STUDIES ON MAMMALIAN VIRAL DNA AND ITS METABOLISM, WITH SPECIAL REFERENCE TO HERPES VIRUS.

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LIST OF ABBREVIATIONS.

Abbreviations used without definition are those recommended by the Editorial Board of the Biochemical Journal (Biochem. J., 102, 1 (1967).

DNA polymerase	- DNA nucleotidyltransferase (E.C. 2.7.7.7.)
DNase	- deoxyribonuclease+
RNase	- ribonuclease+
α-^{32}P-dTTP, etc.	- deoxyribonucleoside 5'-triphosphates of thymine, etc., proximally labelled with ^{32}P.
XpY, etc.	- dinucleotide of the (deoxy)ribonucleosides X and Y, in 3'-5'-phosphodiester linkage.
TdR, FUdR, BUdR	- deoxyribonucleosides of thymine, 5'-fluoro- and 5'-bromouracil.
PPO	- 2,5-diphenyloxazole
POPOP	- 1,4-bis-(2-(5-phenyloxazolyl)-benzene).
SH-EtOH	- 2-mercaptoethanol
TCA	- trichloroacetic acid
PCA	- perchloric acid
SDS	- sodium dodecyl sulphate
BHK 21 cells	- a strain of cultured cells derived from baby hamster kidney (MacPherson and Stoker, 1962).
HEp-2 cells	- human epithelioid carcinoma, No. 2.
RK cells	- rabbit kidney cells.
MOI= x pfu/cell	- multiplicity of infection = x plaque forming units per cell.

+ E.C. reference numbers not used, as they do not adequately describe the diversity of these enzymes (see ChapterI, Section 2.3.3.)

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CHAPTER I

CHAPTER I - INTRODUCTION

1. VIRUSES

1.1. Historical Background.

In the first twenty years of the 20th Century, agents were discovered which infected plants, animals or bacteria, which were smaller than bacteria and required a host cell within which to multiply. These agents became known as viruses. A commonly observed feature of virus infection was the destruction or alteration of appearance of the cells of the host and this, of course, is the cause of the pathogenicity of many of the human viral diseases; the severity and frequency of occurrence of these have always been strong incentives for investigation of the nature of viruses and their growth.

The first major breakthrough in determining the chemical nature of viruses came in 1935, when Stanley crystallised tobacco mosaic virus and showed that it contained only protein and a small amount of RNA (Stanley, 1935). On the other hand, examination of bacterial viruses (bacteriophages) had revealed that they, although also nucleoproteins (Schlesinger, 1936), contained DNA and also possessed a more complex morphology. Analysis of animal viruses revealed that both DNA- and RNA-containing types existed, and it gradually emerged that viruses differed from other organisms

in possessing only one type of nucleic acid.

Within the last two decades, application of biochemical techniques to the study of virus-infected cells has led to tremendous advances in the knowledge and understanding of the processes of viral multiplication, and these in turn have contributed substantially to an understanding of the molecular mechanisms operating in living cells. Cohen (1963) sums up the situation when he declares that, "Virology has emerged..... as a major approach to the study of the physiology and genetics of the synthesis of specific proteins and nucleic acids."

1.2. Characteristics of Viruses.

When viruses are considered as organisms, the fundamental question of whether they are "living" or "non-living" has often been raised in view of the seeming inertness of the virus particle. This knotty problem has been gradually reduced to one of semantics as knowledge of the fundamental processes involved in virus growth has increased, and as it has been realised that viruses are extremely efficient parasites which use the metabolic capability of the invaded cell in order to synthesise the components of the progeny virus. The so-called "inertness" of viruses is true only of the mature virus state which is analogous, in certain respects, to the spore state in other micro-organisms. The almost frantic rate at which

biochemical processes take place during the vegetative state of many viruses, especially bacteriophages, could scarcely be called inert!

In order to distinguish viruses from non-viruses, Lwoff (1953) proposed the following discriminatory characteristics:-

(i) Viruses possess only one type of nucleic acid, either RNA or DNA; other infectious agents possess both types.

(ii) Viruses are reproduced solely from their nucleic acid; other agents are reproduced from the integrated sum of their constituents.

(iii) Viruses are unable to grow (as distinct from multiply) and to undergo binary fission.

Later, he added another two criteria (Lwoff, 1957):-

(iv) Viruses do not possess the genetic information for the synthesis of an energy-producing system.

(v) Viruses make use of the ribosomes of their host cells.

Viral nucleic acids are either single- or double-stranded, all four possible types having been described (Table I.1). An increasing number have been isolated as single molecules, and it seems likely that this is a universal feature of viral, if not all, chromosomes (Josse and Eigner, 1966; Thomas and MacHattie, 1967).

The viral nucleic acid is surrounded by one or two coats. The first and invariable coat is called the capsid and comprises protein subunits or capsomeres surrounding the

Table I.1.

Types of Nucleic Acid Found in Animal Viruses.

Type of nucleic acid	Number of strands	Class of Virus	Example
RNA	one	Picornavirus	Poliovirus
RNA	one	Arbovirus	Semliki forest
RNA	one	Myxovirus	influenza
RNA	two	Reovirus	reovirus[1]
DNA	one	not named	Kilham rat virus[2]
DNA	two	Papovavirus	polyoma[3]
DNA	two	Adenovirus	many (numerical)[4]
DNA	two	Herpesvirus	herpes (simplex)[5]
DNA	two	Poxvirus	vaccinia[6]

References:-

1) Gomatos, P.J. and Tamm, I., Proc.Nat.Acad.Sci., 49,707(1963)

2) J.Miller Whalley, personal communication.

3) Crawford, L.V., Virology, 19,279(1963)

4) Green, M. and Pina,M., Virology,20,199(1963)

5) Russell, W.C. and Crawford, L.V., Virology,21,353(1963).

6) Joklik.W.K., J.Mol.Biol.,5,265(1962).

viral nucleic acid. The number of capsomeres is constant for a given virus and the symmetry of the capsid may be helical or cubic. The viral nucleic acid plus capsid is termed the nucleocapsid and this may exist in naked or enveloped form. The second coat or envelope is termed the peplos and may be composed of viral and/or host cell material.

Various schemes of classification of viruses have been proposed (Lwoff, 1966). For example, the system devised by Lwoff, Horne and Tournier (1962) uses the following "essential integrants":-

(i) Type of genetic material: RNA or DNA

(ii) Symmetry of virion: helical, cubic or binal.

(iii) Nucleocapsid: naked or enveloped.

(iv) Quantitative data:

helical - diameter of nucleocapsid

cubic - number of capsomeres.

At the present state of knowledge, however, such attempts at classification are necessarily preliminary.

1.3. Multiplication of Viruses.

The time course of viral infection may be divided into three stages:-

(i) adsorption and penetration of the virus particle into the host cell;

(ii) a latent or eclipse phase during which no infective virus can be detected;

(iii) release of mature virus particles from the cell.

The characteristics of the first and third stages vary considerably from virus to virus and host to host, while the second, although "in eclipse" as judged by the level of infective virus, is, in fact, a turmoil of biosynthetic activity in which considerable generality can be discerned irrespective of the particular characteristics of host or virus.

During the eclipse phase, the following events must take place:-

(i) the virus loses its protein coat (uncoating);

(ii) the viral DNA is transcribed and the resultant messenger RNA (or viral RNA) translated into virus-specified "early" proteins necessary for -

(iii) the replication of viral nucleic acid;

(iv) a further phase of transcription from progeny and/or parental nucleic acid takes place, leading to -

(v) the synthesis of "late" virus-specified proteins which include protein(s) of the viral coat;

(vi) assembly of the viral particle (maturation).

The eclipse phase is the period most intensively studied from a biochemical viewpoint, and further description of DNA virus multiplication will be found in Section 3 of this Chapter. The information on RNA viruses is voluminous and will not be dealt with here.

1.4. DNA Viruses.

1.4.1. Bacterial DNA viruses.

Many diverse DNA-containing viruses have been described which infect animal or bacterial cells, although none has so far been discovered which infects plants. The DNA virus-host cell interaction which was first examined and which has continued to be the most favoured experimental system is Escherichia coli infected with bacteriophages of the T-series. Seven of these have been described (T1-T7); the closely-related T2 and T4 are undoubtedly the DNA viruses which are best understood in molecular terms.

Virulent bacteriophages are those which have only one mode of interaction with the host cell, namely, one which leads to the multiplication of the virus and the eventual lysis of the host cell, with concomitant release of mature progeny virus particles. Two classes have been described (Whitfield, 1962), the autonomous virulent (e.g. the T-even and T5 bacteriophages) which bring about the rapid destruction of the bacterial genome, and the dependent virulent phages (e.g. T1, T3 and T7) which allow bacterial metabolism to continue for a time and indeed may require the integrity of the bacterial genome.

Another virus which has been the subject of intense experimentation is the temperate bacteriophage λ of Esch.coli

In contrast to the virulent bacteriophages, which can only interact with a cell so as to cause its lysis at the time of release of progeny virus particles, the temperate bacteriophages have the alternatives of initiating a lytic infectious cycle or of lysogenising the bacterial host. In the lysogenic state, the bacterium carries the genome of the virus in a latent condition (prophage) which is located at a specific site on, and multiplies along with the bacterial genome until some stimulus induces the vegetative state. The bacteriophage genome then expresses itself autonomously by initiating virus production and eventually causing lysis of the host cell.

One other class of DNA bacteriophages worthy of note are the small viruses containing single-stranded DNA (molecular weight of DNA, 1.6×10^6 daltons), the first observed and most extensively studied of which is ΦX 174, infecting Esch.coli. The discovery of this virus was the first observation of naturally-occurring, single-stranded DNA.

1.4.2. Animal DNA Viruses.

DNA viruses infecting animal cells display a diversity comparable with that of the DNA bacteriophages. Their size range is large; they multiply in the nucleus (herpes, adeno and papovaviruses) or the cytoplasm (poxviruses) of the host cell; the majority contain double-stranded DNA, but, in a few cases (e.g. Kilham rat virus and the minute virus of mice)

the DNA is probably in the singe-stranded configuration. In addition to these varied characteristics, animal viruses seem to be divisible into two categories, in many ways analogous to those of the virulent and temperate bacteriophages.

Many animal DNA viruses are invariably virulent, e.g. the herpes and poxvirus groups and many members of the adenovirus group, but, in no case has marked destruction of the host genome been demonstrated. At the same time, no definite dependence on the integrity of the host cell genome has been shown and, indeed, the capacity of cells to support the growth of herpes and pseudo-rabies viruses is highly resistant to radiation (Powell, 1959; Kaplan, 1962), suggesting that these at least are autonomous.

The other class of animal DNA viruses is somewhat analogous to the temperate bacteriophages in that the interaction of virus and cell can lead to a state other than cytocidal multiplication of virus. Viruses of this type, termed moderate by Dulbecco (1965), include the papovaviruses and certain members of the human adenovirus group. When these viruses infect a cell, they can either multiply and cause cell lysis or else enter a latent state in which infective virus disappears and the viral genome may become associated with the host cell, while the cell becomes transformed or cancerous and virus-specific antigens appear. Unlike lysogenic bacteria, transformed animal cells only

rarely produce virus if at all (Gerber, 1966). These oncogenic viruses are mostly of small size, the papova viruses have DNA molecular weights in the range $3-5 \times 10^6$ daltons, the oncogenic adenoviruses about 20×10^6 daltons. Thus, by virtue of their smaller content of genetic material, these viruses are inevitably more dependent, during multiplication, on the metabolic processes of the host cell than are the larger, virulent viruses.

2. DEOXYRIBONUCLEIC ACID.

2.1. Primary Structure of DNA.

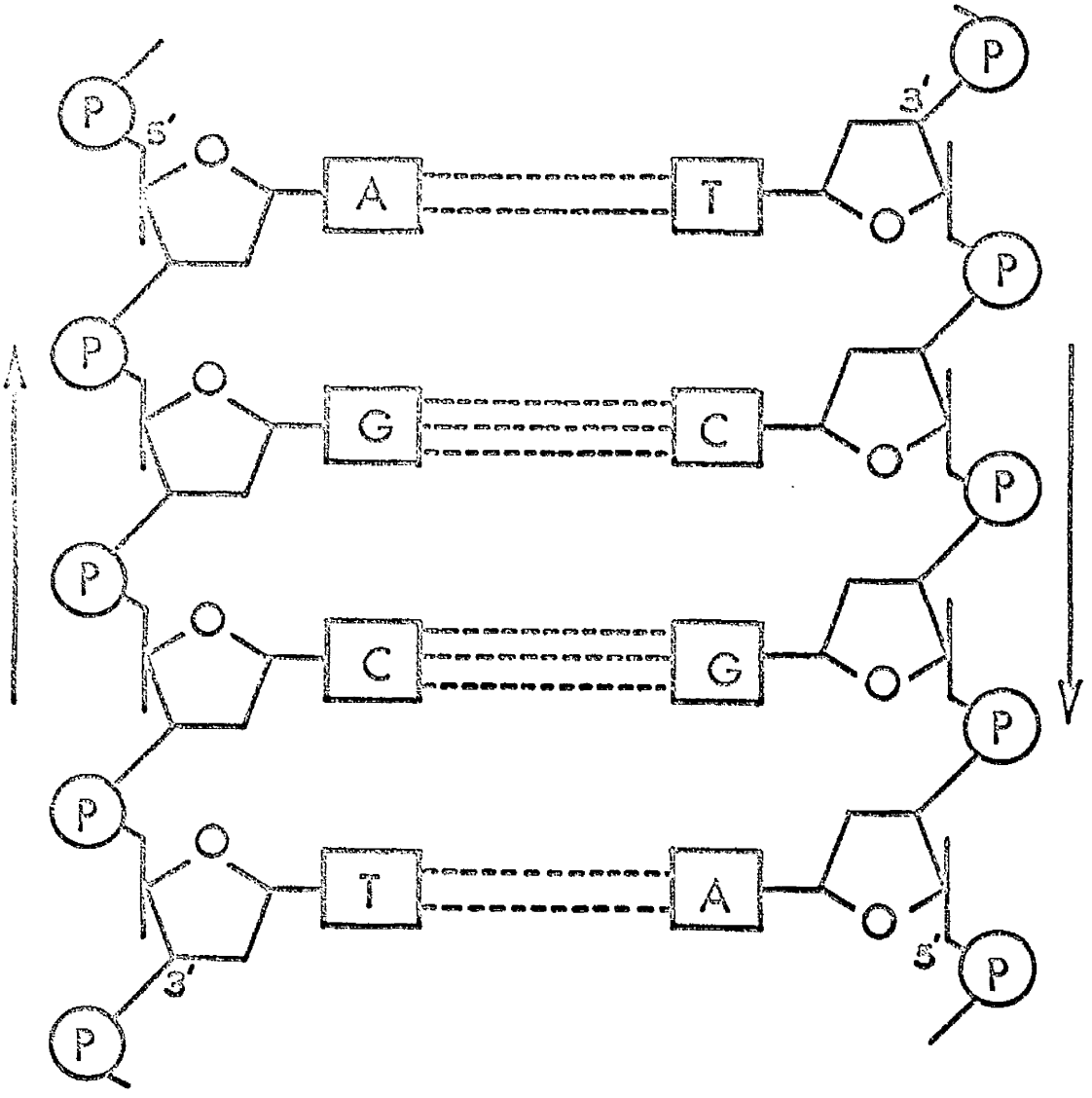
2.1.1. The chemical nature of DNA.

DNA is a linear heteropolymer whose monomers are purine and pyrimidine deoxyribonucleotides. The backbone of the chain comprises alternating 2-D-deoxyribose and phosphate residues, joined by 3',5'-phosphodiester linkages (Figure I.2.). The absence of a 2'-hydroxyl group prevents the formation of 2',3'-cyclic monophosphates, as, for example, happens when RNA is exposed to alkaline conditions. Stability of the primary structure of DNA to alkali is a feature which has long been used to distinguish the two types of nucleic acid. It is the basis of methods such as that of Schmidt and Thannhauser (1945) by means of which tissues may be analysed for their DNA and RNA contents.

The nitrogenous bases, which are normally adenine and

Figure I.2.

Structure of DNA.



guanine (purines) and cytosine and thymine (pyrimidines) are bound to carbon-1 of deoxyribose through their nitrogen-9 and nitrogen-1 atoms respectively. Various other bases have been detected in DNA. These include:-

(i) 5'-methyl cytosine, which occurs to the extent of a few per cent in animal, bacterial and plant DNAs;

(ii) 6'-methyl aminopurine (6'-methyl adenine), which occurs in small amounts in bacterial and bacteriophage DNAs;

(iii) 5'-hydroxymethylcytosine, which completely replaces cytosine in the DNA of the T-even bacteriophages (Wyatt and Cohen, 1953).

(iv) 5'-hydroxymethyluracil, which completely replaces thymine in the DNA of the bacteriophage SP8 of Bacillus subtilis (Kallen et al., 1962).

(v) Uracil, which completely replaces thymine in the DNA of the bacteriophage PBS8 of B. subtilis (Takahashi and Marmur, 1963).

The bacteriophage DNAs containing these unusual bases (iii-v) also contain glucose, but only in the case of the T-even bacteriophages is it directly attached to the unusual base.

Chemical or enzymic hydrolysis of DNA, followed by chromatographic analysis, gives its base composition. Certain quantitative relationships exist among the four bases:-

(a) the molar ratios of adenine to thymine and of guanine

to cytosine are unity, (i.e. A = T; G = C);

(b) the sum of the purine bases equals the sum of the pyrimidine bases, (i.e. A+G=C+T);

(c) The number of 6-amino groups equals the number of 6-keto groups, (i.e. A+C=G+T), (Chargaff, 1955).

Equation (a) is the fundamental one, (b) and (c) follow from it. Recognition of these equalities contributed towards the development of the double-helical structure of DNA (Watson and Crick, 1953). In addition, the description of DNA preparations in terms of their percentage content of G+C has been very useful. (e.g. herpes virus DNA, G+C = 68%; A=T=16%; G=C=34%; Russell and Crawford, (1964)).

2.1.2. Analysis of the primary structure of DNA.

The primary structure of a macromolecule is the linear sequence of the monomeric units. In protein chemistry, techniques have been developed which permit the determination of the amino acid sequence of polypeptide chains of moderate size. Here, the problem is one of an alphabet of twenty and words of a few hundred letters; with the nucleic acids, the alphabet has shrunk to four and the length of words increased to many thousands of letters! Although the nucleotide sequences of some transfer-RNAs (e.g. Holley et al., 1965) and and of 5S RNA (Brownlee et al., 1967) have been determined, it would seem that current techniques are near their limit and sequence determination of larger polynucleotides is some way off, (Review by Burton, 1965).

The picture is not one of unmitigated gloom, however, as several methods exist which give some information on the primary structure of DNA. These fall into two categories:-

(i) Methods involving enzymic or chemical degradation of DNA.

If DNA can be degraded in some specific way, then the oligonucleotides produced can be analysed and the frequency of occurrence of particular nucleotide sequences can be measured. Enzymic hydrolysis has not been widely used because highly bond-specific DNases have not yet been discovered (Laskowski, 1967), but acidic hydrolysis, which produces pyrimidine clusters, hydrazinolysis, which produces purine clusters, and degradation with OsO_4 have yielded useful information (Burton, 1965; Burton et al., 1963; Rudner et al., 1966)

(ii) Methods involving enzymic synthesis on a DNA template.

The DNA polymerase of Esch.coli catalyses the synthesis of DNA from the four deoxyribonucleoside 5'-triphosphates dATP, dCTP, dGTP and dTTP when supplied with a DNA "primer" (Kornberg, 1961), (see Section 2.3.2). The base composition of the synthesised material reflects that of the added primer because the latter acts as template for the production of a new DNA strand by complementary base-pairing (see Section 2.2.1.). Thus, analysis of the enzymic product sheds light on the primary structure of the template DNA. Josse, Kaiser

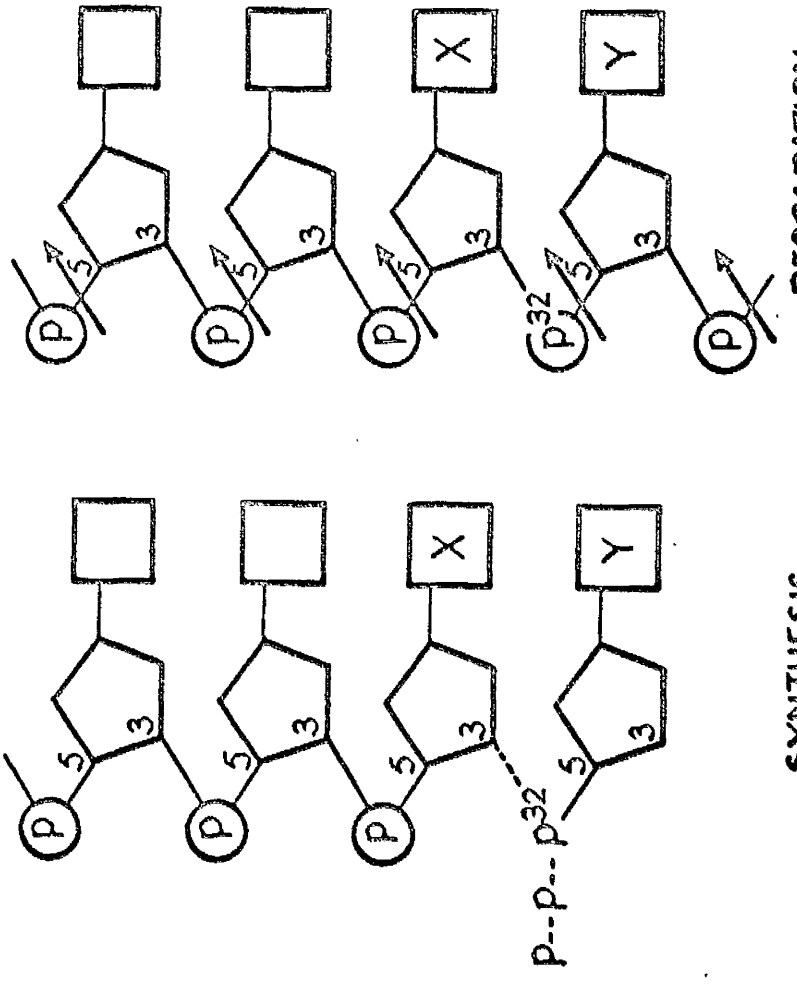
and Kornberg (1961) used this rationale in developing the technique of nearest neighbour frequency analysis of DNA (Figure 1.3.).

Basically, this method involves use of the DNA to be analysed in a DNA polymerase reaction in which only one of the four triphosphates is labelled with ^{32}P in the α-phosphate position, (e.g. dATP). Subsequent hydrolysis of the product to 3'-monophosphates effects the transfer of the ^{32}P phosphate from the deoxyadenosine residue to its nearest neighbour nucleoside on the 5'-side. Isolation of the four 3'-monophosphates and assay of each for radioactivity gives the proportion of each nucleoside neighbouring deoxyadenosine, which is related to the frequency of the dinucleotides ApA, CpA, GpA and TpA. Repetition with the other three triphosphates labelled in turn gives the complete analysis (see Chapter III. Section 2.).

Another method of this type, which has not been widely exploited so far depends on the ability of Esch.coli DNA polymerase to incorporate ribonucleotides into a DNA strand in the presence of Mn^{2+} (Berg et al., 1963). This introduces alkali-labile bonds into the polynucleotide product. If, for example, DNA is incubated with DNA polymerase in the presence of dATP, dGTP, dTTP and α-^{32}P-CTP and the product hydrolysed with alkali, oligonucleotides of composition $(dXp)_nYpCp$ will be produced, with the phosphate on the 5'-side of the C residue labelled and that on the 3'-side labelled only if its neighbour was another C residue.

Figure I.3.

Scheme of Nearest Neighbour Frequency Analysis.



Synthesis of a P^{32}-labeled DNA chain and its subsequent enzymatic degradation to 3'-deoxyribonucleotides. The arrows indicate the linkages cleaved by micrococcal DNase and calf spleen phosphodiesterase, yielding a digest composed exclusively of 3'-deoxyribonucleotides.

Measurement of the ratio of phosphatase-sensitive to total radioactivity for a series of isolated oligonucleotides gives information on the frequency of occurrence of certain sequences (e.g. YpCpC in the above case). This method has been used for the analysis of the DNA component of cytochrome b_2 (Jackson et al., 1965) and is clearly capable of considerable development.

2.2. The Secondary Structure of DNA

2.2.1. The Watson-Crick model.

Once the main features of the primary structure of DNA had been elucidated, the way lay open to use the powerful technique of x-ray diffraction to obtain information on precise stereochemical arrangements of the constituent atoms. The data arising from these studies allowed Watson and Crick (1953) to propose a double-helical structure for DNA.

Their model accommodates both the x-ray diffraction data and the observed quantitative relationships between the bases, and has the following basic features (Figure 1.4.):-

(i) two right-handed, helical polynucleotide chains, coiled on the same axis;

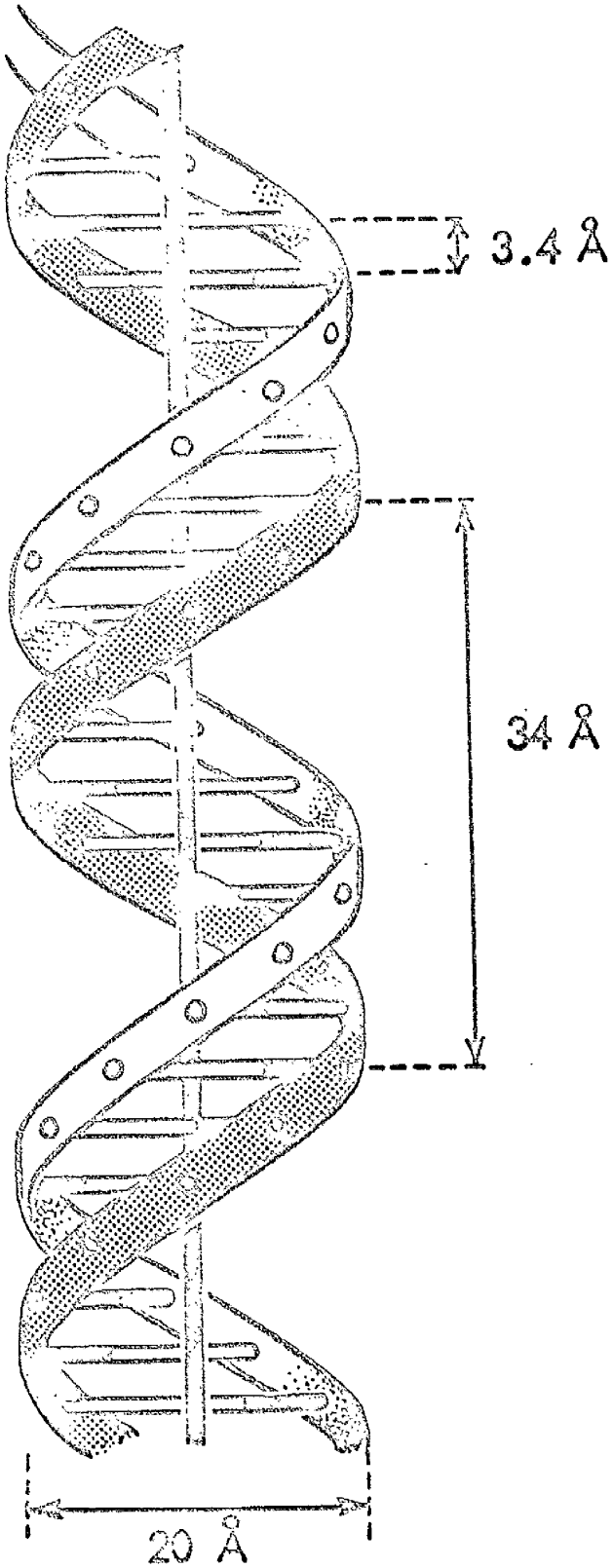
(ii) the diameter of the helices is 20Å and the pitch 34Å (equivalent to about 10 nucleotide pairs per turn);

(iii) the polynucleotide chains are antiparallel;

(iv) the bases lie normal to the long axis and are

Figure I.4.

Watson-Crick Model of DNA.



arranged specifically, so that A in one strand pairs by hydrogen bonding only with T in the other stand, and likewise G with C, (Figure I.5.).

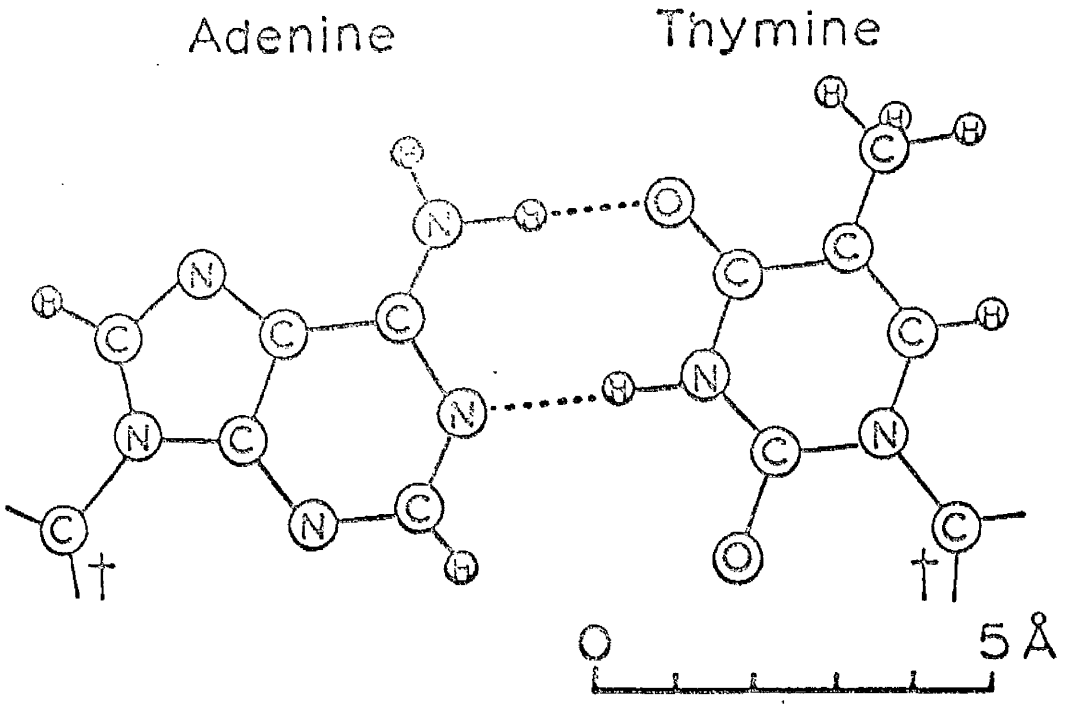
(v) the diameter of the double helix is constant, because all pairs are purine-pyrimidine;

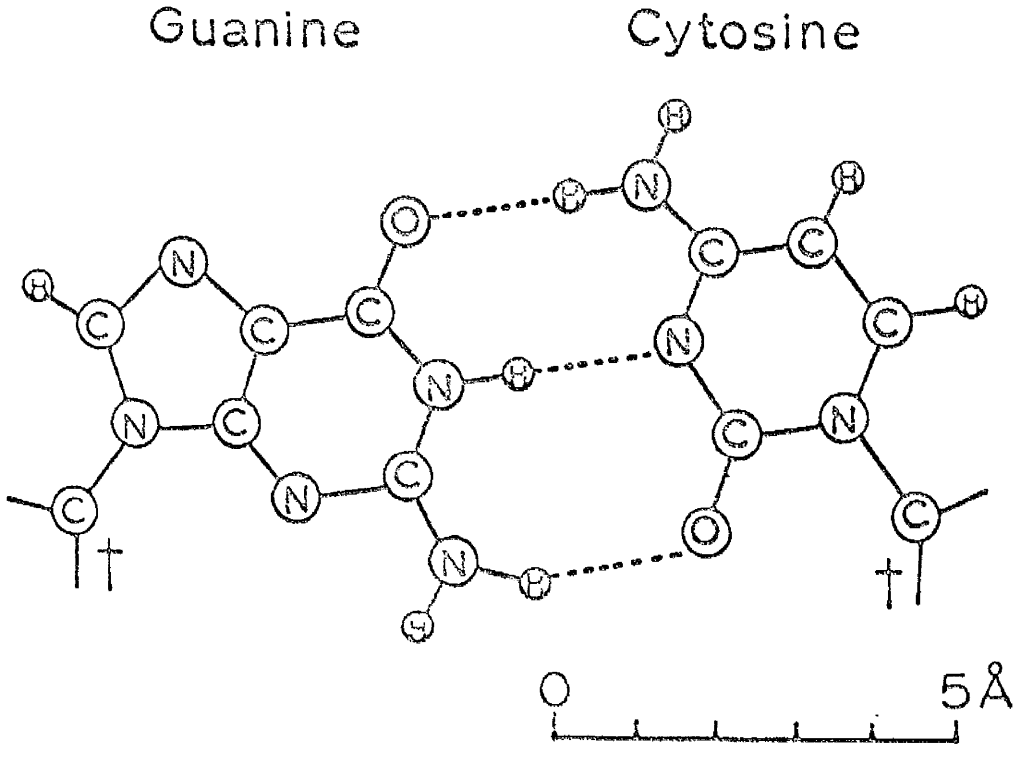
(vi) the deoxyribose-phosphate chains lie on the outside of the chains with alternate wide and narrow grooves between the helices (Figure I.4.).

The Watson-Crick model for the secondary structure of DNA has gained virtually universal acceptance and has proved an extremely fruitful one for modern biologists, implying as it does a mechanism for DNA replication, and, following the discovery of an RNA species complementary to DNA (Volkin and Astrachan, 1956), a mechanism for gene expression. Although the model has remained basically unchanged, some important refinements have been made. The X-ray studies of Wilkins and his colleagues revealed that three configurations of the DNA fibre are possible, with different pitches and different numbers of nucleotides per turn. In addition, recent work (DeVoe and Tinoco, 1965) has shown that although hydrogen bonding between base pairs gives the specificity of chain interaction, it is probably the stacking of the bases which is chiefly responsible for maintaining the double helix.

Figure I.5.

Hydrogen-bonded Base Pairing in DNA.





2.2.2. Molecular weight of DNA and aspects of DNA structure in vivo.

Both the solution properties and the ultracentrifugal properties of DNA have been used to determine its molecular weight, but these methods, while useful for comparative purposes, do not yield absolute values. Use of an autoradiographic technique which gives a direct measure of the number of phosphorus atoms in a DNA without making any assumptions about its structure gave a molecular weight for T2 bacteriophage DNA of $130 \pm 10 \times 10^6$ daltons. This provided a standard against which other methods could be calibrated (see Josse and Eigner, 1966).

Autoradiography and electron microscopy have both been used to determine the length of DNA molecules, and from this measurement, the molecular weight can be obtained, since the mass per unit length of the Na salt of DNA from X-ray diffraction studies is 200 daltons per Å. Electron microscopy, in particular, has proved a useful technique which gives both the molecular weight and the degree of heterogeneity of a given DNA preparation.

When the autoradiographic technique mentioned above was applied to whole T2 virus particles, the same result was obtained as with isolated DNA, indicating that the DNA of the virus is a single molecule. The same conclusion has been reached with several other DNA viruses, both bacterial and animal, and it seems likely that this is a general feature of viral nucleic acids. Other specific features of certain

bacteriophage DNAs are :

(i) the occurrence of single-strand breaks in genetically fixed points on T5 DNA (Thomas, 1966);

(ii) the existence of the DNA of T2 and T4 as collections of circularly permuted molecules with terminal redundancy (Thomas, 1966; Thomas and MacHattie, 1967).

Several viral DNAs have been isolated in circular forms. These include the single-stranded DNA of bacteriophage ØX174 (Tiers and Sinsheimer, 1962) and the double-stranded DNA of the papovaviruses (Crawford, 1964; 1965). The DNAs of the latter group exist in a supercoiled form which is converted to a ring form by a single-strand break, and to a linear form by a double-strand break. Bacteriophage λ DNA can occur in a "pseudo-circular" form because of the complementary, single-stranded regions which exist at either end of the molecule (Hershey et al., 1963). An enzyme has been detected in (Gellert, 1967) and purified from extracts of Esch. coli (Gefter et al., 1967) which will convert these "pseudo-circles" into covalent circular DNA.

Much less is known about the state of the DNA of the chromosomes of animal cells and of the significance of the interactions between DNA and the histones and other proteins of the cell nucleus.

2.3. Metabolism of DNA.

2.3.1. Synthesis of nucleotide precursors.

The reactions involved in nucleotide synthesis can be divided into several groups (Figure 1.6.):-

(i) Purine and pyrimidine biosyntheses take place by way of the well-established pathways from 5-phosphoribosyl-pyrophosphate (PRPP) to IMP, and from aspartate to UMP respectively.

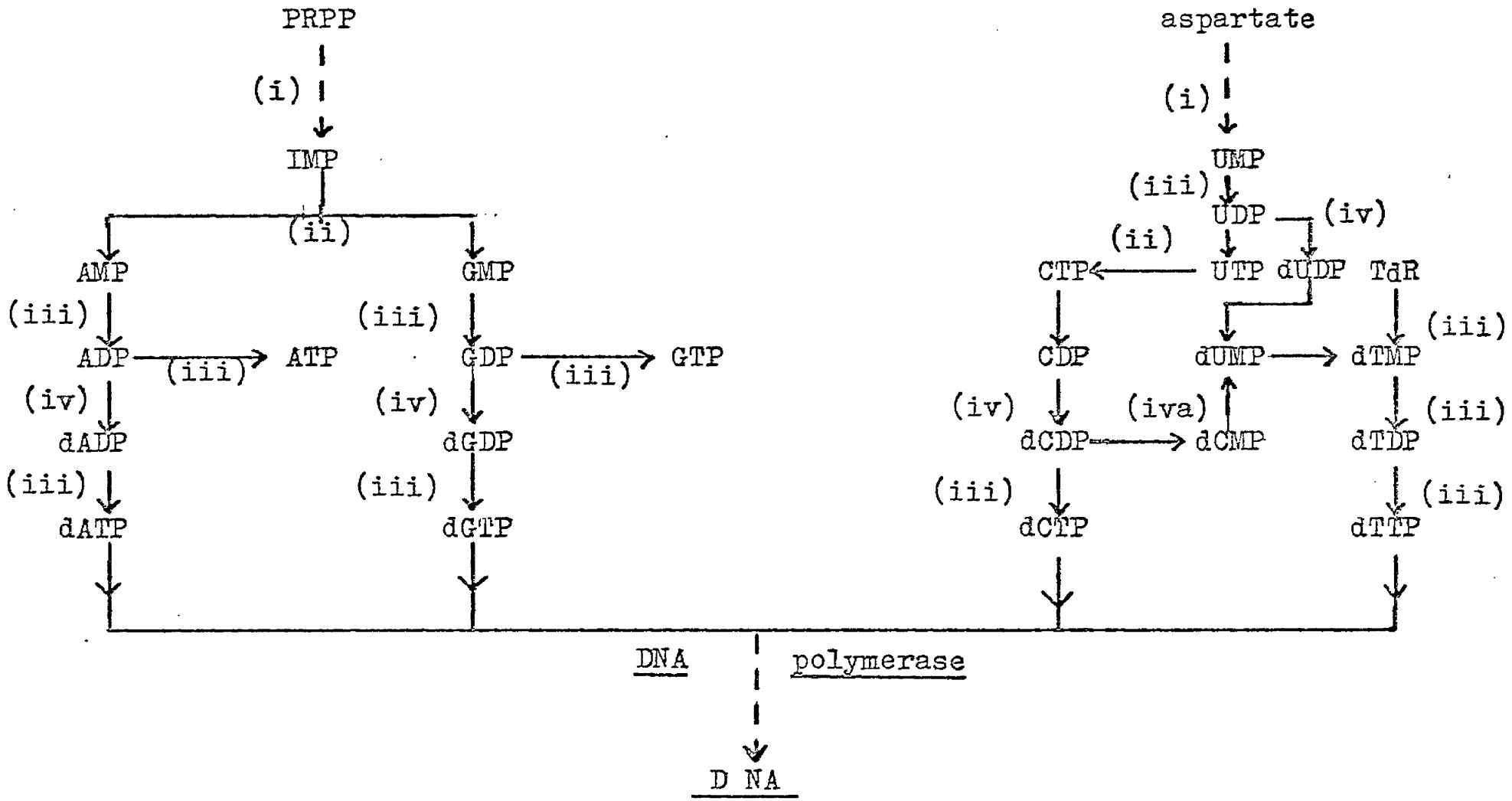
(ii) Ribonucleotide conversions give rise to AMP and GMP from IMP, and to CTP from UTP.

(iii) Phosphorylation of nucleoside monophosphates to the di- and triphosphates is catalysed by various kinases. Other kinases exist which phosphorylate nucleosides to nucleotides. One of the most important of these is TdR kinase, an enzyme which increases markedly following infection of animal cells with many DNA viruses. Many phosphatases of varying degrees of specificity exist which carry out the reverse of these phosphorylations.

(iv) Ribonucleotide reduction occurs at the diphosphate level in mammalian cells and Esch.coli, but at the tri-phosphate level in Lactobacillus leichmanni. It was believed that uridine nucleotides were not reduced and that dUMP was produced from dCMP by dCMP deaminase, (iva) but it is now known that both pathways occur (Moore and Hurlbert, 1966). Incorporation of dUTP into DNA, in

Figure I.6.

Synthesis of Deoxyribonucleotide Precursors.



Esch.coli at least, is prevented by the existence of a potent dUTPase (Greenberg et al.,1962).

(v) dTMP synthesis is catalysed by the enzyme thymidylate synthetase which methylates dUMP, in a reaction involving N^5,N^{10},-methylene tetrahydrofolate.

Alterations in nucleotide metabolism occur in virus-infected cells (Section 3.).

Many of the enzymic reactions of nucleotide synthesis are under the fine control of a negative feedback mechanism in which an end product inhibits an enzyme on its own synthetic pathway, e.g. AMP and GMP inhibit the first step of purine synthesis, and dTTP inhibits TdR kinase, dCMP deaminase and ribonucleotide reductase.

2.3.2. Synthesis of polydeoxyribonucleotides.

All polydeoxyribonucleotide-synthesizing enzymes utilise deoxyribonucleoside 5'-triphosphates as monomeric units and the reaction catalysed involves nucleophilic attack of a 3'-hydroxyl on the α-phosphate of a triphosphate residue to give formation of a3',5'-phosphodiester bond with the release of pyrophosphate (Kornberg, 1961). This reaction forms the basis of the assay method, which measures radioactivity rendered acid-insoluble when the enzyme is incubated with DNA, Mg^{2+} and the four deoxyribonucleoside 5'-triphosphates, one of which is radioactively labelled.

Enzymes of this type, called DNA polymerases (DNA nucleotidyltransferase, E.C.2.7.7.7.), have been detected

in many systems - bacterial, animal, plant and DNA virus-infected cells, and also in DNA-containing sub-cellular components, e.g. mitochondria. The DNA polymerase activities from these sources have several features in common (Keir, 1965; Kornberg, 1967):-

(i) a dependence on added DNA primer;

(ii) a requirement for the four 5'-triphosphates, (dATP, dCTP, dGTP and dTTP) for maximal activity;

(iii) the base composition of the synthesised material is complementary to that of the primer DNA.

A distinction has been drawn (Krakow et al., 1962) between the above type of polymerase activity, termed "replicative", and a homopolymer-synthesising activity called the "terminal addition" reaction which requires only onetriphosphate. The role of the preformed DNA in these two reactions is worthy of comment. In the second case, the DNA acts simply as a "primer" or initiator for the polymerisation reaction; in the case of replicative DNA polymerase, the DNA must not only act as a "primer", but also as a template on which the new strand is synthesised by complementary base pairing. Non-primed synthesis of poly dAT and poly dGdC from the appropriate triphosphates occurs after a lag period with Esch.coli DNA polymerase.

The best characterised DNA polymerases are those of Esch.coli (Richardson et al., 1964a) and calf thymus

(Bollum, 1960; Yoneda and Bollum, 1965); in these, and other systems, the DNA polymerase reaction and its products have been studied in some detail. Use of the Esch.coli DNA polymerase for the nearest neighbour frequency analysis of DNA has already been mentioned (Section 2.1.2.). The results obtained with this technique are consistent with a hydrogen-bonded selection of incoming triphosphates with their complementary nucleotides on the template strand, and also with an anti-parallel alignment of the polynucleotide chains of DNA (Josse, Kaiser and Kornberg, 1961). An examination of the replacement of the normal four deoxyribonucleoside 5'-triphosphates with unusual analogues which can form hydrogen bonds similar to the normal base which they resemble can be incorporated (Kornberg, 1961). A discussion of the role of DNA polymerases in DNA replication in vivo will be foundin Section 2.4.2.

2.3.3. Degradation of DNA

The nucleic acids are susceptible to attack by a class of phosphodiesterases commonly termed nucleases. These enzymes are of widespread occurrence and show considerable diversity in their properties. The main features of the action of nucleases will be described as a basis for classification:-

(i) Specificity towards substrate.

1. non-specific - i.e. hydrolysing both DNA and RNA.

2. specific - i.e.hydrolysing either DNA or RNA.

(ii) Mode of attack on substrate

Polymeric substrates can be attacked by enzymes in two ways, by endolytic attack, i.e. at points within the polymer chain, or by exolytic attack, i.e. stepwise from one end of the chain. Thus, two types of nuclease exist -

1. endonucleases - which produce oligonucleotides, and rapid changes in physical properties (e.g. viscosity) of the nucleic acid.

2. exonucleases - which produce mononucleotides and change the physical properties more slowly.

It should be noted that certain enzymes - micrococcal nuclease (de Meuron-Landolt and Privat de Garilhe, 1964) and bacteriophage T5-induced DNase (Paul and Lehman, 1966) - seem to carry out both endo- and exonucleolytic attack.

(iii) Mode of phosphodiester bond cleavage.

Unlike other polymeric substrates such as proteins and carbohydrates, whose subunits can only be cleaved in one way, the pentose-phosphate backbone of the nucleic acids can be split in two ways, to form products bearing -

1. 5'-phosphoryl groups, by hydrolysis of the bond between the 3'-hydroxyl and the phosphate, or

2. 3'-phosphoryl groups, by hydrolysis of the bond between the 5'-hydroxyl and the phosphate group.

(iv) Specificity towards secondary structure of substrate.

Several nucleases have been described which show absolute specificity towards (1.) single- or (2.) double-stranded

nucleic acids. Others show a strong preference for one configuration (1 or 2) while others (3.) attack both with similar facility.

These four criteria form the basis of a classification of nucleases, so that pancreatic DNase, for example, would be described as 2 (D).1.1.3., i.e. a DNA-specific endonuclease, forming 5'-phosphoryl groups and showing no strong specificity towards secondary structure. Pancreatic RNase would be 2(R).1.2.1. and snake venom phosphodiesterase 1.2.1.3.

Other features of the action of nucleases include (i) existence of a limit to digestion; (ii) with exonucleases, the end of the polynucleotide chain at which attack is commenced; (iii) with endonucleases, preferential bond hydrolysis, e.g. of GpX⟶Gp by RNase T_1; (iv) scission of double-strand DNA by single-hit (e.g. spleen DNase) or multiple-hit (e.g.pancreatic DNase) kinetics.

Although nucleases can be detected in practically every biological system their physiological roles are virtually unknown. Breakage of phosphodiester bonds probably occurs in processes such as recombination and replication of supercoiled, circular DNA but the enzymes involved have not yet been defined.

2.3.4. Alteration of DNA.

Several mechanisms exist which can modify the structure

of whole DNA molecules. Some of these are chemical (e.g. the action of mutagens such as nitrous acid), some are physical (e.g. the formation of intra-chain thymine dimers by ultraviolet light) but only the enzyme-catalysed types will be dealt with here.

(i) Methylation

Non-thymine methyl groups in DNA are added after formation of the DNA chain. The enzymes responsible (DNA methylases) are found in animal, bacterial and bacteriophage-infected systems and cause the methylation of cytosine (animal) or cytosine and adenine (bacterial) by the methyl donor, S-adenosylmethionine. The degree of methylation of a given DNA seems to be determined by the specificity of its homologous methylase, as the characteristic degree of methylation of a DNA can be increased by use of a heterologous enzyme, (see Borek and Srinivasan, 1966).

(ii) Glucosylation.

A proportion of the hydroxymethylcytosine residues of the DNAs of the T-even bacteriophages is glucosylated by virus-induced enzymes which transfer glucose residues from UDPglucose to form α- and β- glucosyl and di-glucosyl hydroxymethylcytosine. Again the nature and degree of the glucosylation depends on the glucosyltransferases induced (Kornberg, Zimmerman and Kornberg, 1961).

(iii) Terminal phosphate metabolism.

Enzymes have recently been detected which catalyse the addition and removal of terminal phosphate residues of nucleic acids. Richardson (1965) has described a bacteriophage T4-induced polynucleotide kinase which phosphorylates 5'-hydroxyl termini of nucleic acids, and more recently (Weiss & Richardson, 1967) a complementary enzyme which repairs single-strand breaks by joining 3'-hydroxy and 5'-phosphoryl termini in a DNA duplex with single-strand breaks. Other enzymes have been found which specifically remove terminal phosphoryl residues from DNA (Becker and Hurwitz, 1967).

2.4. Replication of DNA.

2.4.1. *In vivo* studies of DNA replication.

The double-helical DNA structure and mechanism of replication proposed by Watson and Crick (1953) provided an excellent basis for experimentation on this problem. Convincing proof of the semi-conservative mode of DNA replication predicted by Watson and Crick came from the classic experiment of Meselson & Stahl (1958), in which DNA from *Esch.coli* grown in a "heavy" (N^{15}) medium and subsequently transferred to a "light" (N^{14}) medium was found to pass through a hybrid (N^{15}/N^{14}) state. This would not be predicted by a model which conserved the parental DNA molecule. The idea that the double-helix, rather than the single chain, is the semi-conserved unit (Cavalieri and Rosenberg, 1962) now has little support

(Kornberg, 1967).

The experiments of Meselson and Weigle (1961) showed that the DNA of bacteriophage replicates semi-conservatively as a whole unit and also that genetic recombination occurs by breakage and reunion of DNA duplexes. Demonstration of semi-conservative replication with T4 bacteriophage (Kozinski, 1961) was complicated by the fact that the semi-conserved DNA chains are much intermixed by the high frequency of recombination, and hybrid molecules can only be detected after fragmentation of the progeny DNA.

Another experiment of great significance is that of Cairns (1963) who examined the replication of the Esch.coli chromosome by autoradiography. He showed that the Esch.coli chromosome is circular and is replicated at a growing point which moves round the circle producing two daughter circular double helices. This work showed the necessity for a swivel in a circular replicating chromosome and strikingly confirmed the undirectional synthesis of DNA and the Y-shaped model of the replicating point.

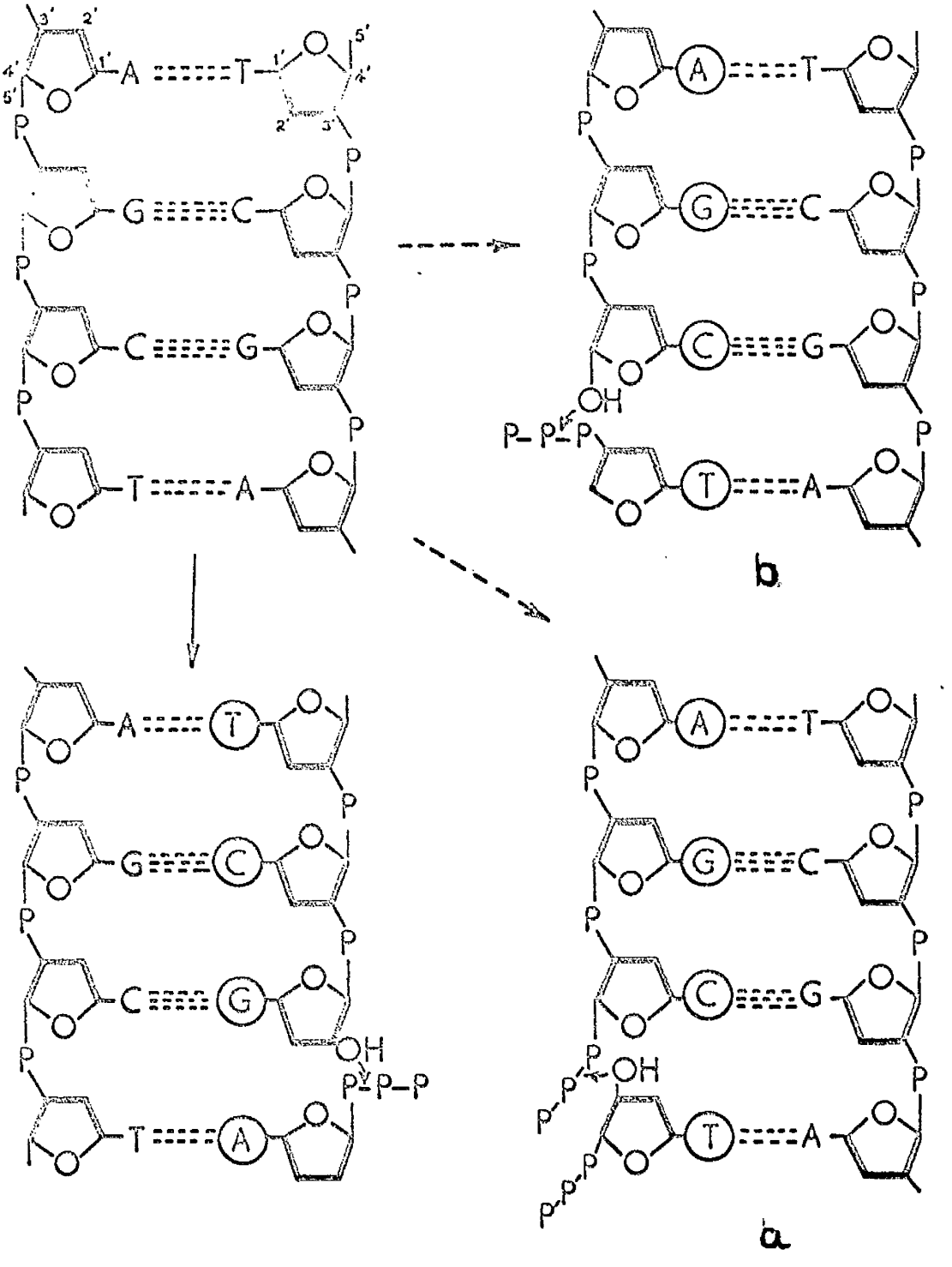
2.4.2. The role of DNA polymerase in DNA replication.

Several problems arise in reconciling the observed properties of DNA polymerase and the known features of DNA replication.

(i) The polymerases found in animal cells and those induced by bacteriophages utilise native DNA templates

Figure I.7.

Possible Mechanisms for the Synthesis of the Antiparallel DNA Strand.



at a much slower rate than denatured DNA, and the reaction stops at or before one replication. On the other hand, bacterial polymerases utilise both native and denatured DNA as template, and do not stop after one replication but perform up to twenty-fold synthesis of added DNA. These two types have been termed initiation- and termination-defective respectively (Bollum, 1966).

(ii) Unidirectional replication of the antiparallel DNA chains also poses a problem. The observed enzymic reaction synthesises the chain growing from a 3'-hydroxyl end, but what of the other, 5'-hydroxyl chain? Two schemes have been proposed (Figure I.7.) involving either (a) attack of the 3'-hydroxyl of the incoming tri-phosphate on a hypothetical 5'-triphosphate on the growing chain or (b) attack of a 5'-hydroxyl of a growing chain on a 3'-triphosphate. No evidence for these reactions has yet been obtained with isolated enzymes (Kornberg, 1967), in fact, recent work on the distribution of P^{32}-phosphate in Esch.coli DNA suggests that only 5'-phosphates are used as DNA precursors (Price et al., 1967). Difficulties such as these have led some to suggest that these isolated enzymes catalyse "repair" of DNA (Richardson et al., 1964b), while the real, DNA-synthesising enzyme is as yet undetected. Perhaps a more realistic viewpoint is that these

isolated activities have been damaged in the process of extraction from the DNA-synthesising complex (see, for example, the model of Hiai and Sibatani (1964)).

Evidence supporting the latter viewpoint has come from a study of conditional lethal mutants of T4 and T5 bacteriophage (de Waard et al., 1965) which showed that certain mutants defective in DNA synthesis failed to induce normal DNA polymerases. This suggests that the isolated enzyme is essential for the synthesis of viral DNA. Related work on bacteriophage mutants (Speyer, 1965) has shown that fidelity of replication is decreased in bacteriophages with altered DNA polymerases. This implies that DNA polymerase can have a mutagenic action and thus that it plays a part in selection of the correct incoming triphosphate (Freese and Freese, 1967). The correlation between the mutagenic action of Mn^{2+} and the defective action of DNA polymerase in its presence (Berg, Fancher & Chamberlin, 1963) supports this view.

3. REPLICATION OF DNA VIRUSES

3.1. DNA Bacteriophages.

Studies on DNA bacteriophages, in particular the T-even bacteriophages of Esch.coli, have been pursued with increasing intensity over the last two decades and knowledge has accumulated to such an extent that these are now the

organisms most precisely understood in molecular terms. The understanding gained from the examination of the biochemical and genetic processes of virus-infected bacteria has contributed significantly towards understanding these processes in more complex systems and, more especially, has provided an extremely useful model for the DNA viruses of animal cells, the study of which is complicated by factors both technical and biological.

The events occurring following infection of Esch.coli by one of the T-even bacteriophages can be split into several stages which will facilitate a comparison between the situation in DNA bacteriophage and animal DNA virus-infected cells.

(i) Attachment and penetration

As the bacterial host is surrounded by a tough, complex cell wall, the bacterial viruses possess an elaborate mechanism for overcoming this barrier. Work on this topic stems from the experiments of Hershey and Chase (1952), who showed that when bacteria were infected with virus doubly-labelled in its protein and nucleic acid, the bulk of the DNA entered the cells, while almost all the protein remained at the cell surface. This segregation of viral components is thought to occur as follows:-

Virus particles attach to specific sites on the bacterial cell wall by fibres at the end of the viral

"tail", whereupon viral lysozyme may weaken the cell wall allowing the viral DNA to be injected lengthwise from the virus head, via the hollow tail, into the host. Injection is achieved by contraction of the tail, the energy for which is supplied by nucleoside triphosphates in the virus particle.

(ii) Interaction with host cell metabolism.

One of the first events observed following infection with an autonomous virulent bacteriophage (see Section 1.4.1.) is a cessation of host DNA, RNA and protein synthesis and the destruction of the host DNA. The first process is independent of the second, but the precise mechanism is not yet understood (Nomura et al., 1962). It is not known whether host or virus-induced enzymes degrade the host DNA, but certain amber mutants of bacteriophage T4 fail to do so (Wiberg, 1966) in the non-permissive host, suggesting the latter.

(iii) Synthesis of early, virus-specified proteins.

In order to express itself and divert the resources of the host to its own ends the viral genome is transcribed, probably by host RNA polymerase, to produce virus-specified messenger RNA (Volkin, 1963) which associates with pre-formed host ribosomes (Brenner et al., 1961) and is translated into the early virus-induced proteins, which include many enzymes of DNA synthesis (see Section 3.3.).

There is a sharp cut-off of early protein synthesis during DNA synthesis (Flaks et al., 1959)

(iv) Synthesis of viral DNA

The virus-induced enzymes ensure the provision of the correct DNA precursors both from host DNA breakdown and de novo synthesis (e.g. by synthesising dHMCTP and degrading dCTP), replicate the parental DNA and glucosylate the hydroxymethylcytosine residues of the progeny DNA molecules. It has recently been shown (Frankel, 1966) that the replicated form of T4 DNA differs from the mature DNA, and Korn (1967) has found that maturation of viral DNA is sensitive to chloramphenicol and a low level of actinomycin D. Recombination of progeny DNA molecules by breaking and joining occurs during DNA synthesis (Kozinski, 1961), is probably also catalysed by virus-induced enzymes, since bacteriophage λ undergoes genetic recombination normally in mutants of Esch. coli defective in recombination (van der Putte and Rorsch, 1967).

(v) Synthesis of late virus-specified proteins.

About the time of cut-off of synthesis of early proteins, a second round of virus-induced protein synthesis occurs, leading to the production of virus structural proteins and enzymes, such as lysozyme and the DNA-maturation factor. The failure of these proteins to appear when bacteria are infected with UV-irradiated virus

(Sekiguchi and Cohen, 1964) whose DNA can be transcribed for the synthesis of early enzyme functions (Dirksen et al., 1960) but which cannot be replicated, suggests that transcription of the RNA for late proteins may well be from progeny DNA. Indeed, from actinomycin inhibition studies (Protass and Korn, 1966) it has been shown that T4 lysozyme mRNA is synthesised late in the infectious cycle. The mechanism of this differential transcription of early and late functions is not known.

(vi) Maturation and release of progeny virus.

While DNA synthesis continues, the molecules of the pool of progeny viral DNA are drawn at random and condensed into polyhedral bodies visible in the electron microscope. The phage internal protein, one of the early virus-induced proteins, may play a part in this condensation. Subsequently, the protein components of the virus head and tail are added in a stepwise manner to form the mature virus particles.

Release of mature virus from the infected cell occurs at the time of cell lysis and an important factor in promoting this is the virus-induced lysozyme which is a general feature of bacteriophage infection. Virus mutants which do not induce lysozyme are unable to lyse cells.

It should be pointed out that the brief outline presented above can only be applied in detail to the T-even

bacteriophages. The course of infections by other bacterial viruses which have been studied follow the same basic pattern, but can differ markedly in many features such as failure to degrade the host genome, induction of fewer enzymes, absence of or presence of other unusual bases in the viral DNA, absence of glucosylation, etc. Many points of difference will doubtless be found as other viruses are studied in greater detail, but the fact remains that the T-even bacteriophage - Esch.coli system has been a most fruitful one and will continue to stimulate other related fields.

3.2. DNA-containing animal viruses.

The replication of animal DNA viruses will be described within the framework of the preceding section. Emphasis will be placed on studies on the larger viruses with special reference to difference from the bacteriophage model and areas of ignorance.

(i) Attachment and penetration.

The initial phase of animal DNA virus infection differs markedly from the bacteriophage model in that specific virus attachment sites probably do not exist and that the energy necessary for penetration of the cell membrane comes, not from the virus particle, but from the pinocytotic mechanisms of the infected cell (Holmes and Watson, 1963). Another major point of difference is that the virus is released from the pinocytotic vesicle into the cytoplasm of the cell

more or less intact; thus the protein coat has to be removed before the viral genome can express itself fully. The uncoating process has been studied intensively only in the case of vaccinia virus (Joklik, 1964).

(ii) Interaction with host cell metabolism.

Following infection of animal cells with viruses of the herpes and pox groups, there is an inhibition of synthesis of host DNA. The mechanism for this inhibition is not clear; in the case of pseudorabies and herpes, protein synthesis is required (Ben-Porat and Kaplan, 1965; Sauer et al., 1964), while with vaccinia this does not seem to be so (Joklik and Becker, 1964). Newton (1967) has recently presented evidence that a non-DNA component of herpes virus is responsible for the inhibition of host cell DNA synthesis. Host RNA and protein synthesis are also inhibited by these viruses (Hay et al., 1966; Roizman et al., 1965; Hamada and Kaplan, 1965; Salzman et al., 1964). Marked degradation of the host DNA in virus-infected animal cells has not been shown; the report that equine abortion virus infection caused degradation of host DNA (Randall and Walker, 1964) has since been shown to be due to the presence of mycoplasma (Randall et al., 1965)

It is possible, however, that, at least in the case of the DNA viruses multiplying in the nucleus, a more subtle destruction may occur, as suggested by the chromatin margin-

ation caused by infection with herpes virus. The inhibition of host DNA synthesis may also be related to effects such as inhibition of mitosis (Stoker and Newton, 1959) and aberrations of chromosomes caused by herpes (Stich et al., 1964). Joklik and Becker (1964) suggest that the agent of inhibition in the case of vaccinia may be a viral structural protein.

Cytocidal infection of cells by papovaviruses on the other hand, appears to stimulate host DNA synthesis (Vogt et al., 1966; Hatanaka and Dulbecco, 1966) possibly because these small viruses are largely dependent on the host's DNA synthetic capability for their own replication. Sheinin (1966) and others, however, have observed a depression of host DNA synthesis following infection with polyoma and Ben-Porat, Coto and Kaplan (1966) have shown that the DNA made after polyoma infection is not normal cellular DNA but is labile and is degraded to material corresponding to viral DNA in size.

(iii) Synthesis of early, virus-specified proteins.

Rapidly-labelled RNA has been detected following infection with vaccinia (Becker and Joklik, 1964; Salzman et al., 1964) herpes (Hay et al., 1966) and adenoviruses (Thomas and Green, 1966) and hybridisation studies have shown that it is virus-specific. In view of the firmly-bound chromosomal location of RNA polymerase, the mechanism

of transcription of the viral DNA is not clear and it is possible that the virus may carry an RNA polymerase molecule. The observation (Kates and McAuslan, 1967a), that transcription starts before uncoating is complete is consistent with this possibility, and the same authors have recently shown that DNA-primed RNA polymerase can be detected in "cores" of vaccinia virus (Kates and McAuslan, 1967b). On the other hand, the demonstration that animal cells have a heat-labile function necessary for the proliferation of cells and DNA viruses, but not RNA viruses (Gharpure, 1965), suggests that the host cell RNA polymerase is used for some, at least, of the viral transcription. Whatever the source of enzyme, however, Munyon and Kit (1966) have shown that protein synthesis is not necessary for vaccinia-induced RNA synthesis.

The translation of virus-specific messenger RNA in the case of viruses multiplying in the nucleus raises the question of the location of protein synthesis. Is it predominantly cytoplasmic, as in non-infected cells? Recent experiments with adenovirus 2 (Thomas and Green, 1966), herpes (Sydiskis and Roizman, 1966) and pseudo-rabies (Fujiwara and Kaplan, 1967) suggest that it is, and that, in the latter case, at least, transfer of protein from cytoplasm to nucleus occurs.

(iv) Synthesis of viral DNA.

When the early enzymes have been made and the DNA precursors synthesised, replication of viral DNA proceeds semi-conservatively (Kaplan and Ben-Porat, 1964). No replicative forms of viral DNA have yet been detected and recombination, if it occurs, is probably much less frequent than in the T-even bacteriophages (Kaplan and Ben-Porat, 1964). Since inhibitors of DNA precursor synthesis such as FUdR (Newton and Tamm, 1959) and cytosine arabinoside (Levitt and Becker, 1967) completely inhibit animal viral DNA synthesis, virtually all the viral DNA precursors must come from de novo synthesis.

(v) Synthesis of late viral proteins.

As in the bacteriophage model, it is postulated that some of these functions are transcribed from progeny, rather than parental DNA. Evidence supporting this comes mainly from studies on the effects of FUdR and BUdR. FUdR, which prevents viral DNA synthesis, prevented the formation of adenovirus antigens (Kjellen, 1962) and the cut-off of vaccinia enzyme induction (McAuslan and Kates, 1966). BUdR, which is incorporated into progeny DNA, rendering it defective, blocks enzyme cut-off (McAuslan and Kates, 1966) and also loss of protein from pseudorabies-infected cells (Kamiya et al., 1965) although it did not prevent the production of herpes antigen (Siminoff, 1964).

(vi) Maturation and release of progeny virus.

The assembly of mature animal virus seems to occur in a stepwise manner, but whereas with bacteriophage, 90% of progeny DNA enters mature particles (Hershey, 1953), only a small proportion (20% or less) of progeny DNA is incorporated into animal viruses (Green, 1962; Ben-Porat and Kaplan, 1963; Russell et al., 1964). Viral protein is also made in excess; this overproduction of viral components may be a general feature of animal DNA virus infection.

Mature virus is released at the time of cell lysis (e.g. vaccinia), but sometimes before. In the case of herpes, electron microscopic studies have shown passage of the virus through nuclear and cell membranes without obstruction of the latter (Morgan et al., 1959) and also transfer by cell-to-cell connections (Hoggan et al., 1961). The leakage of protein from pseudorabies-infected cells mentioned above, may be a symptom of avirus-induced effect on the cell membrane connected with virus release.

3.3. Enzyme induction by DNA viruses.

3.3.1. Bacteriophages.

Cohen (1948) found that net protein synthesis is unchanged following T2 infection and Hershey and colleagues (1954) showed that while 60-70% of the protein made late in the infectious process was incorporated into mature virus, only a small proportion of the early protein

entered the progeny. The function of this early virus-induced protein remained obscure until Flaks and Cohen (1959) and Kornberg, Zimmerman, Kornberg and Josse (1959) showed that bacteriophage infection caused a "virus-induced acquisition of metabolic functions". Since then, more than a score of enzymes have been shown to be induced in Esch. coli by infection with the T-even bacteriophages while other bacteriophages also induce a number of enzymes. These enzymes have been described by Stent (1963) and reviewed by Cohen (1963).

For the present, further mention will be made only of the DNA polymerases induced by bacteriophages T2 (Aposhian and Kornberg, 1962) T4 (Lucas, 1965) and T5 (Orr et al., 1966), and of the studies on the structural genes of the T4- and T5-induced polymerases (de Waard et al., 1965). The viruses inducing DNases include λ (Radding, 1966; Little, 1967), T2 (Oleson and Koerner, 1964; Short and Koerner, 1965; Bose and Nossal, 1964), T4 (Weissbach and Korn, 1964), T5 (Paul and Lehman, 1966), T6 (Stone and Burton 1962) and bacteriophage SP3 of B.subtilis (Trilling and Aposhian, 1965). The observed DNases are not necessarily responsible for the degradation of host DNA (Stone and Burton, 1962). Mutants in genes 46 and 47 of T4 have been shown to be defective in this function (Wiberg, 1966) although they cause dissolution of the bacterial nucleus.

3.2.2. Animal viruses.

Increases in several enzyme activities have been observed following infection of animal cells with DNA viruses.

(i) TdR kinase increases after infection with herpes, pseudorabies, vaccinia, polyoma and SV40 viruses (Keir, 1968). Several of the induced increments have properties which differ from those of the non-infected cell and those of herpes and pseudorabies have been shown to differ immunologically (Klemperer et al., 1967; Hamada et al., 1966) from the enzymes in non-infected cells. Kit and Dubbs (1965) have developed a strain of LM cells which has a very low level of TdR kinase. In these cells herpes and vaccinia viruses induce TdR kinases which differ from one another and from the host cell enzyme. Mutants of herpes and vaccinia which have lost the ability to induce TdR kinase (Kit and Dubbs, 1964) grow normally, suggesting that the enzyme is not essential for virus multiplication.

(ii) DNA polymerase increases after infection with herpes, pseudorabies, vaccinia and polyoma viruses and SV40 (Keir, 1968). The enzymes induced by herpes (Keir et al., 1966a) and vaccinia viruses (Jungwirth and Joklik, 1965) have properties different from and are immunologically unrelated to those of non-infected cells (Keir et al., 1966b; Magee and Miller, 1967).

The pseudorabies-induced DNA polymerase appears to be related to that found in non-infected rabbit kidney cells (Hamada et al., 1966)

(iii) DNase 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 is enzymically and immunologically different and separable from the DNase of non-infected cells (Morrison and Keir, 1966; 1967), while cowpox virus is claimed to induce three DNases different from those of the host cell (Eron and McAuslan, 1966). The report that equine abortion virus infection causes a 9-fold rise in DNase activity (Randall and Walker, 1964) remains to be confirmed because of the presence of mycoplasma in the cells used, (Randall et al., 1965).

dTMP kinase is increased after infection with pseudorabies (Nohara and Kaplan, 1963) and herpes viruses (Newton, 1964).

dCMP deaminase increases following infection with polyoma (Hartwell et al., 1965) and herpes viruses (McGeoch and Keir, unpublished results).

Aspartate transcabamylase (ATCase) is increased 2-3-fold in cells infected with adenovirus 5 (Consigli and Ginsberg, 1964); this was shown to be due to a virus-induced activation of the host enzyme. The adenoviruses seem to

differ from the other animal DNA viruses in that they do not appear to induce enzyme increases (Green et al., 1964), although Ledinko, (1966) reports increases in TdR kinase and dCMP deaminase as well as ATCase.

The induction of enzymes by animal DNA viruses and its significance has been reviewed by Green (1966) and Keir (1968) and will be further discussed in Chapter IV.

4. INFORMATION TRANSFER IN BIOLOGICAL SYSTEMS.

The work of biochemists of the pre-1950 era established proteins as the molecules of pre-eminent biological importance, both in structural and catalytic functions. The theoretical and practical basis for the study of enzymes had been laid and the composition and proteinaceous nature of many of these biological catalysts had been established. It was also appreciated that protein molecules comprised linear arrays of amino acids and that the specific catalytic functions of enzymes probably depended on the correct arrangement of amino acids on the polypeptide chain. Methods of sequence determination were approaching the point when Sanger would determine the sequence of a small protein and Perutz was probing the 3-dimensional structure of crystalline haemoglobin by means of X-ray diffraction.

Thus, a student of proteins might have felt sure that he was investigating the key to the secrets of the cell. There was, however, one cloud on the horizon. How were these specific protein structures synthesised in the cell and how were they propagated from generation to generation? Genetic studies had shown that the hereditary material of cells resided in the chromosomes, and comprised a linear array of genes. Although the concept that each gene carried the information for the synthesis of one enzyme was evolving, the definition of the nature of the genetic material in molecular terms still lay in the future.

The ordering of a sequence of amino acids by enzymes was clearly impracticable, as many enzymes would be needed for the synthesis of each protein and more for their own synthesis etc. Thus, the concept developed of a template on which a protein could be made using a small number of enzymes which would serve for the synthesis of other proteins on other templates. A series of template subunits on the gene, therefore, would serve both for the expression and the duplication of the gene.

What was the chemical nature of the template and its subunits? Protein chemists put forward their candidate for the role, and with considerable justification, since at the time no other seemed plausible. The nucleic acids had been analysed and their components identified,

but, in ridding the purified nucleic acids of protein the analysts had destroyed the highly-polymerised nature of the former, and it was believed for many years that they were tetranucleotides, patently quite unsuitable as the genetic material.

Use of less harsh methods in the isolation of DNA revealed its high molecular weight and the demonstration (Avery et al.,1944) that such DNA could carry permanent genetic characters from one bacterial cell to another (transformation) began to change this viewpoint, although acceptance did not come overnight. The bacteriophage experiments of Hershey and Chase (1953), the double-helical structure for DNA of Watson and Crick (1953), with its implications for replication, and the demonstration of the infectivity of several viral DNAs have brought about virtually universal acceptance of DNA as the genetic material.

Once this had been accepted, it remained tobe shown how a sequence of nucleotides could define a sequence of amino acids in a protein molecule. The solution of this problem has been perhaps the outstanding achievement in the field of biology over the last fifteen years. Stent (1963) traces the development of the concept of the genetic code and the elucidation of the triplet nature of the "codon" from studies on the fine genetic mapping of

bacteriophage mutants. He also describes the discovery of "messenger" RNA, its role as intermediary between DNA from which it is transcribed, the ribosomal sites of protein synthesis and the role of transfer RNA as an "adaptor" between a particular amino acid and its corresponding "anticodon" on the messenger RNA. More recent developments in the field can be found in the Cold Spring Harbor Symposium on Quantitative Biology, Volume 31, "The Genetic Code" (1966).

4.1. Information Transfer in Virus-infected cells.

The relevance of the foregoing section to the present work may be seen when one considers the DNA content of viruses and the potential biological information contained therein.

A virus such as herpes with a DNA content of 68×10^6 daltons (Russell and Crawford, 1964) contains approximately 4×10^4 duplex nucleotide triplets, or enough information for the synthesis of around 200 polypeptide chains of molecular weight 2×10^4 daltons. One approach to investigating this information content more closely is by studying virus-induced proteins or more specifically enzymes. The small number that have been detected so far, along with the virus structural proteins and other possible virus-induced functions (Figure I.8.) do not seem likely to approach this figure.

Figure I.8.

Observed and Potential Functions Induced by Herpes Virus.

a) Early functions

+ 1. Factor(s) causing inhibition of host cell macromolecular synthesis;

+ 2. factor(s) which have effects on host cell chromosome (if different from 1.);

3. herpes-induced TdR kinase;

4. herpes-induced DNA polymerase;

5. herpes-induced DNase;

+ 6. other herpes-induced enzymes; dCMP deaminase, dTMP kinase, etc.;

b) Late functions

+ 7. factor involved in induced enzyme "cut-off";

8. structural components of virion;

+ 9. factor(s) involved in maturation of DNA or virus;

+ 10. factor(s) involved in virus release.

+ signifies a function whose agent has not yet been positively identified in herpes-infected cells.

Assuming then, that all the viral DNA is expressed during infection, what other functions might be transcribed from the viral genome? One possibility is that herpes may induce one or more new t-RNA molecules more suited to the translation of viral genes. The synthesis of t-RNA after infection has been demonstrated (Hay et al., 1966), hybridisation studies suggest that at least a part of this is virus-specific (Subak-Sharpe and Hay, 1965) and evidence for the occurrence of a new arginyl-t-RNA after infection has been obtained (Subak-Sharpe et al., 1966b).

In order to investigate whether there might be a requirement for new t-RNAs in herpes-infected cells, the nearest neighbour frequency analysis (Josse et al., 1961) of a number of animal DNA viruses was undertaken, (Subak-Sharpe et al., 1966a; Morrison et al., 1967). This is one of the few ways of obtaining information on the sequence of bases in DNA and the occurrence of major differences in pattern between viral and host cell DNAs and the significance of the differences will be described and discussed in Chapters III and IV.

CHAPTER II

CHAPTER II - MATERIALS AND METHODS

1. MATERIALS.

1.1. Chemicals.

All chemicals were, where possible, "ANALAR" grade or equivalent. Non-radioactive deoxyribonucleoside 5'-triphosphates were purchased from Calbiochem, Los Angeles, Calif. and P-L Biochemicals, Milwaukee, Wis. These were purified, where necessary, by paper chromatography with isobutyric acid/NH_3/H_2O as solvent.

Puromycin hydrochloride was purchased from Sigma Chemical Co., St.Louis, Mo. and actinomycin D was the generous gift of Merck, Sharp and Dohme, Rahway, N.J. CsCl (optical grade) was purchased from Harshaw Chemical Co., Cleveland, Ohio.

1.2. Radioactive Compounds.

32p-labelled dTMP, inorganic phosphate and 3H- and ^{14}C-labelled TdR were obtained from the Radiochemical Centre, Amersham, Bucks.

α-^{32}P-labelled deoxyribonucleoside 5'-triphosphates of adenine, cytosine and guanine were purchased from International Chemical and Nuclear Corp., City of Industry, Calif. Their purity was checked and they were purified, where necessary, by paper chromatography with isobutyric acid/NH_3/H_2O as solvent.

1.3. Media for chromatography and electrophoresis.

Whatman filter paper Nos. 1, 3MM and DE 81 (DEAE-paper) were purchased from H. Reeve Angel & Co. Ltd., London., as were the ion-exchange celluloses DE 11 and DE 52.

Gel filtration media Sephadex G-100 and G-200 and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Uppsala.

1.4. Enzymes.

Enzymes were purchased from the following suppliers:-

Micrococcal nuclease, pancreatic DNase, snake venom phosphodiesterase, spleen phosphodiesterase and pancreatic RNase from Worthington Biochemical Corporation, Freehold, N.J.;

Spleen phosphodiesterase and snake venom 5'-nucleotidase from Sigma Chemical Co., St. Louis, Mo.;

Esch. coli alkaline phosphatase from Nutritional Biochemicals Corp., Cleveland, Ohio;

Pronase from Calbiochem, Los Angeles, Calif.

Esch.coli DNA polymerase was prepared (see Section 2.3.) from a batch Esch.coli strain B which was the generous gift of Dr. R. Elsworth and colleagues, Microbiological Research Establishment, Porton, Wilts.; Dr. B.J. Gomatos kindly supplied an initial sample of this enzyme.

1.5. Miscellaneous

Ferritin was the kind gift of Dr. J. Drysdale; haemoglobin and cytochrome were purchased from Sigma

Chemical Co., St. Louis, Mo.

2. METHODS.

2.1. General.

2.1.1. Analytical methods.

Protein was measured by the method of Lowry et al., (1951); precipitation with 5% TCA was performed where necessary, e.g. when high levels of KCl or SH-EtOH were present.

DNA was determined by the method of Burton (1956).

Inorganic orthophosphate was measured by the method of Allen (1940).

2.1.2. Chromatography.

(a) isobutyric acid/NH_3 (0.88SG)/H_2O: (66/1/33) was used for the separation of the 5'-mono-, di- and tri-phosphates of the deoxyribonucleosides and for the separation of the deoxyribonucleoside 3'-monophosphates resulting from the enzymic digestion of DNA in the nearest neighbour frequency analysis. The R_f values in this solvent were :- dAMP: 0.7 , dCMP: 0.6 , dTMP: 0.5 , dGMP: 0.4 , P_i: 0.3 (descending run on Whatman No. 1, 25°, 18hr.).

(b) 0.75M NH_4HCO_3, pH 8.6 was used for the separation of the products of enzymic hydrolysis of DNA on DEAE-paper (DE 81). A 4hr. ascending run at 20° gave the following R_f values:- P_i: 0.8, dCMP, dTMP: 0.7, dAMP, dGMP: 0.5,

oligonucleotides: 0-0.2.

2.1.3. Preparation of buffers.

Buffers were prepared by weighing the required amount of solid, dissolving it in less than the required volume of H_2O, titrating the solution to the desired pH with the appropriate acid or base (20°) and adjusting to the final volume. Buffer stocks were normally made up at 1M or 0.5M and the pH checked, and altered if necessary, after dilution. It was found that tris-HCl buffers, prepared at 1M and 20°, gave the same pH when diluted to 0.05M at 37°.

2.1.4. Measurement of radioactivity.

(a) ^{32}P Material containing this isotope was prepared for counting by (i) precipitation on to discs of filter paper with 5% TCA and protein co-precipitant, followed by washing and drying or (ii) drying directly on to stainless steel planchettes, after neutralisation with KOH, if necessary. Radioactivity was then measured in a Nuclear-Chicago gas-flow counter(98.7% helium + 1.3% butane) at an efficiency of nearly 50% and with a background of 15-18 counts/min.

^{32}P-containing spots of 3'-monophosphates which had been separated by electrophoresis or chromatography were thoroughly dried, cut out, immersed in a toluene-based liquid scintillator (0.5% PPO + 0.03% POPOP), and counted in a Nuclear-Chicago liquid scintillation spectrometer.

(b) ^{3}H and ^{14}C. DNA containing these isotopes was precipitated with 5% TCA and denatured DNA co-precipitant and trapped on a "Millipore" membrane which was then washed, dried, immersed in toluene-based scintillator and counted in a Packard liquid scintillation spectrometer. Estimation of ^{3}H and ^{14}C in doubly-labelled samples was performed by reference to standard curves of counting efficiences.

2.2. Techniques Associated with the Study of Enzymes in Virus-infected Cells.

2.2.1. Growth of cells and infection with virus.

BHK 21 cells (C13; MacPherson and Stoker, 1962) and HEp-2 cells were normally grown in monolayer culture in rotating Winchester bottles (House and Wildy, 1965) in a modified Eagle's medium (Vantsis and Wildy, 1962) containing 10% tryptose phosphate broth and 10% calf serum. Infection with herpes virus strain ∝ HFEM (Holmes and Watson, 1963) on pseudorabies virus was performed at an input multiplicity of 10-15 and an absorption period of 30 min.

2.2.2. Harvesting of cells and preparation of cell extracts.

Cells were removed from the glass by scraping or treatment with EDTA, washed twice in cold, phosphate-buffered isotonic saline and the packed cell volume (PCV) measured.

Various modifications of the preparation of cell extracts were employed at different times, but these can

be broadly divided into two methods (all operations being conducted at 0°):-

(i) the extraction procedure described by Keir et al., (1966a) which was used in the earlier stages of the work, especially for studies on the herpes-induced DNA polymerase;

(ii) a concentrated suspension of cells (in 2x PCV) was made to 0.1M tris-HCl pH8, 2mM EDTA and 10mM SH-EtOH(TEM) by the addition of 1/5vol. 10x concentrated buffer, whereupon 4xPCV of TEM buffer was added and the cells were disrupted by ultrasonic vibration (4x15sec. with MSE Ultrasonic Power Unit, small probe). The suspension was then centrifuged for 30min. at 30,000g, the supernatant fluid retained and stored at 0° in a sealed container. The precipitate contained negligible amounts of DNase.

2.2.3. Subcellular fractionation.

Nuclei were prepared from BHK21 cells by homogenisation in 0.25M sucrose, 3mM $CaCl_2$, 20mM tris-HCl pH8, 5mM SH-EtOH (SCTM) after scraping the cells from the glass in the same medium. The nuclei were sedimented (10min. at 800g), resuspended in SCTM by homogenisation and centrifuged again. The two supernatant fractions were pooled, dialysed against 0.15M KCl, 20mM tris-HCl pH8, 1mM EDTA, 5mM SH-EtOH (KTEM) and centrifuged for 30min. at 30,000g to give the cytoplasmic fraction. The washed nuclei were resuspended in KTEM, disrupted by ultrasonic vibration, dialysed against KTEM, centrifuged for 30min. at 30,000g and the resultant

supernate termed the nuclear fraction.

N.B. The nuclei were slightly contaminated by cytoplasmic material as revealed by staining with aceto-orcein/fast green; this was not removed even when the non-ionic detergent Triton X-100 (1% v/v) (Rohm and Haas, supplied by British Drug Houses, Ltd.) was used. Triton X-100 did not inhibit or inactivate DNase.

2.2.4. Preparation of antisera.

Cultures of rabbit kidney cells were infected with herpes virus and harvested with EDTA 6hr. post infection. Extracts were prepared in the cold by disrupting the cells by ultrasonic vibration in KTEM buffer (see Section 2.2.3.) and centrifuging for 1hr. at 100,000g. The supernate was mixed with Freund's Adjuvant and injected intra-muscularly or subcutaneously into rabbits. Second and third doses were administered at intervals of several months. The rabbits were bled (20ml.) after the third dose; the blood was allowed to clot, the resultant serum was freed of blood cells by centrifugation and stored at -20^{o}.

2.2.5. Preparation of substrates.

(i) α-^{32}P-dTTP was prepared according to the method of Gray et al., (1960) starting with ^{32}P-dTTP purchased from the Radiochemical Centre, Amersham, Bucks.

(ii) DNA.

DNA from calf thymus and from Landschutz ascites-tumour cells was prepared essentially according to the method of Kay et al., (1952).

^{32}P-DNA was prepared from Esch.coli grown in the presence of ^{32}P-P_i (Lehman, 1960). The DNA was extracted and purified by the methods of Lehman (1960) omitting the charcoal step or of Thomas et al., (1966). Ratios of E_{260}/E_{280} of the final product were in the range 1.75-1.85 and RNA contamination, as judged by the amount of radioactivity rendered acid-soluble by pancreatic RNase, was less than 5%. One problem was that the acid-soluble background of the DNA was of the order of 2-3%; this could not be reduced even after extensive dialysis in tubing made more porous by treatment with 64% $ZnCl_2$ (Massie and Zimm, 1965) and had the unfortunate effect of reducing the sensitivity of the DNase assay when the DNA was used as substrate.

In calculating the concentration of DNA solutions from E_{260} measurements, an E(P) of 6×10^3 was used (i.e. 50µg. DNA /ml. gives a solution whose E_{260} is unity).

2.2.6. Assay of enzymes.

(i) DNA polymerase: the assay measures the incorporation of ^{32}P- dTMP residues from α-^{32}P-dTTP into denatured DNA

in the presence of dCTP, dGTP and dATP as described by Keir (1962). The standard assay (0.25 or 0.125ml.) contained the following :- denatured DNA - 400µg/ml; dATP, dCTP, dGTP and α-^{32}P-dTTP - 0.2mM (each); Mg^{2+} - 8mM; tris-HCl, pH7.5 - 25mM; KCl - 60mM; SH-EtOH - 5mM; EDTA - 0.4mM.

For specific assay of the herpes-induced DNA polymerase, the KCl concentration was 0.2M; sometimes, e.g. for pH optimum determination, KCl was omitted and the tris concentration was 50mM.

A unit of DNA polymerase is that amount of enzyme which will incorporate 1mµmole dTMP into acid-insoluble material per hr. at 37°.

(ii) DNase: the assay method was measurement of the release of acid-soluble material from DNA, as :-

(a) UV-absorbing material: Calf thymus or ascites-tumour cell DNA was incubated with the DNase, the reaction stopped by cooling on ice, protein added as co-precipitant and the solution made 0.5M with respect to PCA; after 10min. at 0°, the resultant precipitate was removed by centrifugation (10min. at 800g) and the E_{260} of the supernatant measured. In calculating the amount of acid-soluble nucleotide released, the molar extinction coefficient of a solution of mixed nucleotides was taken as 10,000, i.e. a solution of E_{260}=1 contains 1/10,000 mole of nucleotide

per litre or 100mμmoles/ml. (equivalent to 30μg./ml. DNA nucleotide).

The standard UV DNase assay (0.2ml.) contained:- DNA (native or denatured) - 300μg./ml.; Mg^{2+} - 2mM; tris-HCl, pH 9 - 50mM; SH-EtOH - 2mM. Some of the earlier assays were performed at pH 8 and 60mM KCl in an assay volume of 1 ml.

(b) radioactivity: ^{32}P-DNA from Esch. coli replaced the unlabelled DNA of the UV assay and a portion of the acid-soluble material was counted as described in Section 2.1.

The standard radioactive DNase assay (0.2ml.) contained :- ^{32}P-DNA - 50μg./ml.; Mg^{2+} - 2mM; tris-HCl, pH 9 - 50mM; SH-EtOH - 10mM.

A unit of DNase is that amount of enzyme which will ~~hydrolyse~~ render P acid soluble 10 mμmoles of DNA per 30min. at 37°.

Acid DNase was measured at pH 4.5 in 0.1M Na-acetate buffer. The radioactive DNase assay was superior to the UV assay in being more specific, more sensitive, needing fewer controls and allowing more critical studies of the mode of action to be made.

(iii) RNase: the UV assay method (see above) was used with ascites cell RNA (prepared by phenol treatment and EtOH precipitation) as substrate. The standard RNase assay (0.2ml.) contained RNA - 300μg./ml.; Mg^{2+} - 2mM; tris-HCl pH 8 - 50mM; SH-EtOH - 2mM.

(iv) Phosphomonoesterase : the method of assay was measurement of release of p-nitrophenol from p-nitrophenylphosphate. The standard assay (0.2ml.) contained:- p-nitrophenylphosphate - 5mM; Mg^{2+} - 2mM; tris-HCl pH 9 - 50mM; SH-EtOH - 2mM. After 60min. at 37°, 0.2ml. of 0.1N.NaOH and 0.2ml. of H_2O were added and the E_{410} measured. The molar extinction coefficient of p-nitrophenol is 12,000 at pH12.

A unit of phosphomonesterase is that amount of enzyme which will liberate 10mμmoles of p-nitrophenol per hr. at 37°.

(v) Inorganic pyrophosphatase: the method of assay was measurement of the release of P_i from PP_i. The standard assay (0.3ml.) contained:- $Na_4P_2O_7$- 1.7mM; Mg^{2+} - 1.7mM; tris-HCl pH 7.5 - 50mM. After 15min. at 37°, 2ml.cold 10% TCA and 1.7ml.cold H_2O were added and after 10 min. at 0°, any protein precipitate which had formed was removed by centrifugation before Pi was determined by the method of Allen (1940).

A unit of inorganic pyrophosphatase is that amount of enzyme which will hydrolyse 1μmole of PP_i per 15min. at 37°.

2.2.7. Enzyme fractionation.

(i) $(NH_4)_2SO_4$ precipitation: This was carried out at pH 8 and 0°. "Enzyme grade" $(NH_4)_2SO_4$ was added slowly, either as a finely-ground powder or as a saturated

solution containing 20mM tris-HCl pH 8, 1mM EDTA and 5mM SH-EtOH; in the former case, extra EDTA and SH-EtOH were added to the solution. The pH of the solution was maintained at pH 8 by the addition of 2N NH_4OH during the addition of $(NH_4)_2SO_4$. Care was taken to minimise frothing of the protein solution. After standing for 15-30min., the suspension was centrifuged for 10min. at 30,000g. The precipitate was redissolved in a small volume of a suitable buffer or else resuspended and stored in 0.8 saturated $(NH_4)_2SO_4$ solution.

(ii) DEAE-cellulose chromatography: The microgranular type DE 52 was prepared according to supplier's instructions. The standard buffer used was 0.05M tris-HCl, pH 8, 1mM EDTA and 5mM SH-EtOH; elution was achieved by means of KCl solutions in standard buffer. Gradient elution was performed in a 1x10cm. column by applying the enzyme solution (dialysed against standard buffer) to the column, washing with buffer, applying a linear gradient of KCl (100ml. of buffer in the mixing beaker and 100ml. of buffer containing 0.35M KCl in the reservoir beaker) and collecting 5ml.fractions. Stepwise elution was performed by washing the column with buffer containing 0.1M and 0.2M KCl. No DNase was eluted in the 0.1M fraction.

(iii) Gel filtration: Sephadex G-100 and G-200 were prepared and packed into 1x100cm. columns according to

the supplier's instructions. The void volume was measured using Blue Dextran or ferritin and the resolution of the columns tested by their ability to separate mixtures of ferritin, haemoglobin and cytochrome c. Elution was carried out in KTEN or TEM (see Section 2.2.3.) at 2-4°. Flow rate was 3-6ml./hr. at a pressure head of 20 cm. of buffer, and 2 or 3ml. fractions were collected.

2.2.8. Density gradient centrifugation.

This technique was performed in a Spinco No. 40 single-head rotor (Flamm et al., 1966) by centrifuging CsCl solutions (4.5ml., under paraffin, initial density = 1.72g./ml.) for 48 hr. at 33,000 rev./min. at 20°. After unbraked deceleration, the tubes were removed and two-drop fractions collected by puncturing the bottom of the tube. The fractions were diluted with 0.5ml. of H_2O and the E_{260} and radioactivity measured (see Section 2.1.4.).

2.3. Nearest Neighbour Frequency Analysis.

This technique was performed essentially as described by Josse et al., (1961) but with the modifications described below :

(i) The primer-DNAs were "activated" as described by Aposhian and Kornberg (1962) in order to achieve suitably high levels of incorporation of radioactivity. Comparison of the nearest neighbour frequencies of "activated" and "non-activated" DNA from herpes virus demonstrated that

this treatment did not introduce any differences in the frequency pattern. (See also Swarts et al., 1962).

(ii) Certain commercial preparations of spleen phosphodiesterase were found to be contaminated with significant amounts of phosphomonoesterase activity. This activity was inhibited by inclusion of potassium phosphate buffer, pH 7.0 at 10 mM, and NaF at 2 mM in the phosphodiesterase incubation mixtures.

(iii) Deoxyribonucleoside 3'-monophosphates resulting from digestion of the DNA product were separated (a) by electrophoresis on paper at an applied voltage of 3000-4000 (48 to 64 v/cm.) in 0.05 M ammonium formate pH 3.5 for 2 to 2.5 hr., and (b) by descending paper chromatography in isobutyric acid/water/conc. NH_4OH (66:33:1) for 24 hr. The latter method was employed when phosphate and fluoride were present as they interfered seriously with the electrophoretic separation.

The interference with the electrophoresis caused by the presence of the salts was obviated in the later analyses by adsorbing the mononucleotides on to activated charcoal, as described by Josse et al., (1961), eluting them with 50% (v/v) ethanol, containing 0.3% conc. NH_4OH, taking to dryness in a stream of air and applying to paper for electrophoresis. Although recovery was only of the order of 70-80%, a pilot experiment showed that there was no selective retention of any of the mononucleotides.

(iv) Radioactivity in the separated 3'-nucleotides was determined by cutting out the spots, immersing each spot in 8 ml. of a toluene-based scintillation fluid, and counting for ^{32}P in a Nuclear Chicago liquid scintillation spectrometer.

The DNAs of polyoma, human papilloma, Shope papilloma and SV40 viruses were prepared by Dr.E.V. Crawford,. Herpes, pseudorabies, equine rhinopneumonitis (equine abortion), vaccinia and adeno 2 viruses were grown by Dr. H.Subak-Sharpe and the DNAs purified by Dr. J. Hay. All DNAs were shown to be homogeneous by equilibrium density gradient centrifugation in the Spinco Model E ultracentrifuge; their GC contents were measured by comparison with $^{15}N^{2}$ H-DNA from Esch.coli (1.748g./ml.). The methods used are described by Subak-Sharpe et al., (1966a).

DNA polymerase of Esch. coli was prepared by the method of Richardson et al., (1964a), except that steps V and VI were omitted. The final preparation had a specific activity of 330 units/mg protein, showed a linear rate of incorporation up to1hr. and showed absolute dependence on added primer DNA. In all analyses, the percentage hydrolysis to mononucleotides was checked to be greater than 95%; in addition, the areas on the electrophoretograms (or chromatograms) lying between

the mononucleotides and at the origin were counted and found to contain negligible radioactivity (less than 1% of total). The area where inorganic phosphate would be expected was also counted and this was normally less than 2% of the total counts, showing that the recovery of mononucleotides after electrophoresis or chromatography was greater than 97%.

CHAPTER III

CHAPTER III - RESULTS.

1. STUDIES ON ENZYME INDUCTION BY HERPES VIRUS.

1.1. Enzyme levels in Cells Infected with Herpes Virus.

When cultures of mammalian cells are infected with herpes virus, the levels of at least four enzymes rise during the eclipse phase. These are TdR kinase (Kit and Dubbs, 1963), dTMP kinase (Newton, 1964), DNA polymerase and DNase (Keir and Gold, 1963; Russell et al., 1964). This work has been concerned with further studies on the last two enzymes with the aim of establishing whether the increments are virus-specified, and, if so, what role they play in virus multiplication.

1.1.1. Alkaline DNase.

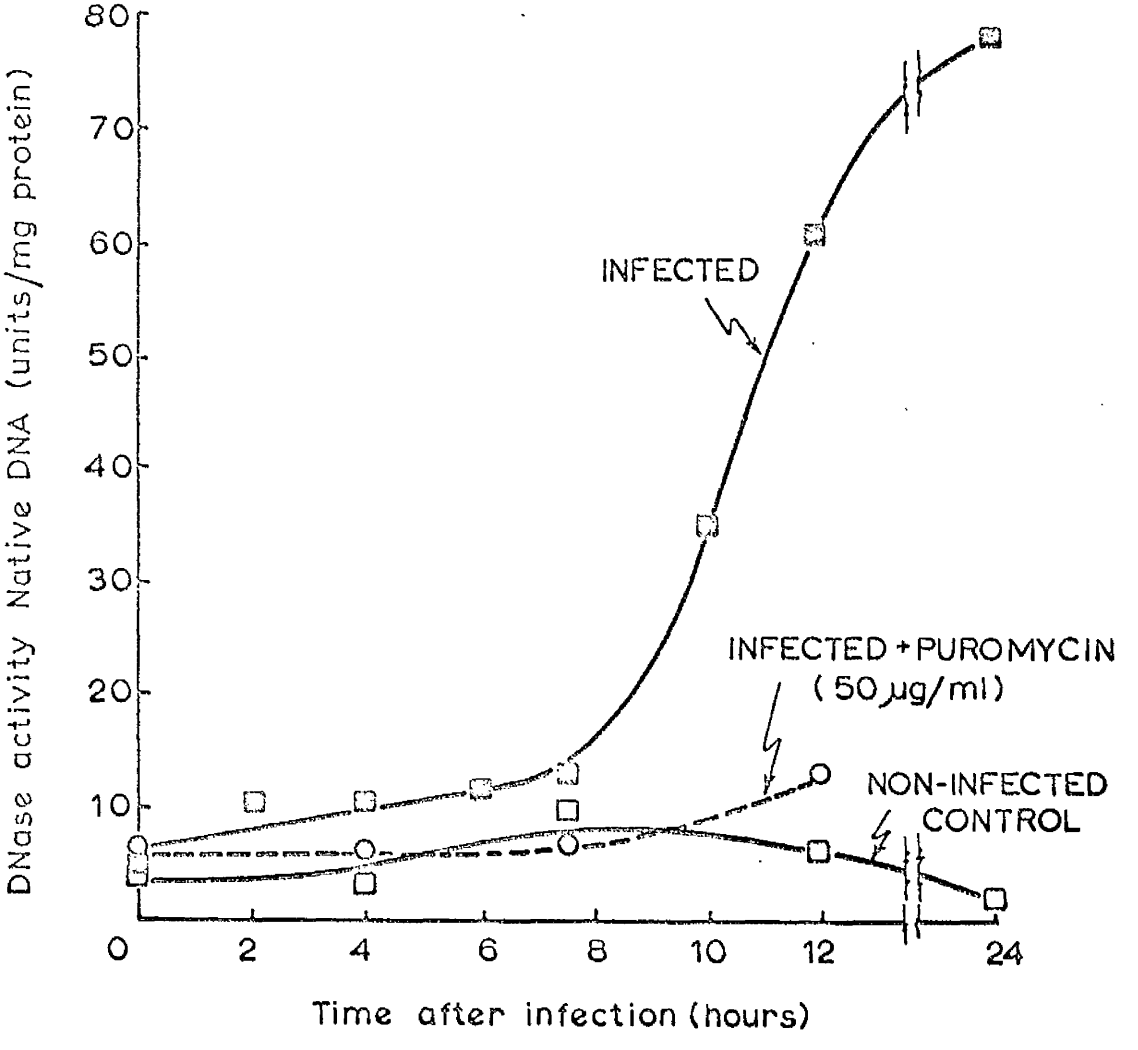
Figure III.1. shows the effect of infection of a growing monolayer culture of BHK 21 cells with herpes virus on the activity of alkaline DNase, with native DNA as substrate. DNase activity rises 6-fold 7-12hr. post infection while infective virus first appears between 8 and 12 hr. DNase with denatured DNA as substrate also increases, but the rise is less marked, as the activity of DNase towards this substrate is higher in uninfected cells, than towards native DNA.

1.1.2. DNA polymerase.

The levels of DNA polymerase following infection

Figure III.1.

INDUCTION of DNase ACTIVITY FOLLOWING INFECTION of BHK-21 CELLS WITH HERPES VIRUS



of growing BHK 21 cells with herpes virus are shown in Figure III.2. In the same experiment as that depicted in Figure III.1., DNA polymerase, assayed under standard conditions, increases 7-fold 5-12hr. post infection. When assayed in the presence of 0.2M KCl, however, the increase is more dramatic, as under these conditions, the host cell enzyme is virtually undetectable, while the herpes-induced activity is stimulated 3-4-fold (see Section 1.4.2.). It should be noted that the time-course of enzyme induction described here is somewhat later than that found by Russell et al., (1964). This is probably due to differences in growth of cells and in the method of infection used.

It is important to note that essentially the same results are obtained with both enzymes when non-growing cultures of BHK 21 cells (Burk, 1966) or growing cultures of HEp-2 cells are infected with herpes virus. Other workers have subsequently noted increases in alkaline DNase in herpes-infected monkey kidney (McAuslan et al., 1965) and L cells (Sauer et al., 1966).

1.1.3. Other enzymes.

Table III.3. shows that levels of acid DNase, alkaline RNase, inorganic pyrophosphatase and alkaline phosphatase do not rise after infection with herpes virus. The absence of increase in acid DNase agrees with the results of McAuslan et al., (1965)and Flanagan (1966)

Figure III.2.

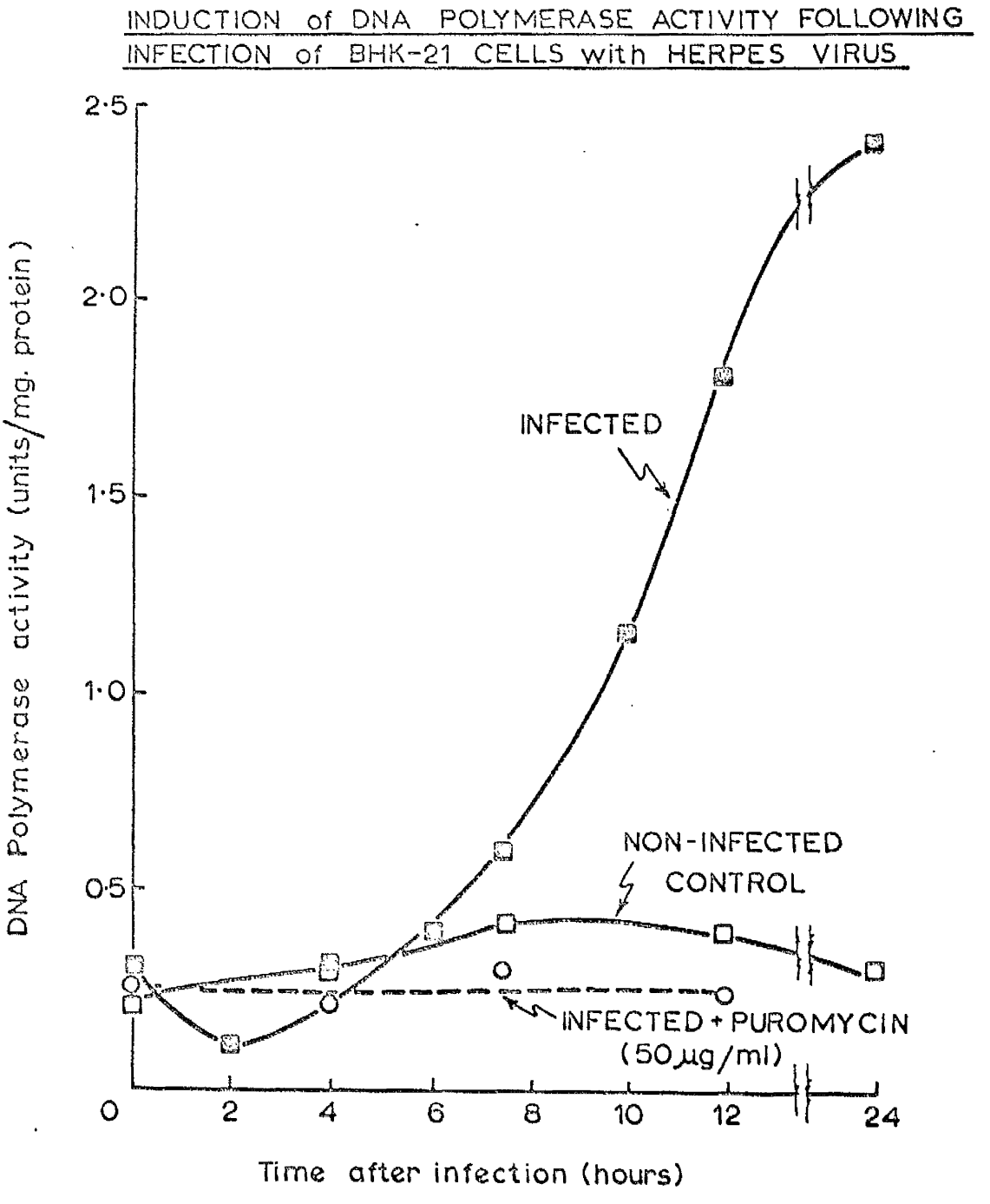

INDUCTION of DNA POLYMERASE ACTIVITY FOLLOWING INFECTION of BHK-21 CELLS with HERPES VIRUS
DNA Polymerase activity (units/mg. protein)
2·5
2·0
1·5
1·0
0·5
0
2
4
6
8
10
12
24
Time after infection (hours)
INFECTED
NON-INFECTED CONTROL
INFECTED + PUROMYCIN (50 µg/ml)

Table III. 3.

Levels of acid DNase, alkaline RNase, inorganic pyrophosphatase and alkaline phosphatase in control and herpes-infected cells.*

Acid DNase

		cytoplasm	nucleus
1)	native DNA		
	BHK 21 cells (control)	38	15
	BHK 21 cells (herpes-infected)	43	18
2)	denatured DNA		
	BHK 21 cells (control)	20	8
	BHK 21 cells (herpes-infected)	21	4

Alkaline RNase

		control	herpes-infected	pseudorabies-infected.
1)	BHK 21 cells	9.1	8.5	8.3
2)	HEp-2 cells	7.4	6.5	3.8

Inorganic pyrophosphatase

	control	herpes-infected
BHK 21 cells	10.3	8.5

Alkaline phosphatase

	control	herpes-infected
BHK 21 cells	4.6	4.8

* See Chapter II for details of assays and enzyme units. All activities expressed as units / mg protein.

but not those of Newton (1964). Growth of cells in suspension in the last-mentioned case may have altered lysosomal stability; it is noteworthy that infection of Hela cells with poxviruses causes an increase in acid DNase (McAuslan et al., 1965). The failure to observe an increase in inorganic pyrophosphatase following herpes infection suggests that the level of this enzyme in the host cell is sufficient to meet the synthetic requirements of the infected cell (see Kornberg, 1962).

1.2. Effect on Inhibitors of Protein and RNA Synthesis on Enzyme Induction.

1.2.1. Puromycin.

Figures III.1 and 2. show that puromycin (50µg./ml.) added at the time of infection, completely inhibits the induction by herpes virus of DNase and DNA polymerase.

1.2.2. Actinomycin D.

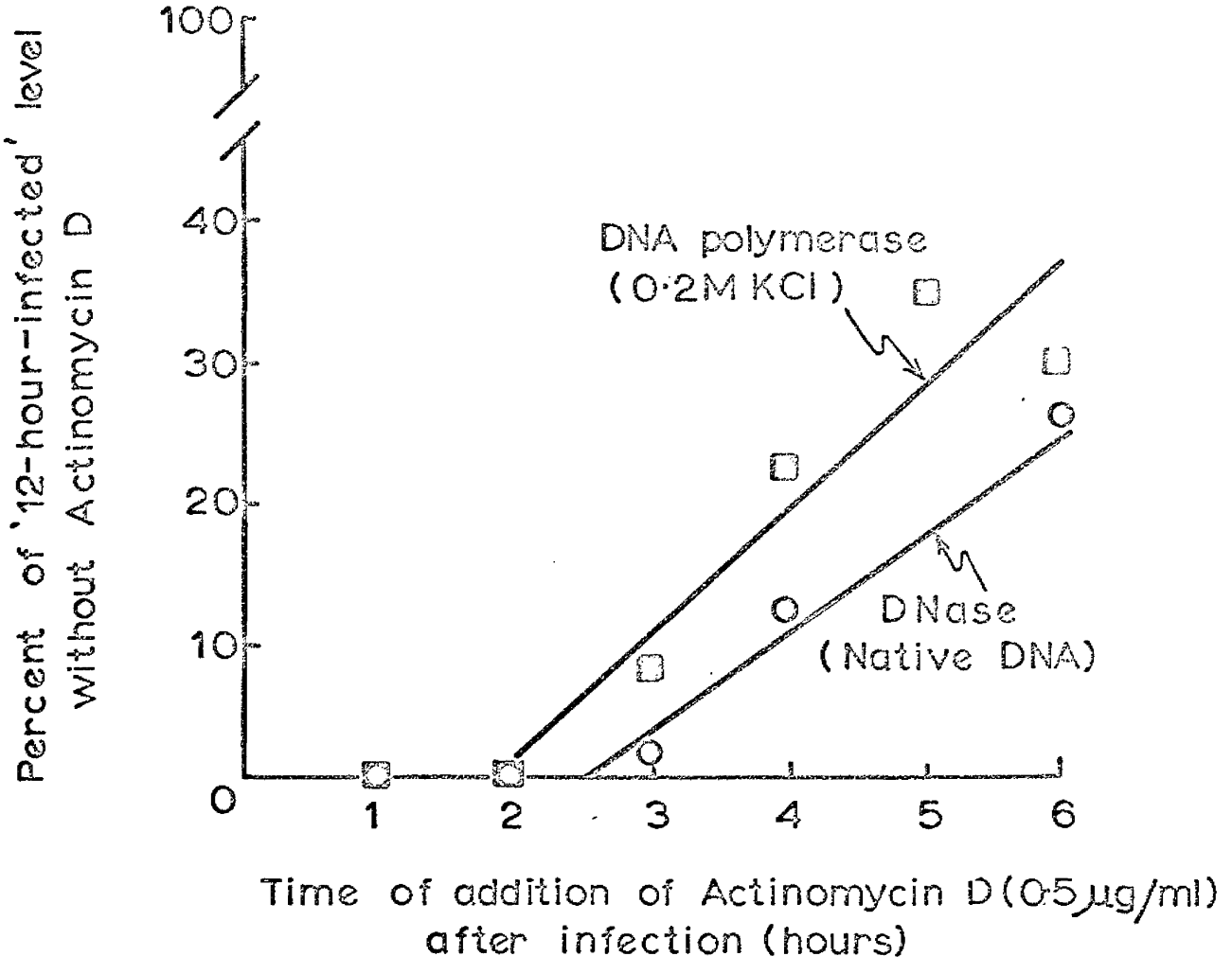
When actinomycin D (0.5µg./ml.) is added at 2hr. post infection or earlier, enzyme induction is completely inhibited; the degree of inhibition diminishes progressively when the actinomycin is added at later times after infection (Figure III.4.).

1.3. Intracellular location of Induced Enzymes.

Nuclear and cytoplasmic fractions were prepared from control and herpes-infected (6hr. post infection) BHK 21 cells and assayed for protein, DNase and DNA polymerase.

Figure III.4.

EFFECT of ACTINOMYCIN D on ENZYME INDUCTION



The nuclei retained a small amount of cytoplasmic material, as judged by aceto-orcein/fast green staining. The distribution of DNase and DNA polymerase is shown in Tables III.5. and III.6. respectively. It can be seen that, while the bulk of the induced DNA polymerase resides in the nuclear fraction, the induced DNase is found predominantly in the cytoplasmic fraction at this time after infection. An examination of the heat lability of the enzymes of the subcellular fractions confirms the distribution of induced enzymes expected from total activities (Figures III.7. and III.8., see also Section 1.4.).

Nuclear/cytoplasmic fractionation was also performed on herpes-infected, non-growing BHK 21 cells harvested at 2 hourly intervals after infection. An increase in DNase and DNA polymerase 4-10hr. post infection was observed; the intracellular distribution was similar to that described above, but indicated a greater proportion of induced DNase in the nuclear fraction at later times after infection.

1.4. Comparison of the Properties of the Induced Enzymes with those of the Host Cell.

1.4.1. Herpes-induced DNase.

The two features which immediately distinguish the DNase of herpes-infected cells from that of the non-infected cell are:-

Table III.5.

Intracellular distribution of DNase in control and herpes-infected BHK 21 cells.

State of cells	Subcellular fraction	State of DNA	DNase (specific activity)	DNase (total activity)
non-infected	cytoplasmic	native	0.02	6
		denatured	0.20	65
	nuclear	native	0.07	8
		denatured	0.52	57
herpes-infected	cytoplasmic	native	0.55	183 (177-86
		denatured	0.78	260 (195-89
	nuclear	native	0.30	37 (29-14%
		denatured	0.65	81 (24-11%

Cells were harvested 6h. post infection, nuclei prepared by homogenisation in 0.25M sucrose, 3mM $CaCl_2$, 5mM 2-mercapto-ethanol, 10mM tris-HCl, pH 8, then disrupted by sonication. The cytoplasmic and nuclear fractions were then dialysed against 0.15M KCl, 1mM EDTA, 5mM 2-mercaptoethanol, 20mM tris-HCl, pH 8 and centrifuged for 30min. at 40,000g. Dialysed fractions were assayed for DNase activity by measuring release of acid-soluble UV-absorbing material after 30min. at 37° (assay medium - 50mM tris-HCl, pH9, 60mM KCl, 2mM $MgSO_4$, 1mM 2-mercaptoethanol, 300ug of DNA/ml.)

Figures in brackets represent the total number of units of DNase induced in each fraction and the percentage of the increment occurring in that fraction.

Table III.6.

Intracellular distribution of DNA polymerase in control and herpes-infected BHK 21 cells.

State of cells	Subcellular fraction	State of DNA primer	DNA polymerase (specific activity)	(total activity)
non-infected	cytoplasmic	native	1.0	326
		denatured	2.9	945
	nuclear	native	0.8	88
		denatured	2.7	297
herpes-infected	cytoplasmic	native	2.2	733 (407-65%)
		denatured	3.5	1166 (221-18%)
	nuclear	native	2.5	310 (222-35%)
		denatured	10.5	1302 (1005-82%

Efficiency of priming--denatured/native

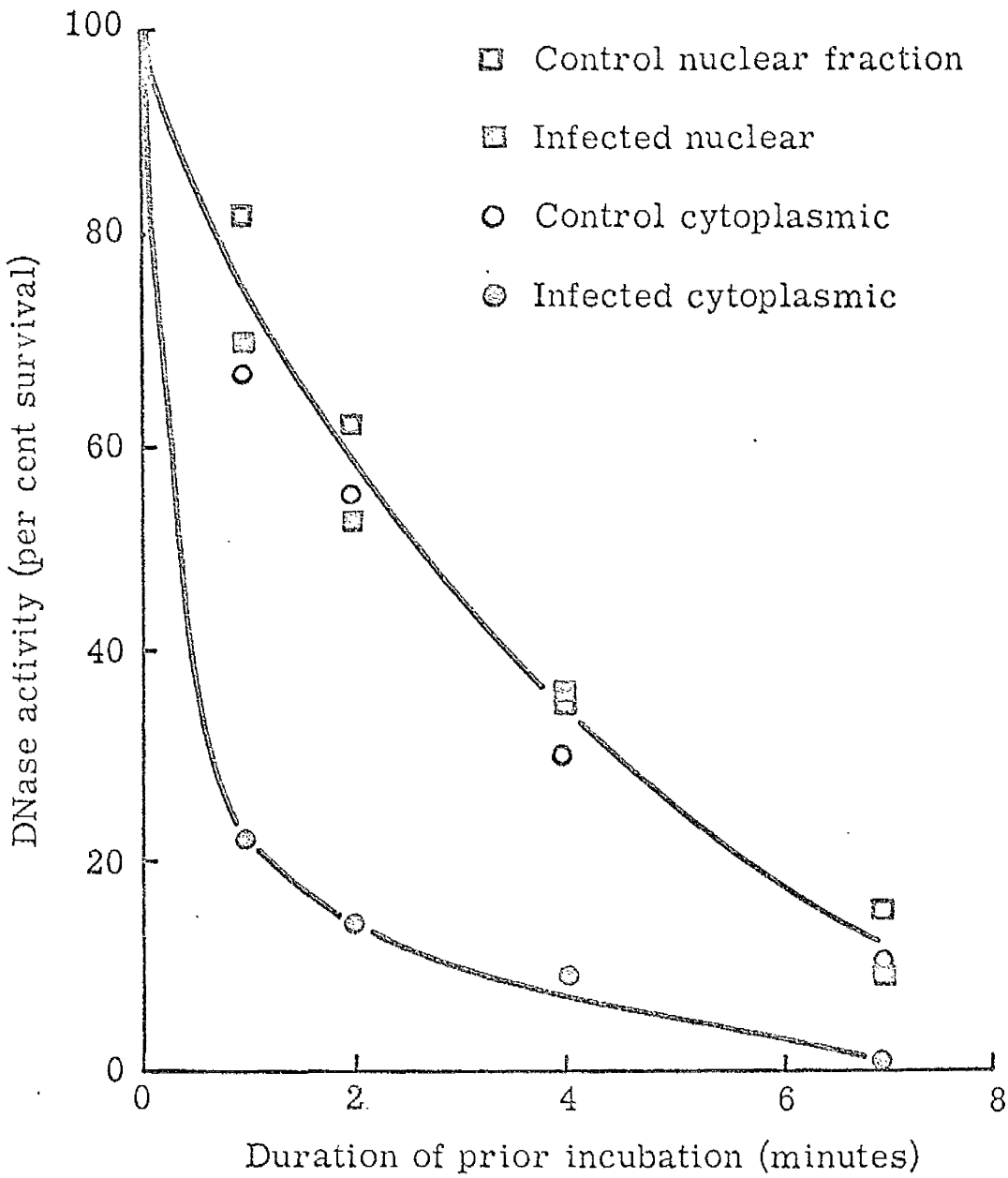
	non-infected	herpes-infected
cytoplasmic	2.9	1.6
nuclear	3.4	4.2

Legend as for table III.5.

Assays performed under standard conditions.

Figure III. 7

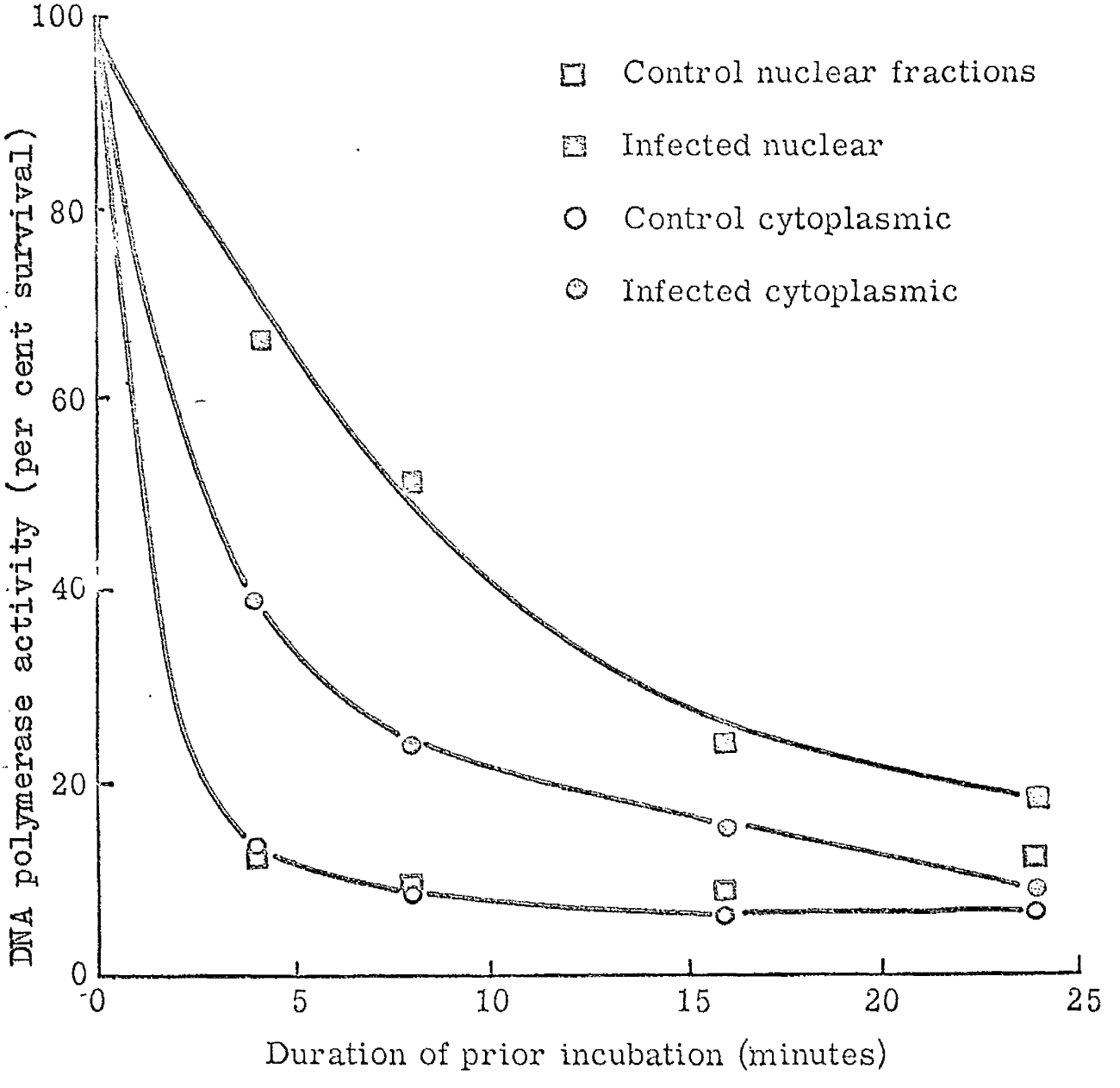
Effect of prior incubation at 50° on the DNase activity (denatured DNA) of nuclear and cytoplasmic fractions of control and herpes-infected BHK 21 cells



80μg of protein of various fractions incubated each at 50° in a volume of 0.04 ml., cooled on ice at time shown, then assayed under standard conditions at 37° (see legend to Table III. 5).

Figure III. 8

Effect of prior incubation at 50° in the presence of denatured DNA on the DNA polymerase activity of nuclear and cytoplasmic fractions of control and herpes-infected BHK 21 cells



80μg of protein of various fractions incubated with 50μg of denatured DNA (volume 0. 1 ml.), cooled on ice at the time shown, then assayed under standard conditions at 37°

(i) Specificity towards state of substrate.

The herpes-induced DNase exhibits a much greater rate of hydrolysis of native DNA than does the host cell enzyme (Tables III.5. and III.9.)

(ii) Stability to heating.

Figure III.7. shows that the DNase which appears after infection with herpes virus is much more sensitive to heating than the existing DNase.

Both these properties are observed not only in crude extracts of infected cells but also in partially purified fractions. Indeed, the herpes-induced DNase becomes even more heat-labile after passage through DEAE-cellulose (see Section 1.7.1.). The different properties are observed when either BHK 21 or HEp-2 cells are infected with herpes virus.

(iii) Effect of 2-mercaptoethanol.

When extracts of infected and non-infected cells are prepared in the presence and absence of SH-EtOH (50mM) and then assayed in its presence (15mM) or absence, it is clear that absence of SH-EtOH from the extracting medium causes a reduction in the DNase activity of infected cell extracts which is only partly restored by adding SH-EtOH before assaying (Table III.9.). A concentration of 10mM gives a 2-fold stimulation of the DNase of herpes-infected cells; the host cell DNase is much less sensitive to the absence of SH-EtOH.

Table III. 9.

Effect of the presence of 2-mercaptoethanol in the extracting medium on the DNase activities of control and herpes-infected BHK 21 cells.

Extracting conditions	Assay conditions	Specific activity of DNase (units/mg.) non-infected	herpes-infected
+SH-EtOH	+SH-EtOH +native DNA	0	27.7
-SH-EtOH	-SH-EtOH +native DNA	0	4.4
-SH-EtOH	+SH-EtOH +native DNA	0	7.3
+SH-EtOH	+SH-EtOH +denatured DNA	3.7	20.5
-SH-EtOH	-SH-EtOH +denatured DNA	5.1	4.4
-SH-EtOH	+SH-EtOH +denatured DNA	3.1	9.5

Extracting medium contained 0.05M tris-HCl pH8, 1mM EDTA and 50mM 2-mercaptoethanol (SH-EtOH) where appropriate. DNase assay was performed by measuring release of acid-soluble radioactivity from ^{32}P-Esch. coli DNA under standard conditions in the presence or absence of 15mM SH-EtOH.

(iv) Effect of monovalent cations.

Studies on the effects of Na^+ and K^+ ions on the DNases of control and infected cells have given somewhat different results depending on whether the UV or radioactive DNase assay was used but, in both cases, the induced DNase was less susceptible to inhibition by high concentrations of Na^+ or K^+ (Figure III.10.).

The other features of the two DNases are similar :-

(v) both act optimally about pH 9 (Figure III.12a.),

(vi) both have an absolute requirement for a divalent cation, 2mM Mg^{2+} or 0.5mM Mn^{2+} giving optimal activity (Figure III.12b.), although the latter seems less effective in activating the induced DNase.

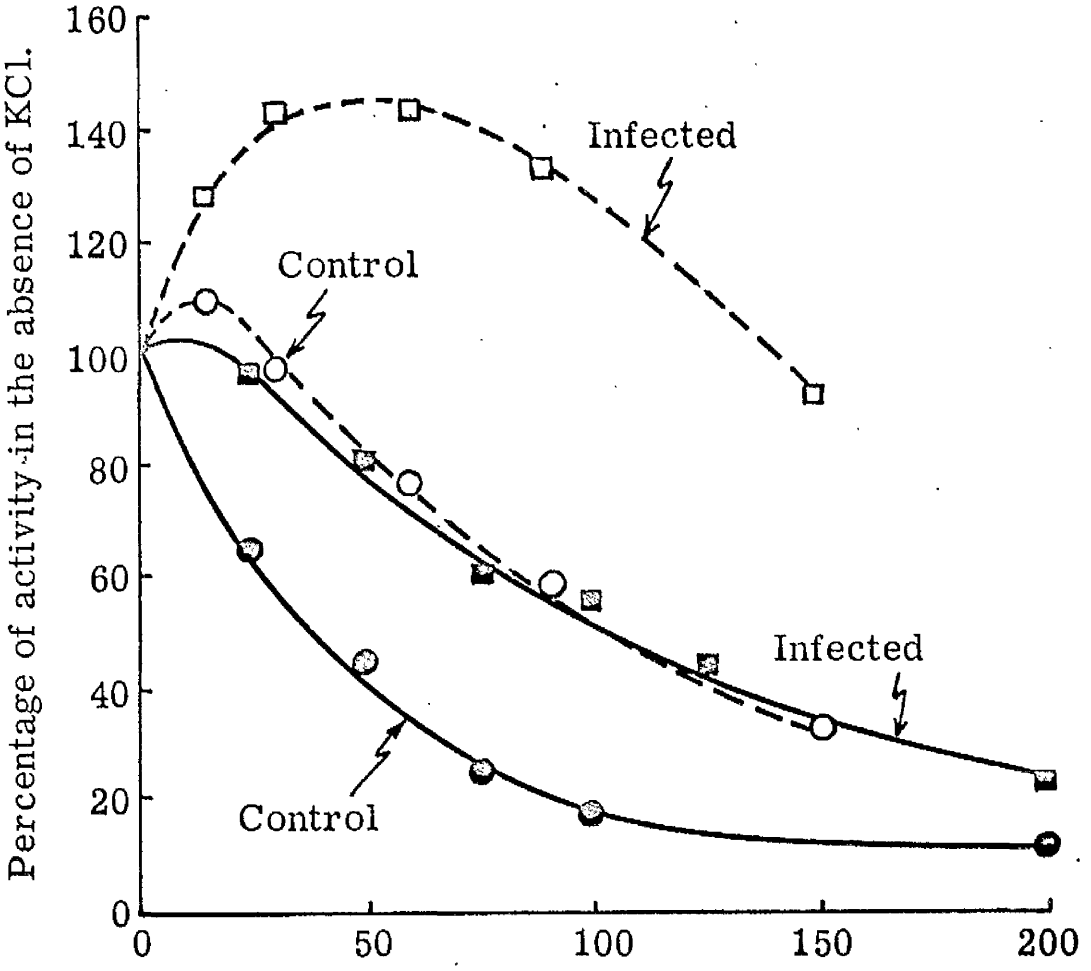
The increase of DNase which occurs in herpes-infected cells could be due to the presence of an inhibitor in non-infected cells which is destroyed during infection. This possibility is made unlikely by the results described in Table III.11.:-

(a) assay of the DNase of non-infected and herpes-infected cell extracts, measured separately and in mixtures, shows that mixing control and infected enzymes gives an additive or super-additive response; an excess of an inhibitor in the control extract would give a sub-additive response;

(b) addition of pancreatic RNase to the assay results in

Figure III.10.

Effect of monovalent cation concentration on the DNase activities of control and herpes-infected BHK 21 cells.



Results of two experiments, with denatured DNA substrate:

(i) broken lines: 100µg. of protein from herpes-infected or 400µg. from non-infected extracts of HEp-2 cells, assayed by release of UV-absorbing material.
(ii) continuous lines: 140µg. of protein from DEAE-eluate or 100µg. from nom-adsorbed DEAE fraction of herpes-infected BHK 21 cells, assayed by release of radioactivity

Table III.11.

Mixing and RNA Inhibition of the DNase Activities of Control and Herpes-infected HK 21 Cells.

(i) Mixing of control and herpes-infected extracts.

Source of enzyme	DNA rendered acid-soluble (counts/min.)	
	native	denatured
non-infected cells	37	279
herpes-infected cells	1692	2045
Sum:	1729	2324
herpes- plus non-infected cells	2743 (159%)	2367 (102%)

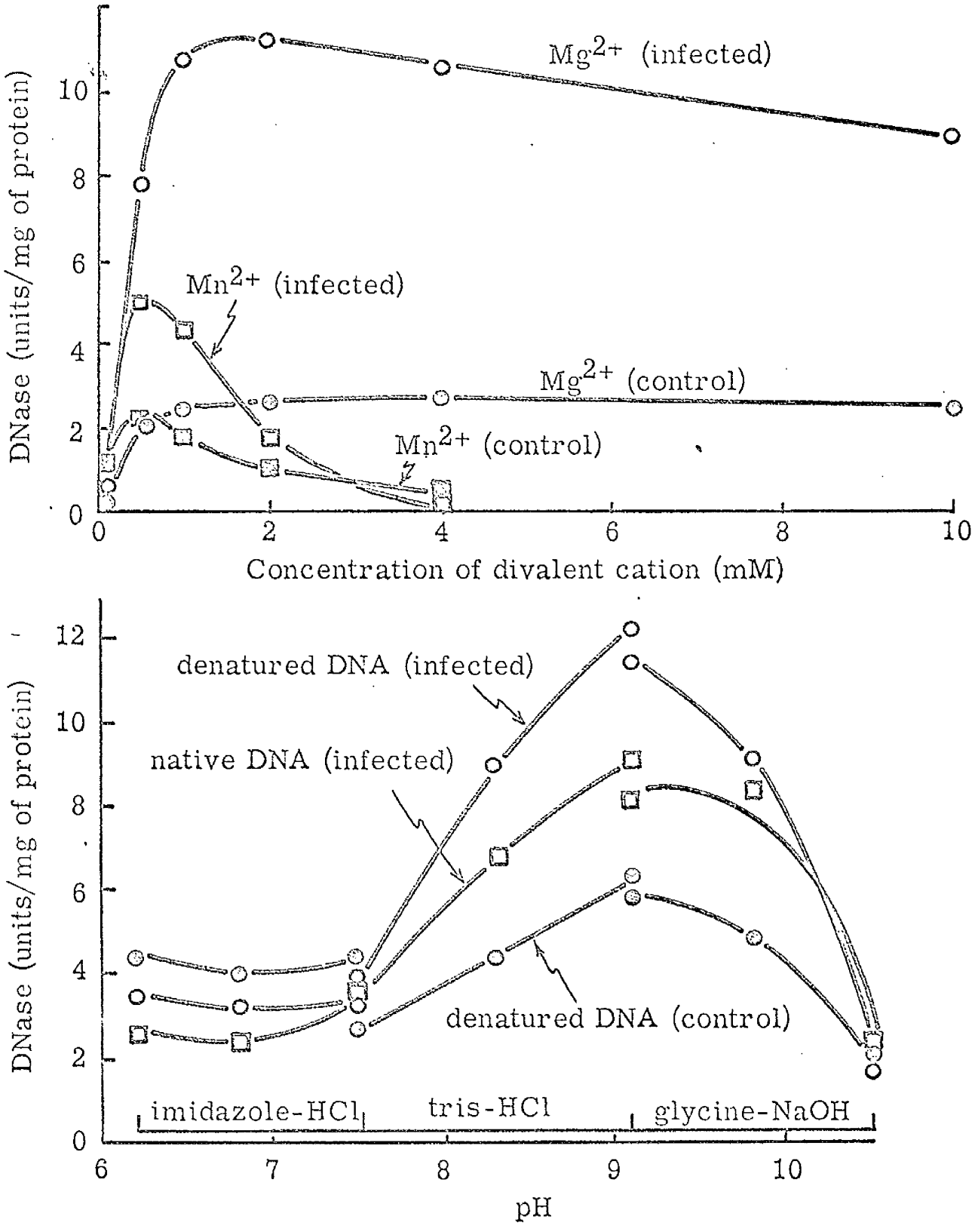
(ii) RNA inhibition.

	native		denatured	
	-RNase	+RNase	-RNase	+RNase
non-infected cells	37	82	279	400
herpes-infected cells	1692	2878	2045	2959

Radioactive DNase assay performed under standard conditions. Pancreatic RNase (50µg./ml.) added where appropriate.

Figure III. 12

Effect of varying pH and divalent cation concentration on the DNase activity of control and herpes-infected HEp-2 cells



Assay performed by release of acid-soluble UV material.

an activation of both control and infected-cell DNases, thus, similar RNA inhibition is occurring in both instances, so that the increase in DNase cannot be due to a reduction in RNA inhibition.

Since RNase activity does not rise in cells infected with herpes virus, it seems likely that the induced DNase is DNA-specific. Analysis of the products of digestion of RNA by extracts of control and infected cells on DEAE-paper (see Section 1.6.), revealed that they were qualitatively and quantitatively similar, only oligo-nucleotides being produced.

1.4.2. Herpes-induced DNA polymerase.

The increased DNA polymerase activity found in herpes-infected cells differs from that of non-infected cells in several respects:-

(i) Increased stability to heating in the presence of DNA.

When extracts of control and infected cells were incubated at 50° before being assayed for DNA polymerase, it was found that the enzyme from infected cells was more stable to heating, especially when the heating was carried out in the presence of DNA (Figure III.8.). Mg^{2+} was not necessary for the protection by DNA, the optimal KCl concentration for protection was 0.05M not 0.2M as for optimal enzyme activity (see below), and native DNA was less effective than denatured DNA (Table III.13.).

Table III.13.

Factors affecting the protective effect of DNA on the DNA polymerase induced by herpes virus.

1) Monovalent cation concentration:

KCl (M)	%activity after 4min. at 50°.
0	23
0.05	33
0.10	21
0.15	9
0.20	1

80µg. of protein from a nuclear fraction of herpes-infected BHK 21 cells incubated with 50µg of denatured DNA (no Mg^{2+}, triphosphates) and the indicated amount of KCl, before assaying under standard conditions (0.2M KCl).

2) Divalent cation concentration:

Mg^{2+} (mM)	% activity after 15min. at 50°.
0	32
3	34
8	11

160µg of protein from a "soluble" fraction of herpes-infected HEp-2 cells incubated with 150µg of denatured DNA (no triphosphates, 0.06M KCl) and indicated amount of Mg^{2+} before assaying under standard conditions (0.06M KCl).

3) Configuration of DNA:	% activity after 20min. at 50°.
native DNA	13
denatured DNA	12

Conditions as for 2), but no Mg^{2+} in pre-incubation medium.

(ii) Activation of herpes-induced DNA polymerase by NH_4^+ or K^+

Figure III.14. shows the different responses to K^+ concentration of the DNA polymerases of control and herpes-infected cells. The virus-induced activity using native DNA primer exhibits the same stimulation, which is still observed after the enzyme has been heated at 50^o in the presence of denatured DNA. NH_4Cl and $(NH_4)_2SO_4$ give a similar activation at 0.2M NH_4^+.

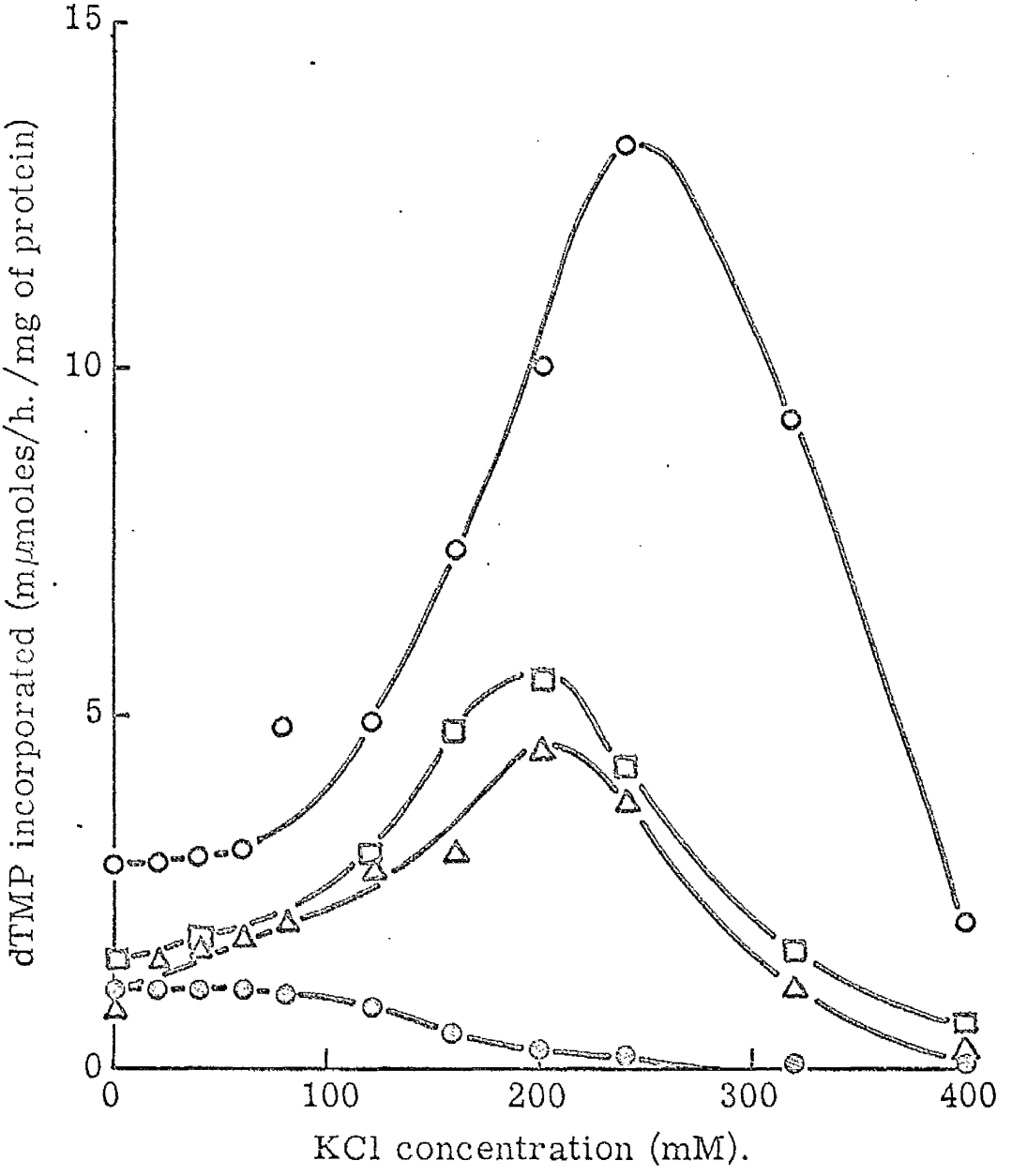
Although this salt concentration and pH (7.5) reduce the DNase levels to less than 10% of the maximum, inhibition of nuclease cannot account for this marked difference between the DNA polymerase of control and infected cells. The induced (or altered) enzyme itself must be affected by the high salt so as to have its enzymic activity increased, and its ability to be so affected must have an order of heat stability not less than that of the enzymic activity itself. The salt effect presumably occurs by an alteration of the tertiary or quaternary structure of the protein. A similar effect has been observed with the DNA polymerase induced by T5 bacteriophage (Paul and Lehman, 1966).

(iii) Increased utilisation of native DNA as primer.

It was observed that, while the DNA polymerase of non-infected BHK21 and HEp-2 cells, like those of other animal tissues (Keir, 1965) used native DNA as primer only

Figure III. 14

Effect of KCl concentration on the DNA polymerase activities of control and herpes-infected BHK 21 cells.



- ○ 80μg of protein from infected nuclear fraction + 50μg of denatured DNA
- △ ditto, but enzyme heated 4 min/50°
- □ ditto, but native DNA primer used
- ⊙ 80μg of protein from control nuclear fraction + denatured DNA

about 10% as efficiently as heat-denatured DNA, the polymerase from herpes-infected cells could use native DNA 50-100% as efficiently as denatured DNA primer. This was at first thought to be a property of the induced polymerase, but subsequent studies suggest that the effect may be due to the herpes-induced DNase converting native DNA into a more effective primer. Two points support this view:-

(a) Figure III. 15. shows thatiincubation of native DNA with extracts of infected cells increases its priming efficiency for the DNA polymerase of control cells; extracts of control cells do not do so;

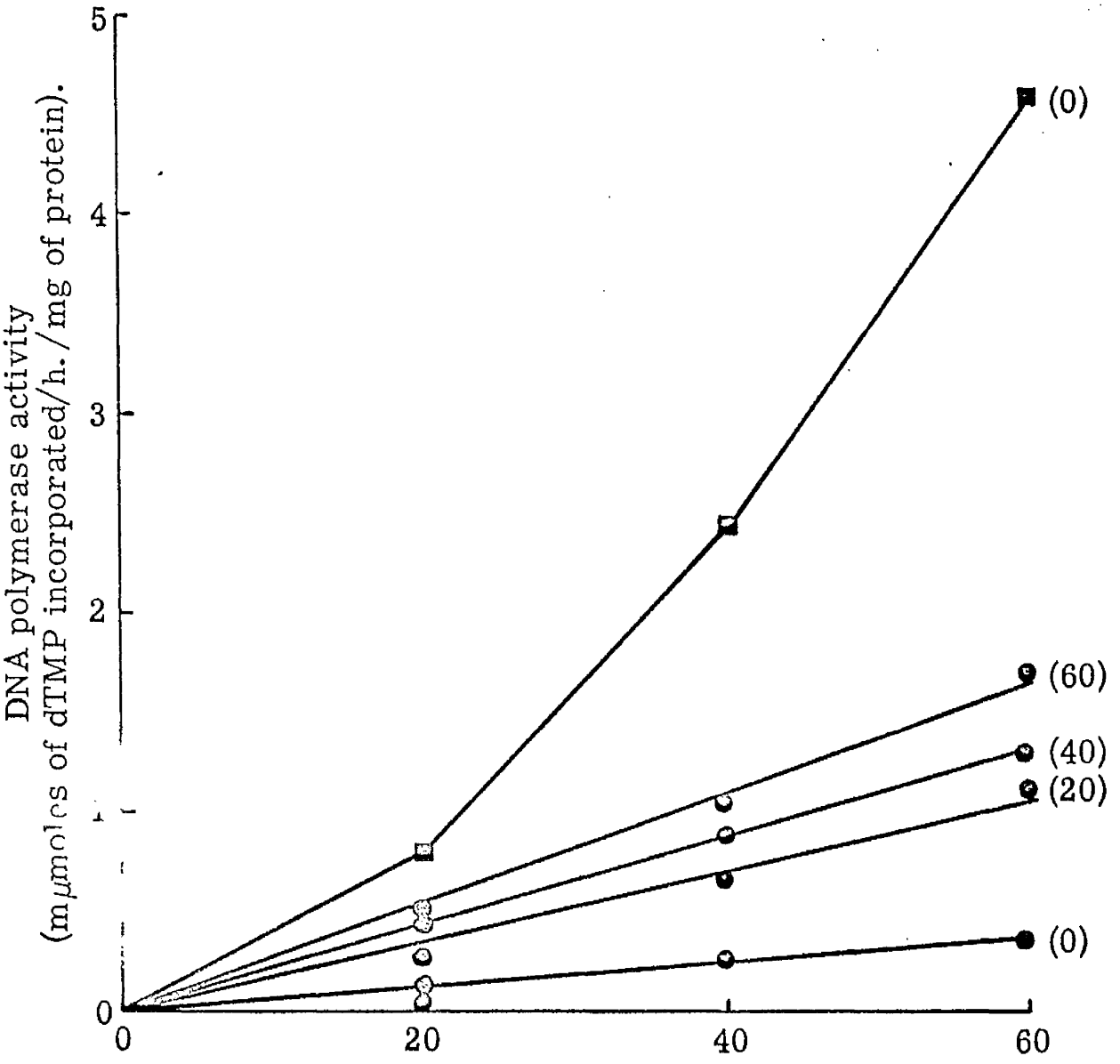
(b) Table III.6. shows that while the bulk of the induced DNA polymerase is in the nucleus, the efficiency of priming by native DNA is low. In the cytoplasm, on the other hand, where most of the induced DNase is found at this time after infection, a much higher priming efficiency by native DNA is observed.

(iv) Other properties.

The herpes-induced DNA polymerase also differs from that found in non-infected cells in its response to varying Mg^{2+} ion and DNA substrate concentrations (Keir et al., 1966a). Studies on the response of the two DNA polymerases to varying concentrations of deoxyribonucleoside 5'-triphosphates have not so far yielded any significant differences. No major difference in pH optimum (7.5-8.5)

Figure III.15.

Increased priming efficiency of native DNA previously incubated with extracts of herpes-infected cells.



Native DNA was incubated with an extract of herpes-infected cells (minus triphosphates) for the times shown in brackets (min.). After heating to destroy added polymerase, the DNA was incubated with extracts of control (circles) and infected HEp-2 cell extracts (squares), in the presence of triphosphates.

was observed.

That the herpes-induced DNA polymerase is a bona fide replicative-type enzyme is shown by the results in Table III.16. Omission of exogenous DNA primer gives low activity (enzyme prepared from cell nuclei, of course, did not show an absolute requirement for DNA) and omission of the three non-radioactive triphosphates drastically reduces the rate of incorporation of the fourth, radioactive triphosphate. The activity observed in 0.2M KCl is also clearly not of a terminal-addition type.

1.4.3. Pseudorabies-induced enzymes.

Pseudorabies virus has been found to induce increases in both DNA polymerase (Hamada et al., 1966) and DNase (Table III.17.). These activities have not been closely studied as yet, but the pseudorabies-induced DNA polymerase does not seem to be activated by 0.2M KCl as strongly as the herpes-induced enzyme. The pseudorabies-induced DNase closely resembles the herpes-induced enzyme in being an exonuclease attacking both native and denatured DNA to produce 5'-monophosphates. (Section 1.6.2.2.). The storage properties of the enzyme suggest that it may be less heat-labile than the herpes-induced DNase. RNase activity does not increase in pseudorabies-infected cells. (Table III.3.).

Table III.16.

Requirements of the DNA Polymerases of Control and Herpes-infected Cells.

(i) DNA primer:	no DNA	native DNA	denatured DNA
control cells			
cytoplasmic	0	0.8	3.1
nuclear	0	0.8	2.8
herpes-infected cells			
cytoplasmic	0	2.3	3.4
nuclear	0.3	2.6	10.9

(ii) deoxyribonucleoside 5'-triphosphate:

control cells		dATP, dCTP, dGTP and ^{32}P-dTTP	^{32}P-dTTP only
cytoplasmic		0.36	0.12
nuclear		0.49	0.22
herpes-infected cells			
cytoplasmic	(60mM KCl)	0.51	0.09
	(200mM KCl)	1.28	0.14
nuclear	(60mM KCl)	9.6	0.52
	(200mM KCl)	20.3	0.57

Activity expressed as units/mg. protein.

Table III.17.

Effect of infection with herpes and pseudorabies viruses on the DNase activities of BHK 21 and HEp-2 cells.

State of cells	DNase specific activity (units/mg).	
	BHK 21 cells	HEp-2 cells
non-infected	0.8	1.8
herpes-infected (8h.post-infection)	50.0	70.0
pseudorabies-infected (7h.post-infection)	20.0	14.7

Cells harvested and washed in isotonic saline, then disrupted ultrasonically in 0.05M tris-HCl buffer containing 1mM EDTA and 10mM 2-mercaptoethanol. Extracts assayed under standard conditions with native ^{32}P-Esch. coli DNA as substrate.

1.5. Immunological Analysis of Herpes-induced Enzymes.

1.5.1. Experimental scheme.

In order to investigate whether the enzymic activities observed to increase after infection with herpes virus are, in fact, specified by the viral genome, the following immunological experiment was performed. (Figure III.18.).

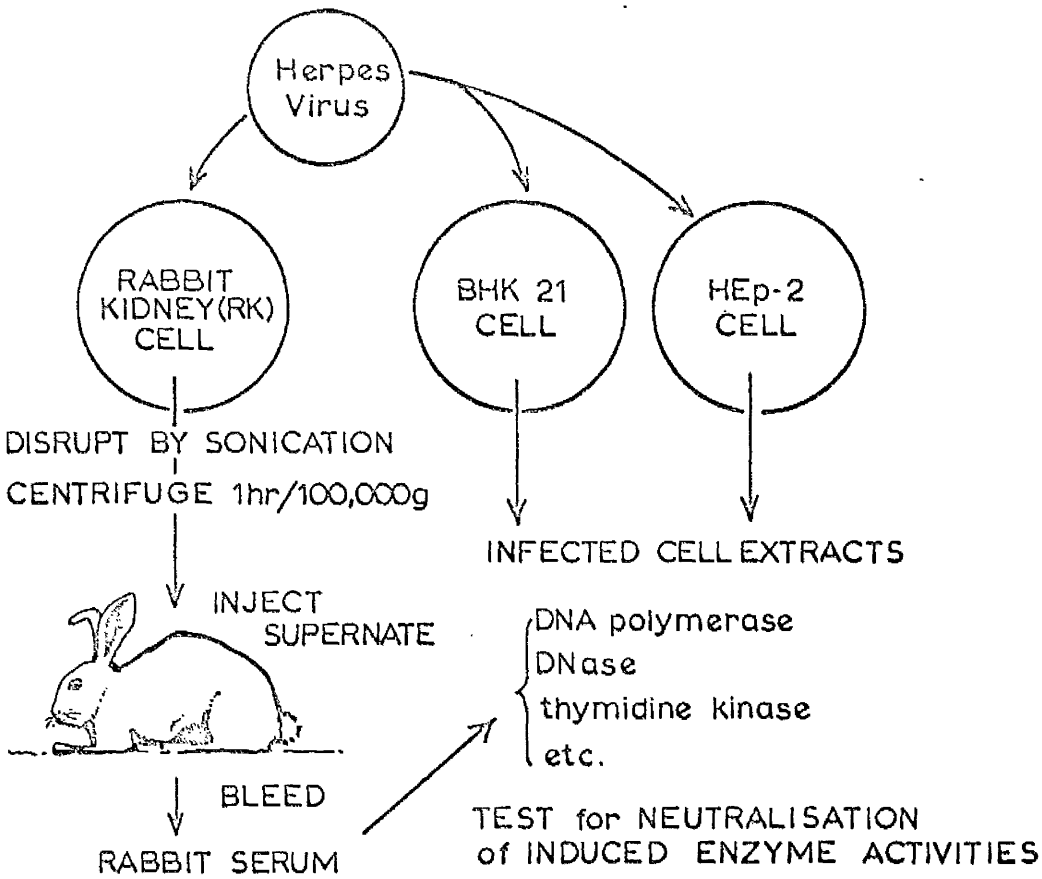
Rabbits were inoculated with high-speed supernatant fractions (1hr. at 100,000g) prepared from growing cultures of rabbit kidney cells which had been infected for 6hr. with herpes virus (MOI = 10-15p.f.u./cell). The homologous rabbit-rabbit system was used so that antibody produced against antigens other than those induced by virus infection would be kept to a minimum. The herpes-infected-cell-supernatant antisera (HICS antisera) were dialysed extensively against 0.02M KCl before use in enzyme neutralisation tests. Heat treatment of the sera (10 min. at 60°) to inactivate complement was not used, as this was found to activate serum DNase, presumably by inactivating serum DNase inhibitor.

1.5.2. Enzyme neutralisation studies with HICS antisera.

Enzyme neutralisation tests were carried out by incubating antisera prepared as described above together with extracts of herpes-infected, pseudorabies-infected or non-infected BHK 21 and HEp-2 cells for 10 min. at 37° prior to enzyme assay.

Figure III.18.

PLAN of IMMUNOLOGICAL EXPERIMENTS



1.5.2.1. Herpes-induced DNase.

When HICS antisera from three rabbits were tested for inhibition of the DNase induced by herpes virus in BHK 21 cells, all three were found to be active (Table III.19.), but one in particular (rabbit No. 5706) was strongly inhibitory. Studies with this serum were pursued further and of several different infected-cell extracts tested (both BHK 21 and HEp-2 cells - see Section 1.5.2.3.) all have been inhibited.

Figure III.20. shows the inhibitory effect of two levels of No. 5706 antiserum against DNase induced in BHK 21 cells by herpes virus. Use of native DNA as substrate makes the test specific for the induced DNase because of the low activity of non-infected cells towards this substrate. Neutral serum (i.e. from a non-immunised rabbit) was used as control because pre-immune serum from rabbit 5706 was in very short supply. This had, however, no specifically inhibitory activity (Table III.19.) on herpes-induced DNase.

In order to verify the virus-specific nature of the antiserum, a similar experiment was carried out using denatured DNA as substrate. In this case (Figure III.21.), it is clear that, although the virus-induced DNase is inhibited, HICS antiserum has no effect on the activity of non-infected cells. The reasons for the somewhat different effects of non-immune serum on DNase activity

Table III.19.

Enzyme Inhibitory Effects of HICS and Control Sera.

(i) Herpes-induced DNase:

	DNase activity (%)
herpes-infected BHK cell extract	100
+ HICS antiserum 5705	67
+ HICS antiserum 5706	11
+ HICS antiserum 5706a	41
+ 5706 serum (pre-immune)	87
+ neutral serum	90
+ DEAE-treated 5706 antiserum	27
+ DEAE-treated neutral serum	105
herpes-induced DNase (DEAE eluate)	100
+ HICS antiserum 5706	41
+ 5706 antiserum (pre-immune)	77
+ neutral serum	77

(ii) Herpes-induced DNA polymerase:

	DNA polymerase activity (%).
non-infected BHK 21 cell extract	
+ neutral serum	100
+ HICS antiserum 5706	95
herpes-infected BHK 21 cell extract	
+ neutral serum	100
+ HICS antiserum 5706	56

Figure III.20.

Effects of HICS Antiserum on the DNase Activity (native DNA) of Herpes-infected BHK 21 Cells.

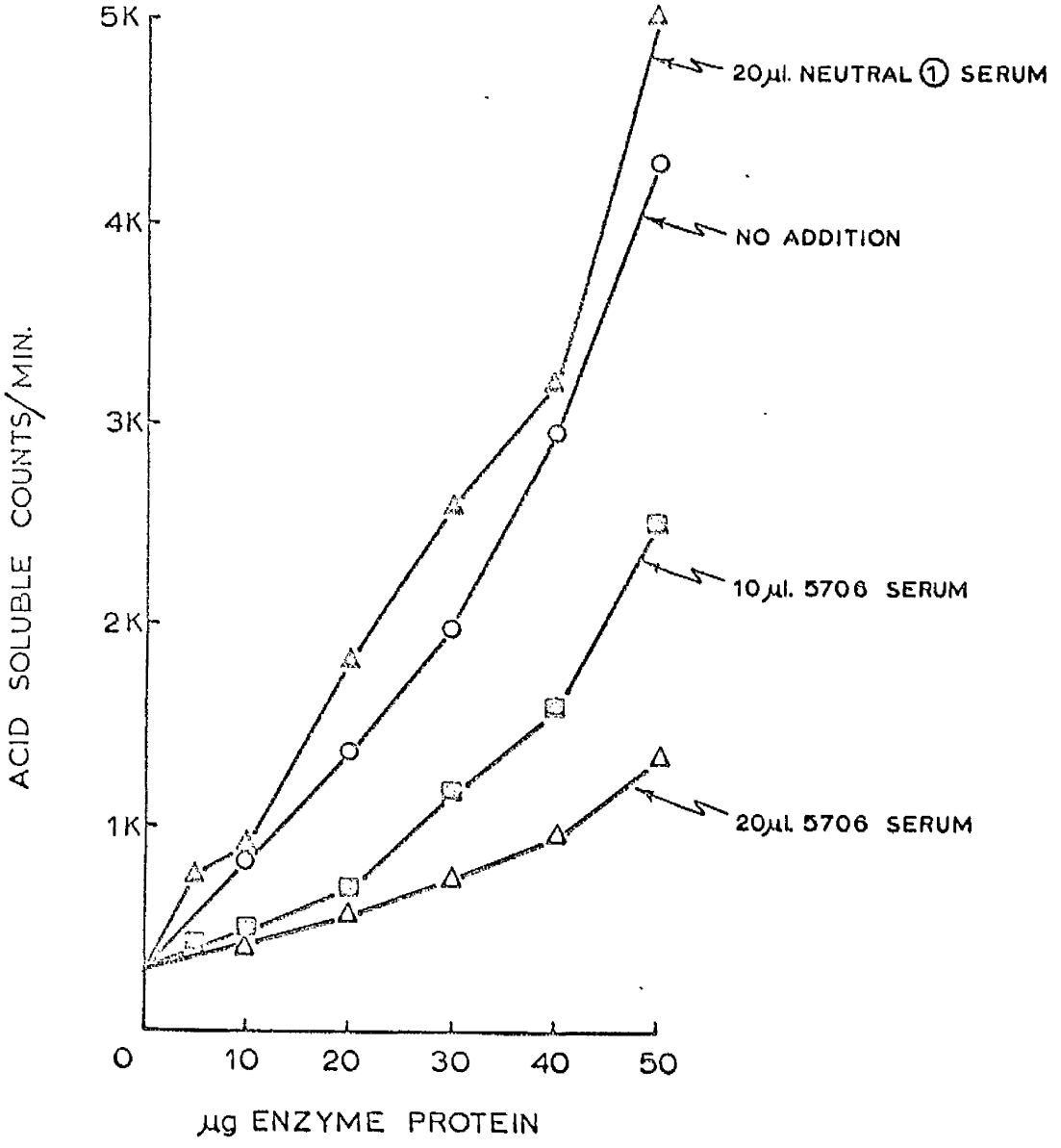
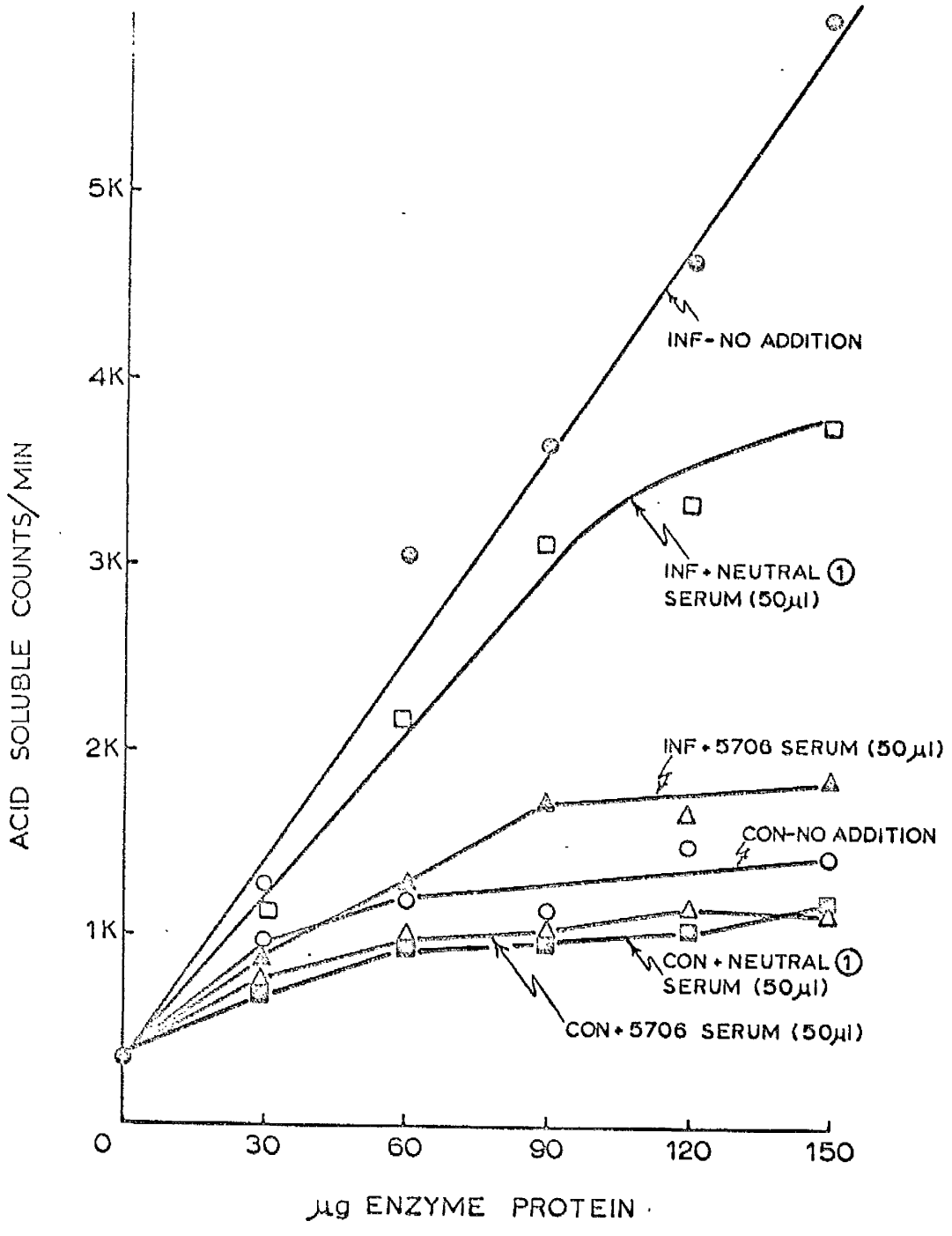


Figure III.21.

Effects of HICS Antiserum on the DNase Activities (denatured DNA) of Control and Herpes-infected BHK 21 Cells.



(stimulation in Figure III.20., inhibition in Figure III.21. and Table III.19.) are not clear, but may be due to a balance between the protective effect of a high protein concentration (stimulation of both DNases) and the occurrence of serum DNase inhibitor (inhibition of host cell DNase).

When HICS antiserum was passed through DEAE-cellulose under conditions (0.0175M $Na-PO_4$, pH6.5) where only the γ-globulins are not adsorbed (Sober and Peterson,1958), the non-adsorbed fraction still inhibited the herpes-induced DNase; this was not so with non-immune serum (Table III.19.). In addition, batchwise fractionation of the DNases of herpes-infected BHK 21 cells (see Chapter II, Section 2.2.7.) on DEAE-cellulose showed that, while the herpes-induced DNase, eluted from the DEAE was inhibited by HICS antiserum, the non-adsorbed, host cell DNase was not (Table III.19.). These observations support the hypothesis that immunisation of rabbits with extracts of herpes-infected rabbit cells has caused the production of a herpes-specific antibody which neutralises the activity of the herpes-induced DNase.

1.5.2.2. Herpes-induced DNA polymerase.

HICS antiserum causes an inhibition of the DNA polymerase activity of herpes-infected BHK 21 and HEp-2 cells but not the DNA polymerase of non-infected cells (Table III.19.); this is in agreement with the results of Keir et al., (1966b). Moreover, the DNA polymerase

of pseudorabies-infected cells was not inhibited by HICS antiserum (Table III.19.), thus lending further support to the hypothesis that virus-specific antibodies are present in the antiserum.

1.5.2.3. Pseudorabies-induced enzymes.

Figure III.22. shows the effect of increasing amounts of HICS antiserum on the DNase activities (native DNA substrate) of herpes- and pseudorabies-infected BHK 21 cells. It is clear that while the herpes-induced DNase is potently inhibited, the very similar DNase induced by pseudorabies virus (see Section 1.4.2.) is not inhibited at all, at the antiserum concentrations tested. Identical results were obtained when the DNase of extracts of virus-infected HEp-2 cells was tested.

Antisera are currently being prepared against supernatants of pseudorabies-infected rabbit kidney cells and non-infected BHK 21 cells. As yet, no anti-enzymes have beenedetected, but when available these should provide additional information on the origin of the virus-induced enzymes.

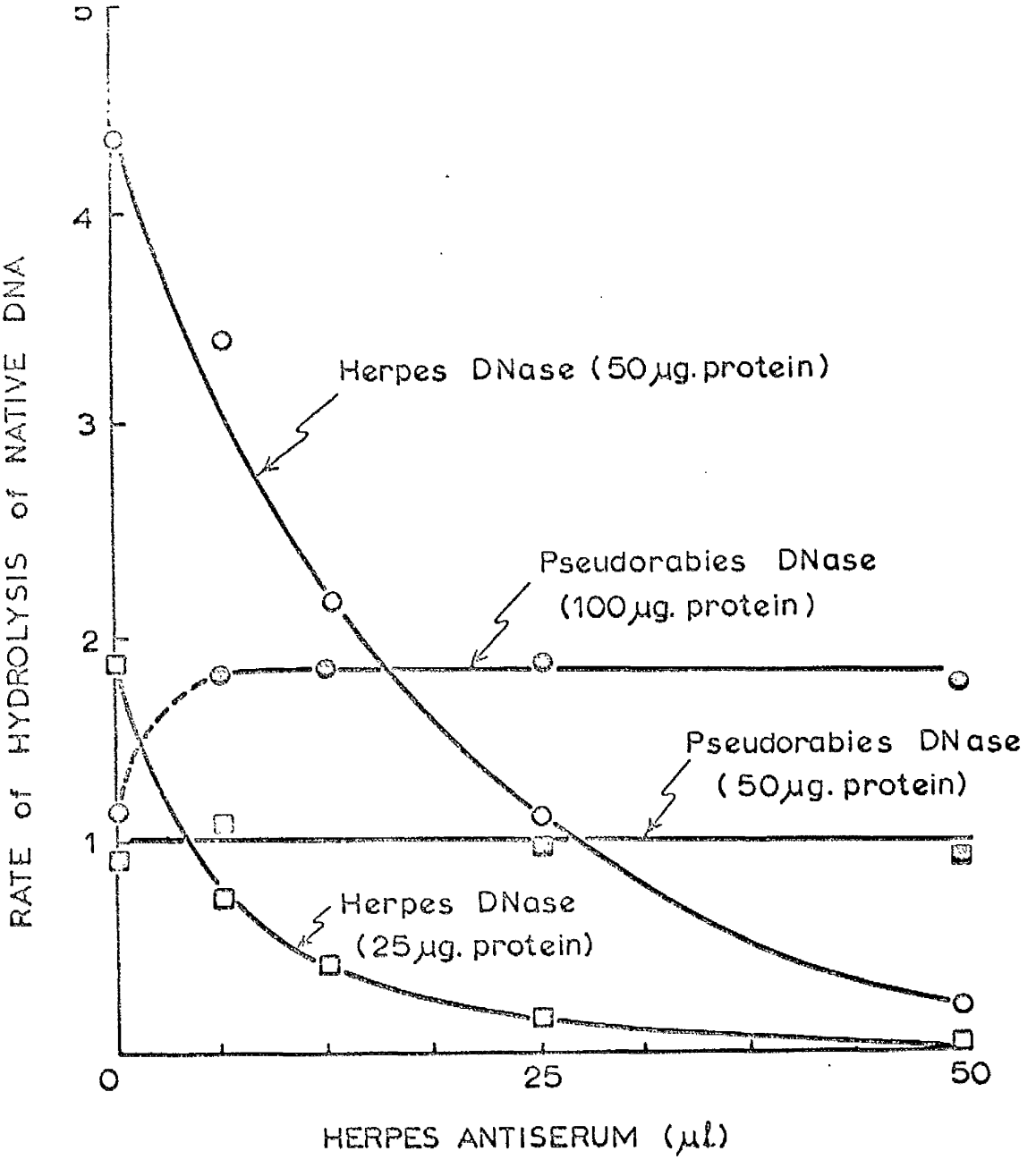
1.6. Mode of Action of DNases

1.6.1. Calibration of method for characterisation of mode of action of DNase.

In order to examine the modes of action (i.e. endo- or exolytic) of the various DNases in the DNA virus-animal

Figure III.22.

Effects of HICS Antiserum on the DNase Activities (native DNA) of Herpes- and Pseudorabies-infected BHK 21 Cells.



cell system, it was decided to examine the products of enzymic hydrolysis of ^{32}P-DNA by chromatography on DEAE-paper followed by measurement of radioactivity in a gas-flow chromatogram scanner. (See Chapter II, Section 2.1.2.). The products of hydrolysis were characterised as follows:-

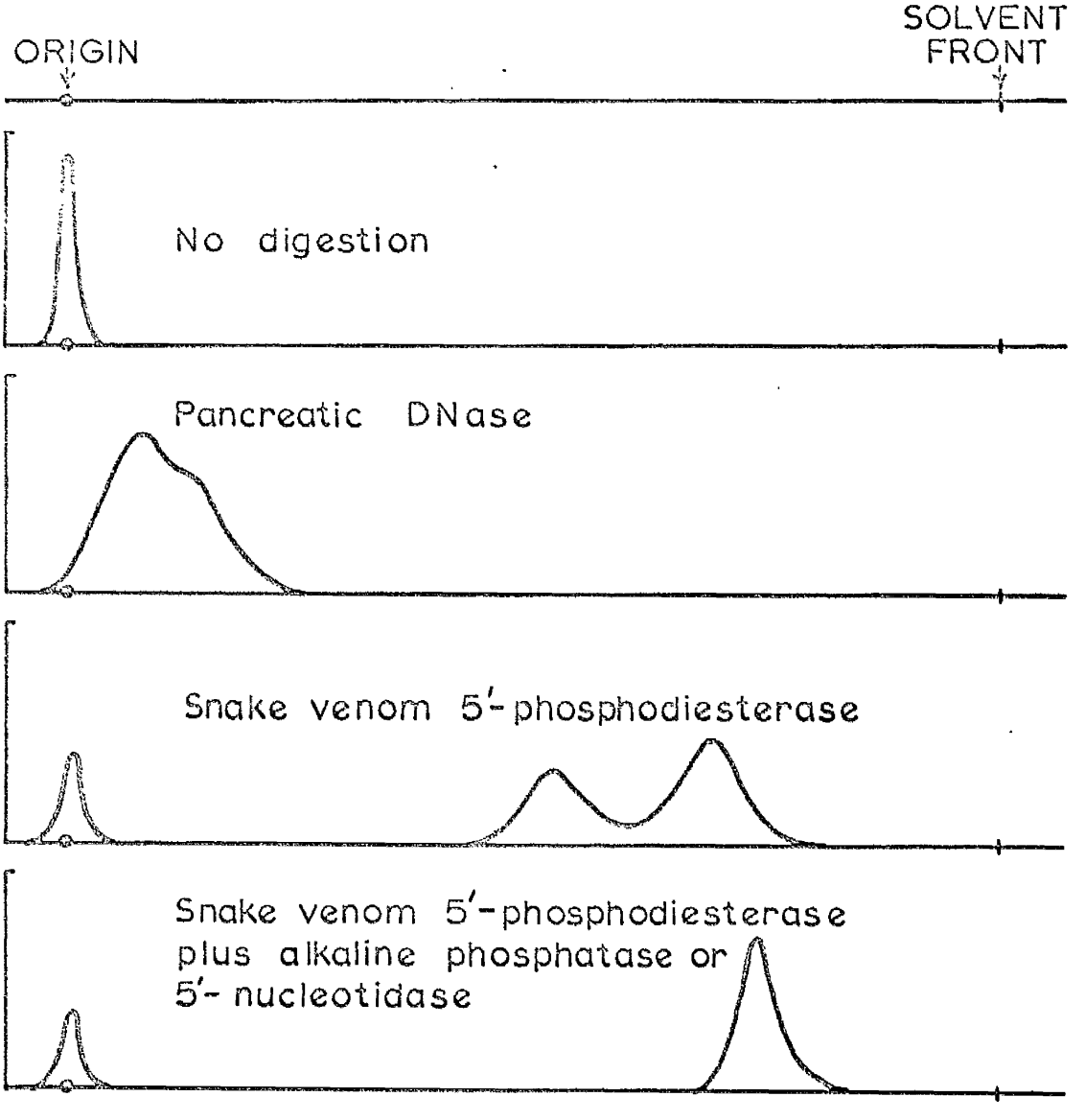
(i) A known endonuclease (pancreatic DNase) and a known exonuclease (snake venom phosphodiesterase) were used to produce partial DNA hydrolysates which were then subjected to chromatography. The characteristic patterns obtained are shown in Figure III.23. High molecular weight DNA remains at the origin; Furlong (1966) has shown that, under the conditions used, oligonucleotides of chain length greater than 30 remain at the origin. The oligonucleotide products of pancreatic DNase digestion move a short distance (R_f 0-0.2) from the origin while the mononucleotides produced by snake venom diesterase have discrete R_f values of 0.5 and 0.7 respectively.

(ii) Commercial preparations of dAMP, dCMP, dGMP and dTMP were run on DEAE-paper under standard conditions and it was found that the substances which had R_f values of 0.5 and 0.7 corresponded to the purine and pyrimidine monophosphates respectively. A slight separation of dGMP and dAMP occurred which explains the somewhat broader shape of the purine peak.

Figure III.23.

Chromatography of DNA on DEAE - paper after partial enzymic digestion.

SOLVENT: 0·75 M NH_4HCO_3, pH 8·6



(iii) When the mononucleotide products of digestion of DNA with snake venom phosphodiesterase were treated with Esch.coli alkaline phosphatase or snake venom 5'-nucleotidase before being applied to the DEAE-paper, the purine and pyrimidine mononucleotide peaks were replaced by an inorganic phosphate peak (R_f - 0.8) identified by comparison with commercial ^{32}P-phosphate.

Thus, by subjecting the products of hydrolysis of DNA resulting from the action of a DNase to chromatography in this system, the mode of action of the DNase may be determined. Furthermore, by use of the specific 5'-nucleotidase, exonucleolytic products may be further characterised as 3'- or 5'-monophosphates.

1.6.2. Mode of action of DNases in herpes virus-animal cell system.

1.6.2.1. Host cell DNase.

When extracts of BHK 21 or HEp-2 cells were incubated with ^{32}P-DNA under standard DNase assay conditions and the digestion products analysed by the above method, only oligonucleotides were observed. The same result was obtained both with native and denatured DNA as substrate, although the latter is attacked more rapidly. Addition of pancreatic RNase to the incubation medium stimulated DNase activity (presumably by destroying inhibitory RNA) but did not alter the nature of the products. Thus, the alkaline DNases of BHK 21 and HEp-2 cells are endonucleases.

1.6.2.2. Herpes-induced DNase.

When herpes-infected BHK 21 cells were examined, the products obtained from native and denatured DNA indicated an exonucleolytic mode of attack for the induced DNase. Digestion with 5'-nucleotidase showed that the hydrolytic products are 5'-monophosphates.

Although the predominant DNase of herpes-infected cells is an exonuclease, the possibility that herpes also induces an endonuclease (cf. bacteriophage T2, Bose and Nossal (1964)) cannot be excluded. There was no increase in the amount of oligonucleotide produced by infected cell extracts compared with those of control cells, but it is highly likely that such oligonucleotides, if produced, would be very efficiently hydrolysed by the herpes-induced exonuclease (see Section 1.6.3.). Assay of endonuclease in infected cell extracts by the method of Geiduschek (1965) did not yield meaningful results because of the high level of exonuclease. It is hoped that subsequent experiments with supercoiled polyoma DNA and host cells with very low levels of endonuclease will help clarify this point.

The results from herpes-infected HEp-2 cells gave similar results, but the system was less satisfactory because of the higher alkaline phosphatase (or 5'-nucleotidase) levels in HEp-2 cells. Thus the main

hydrolytic product of digestion of DNA by herpes- (or pseudorabies) infected HEp-2 cells was inorganic phosphate.

1.6.2.3. Pseudorabies-induced DNase.

The results obtained with extracts of pseudorabies-infected cells were identical with those with herpes virus. Two viruses of the herpes group therefore, induce exonucleases producing 5'-monophosphates during the infective process.

1.6.3. Direction of attack of herpes-induced DNase.

Most exonucleases which produce 5'-monophosphates attack the DNA substrate sequentially from the 3'-hydroxyl end of the polynucleotide chain (Lehman, 1964). Recently, however, it has been shown that the exonuclease induced by bacteriophage λ produces 5'-monophosphates by attacking from the 5'-phosphoryl end of the chain (Little, 1967). Thus, it was of interest to establish the direction of attack of the herpes-induced exonuclease.

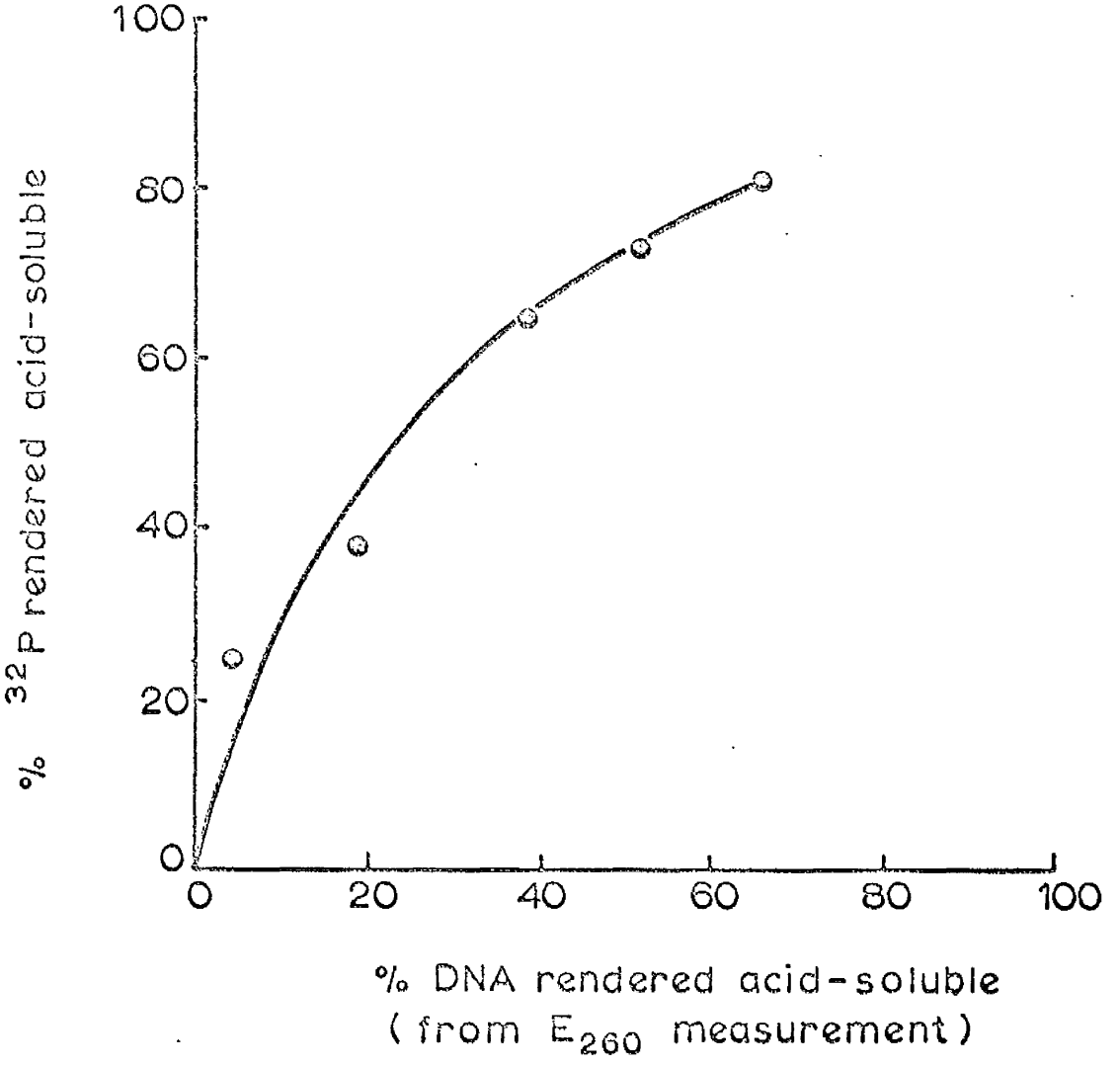
Increasing digestion of the DNA substrate with pancreatic DNase increases the rate of attack by the herpes-induced exonuclease, but this does not distinguish the two possibilities since both 3'-hydroxyl and 5'-phosphoryl groups are produced. The fact, however, that treatment of native DNA with extracts of herpes-infected cells, under conditions where the induced exonuclease

is active, increases the priming efficiency of the DNA for DNA polymerase (Figure III.15.), suggests that 3'-hydroxyl, rather than 5'-phosphoryl groups are being produced on the remaining polynucleotide.

Clear proof of the direction of attack can be obtained using the method of Lehmann and Nussbaum (1964). "Activated" DNA (Aposhian and Kornberg, 1962) was incubated at pH 7.5 and 0.2M KCl (conditions optimal for herpes-induced DNA polymerase, with minimal DNase activity) in the presence of one triphosphate (α-^{32}P-dCTP of high specific activity) and an extract of herpes-infected non-growing BHK 21 cells (low endonuclease activity). After 60 min. at 37°, 500 µg. of denatured DNA was added and the DNA precipitated in the cold with PCA. The precipitate was redissolved in 0.1N NaOH and dialysed extensively against 0.02M KCl. The ^{32}P-DNA (labelled with a dCMP residue(s) at the 3'-end of the DNA chain) was then incubated with more of the same herpes-infected cell extract at pH9 with no KCl (optimal conditions for herpes-induced DNase) and the release of acid-soluble radioactivity and UV-absorbing material was followed. The results (Figure III.24.) suggest attack from the 3'-hydroxyl end, but are not completely conclusive because of low levels of radioactivity and possible endonuclease action during DNase incubation. The weight of evidence, however, remains in favour of this

Figure III. 24.

Hydrolysis of DNA labelled with ^{32}P-dCMP at the 3'-terminus by an extract of herpes infected BHK 21 cells (non-growing)



direction of attack.

1.7. Partial Purification of Herpes-induced Enzymes.

Because the amounts of virus-infected cells available are limited by technical considerations, enzyme purification has been undertaken with the aims of : (i) specifically removing interfering enzymes and other factors, and (ii) separating the enzymes of interest, rather than simply trying to obtain a high specific activity. In this, a degree of success has been attained, particularly with the herpes-induced DNase.

1.7.1. DNases of herpes-infected cells.

1.7.1.1. Chromatography on DEAE-cellulose.

The most useful purification procedure for the herpes-induced DNase has been chromatography on DEAE-cellulose. Use of this ion-exchanger was indicated by the finding of Curtis et al., 1966) that a DNase of rat liver, preferentially hydrolysing denatured DNA, was not adsorbed by DEAE under conditions which caused most other proteins to be retained. Preliminary experiments with extracts of herpes-infected cells showed that the activity towards native DNA could be removed from the extract by treatment with DEAE-cellulose in 0.05M tris-HCl, pH8. DNase acting on denatured DNA and at a level of activity comparable to that found in non-infected cells was left in solution.

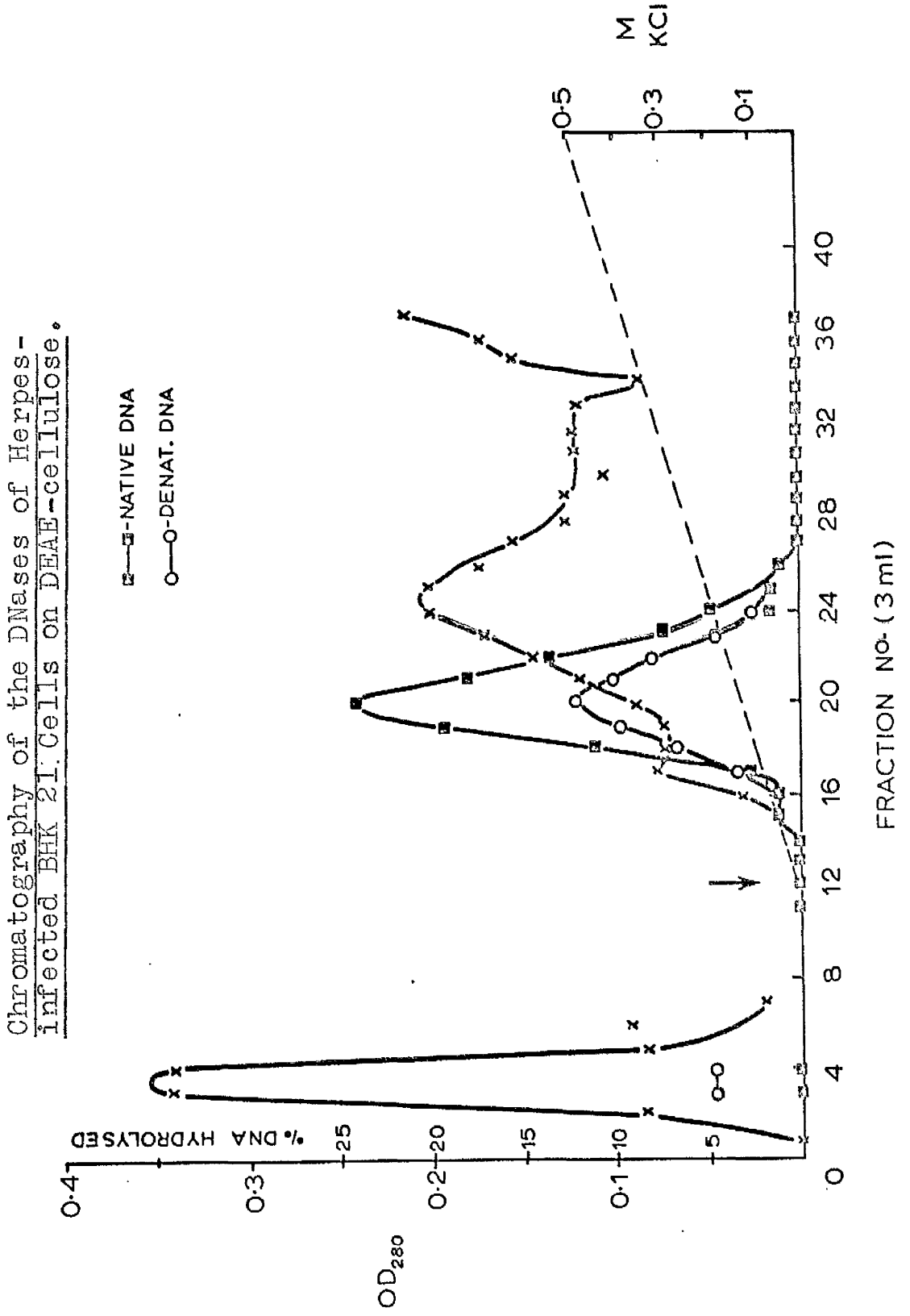
When DEAE-cellulose chromatography of extracts of herpes-infected cells is performed, with elution by a

linear KCl gradient, two distinct DNase activities are resolved (Figure III.25.): peak I, which is not retained by the DEAE and which is most active on denatured DNA, and peak II, which is adsorbed, is eluted between 0.1 and 0.15 M KCl and shows highest activity towards native DNA. The same pattern obtains with cytoplasmic and nuclear fractions of infected cells, but with extracts of non-infected cells, only Peak I is observed. Alkaline phosphatase, levels of which are low in BHK 21 cells, is eluted before Peak II. Thus, in one step, the herpes-induced exonuclease is removed from its two most serious contaminants. Further purification is complicated by the lability of the enzyme after elution from DEAE-cellulose.

1.7.1.2. Gel filtration.

Another technique which has been of some value is gel filtration on columns of Sephadex G-200. Again, a separation of host cell andherpes-induced DNases is effected, the virus-induced enzyme appearing with, or just behind, the void volume, while the host cell DNase elutes at the same position as haemoglobin, suggesting a molecular weight of 60-70,000 (cf. Curtis, et al., 1966). The herpes-induced DNase is probably genuinely of high-molecular weight (i.e. not aggregated or associated with other high molecular weight material

Figure III.25.



e.g. nucleic acids), since treatment of the cell extract with the non-ionic detergent Triton X-100 or passage through DEAE-cellulose, prior to gel filtration, does not alter its elution volume. Only a part of the alkaline phosphatase is removed from the herpes-induced DNase on Sephadex G-200, since the former is resolved into two peaks, one of which elutes with the void volume.

1.7.1.3. Other methods.

Fractional precipitation with ammonium sulphate failed to achieve any separation of components or removal of contaminants, but the method has remained useful for concentration of the enzyme and 2-3-fold purification, especially after DEAE-cellulose chromatography. Storage of the enzyme as a suspension in 0.8 saturation ammonium sulphate has proved more satisfactory than storing in solution.

1.7.2. DNA polymerases of herpes-infected cells.

Purification attempts with DNA polymerase have been less successful than those with DNase. It has not yet been possible to separate the herpes-induced polymerase from either the host cell enzyme or the virus-induced DNase.

1.7.2.1. DEAE-cellulose.

Both the herpes-induced and host cell polymerases elute at the same position as the herpes-induced DNase.

Becausecthe leading edge of the polymerase peak showed greater activity in 0.2M KCl than did the trailing one, there may be a possibility of separating the host and virus-induced enzymes with different eluting conditions.

1.7.2.2. Gel filtration.

Both polymerases eluted with, or just behind, the void volume on Sephadex G-200. Pretreatment with DEAE-cellulose did not alter the elution volume, so that the enzyme proteins probably have molecular weights in the region 200,000-500,000 (Andrewes, 1965). Recoveries from Sephadex columns have not generally been good, probably because of the long running time involved (30-50 hours) with the columns used (80-100 x 1 cm.).

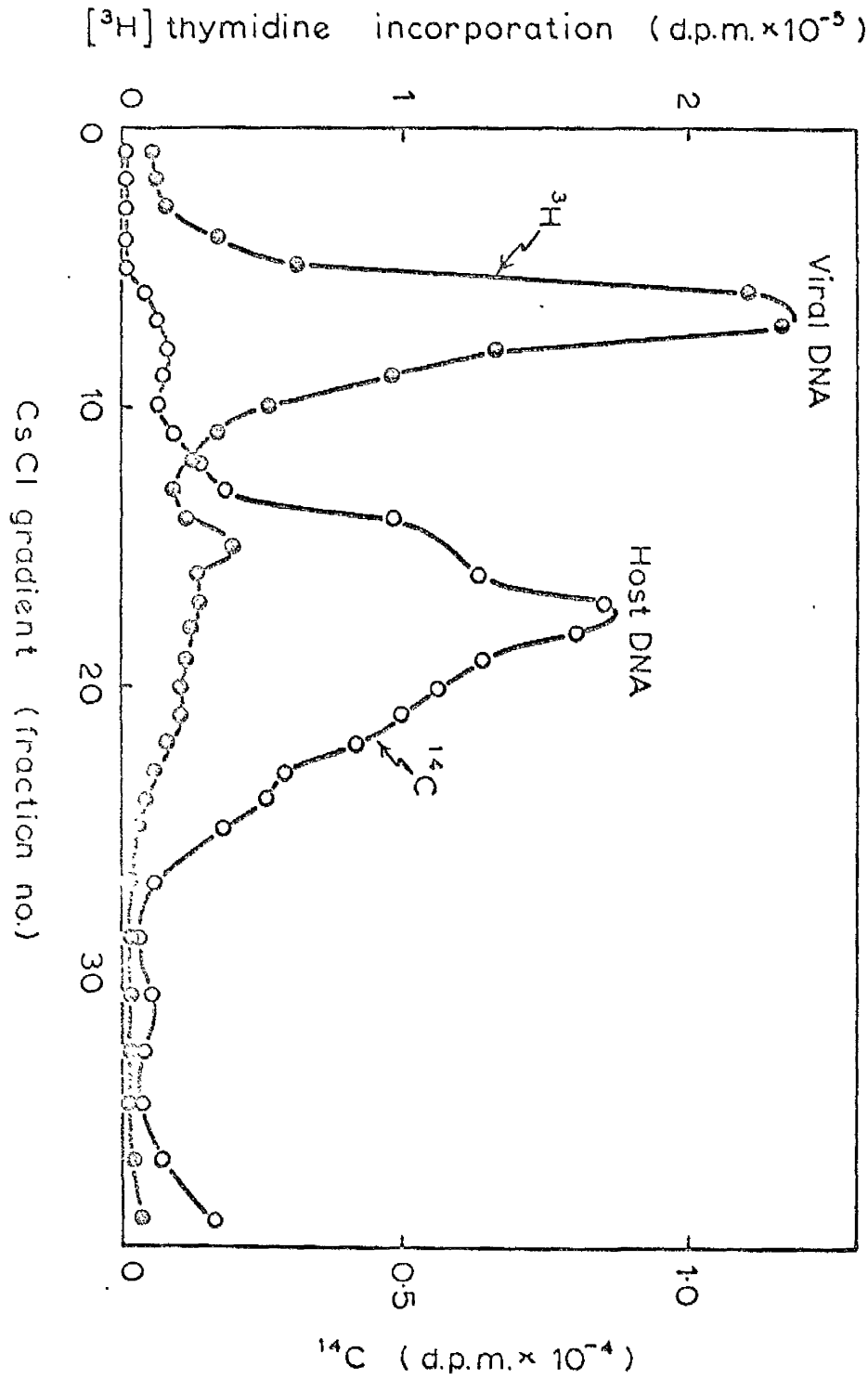
1.7.2.3. Other methods.

As for Section 1.7.1.3.

1.8. DNA Metabolism in Herpes-infected Cells.

In order to investigate the role of these two herpes-induced enzymes in the infective process, the metabolism of host cell and viral DNAs after infection with herpes virus has been studied by labelling the DNAs with radioactive thymidine and separating them by equilibrium density gradient centrifugation in an angle-head rotor (Flamm et al., 1966). Figure III.26. shows the degree of separation of viral and cellular DNA achieved by this technique.

Figure III.26.



[3H] DNA synthesis in cells 9-12 hr. after herpes infection of cells previously labelled with [14C] thymidine

BHK 21 cells were pre-labelled with ^{14}C-TdR and maintained in a low serum (0.5%) medium which reduces cell metabolism and division to a low level (Burk, 1966). When these cells were infected with herpes virus and pulsed with ^{3}H-TdR for 3 hour periods, the following results were obtained (Table III.27. and Figure III.28.):-

(i) the incorporation of ^{3}H-TdR into viral DNA is most active 6-12 h. post-infection; since only 1-5% of the added ^{3}H-TdR was actually incorporated, these figures probably reflect the rates of DNA synthesis fairly accurately;

(ii) the incorporation of ^{3}H-TdR into cellular DNA is inhibited after infection; this is in agreement with the results of Sauer et al., (1966) and Roizman and Roane (1964) with herpes- and of Kaplan and Ben-Porat (1963) with pseudorabies-infected cells;

(iii) the amount of ^{14}C-label in the cellular DNA peak does not fall in infected cells, indicating that the host DNA is not degraded extensively after infection; the fact that no broadening of the cellular DNA peak is observed, suggests that little endonucleolytic breakdown occurs, even at 24hr. post infection;

(iv) only a small proportion ($< 5\%$) of the ^{14}C-label appears in the viral DNA peak, and it did not show the sharpness of the ^{3}H peak; the quantitative significance of the ^{14}C-label

Table III.27.

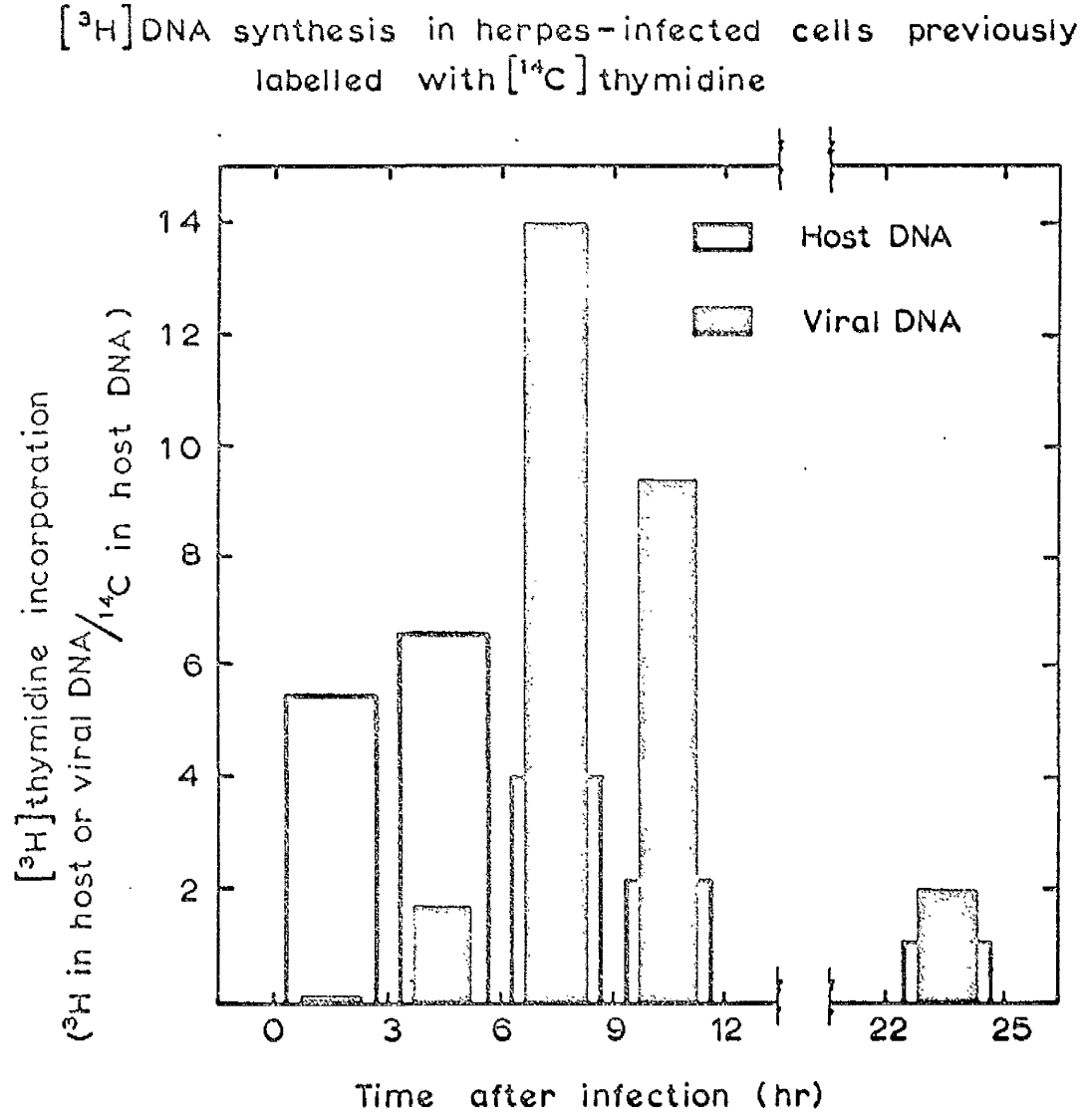
Distribution of Radioactivity between Viral and Cellular DNAs, Following Infection with Herpes Virus and 3H-thymidine labelling of BHK 21 Cells, Previously Labelled with ^{14}C-thymidine.

Duration of exposure to ^{3}H-TdR. (Hr. post-infection).	Radioactivity in DNA Species.			
	Viral DNA		Cellular DNA	
	^{3}H d.p.m. (x10-5)	^{14}C d.p.m. (x10-4)	^{3}H d.p.m. (x10-5)	^{14}C d.p.m. (x10-4)
0 - 3	-	-	4.2	7.8
3 - 6	1.3	-	5.1	7.6
6 - 9	9.1	0.12	2.7	6.4+
9 - 12	7.7	0.14	1.9	8.1
22 - 25	1.5	0.18	0.9	7.3
non-infected cells	-	-	5.9	8.1

BHK 21 cells, labelled with ^{14}C-thymidine during growth, placed in plastic Petri dishes ($5x10^{6}$ cells/dish) and subsequently maintained in low serum medium for 4 days at 37^{o}, were infected at time zero with herpes virus (MOI - 15p.f.u./cell). ^{3}H-thymidine (0.5μcurie/dish) was added at the times indicated and incubation stopped after 3hr. by removing the medium and washing with cold isotonic saline. 1mg./ml. pronase in 1% SDS was added and the dishes incubated overnight at 37^{o} before the contents (2 dishes per point) were subjected to buoyant density centrifugation (see Chapter II, Section 2.2.8.).

+ A part of the cellular DNA in this experiment was lost at the top of the gradient.

Figure III.28.



in the viral peak is uncertain since the specific activities of the DNA peaks could not be determined accurately, owing to the high UV-absorbing background caused by the pronase digestion products. The lack of sharpness in the ^{14}C-label in the viral DNA fraction suggests that this may not represent true incorporation into herpes DNA but merely background radioactivity.

Further experiments are being carried out to determine the quantitative and dynamic relationships between host and viral DNAs in infected cells, with due attention to possible effects of changes in the pool size of TdR and its phosphorylated derivatives (Newton et al., 1962).

2. NEAREST NEIGHBOUR FREQUENCY ANALYSIS OF THE DNAs OF SEVERAL MAMMALIAN VIRUSES.

2.1. A Consideration of the Technique and the Results which it Yields.

This technique, described in Chapter I, Section 2.1.2. and Chapter II, Section 2.3., measures the frequency of occurrence of each of the sixteen possible dinucleotides in the DNA being analysed (Josse et al., 1961; Swartz et al., 1962). The actual measurements made are of the radioactivity in the four 3'-mono-nucleotides obtained after hydrolysis of the enzymically synthesised product. From these figures, the

radioactivity occurring in each of the four mono-nucleotides may be expressed as a fraction of the total; when these fractions have been obtained for all sixteen dinucleotides, the base composition of the synthesised material may be computed. The product of the dinucleotide fraction and the base fraction of the nucleotide which was originally labelled with ^{32}P corresponds to the frequency of occurrence of the dinucleotide.

The final results may be checked in two ways:-

(i) the base composition of the synthesised material, obtained from the analysis, can be compared with that of the primer DNA, which has been measured by some other method, e.g. chemical analysis of buoyant density measurements (Schildkraut et al., 1962);

(ii) an anti-parallel, base-paired DNA duplex (Watson and Crick, 1953) will contain equal amounts of complementary dinucleotides (e.g. TpG and CpA) and comparison of the frequencies of these (identified by Roman numerals in Table III.29.) will give an indication of the fidelity of the analysis.

2.2. The Results Obtained with Mammalian DNA Viruses.

Table III.29. shows a typical set of results, in this case, a duplicate analysis of the DNA of pseudorabies virus. The base composition obtained (72.4% GC content)

Table III.29.

Nearest Neighbour Frequency Analysis of the DNA of Pseudorabies Virus.

Reaction number	Labelled triphosphate	Tp	Ap	Cp	Gp
1	dATP	TpA	ApA I	CpA II	GpA III
		0.013	0.017	0.044	0.055
2	dTTP	TpT I	ApT	CpT IV	GpT V
		0.023	0.018	0.054	0.052
3	dGTP	TpG II	ApG IV	CpG	GpG VI
		0.055	0.049	0.146	0.117
4	dCTP	TpC III	ApC V	CpC VI	GpC
		0.064	0.046	0.115	0.132
	Sums:	0.155	0.130	0.359	0.356

	X=A		X=T		X=G		X=C		
	c./m.	fr.	c./m.	fr.	c./m.	fr.	c./m.	fr.	base fraction
TpX	4496 6355	0.102	653 709	0.155	10324 10685	0.151	9686 9386	0.178	t = 0.147
ApX	5405 8828	0.134	537 567	0.125	8752 9681	0.132	7113 6541	0.128	a = 0.130
CpX	13712 22500	0.342	1608 1613	0.366	25688 29612	0.397	18531 16030	0.323	c = 0.357
GpX	17355 27346	0.422	1533 1590	0.355	21092 23511	0.320	20688 18976	0.371	g = 0.367

c./m. = counts per min. in isolated mononucleotide.
fr. = fraction of total counts in each mononucleotide.

compares well with a figure of 74% from buoyant density measurement, and the complementary dinucleotides are :

TpT/ApA-0.023/0.017; TpG/CpA-0.055/0.044; TpC/GpA-0.064/0.055; ApG/CpT-0.049/0.054; ApC/GpT-0.046/0.051; CpC/GpG-0.115/0.117. The ratios G/C and A/T are 1.03 and 0.88 respectively.

Table III.30. shows the results obtained from the analyses of nine viral and one cellular DNAs; unlike Table III.29., the frequencies are expressed as parts per thousand. When compared with the results of Josse et al., (1961) and Swartz et al., (1962) the complementary dinucleotides are somewhat less well matched. In addition, the base compositions calculated from the analyses are lower than those obtained from buoyant density determinations by 0-3%. No reason has been found for this discrepancy, but Swartz et al., (1962) state that Esch. coli endonuclease I can alter the nearest neighbour pattern; (in order to eliminate this possibility, Esch. coli sRNA, a potent inhibitor of endonuclease I, has been used in subsequent analyses). Nevertheless, the analyses are felt to be sufficiently precise to allow adequate comparison of the patterns of viral and cellular DNAs.

2.3. Treatment of the Results.

In order to compare the nearest neighbour patterns

Table III.30.

Nearest Neighbour Frequency Analysis of the DNAs of Nine Animal Viruses.

DNA:	Human spleen	Herpes	Pseudorabies	Polyoma	Shope
ApT	81	34	18	67	70
TpA	67	34	13	60	55
ApA TpT	97 97	35 36	17 23	74 85	73 89
GpT ApC	49 54	59 58	52 46	61 47	63 50
TpG CpA	74 74	62 65	55 44	78 71	86 75
GpA TpC	61 57	55 49	55 64	60 62	58 63
ApG CpT	70 71	50 45	49 54	77 72	67 73
GpG CpC	50 47	110 100	117 115	65 52	55 46
GpC	43	108	132	52	55
CpG	10	109	146	18	24
(G + C)% from freq. analysis	40.5%	65%	72.4%	45%	45%
(G + C)% from buoyant density determ.	—	68%	74%	48%	47%

BHK21/C13	sv40	Human papilloma	Adenovirus type 2	Equine rhino-pneumonitis	Vaccinia
82	74	79	48	48	124
73	68	72	44	50	111
98 108	105 116	91 96	64 68	58 57	106 112
60 52	58 48	57 54	59 56	63 58	53 49
79 68	77 72	73 69	71 66	67 58	57 52
62 57	54 50	57 55	55 55	60 57	65 61
69 68	73 62	65 64	62 64	62 62	55 53
44 40	49 44	50 45	72 72	79 73	28 26
35	44	48	82	77	22
8	6	24	62	72	28
38·2	39·0	41·4	53·2	54·4	32·5
42	41	41	57	55	35

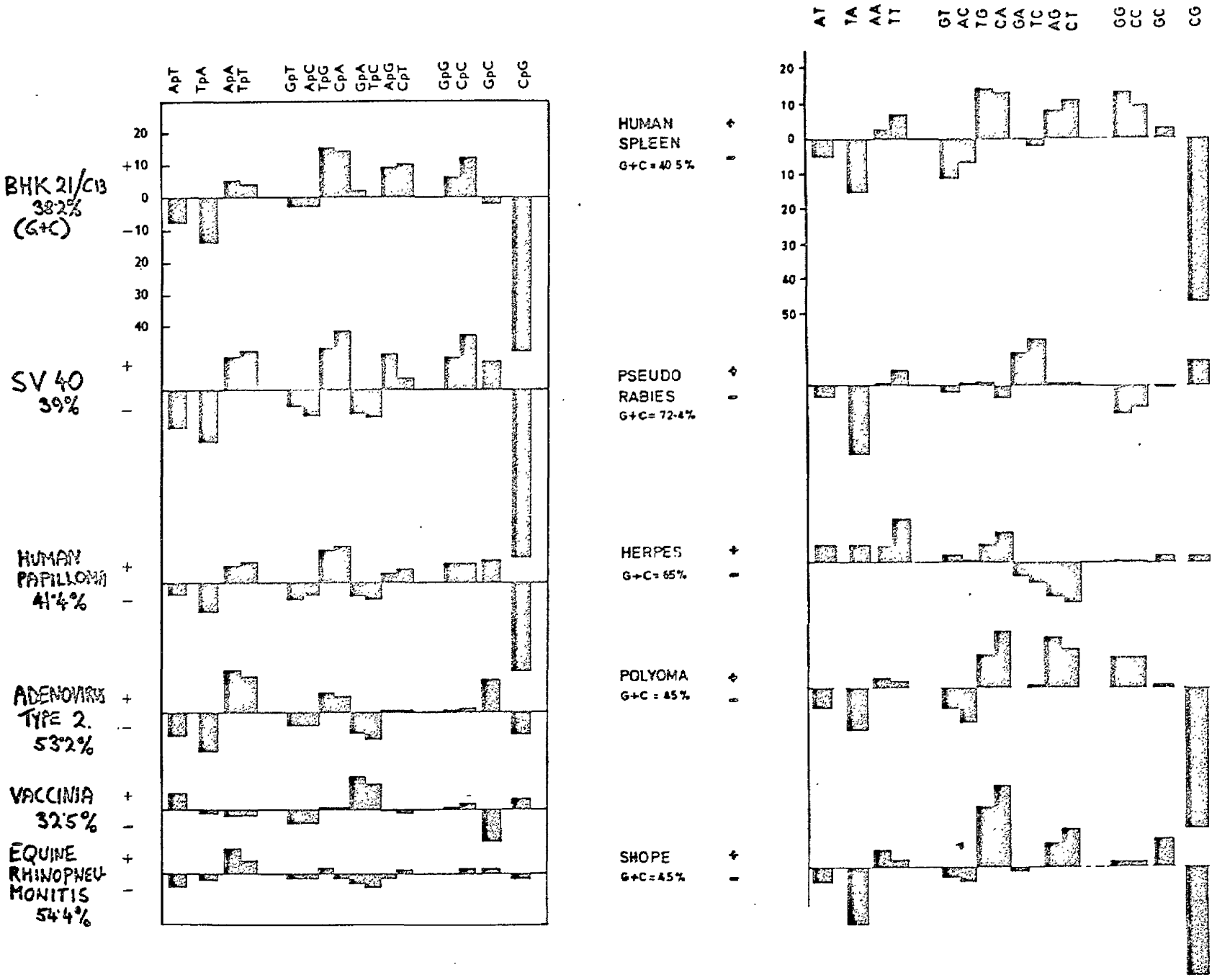
Experimentally obtained values for the dinucleotide frequencies of the DNAs of nine animal viruses. Each DNA was analysed at least twice; the average values are given; the dinucleotide frequencies are expressed as parts per thousand. The analyses of BHK 21 cell and human spleen DNAs (the latter from the results of Swartz et al., (1962)) are included for comparison.

of DNAs of widely differing base compositions, the observed frequencies may be "normalised" to the values which they would have if the DNA base composition were 50% GC content. This is achieved by dividing the observed value z, by the expected frequency assuming randomness (i.e. x.y where x and y are the base ratios of the components of the dinucleotide) and multiplying the quotient by 0.0625 (the expected frequency of every dinucleotide in a DNA of 50% GC content, assuming randomness). Thus, the normalised frequency of a dinucleotide XpY is 0.0625z/x.y. The results obtained, normalised in this way, can be found in Table 2 of Subak-Sharpe et al.,(1966a) and Table 2 of Morrison et al., (1967); Figure III.31. expressed the same results digramatically as the deviation from random expectation of each dinucleotide.

The outstanding feature of the nearest neighbour pattern of mammalian, indeed all vertebrate, DNAs is the rarity of occurrence of the dinucleotide CpG. From Figure III.31., it can be seen that the DNAs of mammalian viruses fall into two groups: (i) the papovaviruses (SV40, polyoma and the Shope and human papillomas) which closely resemble the pattern of the host cell, particularly in the scarcity of CpG; and (ii) comprising three herpes-viruses, an adenovirus and a poxvirus, which

Figure III.31.

Results of Table III.30., Normalised to a GC Content of 50% and Expressed Diagramatically as Deviation from Random Expectation.


ApT TpA ApA TpT GpT ApC TpG CpA GpA TpC ApG CpT GpG CpC GpC CpG
AT TA AA TT GT AC TG CA GA TC AG CT GG CC GC CG
BHK 21/C13
38·2%
(G+C)
SV 40
39%
HUMAN
PAPILLOMA
41·4%
ADENOVIRUS
TYPE 2.
53·2%
VACCINIA
32·5%
EQUINE
RHINOPNEU-
MONITIS
54·4%
HUMAN
SPLEEN
G+C = 40·5%
PSEUDO
RABIES
G+C = 72·4%
HERPES
G+C = 65%
POLYOMA
G+C = 45%
SHOPE
G+C = 45%

deviate much less from random expectation.

To facilitate comparison between the frequency of occurrence of dinucleotides in the DNA of the host cell and that of the invading virus, a "shortage histogram" has been constructed to show which dinucleotides occur less frequently in the host DNA than they do in the viral DNA and to indicate the magnitude of the deficiency.

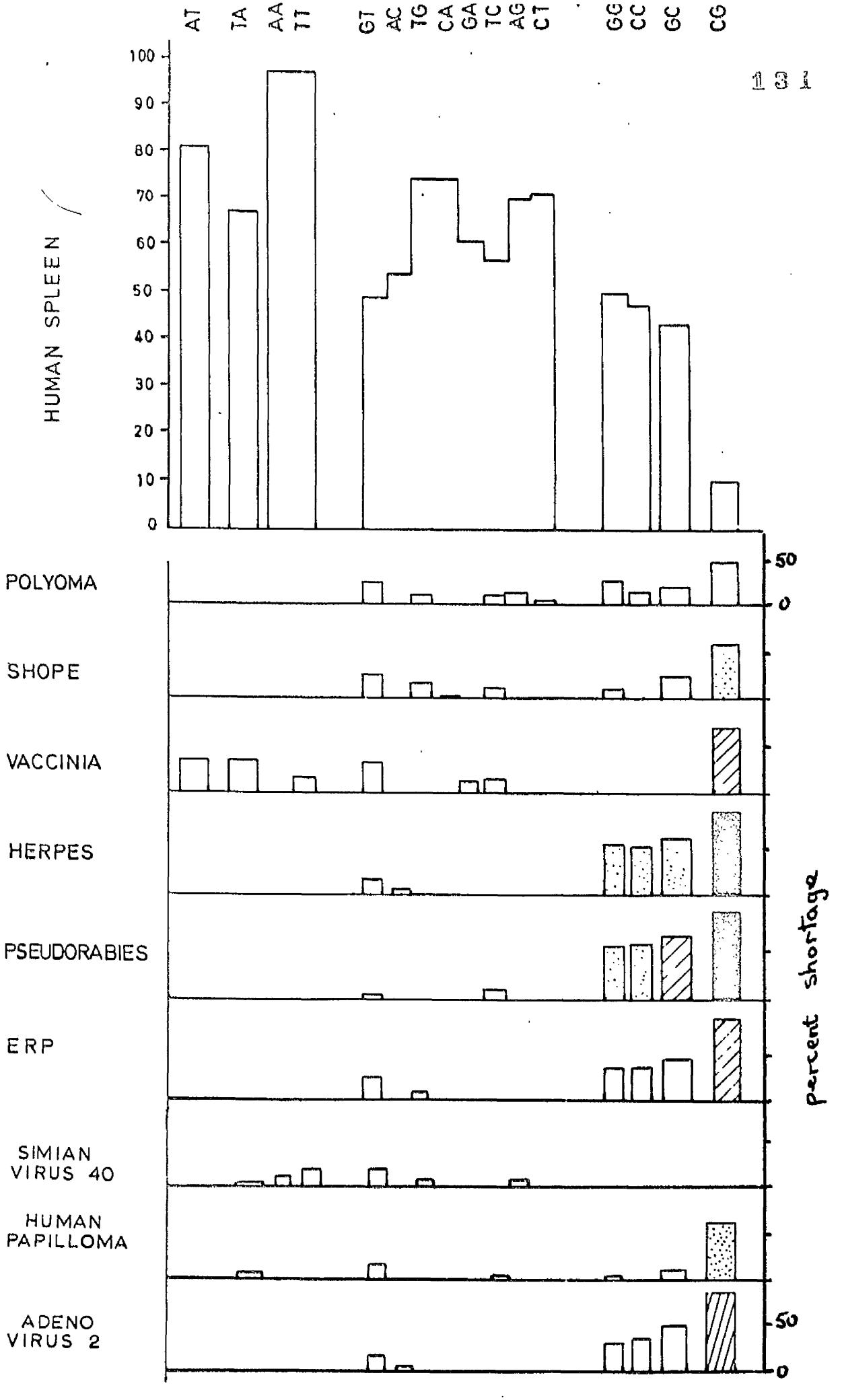
The percent shortage of each dinucleotide is expressed as follows:-

$$\left(100 - \frac{\text{dinucleotides per 1000 in host DNA}}{\text{dinucleotides per 1000 in viral DNA}} \right) \times 100\%$$

Figure III.32. shows the shortage histograms for the viruses which have been analysed. The results described in this Section will be discussed and their significance evaluated in Chapter IV, Section 2.

Figure III.32.

Histograms of the Relative Shortage of Dinucleotides in the DNA of Human Spleen Compared with their Frequency of Occurrence in the DNAs of Animal Viruses.


AT
TA
AA
TT
GT
AC
TG
CA
GA
TC
AG
CT
GG
CC
GC
CG
HUMAN SPLEEN
100
90
80
70
60
50
40
30
20
10
0
POLYOMA
SHOPE
VACCINIA
HERPES
PSEUDORABIES
ERP
SIMIAN VIRUS 40
HUMAN PAPILLOMA
ADENO VIRUS 2
50
0
50
0
percent shortage

CHAPTER IV

CHAPTER IV - DISCUSSION.

1. ENZYME INDUCTION BY HERPES VIRUS.

In this section, possible mechanisms for the induction of increased levels of DNase and DNA polymerase will be described, the salient features of the induction of these enzymes will be summarised and the hypothetical mechanisms and experimental observations correlated as far as possible.

1.1. Possible Mechanisms Causing Increase of Enzyme levels after Infection with Herpes Virus.

I. Activation of existing enzyme molecules.

a. "External factors".

These are factors which do not directly affect the structure of existing enzyme molecules but which interact with the enzyme so as to alter its activity. The factors could be:-

(i) removal of an enzyme inhibitor, or

(ii) appearance of an enzyme activator;

both of these would lead to an apparent increase in enzyme activity following virus infection.

b. Alteration of enzyme structure.

Existing enzyme molecules could have their activity and properties changed or existing, inactive proteins could acquire enzymic activity by:-

(i) alteration of the tertiary or quaternary

structure of a protein, or

(ii) addition of avirus-specified oligopeptide to an existing protein molecule.

II. Synthesis of new enzyme molecules.

This could occur in at least three ways:-

a. synthesis of existing-type molecules.

b. synthesis of a different, host cell enzyme, not normally made (e.g. by derepression of a host gene).

c. synthesis of a different enzyme, specified by a viral gene.

1.2. Features of Enzyme Induction by Herpes Virus.

(i) Levels of DNase and DNA polymerase rise after infection of animal cells with herpes virus at a time when they can participate in the processes of virus multiplication.

(ii) Synthesis of mRNA and protein is necessary for enzyme induction.

(iii) Addition of extracts of control cells to extracts of infected cells does not cause inhibition of the induced enzymes.

(iv) Mixing of control and infected cell extracts may give a super-additive response of enzyme activity.

(v) Both induced enzymes differ from the corresponding activities in control cells in several enzymic properties.

(vi) The induced DNase is physically separable from the DNase of control cells.

(vii) Antiserum prepared against extracts of herpes-infected RK cells neutralises the enzymes induced by herpes virus in BHK 21 and HEp-2 cells, but not the host cell enzymes or the corresponding enzymes induced by pseudorabies virus; the serum inhibitor is not present in pre-immune serum and behaves like γ-globulin on DEAE-cellulose.

1.3. Correlation of Observed Features and Possible Mechanisms of Enzyme Induction by Herpes Virus.

Correlation of Observed Features and Possible Mechanisms of Enzyme Induction by Herpes Virus.

Pc[illegible] importance since, if an enzyme was induced at a time when it could no longer function in virus multiplication, its appearance would probably be due to some indirect effect, such as viral cytotoxicity, rather than virus-directed enzyme induction.

A requirement for protein synthesis would apparently rule out all the possibilities of group I, except I.b.ii., but synthesis of new proteins might well be necessary for the other mechanisms, e.g. synthesis of an enzyme activator.

Feature (iii) means that there can be no excess of an inhibitor(s) of the induced enzymes present in non-infected cells; this makes possibility I.a.i. unlikely.

Feature (iv) appears to favour possibility I.a.ii.,

but the super-summation can be explained in terms of the known properties of the enzymes of control and infected cells. In the case of the DNases, adding control extracts to those of infected cells will increase the amount of endonuclease; this will convert more of the high-molecular weight DNA substrate into oligonucleotides which are better substrates for the herpes-induced exonuclease. With the DNA polymerases, the ability of the herpes-induced exonuclease to increase the priming efficiency of DNA could make the host cell enzyme more active than it is in extracts of non-infected cells. Since, additionally, it is difficult to explain the different properties of the induced enzymes in terms of an activator of existing enzymes, possibility I.a.ii. also seems unlikely.

Possibility I.b.i. can be virtually ruled out in the case of the induced DNase because of the separation which can be achieved on DEAE-cellulose, but it is still possible that a protein which is inactive in non-infected cells might be suitably altered. The strongest evidence against this likelihood is the immunological data (see below), but the interpretation cannot be unequivocal, since alteration of protein structure might alter the immunological specificity of a host enzyme.

The alteration of existing protein molecules by addition of a virus-specified oligopeptide is even more

difficult to eliminate, because such an addition could explain the immunological evidence. In the case of the induced DNase, of course, the physical separation makes this explanation less likely. In any event, the dividing line between possibilities I.b.ii. and III.c. is bound to be diffuse.

Of the mechanisms involving synthesis of new enzyme molecules, II.a. is clearly eliminated by the different properties of the induced enzymes compared with those of the host cell. II.b. is made rather implausible by the immunological evidence, since in order for this mechanism to operate, herpes virus must induce or derepress immunologically identical enzymes in three different cell lines, while another virus of the herpes group, pseudorabies, must induce or derepress immunologically different enzymes, but with similar enzymic properties, in the same three cell lines.

Thus, the only possibility which is wholly compatible with all the available evidence is II.c. It can be seen, however, that much of the argument against the other mechanisms is indirect, and that I.b.i. and, especially I.b.ii., cannot be completely eliminated. Further evidence of the virus-specified nature of the induced enzymes may be obtained by experiments involving selective inactivation of host and viral genomes and with

conditional lethal mutants of herpes virus, but unequivocal proof can only come from the use of viral DNA in conjunction with *in vitro* RNA and protein synthetic systems; recent synthesis of bacteriophage T4 lysozyme *in vitro* from *in vivo* T4 mRNA (Salser *et al.*, 1967) suggests that there is cause for optimism about the practicability of this approach.

1.4. Speculation on the Roles of the Herpes-induced Enzymes.

1.4.1. Herpes-induced exonuclease.

Induction of DNases has been observed with many DNA viruses (see Chapter I, Section 3.3.), but only in the case of bacteriophage T2 has it been possible to correlate the appearance of the induced DNase with a known feature of the infective process (Lehman, 1967). In this case, it seems likely that the exonuclease (Oleson and Koerner, 1964) and/or the endonuclease (Bose and Nossal, 1964) observed to increase at the same time as the "early" enzymes, are responsible for the breakdown of the host cell DNA (Hershey *et al.*, 1954) which occurs progressively after infection. The discovery (Wiberg, 1966) that conditional lethal mutants of bacteriophage T4 in genes 46 and 47 are unable to degrade the bacterial genome to acid-soluble fragments should enable this hypothesis to be tested. In the case of bacteriophage T5, however, the induced DNase (Paul and Lehman, 1966) appears much too

late to be responsible for the very rapid breakdown of bacterial DNA which occurs with this virus (Crawford, 1959). Virus-induced DNases may also be involved in such phenomena as the breakdown of the DNA of superinfecting bacteriophage (French et al., 1951).

In the case of the herpes-induced DNase, it is not yet possible to establish the role of the enzyme in the infective process, but various possibilities can be examined in the light of existing evidence:-

(i) Degradation of host DNA.

As described in Chapter III, Section 1.8., no extensive breakdown of host cell DNA occurs after infection with herpes, or for that matter, any animal virus so far examined. This fact would seem to exclude a purely degradative role for the induced DNase, but, because of the vast difference in size between the DNAs of an animal cell and herpes virus ($2x10^{12}$ and $7x10^{7}$ daltons), degradation of only 1% of the host DNA would provide enough material for the synthesis of about 300 viral DNA molecules. From the results of Russell et al., (1964), each infected cell produces about 50 infective virions, 10^3 particles and $5x10^3$ equivalents of viral DNA. This last figure would require the degradation of 15% of the host DNA, if the latter were the sole source of viral DNA precursors; if, however, less than half the viral DNA

was derived from this source (as is the case with the T-even bacteriophages), it would be difficult to exclude the possibility that such an amount of breakdown had occurred in the experiment described in Chapter III. Very little label, however, derived from the host cell DNA, can be detected in the viral DNA and although this experiment cannot be completely quantitated, the recent results of Munk and Klamerth (1967) show that no host DNA material appears in mature herpes virus or in viral DNA.

There remains, however, the effect of herpes virus which manifests itself as a margination of the chromatin of the infected cell. It is not inconceivable that a DNase might play a part in this process, but an endonuclease would seem a more likely candidate for the role. As was discussed in Chapter III, it has not yet been possible to demonstrate or eliminate the possibility that herpes virus induces an endonuclease.

(ii) Degradation of viral DNA.

At first sight, this seems a highly improbable suggestion, but is worth considering because of two facts. Firstly, there is a considerable excess of viral DNA produced (see above). This may be an obligatory feature of animal DNA virus multiplication, as it is also observed with vaccinia, pseudorabies and adenoviruses, and may not be as wasteful as it seems. Hoggan et al.,(1961) observed inter-cell "bridges" in herpes-infected cells and so it is

possible that excess viral DNA, hydrolysed by a virus-induced DNase, may pass into the next cell with the infecting virus and serve as a source of preformed nucleotide for further DNA synthesis. This may be of importance to the virus because of the low rate of metabolism in the cells which it normally infects in the whole animal.

Secondly, the recent observation of Becker et al., (1967) that the DNA of herpes virus is circular may mean that much of the excess DNA which is produced is non-infective because of lack of circularity. It might, therefore, be of advantage to the virus to eliminate this material which might interfere with the assembly of mature, infectious virions. An exonuclease would be necessary for such a task.

(iii) "Parasynthetic" roles.

The idea that DNases may have more than a purely degradative role has grown in recent years and has been reviewed by Lehman (1967). The frequent physical association of exonucleases with DNA polymerases and their appearance at times of DNA synthesis have provided suggestive evidence for this hypothesis in bacterial and bacteriophage systems. Herpes-infected cells also show both these features, since the herpes-induced exonuclease co-chromatographs with DNA polymerase on DEAE-cellulose and is induced slightly later than that enzyme, but about

the time of DNA synthesis.

Other "parasynthetic" roles might be:-

(a) the "trimming" of the ends of newly-synthesised DNA molecules, although, clearly, some form of control would be necessary to prevent excessive hydrolysis;

(b) the maturation of replicative forms of newly-synthesised DNA, such as that described by Frankel (1966) for bacteriophage T4;

the precise nature of these parasynthetic roles must necessarily remain speculative, but, of course, much remains to be learned about the mechanism of DNA synthesis itself.

1.4.2. Herpes-induced DNA polymerase.

The role of this enzyme is self-evident, but the necessity for its production during virus infection is worthy of some discussion.

In the case of T4 bacteriophage, the occurrence of conditional lethal mutants in gene 43 which do not induce the synthesis of DNA polymerase or make any viral DNA, shows that the Esch.coli DNA polymerase is not capable of replicating T4 DNA in vivo. Since the bacterial DNA polymerase is firmly bound to DNA, the reason may be purely one of physical separation, but this is unlikely to be the case in the mammalian cell, where the DNA polymerase molecule, if not actually the whole DNA-synthesising complex, seems not to be chromosomally bound.

This suggests that the DNA polymerase of the host cell is incapable of replicating the herpes DNA molecule. One obvious possibility is that there is a site on the herpes DNA molecule which is recognised by the virus-induced, but not the host cell DNA polymerase. No highly specific interaction between herpes DNA and the induced polymerase has yet been observed, although, clearly, intact viral DNA should be used for definitive enzymological and binding studies. The precedent for such a recognition site has been clearly established in the case of the small RNA bacteriophages Q_β and MS2 by the work of Spiegelman and his colleagues (Haruna and Spiegelman, 1965).

Another possibility is that the inhibition of host cell DNA synthesis which occurs after infection with herpes virus may be due, at least in part, to an inhibitory effect on the DNA polymerase of the host cell. The herpes-induced polymerase cannot be susceptible to this putative inhibition. The small, but consistently observed decrease in DNA polymerase activity 2h. post-infection (see Figure III.2.) may be due to the production of such an inhibitor. Newton (1967) has recently presented evidence that a non-DNA viral component (presumably a structural protein) is responsible for the inhibition of host cell DNA synthesis, and, if this is so,

a differential effect of viral protein on the DNA polymerases of control and herpes-infected cells should be demonstrable.

In conclusion, the results presented here show that two enzymes, DNase and DNA polymerase, are synthesised following infection of mammalian cells with herpes virus. These enzymes differ in their properties from the corresponding activities present in non-infected cells and are probably specified by the viral genome. The evidence does not permit a precise definition of the roles of the induced enzymes in the infective process.

2. NEAREST NEIGHBOUR PATTERNS OF MAMMALIAN DNA VIRUSES.

2.1. Possible Translation Difficulties Experienced by Viruses in the Infected Cell.

2.1.1. Rationale.

It has been generally assumed that virus-specified protein synthesis in the infected cell is performed using the existing translation apparatus (ribosomes, activating enzymes, transfer RNAs, etc.) of the host cell. It will be shown that, in the case of herpes and certain other animal DNA viruses, this is probably not so, at least as far as the population of tRNA molecules is concerned.

As described in the preceding Chapters, the technique of nearest neighbour frequency analysis gives information on one aspect of the primary structure of DNA molecules, namely the occurrence of the sixteen possible dinucleotides. It thus provides a means of comparing the DNAs of different organisms. The results of Chapter III, Section 2. show that, of the nine viral DNAs analysed, four (all papovaviruses) have patterns which closely resemble that of the mammalian host cell, especially in the rarity of occurrence of the dinucleotide CpG, while the dinucleotide frequencies of the other five (three viruses of the herpes group, one adenovirus and one poxvirus) deviate much less from random, and, in particular, show no scarcity of CpG.

The rationale which will be used to examine possible difficulties of translation is as follows:-

If the assumption is made that the greater part of the DNA of an organism specifies polypeptides (or, if this is not so, that the polypeptide-specifying part has the same nearest neighbour pattern as the whole DNA), then the frequency of dinucleotides in the DNA will reflect their frequency of occurrence in the codons used for protein synthesis. By this token, the rarity of the CpG dinucleotide in the DNA of mammalian cells implies that codons containing this dinucleotide must be infrequently used for translation in these cells. Indeed,

if one allows for the intercodon position, (XXX)(XXC)(GXX)(XXX), CpG may never occur within a codon in mammalian cells. An examination of the codon assignments reveals that the amino acids affected by such a restriction are ⊖ arginine (CGA, CGC, CGG and CGU), serine (UCG), proline (CCG), threonine (ACG) and alanine (GCG).

If a second assumption is now made that, in any given cell, the population of tRNA species is optimally adapted to the translation requirements of the genome of that cell, then, clearly, a foreign genome, such as that of an invading virus, will find the existing tRNA population optimally adapted to its own needs, only if these are closely similar to the needs of host cell translation. In other words, if the virus uses the same codons as the host and with approximately the same frequency, the translation requirements of the two will coincide.

2.1.2. Consideration of specific virus-cell interactions.

One must now consider which of the viruses studied are likely to encounter shortages of tRNA species with CpG-containing codons during the translation of virus-specific messenger RNA in the infected cell. An examination of Figure III.32. (see Chapter III, Section 2.3.) reveals that the four papovaviruses are unlikely to experience any major deficiency of such tRNA species;

on the other hand, vaccinia and adenovirus 2 and more especially the three viruses of the herpes group will encounter serious shortages of tRNA molecules required for the translation of CpG-containing codons.

Thus, a rationale has been developed which leads to the conclusion that the translation mechanisms of the mammalian cell are not wholly adequate for the protein synthetic requirements of certain DNA viruses. If this is the case, presumably the virus must alter the translation apparatus to its own requirements. Is there any evidence for such alteration?

The work of Subak-Sharpe et al.,(1966b) strongly suggests that one or more species of arginyl-tRNA, different from that found in non-infected cells, are synthesised after infection with herpes virus. Since four of the six codons for arginine contain the dinucleotide CpG, and so are probably not used in mammalian cells, this evidence supports the hypothesis that herpes virus induces the synthesis of one or more tRNA species necessary for its own translation requirements.

From the foregoing paragraphs, it can be seen that the technique of nearest neighbour frequency analysis yields results which are relevant both to the information content of viral DNA molecules and to the mechanisms of viral replication.

SUMMARY

SUMMARY.

The information content of the DNA of herpes virus, and its expression in the infected cell, have been studied by two main approaches.

The first approach has been the study of two enzymes, alkaline deoxyribonuclease (DNase) and DNA polymerase, which are found in markedly increased levels in herpes-infected cells. It has been shown that the induction of these enzymes after infection requires synthesis of mRNA and protein, and that the induced enzymes differ from those found in the non-infected cell in several of their enzymic properties.

Partial purification of the induced enzymes has been undertaken and, in the case of the herpes-induced DNase, separation from the corresponding host cell enzyme activity has been achieved. The induced DNase is a DNA-specific exonuclease attacking both native and denatured DNA, and releasing 5'-monophosphates, probably from the 3'-hydroxyl terminus. It has been shown that pseudorabies virus induces a similar enzyme. The alkaline DNase of the host cell is an endonuclease, preferentially attacking denatured DNA.

Immunological methods have been used to determine the specificities of the induced enzymes. Serum from rabbits

immunised against high-speed supernatant fractions of herpes-infected rabbit kidney cells inhibits the DNase and DNA polymerase induced by herpes virus in BHK 21 and HEp-2 cells, but not the corresponding enzymes of non-infected or pseudorabies-infected cells.

The significance of these results and of experiments on the synthesis of cellular and viral DNA is discussed in terms of two problems:-

(i) Does the information for the amino acid sequence of the induced enzymes reside in the nucleotide sequence of the genome of the host cell or of the invading virus?

(ii) What roles do the induced enzymes play in the infective process?

The results obtained suggest that the enzymes are virus-specified. The possible roles of the induced enzymes are discussed in the light of this and other evidence.

The second approach has been to examine possible difficulties in translation encountered by the virus when using the protein-synthetic mechanisms of the host cell.

One of the few methods of obtaining information on the primary structure of DNA molecules is the technique of nearest neighbour frequency analysis. Analyses of nine animal DNA viruses have been performed

and the patterns obtained compared with that of the mammalian host. The most outstanding feature of the nearest neighbour pattern of the DNA of mammalian cells is the marked rarity of the dinucleotide CpG. The viruses examined were broadly divisible into two groups; one, comprising members of the papovavirus group, which closely resembled the host cell pattern, especially in the rarity of CpG, and the other - comprising three viruses of the herpes group and one member each of the adenovirus and poxvirus groups - had patterns which deviated much less from random expectation.

If the assumption is made that the greater part of DNA specifies polypeptides, then the nearest neighbour pattern of the DNA will reflect the frequency of occurrence of the various dinucleotides in the codons being used for protein synthesis. Thus, in the mammalian cell, CpG-containing codons must be infrequently used, if at all, while, during virus-specific protein synthesis in cells infected with, for example, herpes virus, such codons will frequently require translation. If one now makes a second assumption, namely that, in a given cell, the transfer RNA population is optimally adapted to the translation of the genome of that cell, then entry into that cell of a virus such as herpes whose DNA contains certain

codons rarely, if ever, found in the non-infected cell will present the virus with a situation in which the existing tRNA population is not optimally adapted to the translation requirements of the viral genome.

Granted the above rationale, the synthesis of virus-specified tRNAs in the infected cell is a reasonable proposition. Evidence for the occurrence of a new arginyl-tRNA in herpes-infected cells has been obtained by other workers. Four of the six codons for the arginine are the CpG-containing triplets CGA, CGC, CGG and CGU.

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