g for 30 min. and concentrated using a Diaflo membrane filter. The concentrate was treated with a Dawe Soniprobe for 2 min. at 2.5 amps. and centrifuged at 105,000g for 30 min. The supernate was stored at -20°C. This crude preparation of phagicin was partially purified by gel filtration as described by Centifanto (1968).

2.3. Analytical Methods

2.3.1. Estimation of Protein

20 to 100 µl. samples of protein solution were precipitated by addition of 1 ml. of ice-cold 5% (w/v) TCA and leaving at 0 - 4°C for 1 hr. The protein was separated by centrifugation at 1,000g for 15 min. Protein in the precipitate was then estimated using crystalline bovine serum albumin as standard (Lowry et al., 1951).

2.3.2. Estimation of DNA

DNA from CsCl fractions was estimated using a modification of the method of Burton (1956). 20 µl 70% PCA was added to 0.26 ml. of a solution containing 1 - 10 µg. DNA and the solution incubated at 70°C for 30 min. After cooling, the solution was made up to 0.5 ml. with 5% PCA and centrifuged at 1,000g for 15 min., 0.25 ml. of the supernatant was taken for the estimation of DNA.

2.4. Autoradiography and Labelling of Cells

2.4.1. Autoradiography

Cells were grown under appropriate conditions in 50 mm. Petri dishes containing 18 x 18mm. coverslips. To estimate the percentage of cells capable of being infected, cultures were infected at different multiplicities of exposure using standard procedures. After virus adsorption, medium was added and the cultures incubated for 7 hr. 5 µc. of ^{3}H-dThd (specific activity = 38.8Ci/m Mole) was added to each culture and incubation continued for 1 hr. The medium was then

removed and the cell sheets washed once with ice-cold 0.9% (w/v) NaCl. The cells were fixed with formal saline and washed four times in ice-cold 5% TCA. The coverslips were rinsed in 70% ethanol and allowed to dry prior to autoradiography. Kodak NTB-3 photographic emulsion was diluted 1 in 3 with water in the dark room. Coverslips were dipped in the emulsion and drained free of excess. After 5 days coverslips were immersed in Kodak D19 developer for 2 min. and rinsed with water. Coverslips were then fixed in Amfix (diluted 1 in 5 with water) for 2 min. and thoroughly rinsed in water. After drying, coverslips were stained with haematoxylin for 10 min., rinsed in water and treated with a drop of dilute ammonium hydroxide to stain the cells blue. The preparation was then ready to be examined under the microscope to determine the percentage of labelled cells.

2.4.2. Pulse Labelling Technique

The rate of DNA synthesis of C-13 cells following viral infection was measured by pulse labelling cells for 1 hr. in the presence of 1 μc of ^{3}H-dCyd (10^{-5}M) at different times after infection. At the end of the labelling period the medium was removed and stored at 4°C in screw capped bottles, while the cell sheet was washed twice, scraped into conical tubes with cold BSS and centrifuged at 500g for 10 min. The cell pellet was suspended in 2 ml. of 5% TCA and left in ice for 15 min. After centrifugation at 1,000g for 15 min., the supernatant was stored at 4°C.

The pellet was mixed with 2 ml. 5% TCA containing Hyflo Supercel (20g./l.) and filtered through a pad of Hyflo Supercel supported on scintered glass. The precipitate was washed thoroughly with 3 x 10 ml. ice-cold 5% TCA and dried by washing with 3 x 10 ml. absolute alcohol followed by 2 x 5 ml. ether. The dried material was transferred directly into a scintillation vial and heated for 30 min. at 65°C with 0.5 ml. 1M-hyamine hydroxide in methanol. The vial was cooled and 10 ml. toluene-based scintillator fluid added prior to counting. 200 µl samples of the supernatants were mixed with 3 ml. absolute alcohol and 10 ml. toluene-based scintillator and counted. When large volumes of supernatants were required to be counted, dioxan-based scintillator was used.

To estimate the amount of radioactivity left in the medium, 0.5 ml. 10% TCA was mixed with 0.5 ml. medium and the precipitate removed by centrifugation. 20 µl. of the supernatant was dissolved in 3 ml. alcohol and 10 ml. toluene-based scintillator and counted.

2.5. Assay of Enzymes

2.5.1. DNA polymerase

The assay measured the incorporation of ^{32}P-dTMP residues from α-^{32}P-dTTP into acid-insoluble DNA in the presence of a primer and all four deoxynucleoside triphosphates as described by Keir (1962). The standard assay mixture contained 400 µg./ml. denatured DNA,

0.2mM-dCTP, dGTP, dATP and α-^{32}P-dTTP, 8mM-$MgSO_4$, 25mM-tris-HCl buffer p^H 7.5, 60mM-KCl, 5mM-mercaptoethanol, 0.4mM-EDTA and enzyme in a total volume of 0.25 ml. For specific assay of the herpes-induced DNA polymerase the KCl concentration was increased from 60mM to 0.2M.

The specific activity of DNA polymerase is the number of n moles of dTMP incorporated into acid-insoluble material per mg. protein in 30 min. at 37°C.

2.5.2. DNase

The assay measured the release of acid-soluble material from ^{32}P-labelled E. coli DNA as described by Morrison and Keir (1968). The standard assay mixture contained 50 µg./ml. ^{32}P-DNA, 2mM-$MgSO_4$, 50mM-Tris-HCl buffer pH 9.0, 10mM-mercaptoethanol and enzyme in a total volume of 0.2 ml.

The specific activity of DNase is the number of µg. DNA degraded to acid-soluble material per mg. protein in 30 min. at 37°C.

2.5.3. Purine Deoxyribonucleoside Kinases

The assay measured the conversion of ^{14}C-deoxyribonucleoside to deoxyribonucleotide by paper chromatographic separation. The incubation mixture contained 5µ moles of BES buffer pH 7.0, 400 n moles ATP, 400 n moles $MgCl_2$, 60 n moles ^{14}C-deoxynucleoside (1 µc/µ mole), 0.1 µ mole mercaptoethanol and 2 - 20 µg. enzyme protein in a total volume of 70 µl. After 30 min. incubation at 37°C, the reaction was stopped by

heating for 2 min. in a boiling water bath and cooling in ice. The suspension was centrifuged at 1,000g for 10 min. at 0 - 4° and the supernatant kept. A 20 µl. sample of the supernatant was mixed with 20 µl. of carrier mixture (containing nucleoside and nucleotide mono, di- and triphosphates, each at a concentration of 10mM) and applied to Whatman No. 1 or 3MM paper.. The nucleoside was separated from the nucleotides by paper chromatography or electrophoresis (see below). The nucleoside and nucleotide spots were cut out, immersed in toluene-based scintillator solution and counted.

The specific activity of the enzyme was defined as the number of n moles of deoxyribonucleoside phosphorylated per mg. protein in 30 min. at 37°C. In the early work, imidazole buffer was used instead of BES buffer.

2.5.4. Pyrimidine Deoxyribonucleoside Kinases

The assay was similar to that for the purine kinases, except that the nucleoside concentration was lower. The incubation mixture consisted of 5 µ moles of BES buffer (pH = 7.0), 100 n moles of ATP, 100 n moles of $MgCl_2$, 10 n moles of ^{14}C-labelled deoxynucleoside (4 µc/µ mole), 0.1 µ mole of mercaptoethanol and 5 to 100 µg. enzyme protein in a volume of 70 µl.

The specific activity was defined as the number of n moles of deoxynucleoside phosphorylated per mg. protein in 30 min.. at 37°C. The percentage of deoxyribonucleoside phosphorylated did not normally

exceed 50%, thus ensuring that all assays were within the range of linear response to time.

2.5.5. ATPase

The assay was performed under conditions similar to the pyrimidine kinase assay, the exceptions being the inclusion of ^{3}H-ATP at 10 n moles/assay (0.5 μc/n mole) instead of cold ATP [illegible]

2.5.6 Heat Inactivation Conditions

100μg amounts of host and virus infected high speed supernatant fractions were subjected to heat treatment for 3 mins in 0.1ml 20mM-BES buffer containing 5mM-mercaptoethanol both in the presence and the absence of the substrate. The cell extracts were cooled in ice and later analysed for enzyme activity.

2.6. Enzyme Purification

2.6.1. DEAE-Cellulose Chromatography

Microgranular type DE-52 was prepared according to suppliers instructions and packed under gravity in a short Whatman column (1 x 12.5 cm) using 0.05M-BES buffer, pH 7.0, containing 1mM-EDTA, 5mM-mercaptoethanol and 10^{-6}M-dCyd. After equilibration overnight at 4°C the column was washed with 100 ml. buffer. The high-speed supernate of a cell extract was dialysed against the buffer, applied to the column and washed through with 70 ml. buffer. 5 ml. fractions were collected and the protein concentration of the fractions adjusted to 1 mg./ml. by addition of bovine serum albumin prior to

storage at -70°. Elution was then performed using a linear gradient of KCl in buffer (0-0.35M, 200 ml.). Fractions were collected and stored as above.

2.6.2. Gel Filtration

Pre-swollen Sephadex G-25, G-50 and G-100 were packed into columns according to suppliers' instructions. Void volumes were estimated using Dextran Blue. Columns of G-25 were often used for desalting protein extracts for rapid enzyme assays and were usually equilibrated and eluted with 20mM BES buffer, pH 7.0, containing 10mM-mercaptoethanol.

2.7. Partial Purification of Phagicin

45 mg. protein of a crude phagicin preparation (5 mg./ml.) was applied to a 1.5 x 40 cm. Sephadex G-100 column and eluted with 0.1M phosphate buffer, pH 7.5 (Centifanto, 1968). The material eluting at the void volume was collected and centrifuged at 105,000g. for 7 hr. The supernatant was stored at -20°C.

2.8. Paper Chromatography

Deoxyribonucleosides were separated from their phosphorylated derivatives on Whatman No. 1 or 3MM paper by descending chromatography with the solvent isobutyric acid/H_2O/NH_3/0.1M-EDTA (100:55:8:4.2:1.6) for 20 hrs. at 20°C. The paper was dried in a fume cupboard and spots

corresponding to different nucleosides and nucleotides located under U. V. light (Thomson, 1969).

DEAE-Paper Chromatography

The products of the enzymatic hydrolysis of DNA was separated by ascending chromatography on DE-81 paper using 0.75M NH_4HCO_3, pH 8.6 The R_f values at 20°C were as follows: P_i:0.8, dCMP, dTMP:0.7, dAMP, dGMP:0.5, oligonucleotides 0 to 0.2. Radioactivity was located and measured using an Actigraph III gas flow counter.

2.9. Electrophoresis

Paper electrophoretic separation of nucleosides and nucleotides were carried out on Whatman 3MM paper in 0.05M citrate buffer, pH 3.5, at a potential gradient of 40 volts/cm. for 45 min.

2.10. Density Gradient Centrifugation

Labelled host and viral DNA preparations were pre-incubated for 24 hr. with 1% sodium dodecyl sulphate and pronase in SSC at 37°C. The viscous solutions were then transferred carefully into glass tubes and the volumes made up to 3.4 ml. with SSC. 4.375g. of CsCl was then dissolved in each tube and the solution clarified by centrifugation at 10,000g. for 30 min. The floating material was removed and the refractive indices of the supernatants adjusted to 1.400 using SSC. The resulting solutions were transferred to polyallomer tubes layered with paraffin oil and centrifuged for 65 - 70 hr. at 33,000 r.p.m. at

20°C in the Spinco No. 50 aluminium angle head rotor (Flamm et al., 1966). Tubes were punctured and 2 drop fractions collected. Fractions were diluted with 0.5 mls. of $^{1}/_{10}$ SSC and E_{260} and radioactivity measured.

2.11 Assay of the Distribution of Radioactive Precursors in Acid-Soluble and Insoluble Pools of DNA and RNA

18 hr. old cultures of BHK cells (4×10^6 cells/50mm.dish) were infected. After 5 hr. incubation at 37° half the number of cultures were treated with 2mM hydroxyurea. After a lapse of 15 min. half of the cultures were pulse-labelled with 10 μc of ^{3}H-dCyd (15.5Ci/m Mole) and the rest with 10 μc of ^{3}H-Cyd (4.5Ci/m Mole) for 1 hr. Non infected control cells were similarly treated. The total amount of radioactivity left in the medium of each culture and the distribution of radioactivity in acid-soluble and insoluble pools of DNA and RNA were estimated.

Radioactivity incorporated into DNA and RNA was estimated using a modified Schmidt-Thannhauser method. The cells were thrice washed with ice-cold BSS and transferred into conical tubes. They were then centrifuged at 600g for 10 min. and the supernatant decanted. The cell pellet was treated with 4 ml. 5% ice-cold TCA and the precipitate collected by centrifugation at 1,000g for 10 min. The supernatant was removed for the estimation of acid-soluble radioactivity. The precipitate was treated with 2 ml. 0.2 N PCA containing Hyflo Supercel and washed

thoroughly with ice-cold 5% TCA on filters. TCA was removed by washing with alcohol followed by ether. The precipitate was transferred to a conical tube and treated with 2 ml. 0.3 N KOH at 37°C for 18 hr. 0.2 ml. 6 N PCA was added and the suspension centrifuged at 1,000g for 10 min. The supernatant contained hydrolysed RNA and protein. Assuming that the amount of radioactive label incorporated into protein was negligible, this soluble material represented the total acid-insoluble RNA. The precipitate was washed with 5% TCA. alcohol and ether. This precipitate contained all acid-insoluble DNA. The radioactivity of different fractions was measured by liquid scintillation.

2.12. Measurement of Radioactivity

(i) ^{32}P was measured in a Nuclear Chicago low background, gas-flow counter with 50% efficiency.

(ii) ^{14}C- and ^{3}H-containing spots from chromatographic or electrophoretic separation were dried, cut out, immersed in 10 ml. toluene-based scintillation fluid and counted in a scintillation spectrophotometer with efficiencies of 75 and 25% respectively.

(iii) DNA containing ^{3}H or/and ^{14}C from CsCl gradients was precipitated with 5% TCA in the presence of denatured DNA carrier (50 μg./sample) and trapped on a Millipore membrane (pore size: 0.45 μ). The precipitate was washed with 3 x 10 ml. cold 5% TCA, dried and counted using toluene-based scintillator.

(iv) Whenever large volumes of radioactive liquid had to be counted dioxan-based scintillator was used instead of toluene-based scintillator.

IV. RESULTS

1. Induction of Enzymes Following Infection of BHK Cells by Herpes Virus

1.1. General Background

Following herpes virus infection, the levels of activities of DNA polymerase, DNase (Keir and Gold, 1963), dCMP deaminase (McGeoch and Keir, 1968), deoxythymidine kinase (Kit and Dubbs, 1963) and dTMP kinase (Newton, 1964) have been shown to increase. On the other hand, the activities of the kinases for dAMP, dCMP and dGMP remained relatively high and unaltered after infection with pseudorabies, a virus related to herpes simplex (Hamada, Kamiya and Kaplan, 1966).

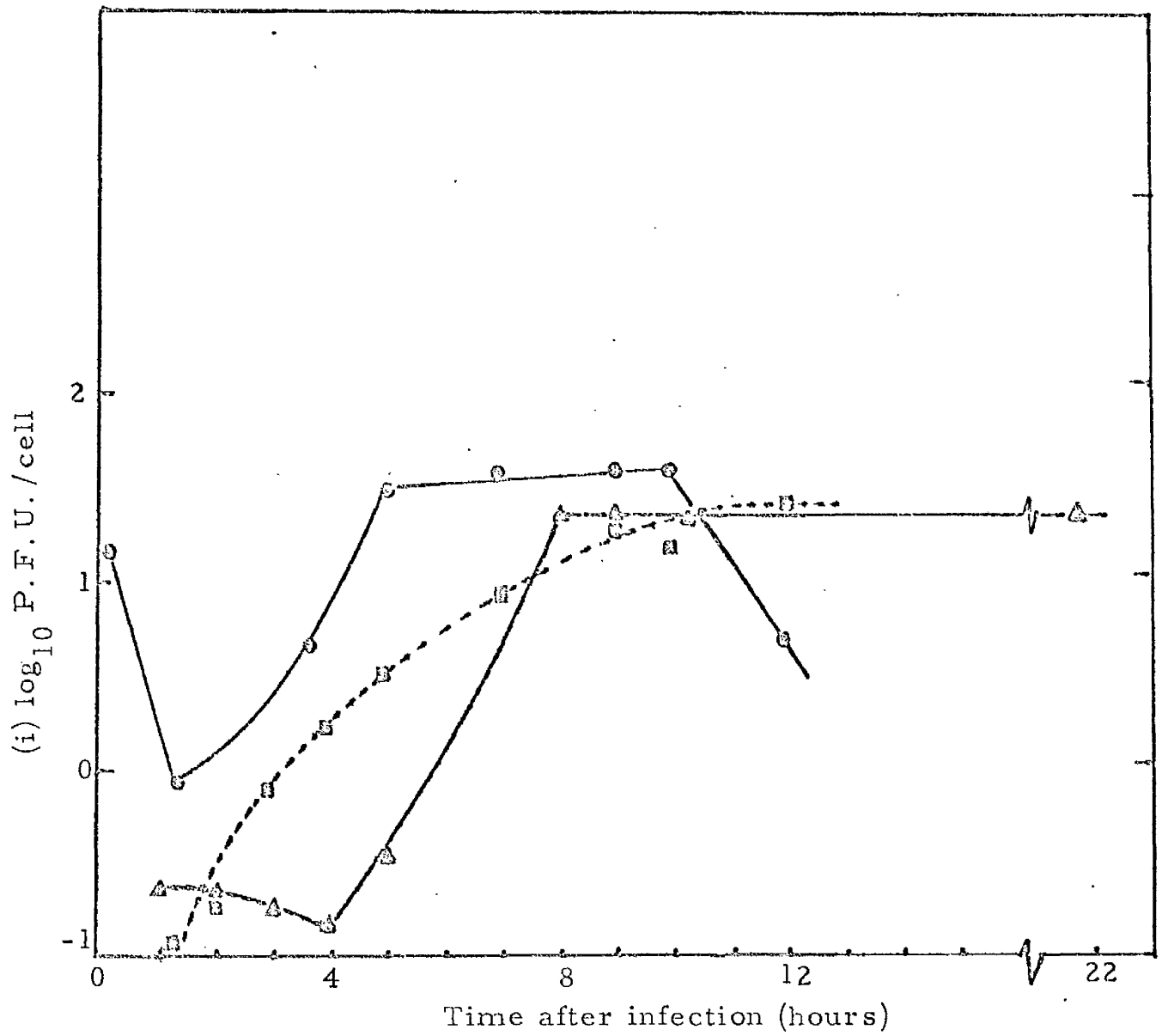
1.2. Purine Deoxyribonucleoside Kinases

The levels of activity of purine deoxyribonucleoside kinases, especially that for deoxyadenosine, were high both in high serum and low serum BHK cells. On infection of high serum cells, the levels of activity of these kinases remained unaltered.

On infection of low serum cells the activity of deoxyadenosine kinase dropped about 50% from its initial level of activity at a time corresponding to entry and uncoating of virions (Fig. IV. 1). Enzyme activity recovered after a short period. The time of recovery coincided with the lowest level of infectivity. The decrease in enzymatic activity could have been a consequence of partial inactivation of the enzyme by a viral component. On the other hand, the activity of dGuo kinase

Figure IV. 1.

Changes in the Levels of Activity of DNA Polymerase and Deoxyadenosine Kinase Following Herpes Infection of Resting BHK Cells



(i) Infectivity

(ii) DNA polymerase

(iii) dAdo kinase

decreased rapidly within the first four hours after infection and remained constant thereafter (Fig. IV.2). This differential effect of virus infection on dAdo and dGuo kinases suggests that two distinct proteins are responsible for the two kinase activities.

1.3. Pyrimidine Deoxyribonucleoside Kinases

On infection of high serum cells, the levels of activity of pyrimidine deoxyribonucleoside kinases were increased 10 -15 fold at 10 - 12 hr. post-infection, whilst low serum cells possessing undetectable amounts of pyrimidine deoxyribonucleoside kinases increased their enzyme activities to levels comparable with those of herpes infected high serum cells (Fig. IV.2). In these low serum cells the activities of dThd and dCyd kinases increased progressively till 8 hr. post-infection and then levelled off. The level of activity of dCyd kinase was higher than dThd kinase activity throughout the virus replication cycle. A close examination of the profiles of virus-induced pyrimidine deoxyribonucleoside kinases suggests that dCyd kinase activity may precede dThd kinase activity in the sequence of enzyme induction.

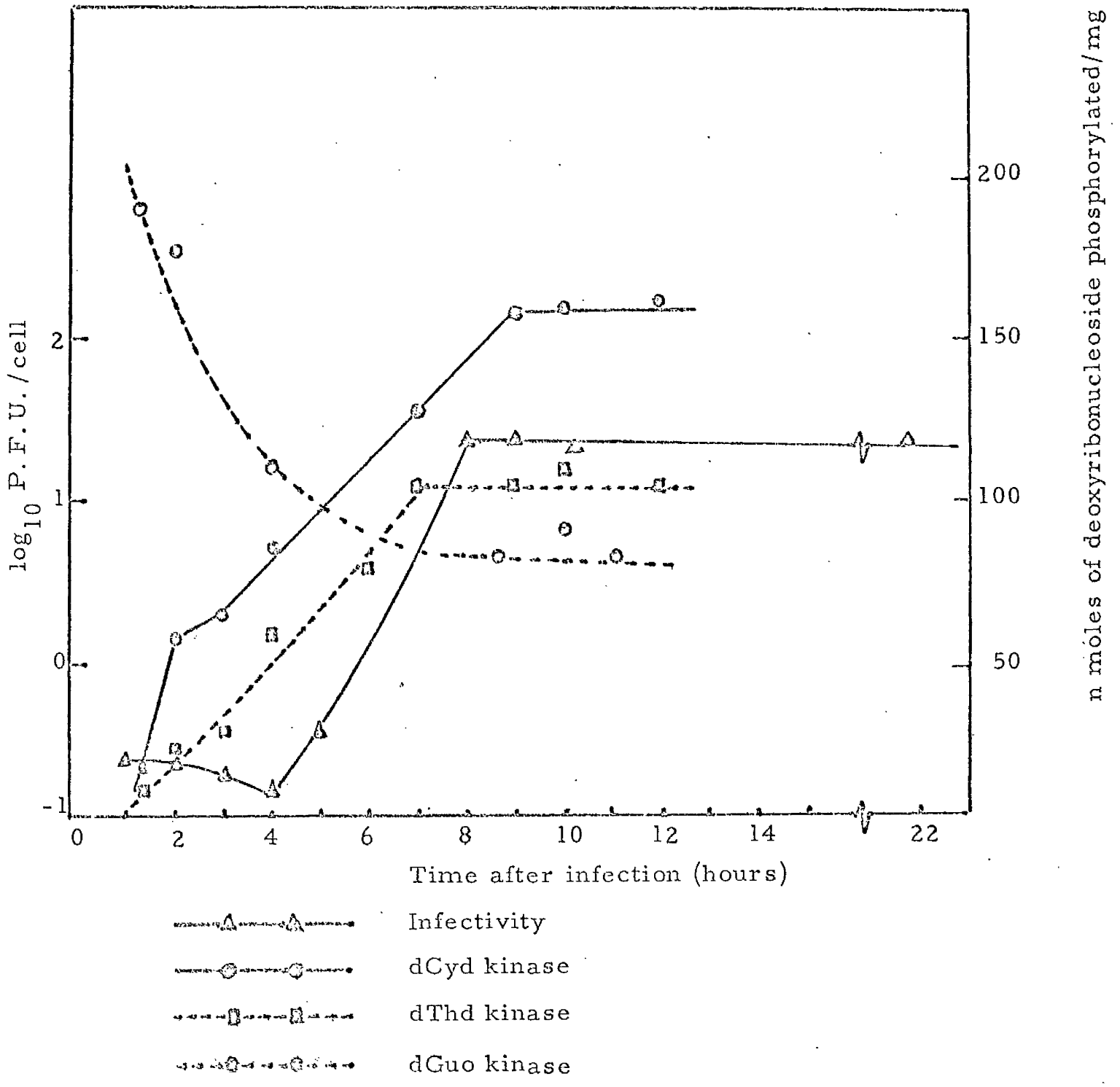
A two stage increase in enzymatic activity of dCyd kinase was observed between 1 - 2 and 3 - 4 hr. post-infection. This differential increase in dCyd kinase activity was repeatable. The significance of these two activities will be discussed later.

1.4. DNA Polymerase

The level of activity of DNA polymerase was very low in low

Figure IV. 2.

Changes in the Levels of Activity of Pyrimidine Deoxyribonucleoside Kinases and Deoxyguanosine Kinase Following Herpes infection of Resting BHK Cells



serum cells. On infection the level of activity rose 15 to 20 fold at 8 - 10 hr. post-infection (Fig. IV. 1). Further induction of enzyme was shut off after this period.

1.5. Other Enzymes

The level of activity of ATPase in high speed supernatant fractions of control and infected cells was too low to be detected under pyrimidine deoxyribonucleoside assay conditions. It has been established that actively growing cells of a variety of tissues contain lower levels of phosphatase than do non growing cells (Fiala et al., 1962; Eker, 1965).

1.6. Infectivity

In a typical viral growth curve, a lag of 4 hr. in the appearance of infectivity was observed corresponding to the uncoating of virions and the transcription of early functions of the viral genome (Fig. IV. 1). There was a rapid synthesis of infective particles from 5 to 8 hr. after infection. The maximum yield of infective particles in low serum cells was about one log unit lower and was reached 2 to 4 hr. earlier than that for high serum cells. The infectivity to particle ratio for herpes virions synthesised was approximately 1:10. Thus, the amount of viral DNA synthesised was at least 10 fold higher than that represented by infectivity measurements.

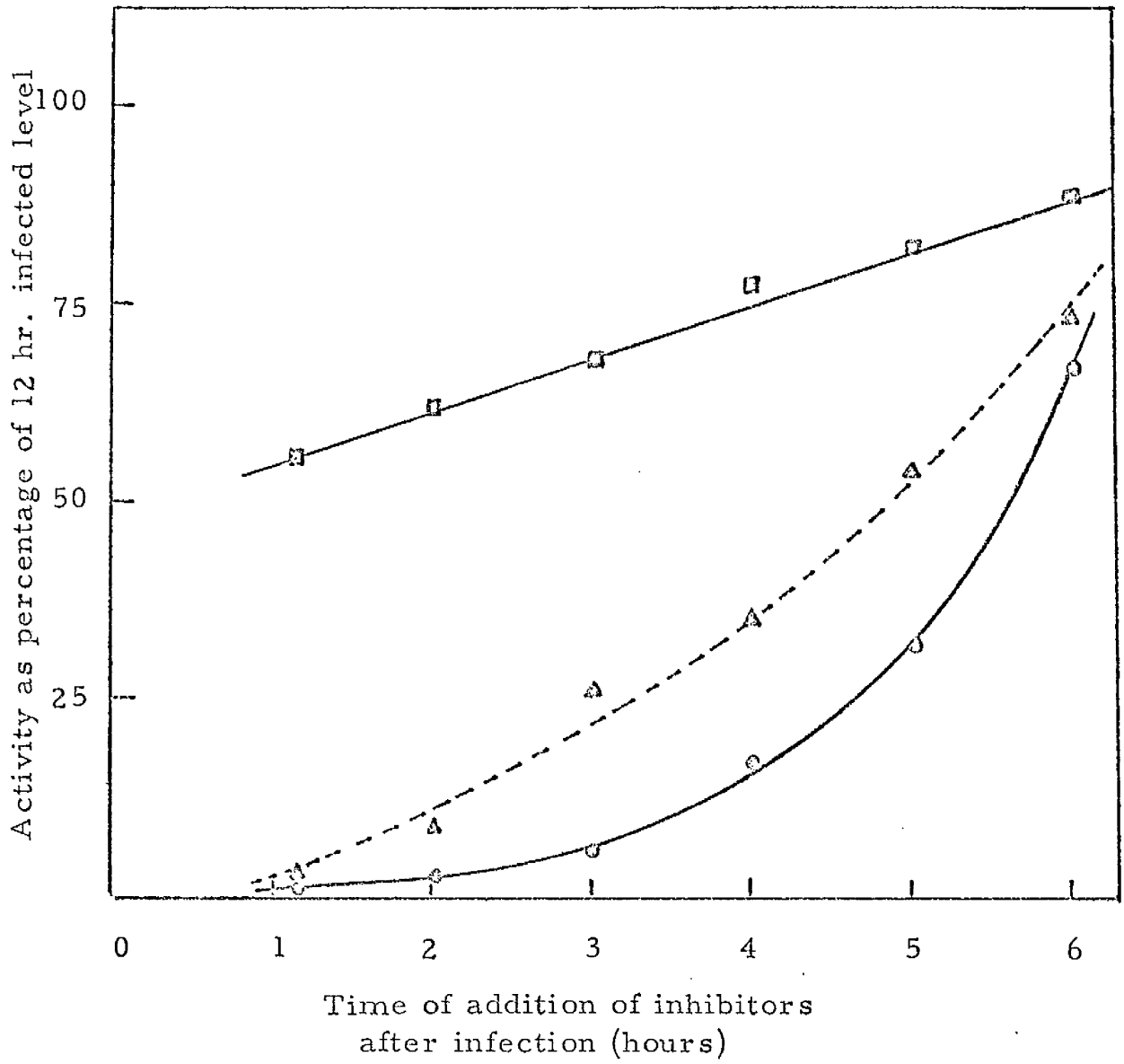
2. Effect of Inhibitors of Macromolecular Synthesis on Enzyme Induction

2.1. DNA Synthesis

Hydroxyurea, a potent inhibitor of DNA synthesis, has little

Figure IV. 3.

Effect of Time of Addition of Hydroxyurea, Actinomycin D and Puromycin on the Induction of Alkaline DNase by Herpes Virus



—□—□— 2mM Hydroxyurea

—Δ—Δ— Actinomycin D (0.5 μg/ml)

—o—o— Puromycin (50 μg/ml)

or no effect on RNA or protein synthesis (Yarbo, Kennedy and Barnum, 1965). The inhibition of DNA synthesis has been suggested as being due to inhibition of de novo synthesis of deoxyribonucleoside diphosphates from their respective ribonucleoside diphosphates via the ribonucleotide reductase pathway (Krakoff, Brown and Reichard, 1968).

2.1.1. Effect of Hydroxyurea on DNase Activity

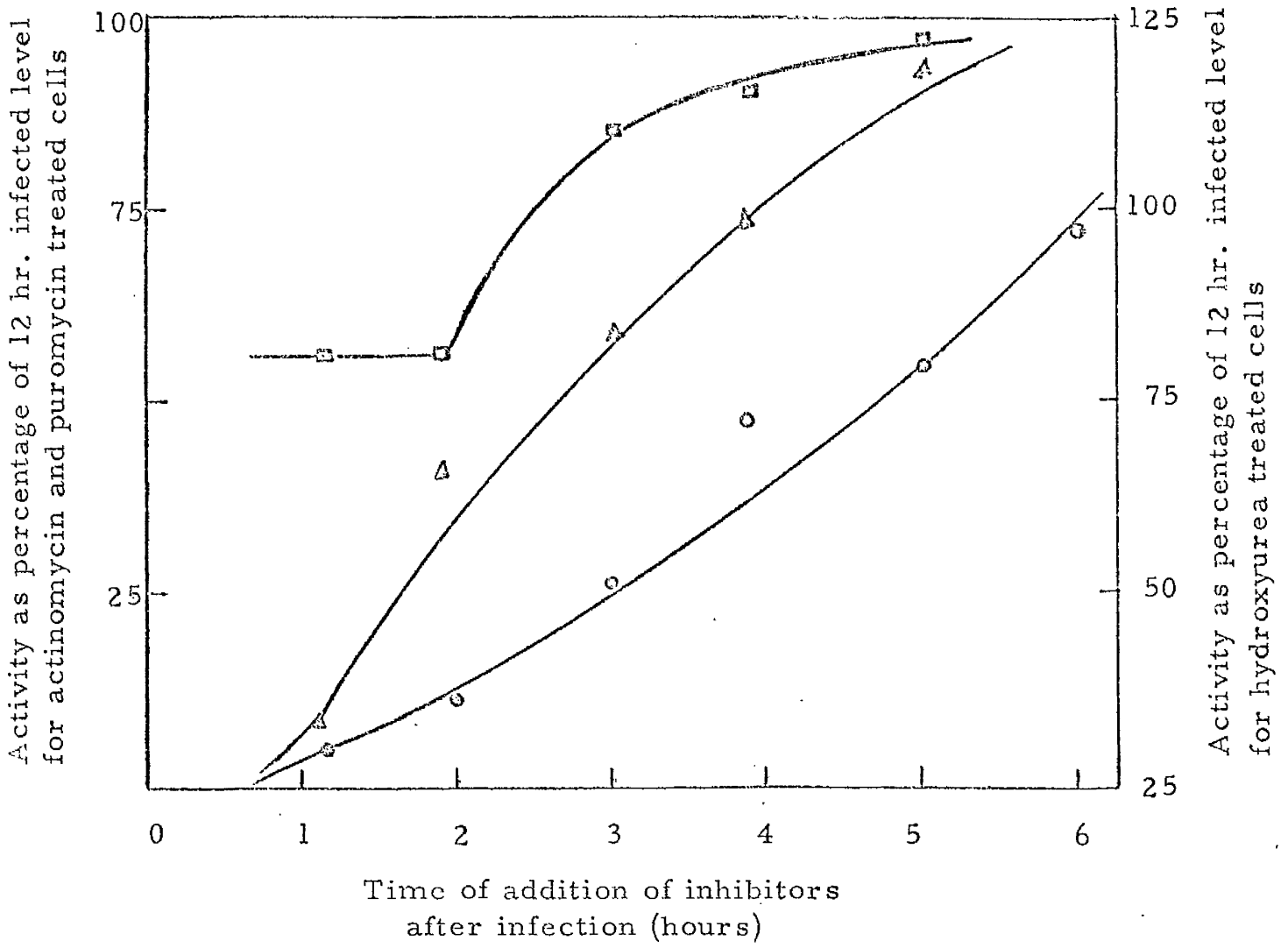
Uncoating of viral capsid is almost complete at 3 hr. post-infection (Holmes and Watson, 1963). Addition of 2mM-hydroxyurea at this juncture reduced the 12 hr. enzyme activity by only about 30% (Fig. IV.3), suggesting, that DNA synthesis is not necessary for the induction of alkaline DNase.

2.1.2. Effect of Hydroxyurea on Deoxycytidine Kinase Activity

Addition of hydroxyurea at 1 - 2 hr. post-infection reduced the 12 hr. enzyme activity of dCyd kinase by 20% (Fig. IV.4). Addition of the inhibitor later than 3 hr. post-infection increased the activity of dCyd kinase at 12 hr. post-infection, suggesting a possible regulatory role of deoxynucleoside-triphosphates on the level of virus-induced dCyd kinase activity. If hydroxyurea had inhibited the synthesis of deoxy-ribonucleoside diphosphates via the ribonucleotide reductase pathway, then a fall in the level of deoxyribonucleoside triphosphates in the acid-soluble pool would be inevitable. This could relieve any inhibitory effect imparted by dTTP and dCTP on the regulation of dCyd kinase synthesis and hence impart an indirect stimulatory effect.

Figure IV. 4.

Effect of Time of Addition of Hydroxyurea, Actinomycin D and Puromycin on the Induction of Deoxycytidine Kinase by Herpes Virus



2.2. RNA Synthesis

Actinomycin D inhibits DNA-dependent RNA synthesis (Goldberg et al., 1962) by binding to the DNA template and thus inhibiting transcription. Among the four bases of DNA, deoxyguanosine shows the strongest affinity towards the inhibitor (Kersten, 1961). Since the guanine content of viral DNA is significantly higher than that of cellular DNA, a higher number of binding sites would be available for stable complex formation with actinomycin.

2.2.1. Effect of Actinomycin D on DNase Activity

Addition of actinomycin D (0.5 μg./ml.) at 1 hr. post-infection completely inhibited the induction of alkaline DNase activity (Fig. IV.3). The degree of inhibition decreased progressively thereafter when actinomycin was added at later times after infection. In the 4 - 6 hr. period the rate of release of DNase activity from inhibition as a function of the 12 hr. enzyme level was a maximum.

2.2.2. Effect of Actinomycin D on Deoxycytidine Kinase Activity

Addition of actinomycin D at 2 hr. post-infection reduced the 12 hr. level of dCyd kinase activity to only 40% (Fig. IV.4), unlike alkaline DNase activity which was decreased to 8%. This remarkable difference in the pattern of induction possibly reflects the order of transcription of genes corresponding to the various virus-induced enzymes. The rate of release of dCyd kinase activity from inhibition

measured as a function of 12 hr. level of activity was a maximum between 1 - 2 hr. after infection. In addition, there was another reproducible increase in activity between 3 - 4 hr. post-infection. The possible significance of this two-stage increase will be discussed later.

2.3. Protein Synthesis.

It has been suggested that puromycin inhibits protein synthesis by causing the premature release of incomplete polypeptide chains from ribosomes (Rabinovitz and Fisher, 1962).

2.3.1. Effect of Puromycin on DNase Activity

Puromycin completely prevented the induction of alkaline DNase when added at 1 - 2 hr. post-infection (Fig. IV.3). Thereafter, the amount of inhibition decreased progressively with the addition of inhibitor at later times after infection. The maximum rate of release from inhibition was achieved between 5 - 6 hr. post-infection. This increase in enzyme activity appears to follow transcription of messenger for the virus-induced DNase. (Fig. IV.3).

2.3.2. Effect of Puromycin on Deoxycytidine Kinase Activity

Addition of puromycin at 1 hr. post-infection reduced the 12 hr. level of dCyd kinase activity to 5% of control. The maximum rate of release from inhibition took place between 3 - 4 hr. after infection, although the rate of release from inhibition between 5 - 6 hr. post-infection was also relatively high and may be significant in terms

of enzyme induction. (see discussion).

3. Distribution of ^{3}H-Deoxycytidine and ^{3}H-Cytidine in Control and Herpes-infected Cells

3.1. Distribution of ^{3}H-Deoxycytidine

The uptake of ^{3}H-dCyd into the acid-soluble and insoluble fractions of BHK cells increased about 4-fold at 6 hr. post-infection (Table IV. 5). Addition of 2mM-hydroxyurea did not appreciably alter the uptake of this labelled precursor into the acid-soluble fraction of infected cells when compared with the 8-fold stimulation it caused in control cells. The inhibitor, however, did cause inhibition of the rate of DNA synthesis in both control and infected cells. These results taken together indicate the possibility that (i) virus-induced dCyd kinase may be less susceptible to feedback control mechanisms involving deoxynucleoside triphosphates, and/or (ii) degradation of host DNA may provide precursors for at least part of the deoxyribonucleoside triphosphat pool.

3.2. Distribution of ^{3}H-Cytidine

The uptake of ^{3}H-Cyd into acid-soluble and insoluble fractions of control and infected cells was not affected by treatment of cells with 2mM-hydroxyurea. The rate of RNA synthesis of control cells dropped to 50% at 6 hr. after infection with herpes virus. Treatment of infected cells with hydroxyurea reduced the incorporation of cytidine into DNA by 90% (Table IV. 6). These results suggest that hydroxyurea had

Table IV. 5.

Distribution of ^{3}H-dCyd into Acid-Soluble and Insoluble Fractions Following Treatment of Control and Infected Cells with Hydroxyurea

Sample	Radioactivity in medium	Acid-soluble material	Acid-Insoluble Material	
			DNA	RNA
	x 10^{5} d.p.m.	x 10^{5} d.p.m.	x 10^{5} d.p.m.	x 10^{5} d.p.m.
Control	228	1.59	0.47	0.08
"	227	1.72	0.45	0.07
Infected	228	7.77	1.99	0.13
"	216	7.75	1.74	0.12
Control + hydroxyurea	219	13.17	0.18	0.09
"	231	13.26	0.19	0.09
Infected + hydroxyurea	235	8.62	0.58	0.06
"	231	8.36	0.57	0.06

Table IV. 6.

Distribution of ^{3}H-Cyd into Acid-Soluble and Insoluble Fractions Following Treatment of Control and Infected Cells with Hydroxyurea

Sample	Radioactivity in medium	Acid soluble material	Acid-Insoluble Material	
			RNA	DNA
Control	x 10^5 d.p.m. 150 146	x 10^5 d.p.m. 23.0 19.7	x 10^5 d.p.m. 42.3 40.5	x 10^5 d.p.m. 0.33 0.32
Infected "	161 160	29.7 28.9	20.6 20.4	0.65 0.50
Control + hydroxyurea "	150 152	24.5 24.3	37.9 41.1	0.06 0.06
Infected + hydroxyurea "	148 147	33.2 33.0	19.9 19.8	0.06 0.06

specifically affected the ribonucleotide reductase pathway in its inhibitory action on the DNA synthesis in both control and virus-infected cells.

4. Effect of Multiplicity of Infection

4.1. High Serum Cells

On infection of BHK cells with herpes virus, the levels of DNA polymerase, alkaline DNase and deoxycytidine kinase increased progressively with both the multiplicity of infection and the time period after infection (Figs. IV, 6, 7 and 8). At 12 hr. post-infection, the levels of activities of DNA polymerase and alkaline DNase had reached a maximum at an input multiplicity of 10 P. F. U. / cell and were not increased by further increases in multiplicity or in time after infection. This would seem to indicate that the expression of viral genome for these early enzymes was complete at 12 hr. post-infection and that further synthesis had been shut off. The inability of large populations of m RNA coding for early enzymes to boost up enzyme activity at 12 hr. post-infection may reflect the saturation of polyribosomes with m RNA. At 6 hr. post-infection, however, the increase in the levels of activities of these enzymes was related to the input multiplicity, suggesting that the most likely effect of the presence of a large number of viral genomes per cell is to provide larger populations of messengers for either activators or virus induced enzymes.

Figure IV. 7.

Effect of Multiplicity of Infection on the Induction of DNA Polymerase in High Serum Cells

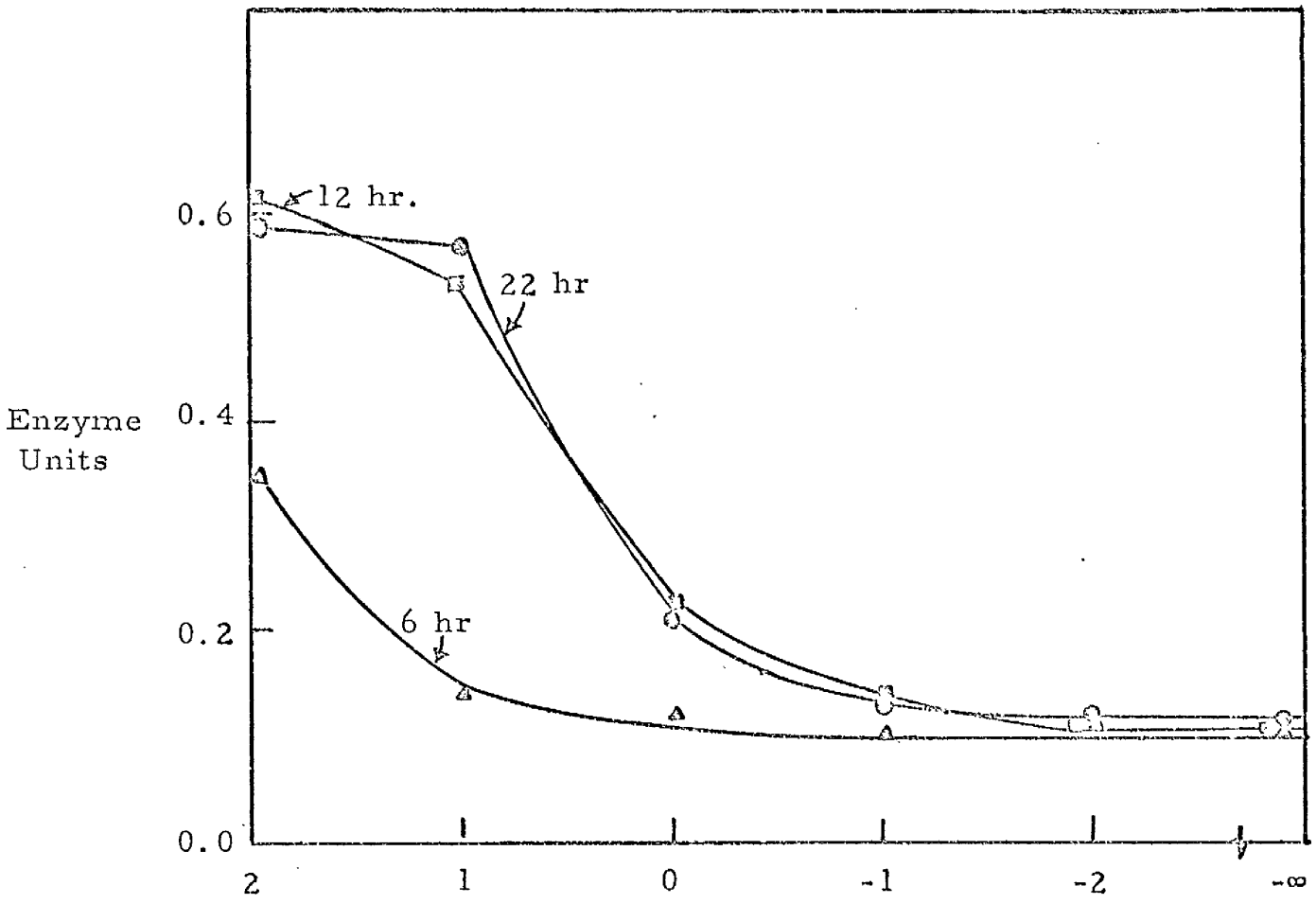


Figure IV. 8.

Effect of Multiplicity of Infection on the Induction of Alkaline DNase in High Serum Cells

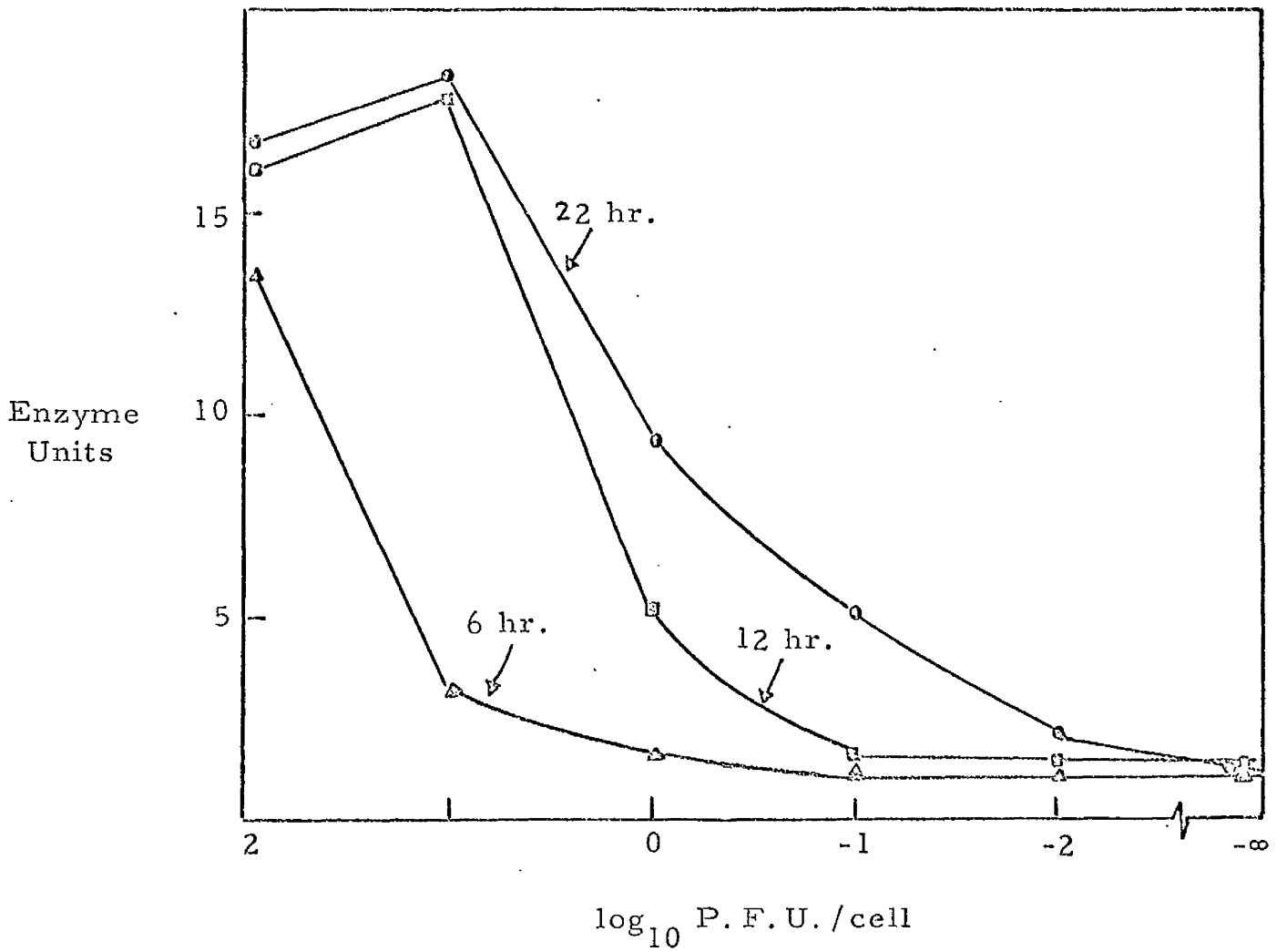
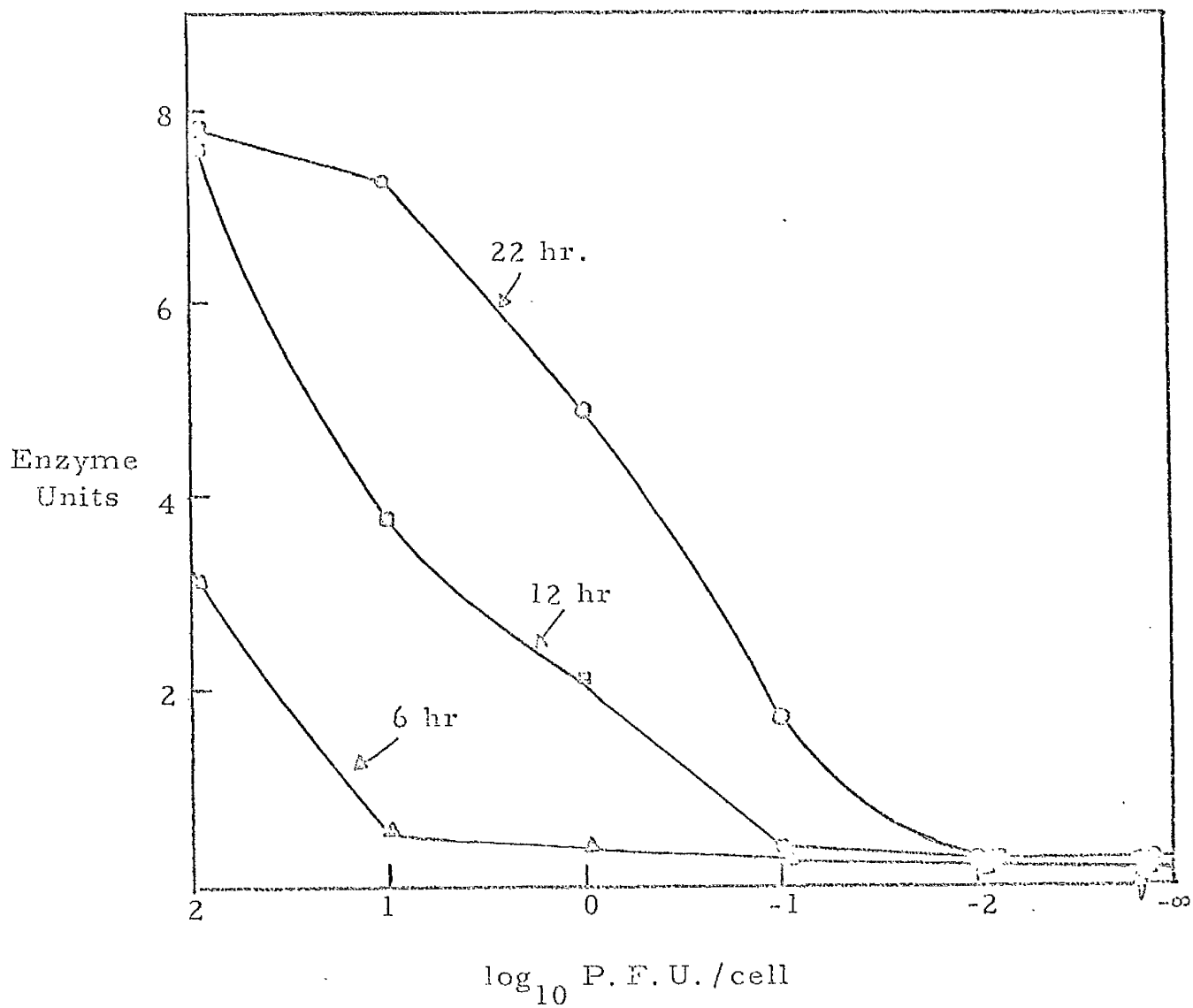


Figure IV. 9.

Effect of Multiplicity of Infection on the Induction of Deoxycytidine Kinase in High Serum Cells



NOTE:- The activity of dCyd kinase at 10 P.F.U./cell at 12hr. post infection in this experiment does not support the proposition of a cut off mechanism for dCyd kinase activity at 12hr. post infection. But it is possible that "cut off" mechanism for dCyd kinase in this experiment has been delayed.

In the case of virus-induced deoxycytidine kinase (Fig. IV. 9) the increase in activity of enzyme was related to the multiplicity of exposures up to a period of 12 hr. But thereafter, no further increase in enzyme activity was observed with increasing multiplicity of infection (> 10 P. F. U. / cell). At low multiplicities of infection, the time taken to acquire the maximum level of enzyme activity varied with the multiplicity of exposure of cells to virus infection, suggesting the possibility that the shut off mechanism was either dependent on the level of enzyme activity or the density of population of a virus specified late m RNA species.

4.2. Low Serum Cells

The maximum levels of virus-induced enzymes were obtained at 12 hr. post-infection at a multiplicity of exposure of 10 P. F. U. / cell (Figs. IV, 10, 11 and 12). Low serum cells were low in metabolic activity and were relatively fragile. At high doses of infectivity the cells were damaged and the induction of enzymes in such systems was very low and erratic, probably due to leakage of protein. This was clearly indicated by the enzyme activities observed at 6, 12 and 22 hr. post-infection after exposure of cells to high multiplicity of infection (100 P. F. U. / cell). These observations suggest that induction of enzymes by herpes was not only dependent on the multiplicity of infection but also on the state of cells in culture and their immediate

Figure IV. 10

Effect of Multiplicity of Infection on the Induction of DNA Polymerase in Resting Cells

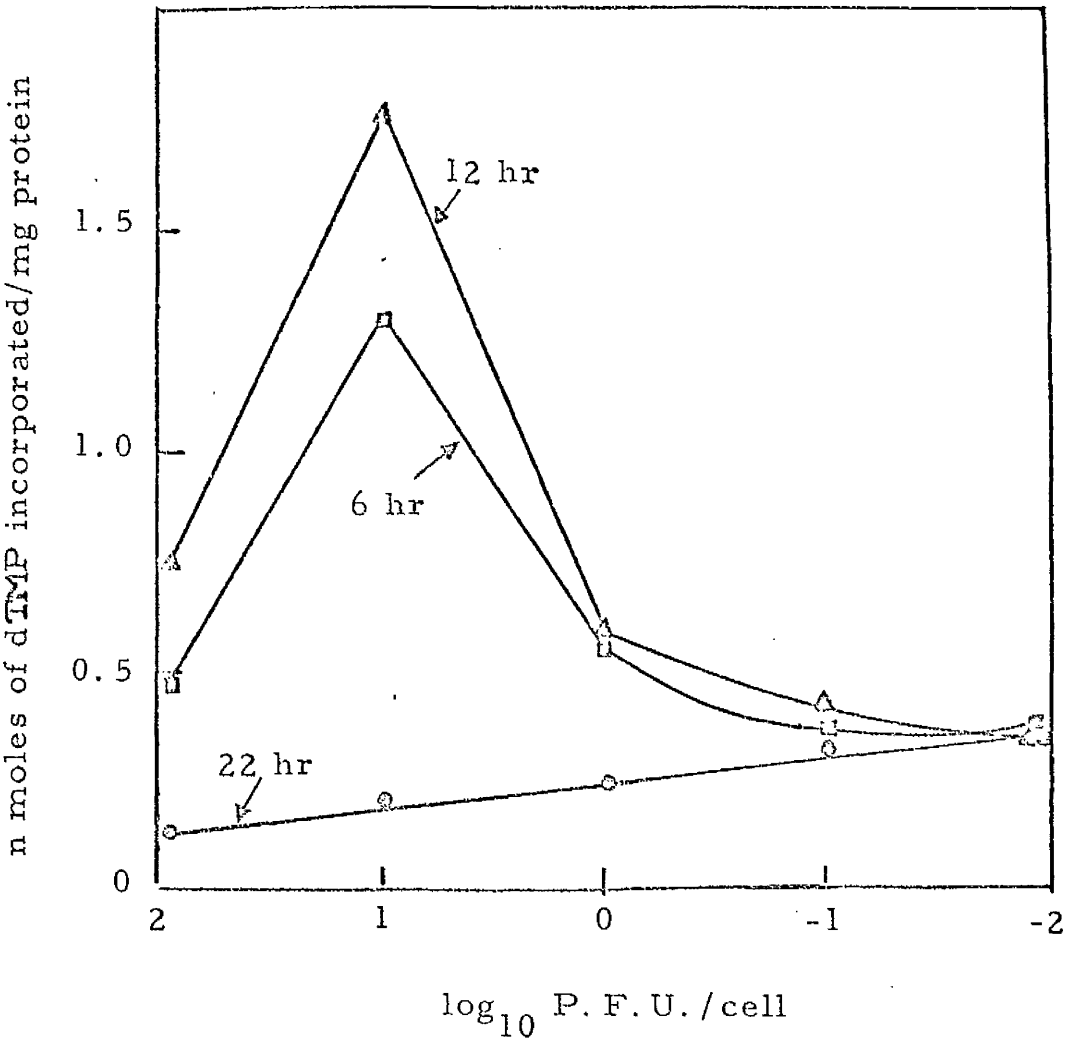


Figure IV. 11

Effect of Multiplicity of Infection on the Induction of Alkaline DNase in Resting Cells

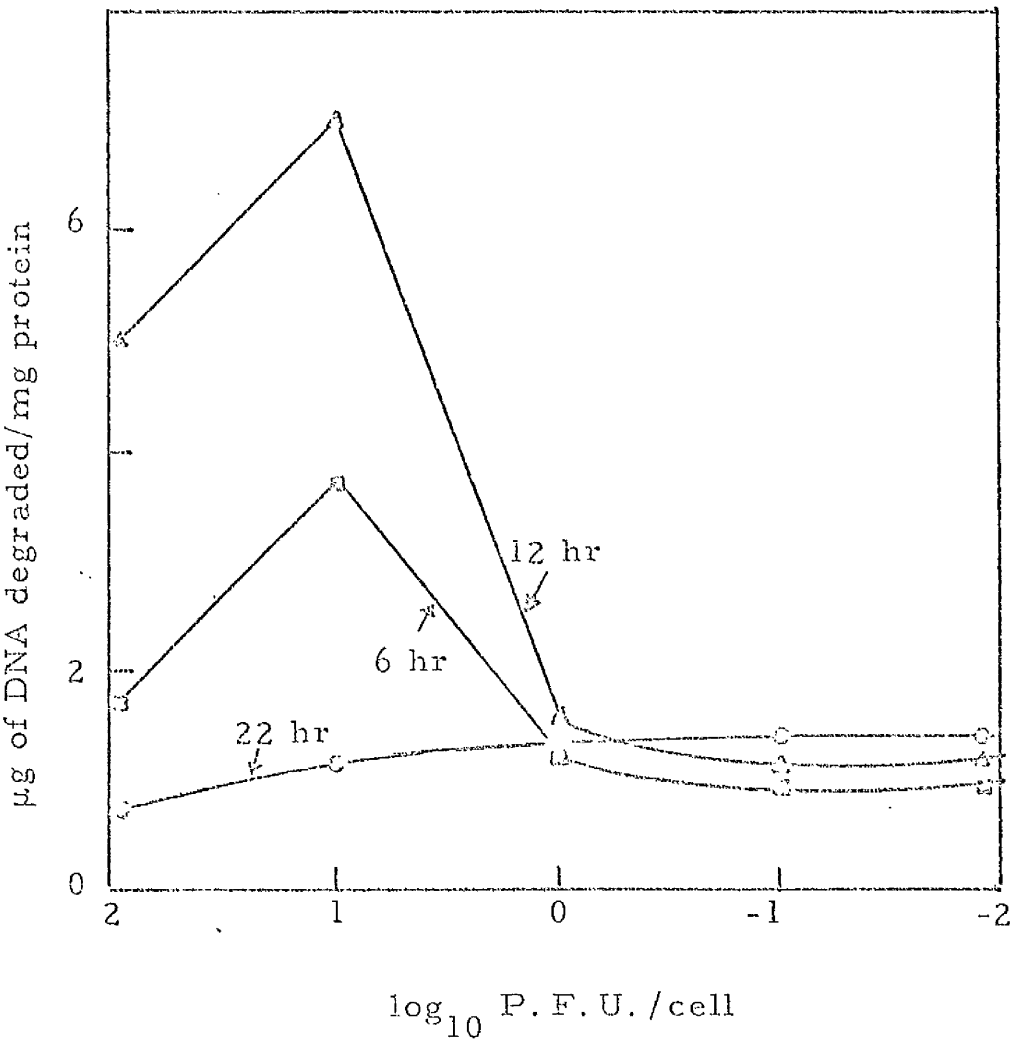
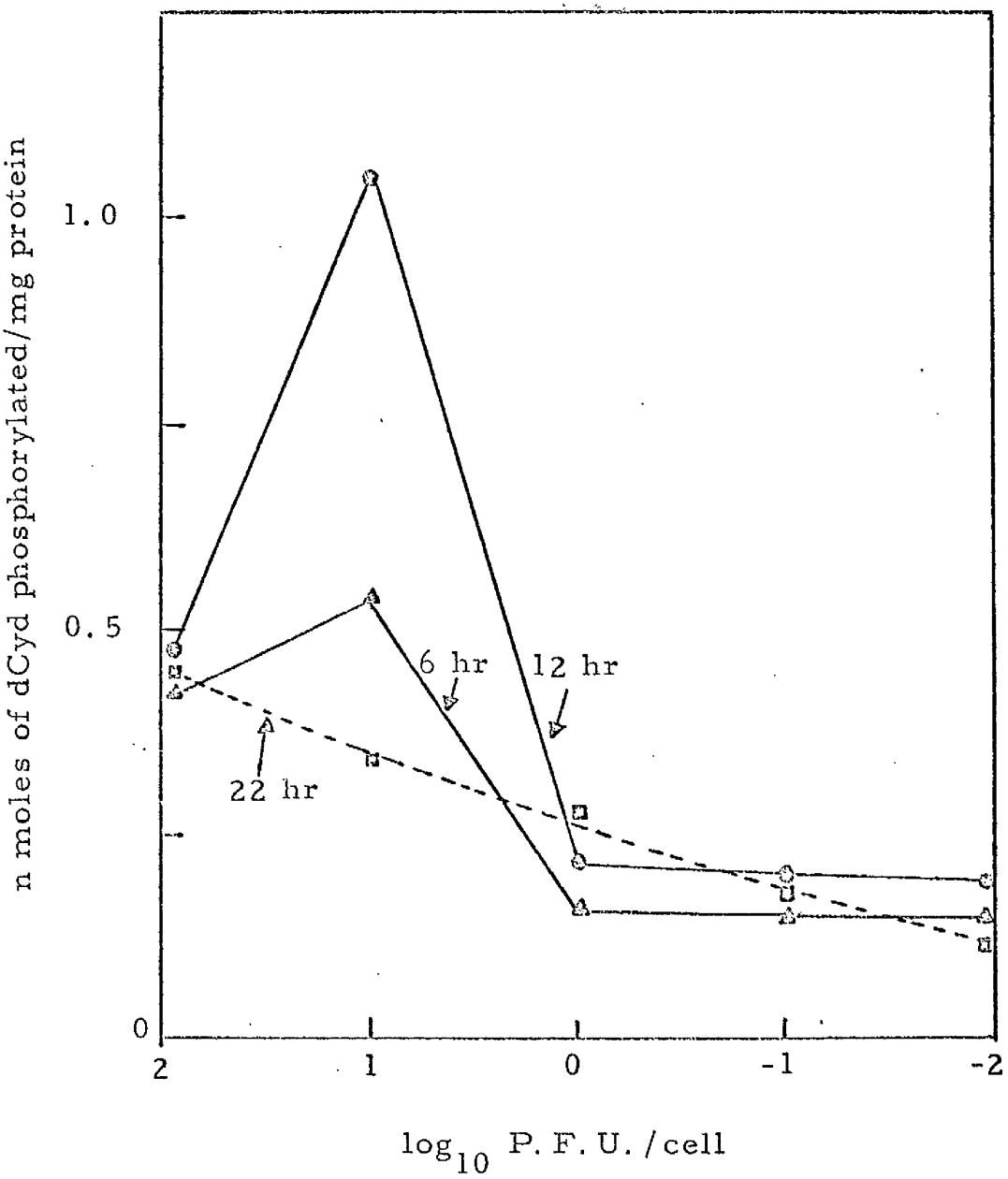


Figure IV. 12.

Effect of Multiplicity of Infection on the Induction of Deoxycytidine Kinase in Resting Cells



nutrient environment. The inhibition of enzyme activity of early enzymes at high multiplicity of infection may be a direct consequence of the presence of large amounts of capsid proteins which may damage the cell membrane and cause the leakage of these enzymes.

Results from both high serum and low serum cells suggest that the primary cause for the induction of enzymes is the invasion of the host cell by infectious virus particles and that the contribution of non infectious particles is less significant in this respect. Therefore, it seems likely that a large proportion of non infectious particles are incapable of carrying out such viral functions.

5. Kinetic Studies on Purine Deoxyribonucleoside Kinases

5.1. Time Course

The enzymatic phosphorylation of dAdo was linear with respect to time up to about 40 min. under the standard assay conditions (Fig. IV. 13). The stepwise phosphorylation of dAMP to dADP and dATP was relatively low under the assay conditions and did not affect the activity of dAdo kinase.

5.2. pH Optimum

The optimum for the assay of dGuo kinase of control cells was pH 7.0 (Fig. IV. 14). There was no change observed in the profile of this curve after virus infection. Unlike dGuo kinase, dAdo kinase had a broad optimum with a maximum at pH 7.2 (Fig. IV. 15). After

Figure IV. 13.

Phosphorylation of Deoxyadenosine by Control Cell Extracts

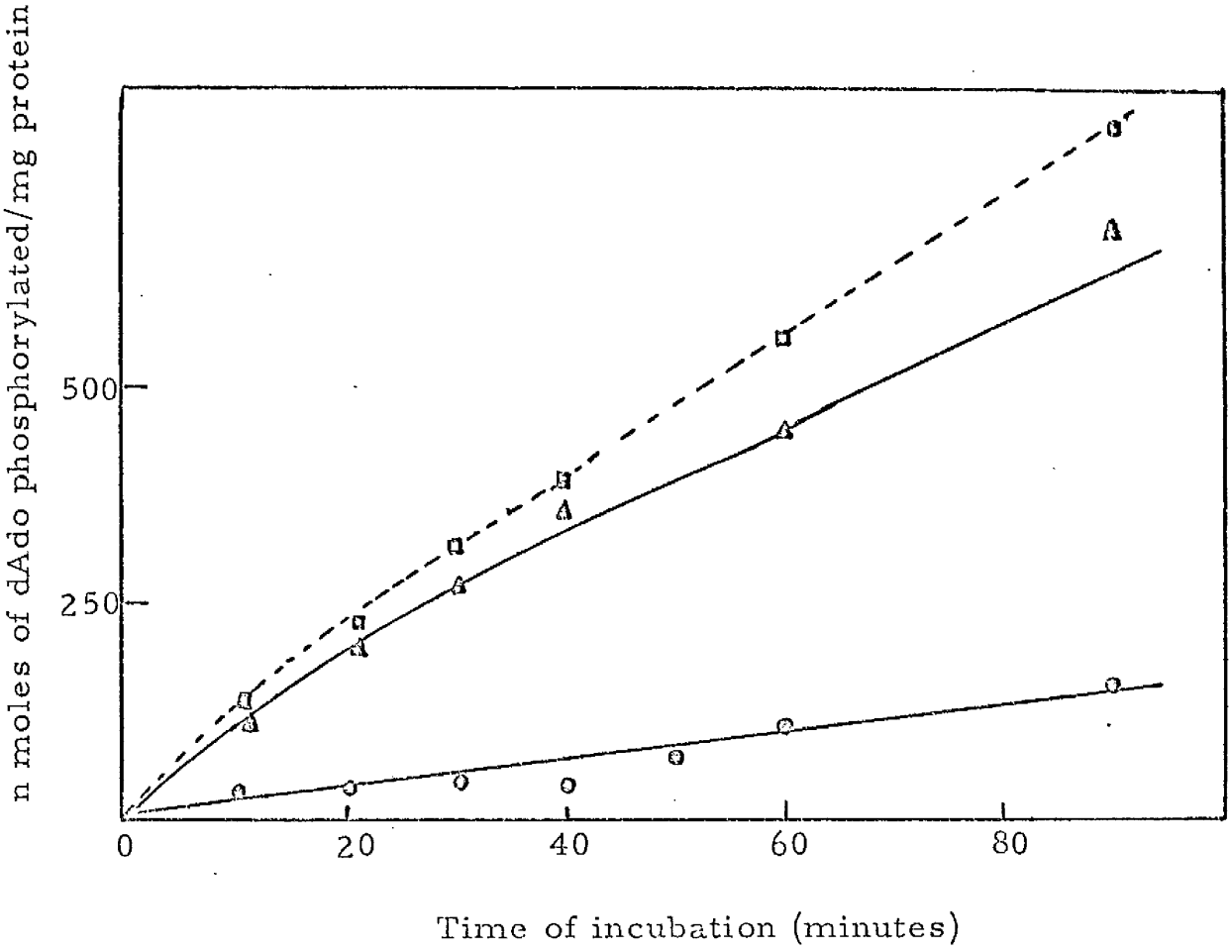


Figure IV. 14.

pH Optimum for Deoxyguanosine Kinase Activity of Control Cells

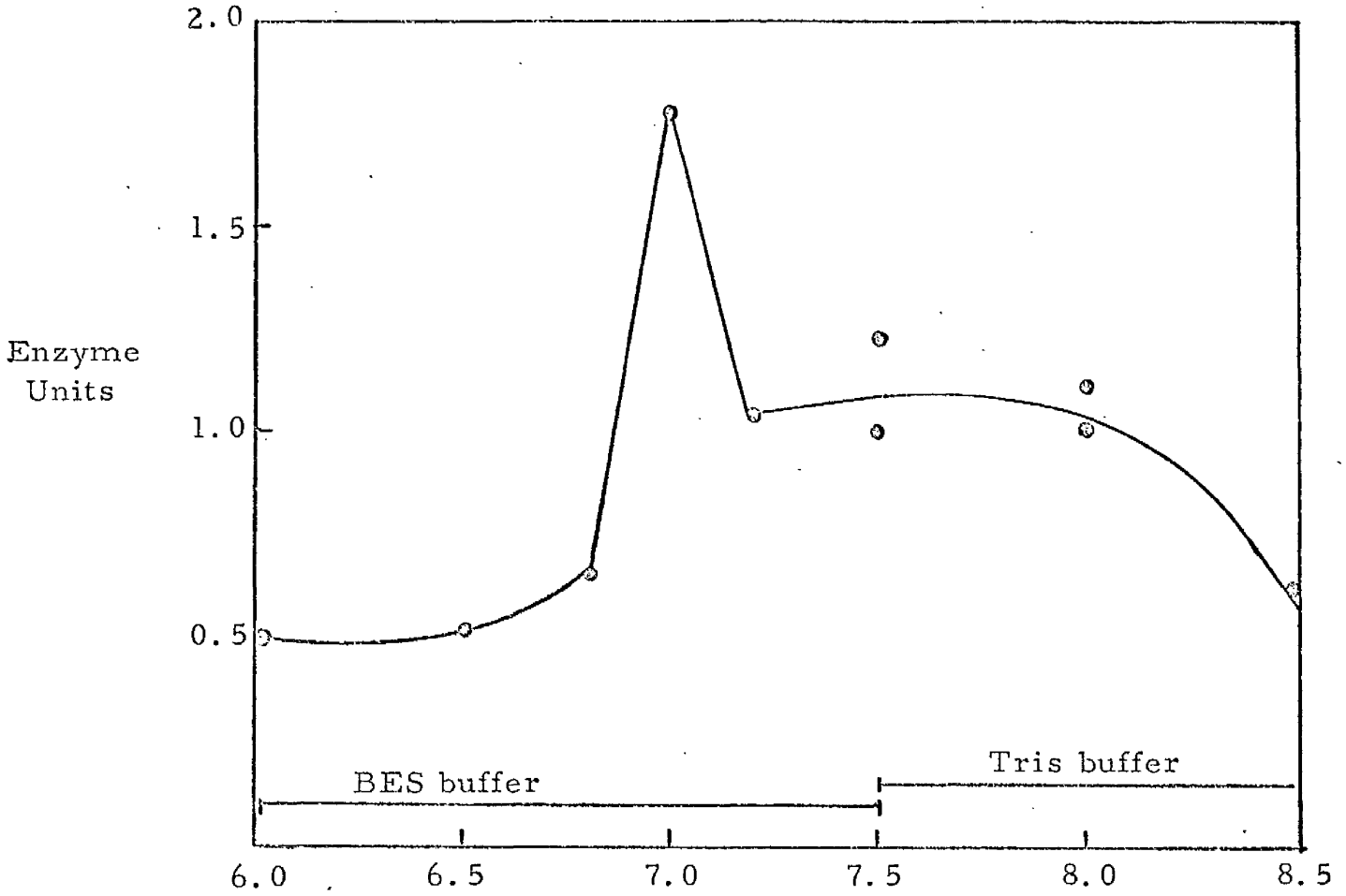
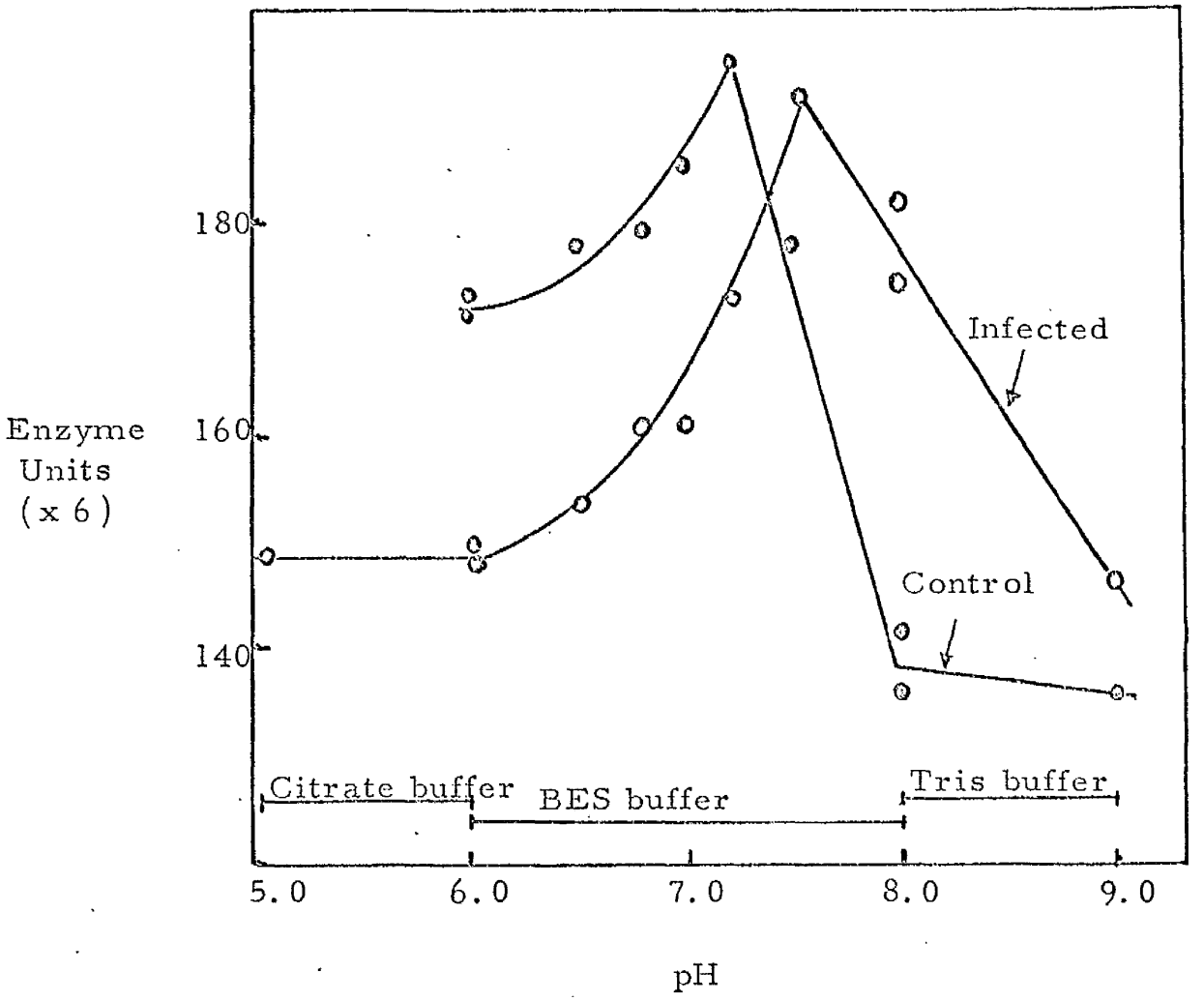


Figure IV. 15.

pH Optima for Deoxyadenosine Kinase Activities of Control and Infected Cells



infection, the pH optimum was shifted slightly to pH 7.5; the significance of this change is not clear.

5.3. Mg^{2+} Requirement.

High speed supernatant fractions which had been extensively dialysed and passed through G-25 Sephadex showed no requirement for Mg^{2+} ions in the purine deoxyribonucleoside kinase assay (Fig. IV.16). This finding was similar to the observation made by Schnebli, Hill and Bennett (1967) on purified adenosine kinase.

After treatment of a cell extract with 0.1mM p-chloromercuric-benzoate at 4° for 10 min. followed by dialysis against 200 volumes of 50mM-BES buffer pH 7 containing 1mM-EDTA, the enzyme showed a requirement for Mg^{2+} for optimal activity. Addition of Mg^{2+} alone gave only 20% activity. But in the presence of 1mM-mercaptoethanol and Mg^{2+} 2/3 of the original activity was recovered (Murray, 1968). This would seem to indicate that the enzyme had been partially inactivated during pCMB treatment with the release of enzyme-bound divalent cations. The optimum Mg^{2+} concentration for the assay of deoxy-purine kinases was equal to the concentration of ATP used in the assay mixture (6mM).

5.4. ATP Requirement

It was observed that even in the absence of exogenous ATP, crude dialysed enzyme preparations phosphorylated deoxyribonucleosides to their respective deoxyribonucleotides. This suggested the presence

Figure IV. 16.

Effect of Exogenous Mg^{2+} on the Deoxyadenosine Kinase Activity of Dialysed Control Cell Extracts

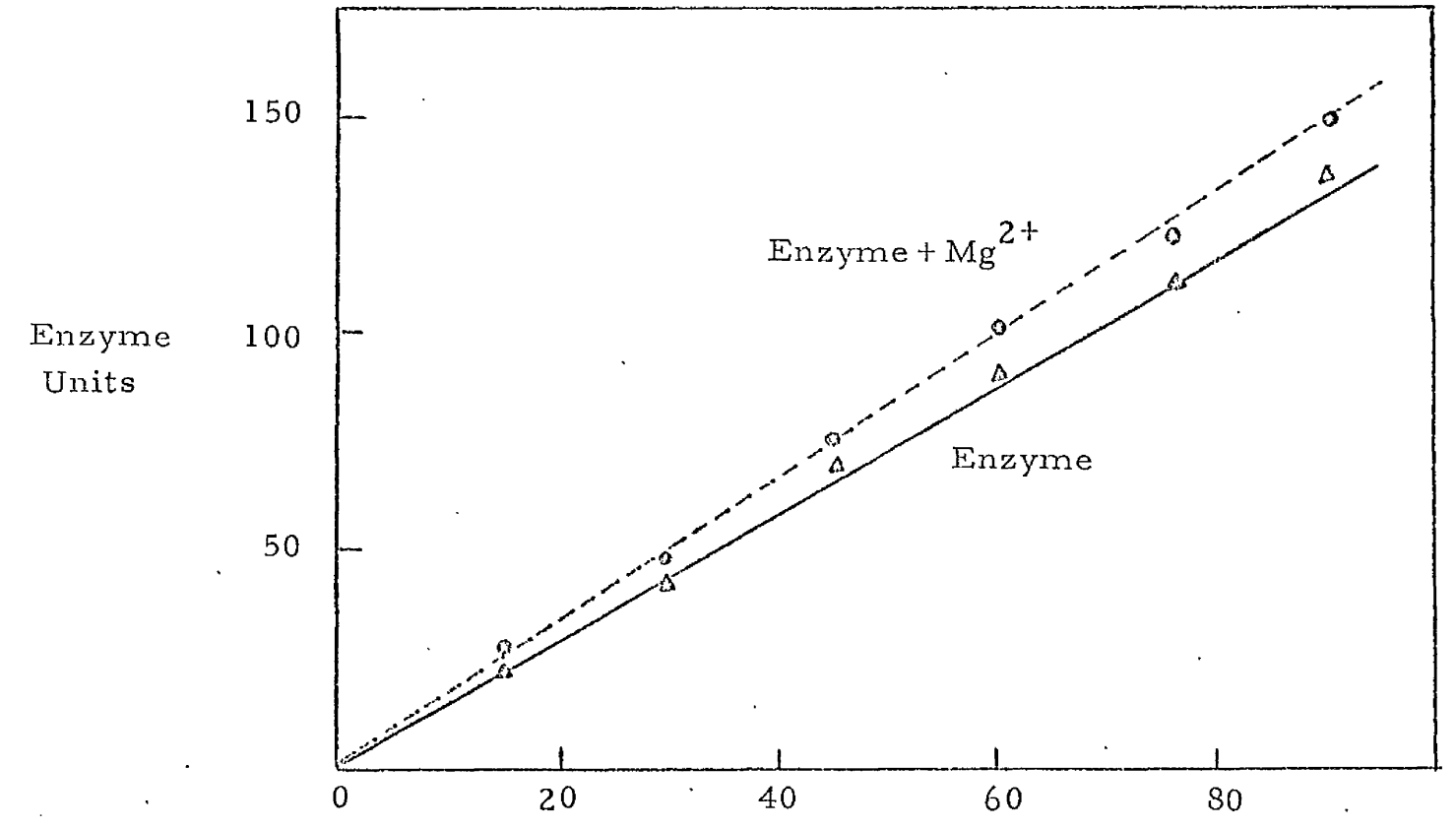


Figure IV. 17

Chromatographic Separation of ATP-bound protein and ATP using a Sephadex G-50 Column

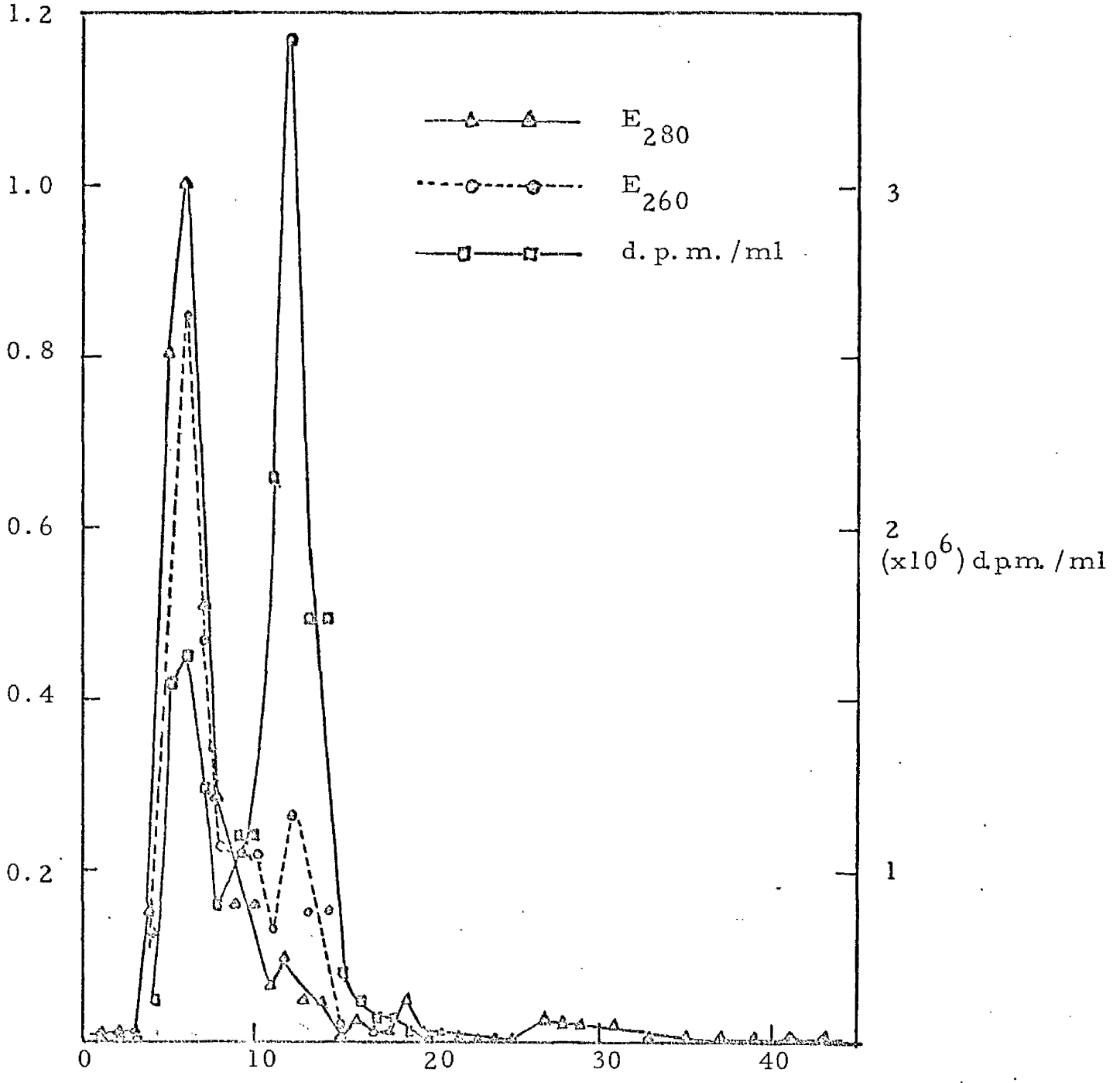
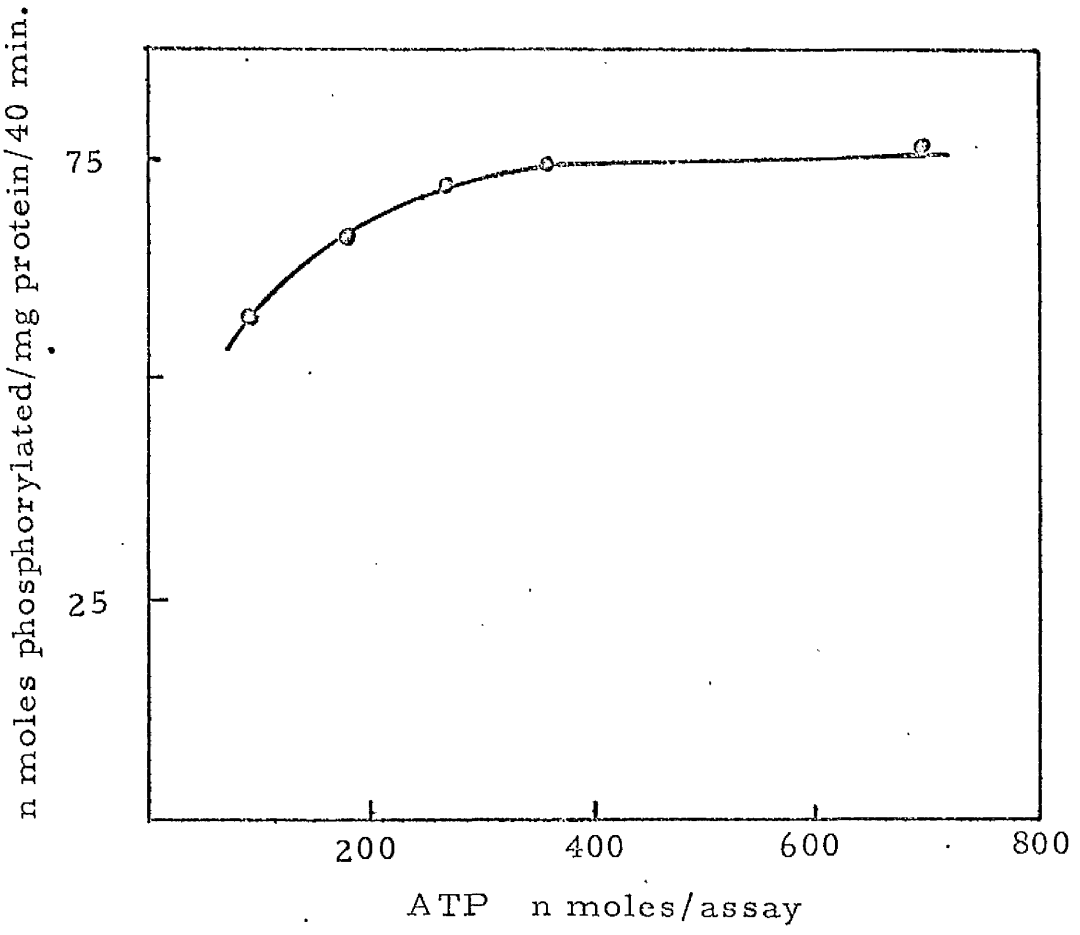


Figure IV. 18.

Effect of ATP Concentration on the Deoxyadenosine Kinase Assay



90 n moles of dAdo was used/assay

of a protein with associated ATP, which could be used for phosphorylation. To test this postulate an extensively dialysed enzyme preparation was incubated with ^{3}H-ATP for 1 hr. at 4°, after which part of it was subjected to gel filtration on G-50 while the rest was dialysed overnight against 0.05M-BES buffer containing 5mM-mercaptoethanol. In both cases, 30% of ^{3}H-ATP was bound to protein in both control and infected cell extracts; 0.1 n mole ATP per mg. protein being bound under these conditions.

The rate of phosphorylation with ATP concentrations below 1mM was less than the optimum. But at concentrations above 1mM the rate of phosphorylation was independent of ATP concentration. For assaying purine deoxyribonucleoside kinases 6mM-ATP was used in the final assay mixture. During all kinase assays percentage phosphorylated was assessed after making necessary corrections for the activity at zero time.

5.5. Michaelis Constant

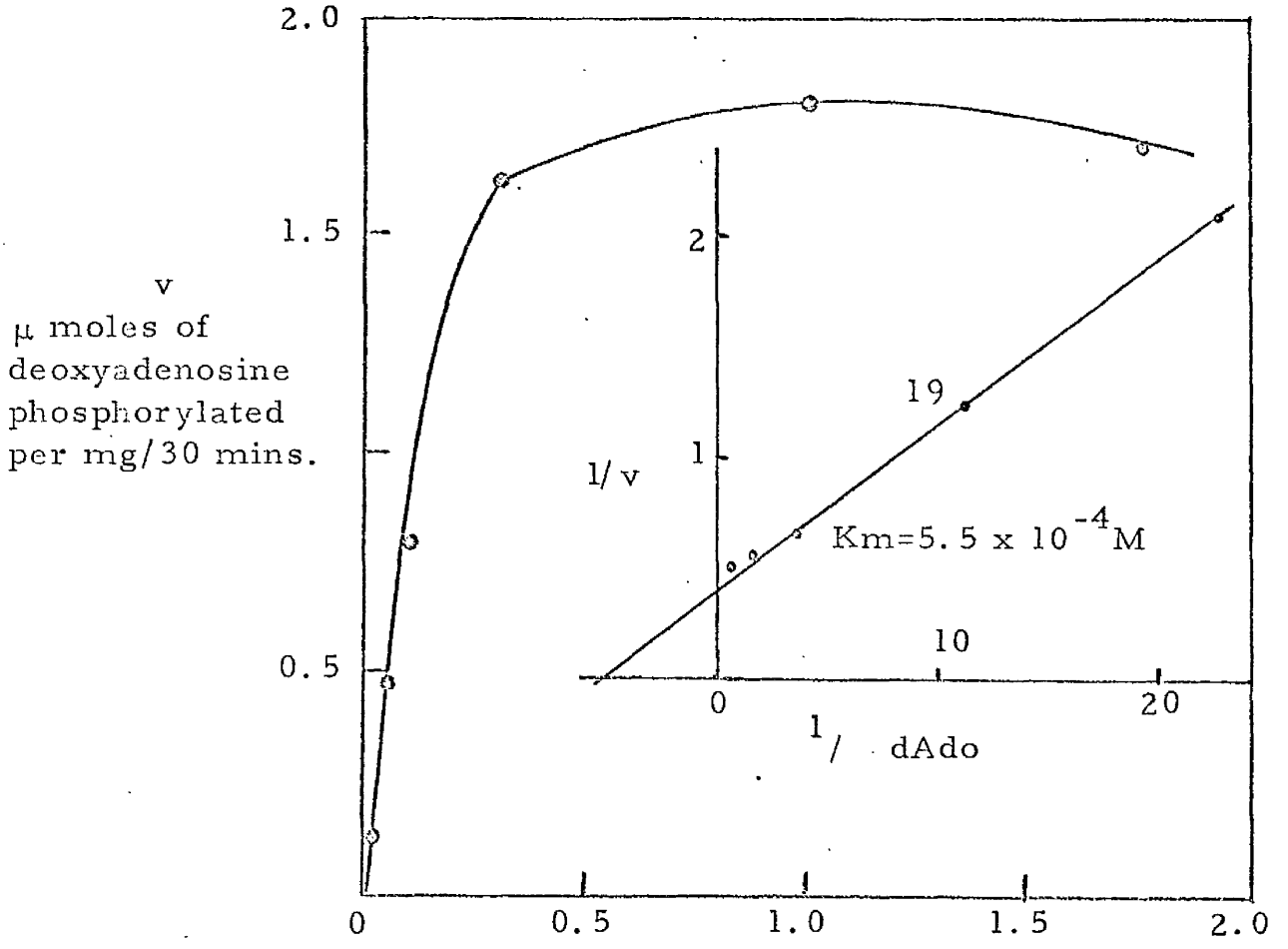
The maximum rate of phosphorylation by dAdo kinase took place at a substrate level of 1mM. The Michaelis constant for dAdo kinase at 37° was $5.5. \times 10^{-4}$M (Fig. IV.19).

6. Stability of Purine Deoxyribonucleoside Kinases

Both purine deoxyribonucleoside kinases were extensively stabilized in the presence of 20% glycerol, during both long term storage and freezing and thawing experiments. Unfortunately, the

Figure IV. 19.

Enzyme Constants of Deoxyadenosine Kinase



presence of glycerol gave rise to difficulties in the separation of deoxyribonucleotides from deoxyribonucleosides due to the tailing it produced both in chromatographic and electrophoretic separations. Addition of protein (Bovine serum albumin) to crude enzyme extracts did not increase stability during enzyme assay or long term storage.

6.1. Heat Stability of Deoxyadenosine Kinase

Deoxyadenosine kinase showed considerable thermostability. Heat treatment of the enzyme for 3 min. at different temperatures in the presence of 20mM-BES buffer, pH 7.0, clearly indicated the stability of the enzyme up to 70° (Fig. IV.20). This is similar to the observations of Okazaki and Kornberg (1964) on the dThd kinase of E. coli. The heat stability of the enzyme remained unaltered before and after infection. No stabilization of this enzyme by substrate was observed during heat inactivation experiments.

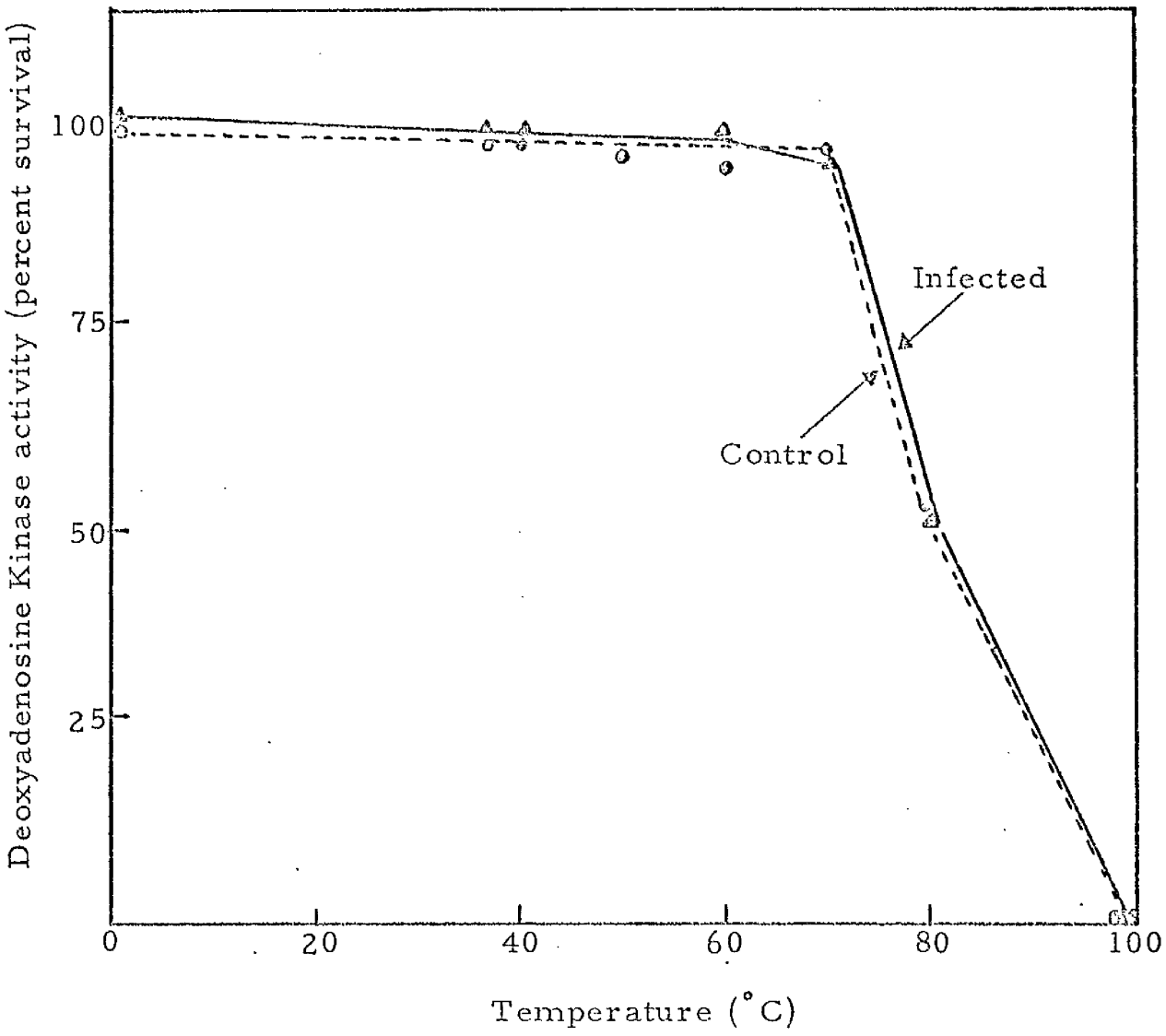
7. Induction of a New Deoxycytidine Kinase by Herpes Virus

7.1. Induction of Pyrimidine Deoxyribonucleoside Kinases

The levels of activity of dCyd and dThd kinases were increased 10 -15 fold by 12 hr. after herpes infection (Fig. IV.2). No such increases were seen when low serum cells were mock infected with EC_{10}, suggesting that elevation of enzyme activity is a result of virus infection.

Figure IV. 20.

Effect of Prior Incubation of Control and Infected Cell Extracts on the Activities of Deoxyadenosine Kinase



7.2. Requirement for New Protein Synthesis

Experiments with actinomycin D (Fig. IV.4) showed clearly that RNA synthesis was required for the appearance of the virus-induced increment in dCyd kinase activity. Studies with puromycin indicated that protein synthesis was also necessary for the induction of this new enzyme (Fig. IV.4). The time profiles of the curves of enzyme activities obtained in the presence of the two inhibitors indicate as might be expected that the effect of inhibition of RNA synthesis on the induction of dCyd kinase activity precedes that of puromycin.

The increase of dCyd kinase observed in herpes cells could be due to the presence of an inhibitor in non-infected cells which is destroyed during infection. This possibility is ruled out by the results obtained (Table IV.21) with mixed extracts of control and infected cells, which show an additive response.

7.3. Properties of Virus-Induced Kinases

7.3.1. Heat Stability Studies

The pyrimidine deoxyribonucleoside kinases of BHK cells were markedly less heat stable than the purine kinases (Fig. IV. 20, 22, 23). Cellular dCyd kinase was so thermolabile that it had to be stabilized by its substrate during heat sensitivity experiments (Fig. IV. 22). The virus-induced enzyme was stable when heated for 3 min. at 40°, whereas, the host enzyme lost over 80% of its activity at the same temperature.

Table IV. 21.

Mixing of Deoxycytidine Kinase Activities
of Control and Infected Cell Extracts

		Enzyme Units
Infected Cell Extract	-	26.7
Low Serum Cell Extract	-	0.0
High Serum Cell Extract	-	2.1
Infected Cell Extract	Low Serum Cell Extract	25.9
Infected Cell Extract	High Serum Cell Extract	28.5

Equal volumes of enzyme extracts were mixed and their activities determined.

In order to differentiate between the dCyd kinase and dThd kinase activities an investigation of the heat stability of dThd kinase was carried out (Fig. IV.23). In this experiment it became very clear that thymidine at 70 μM imparted maximum stability to the virus-induced dThd kinase activity at 50°, whilst the host enzyme was completely inactivated at this temperature even in the presence of substrate. The heat sensitivities of virus-induced dThd and dCyd kinases, both in the presence and in the absence of their respective substrates were quite distinct.

Extracts of infected cells were stored for 4 weeks at -70° in 20mM-BES buffer, pH 7.0, containing 5mM-mercaptoethanol and 10^{-6} M-dCyd. On assaying for dCyd kinase and dThd kinase after 15 min. prior incubation at 37°C, it was found that dCyd kinase had retained 70% activity whilst dThd kinase had lost nearly 90% of its original activity.

7.3.2. Effects of Deoxyribonucleoside Triphosphates

dCTP and dTTP are well known feedback inhibitors of the synthesis of deoxyribonucleotides (Grav, 1967; Maley, 1962b). At a concentration of 2×10^{-5} M dCTP, host dCyd kinase activity was reduced to 15% whereas the virus-induced dCyd kinase activity was little affected (Fig. IV.24). At higher concentration, however, dCTP (2×10^{-4} M) inhibited the virus-induced enzyme by 80% whilst the host

Figure IV. 22

Effect of Prior Incubation of Control and Infected Cell Extracts on the Activities of Deoxycytidine Kinase

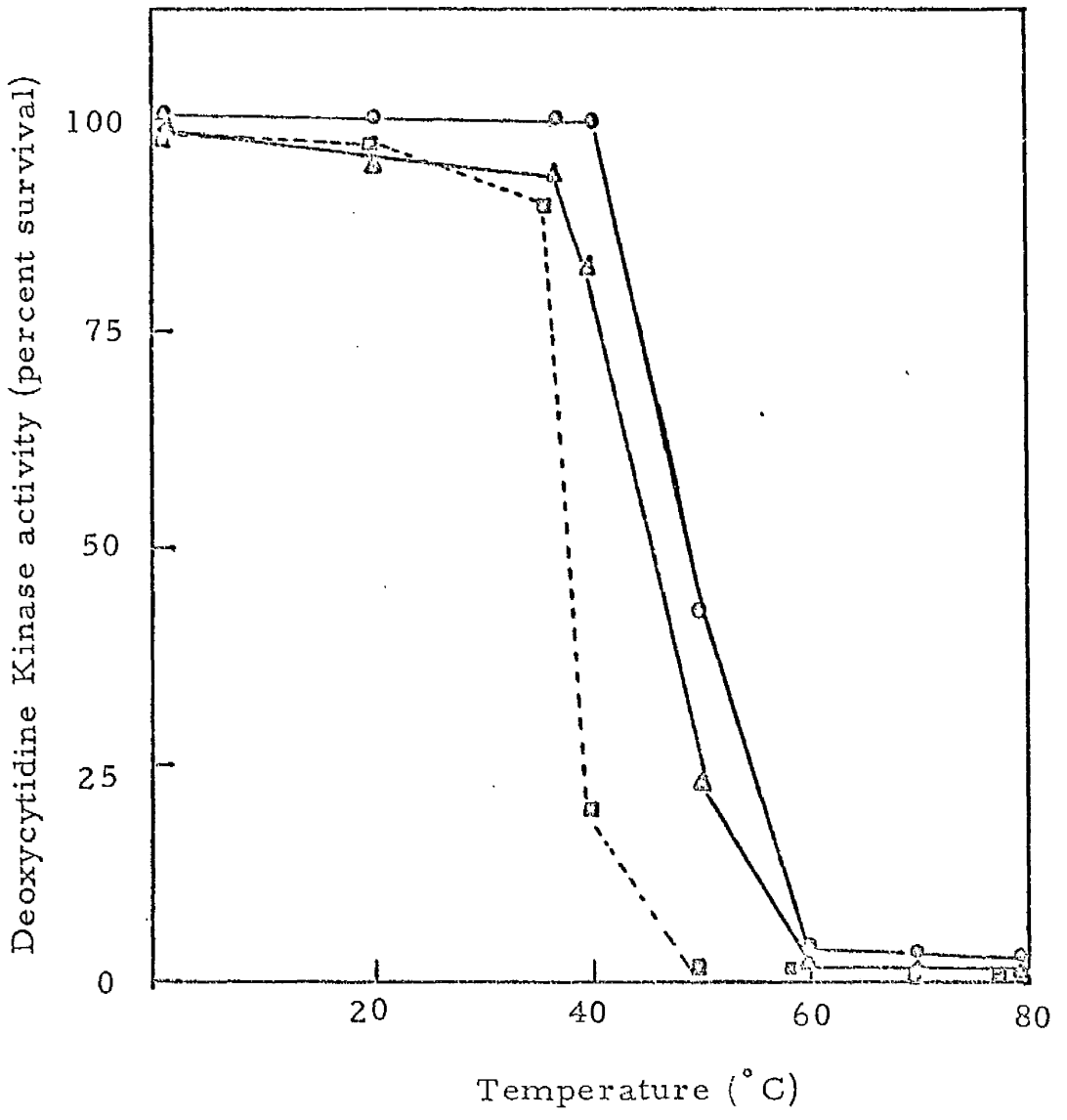
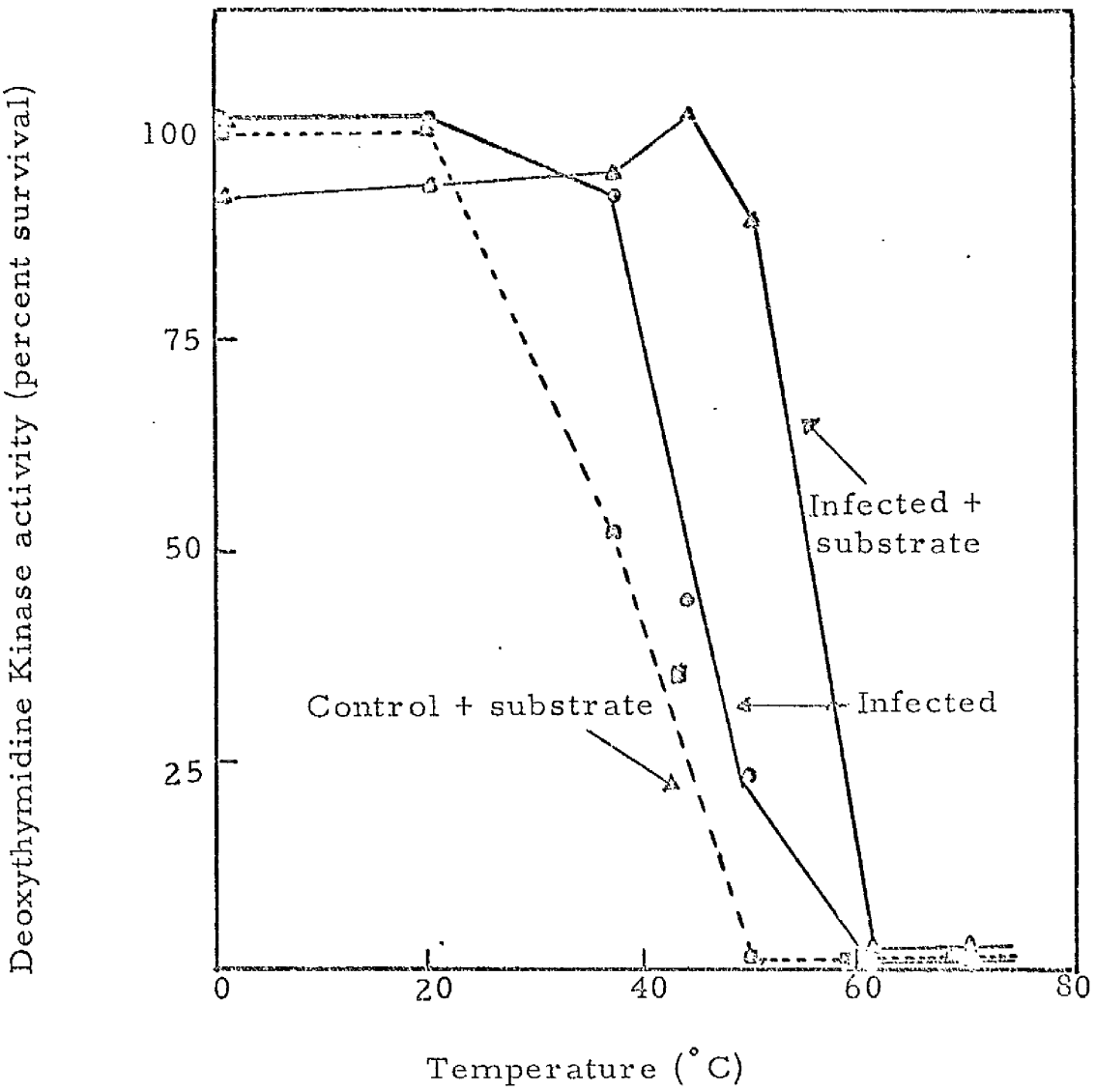


Figure IV. 23

Effect of Prior Incubation of Control and Infected Cell Extracts on the Activities of Deoxythymidine Kinase



enzyme was completely inhibited. In contrast, dCTP at 2×10^{-4} M increased the activity of virus-induced dThd kinase by over 40%. This further differentiates the virus-induced dCyd kinase and dThd kinase activities.

dTTP was a rather more potent inhibitor of dCyd and dThd kinases than dCTP. At 2×10^{-4} M, dTTP completely inhibited the activities of both host dCyd and dThd kinases (Table IV. 25). The fact that 2×10^{-4} M-dTTP completely inhibited the virus-induced dCyd kinase whereas virus-induced dThd kinase activity was unaffected further supports the hypothesis that these two virus-induced enzymes are separate entities.

7.4. Distribution of ^{3}H-Deoxycytidine in Virus-Infected Cells

Following infection of BHK cells with herpes virus the uptake of ^{3}H-dCyd (10^{-5} M) into the acid-soluble pool increased progressively, closely following the induction of dCyd kinase (Fig. IV. 2, 26). The uptake of ^{3}H-dCyd into the acid soluble levelled off at 10 hr. post-infection and remained at approximately the same level during the rest of the virus replication cycle. The shut off of enzyme synthesis coincided with the levelling off of the uptake of dCyd into acid-soluble pool.

The rate of uptake of ^{3}H-dCyd into the acid-insoluble fraction increased exponentially from 2 to 6 hr. post-infection followed by a levelling off period from 7 to 10 hr. Thereafter, the rate fell off

Figure IV. 24

Differential Effect of dCTP on Virus-Induced Pyrimidine Deoxyribonucleoside Kinases and Host dCyd Kinase

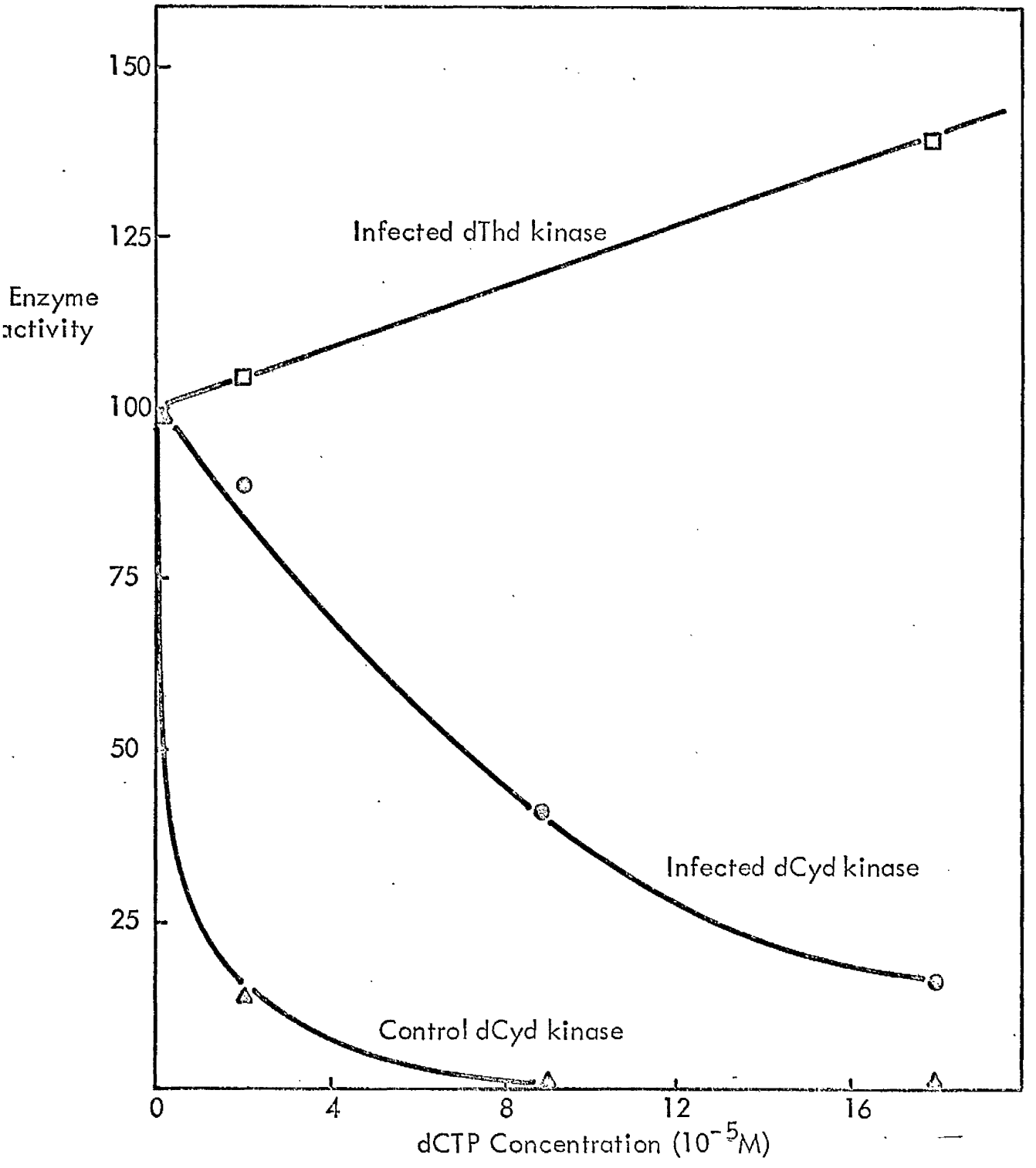


Table IV.25

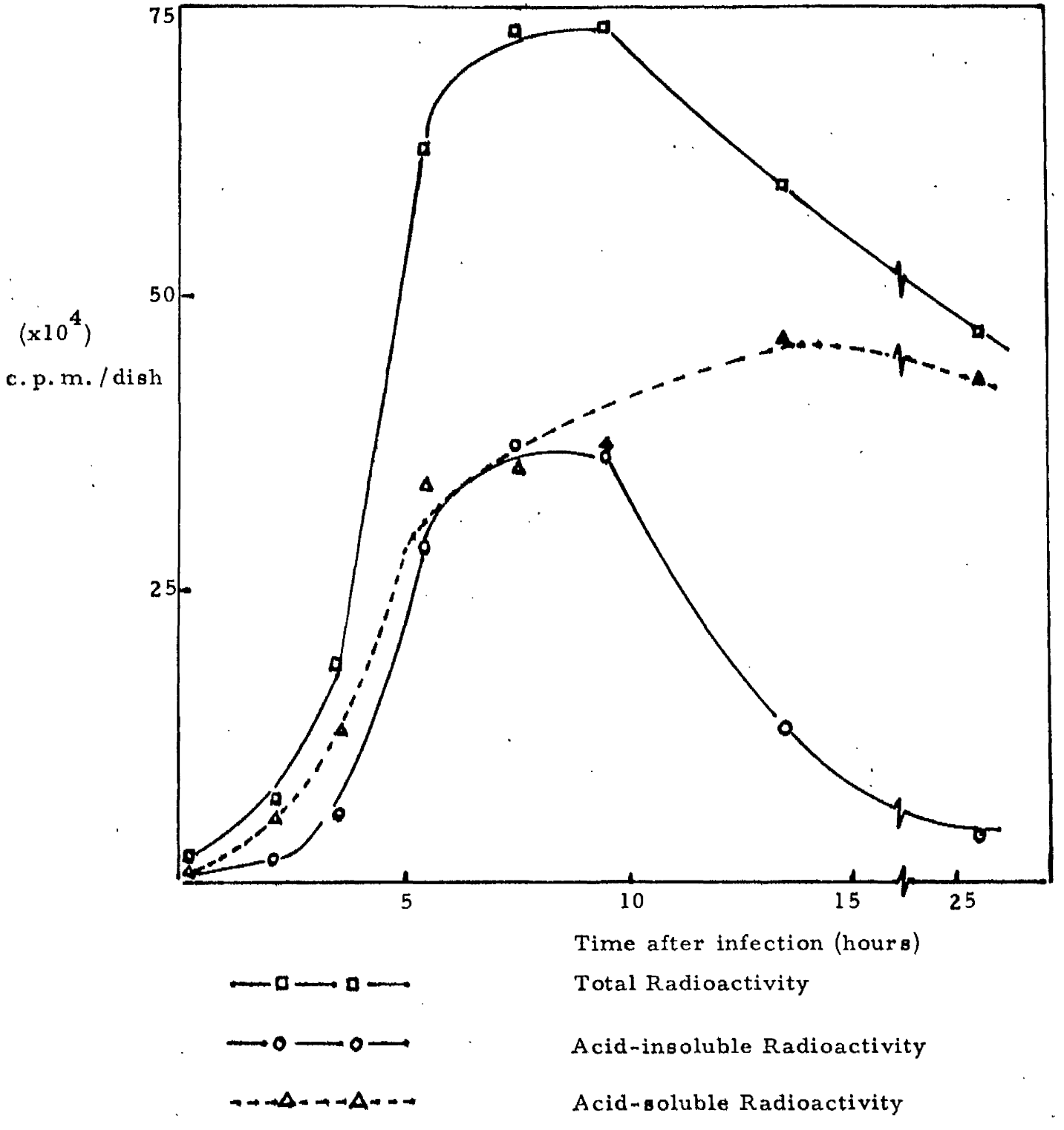
Effects of dCTP and dTTP on Host and Virus-Induced Pyrimidine Deoxyribonucleoside Kinases

Enzyme activity	no addition	2×10^{-5} M dCTP	2×10^{-4} M dCTP	2×10^{-5} M dTTP	2×10^{-4} M dTTP
Host dCyd kinase	1.2	0.0	0.0	0.2	0.0
Virus-induced dCyd kinase	26.1	22.8	3.8	3.3	0.0
Host dThd kinase	3.3	0.0	0.0	0.0	0.0
Virus-induced dThd kinase	13.3	13.6	18.2	13.4	11.6

The enzyme activities are expressed as n moles of deoxyribonucleoside incorporated per mg. protein.

Figure IV 26

Distribution of ^{3}H-dCyd into Acid-Soluble and Acid-Insoluble Fractions Following Pulse Labelling of Infected High Serum Cells for 1hr.



rapidly to a low level at a time corresponding to the maturation and release of virus particles. The maximum rate of uptake of dCyd into DNA was observed between 5 and 6 hr. post-infection. This coincided with the maximum rate of release of activity of DNase from inhibition by puromycin and hence its relationship to DNA polymerase (see discussion).

8. Partial Purification of Virus-Induced Enzymes

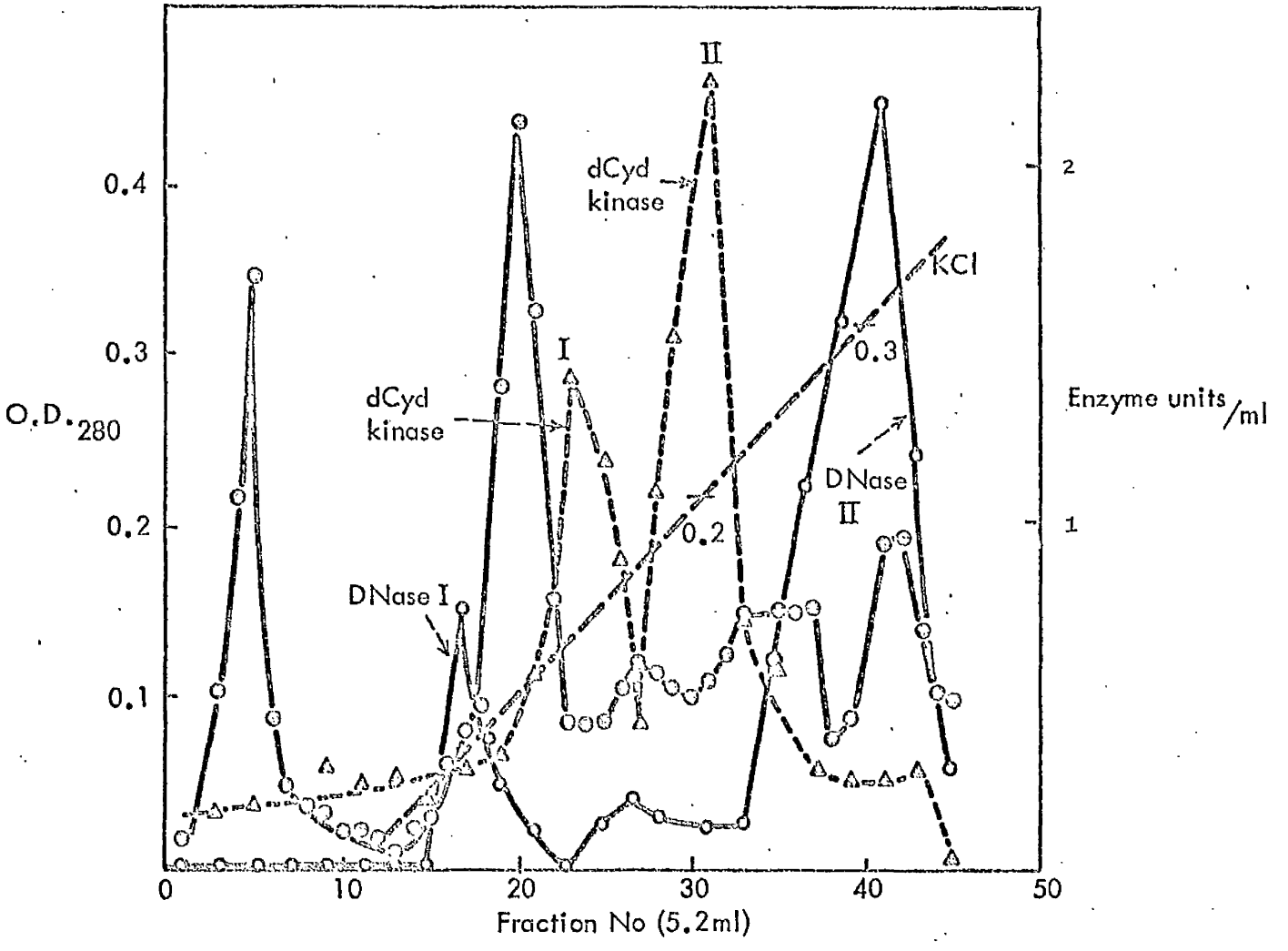
The high speed supernate of a sonicate extract of infected high serum cells was dialysed against 50mM-BES buffer containing 5mM-mercaptoethanol, 10^{-6} M-dCyd and 1mM-EDTA. The dialysed extract was fractionated on a column of DEAE-cellulose with elution by a linear gradient of KCl.

Estimation of protein in different fractions by measurement of extinction at 280 nm revealed two main protein peaks (Fig. IV. 27). One corresponded to the excluded fraction obtained during the washing stage and the other to the adsorbed fraction eluted by KCl. The adsorbed protein peak was eluted around 0.08M KCl concentration. Nucleic acids did not elute until higher KCl concentrations were reached.

Two peaks of dCyd kinase activity appeared at the troughs of the protein curve ensuring a large degree of purification. Two peaks of alkaline DNase appeared on either side of the two kinase peaks.

Figure IV. 27.

Partial Purification of Virus Induced Enzymes on DEAE Cellulose



DNase Fraction	Substrate: Native *E. coli* DNA	Substrate: Denatured *E. coli* DNA
DNase peak I	0.15 units	1.78 units
DNase peak II	1.98 units	0.52 units

Native DNA was a better substrate for DNase peak II than denatured DNA, whereas denatured DNA was the preferred substrate for DNase peak I. The modes of action of the DNase peaks were determined by chromatographic separation of digestion products of ^{32}P-*E. coli* DNA on DEAE paper. Peak I displayed endonucleolytic activity while peak II showed exonucleolytic activity. Peak II was found to be associated with DNA polymerase activity.

Partially purified dCyd kinase from both peaks was more heat sensitive than crude enzyme and was not activated by 2×10^{-4} M dCTP unlike virus-induced dThd kinase.

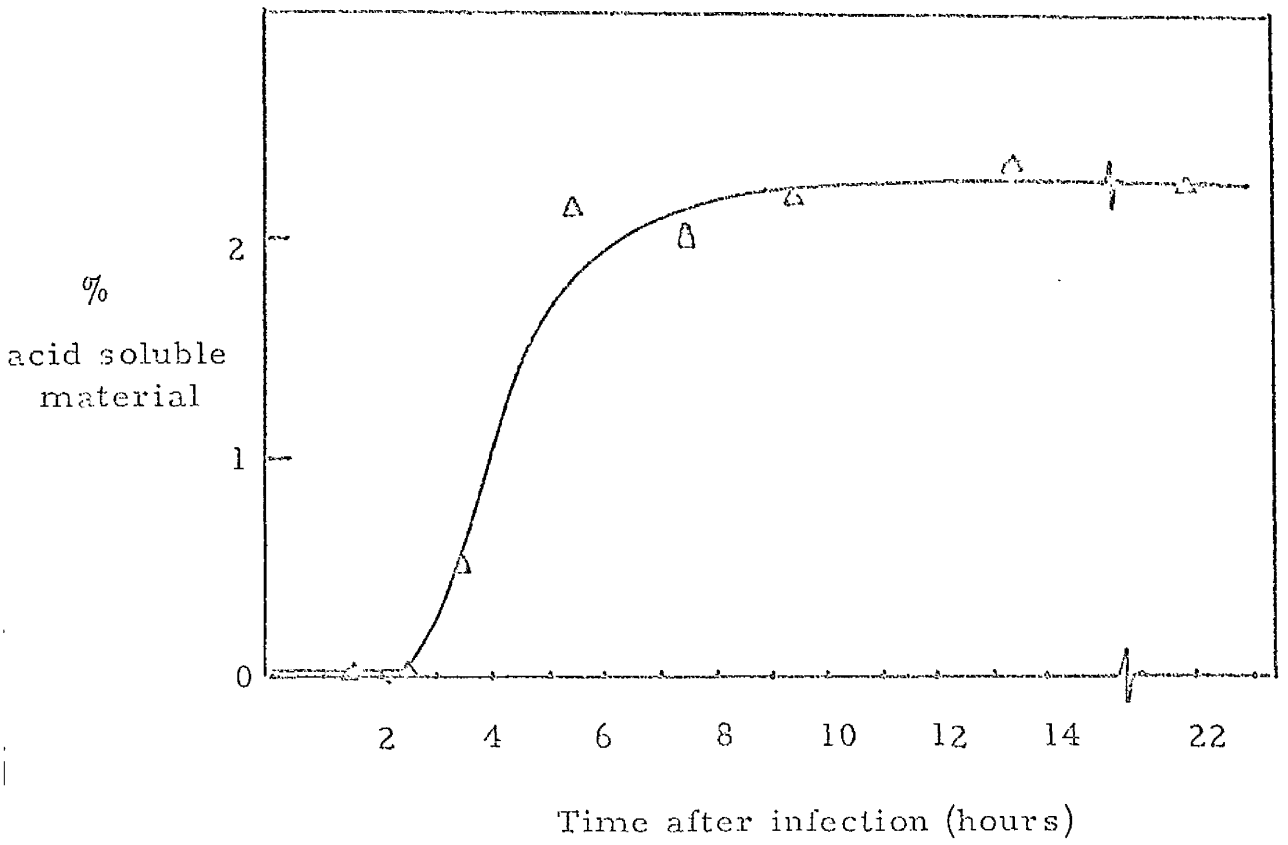
9. DNA Metabolism in Herpes Infected Cells

9.1. Degradation of Host DNA Following Viral Infection

On infection of ^{14}C-dCyd labelled high serum cells (3.5×10^3 d.p.m./10^6 cells) at a multiplicity of exposure of 10 - 15 P.F.U./cell a small percentage of the radioactivity appeared in the acid-soluble fraction between 2 and 6 hr. after infection (Fig. IV.28). The increase in acid-soluble radioactivity was less rapid thereafter

Figure IV. 28

Appearance of Host Cell Deoxyribonucleotides in Acid-soluble Pool Following Infection of High Serum Cells



until it finally levelled off at 9 hr. post-infection. To minimise any radioactivity being drawn into the acid soluble pool during extraction 10^{-5} M-dCyd was added into the medium 1 hr. before the collection of cultures. The percentage of radioactive acid-soluble material in mock-infected cells never exceeded 0.1% of acid-precipitable DNA material.

9.2. Incorporation of Cellular DNA Material into Viral DNA

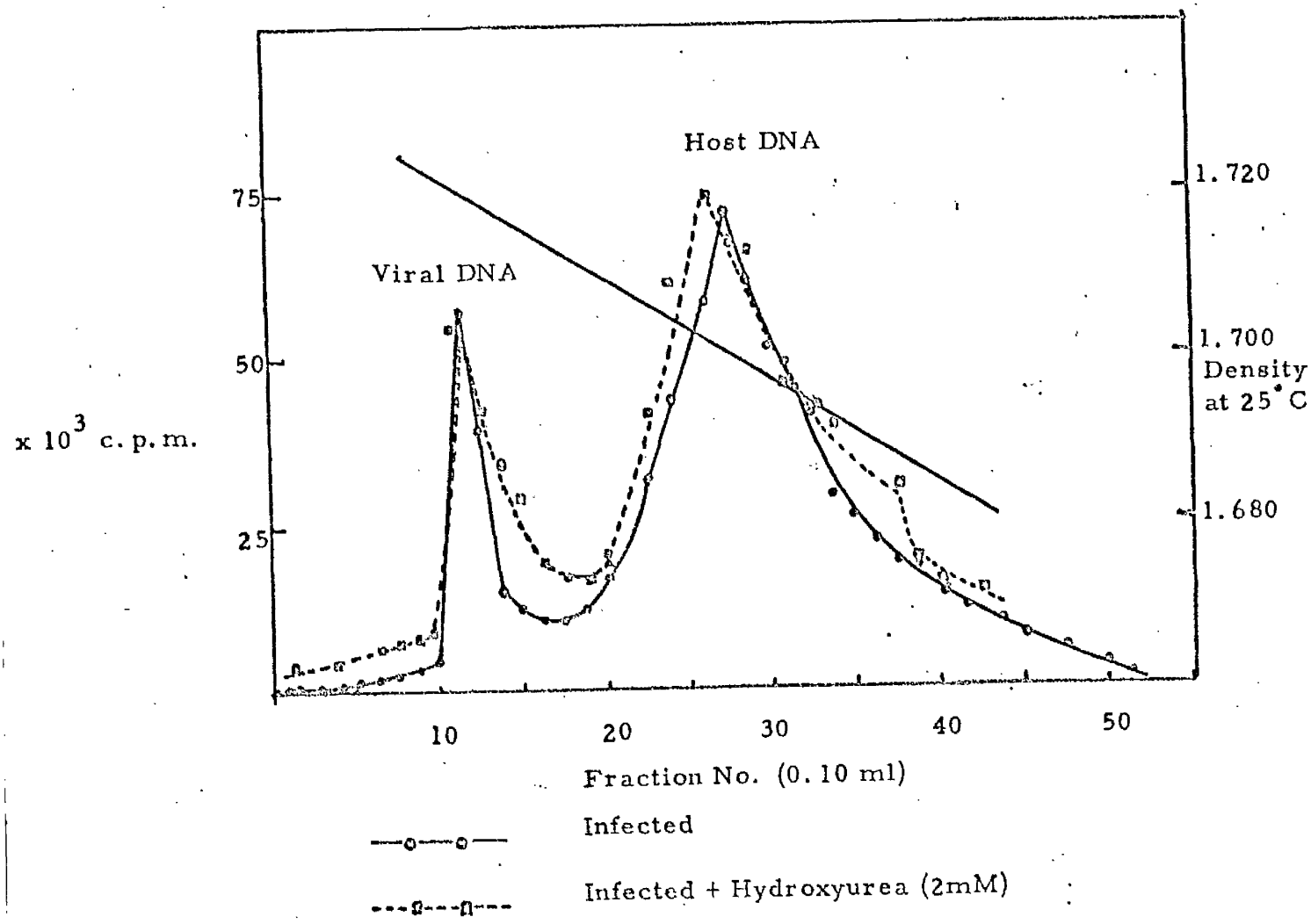
Cells pre-labelled with ^{3}H-dThd were infected at a high multiplicity (= 100 P.F.U./cell) in Eagle's medium unsupplemented by calf serum. Under these conditions it was possible to demonstrate the incorporation of host cell deoxyribonucleotides into viral DNA (Fig. IV.29). 2mM-hydroxyurea or 10^{-6} M-F dUrd had very little effect on the specific activity or amount of viral DNA synthesised from host cell material. This suggests that the contribution of the de novo pathway towards the synthesis of viral DNA was rather small under the above conditions. All cultures were collected at 10 hr. post-infection and host and viral DNA separated on neutral CsCl isopycnic gradients as described in Methods.

9.3. Progressive Incorporation of Cellular DNA Material into Viral DNA

On infection of cells prelabelled with ^{3}H-dThd (1.5×10^{6} d.p.m./10^{6} cells) at a high multiplicity of infection (= 100 P.F.U./cell) as in section 9.2.there was a progressive increase in the radioactivity

Figure IV.29

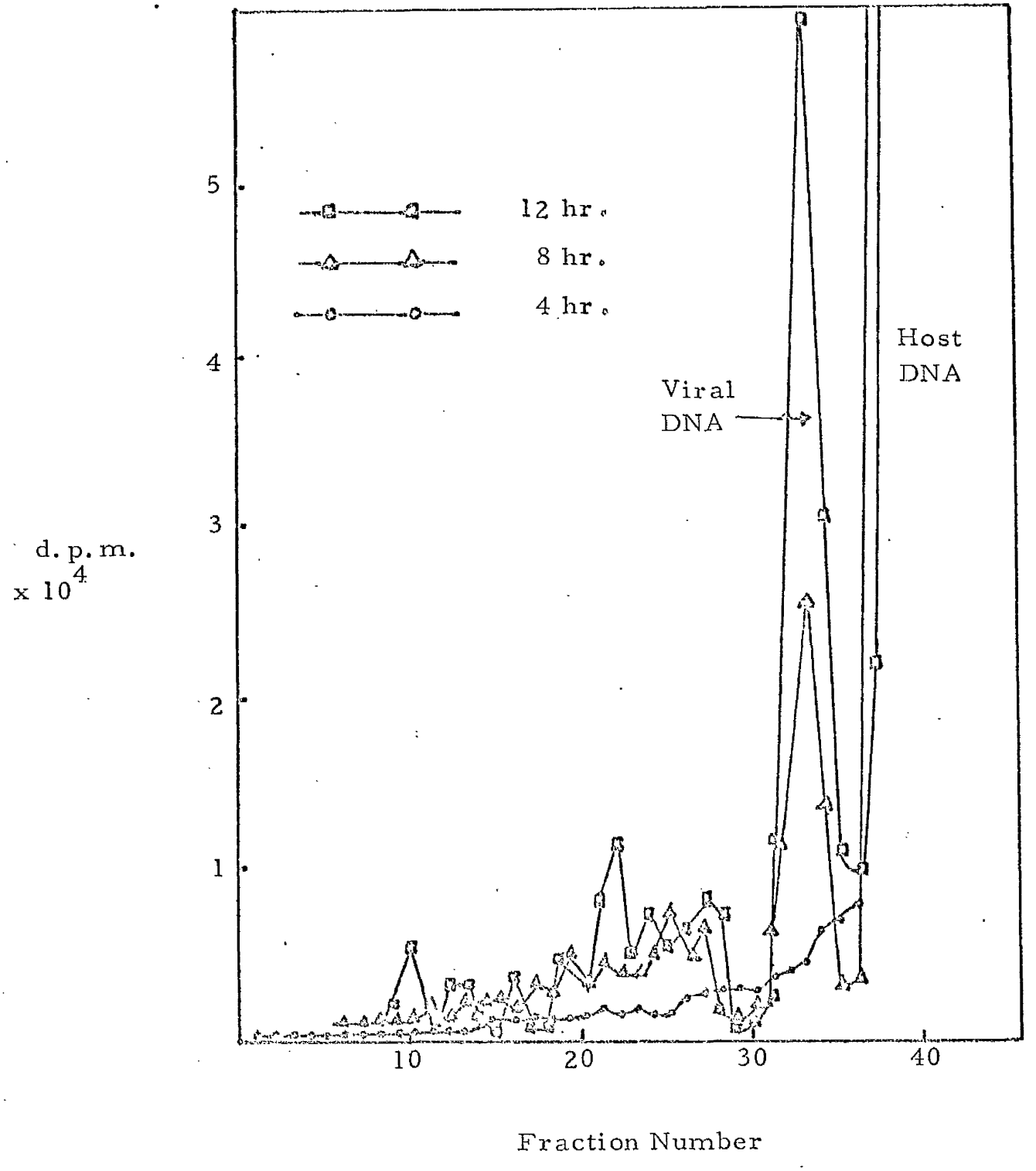
Incorporation of Host Cell Deoxyribonucleotides into Herpes Virus DNA Following Multiple Infection of High Serum Cells and the Effect of Hydroxyurea.



Cells were pre-labelled with ^{3}H-dThd for 18hr. using 100μC per roller bottle. The Cells were washed, trypsinised and sub cultured in roux bottles at 40 x 10^{6} cells/ bottle. After 18hr. of growth the monolayer of cells was washed and infected at 100 P.F.U./cell and incubated with Eagles medium for 10hr. 2mM-hydroxyurea and 10^{-6}M FdUrd were added separately to roux bottles and incubated with Eagles medium after infection. Cells were collected after 10hr. and subjected to neutral CsCl density gradient centrifugation after treatment of DNA with 1% S.D.S. and pronase (1mg/ml).

Figure IV. 30

Progressive Incorporation of Host Deoxyribonucleotides into Viral DNA Following Multiple Infection

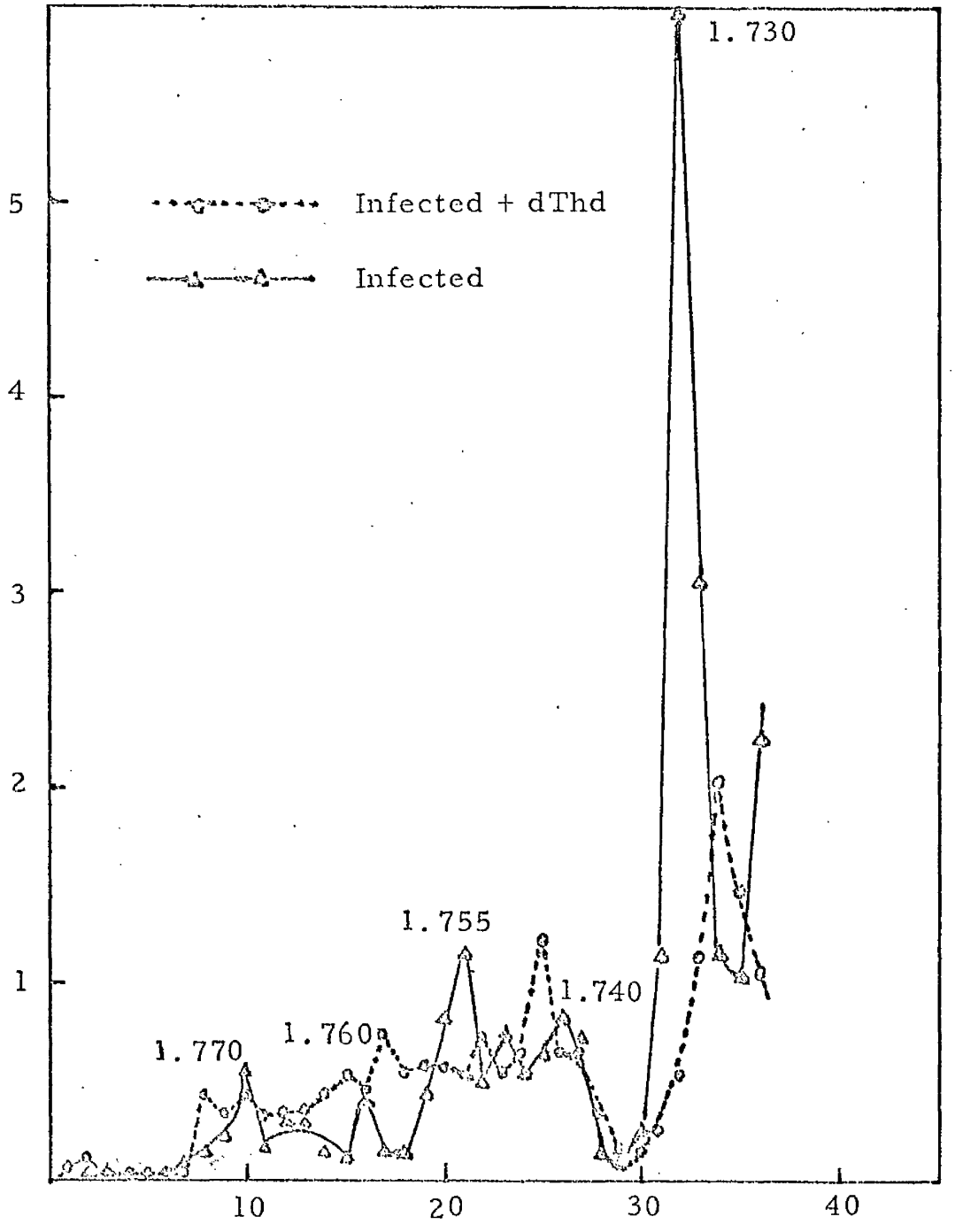


of the viral DNA peak (Fig. IV. 30). The buoyant density of herpes DNA was 1. 728g./cm.^{3} on neutral CsCl isopycnic gradients. No viral peak was observed prior to 4 hr. post-infection. From 8 hr. onwards about 4 extra peaks appeared at densities greater than the double-stranded viral DNA peak. The positions of the last 3 peaks on the gradient suggest that they may possibly be single-stranded DNA of high G+C content. The 4 peaks increased with time after infection. One of the peaks had a buoyant density of 1. 740g./cm^3. Its position corresponds to single-stranded herpes virus DNA (Sinsheimer and Lawrence, 1964). This single-stranded viral DNA may have acted as an intermediate of viral DNA replication. The significance of the other 3 peaks is at the present time unknown, but the effect is nevertheless quite reproducible.

Infection of prelabelled cells in the presence of 5×10^{-6} M-dThd reduced the incorporation of radioactive material into the double-stranded viral DNA (Fig. IV. 31). The 4 dense peaks were still observed under these conditions. The high buoyant densities of these peaks indicate that they could be DNA-RNA type hybrids which could be involved in transcription of late functions of progeny viral DNA.

Figure IV. 31

Effect of Deoxythymidine on the Incorporation of Host Deoxyribonucleotides into Viral DNA Following Multiple Infection of High Serum Cells



10. Phagicin as a Tool for the Study of DNA Metabolism of Herpes-Infected Cells

10.1. Inhibition of Plaque Formation

50mm Petri dishes containing 4×10^6 cells/dish were infected with herpes using approximately 250 P.F.U./dish. Half the number of the plates were treated with 0.1 ml. of crude phagicin (70 μg/ml.) after the adsorption of virus. Cultures were checked for plaques on the third day after infection. Cultures treated with phagicin were found to be clear of any plaques whilst the rest showed an average plaque count of 265.

10.2. Effect of Phagicin on Uptake of ^{3}H-dThd into DNA

Petri dish cultures of 3 days-old low serum cells (2×10^6 cells/dish) were infected with herpes while others were mock-infected. After an hour of virus adsorption different extracts were added in duplicate into both control and infected cells and the amount of DNA synthesised determined. Cultures were exposed to 10 μc of ^{3}H-dThd (10^{-6} M) from 3 to 8 hr. post-infection and the acid-soluble radioactivity measured.

Incorporation ^{3}H-dThd into DNA

Mean d.p.m./dish

Sample	0.2 ml. PBS Sol. A	0.2 ml. λ lysate	0.2 ml. T_2 lysate
Control	9,250	9,103	——
Infected	111,234	26,843	111,462
Sample	0.2 ml. PBS Sol. A	0.2 ml. E. coli W3110 Extract	0.2 ml. E. coli R_2 Extract
Control	9,250	9,402	7,610
Infected	111,234	38,243	108,450

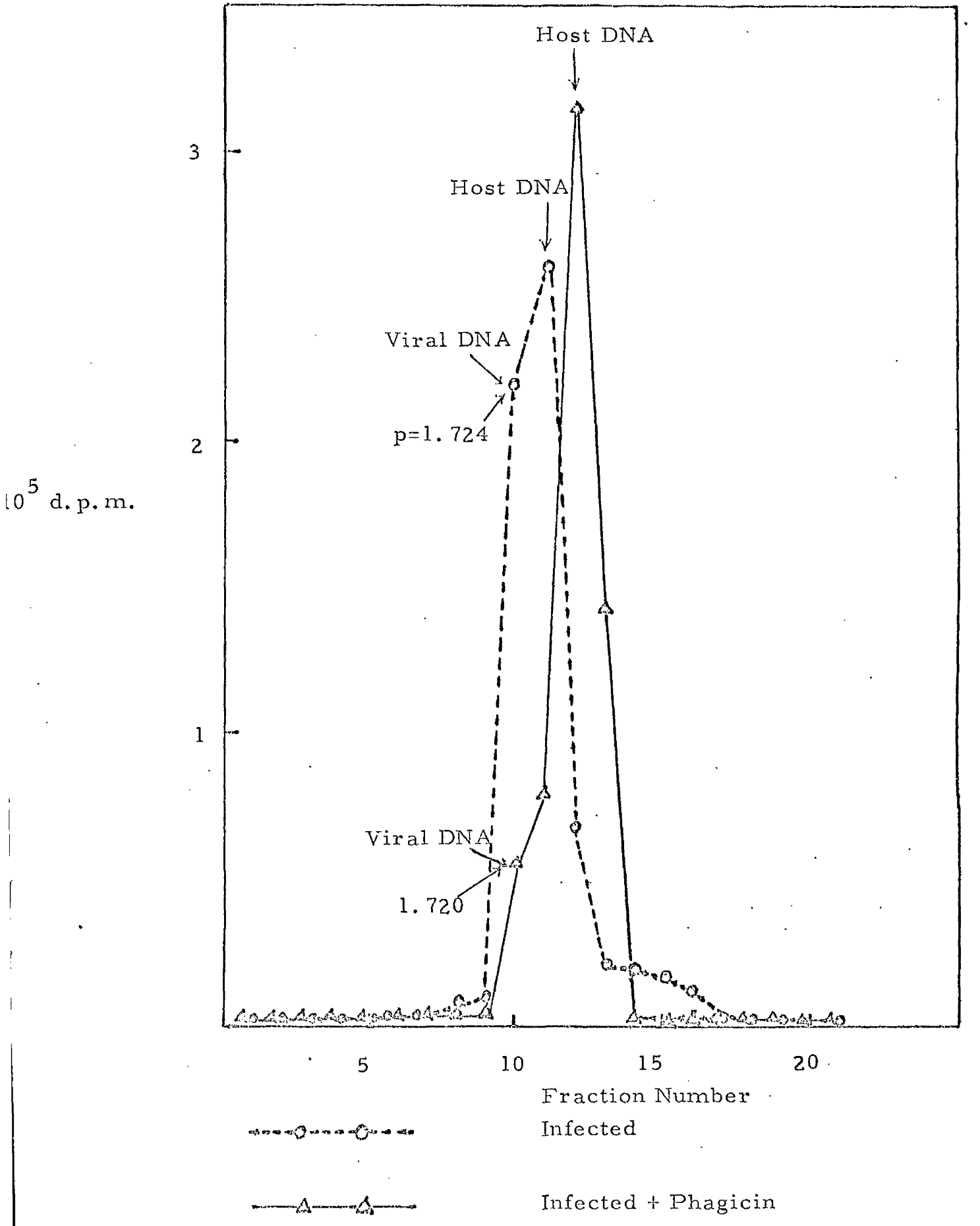
Phage λ lysate and E. coli W3110 extract both inhibited DNA synthesis following viral infection while phage T_2 lysate and extracts of E. coli R_2 did not affect the DNA synthesis. None of the extracts had any significant effect on the DNA synthesis of low serum cells.

10.3. Effects of Phagicin on the Synthesis of Viral DNA

90mm. Petri dish cultures were infected at 10 P.F.U./cell. EHu_{10} and 0.5 ml. of phagicin stock (70 μg./ml.) were added after an hour of virus adsorption. The cells were exposed to 10 μc of ^{3}H-dThd at 10^{-6} M, from 1 to 11 hr. post-infection after which the cells were scraped off, washed with PBS solution A, suspended in 0.01M-Tris

Figure IV. 32

Inhibition of the Synthesis of Herpes Virus DNA Following Treatment of Infected Cells With Phagicin



buffer pH 8.0 and treated with 1% SDS at 37°C for 30 min. Host and viral DNA was separated by isopycnic centrifugation. The amount of viral DNA was reduced by 70% in the phagicin-treated culture (Fig. IV.32).

Incorporation of ^{3}H-dCyd into acid insoluble material showed that there was a 85% inhibition of DNA synthesis of infected cells and a 10% inhibition of DNA synthesis of control cells following treatment with phagicin (50 μg/ml.).

10.4. Effect of Phagicin on the Induction of Enzymes

Addition of phagicin (50 μg/ml.) into herpes-infected cells at 1 hr. post-infection did not alter the levels of activity of virus-induced enzymes measured at 10 hr. post-infection. Thus suggesting the possibility that inhibition of DNA synthesis may have taken place at the level of DNA replication itself rather than at the transcriptional or translational level of gene expression.

V. DISCUSSION

1. Selection of Tissue Culture for Metabolic Studies

Tissues dissociated into single cells and grown in monolayer or suspension culture provide easily accessible and reproducible systems for metabolic studies. The BHK cell used in this project is a standard line obtained from an explant of baby hamster kidney. Since its adaptation to the present nutritional environment the cell has probably undergone dedifferentiation. Although this system cannot completely simulate virus-cell interaction in the whole organism, it is much more amenable to experimentation and is a very useful first approximation to the in vivo state.

1.1. Viruses as Probes in the Study of Control Mechanisms

The molecular weight of herpes DNA is 100×10^6 daltons (Becker, Dym and Sarov, 1968) and is thus about ten thousand-fold smaller than the total DNA content of BHK cells. The coding potential of herpes virus is about 130 proteins of 50,000 molecular weight. This number is relatively small compared with the wide range of proteins necessary for the upkeep and the propagation of the cell. At present, it is impossible to isolate and characterise a specific mammalian gene, its corresponding m RNA and its protein product. In E. coli, however, use of an ingenious genetic approach coupled with DNA hybridization has allowed the isolation of the operon

for β-galactosidase (Shapiro et al., 1969). The great potential of herpes is that it can be used to introduce a discrete amount of genetic information into a cell. Therefore this system provides a useful means of introducing and expressing foreign genes within a mammalian cell - hence the usefulness of herpes virus as a probe for the study of control mechanisms.

1.2. Low Serum Cells as a System for Virus Infection

High serum cells are metabolically active and provide a suitable system for the study of inhibition of host metabolism following virus infection. But such systems are not advantageous to investigations of increases in enzyme activity, where the background level of the corresponding host enzyme may be high. The metabolically less active or resting cell provides a suitable biochemical environment for such studies (Burk, 1966).

The resting state is achieved by plating cells in low serum and leaving them for four days at 37°. After this treatment, only 5% of the cells synthesise DNA, but all are capable of supporting virus growth. The very low levels of most host enzymes present in these cells enable the virus-induced enzymes to be detected more easily and also obtained with less contamination by host enzymes. Therefore, low serum cells are a suitable source for the extraction and purification of such enzymes.

Extracts of low serum BHK cells are known to inhibit the growth of polyoma-transformed cells (Burk, 1966). This inhibition can be overcome by addition of calf serum. The inhibition of growth of BHK cells in low serum cells may be due to the production of this same inhibitor. Since this type of growth inhibition unlike contact cell inhibition (Abercrombie, 1965) is apparently related to nutrient starvation a precise control of BHK cells in the "resting state" seems unlikely.

2. Effects of Herpes Virus on Host Cell Metabolism

2.1. DNA Synthesis in Virus-Infected Cells

There is an increased uptake of deoxyribonucleoside precursors into the acid-soluble pool of BHK cells following herpes infection (Fig. IV.26). This could be the outcome of a physiological change in membrane structure allowing increased permeability, similar to the phenomenon observed during the action of oestradiol-17β on immature rat uterus (Billing, Barbiroli and Smellie, 1968), but is more likely to be due to the synthesis of virus-induced deoxyribonucleoside kinases. The parallel between the increase in uptake of ^{3}H-dCyd and the induction of dCyd kinase (Fig. IV.2, 26) indicates the latter possibility.

The rate of DNA synthesis increases following herpes infection.

The increase in the rate of DNA synthesis in the infected cell is immediately followed by the appearance of viral DNA and a simultaneous decrease in the synthesis of host DNA (J. M. Morrison, Ph. D. thesis). The inhibition of cellular DNA synthesis increases with the time and multiplicity of infection.

2.1.1. Inhibition of Host DNA Synthesis

The inhibition of host DNA synthesis following herpes infection may be due to one or more of the following possible mechanisms.

(i) The population of virus structural protein which is introduced into the cell during infection could inhibit host DNA synthesis by:-

(a) Complexing with host DNA template to block replication, perhaps in a manner analogous to the inhibition of RNA polymerase by histones (Spelsberg and Hnilica, 1969),

(b) reacting with the nuclear membrane and inhibiting DNA synthesis in a manner analogous to that induced by "ghosts" of T-even phages with E. coli (French and Siminovitch, 1955).

Studies with phagicin (Centifanto, 1968) demonstrate the capability of a dialysable polypeptide to inhibit viral DNA synthesis without interfering with the induction of new enzymes (Section IV.10).

In 1960, Pereira reported that adenovirus C antigen, now termed the fibre antigen, could inhibit the multiplication of adenoviruses, poliovirus and vaccinia virus without affecting the adsorption of the challenge virus to their host cells. Later, Levine and Ginsberg (1965) found that the production of DNA, RNA and protein was inhibited about 20 hr. after the fibre antigen was added to either uninfected or infected cells. These results suggest that fibre antigens might have a general inhibitory effect on the biosynthesis of macromolecules in mammalian cells. Extending this hypothesis to herpes infected cells, it is possible that inhibition of host DNA synthesis occurs when the intracellular pool of structural proteins attains an adequate concentration. In fact, Newton (1967) has shown that a non-DNA component is responsible for the inhibition of host cell DNA synthesis following herpes infection.

(ii) Inhibition of host DNA synthesis could also be mediated through the induction of a virus coded protein. An endonuclease, perhaps specific for cellular DNA, might be a suitable candidate for such a function.

2.1.2. Degradation of Host DNA

During infection of E. coli by phage T_4, the inhibition of host DNA synthesis is at least partly due to the degradation of host DNA by phage induced DNases (Sadowski and Hurwitz, 1969). Wiberg

(1966) has shown that mutants of phage T_4 with defects in genes 46 and 47 are unable to degrade the host DNA. After herpes infection of BHK cells the activity of DNA polymerase is found to decline from its control level at 1 - 2 hr. post-infection followed by an increase above the control level 2 - 4 hr. later (Keir, 1968). This decrease in the activity of DNA polymerase could be caused by the action of a virus-induced DNA endonuclease. But no such activity has yet been recognised as being virus coded probably because of difficulties in the separation of host and virus-induced endonucleases.

Evidence for the degradative action of virus-induced DNases comes from the investigations on the breakdown of host DNA into the acid-soluble pool of the cell (Section IV. 9). The increase in the degradation of host DNA follows the time scale after infection until it levels off at 8 hr. post-infection (Fig. IV. 28). In addition there is further evidence which supports this point of view.

(i) On infection of mammalian cells with herpes margination of chromatin and chromosome breakage are observed (Morgan, et al., 1954; Stich et al., 1964). The distribution of chromosomal lesions is non-random and they do not appear in cells infected with inactivated virus (Kaplan, 1969).

(ii) There is a marked increase in the activity of a DNA exonuclease producing 5'-mononucleotides following herpes infection of BHK cells (Morrison and Keir, 1968). The time of appearance of this enzyme is compatible with a role in host DNA degradation.

(iii) Inhibition of cellular DNA synthesis parallels the induction of early enzymes.

2.1.3. Viral DNA Synthesis

The synthesis of viral DNA is preceded by the induction of early enzymes which are required for efficient synthesis of viral progeny DNA (Fig. IV.1, 2, 30). Mature viral particles begin to appear only after 4 hr. post-infection. The virus-induced DNA polymerase probably synthesises viral DNA in preference to host DNA. The DNA exonuclease (Morrison and Keir, 1968) induced after virus infection may provide 5'-deoxyribonucleoside triphosphate precursors from degraded host DNA fragments. Of the total amount of viral DNA synthesised only about 10 - 15% is encapsulated (Kaplan, 1969). The reason for this is not clear. But, in the in vivo situation, excess DNA formed could provide a rich source of precursors for the synthesis of DNA in adjacent uninfected cells which subsequently become infected by the progeny from those cells which were first infected.

2.2. Protein Synthesis

Following herpes infection of BHK cells the levels of activity of dGuo kinase (Fig. IV.2) and DNA polymerase (Keir, 1968) decline in a manner characteristic of each enzyme. Hsu and Weiss (1969) report that during infection of E. coli by phage T_4 the bacterial ribosomes are altered in a way that restricts translation of bacterial m·RNA but allows normal translation of T_4 phage m·RNAs. In addition, disaggregation of polyribosomes has been observed following infection of canine kidney cells with herpes virus (Sydiskis and Roizman, 1967). The decrease in activity of dGuo kinase and DNA polymerase may therefore be a result of interference with host cell translation. The levelling off of the activity of dGuo kinase observed at 4 hr. post-infection (Fig. IV.2) may be a consequence of the inability of host m·RNA for dGuo kinase to translate efficiently on virus-induced aggregates of polyribosomes which have different sites and affinities towards host m·RNAs in general.

3. Induction of Enzymes After Herpes Infection

3.1. Possible Mechanisms Involved in the Induction of Enzymes

On infection of BHK cells the levels of DNA polymerase, alkaline DNase, dCyd kinase and dThd kinase are increased. The increases in enzyme activities precede the production of infectious

virus particles, thus suggesting the necessity for the inhibition of the host cell's DNA synthesising apparatus and its replacement by viral functions for the efficient production of progeny virus. Furthermore, the inhibition of host protein synthesis immediately after infection prevents the increase in activity of host cell enzymes. The increase in the activities of the above enzymes may be due to one or more of the following reasons:-

(i) Synthesis of an activator or the inactivation of an inhibitor of a host enzyme;

(ii) modification of a host enzyme;

(iii) derepression of a host gene corresponding to a new enzyme

(iv) synthesis of a virus-coded enzyme.

When infected cell extracts are mixed with different proportions of control cell extracts, the total enzyme activity obtained for dCyd kinase is equal to the sum of the activities of each extract assayed separately (Table IV.21). This result eliminates the first possibility. Similar results are obtained with virus-induced dThd kinase.

DNA polymerase and alkaline DNase present in infected cells differ in their antigenic properties from their respective counterparts in control BHK-21 and HEp-2 cells, but the antigenic properties of the enzymes induced by herpes virus in both BHK-21 and HEp-2 cells

are similar (Keir, 1968). This rules out the third possibility provided the antigenic similarity between virus-induced DNase activities in BHK-21 and HEp-2 cells is significant. Similar observations have been made by other workers for dThd kinase (Klemperer et al., 1967).

The different antigenicity displayed by enzymes from infected and non-infected cells appear to indicate the de novo synthesis of new and different enzyme proteins. But this criterion is not completely unequivocal. Thus an alteration in the antigenicity of a protein can occur as the result of a change in the state of aggregation of the enzyme. It has been shown that under certain conditions, the molecular form of crystalline glutamic dehydrogenase is changed from a polymer to a monomer, thereby changing the antigenicity of the enzyme (Talal et al., 1964).

There is suggestive evidence that dThd kinase may be coded by the viral genome. This comes from the work of Kit et al. (1967b), who showed that dThd kinase induced in LM (TK^-) cells by vaccinia virus and herpes simplex virus have different properties and that viral mutants which did not induce the enzyme could be obtained. A mutant strain of BHK cells lacking dCyd kinase has been demonstrated to incorporate ^{3}H-dCyd into DNA following herpes infection (H. Subak-Sharpe, unpublished results). This may be taken as circumstantial evidence for the induction of dCyd kinase.

3.2. Evidence for the Induction of a New Deoxycytidine Kinase

1. Synthesis of protein is a prerequisite for the induction of dCyd kinase.

2. There is a sharp cut off of enzyme activity at its peak level at 10 - 12 hr. post-infection. This agrees with the behaviour of other virus-induced "early enzymes".

3. Synthesis of RNA precedes the synthesis of dCyd kinase after infection. Inhibition of the synthesis of early RNA prevents the induction of the enzyme.

4. Mixing experiments with control and infected cell extracts show an additive response for dCyd kinase.

5. The increase in enzyme activity parallels the increase in the uptake of dCyd into the acid soluble pool.

6. The virus-induced enzyme is more thermostable than the host enzyme and its substrate stabilization profile differs from that of the virus-induced dThd kinase.

7. At 2×10^{-5}M, dCTP inhibits host dCyd kinase markedly but has little effect on virus-induced dCyd kinase.

8. At 2×10^{-4}M, dCTP inhibits both host and virus-induced dCyd kinases but activates virus-induced dThd kinase by 40%.

9. At 2×10^{-4}M, dTTP inhibits both host and virus-induced dCyd kinases and host dThd kinase, but has little effect on virus-induced

dThd kinase.

10. The presence of deoxycytidine at 10^{-6}M during storage does not stabilise virus-induced dThd kinase against heat inactivation to the same extent as dCyd kinase.

11. The incorporation of host cell deoxyribonucleotides into viral DNA has been shown (Section IV. 9) and a requirement for virus-induced pyrimidine deoxyribonucleoside kinases seems likely.

The appearance of two deoxycytidine kinase peaks during partial purification of virus-induced enzymes on DEAE columns (Fig. IV. 27) and the consistent two progressive stepwise increments observed for enzyme activity following infection (Fig. IV. 2, 4) suggest the possibility of two virus-induced dCyd kinases. Since the two enzyme activities separate on DEAE cellulose, it is possible that they represent two distinct proteins. Further critical analysis is necessary before the acceptance of this hypothesis.

3.3. Analysis of Evidence for the Absence of Virus-Induced Purine Deoxyribonucleoside Kinases

The levels of purine deoxyribonucleoside kinases in control cells are considerably higher than those of pyrimidine deoxyribonucleoside kinases. Their activities are probably capable of handling large increases in deoxyribonucleoside pools within infected cells. Following infection of low serum cells dAdo kinase and dGuo kinase activities

decrease. But dAdo kinase activity recovers after a short lapse of time whilst dGuo kinase activity remains constant after 4 hr. post-infection. The behaviour of dAdo kinase and dGuo kinase after infection rules out the possibility of virus induction of these kinases. The final yield of infective virus particles in low serum cells is about one log unit of infectivity less than in high serum cells. This is an indication that cellular enzymes in such systems may not be able to provide sufficient precursors for optimal synthesis of viral progeny. But with high serum cells no such situation arises as adequate amounts of enzymes are available within the cell.

Synthesis of DNA in mammalian cells is inhibited by high concentrations of dAdo in the medium. This inhibition of DNA synthesis may be due to the accumulation of dATP within the cell, which acts as an allosteric inhibitor of ribonucleotide reductase in mammalian cells at concentrations in the order of 8 μM (Moor and Hurlbert, 1966). Addition of 8 mM dAdo to HEp-2 cells, immediately after infection with herpes simplex, prevents the synthesis of deoxythymidine kinase (Borman and Roizman, 1965). The enzyme continues to be made if dAdo is added after the onset of the synthesis of the enzyme but the final yield of virus is reduced. Pulse labelling experiments indicate that dAdo also inhibits RNA synthesis. The fact that the levels of

dAdo and dAMP kinases of BHK cells are high and that further increases could possibly give rise to inhibition of viral DNA synthesis, tends to rule out any necessity for the induction of dAdo or dAMP kinases.

Heat sensitivity and pH optima of purine deoxyribonucleoside kinases remain unaltered after herpes infection. Compared with cellular pyrimidine deoxyribonucleoside kinases, purine deoxyribonucleoside kinases are much more thermostable. This "robustness" and the high levels of the purine kinases in uninfected cells explains the absence of a requirement for the induction of these enzymes.

3.4. The Probable Order of Enzyme Induction

Soon after herpes infection several enzymes are induced. Their activities increase in an orderly manner during early part of the virus replication cycle. If it is assumed that an increase in enzyme activity parallels the initiation of gene expression, then a tentative order of gene expression for early enzymes can be proposed. By examining the profiles of enzyme induction at different times after infection (Figs. IV. 2, 3, 4) the following deductions regarding the order of enzyme induction can be made.

(i) Deoxycytidine Kinase

(ii) Deoxythymidine Kinase

(iii) dTMP Kinase ?

(iv). DNA Polymerase/Exonuclease

(v) DNA Exonuclease ?

(vi) Deoxycytidine Kinase ?

Position (iii) has been allocated to dTMP in view of the observations made by Newton (1964). It is reported that an increase in dTMP kinase activity commences about 2 hr. later than that of dThd kinase and somewhat earlier than the overall DNA synthesising reaction. But this finding should be treated cautiously as it has been reported that dTMP kinase in pseudorabies infected rabbit kidney cells is not a virus-induced enzyme but a virus stabilised host enzyme whose configuration has been altered (Kaplan et al., 1967b).

After herpes infection an elevation of alkaline DNase is observed. On partial purification of the enzyme on DEAE cellulose an "adsorbed peak" of enzyme activity appears at a salt concentration of about 0.12M-KCl. On further purification of this fraction on hydroxyapatite it is resolved into two exonucleases (Paton and Morrison, 1969). One exonuclease fraction shows properties characteristic of DNA polymerase, while no such activity is seen in the other exonuclease. It has been suggested that DNA polymerase is associated with exonuclease in view of their association after many purification steps (Paton and

Morrison, 1969). This requires the induction of DNA polymerase and DNA exonuclease activities together, but induction of DNA polymerase precedes the induction of alkaline DNase in the order of appearance of enzyme activity (Morrison and Keir, 1968). In view of these results the induction of a second virus-induced DNA exonuclease after the induction of DNA polymerase-associated exonuclease seems a possible explanation of this difference in the induction of DNA polymerase and exonuclease.

4. Speculation on the Role of Herpes-Induced Enzymes on Viral Multiplication

4.1. Introduction

Virus induced early enzymes appear in an orderly manner following the transcription of early m RNA. The regular 10 - 15 fold increases in enzyme activity observed for virus-induced enzymes at 10 - 12 hr. post-infection indicate a possible co-ordinated form of action in control and regulation of the synthesis of progeny virus. Cells irradiated with U. V. light so that they are unable to replicate DNA can still support growth of herpes virus (Kaplan, 1957), indicating a high degree of viral autonomy as compared with, say, polyoma virus. The replacement of thymidine in pseudorabies progeny viral DNA with Br dUrd or Id Urd is known to interfere with the synthesis of a "late

protein" responsible for the "cut off" of early enzyme synthesis (Kaplan et al., 1965). Since, pseudorabies is a closely related virus of the herpes group, this same hypothesis can be extended to herpes simplex. The three extra peaks of high buoyant density which appear by 8 hr. post-infection (Fig. IV.31) may well represent DNA-RNA hybrids of herpes corresponding to transcription of late m RNA from progeny viral DNA template. It is envisaged that the progeny viral genome codes for the synthesis of specific proteins which control production of induced enzymes and other late functions.

4.2. DNA Polymerase

Virus-induced DNA polymerase differs from host DNA polymerase in the template and ionic requirements for optimal activity (Keir, 1968). The minimum template requirement for optimal activity of virus-induced enzyme is about 6 fold higher than control cell enzyme. This may either represent a larger population or a higher specific activity for virus induced enzyme in the partially purified enzyme preparation. Until a homogenous preparation of this enzyme is available it will not be possible to analyse this requirement. The isolation of single stranded herpes DNA on CsCl isopycnic gradients suggests that large single stranded regions of herpes DNA

which have come apart may well be a requirement for herpes DNA replication. In vitro studies, herpes-induced DNA polymerase did show a preference for denatured DNA as a primer over native DNA. But this could well be an outcome of damage done to DNA polymerase during extraction.

4.3. DNA Exonuclease

Virus-induced DNA polymerase is found to be associated with exonucleolytic activity. Like fatty acid synthetase (Lynen, 1967) this may well be a multi-enzyme complex composed of subunits of different enzyme activity. In Salmonella typhimurium genes controlling biosynthesis of histidine are found to be clustered together in one operon (Goldberger et al., 1967). If a cluster is to persist, gene arrangement must offer some selective advantage to the organism. In fact it is observed that when proteins are synthesised in such systems, instead of synthesising each individual enzyme separately, some enzymes are synthesised together as a string of protein. So that, end product of one could be handed over to the next in line with least effort and maximum efficiency. This may well be the case with DNA polymerase associated exonuclease. Exonuclease could provide two parasynthetic functions.

(i) It could degrade double stranded viral DNA from 3' end to provide suitable single stranded regions to initiate the replicative process.

(ii) It could trim off excess nucleotides from the ends of the finished product.

These functions clearly need careful regulation which could involve high G + C regions on the viral genome. Novak (1969) has demonstrated that transcription of RNA *in vitro* from T_7 phage DNA template was terminated in a region whose GC content was high. In fact micrococcal RNA polymerase cannot transcribe either strand of poly dG. dC (Steck *et al.*, 1968). Thus it is possible that virus-induced DNA polymerase may prefer viral DNA of high G+C content as template whilst exonuclease may prefer host DNA of low G+C content as a substrate.

To understand the vital functions of exonuclease it is necessary to obtain a mutant of herpes simplex lacking exonuclease activity in the enzyme complex. The production of such a mutant could be difficult if DNA polymerase and exonuclease genes are in the proximity of each other. The other alternative is to purify exonuclease to homogeneity and to analyse the products obtained after digestion of

viral and host DNAs with the same. The discovery of breakdown of host DNA into soluble DNA precursors and its reincorporation into viral DNA in in vivo studies has provided a degradative role for the exonuclease.

4.4. Pyrimidine deoxyribonucleoside Kinases

Any increase in DNA synthesis in eukaryotic cells is usually accompanied by an increase in the activity of dThd kinase. The only known function of this enzyme is the phosphorylation of exogenous deoxythymidine, probably, arising from degraded cellular DNA from dead cells. It is for this reason that it is often referred to as a "salvage enzyme". The same argument applies to dCyd kinase. The fact that rapidly proliferating tissues (Durham and Ives, 1969) and virus-infected mammalian cells (Kaplan and Ben-Porat, 1968) have relatively high levels of pyrimidine kinases, suggests the possibility that these enzymes may also participate in some other pathway for the synthesis of pyrimidine deoxyribonucleoside triphosphates (Grav, 1967) in addition to a salvage function.

In herpes-infected BHK cells the activity of dCyd kinase is about twice that of virus-induced dThd kinase (Fig. IV.2). The fact that deoxycytidine can act as a precursor of dTTP via the dCMP

deaminase pathway, as well as dCTP; and that dCyd kinase is more vulnerable to feedback inhibition than dThd kinase (Table IV. 21), makes this relatively large increment of dCyd kinase in virus-infected cells more significant. Virus induced pyrimidine deoxyribonucleoside kinases may have to deal with large increases in pool size of deoxyribonucleosides (Fig. IV. 26) within the cell. Cellular enzymes are low in activity and may not be able to deal with this situation. Furthermore, dihydrofolate reductase and dTMP synthetase are not increased after herpes infection (Frearson et al., 1966). Hence the requirement for efficient phosphorylating agents for preformed deoxyribonucleosides.

Treatment of virus-infected BHK cells with hydroxyurea inhibits the ribonucleotide reductase pathway and hence depletes the pool of deoxyribonucleoside triphosphates. This may stimulate the synthesis of pyrimidine deoxyribonucleoside kinases. Interestingly, it is found that hydroxyurea does enhance the activity of viral-induced dCyd kinase when added at any time after 3 hr. post-infection (Fig. IV. 4). In view of the reports that modification of histones by acetylation (Allfrey, 1966) or phosphorylation (Stevely and Stocken, 1966) can alter their ability to inhibit DNA-directed synthesis of RNA, it is not impossible that an interaction between an intracellular nucleotide and

the histone part of the DNA-histone complex could modify the repressing ability of the latter. Preliminary experiments (Eker, 1968) have shown that deoxythymidine is bound to histones in vitro.

Non-growing cells are reported to contain higher levels of phosphatase than do growing cells (Eker, 1965). Although pyrimidine deoxyribonucleoside kinases may have little or no survival value if infection occurs in growing cells, it may be of importance if viral DNA synthesis occurs in resting cells such as nerve cells which seems to be the natural host cell for herpes viruses (Fenner, 1968). To overcome the catabolic enzymes present in stationary phase cells the infecting virus may have to establish a mechanism to ensure the accumulation of deoxyribonucleoside triphosphates necessary for the synthesis of herpes DNA.

Out of all functions that would require the induction of herpes-induced pyrimidine deoxyribonucleoside kinases the most important function appears to be the necessity to utilise material from degraded host DNA for viral DNA synthesis.

5. Evolution

Herpes DNA with a G+C content of 68% differs markedly from the nearest neighbour base sequences of the host genome, whose

G+C content is 42%. In fact, the doublet frequency pattern of herpes DNA does not differ much from that of E. coli DNA (Subak-Sharpe et al., 1966b). It is tempting to speculate that herpes DNA could have arisen by fragmentation of a bacterial genome. Some of the general properties of virus-induced enzymes appear to support this point of view.

(i) Virus-induced DNA polymerase is associated with exonuclease, unlike typical mammalian DNA polymerases (Yoneda and Bollum, 1965).

(ii) Virus-induced DNA polymerase has a relatively higher affinity for native DNA as template than host DNA polymerase.

(iii) Virus-induced pyrimidine deoxyribonucleoside kinases like the bacterial enzymes are more stable than the corresponding host enzymes.

On the other hand, there are properties such as the presence of large nuclear viral RNA which have to be fragmented before being transported across the cytoplasm, which resemble eukaryotes (Wagner et al., 1969). It is possible that the "early herpes virus" which was in a quiescent stage like the prophage stage in bacteria may have adapted itself to the host cell environment. Ultra violet radiation or a hormonal imbalance may have then caused the release

of viral genome. The fact that we have been able to identify two activities of dCyd kinase may mean that gene duplication with translocation may have taken place during evolution of herpes DNA.

6. Horizons

Herpes DNA is large enough to code for about 130 proteins of 50,000 average molecular weight. Polyacrylamide gel electrophoresis of proteins of herpes infected HEp-2 cells reveals only about 25 protein bands (Spear and Roizman, 1968). This leaves about 75% of its coding potential unaccounted for. Viral RNA of sedimentation coefficients 10 - 20 S extracted from the cytoplasmic polyribosomes of HEp-2 cells competed to a level of at least 80% in viral DNA-RNA hybridisation tests with >50 S RNA extracted from nuclei of infected cells (Wagner and Roizman, 1969). This is consistent with the hypothesis that viral m RNA is produced by cleavage of precursor RNA molecules. Under these conditions, some of the large segments of RNA transcribed may not be able to enter the cytoplasm without modifications and hence may not be translated at the cytoplasmic ribosomes.

Herpes infection alters the populations of t RNA in BHK cells (Hay et al., 1966). A new or modified t RNA specific for arginine is found among altered t RNA populations (Subak-Sharpe et al., 1966a).

Chromatography of digestion products of arginyl-t RNA's suggests that the virus induced arginyl-t RNA is different from host arginyl-t RNA and is probably virus coded. Cellular arginyl-t RNA synthetase may not be made in sufficient amount to meet the amino acid coding requirements of viral arginyl-t RNA. Hence it is possible that the virus genome may code for the synthesis of aminoacyl synthetases. These possibilities were predicted earlier from analysis of nearest neighbour base sequences.

In conclusion it must be said that herpes has the potential to code for many more enzymes other than those already recognised as being virus coded. An understanding of the complete co-ordinated action of virus-coded enzymes with the virus induced modifications in the transcriptional and translational apparatus of the host cell may provide invaluable guidance for the understanding of control mechanisms of the mammalian host cell, just as bacteriophages has helped in the study of control mechanisms of bacteria.

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