ENZYMES OF DNA SYNTHESIS AND DEGRADATION IN CELLS INFECTED WITH HERPES SIMPLEX VIRUS.

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LIST OF ABBREVIATIONS.

Abbreviations and symbols used without definition in this thesis are, in general, those recommended by the Editorial Board of the Biochemical Journal (Biochem. J.(1971) 121, 1.).

In the case of nucleic acids, polynucleotides and their constituents, however, the symbols and abbreviations are as set out by the IUPAC-IUB Commission on Biochemical Nomenclature (Eur. J. Biochem.(1970) 15, 203).

Other contractions are as follows:-

ApC, CpC, GpC, TpC	Dinucleotide sequences (from DNA) in which deoxycytidine residues are linked in 5'-3'-phosphodiester linkage via the nucleotide's 5'-C to deoxyadenosine, deoxycytidine, deoxyguanosine and thymidine respectively.
BHK 21 (C13) cells	Clone 13 of a strain of cultured cells derived from baby hamster kidney(MacPherson and Stoker,1962).
DEAE-starter buffer	0.05M-tris-HCl buffer(pH 8.0)-1mM-EDTA-10mM-2-mercaptoethanol.
DNA polymerase	DNA nucleotidyltransferase (E.C.2.7.7.7).
DNA's	Deoxyribonucleic acids.
*DNases	Deoxyribonucleases
HSV	Herpes simplex virus.
dNTP's	Deoxyribonucleoside 5'-triphosphates.
PBS(A)	Phosphate buffered saline solution (A) (Dulbecco and Vogt,1954).
PMSF	Phenylmethanesulphonyl fluoride.
RK cells	Rabbit kidney cells.

SAM	S-adenosylmethionine.
SCTM buffer	0.25M-sucrose-1.5mM-$CaCl_2$-10mM-tris-HCl(pH 8.0)-10mM-2-mercapto-ethanol.
SSC	Standard saline citrate.
TEM buffer	0.10M-tris-HCl buffer(pH 8.0)-1mM-EDTA-10mM-2-mercaptoethanol.
TEMED	NNN'N'-tetramethylethylenediamine.

* E.C. reference numbers not used throughout as they do not adequately describe the diversity of these and other enzymes of nucleic acid metabolism.

Footnote- DNA primer.

DNA polymerase activity in the case of the HSV-induced DNA polymerase functions in a manner which suggests that added DNA is a template for the enzyme. The term template is, however, very specific and implies that polymerisation is proceeding by specific base-pairing with formation of new DNA strands complementary to the added DNA; it also implies the absence of non-specific addition to the termini of DNA chains. For this reason the more general term DNA primer has been adopted in this context to mean simply an initiator of polymerisation without defining exactly the type of polymerisation (non-specific and/or template directed) which may be occurring.

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by

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The main aim of the project was to purify the virus-induced DNA polymerase from herpes simplex virus-infected baby hamster kidney cells grown in culture. If this could not be carried to homogeneity, then the aim would be at least to separate host DNA polymerase and DNase from the enzyme in order to clarify the relationship of the induced DNA polymerase and DNase and eliminate the interfering effects of the other enzymes.

In the course of the present purification studies involving column chromatography on DEAE-cellulose, hydroxylapatite, Sephadex G-150, and phosphocellulose, and fractionations using sucrose gradient sedimentation and polyacrylamide gel electrophoresis, evidence was obtained that at least part of the virus-induced DNase activity could be separated from DNA polymerase especially on hydroxylapatite columns. The question arose whether there were one

or two new virus-induced DNA exonuclease(s) because, while one of the peaks of DNase from hydroxylapatite was polymerase-free, the other was closely associated with polymerase and remained so throughout several purification steps.

The two virus-induced exonuclease peaks were compared using several criteria including: sedimentation coefficients, substrate specificities, heat stability and rechromatography. The results showed that the enzymes were in the main similar by these criteria although differences detected by the last two criteria may be significant. On the whole these and other characterisation studies coupled with consideration of earlier work suggested that there was probably only one virus-induced DNA exonuclease, and that this was distinct from the DNA polymerase purified protein. Nevertheless, there is also substantial evidence in favour of there being two distinct virus-induced exonucleases, one polymerase-free, the other polymerase-associated. A further possibility that the polymerase-free exonuclease is a breakdown product of a single virus-induced DNA polymerase-DNA exonuclease protein has not been eliminated completely by studies carried out with a protease inhibitor. The final answer rests upon further purification studies.

Using hydroxylapatite column chromatography, enzyme purification of some 200-fold was obtained using activated DNA which was a particularly effective primer after this step. Further purification was hampered by instability of the enzyme but

studies on protecting agents revealed that storage at -70°C in high glycerol concentrations allowed retention of activity over long periods, opening the way to purification to homogeneity.

Studies on the molecular weight of the herpes DNA polymerase indicated values in the range 50,000 - 200,000, probably due to the occurrence of aggregation. The true value probably lies in the lower part of this range.

Characterisation studies on the hydroxylapatite-purified virus-induced polymerase revealed that the enzyme was of the replicative type, requiring all four deoxyribonucleoside 5'-triphosphates and DNA for activity. Nearest-neighbour frequency analysis using the herpes polymerase gave results consistent with faithful copying of template DNA's. Heat inactivation revealed different profiles for the polymerase and exonuclease activities but this does not necessarily preclude their being in the same protein. Evidence was also obtained showing a preference for native or activated herpes DNA as primer over DNA's of corresponding secondary structures from other sources including the host cell.

Chromatographic studies carried out using extracts of host cells revealed that the host DNA polymerase is of the replicative type and appears to exist in at least two separable forms concurring with the results obtained from other mammalian cell sources.

Finally, comparison of virus-induced DNA polymerase and DNase with the corresponding host activities using a wide range of

criteria clearly indicates their non-identity and supports a viral origin for the new DNA polymerase and DNA exonuclease(s). The results from the project confirm and amplify earlier research, and have achieved the aim of separating the host and virus-induced activities and laid a substantial base for continued purification and characterisation studies on the viral enzymes.

CHAPTER I - INTRODUCTION.

1. DNA AND ITS METABOLISM.

1. 1. The primary and secondary structure of DNA.

1. 1. 1. Primary structure.

It is now firmly established that DNA is the genetic material for all forms of life except the RNA viruses. The sequence of the bases in DNA chains contains, in coded form, the messages of the genes, which are ultimately expressed in the primary structure of RNA and protein molecules. Some DNA sequences, however, are not thus expressed but act as "recognition" or binding sites for both regulatory proteins, such as repressors, and RNA polymerase (Yarus, 1969).

The linear sequence of bases in a DNA molecule, the primary structure, is thus a matter of great importance and interest. Unfortunately, its determination is an extremely difficult exercise because of: (i) the difficulty in obtaining intact, monodisperse DNA, (ii) the great length of the molecules, (iii) the small number of possible bases at each position and (iv) the fact that the techniques for DNA sequencing are poorly developed as yet. Thus, generally, only short sequences of DNA molecules have been absolutely defined.

Nevertheless, methods for providing useful, if incomplete, information about DNA primary structure are available, one of the

most extensively utilised of these being the method of nearest-neighbour sequence analysis (Josse, Kaiser and Kornberg, 1961). This yields the frequency of the sixteen possible dinucleotide sequences for the DNA of any species examined (Methods section 2. 14).

Nearest-neighbour studies on DNA's from a wide variety of organisms have shown distinct, non-random patterns for each species, and similar patterns for closely related organisms. One feature of particular interest is the markedly low frequency of occurrence of the sequence CpG in mammalian DNA's. The results in general hint at the almost certainly unique sequences of bases which we should expect to find in the DNA molecules of each different species.

A related technique (Berg, Fancher and Chamberlin, 1963) permits extension of the sequence analysis of DNA to tri-, tetra- and pentanucleotide sequences.

The partial degradation of DNA by acid, followed by alkali (Shapiro and Chargaff, 1964) or by diphenylamine in acid conditions (Lunt, Siebke and Burton, 1964) has revealed that pyrimidines often occur as clusters in DNA: an analogous technique yields purine clusters which may be similarly examined (Jones and Walker, 1964).

The technique of DNA-DNA hybridisation has been highly developed to provide the most sensitive index of homology between different DNA molecules (McCarthy and Church, 1970).

Similarly, DNA denaturation and renaturation studies have revealed that there are 10^2 - 10^3 copies of certain nuclear

nucleotide sequences and 10^4 - 10^5 copies of others in the genome: sometimes these puzzling sequences are less than 300 nucleotides in length (Britten and Kohne, 1970).

Thus we see that there are several approaches available, yielding largely fragmentary information on DNA primary structure: the complete analysis of chromosomal DNA sequences seems a long way off at the present time.

1. 1. 2. Secondary structure.

The term "secondary structure" refers to the regular arrangement in space of the DNA chain along its length. Our knowledge of this comes from both the evidence of X-ray diffraction studies on DNA (Astbury, 1947; Franklin and Gosling, 1953a,b, Wilkins, Stokes and Wilson, 1953; Wilkins, 1963; Fuller, 1964) and estimations of the molar proportions of bases in DNA (Chargaff, 1950,1951) together with work involving molecular model building.

DNA gives two main X-ray patterns: the crystalline pattern of the A structure, and the paracrystalline pattern of the B structure produced when the water content is higher; examination of these pictures allowed important inferences to be made regarding DNA structure, including the presence of several chemical repeats of the phosphate-sugar chain in one structural repeat.

Chargaff (1950, 1951) emphasised the occurrence of certain regularities in the base composition of DNA from most sources. His most important finding was that adenine and thymine are present

in equal molar quantities and likewise cytosine and guanine: the other relationships he revealed follow from this one.

Watson and Crick (1953a) accounted for these findings by advancing the view that the DNA molecule is a double, right-handed helix, consisting of two polynucleotide chains winding round the same axis and held together by hydrogen-bonds between A and T, and G and C specifically.

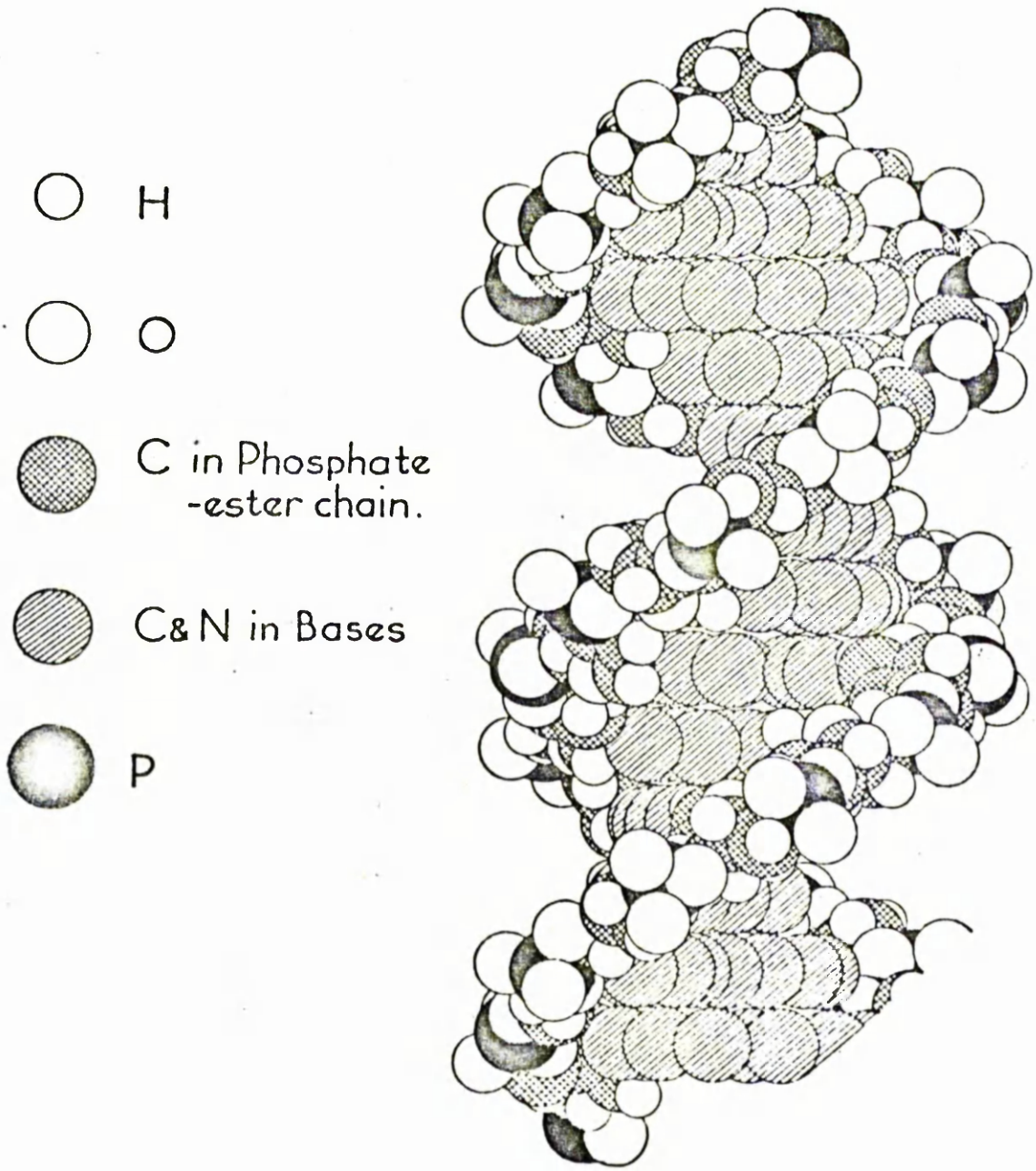
On the basis of this double-helical structure, Watson and Crick (1953a,b) suggested that the DNA molecule might duplicate itself through each strand's acting as template for the synthesis of its complement in accordance with the rules of base pairing.

A similar mechanism might explain how DNA transcription might occur.

The finer details of the DNA structure put forward by Watson and Crick (1953a) were later corrected by Wilkins and his colleagues and a section of a model of the revised B structure is depicted in Fig. I. 1. It may be noted that the pairs of bases are flat and stacked one above the other, the stacking distance between the bases being 3·4Å. The individual strands are of opposite polarity - in the sense that the internucleotide linkage in one strand is 5' → 3' while, in the other, it is 3' → 5'. There are ten base pairs per turn of the double helix and each turn (structural repeat) has a height of 34Å - the crystallographic repeat distance. Between the two strands are a shallow groove and a deep groove and

Fig. I. 1.

Model of the Revised B Structure of DNA.



the strongly polar, hydrophilic phosphodiester backbone faces outwards into the solvent while the more hydrophobic bases are located centrally. The width of the double helix is 20Å.

Similar X-ray patterns, corresponding to the B structure, are also obtained from DNA in solution and from intact biological material such as sperm heads and T-even phage, suggesting that the double-helix exists *in vivo* as well as *in vitro* (Wilkins *et al.*, 1953; Fuller, 1964).

However, exceptions exist to this double-helical structure for DNA; these include the single-stranded DNA chromosomes of certain bacteriophages and animal viruses.

Further exceptions to the general double helical structure have recently been described (Langridge, 1969; Mitsui *et al.*, 1970). These concern the secondary structures of synthetic DNA analogues such as poly (dA).poly(dT), poly(dC).poly(dG), poly[d(T-C)].poly[d(G-A)] and poly[d(I-C)].poly[d(I-C)]. Each of the first three polymers has pyrimidine bases in one strand and purine bases in the other, and such polynucleotides give X-ray diffraction patterns distinct from those of both native DNA and synthetic polynucleotides with mixed purine and pyrimidines in each strand. The results suggest that the localised regions in natural DNA's where pyrimidine clusters exist may have distinct secondary structures which might conceivably act as "recognition" sites. The X-ray diffraction results from the fourth polymer suggest a left-handed helix with distinct dimensions. Any *in vivo* significance for this latter structure,

however, is dubious since inosine is not believed to exist in natural DNA.

Despite these minor exceptions, it seems unlikely that any structure, other than the "Watson-Crick" one, represents the main bulk of DNA *in vivo* (Donohue, 1969; Arnott, 1970; Crick, 1970; Donohue, 1970; Wilkins, Arnott, Marvin and Hamilton, 1970).

1. 2. The detailed structure and location of DNA in vivo.

From the following examples, in which aspects of the tertiary and quaternary structure of DNA's are outlined together with other aspects of their primary structure, it may be seen that the secondary structure of DNA forms but one feature of the large variety of yet more complex complete structures of the DNA of organisms.

1. 2. 1. Bacterial DNA.

The genome of the bacterium *Escherichia coli* consists of a single chromosome which takes the form of a cyclic molecule of double-stranded DNA of molecular weight $2.5 \times 10^9 \pm 0.5 \times 10^9$ (Cairns, 1963). It is likely that this kind of chromosome structure is characteristic of other bacterial species. The bacterial chromosome appears to be attached to the cell-membrane, apparently at a mesosome (Ryter and Landman, 1964) and seems to exist as a fairly compact mass in direct contact with the cell contents and, unlike eucaryote

DNA, complexed with polyamines and not forming part of a true nucleus. Bacteria often contain extra genetic material which may exist free, or integrated with the main chromosome , such structures being called plasmids or episomes.

1. 2. 2. Eucaryotic DNA.

In eucaryotes, the DNA is located mainly within the nuclei, arranged in chromosomes in a complex fashion which is still incompletely understood, although it is known to be associated with large amounts of basic proteins (histones) and acidic proteins, together with amounts of RNA which apparently vary with the transcribing activity of the chromosomal genes (Hearst and Botchan, 1970).

In addition to the chromosomes, the mitochondria and centrioles of eucaryotes and the chloroplasts of plant cells have also been shown to contain DNA (Granik and Gibor, 1967).

Mitochondrial DNA, from mammals, has been demonstrated to be covalently cyclic, double-stranded and sometimes supercoiled - a phenomenon caused by constraint in these cyclic molecules owing to the existence of less than the normal number of turns in their double-helical structure - and the DNA can exist as a system of interlocking rings (Clayton and Vinograd, 1967).

The satellite DNA of some species appears to contain repeating sequences and to have a base composition different from the bulk of the nuclear DNA. In addition, extrachromosomal genetic material with several tandem copies of a single gene has been

described in vertebrate oocyte nucleoli (Watson, 1970).

1. 2. 3. Viral DNA.

Among viral DNA molecules - the best characterised of all DNA species (Thomas and MacHattie, 1967) - we find many features of interest. These include instances of single-stranded cyclic DNA molecules (e.g. ØX174 DNA) which have double-stranded, cyclic, supercoiled replicative forms. We find genomes, like that of bacteriophage λ which have complementary, single-stranded ends (cohesive ends) which allow cyclisation by artificial or natural means. There are genomes, like those of bacteriophages T2, T4 and P22, which are apparently linear duplices with terminal redundancy and circular permutation. Bacteriophage T7 DNA is a linear duplex which is terminally redundant but not circularly permuted, and bacteriophage T5 DNA appears to carry specific single-strand scissions (nicks) in its linear duplex molecule (Thomas, 1966).

1. 2. 4. Variations in the purine and pyrimidine bases of DNA.

(a) Cellular organisms.

Methylated bases occur widely in nature, methylation of the normal bases occurring after synthesis of the DNA polynucleotide. This phenomenon predominantly affects adenine and cytosine producing 6-methylaminopurine and 5-methylcytosine respectively. Any given DNA carries a characteristic amount and distribution of methylated base.

(b) Viruses.

Cytosine may be totally replaced by 5-hydroxymethylcytosine- as in the DNA of T-even bacteriophages (Wyatt and Cohen, 1950). α - and β - glucosyl and gentiobiosyl residues may be attached to the unusual cytosine analogues in this group of phages (Lehman and Pratt, 1960).

Uracil replaces thymine in the DNA of bacteriophage PBSI (Takahashi and Marmur, 1963) and 5-hydroxymethyluracil wholly replaces thymine in the DNA of bacteriophage SP8 of Bacillus subtilis (Kallen, Simon and Marmur, 1962).

1. 3. Metabolism of DNA.

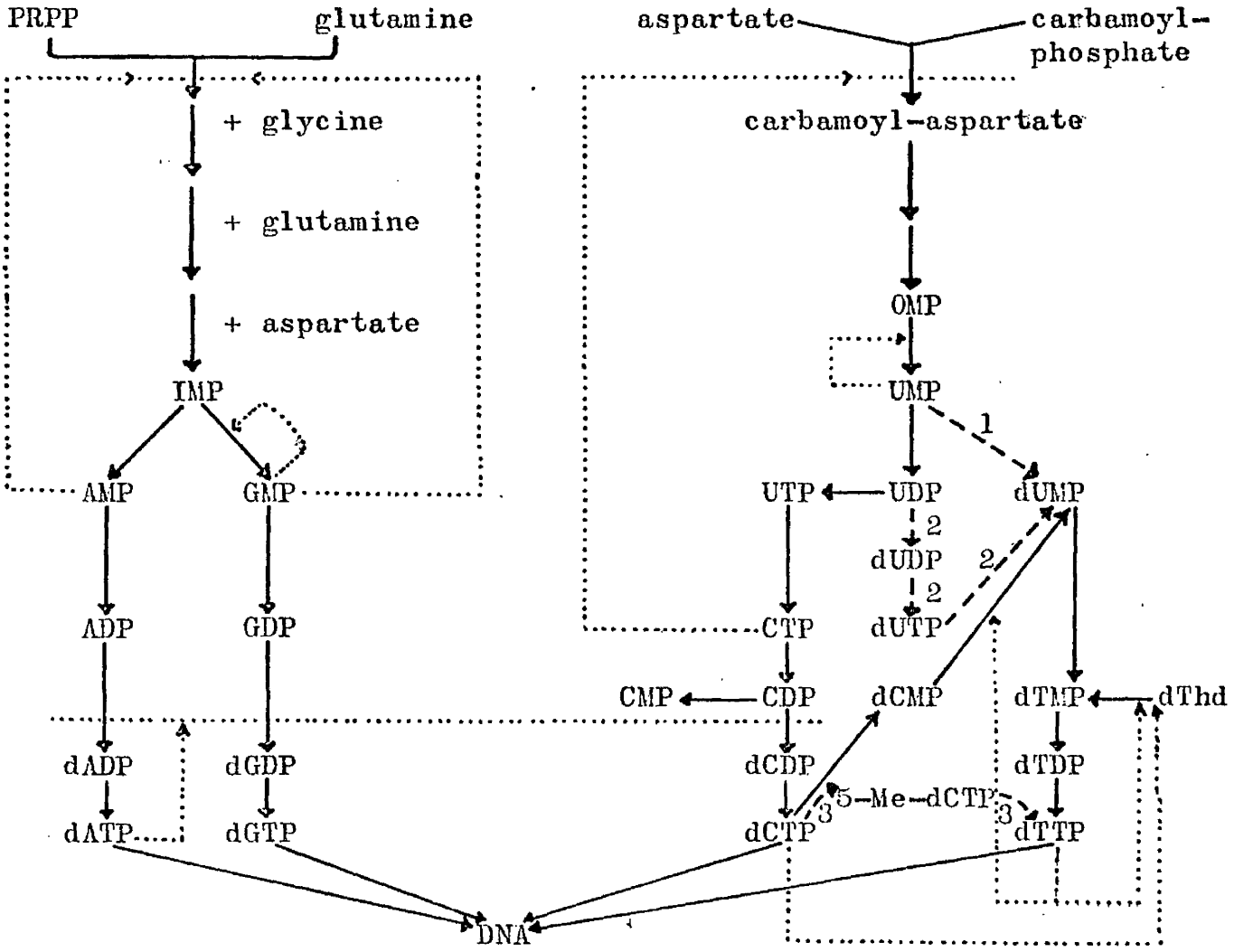
1. 3. 1. Biosynthesis of the deoxyribonucleoside 5'-triphosphates.

The biosynthesis of the deoxyribonucleoside 5'-triphosphates - the precursors for polymerisation into DNA in vitro and probably in vivo - may be considered to occur in 3 stages: (i) The biosynthesis of purine and pyrimidine ribonucleoside monophosphates and their phosphorylation (Schulman, 1961); (ii) the reduction of these ribonucleotides to the corresponding deoxyribonucleotides and (iii) the phosphorylation of the deoxyribonucleotides to the 5'-triphosphate level.

These three stages and their control are summarised in Fig. I.2. and are described concisely by Davidson (1969) who also discusses other pathways leading to the synthesis of nucleosides

Fig. I. 2.

Pathways involved in the biosynthesis of the deoxyribonucleoside triphosphates.



·····> Feed-back mechanisms.
——→ Well-known pathways.
– – –→ Less well-known pathways.

1. Known in Salmonella typhimurium and chick embryos.
2. Known in Escherichia coli.
3. Known in T-even bacteriophage-infected Esch. coli.

and nucleotides from the level of bases, so utilising products of degradation of nucleic acids.

As hinted earlier, while the deoxyribonucleoside 5'-triphosphates are the monomer precursors for *in vitro* DNA polymerisation reactions, the nature of the *in vivo* DNA precursor is at present controversial: there is a suggestion of separate pools of precursors for repair and replicative DNA synthesis since the two processes seem to utilise thymine and thymidine to different extents (Werner, 1971) and there is now an indication that deoxyribonucleoside 5'-diphosphates may be the immediate precursors for DNA biosynthesis *in vivo* (Werner, 1971; J. Cairns, personal communication).

1. 3. 2. In vivo DNA biosynthesis.

(a) Bacterial and bacteriophage systems.

(i) Origin, direction, rate and mode of DNA biosynthesis.

Insight into the mechanism of *in vivo* DNA synthesis in procaryote systems was much enhanced by the experiment of Meselson and Stahl (1958) which demonstrated that DNA is replicated by a semi-conservative mechanism, the conserved unit being a single strand. The experiment also showed that DNA molecules are replicated in a sequential manner.

Cairns (1963) produced autoradiographs of the *E. coli* chromosome by labelling with [^{3}H] thymidine prior to gentle isolation of the structure. The experiment demonstrated the cyclic

nature of the DNA molecules with a single replicating loop and a growing point proceeding apparently unidirectionally. The replicating fork was visible and clearly showed the mode of synthesis of the DNA to be semi-conservative and sequential. The rate of replication was estimated to be 1000-1400 nucleotides per second.

Early work by Cooper and Helmstetter (1968) showed that, under normal conditions, the time for the replication point to traverse the E. coli genome and the time between the end of a round of replication and cell division were constant and they predicted gaps in DNA synthesis during slow growth and multiple replication forks during rapid growth. Helmstetter (1968) was able to demonstrate the initiation of new rounds of replication before the first were completed in rapid growth as confirmed by Ward and Glaser (1969a,b,c).

Helmstetter (1968) and Nagata (1963) presented evidence for sequential replication of the bacterial genome from a fixed point which they localised; and Helmstetter (1968) traced the sequence which was strain specific and appeared to proceed clockwise.

Recent experiments, however, have demonstrated bidirectional DNA replication in E. coli (Masters and Broda, 1971), in phage λ (Schnös and Inman, 1970), and probably also in bacteriophage T4 (J. Cairns, personal communication) - the latter finding being in conflict with the work of Werner (1968) and Mosig (1970). B. subtilis DNA, however, is apparently replicated unidirectionally (Sueoka and Quinn, 1968).

(ii) Association of DNA replication with the cell membrane.

A large body of evidence has accumulated to support the idea advanced by Jacob, Brenner and Cuzin (1963) that the DNA replication unit or "replicon" is attached to the cell membrane.

Goldstein and Brown (1961), using E. coli protoplasts, showed the occurrence of DNA synthesis in a particulate complex such as that isolated and characterised by Fuchs and Hanawalt (1970). A similar association of DNA was demonstrated in E. coli by Smith and Hanawalt (1967) and in B. subtilis (Ganesan and Lederberg, 1965); in these experiments it appeared that the DNA was attached to the membrane only at the time of replication. On the other hand, there is evidence of a stable attachment of DNA to membrane at the replication origin of B. subtilis and E. coli (Sueoka and Quinn, 1968; Fielding and Fox, 1970).

Smith and Hanawalt (1967) showed that the growing point region of the bacterial chromosome had properties suggestive of Y-shaped, partially replicated DNA units: Fuchs and Hanawalt (1970), however, found no evidence of lipid associated with the growing structure.

Inouye and Pardee (1970) and Shapiro, Siccardi, Hirota and Jacob (1970) have shown membrane protein alteration associated with aberrations in DNA synthesis.

Thus, although there is much evidence (supplemented by in vitro work to be discussed later) to support the association of

replicating DNA with membrane structures, some findings conflict with this view and the matter cannot be regarded as settled as yet.

(iii) Fragmentary intermediates in DNA biosynthesis.

In 1966, Sakabe and Okazaki suggested that DNA replication proceeds by way of fragmentary intermediates: newly replicated DNA seems to exist as short, single-stranded sections which later become incorporated into macromolecular double-stranded DNA. Oishi (1968a,b,c) verified this finding for both E. coli and B. subtilis.

Okazaki et al. (1970a) have recently reviewed the progress in this field and the findings are as follows.

(a) The situation in bacteriophage T4 DNA replication closely resembles that for E. coli (Okazaki et al., 1968) and upon incubating the single-stranded regions with DNA polymerase and polynucleotide ligase in vitro the short strands join together with a marked increase in their sedimentation rate (Yudelevich, Ginsburg and Hurwitz, 1968).

(b) In ligase-deficient bacteriophage T4 the newly-synthesised short chains accumulate (Sugimoto, Okazaki and Okazaki, 1968).

(c) The short strands are synthesised in the 5' → 3' direction in vivo (Okazaki et al., 1968).

(d) Nascent short chains isolated from T4-infected E. coli under a variety of conditions anneal equally to the separated complementary phage DNA strands - consistent with the hypothesis that both strands are synthesised discontinuously (Sugimoto, Okazaki,

Imae and Okazaki, 1969).

Recent work, however, with B. subtilis reveals that the "Okazaki fragments" anneal to only one of the strands of the bacterial DNA, which replicates unidirectionally; so the fact that the fragments anneal to both strands in DNA species which are bidirectionally replicated would be expected, even if, as the B. subtilis results suggest, only one strand of the DNA is replicated by way of fragmentary intermediates in each direction (Okazaki, Sugimoto, Okazaki, Imae and Sugino, 1970b).

(iv) Control of DNA replication and the association of protein synthesis with DNA replication.

Lark (1969) showed that the processes of initiation and control of DNA synthesis are complex, involving in the case of bacteriophage T4 DNA replication, the products of some twenty genes, many of which have as yet uncharacterised products.

Studies with such inhibitors as chloramphenicol and phenethyl alcohol indicate that protein synthesis is necessary for the initiation of DNA synthesis: at least two distinct proteins seem to be involved (Kohiyama, Lanfrom, Brenner and Jacob, 1963; Ward and Glaser, 1969c; Hirota, Mordoh and Jacob, 1970).

Rosenberg, Cavalieri and Ungers (1969) produced evidence for a negative control of E. coli DNA replication, involving a periodically synthesised replication repressor and a continuously synthesised anti-repressor protein.

(v) Bacteriophage DNA replication.

DNA replication in bacteriophages has many common features recurring among the different species.

Intermediate long forms of DNA, equivalent to several mature genomes in length, are sometimes found as in T4 and T7 DNA replication (Frankel, 1968; Kelly and Thomas, 1969). Circular intermediates are found in phage λ replication (Young and Sinsheimer, 1967a,b) and double-stranded circular replicative forms in ØX174 DNA replication. In both these cases there is evidence for the occurrence of a "rolling circle" mechanism of DNA replication (Fig. I. 3.) (Dressler, 1970; Kiger and Sinsheimer, 1971) and, in the case of ØX174 DNA, a brief, transitory membrane association may occur during replication.

(b) Eucaryote systems.

(i) The cell cycle.

Howard and Pelc (1953), by using stripping film autoradiography, studied the time course of DNA biosynthesis in bean root cells and demonstrated a pre-synthetic period (G_1) in early interphase after mitosis, followed by a synthetic period (S) and then a post-synthetic gap (G_2) immediately before mitosis (M).

The same sequences (cell cycles) exist in other eucaryotic cells, the lengths of the different phases varying and being characteristic of the cell types.

Fig. I. 3.

The "Rolling Circle Model" for DNA replication [with inset speculative scheme for unidirectional replication of the duplex chain ("Knife and Fork Model")].

(I.) Replication is initiated upon a closed DNA circle.

(II.) A sequence-recognizing endonuclease inserts a nick into the positive strand.

(III.) The DNA polymerase adds nucleotides on to the 3'-end of the open strand, displacing the positive strand at the tail. The 5'-end of the tail may be fixed to some structure - perhaps membrane. The correct nucleotides are chosen by hydrogen-bonding to the negative strand template. As new nucleotides are chosen, the positive strand becomes longer than unit length.

(IV.) Complementary fragments begin to be synthesized on the elongating tail thereby converting it to a double-helical form. This may be achieved by the "Knife and Fork" mechanism (see inset diagram and legend below).

(a) Nicked region

(b) Covalent extension

(c) Formation of fork

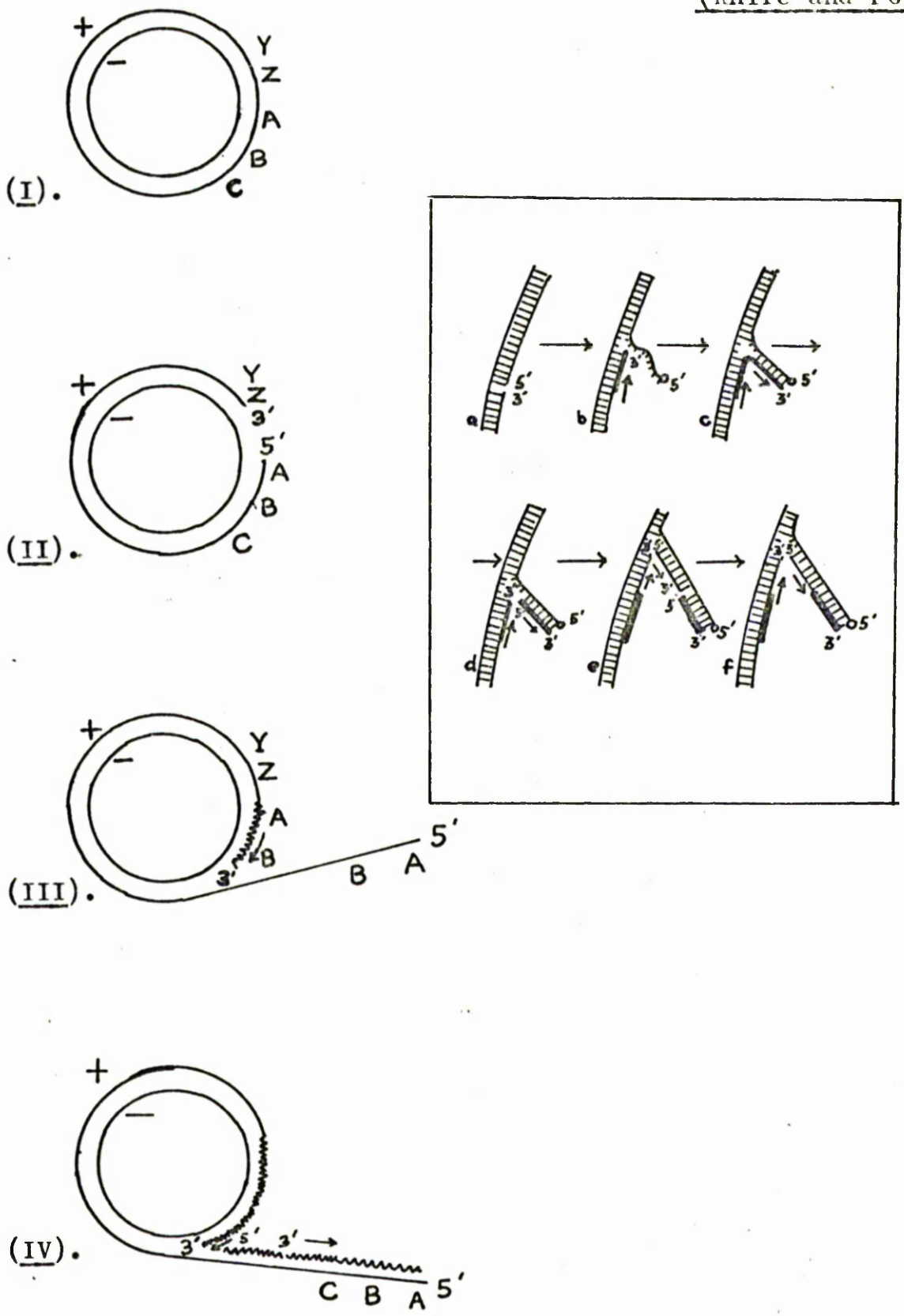
(d) Cleavage by nuclease

(e) Further covalent extension and nuclease-cleavage

(f) Ligase action

Fig. I. 3.

The Rolling Circle Model for DNA Replication with Inset Speculative Scheme for Unidirectional Replication of the Duplex Chain (Knife and Fork Model).



(ii) Origin, direction, rate and mode of DNA biosynthesis.

The semi-conservative mode of DNA replication also holds in eucaryotes (Taylor, Woods and Hughes, 1957; Taylor, 1960) so that each daughter chromosome receives half of each molecule that was present in the parent chromosome.

Cairns (1966), from autoradiographic studies on HeLa cell DNA, deduced that the DNA of mammalian chromosomes is arranged in the form of long fibres up to 500μm in length. He estimated the rate of replication as 0.5μm per minute and postulated at least 100 sites of replication per chromosome, with the units of DNA joined in tandem (Painter, Jermany and Rasmussen (1966) estimated the number of replicating sites per cell as 10^3 or 10^4). Huberman and Riggs (1968) provided evidence in Chinese hamster cells that sections of replicating DNA are contiguous, appear to be less than 30μm long, and are replicated at the rate of 2.5μm or less per minute. Replication seems to proceed in opposite directions at adjacent growing points and these diverging growing points appear to initiate replication at the same time: this mode of synthesis is strikingly similar to the bidirectional synthesis recently revealed in procaryotes. Painter and Schaefer (1969b) found that the rate of DNA synthesis along replicons of 5 different kinds of mammalian cells is remarkably constant.

Comings (1968) and Comings and Kakefuda (1968) have presented evidence for the ordered arrangement of chromatin in the

nucleus and the attachment of specific sites on the chromatin to specific sites on the nuclear membrane; they also showed that initiation of DNA replication occurs at the nuclear membrane but that, later in S-phase, the replication points are not attached to the membrane.

Tobia, Schildkraut and Maio (1970) showed that, in synchronised cultured mammalian cells, the synthesis of regions of DNA of different base composition and satellite DNA occurs at specific times in S-phase.

(iii) Fragmentary intermediates in DNA biosynthesis.

Observations similar to those of Okazaki and his colleagues have been made in eucaryotes and intermediates in DNA synthesis similar to the "Okazaki fragments" appear to exist in these higher forms (Taylor, 1968; Painter and Schaefer, 1969; Berger and Irvin, 1970).

(iv) Factors influencing the initiation and maintenance of DNA biosynthesis.

Lieberman and Ove (1962) demonstrated a requirement for Zn^{2+} in the development of the ability of certain cells in tissue culture and other systems (Lieberman, Abrams, Hunt and Ove, 1963; Fujioka and Lieberman, 1969) to initiate the DNA synthesis. A similar Ca^{2+}-dependent initiation of DNA synthesis has been described in thymocytes and their isolated nuclei (Burgoyne, Wagar and Atkinson, 1970).

Prescott and Goldstein (1967) presented clear evidence

for the implication of one or more cytoplasmic factors in the initiation of DNA biosynthesis. Furthermore, protein synthesis is required for nuclear DNA synthesis in Chlorella (Wanka and Moors, 1970) and in yeast (Grossman, Goldring and Marmur, 1969); this seems to be true also in Physarum polycephalum (Brewer and Rusch, 1968) and in Chinese hamster cells (Ley and Tobey, 1970).

A number of factors have been shown to initiate or stimulate DNA biosynthesis in both tissue culture and whole animal systems: these include factors from used cell or tissue culture media (Kasakura and Lowenstein, 1965); serum - the active factor being associated with the γ-globulin fraction - (Clarke, Stoker, Ludlow and Thornton, 1970; Dulbecco, 1970); hormones such as erythropoietin; and an apparently humoral factor triggering DNA synthesis after partial hepatectomy in the rat (Sakai, 1970). Whether these factors and the tissue-specific inhibitors called chalones (Bullough, Lawrence, Iverson and Elgjo, 1967) have direct or more or less indirect effects upon DNA biosynthesis in vivo is uncertain; in some cases the effect on DNA synthesis may be merely a remote consequence of some other growth or cell-division regulating reaction.

In conclusion, it can be seen that, despite intensive study, our knowledge of in vivo DNA synthesis in eucaryotes and even in procaryotes, consists of many fragmentary items of evidence and isolated facts and (as will be further discussed in Introduction sections 1. 3. 3. and 1. 4.) the complete DNA

replication process, at the molecular level, remains largely a mystery.

1. 3. 3. In vitro DNA biosynthesis.

(a) DNA polymerase (DNA-dependent).

The *in vitro* reaction $\left[(DNA)_n + dNTP \rightleftharpoons (DNA)_{n+1} + PPi\right]$ (Englund *et al.* 1968), in which the four deoxyribonucleoside 5'-triphosphates are polymerised to the level of macromolecular DNA in the presence of Mg^{2+} and DNA, has been known and studied intensively over the course of the last thirteen years (Davidson, 1969).

The activity responsible - DNA polymerase - has been studied in extracts of bacteria, phage-infected bacteria, moulds, plants, invertebrates, vertebrates - including a large variety of mammalian sources - and a number of virus-infected animal tissues.

(i) Bacterial DNA polymerases.

The Kornberg E. coli DNA polymerase (DNA polymerase I).

This enzyme, the best characterised of all DNA polymerases, has been purified to homogeneity and appears to consist of a single polypeptide chain of mol.wt.109,000 daltons (Englund *et al.*, 1968; Jovin, Englund and Bertsch, 1969).

The enzyme has an active site which comprises the following: a single binding site for deoxyribonucleoside 5'-triphosphates for which all four triphosphates compete (Englund, Huberman, Jovin and Kornberg, 1969a); a single binding site for

DNA template (Englund, Kelly and Kornberg, 1969b) and a binding site for oligonucleotide - or DNA - primer which must have a 3'-OH group in the ribo configuration (Huberman and Kornberg, 1970). Models for this active site have been proposed (Beyersmann and Schramm, 1968; Kornberg, 1969).

The enzyme catalyses several reactions: the polymerisation of the four deoxyribonucleoside 5'-triphosphates to DNA in the presence of a DNA template and Mg^{2+}, the closely related pyrophosphate exchange, the degradative reactions of pyrophosphorolysis and the two exonucleolytic hydrolytic reactions, one occurring in the 3' → 5' direction, the other in the 5' → 3' direction, both releasing deoxyribonucleoside 5'-monophosphates (Klett, Cerami and Reich, 1968; Deutscher and Kornberg, 1969a,b). The enzyme also excises thymine dimers by a reaction in which di-, tri- and even longer nucleotides containing these pyrimidine dimers are released (Kelly, Atkinson, Huberman and Kornberg, 1969).

Studies on the binding of DNA polymerase to DNA revealed: binding to bihelical DNA only at nicks or ends, no binding to double-stranded covalently cyclic DNA, and binding at multiple sites along single-stranded DNA (Englund et al., 1969b).

For all replicative DNA polymerases studied there seems to be an absolute requirement for a template and for a primer with a 3'-OH group to which latter, the deoxyribonucleoside 5'-triphosphate attaches with loss of pyrophosphate in the polymerisation, using the template strand as a guide to which of the four tri-

phosphates is to be incorporated.

The E. coli DNA polymerase I will accept single-stranded DNA, native double-stranded linear DNA and poly[d(A-T)] or poly(dC). poly(dG) as template; but it is completely inactive on double- or single-stranded covalently cyclic DNA.

Activation of double-stranded DNA (Aposhian and Kornberg, 1962) involves the introduction, by DNase I, of single-strand scissions into the structure producing adjacent 3'-OH and 5'-phosphoryl groups: this treatment greatly enhances all polymerase functions. The enzyme attaches at these nicks and polymerisation then occurs in the 5' → 3' direction in conjunction with 5' → 3' exonuclease action so that nick-translation occurs with no net synthesis; later events whereby net synthesis occurs with production of a non-denaturable, branched, biologically inactive product are not understood (Kelly, Cozzarelli, Deutscher, Lehman and Kornberg, 1970).

To replicate single-stranded DNA, either the nucleic acid must bend back upon itself to form a hairpin-like structure thus providing a priming 3'-OH group and a template from one strand of DNA, or a small oligonucleotide initiator is required to pair with the single strand and provide the required 3'-OH group Goulian, 1968a,b).

The polymerase will also carry out a repair-type of synthesis with double-stranded DNA possessing single-stranded ends - converting this to a totally double-stranded structure (Richardson,

Inman and Kornberg, 1964).

In the presence of Mn^{2+}, a single ribonucleoside 5'-triphosphate may be incorporated into synthesised DNA along with the three other deoxyribonucleoside triphosphates (Berg et al., 1963).

The enzyme, in an incomplete state of purity, may also carry out de novo (unprimed) synthesis of poly[d(A-T)].poly[d(A-T)] and poly(dG).poly(dC) in the presence of the appropriate precursors and defined ionic conditions (Schachman, Adler, Radding, Lehman and Kornberg, 1960; Radding, Josse and Kornberg, 1962; Radding and Kornberg, 1962; Burd and Wells, 1970).

RNA, synthetic RNA and synthetic RNA-DNA hybrids have also been shown to act as templates for the polymerisation of deoxyribonucleoside 5'-triphosphates by the enzyme (Lee-Huang and Cavalieri, 1965; Cassidy, 1966; Cavalieri and Carroll, 1970, 1971).

It has been shown that the intact DNA polymerase molecule may suffer proteolytic cleavage to produce enzymically active fragments (Brutlag, Atkinson, Setlow and Kornberg, 1969; Brutlag and Kornberg, 1970; Klenow and Overgaard-Hansen, 1970). A fragment of molecular weight 76,000 has polymerase and 3' → 5' exonuclease function but no 5' → 3' exonuclease activity and, in some circumstances, the smaller fragment of molecular weight 35,000 is found to have 5' → 3' exonuclease activity. This proteolytic cleavage may explain numerous reports of the finding of multiple molecular species of DNA polymerase from E. coli, with a wide

variety of molecular weight values from 10,000 - 150,000 from which evidence several authors have postulated a subunit structure for the enzyme (Lee-Huang and Cavalieri, 1965; Lezius, Hennig, Menzel and Metz, 1967; Cavalieri and Carroll, 1968; Yoshida and Cavalieri, 1970).

The detailed understanding of this enzyme is now impressive; but unfortunately, as will be discussed later, it appears likely that DNA polymerase I has some role in repair rather than in DNA replication.

E. coli DNA polymerase II.

This apparently membrane-associated enzyme solubilised, partially isolated, and characterised from E. coli by Knippers (1970) in many respects resembles the E. coli DNA polymerase I eg. deoxyribonucleoside 5'-triphosphates are the immediate precursors for DNA synthesis and Mg^{2+} and DNA are required; but there are clear differences too.

In the case of polymerase II, sonicated native DNA is the favoured template or primer, denatured DNA is not accepted and "activation" by DNase I does not enhance priming with native DNA. The rate of DNA synthesis in vitro with this partially purified polymerase II preparation is close to the in vivo rate in contrast to polymerase I's rate of DNA synthesis which is 100 times more slow than the in vivo reaction. The molecular weight of polymerase II is 60,000 - 90,000 and there are differences in the effects of ions and organic agents upon polymerase I and II (Knippers, 1970).

Similar activities, probably identical to DNA polymerase II, have been studied by other workers (Kornberg and Gefter, 1970, 1971; Moses and Richardson, 1970a,b, 1971; Loeb, Slater, Ewald and Agarwal, 1971) with many consistent findings although one group found "activated" DNA to be the preferred template or primer. Notably, Kornberg and Gefter (1971) have purified DNA polymerase II to homogeneity and have detected yet a third DNA polymerising activity apparently distinct from DNA polymerases I and II.

It is possible that DNA polymerase II may emerge as the true E. coli DNA replicase but, equally possibly, some other enzyme, or multienzyme complex, as yet undetected or (owing to a requirement for structural integrity) undetectable in vitro, may be the true replicase (Becker and Hurwitz, 1971)(see Introduction section 1.4.1.).

The DNA polymerase of B. subtilis.

The DNA polymerase of both vegetative and spore forms of this organism has been studied (Okazaki and Kornberg, 1964; Falaschi and Kornberg, 1966) and the enzyme appears to be the same in both, being similar in many respects to the E. coli DNA polymerase I, although the enzyme of B. subtilis lacks associated DNA exonuclease activity and has a molecular weight of 70,000 (originally reported as 46,000).

The DNA polymerase of Micrococcus luteus.

This enzyme has been quite extensively studied (Zimmerman, 1966; Litman, 1968; Harwood, Schendel and Wells, 1970; Harwood and Wells, 1970) and it is broadly similar to the E. coli DNA polymerase I: it also has a DNA exonuclease activity (5' → 3' direction)

associated with it but this differs from the corresponding E. coli activity in several respects (Harwood, Shendel, Miller and Wells, 1970; Litman, 1970).

(ii) Bacteriophage - specific DNA polymerases.

In bacteriophage T2-infected E. coli a new DNA polymerase, quite distinct from the host enzymes, has been detected, isolated and characterised (Aposhian and Kornberg, 1962). In contrast to the E. coli DNA polymerase I, the phage-specific enzyme has an absolute requirement for single-stranded DNA as template and it has an associated DNA exonuclease activity which acts only in the 3' → 5' direction. No de novo unprimed synthesis occurs with the phage enzyme and net synthesis with single-stranded DNA never exceeds 100%, producing a hairpin-like double-stranded DNA.

Bacteriophage T4-infected E. coli yields a very similar enzyme with equivalent priming and product characteristics to the T2 enzyme. It has a mol.wt. of 112,000 (Goulian, Lucas and Kornberg, 1968). Genetic evidence (De Waard, Paul and Lehman, 1965; Warner and Barnes, 1966) has shown that this enzyme is specified by phage gene 43 and is essential for phage DNA replication being very probably the phage DNA replicase. The work of Speyer (1965), Freese and Freese (1967) and Drake and Greening (1970), with mutants of gene 43 which resulted in mutator and antimutator gene effects, strongly suggests that the T4 polymerase assists in the selection of the correct base introduced in DNA synthesis, in collaboration with base pairing selection effects.

Bacteriophage T5-infected E. coli also yields a new virus-specific DNA polymerase (mol.wt. 96,000) with similar priming characteristics to the T2 and T4 enzymes (Orr, Herriott and Bessmann, 1965; Steuart, Anand and Bessmann, 1968a). Again DNA exonuclease activity is associated, and T5 gene 53 appears to specify the viral DNA polymerase (De Waard et al., 1965): the enzyme is again essential for phage DNA replication and can synthesise transforming DNA in vitro (Steuart, Anand and Bessman, 1968b).

(iii) Vertebrate DNA polymerases.

Of this group, the enzymes of mammalian tissues have been best characterised.

The calf thymus replicative DNA polymerase.

This enzyme has been partially purified and characterised (Yoneda and Bollum, 1965) and has been the most extensively studied mammalian polymerase.

The enzyme is typical of DNA polymerases in general in the usual requirements for triphosphates, Mg^{2+}, and DNA template or primer. Like the bacteriophage polymerases, the calf thymus enzyme shows a requirement for single-stranded DNA as primer (Bollum, 1959; Wang, 1967): the maximum yield of product is 100% this being of the hairpin, non-denaturable, double-helical type. The enzyme is endonuclease-free but a small amount of exonuclease activity is associated. The molecular weight of the polymerase is 110,000 but physical studies have not been extensive as the enzyme

is not yet pure. Interestingly, this polymerase does not readily form stable complexes with DNA like the E. coli DNA polymerase I (Yoneda and Bollum, 1965).

Calf thymus terminal DNA nucleotidyltransferases.

Calf thymus tissue is noteworthy on account of its containing two terminal DNA nucleotidyltransferase activities; one is associated with the nuclei, the other is cytoplasmic. These enzymes carry out a terminal addition reaction adding deoxyribonucleoside 5'-triphosphates to a 3'-OH group of the initiator (DNA) without any direction from a template: Mg^{2+} is required and optimal activity occurs with a lone species of deoxyribonucleoside 5'-triphosphate (Krakow, Kammen and Canellakis, 1961; Krakow, Coutsogeorgopoulos and Canellakis, 1962; Keir, Shepherd and Hay, 1963; Yoneda and Bollum, 1965; Gottesman and Canellakis, 1966; Kato, Gonçalves, Houts and Bollum, 1967; Chang and Bollum, 1971).

The cytoplasmic enzyme appears to be a unique type of enzyme found only in thymus gland and utilises an initiator which may be denatured DNA or an oligonucleotide which may be as small as a trinucleotide with a 3'-OH and a 5'-phosphoryl group.

The nuclear enzyme, in contrast, cannot utilise oligonucleotides: it requires denatured DNA as initiator.

Terminal DNA nucleotidyltransferase activity in general seems to be fairly common in mammalian nuclear preparations (Rothschild, Halpern and Smith, 1968; Lindsay, Berryman and Adams,

1970) but the *in vivo* function of these activities is unknown although it has been suggested that they may represent a catalytic subunit of replicative DNA polymerase (Keir, 1965).

Rat liver DNA polymerase (nuclear and soluble cytoplasmic activities).

De Recondo and Fichot (1969) showed the presence of two species of rat liver cell-supernatant DNA polymerase activity; one with a preference for native DNA, the other for denatured DNA as primer. This heterogeneity of DNA polymerase in rat liver and hepatomas has been confirmed by other workers (Bellair, 1968; Iwamura, Ono and Morris, 1968; Ove, Brown and Laszlo, 1969).

Rat liver mitochondrial DNA polymerase.

A third species of rat liver DNA polymerase was discovered when it was shown that mitochondrial DNA polymerase was chromatographically distinct from the nuclear ensyme(s) and free from DNase and terminal DNA nucleotidyltransferase. The new polymerase preferred native mitochondrial DNA as template and its molecular weight was about 100,000 (Kalf and Ch'ih, 1968).

In 1970, however, Meyer and Simpson reported a 14,000 fold purification of the enzyme: they showed that the polymerase now preferred single-stranded DNA as template - again with a preference for rat liver mitochondrial DNA - but they advised caution regarding any claim for an absolute specificity for the homologous DNA.

A large variety of other mammalian sources of DNA

polymerase have been explored (Keir, 1965) but the enzymes all appear to have broadly similar properties.

Nuclear systems for DNA replication, and associated studies on nuclear DNA polymerase activity.

Friedman and Mueller (1968) described a nuclear system for DNA replication from synchronised HeLa cells: this appeared to continue the *in vivo* replication process and, interestingly, like the *E. coli* membrane DNA replication system of Smith, Schäller and Bonhoeffer (1970), all four deoxyribonucleoside 5'-triphosphates (cf. Werner, 1971), Mg^{2+} and ATP were required. In addition, a heat-labile factor was needed from the cytoplasmic fraction. A comparison of the nuclear system and the cytoplasmic DNA polymerase revealed several different requirements and Teng, Bloch and Roychoudhury (1970) described an analogous nuclear system from Ehrlich ascites cells from which they concluded that the cytoplasmic DNA polymerase is not involved in the normal replication of DNA.

Littlefield, McGovern and Margeson (1963), Gold and Helleiner (1964) and Adams and Lindsay (1969) have conducted experiments which suggest migration of DNA polymerase from the cytoplasm to the nucleus where it may assume an active conformation for nuclear DNA synthesis and has a preference for native DNA as primer: then, apparently after S-phase, the structure of the enzyme is altered and it is released into the cytoplasm as a denatured DNA-preferring enzyme.

(b) RNA-dependent DNA polymerases.

In addition to these DNA-dependent DNA polymerases, instances of RNA-dependent DNA polymerases isolated from certain whole tumour-virus particles have come to light (Baltimore, 1970; Temin and Mizutani, 1970) and have been implicated in transformation by these viruses: similar activities, however, have also been shown to be present in normal cells (Scolnick, Aaronson, Todaro and Parks, 1971).

(c) Other enzymes with possible roles in DNA synthesis.

(i) Polynucleotide ligase.

In 1967, Gellert reported the detection of an *in vitro* polynucleotide-joining reaction: in this case, by an extract of *E. coli*. Since then, ligase activity has been demonstrated in mammalian cells (Lindahl and Edelman, 1968) and polyoma virus-infected mouse cells (Beard and Pitts, 1968) and new ligases have been described in T3, T4 and T7 phage-infected *E. coli* (Becker, Lyn, Gefter and Hurwitz, 1967).

In all cases investigated, the reaction involves the sealing of single-strand gaps by the formation of 3'-5' phospho-diester bonds in double-stranded polynucleotides which have 3'-OH and 5'-phosphoryl termini at the gaps.

A common mechanism appears to exist in the reaction (Little, Zimmerman, Oshinsky and Gellert, 1967; Olivera and Lehman, 1967; Olivera, Hall and Lehman, 1968; Weiss, Jacquemin-Sablon, Live, Fareed and Richardson, 1968).

Recently, a novel joining reaction involving end-to-end joining of double-stranded DNA molecules has been reported - catalysed apparently by the same T4 phage-specific polynucleotide ligase that carries out the nick-sealing reaction (Sgaramella, Van de Sande and Khorana, 1970).

It appears, from studies with ligase-defective mutants, that ligase is needed for DNA recombination and for normal DNA synthesis in bacteriophage T4 (Anraku and Lehman, 1969). In E. coli, ligase seems to be necessary for DNA repair; the enzyme's necessity in normal DNA replication is less certain (Pauling and Hamm, 1969; Gellert and Bullock, 1970).

(ii) Polynucleotide kinase.

This enzyme, detected in bacteriophage T2- and T4-infected E. coli and also in rat liver nuclei (Richardson, 1965; Becker and Hurwitz, 1966), catalyses the phosphorylation by ATP of 5'-OH groups of nucleotides ranging from the level of nucleoside 3'-monophosphates to ribo- and deoxyribo-polynucleotides. Therefore, with alkaline phosphatase or the specific 3'- and 5'-polynucleotide phosphatases such as those induced by bacteriophage T4 (Becker and Hurwitz, 1966) interplaying with polynucleotide kinase, single-strand scissions with various termini could be sealed by polynucleotide ligase.

1. 3. 4. DNA degradation - Deoxyribonucleases.

DNA may be degraded in vivo by a large variety of enzymes

called deoxyribonucleases. These enzymes exhibit various specificities and are classified accordingly. For example, deoxyribonucleases which degrade macromolecular DNA from the ends of the molecule hydrolysing nucleotides sequentially, one or two at a time, are called DNA exonucleases. In contrast, the enzymes which attack the DNA molecule along its length, hydrolysing bonds at non-terminal positions, are called DNA endonucleases.

(a) DNA exonucleases.

These show further specificities in their activity eg. in E. coli there exist at least five DNA exonucleases with distinct characteristics (Koerner, 1970). Exonuclease I will attack only denatured DNA up to a final dinucleotide. Exonuclease II, the DNA polymerase I - associated exonuclease, has been described already. Exonuclease III, the DNA phosphatase-exonuclease, will attack only native DNA and exonucleases IVA and IVB are chromatographically distinct oligonucleotide diesterases.

New exonucleases may be induced upon bacteriophage infection, eg. bacteriophage λ has been shown to specify a new DNA exonuclease which attacks from the 5'-end of the DNA (Little, 1968; Little, Lehman and Kaiser, 1968). All the exonucleases so far mentioned produce the same product - deoxyribonucleoside 5'-monophosphates. Bacteriophage T5 induces a DNase with mixed exonucleolytic and endonucleolytic action (Paul and Lehman, 1966) and poxviruses have been shown to specify two exonucleases: one has an acidic pH optimum (McAuslan and Kates, 1966) and the other

has an alkaline pH optimum (Jungwirth, Launer, Dombrowski and Horak, 1969). A DNA endonuclease seems to be specified too and encapsulated in the mature virion together with acid DNA exonuclease (Pogo and Dales, 1969).

(b) DNA endonucleases.

These show analogous specificities to those of the DNA exonucleases. E. coli contains at least four endonucleases (Koerner, 1970). Endonuclease I, for example, attacks both single- and double-stranded DNA to yield oligonucleotides with 5'-phosphoryl termini; endonuclease II specifically nicks alkylated DNA; endonuclease IIIK of E. coli K functions in DNA restriction (Arber and Linn, 1969); a fourth E. coli DNA endonuclease (Introduction section 1. 3. 5.a) is specific for u.v.-irradiated DNA.

Several bacteriophages of E. coli produce new DNA endonucleases. Bacteriophage T4, for example, induces at least three, and possibly five or six new endonucleases (Koerner, 1970). T4 endonuclease II is an early enzyme introducing predominantly single-strand breaks into native DNA; but the enzyme has no effect on T4 DNA. T4 endonuclease IV preferentially introduces breaks into single-stranded DNA to produce oligonucleotides (150 nucleotides long). This enzyme introduces scissions exclusively next to cytosine residues and again is inactive on T4 DNA. These two endonucleases, together with the products of phage genes 46 and 47 and possibly with host and phage-induced DNA exonucleases, have been implicated in host DNA breakdown which produces deoxyribo-

nucleoside 5'-monophosphates for phage DNA synthesis (Sadowski and Hurwitz, 1969). T4 endonuclease V, specific for u.v.-irradiated DNA, to be discussed later (Introduction section 1. 3. 5.a), and two DNA-nicking enzymes - T4 endonucleases A and B - have been reported to produce single-strand breaks in double-stranded DNA (Ando, Takagi, Kosawa and Ikeda, 1970). Endonuclease A seems to be a completely new enzyme: endonuclease B, however, may be identical to T4 endonuclease II.

We see, even from the small selection of examples cited, that a great variety of deoxyribonucleases exist with widely different specificities. In some cases their in vivo function is clear, as in the case of the phage-induced endonucleases: the in vivo significance of many others, however, and the reason for their association with DNA synthesis remains obscure (Lehman, 1967).

1. 3. 5. DNA repair and recombination.

(a) DNA repair.

DNA repair is the process by which regions of damaged DNA in an organism are restored to their original intact state.

The main types of lesion in damaged DNA are as follows:-

(i) Intra-strand pyrimidine dimers - the main lethal consequence of u.v.-irradiation of DNA.

(ii) Single-strand or double-strand scissions in the DNA, which may result from its interaction with DNases, alkylating agents or ionising radiation.

(iii) Inter-strand cross-linking which may be caused by u.v.-irradiation or by agents such as mitomycin C and the bifunctional alkylating agents nitrogen- and sulphur mustard.

(iv) Mis-pairing of bases, caused by the incorporation of base analogues or incorrect bases in the course of DNA replication; this may produce localised deformations in the DNA.

DNA repair following u.v.-irradiation.

This is the best-studied system and involves the repair of DNA-containing pyrimidine dimers. There appear to be three main mechanisms available for the repair of this type of lesion: photoreactivation; excision-repair; and post-replicative repair involving recombination (Howard-Flanders, 1968).

Photoreactivation.

In this enzymic reaction, transformation of the dimer to monomers occurs in the presence of visible light. The other two processes constitute dark-reactivation.

Excision-repair.

This has been proposed to involve firstly, an endonucleolytic single-strand scission near the thymine dimer; secondly, exonucleolytic removal of the dimer and surrounding nucleotides; thirdly, repair synthesis using the intact strand as template and finally, a ligase-type of sealing reaction which completes the process.

Several enzymes which might carry out these steps in vivo have been reported. An endonuclease specifically introducing

single-strand nicks into u.v.-irradiated DNA and an exonuclease which, acting with the former enzyme, causes specific excision of thymine dimers, have been described in M. luteus (Kushner, Kaplan and Grossman, 1970; Nakayama, Okubo and Takagi, 1971; Okubo, Nakayama and Takagi, 1971) and an enzyme similar to the M. luteus endonuclease exists in E. coli strain 1100 (Takagi et al, 1968).

As already mentioned, the Kornberg DNA polymerase I of E. coli has been shown to have a 5' → 3' combined exonuclease and endonuclease action on u.v.-irradiated DNA resulting in thymine dimer excision. A model for DNA excision-repair in E. coli, involving only three enzymes: a u.v.-specific endonuclease, the Kornberg DNA polymerase I, and polynucleotide ligase, has thus been proposed (Kelly et al., 1969). In support of this model, a ligase-defective mutant (Pauling and Hamm, 1969) and a polymerase I-defective mutant (De Lucia and Cairns, 1969) of E. coli have both been shown to be u.v.-sensitive.

In support of the involvement of DNA polymerase in excision-repair in eucaryotes, one of two species of DNA polymerase is strongly induced in Tetrahymena cells following excision-repairable radiation damage to their DNA (Westergaard, 1970; Keiding and Westergaard, 1971).

The v gene of bacteriophage T4 appears to specify a u.v.-specific endonuclease which may carry out the first step in excision-repair of T4 DNA (Sekiguchi et al., 1970: Yasuda and Sekiguchi, 1970a,b).

There therefore appears to be considerable experimental support for the proposed scheme for excision-repair.

Post-replicative repair, involving recombination.

It appears that, when thymine dimers exist in DNA, replication of the DNA leaves gaps opposite the dimers; these cannot be repaired by the excision-repair mechanism and it requires a recombinational event, probably involving an ATP-dependent exonuclease (Barbour and Clark, 1970; Oishi and Rola, 1970) to produce an intact DNA.

(b) DNA recombination (Genetic recombination).

Closeness and homology granted, double-stranded DNA molecules *in vivo* may interact, probably by a common mechanism, to produce recombinant versions of themselves ie. DNA molecules of mixed parentage.

A number of models for recombination have been proposed (Davern, 1971) but for a long time two main theories thrived: (i) breakage and reunion and (ii) copy choice.

(i) In the breakage and reunion model the paired strands line up exactly opposite each other - by a totally unknown mechanism - then breaks appear at the same positions in each molecule and a crossing over occurs: the resulting strands consist of the original molecules up to the breakage point and, after this, the remainder of the opposite molecules.

(ii) In the copy choice scheme, the cross-over arises owing to replication and the theory has fallen into disfavour because of

the demonstrated absence of DNA synthesis during recombination (Frey and Melechen, 1965; Simon, 1965). The breakage and reunion model is thus now favoured; although even it does not seem to account for all recombination (Fox, 1966).

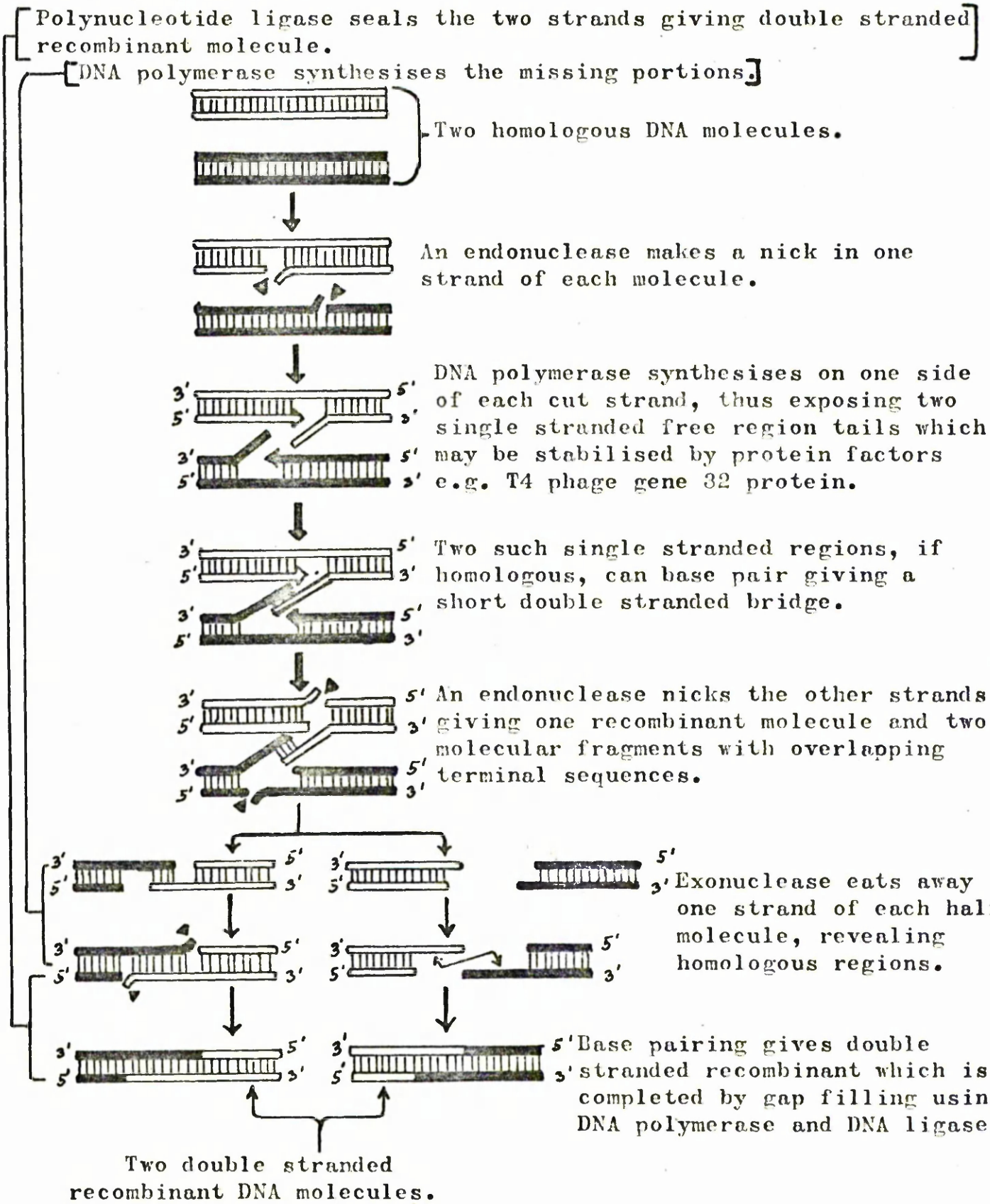
Several mechanistic variants have been proposed for the break-reunion model including the hybrid-overlap model (Fig. I.4.). Considerable supportive evidence from *in vitro* studies now exists for such models. For example, in *E. coli*, normal recombination appears to involve the interaction of the rec A gene product (Hout, Van de Putte, De Jounge, Schuite and Oosterbaan, 1970) with an ATP-dependent DNA exonuclease (Buttin and Wright, 1968; Barbour and Clark, 1970: Oishi and Rola, 1970). *E. coli* also possesses appropriate endonucleases (Friedberg and Goldthwait, 1968) and, of course, DNA polymerase I and polynucleotide ligase: together these enzymes would be sufficient, in theory, to allow the breakage and reunion hybrid-overlap mechanism to operate.

In bacteriophage T4, the exact enzymes involved in recombination are unknown, but the T4 genes 46, 47 and x are necessary for normal levels of recombination and genes 46 and 47 seem to specify DNases; in addition, the gene 32 protein is indispensable (Fig. I.4.).

The bacteriophage λ *red* system seems to comprise two or three cistrons which code for the λ-exonuclease and β-protein which together operate as an aggregate with a definite

Fig. I. 4.

Speculative Mechanism of Genetic Recombination in Accordance with the Breakage and Reunion Model.



function in recombination (Carter and Radding, 1970, 1971; Radding, 1970: Cassuto and Radding, 1971; Davern, 1971; Radding, Rosenzweig, Richards and Cassuto, 1971).

Thus several deoxyribonucleases, polynucleotide ligase and a denatured DNA-stabilising protein (gene 32 protein) have experimental support for their implication in DNA recombination.

1. 3. 6. Other aspects of DNA metabolism.

Macromolecular DNA is involved in other metabolic processes which include: (a) methylation of the polynucleotide structure (Borek and Srinivasan, 1966; Srinivasan and Borek, 1966); (b) glucosylation of the hydroxymethylcytosine residues in the case of the T-even phage DNA molecules; (c) host-controlled modification and restriction, involving both methylation and nucleolytic processes (Arber and Linn, 1969) and, of the utmost importance, (d) the expression of the genetic message involving transcription and translation, the processes of which have been recently described and discussed concisely by Davidson (1969) and Watson (1970).

1. 4. Correlation of *in vivo* and *in vitro* observations on DNA biosynthesis and replication.

1. 4. 1. The *in vivo* significance of DNA polymerases, ligase and DNases.

The Kornberg DNA polymerase I of *E. coli* seemed likely

to be the DNA replicase of that organism for many years although, from time to time, items of evidence caused minor doubts eg. the enzyme seemed unable to make transforming DNA *in vitro* from a native DNA template, the product being unnatural by several criteria (Inman, Schildkraut and Kornberg, 1965; Kornberg, 1967) although total *in vitro* synthesis of infective ØX174 DNA was subsequently achieved with the help of polynucleotide ligase (Goulian, Kornberg and Sinsheimer, 1967).

The reassurance that this latter impressive achievement produced, however, was dispelled by a series of reports on *E. coli* mutants lacking DNA polymerase I. De Lucia and Cairns (1969) and Gross and Gross (1969) studied such a mutant which had less than 1% of the normal level of DNA polymerase I: the variant multiplied normally but it had an increased sensitivity to u.v. light. Later, Kohiyama and Kolber (1970) reported that, although several classes of *E. coli* mutants had been isolated which were temperature-sensitive for DNA replication, none of them possessed a temperature-sensitive DNA polymerase I. Finally, the Okazaki group demonstrated the normal formation of the fragmentary intermediates of DNA synthesis *in vivo* in the polymerase-less mutant described by De Lucia and Cairns (Okazaki *et al.*, 1970).

The data of these and other groups of workers suggest that there are at least two DNA-synthesising activities in the bacterial cell: the classical DNA polymerase I - now believed to have a role in DNA repair rather than replication - and a membrane-

associated enzyme system (Smith, Schaller and Bonhoeffer, 1970; Knippers and Stratling, 1970; Knippers, 1970) which appears to contain a distinct DNA-synthesising activity-DNA polymerase II - which may be a component of, or the complete, E. coli DNA replicase.

The recent indications that the DNA polymerase I of E. coli is an enzyme of DNA repair rather than the true DNA replicase of this bacterium and the general resemblance of the enzyme to the DNA polymerases of most sources have laid open to question the in vivo roles of the multitude of DNA polymerases isolated not only from other bacteria, but also from the many eucaryote sources: the in vitro experiments with isolated nuclei described earlier considerably add to the doubts. Only the phage-induced DNA polymerases, as discussed earlier, seem to be, almost certainly, true replicases - although conceivably even they might only function in repair or "editing" steps essential for phage DNA replication. It is encouraging, nevertheless, to recall that these phage polymerases, in many respects, are very similar to the mammalian DNA polymerases.

The experiments with ligase-defective mutants mentioned earlier have implicated polynucleotide ligase in DNA replication and likewise Lehman (1967) has suggested that DNases must have some role in DNA replication, from the many strong correlations found between DNA synthesis and DNase activity: DNases are also indispensable in many of the proposed models for DNA synthesis.

1. 4. 2. Models for DNA synthesis.

In order to correlate the *in vivo* findings on DNA replication with the *in vitro* properties of the Kornberg DNA polymerase I a large number of models for DNA synthesis were proposed.

These include the "pre-fork"model of Haskell and Davern (1969) and the "knife and fork" model (Guild, 1968; Richardson, 1969) - a model designed to overcome the difficulty of the simultaneous growth of two strands from the replicating point using an enzyme which synthesises DNA in the 5' → 3' direction only, and which does not utilise deoxyribonucleoside 3'-triphosphates. The latter model can be incorporated into the "rolling circle" model (Gilbert and Dressler, 1968; Richardson, 1969) (Fig. I.3.) - a scheme which has considerable experimental support. The master strand model for DNA replication (Kubitschek and Henderson, 1966) has recently been shown to be invalid (Russo, Stahl and Stahl, 1970; Becker and Hurwitz, 1971).

A major problem in DNA replication, often neglected in such models, is the apparent need for unwinding the double helix (the only alternative being frequently-produced single-strand breakages). In support of this seeming requirement, the DNA polymerases of many sources have either an absolute requirement or a preference for single-stranded DNA (Bollum, 1959; Aposhian and Kornberg, 1962). Cairns (1963) proposed that a linker in the form of a wheel might exist in the *E. coli* cyclic chromosome; this

would allow rotation, and so unwinding, of the DNA molecule. Erhan (1968) proposed that DNA polymerase molecules were made up of at least three structural units, one of which he postulated to act as a "wedge" - an initiator which separated the strands which were subsequently replicated using the other two subunits. Later, he described a low molecular weight factor from ascitic fluid, the properties of which were consistent with those expected of the "wedge" (Erhan, Reischer, Franko, Kamath and Rutman, 1970). Alberts and Frey (1970) showed that the product of bacteriophage T4 gene 32 - a structural protein essential for replication and recombination of DNA, facilitated DNA denaturation and renaturation by binding stoichiometrically to single-stranded DNA and maintaining it in an extended, non-hydrogen-bonded form. Its usefulness in recombination and replication can easily be envisaged (Fig. 1. 3.). The additional fact that it forms complexes with T4 DNA polymerase encourages the view that it may indeed be the "unwinder" of the double helix. Similar kinds of factors have been described from other sources (Paetkau, 1969; J.M. Morrison, personal communication).

The fact that the E. coli DNA polymerase II, the T-phage DNA polymerases, and other polymerases which may be true DNA replicases, have such similar properties to DNA polymerase I mitigates the recent indications that the latter enzyme could not be the replicase involved in most of the models proposed, and it seems likely that e.g. the "rolling circle - knife and fork" combination of models could still be viable, as suggested by experiment, using

a replication complex in which DNA polymerase I is replaced by DNA polymerase II or some other similar activity.

2. VIRUSES AND THEIR REPLICATION - WITH PARTICULAR REFERENCE TO HERPES SIMPLEX VIRUS.

2. 1. The general nature of viruses.

Viruses are potentially pathogenic entities with an infectious phase, replicating strictly intracellularly, their genomes consisting of an element of either DNA or RNA. Viruses multiply in the form of their genetic material and are unable to grow or to undergo binary fission. They are devoid of a system of enzymes for energy production and they reproduce inside living cells using the host's synthetic machinery, ribosomes included, to direct the synthesis of the virus particle or virion which contains the viral genome and transfers it to other cells (Lwoff, 1957; Luria and Darnell, 1967). Fenner (1968) classified animal viruses into two main groups: the deoxyriboviruses and the riboviruses. The herpesvirus group, to which herpes simplex virus belongs, forms one of the five main groups of deoxyriboviruses of vertebrates that Fenner proposed and defined: it embraces a large and growing number of viruses (Andrewes, 1964).

2. 2. The structure and chemical composition of herpes simplex virus.

Basically, all viruses consist of a molecule of nucleic acid enclosed within a protective coat - the capsid - made up of repeating subunits of protein - the capsomeres. The capsid, which may be composed of several layers and which may be of helical, isometric (quasi-spherical or cubic) or complex symmetry, when considered with its enclosed nucleic acid, is called the nucleocapsid which, in turn, may be enclosed within a lipoprotein envelope called the peplos.

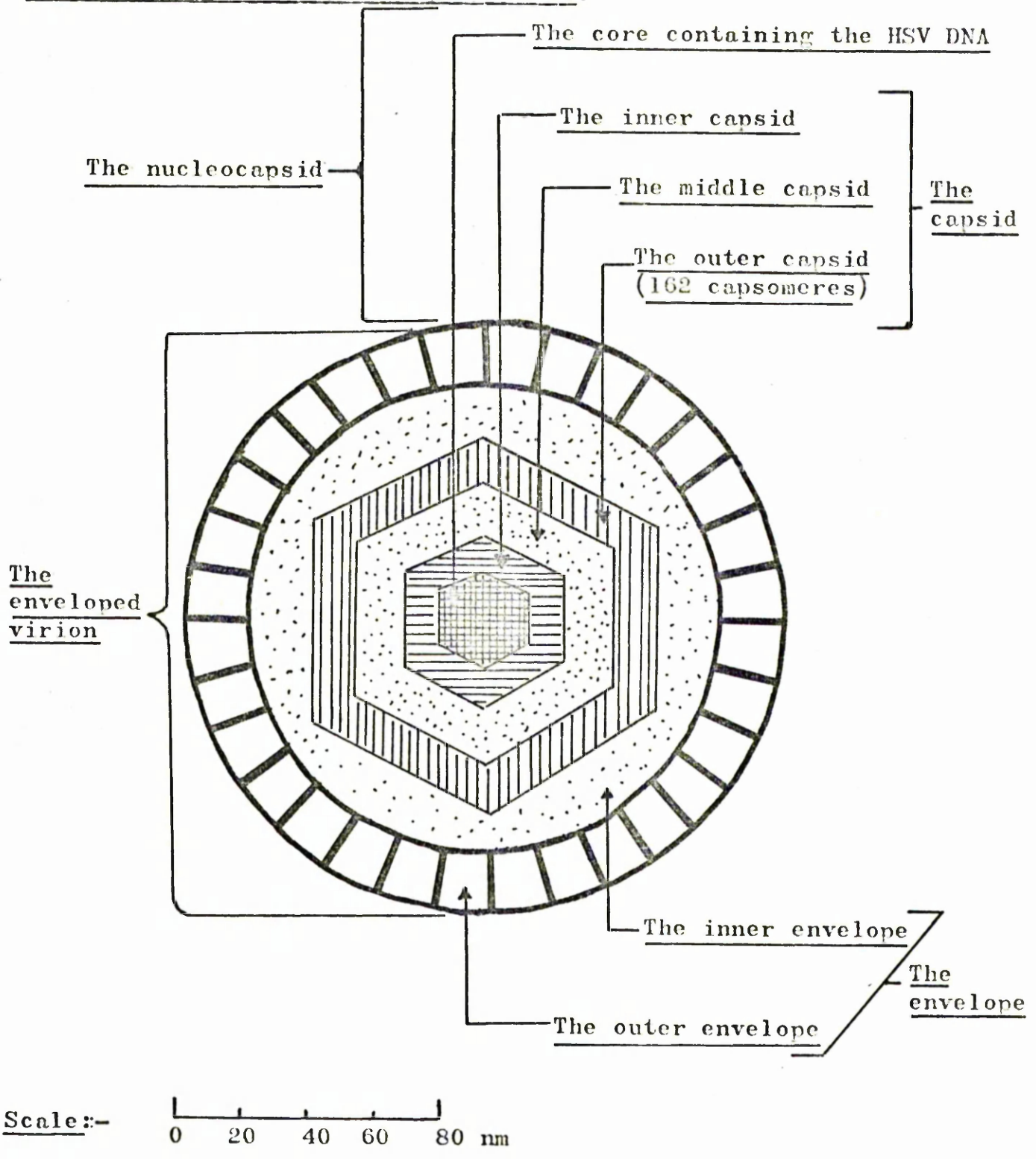
In particular, herpes simplex virus appears to consist of a core and a three-layered icosahedral capsid with 162 capsomeres in the outer layer, the whole being enclosed by a two-layered envelope (Roizman, 1969). The overall structure and dimensions of the virion are shown in Fig. I.5.

A chemical analysis of purified suspensions of the virus revealed the following: Protein, 70%; phospholipid, 22%; carbohydrate, 1.5%; DNA, 6.5%; RNA, less than 0.1% (Russell, Watson and Wildy, 1963).

The genome appears to consist of a single molecule of double-stranded DNA of molecular weight $(70 - 100)\times10^6$ (Russell and Crawford, 1964; Becker, Dyrn and Sarov, 1968). The base composition is 68% (G+C)(Russell and Crawford, 1964), and so is widely different from that of the DNA of the mammalian cells which the virus infects (40-44%(G+C)) (Davidson, 1969). The nearest-neighbour frequency pattern of the viral DNA is much closer to those of bacteria than to those of mammals (Subak-Sharpe et al.,

Fig. I. 5.

The Structure of Herpes Simplex Virus.



1966b). Furthermore, there are no known unusual bases in the DNA and the nucleic acid is not methylated (Low, Hay and Keir, 1969).

The structural proteins of purified HSV, from polyacrylamide gel electrophoresis studies with labelled virus, appear to number at least seven and possibly eight or nine. These have been numbered I - IX from the origin and their molecular weights range from 100,000 - 24,000. Two of the proteins constitute the viral capsid, two are the viral core proteins and three glycoproteins are present in the viral envelope (Olshevsky and Becker, 1970a,b).

The phospholipid of the envelope has not been chemically analysed but it is likely that the lipids are specified by the host cell. The protein moiety of the envelope appears to be derived both from host and from virus-specified proteins and so the envelope contains both host- and virus-specific antigenic determinants (Watson and Wildy, 1963; Roizman and Spring, 1967).

2. 3. The Replication of herpes simplex virus.

2. 3. 1. The relationships between HSV and its host.

Several possible relationships between HSV and its host seem to be possible (Kaplan 1969; Roizman 1969).

(i) A lytic infection is common in which the host cell is finally killed and lysed as the virus productively infects the cells. A large number of cytopathic effects are found including

giant-cell formation, rounding-off of cells, margination of the chromatin, nucleolar disruption, chromosome aggregation and breakage, and prevention or abortion of mitosis.

(ii) Latent relationships are possible in which eg. recurrent herpetic eruptions occur at a site: they heal, the virus apparently disappearing from the epithelia and seeming to lie latent in the sensory nerves or ganglia supplying the area until the next episode occurs.

(iii) A case is growing for the capability of HSV to cause neoplasms in the lips (HSV type I) or in the cervix uteri (HSV type II) (Kvasnicka, 1964; Josey, Nahmias and Naib, 1968).

2. 3. 2. The Infective cycle of HSV.

Knowledge of the events in the infective cycle of HSV is much less advanced than is the case with eg. bacteriophage T4 (Luria and Darnell, 1967; Watson, 1970): nevertheless a large amount of information exists, the process appearing to be broadly typical of that of DNA viruses in general (Fenner, 1968; Watson, 1970) but with some special features (Fenner, 1968; Roizman, 1969).

For the purposes of description, the events have been divided into 5 stages: (a) adsorption; (b) penetration and uncoating; (c) eclipse period - early events; (d) eclipse period - late events (HSV assembly and maturation) and (e) release of the virus.

(a) Adsorption.

Adsorption of both enveloped and non-enveloped virus to host cells seems to occur although the enveloped virions adsorb more readily (Holmes and Watson, 1963).

(b) Penetration and uncoating of HSV.

Penetration is temperature-dependent and requires the expenditure of energy by the cell; but, once the virus is adsorbed, penetration is relatively rapid. Many workers reported from electron microscopic evidence that immediately after penetration, nucleocapsids could be discerned free in the cytoplasm and in "vacuoles" from which they concluded that the virus was taken into the cell by pinocytosis. Morgan, Ellison and Mednis (1968), however, proposed that infection begins with fusion of the viral envelope with the cell-membrane leading to the release into the cytoplasm, and the uncoating of the nucleocapsid, the process occurring in five stages: (i) attachment; (ii) digestion of the viral envelope; (iii) digestion of the cell wall; (iv) passage of the nucleocapsid directly into the cytoplasm and (v) digestion of the capsid with release of the core whence the nucleic acid migrates into the nucleus.

(c) The eclipse period - early events.

The previous two stages comprise the process of infection: they are followed by the eclipse period, in which no whole infective virus exists. This period is characterised by intense biochemical activity in which the synthesis of viral mRNA, virus-induced

enzymes and other proteins, viral DNA and virus structural components, occurs together with the inhibition of host-cell macromolecular synthesis: late in this period the assembly of new virions and their maturation at the nuclear membrane then completes the replication of the virus and marks the end of the eclipse phase.

RNA metabolism in HSV-infected cells.

Evidence exists that transcription of DNA is needed for viral multiplication (Sauer and Munk, 1966). In support of this, RNA with the characteristics of mRNA has been demonstrated in HSV-infected cells 1.5-14h after infection: the RNA is moderately large (12-36S), it anneals to HSV DNA, resembles HSV DNA in base composition, and is rapidly labelled (Hay, Kŏteles, Keir and Subak-Sharpe, 1966; Flanagan, 1967). As yet, however, no demonstration has been achieved of different species of mRNA corresponding to the "pre-early", "early" and "late" mRNA of T4-infected cells (Watson, 1970).

The synthesis of total RNA and of most of the individual species of RNA in HSV-infected cells falls on initiation of infection (Fig. I.7.). Ribosomal RNA synthesised before infection is stable up to 6h after infection and rRNA continues to be synthesised but at a linearly decreasing rate (Hay et al., 1966).

The origin of 4S RNA, the synthesis of which declines but continues at a low rate after infection has been investigated. Some evidence suggests that it may be at least in part viral and,

as in T-phage infection (Daniel, Sarid and Littauer, 1968; Weiss, Hsu, Foft and Scherberg, 1968), that the RNA synthesised might include new virus-specified species of tRNA (Subak-Sharpe, Shepherd and Hay, 1966a; Hay, Subak-Sharpe and Shepherd, 1967; Morris, Wagner and Roizman, 1970).

There is evidence that viral RNA is made in the nucleus (Wagner and Roizman, unpublished observations) but nothing is known of the source of the RNA polymerase producing the RNA (Fig. 1.8.).

Methylation of rRNA and tRNA also declines in HSV-infected cells but not owing to an SAM-cleaving enzyme as in T3 phage infection (Hay and Low, 1970).

Protein synthesis in HSV-infected cells.

Sydiskis and Roizman (1966) showed that, in HSV-infected cells, the rate of protein synthesis at first declines, then is stimulated between 4 and 8h after infection, before finally irreversibly declining from 9-16h (cf. Fig. I.7). The shift in the general synthesis of proteins from cellular to viral in origin suggested above is reflected in the profiles of polysomes in sucrose gradient centrifugation. As yet, however, no demonstration of the virus-specificty of the RNA and nascent peptides associated with the polysomes has been reported. In support of the cytoplasmic locus of synthesis of viral proteins, an intracellular flow of structural viral proteins from cytoplasm to nucleus has been demonstrated (Olshevsky, Levitt and Becker, 1967).

Among the proteins synthesised in HSV-infected cells are the virus-induced enzymes the known ones of which catalyse reactions associated with DNA metabolism (Introduction section 2. 4. 3.) (Fig. I.8.). Detailed examination of the HSV-induced proteins (Spear and Roizman, 1968) has revealed the appearance of 25 discrete species of protein in the cytoplasm after infection, most showing migration to the nucleus and some being ascribed to parts of the structure of the virions. Similar aspects of viral antigens have been investigated (Watson et al, 1966; Nii, Morgan, Rose and Hsu, 1968).

The synthesis of HSV DNA.

The overall pattern of total DNA synthesis in HSV-infected cells is shown in Fig. I.7. The pattern is largely owing to viral DNA synthesis since within a few hours of infection, HSV DNA synthesis almost completely replaces cellular DNA synthesis which declines from 0-2.5h, levels off between 2.5 and 6h, then irreversibly declines (Roizman and Roane, 1964; Russell et al., 1964; Roizman, 1969). Viral DNA synthesis takes place in the nucleus (Newton and Stoker, 1958; Munk and Sauer, 1964; Sydiskis and Roizman, 1966).

The DNA of HSV is not a substrate for DNA methylation in vivo (Low et al., 1969) but the nucleic acid seems to undergo recombination allowing the genetic analysis of the virus to be undertaken (Hay, Perera, Morrison, Gentry and Subak-Sharpe, 1971).

(d) The eclipse period - late events (the assembly and maturation of HSV).

Encapsidation.

Viral DNA seems to be randomly withdrawn from the DNA pool, slowly and inefficiently and first becomes incorporated into a structure impermeable to DNase but distinct from the virion (Roizman, 1969). From the evidence of some 100 papers involving electron microscopic examination (Roizman, 1969), the process seems to occur by sequential addition of capsid layers to cores since possible intermediate forms with what appear to be one or all three capsid layers have been discerned, together with nucleocapsids with what seem to be inner envelopes added to them.

Envelopment of the HSV nucleocapsids (maturation).

It is now widely accepted that the nucleocapsid is enveloped mainly by the inner lamellae of the nuclear membrane as the virus leaves the nucleus; it seems however that it may also be enveloped by other cell membranes (Epstein, 1962; Siminoff and Menefee, 1966; Nii, Morgan and Rose, 1968). The envelope seems to be required for maximum infectivity of the virus (Smith, 1964; Watson et al., 1964; Stein, Todd and Maloney, 1970).

(e) The Release of HSV from infected cells.

Virus release is temperature-dependent, and the viruses issuing from the infected cells consist largely of enveloped nucleocapsids. The mechanism by which the virions move from nucleus to extracellular fluid had been controversial until recent

studies by Schwartz and Roizman (1969) showed that a network of branched tubules, continuous with both nuclear and cytoplasmic membranes at their extremities and absent in uninfected cells, become apparent in HEp-2 cells eight hours after infection with the virus. At this time enveloped nucleocapsids appeared at the junction of the tubules with the nucleus and at 16h after infection, virions filled the tubules and appeared outside the cell.

2. 4. Virus-induced enzymes.

2. 4. 1. Bacteriophage-induced enzymes.

The demonstration of the presence of a new enzyme activity - dCMP hydroxymethylase (Flaks and Cohen, 1957) - in T2 phage-infected *E. coli* laid the foundations for a vast new area of research - the study of virus-induced enzymes.

The problems involved in the determination of the origin of the new dCMP hydroxymethylase were recognised and stated and the genetic origin rigorously examined: this has served as a model for subsequent work (Cohen, 1968).

Virus-induced increases in enzyme activity might reflect: (i) removal of an inhibitor from, or addition of a stimulator to, an existing host enzyme; (ii) increased stabilisation or decreased destruction of a host enzyme; (iii) the addition of a peptide to, or the removal of a peptide from, an existing host enzyme; (iv) the derepression of the synthesis of a host enzyme not usually

expressed or (v) the synthesis of a new enzyme specified by the viral genome. Clearly then, although we may suspect that an enhanced or new activity represents a virus - specified enzyme, much effort may be involved in establishing this.

The enzymes induced by T-even phages are the best-characterised and understood of the large number of virus-induced enzymes now known: already over thirty phage-induced enzymes and other new proteins have been recognised in T4-infected cells: these are summarised in Fig. I.6. It may be seen that all the enzymes, with the exception of the proteinase, are concerned with either the synthesis, degradation or modification of DNA, and that the synthetic enzymes all act at stages at or beyond the nucleoside monophosphate level. The T-even phage enzymes can be seen to effect the cessation of synthesis and the degradation of host DNA together with the channelling of host DNA breakdown products to DNA synthesis pathways for viral DNA: viral DNA synthesis is selectively highly favoured.

2. 4. 2. Enzymes induced by deoxyriboviruses of animals.

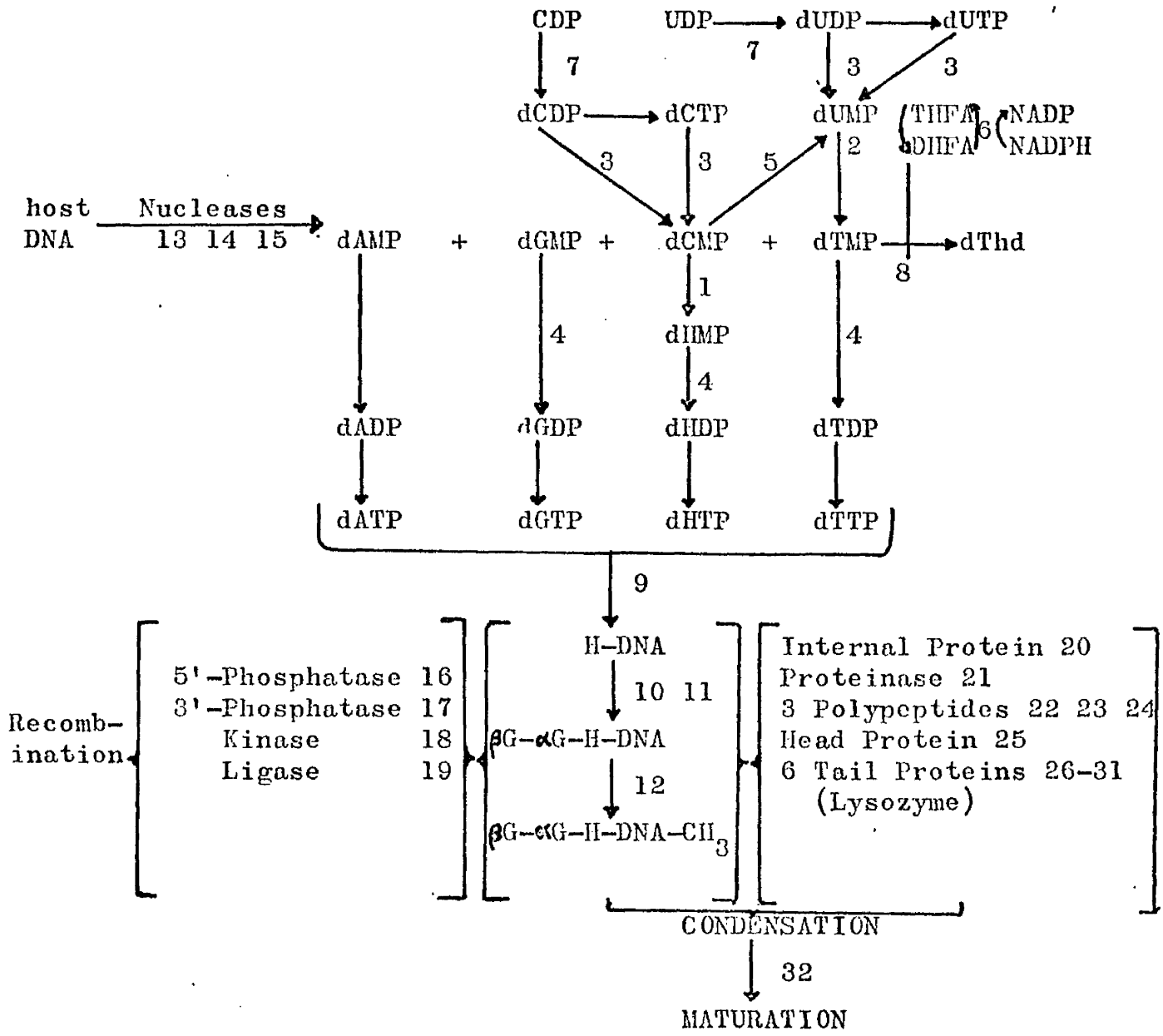
Increased levels of DNA polymerase appear to be induced, not only by infection by bacteriophages T2, T4 and T5, but also by vaccinia virus, HSV, pseudorabies virus, adenovirus SV15, polyoma virus and SV40. The first six mentioned appear to be new virus-specified enzymes; the latter three have less certain genetic origins, although most probably host.

Fig. I. 6.

List of T4 phage-induced enzymes and other proteins.

(1) Deoxycytidylate hydroxymethylase
(2) Thymidylate synthetase
(3) dCTPase (Deoxycytidine triphosphatase)
(4) Deoxynucleotide Kinase
(5) Deoxycytidylate deaminase
(6) Dihydrofolate reductase
(7) Ribonucleotide reductase
(8) dTMPase inhibitor ?
(9) DNA polymerase
(10) α-glucosylating enzymes
(11) β-glucosylating enzymes
(12) DNA methylase
(13) Exonuclease A
(14), (15) DNA endonucleases including: endonuclease II; endonuclease IV; endonuclease V; endonuclease A; ? endonuclease B
(16) Polynucleotide 5'-phosphatase
(17) Polynucleotide 3'-phosphatase
(18) Polynucleotide Kinase
(19) Polynucleotide ligase
(20) Internal protein
(21) Proteinase
(22), (23), (24) 3 polypeptides
(25) Head protein
(26) – (31) 6 tail proteins including lysozyme
(32) Maturation factor
(33) Gene no.32 product for DNA replication and recombination
(34) σ factor

Fig. I. 6.


CDP
UDP
dUDP
dUTP
7
7
3
3
dCDP
dCTP
dUMP
THFA
DHFA
6
NADP
NADPH
3
3
5
2
host DNA
Nucleases
13 14 15
dAMP
+
dGMP
+
dCMP
+
dTMP
dThd
8
1
4
dIMP
4
4
dADP
dGDP
dHDP
dTDP
dATP
dGTP
dHTP
dTTP
9
Recomb-
ination
5'-Phosphatase 16
3'-Phosphatase 17
Kinase 18
Ligase 19
H-DNA
10 11
βG-αG-H-DNA
12
βG-αG-H-DNA-CH3
Internal Protein 20
Proteinase 21
3 Polypeptides 22 23 24
Head Protein 25
6 Tail Proteins 26-31
(Lysozyme)
CONDENSATION
32
MATURATION

New DNases are specified by bacteriophages T2, T4, T5 (Introduction section 1. 3. 4.) and probably by T3 in addition to bacteriophage λ (an exonuclease and an endonuclease). Vaccinia virus seems to code for three DNases; HSV and the related pseudorabies virus appear to specify at least one new exonuclease each.

After infection by certain animal viruses, several other enzymes of nucleic acid metabolism appear to be synthesised in increased amounts (Kit and Dubbs, 1969), eg. (i) thymidine kinase (ii) deoxycytidylate deaminase and (iii) dTMP kinase.

DNA-dependent RNA polymerase activity has been shown to increase owing to a new virus-specific RNA polymerase enzyme in T7 and T3 infection of E. coli (Chamberlin, McGrath and Waskell, 1970; Maitra, 1971) and new virus-specified σ factors have been demonstrated in T-even phage infection (Travers, 1969; Watson, 1970): no convincing cases however of new animal DNA virus-specified DNA-dependent RNA polymerases have been demonstrated, although vaccinia virus has been shown to contain DNA-dependent RNA polymerase, of unknown genetic origin, encapsulated in the virion. (Kates and McAuslan, 1967).

2. 4. 3. HSV- Induced enzymes.

HSV has been reported to cause elevations in the following enzyme activities: - DNA polymerase, alkaline deoxyribonuclease, thymidine kinase, dCMP deaminase, deoxycytidine

kinase and dTMP kinase (Keir, 1968; Perera and Morrison, 1970).

The following enzymes have been shown not to increase in level:- acid DNase, alkaline phosphatase, RNase and pyrophosphatase (Morrison, 1967; Morrison and Keir, 1968a); dTMP synthetase and tetrahydrofolate dehydrogenase (Frearson, Kit and Dubbs, 1965, 1966); deoxyadenosine- and deoxyguanosine kinases (Perera and Morrison, 1970): the position with regard to DNA-dependent RNA polymerase is less certain (Keir, 1968).

A summary of the time course of induction of some of the known HSV-induced functions is presented in Fig. I. 7: Fig. I. 8. summarises both established and hypothetical functions induced by HSV-infection - some, or all of which, may be specified by the HSV genome.

(a) Thymidine kinase.

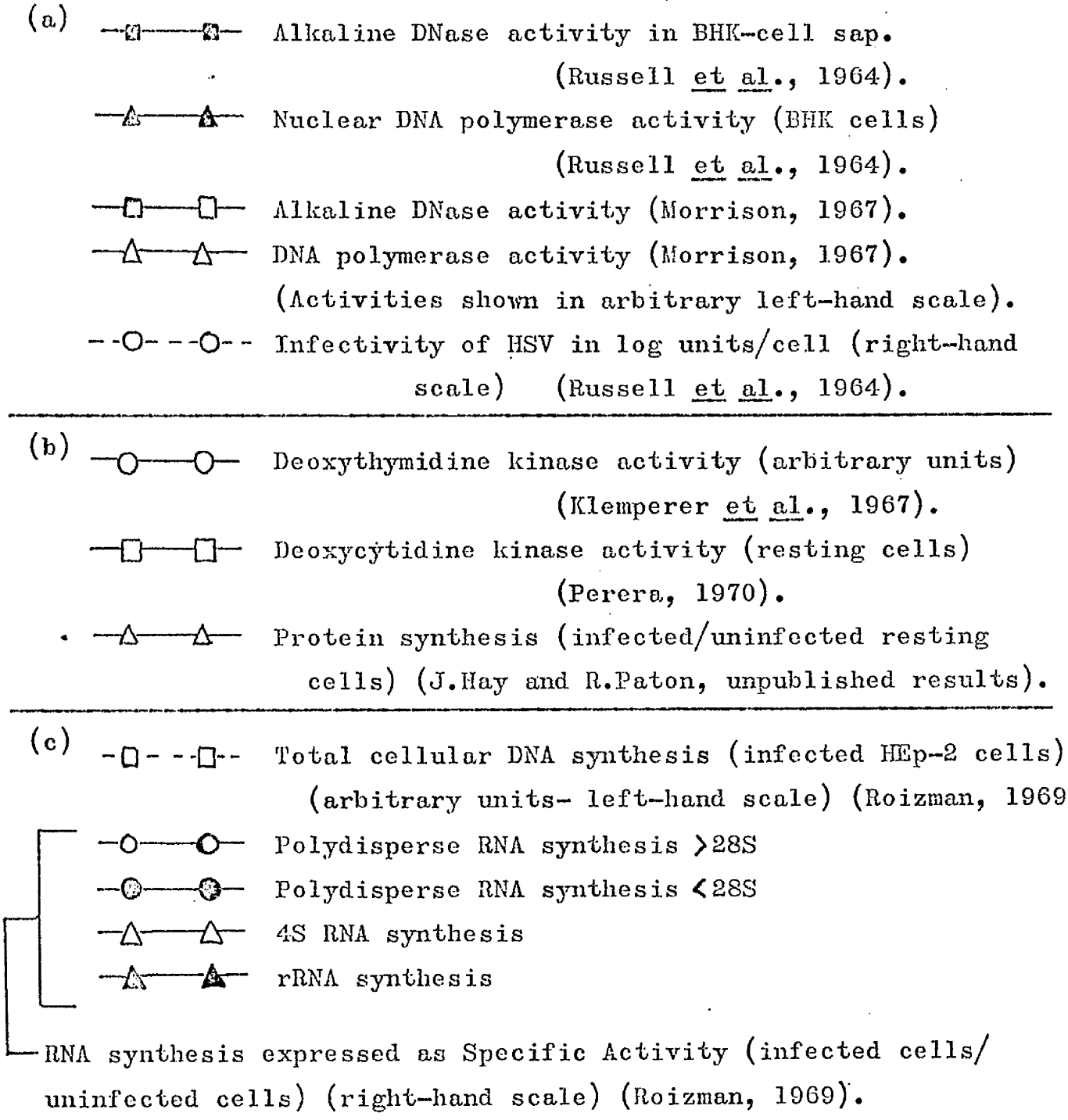
The increase in activity of this enzyme has been convincingly shown to represent the induction of a new protein different from the host enzyme, on the basis of several criteria (Klemperer, Haynes, Shedden and Watson, 1967). Furthermore, a strain of cells lacking thymidine kinase acquires the activity after infection with the virus (Kit and Dubbs, 1963a,b; Dubbs and Kit, 1964, 1965) and the induction of the enzyme requires RNA and protein synthesis. Taken together, the evidence is strongly suggestive of a viral origin for the enzyme.

(b) dTMP kinase.

Levels of this enzyme were reported to be increased in

Fig. I. 7.

Time course of events after HSV-infection of mammalian cells grown in culture.



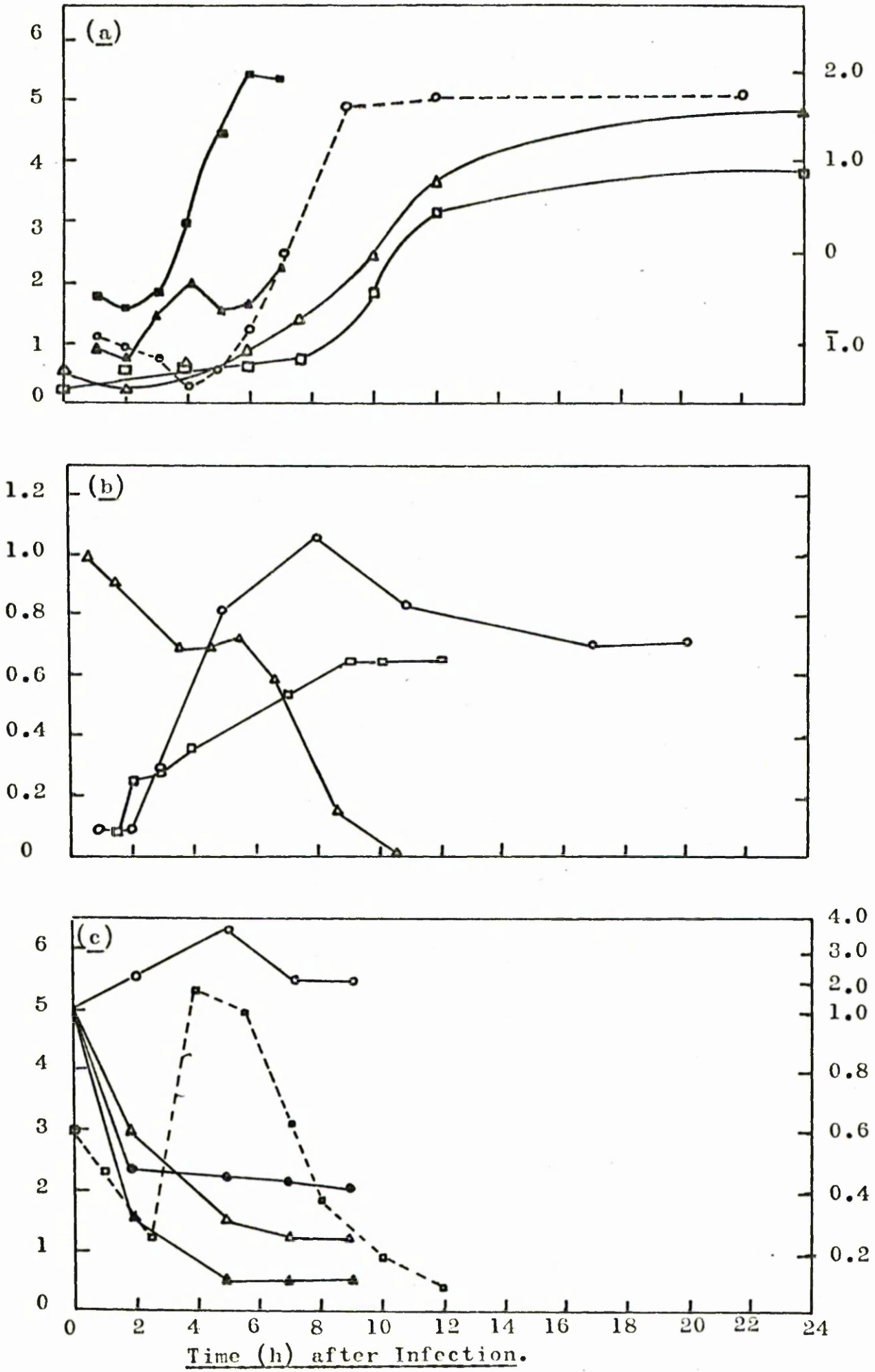
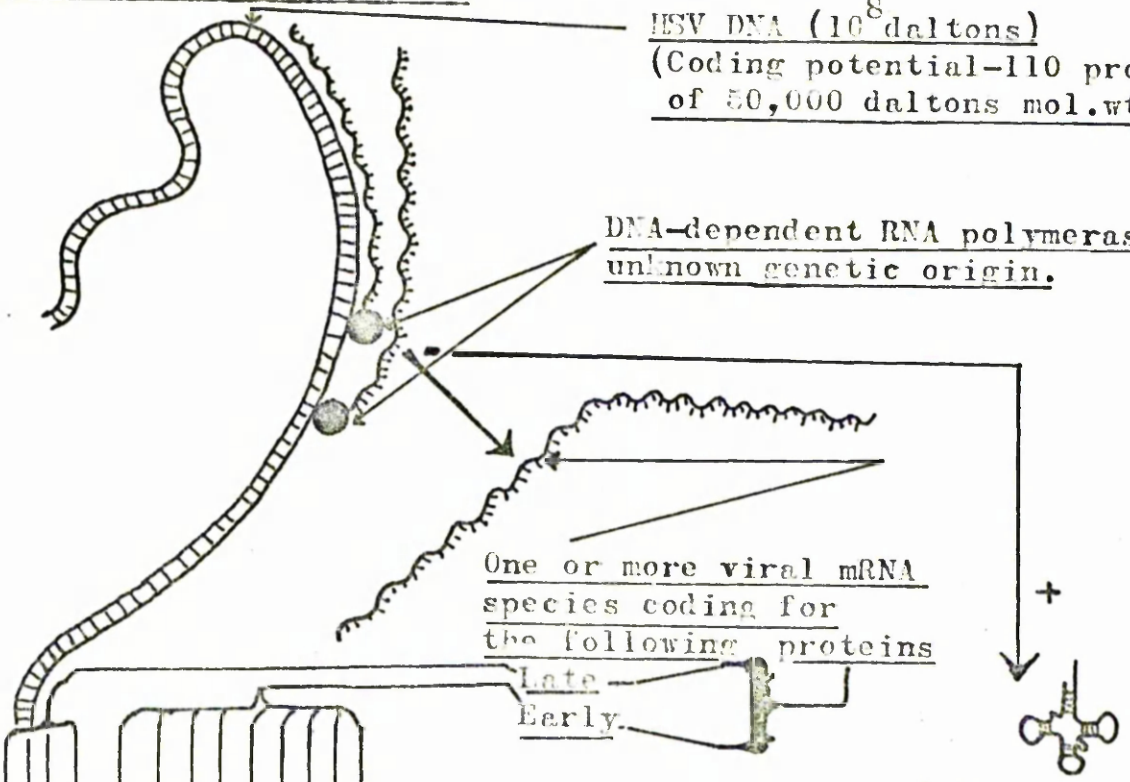

(a)
(b)
(c)
Time (h) after Infection.

Fig. 1. 6.

Diagrammatic Summary of the Virus-Induced Products which may be Specified by HSV DNA.



HSV DNA (10^8 daltons)
(Coding potential-110 proteins of 50,000 daltons mol.wt.)

DNA-dependent RNA polymerase of unknown genetic origin.

One or more viral mRNA species coding for the following proteins

Late

Early.

? Some new virus-coded tRNA species.

(1). HSV-induced DNA polymerase.

(2). HSV-induced Alkaline DNase (Exonuclease).

(3). HSV-induced dThd kinase.

(4). HSV-induced dCyd kinase.

(5).? HSV-induced dCMP deaminase.

(6).? HSV-induced dTMP kinase.

(7).? HSV-induced DNA-Endonuclease.

(8).? One or more protein factors shutting off host macromolecular (RNA, DNA, protein) synthesis.

(9). HSV Structural proteins (2 in the capsid, 2 in the core, 3 in the envelope).

(10).? Factor(s) having effects on host cell chromosomes { if different from (8) }.

(11).? Factor involved in induced enzyme cut off.

(12).? Factor(s) involved in maturation (DNA or virus).

(13).? Factor( ) involved in virus release.

HSV-infected L-cells (Newton, 1964); but other evidence suggests that the effects may arise from increased stabilisation of the host enzyme (Prusoff, Bakhle and Sekely, 1965; Kaplan, Ben-Porat and Coto, 1967).

(c) Deoxycytidine kinase.

The increase in this activity following HSV-infection has been shown to require RNA and protein synthesis and the properties of the activities in extracts of infected and uninfected cells have been found to differ in several respects, suggesting that the virus induces a new kinase distinct from the corresponding host enzyme (Perera and Morrison, 1970).

(d) dCMP deaminase.

Preliminary studies on the HSV-induced increases in the level of this enzyme activity have not yet established whether the enhancement reflects the synthesis of a new virus-specified enzyme or increased activity of the host enzyme (Keir, 1968).

(e) Alkaline deoxyribonuclease.

The early observations revealing that DNase activity increased markedly in cells infected with HSV were made by Keir and Gold (1963) and Russell et al., (1964). The induced DNase, and the DNA endonuclease which forms the main bulk of host (BHK 21 (C13)) cell DNase activity are clearly distinguishable by several criteria:-

(i) Chromatographic behaviour on DEAE-cellulose, allowing separation of the two activities (Morrison and Keir, 1967, 1968a).

(ii) Response to a range of concentrations of monovalent or divalent cations (Morrison and Keir, 1966).

(iii) Heat-inactivation behaviour (Morrison and Keir, 1966).

(iv) Substrate specificities - the induced enzyme being equally active upon denatured or native DNA and the host enzyme being very much less active upon native DNA.

(v) The mode of action of the enzymes - the host enzyme being an endonuclease, the virus-induced activity being exonucleolytic, producing deoxyribonucleoside 5'-monophosphates.

(vi) Effects of antibodies specific for herpes-induced proteins, the host enzyme being uninhibited and the induced enzyme being strongly inhibited.

Both RNA and protein synthesis are obligatory for induction of the new DNase activity as deduced from studies with actinomycin D and puromycin (Morrison, 1967).

Thus the results, as a whole, demonstrate clearly that the synthesis of a new DNase activity, distinct from the main host-cell DNase, is induced in HSV-infected cells, and the evidence strongly suggests that the enzyme is genetically virus-specified.

(f) DNA polymerase.

The initial observations on the increased levels of DNA polymerase were carried out by Keir and Gold (1963), and Russell et al., (1964). The time courses of the induction of DNA polymerase found by the latter group and subsequent workers are shown in Fig. I. 7.

As is the case with the induced DNase, the time and mechanism of termination of enzyme increase has not yet been ascertained.

Although the distribution of polymerase in control and infected-cell fractions (nuclei, small particles and soluble cell sap) is compatible with a nuclear site for viral DNA replication (Introduction section 2. 3. 2.c.), the site of synthesis of the induced polymerase is probably cytoplasmic since the small particle fraction shows appropriate increases in the enzyme after infection (Keir and Gold, 1963; Sydiskis and Roizman, 1966).

Conditions can be selected in which the harvested infected cells show high activity of DNA polymerase and minimal DNase activity (Keir, Hay, Morrison and Subak-Sharpe, 1966a) and properties of the two activities have been determined which allow the assay of one with essentially no interference from the other (Keir, Subak-Sharpe, Shedden, Watson and Wildy, 1966b; Morrison and Keir, 1967).

The DNA polymerase activities of uninfected and HSV-infected cells show several different properties:-

(i) Distinct responses to a range of concentrations of monovalent (Keir et al., 1966b; Morrison, 1967) or divalent (Keir, 1965) cations.

(ii) Different responses to thiol-group inhibitors.

(iii) Distinct heat-inactivation effects - the induced enzyme being more heat-stable in the presence of DNA and less heat-stable

in the absence of DNA, than the host activity (Keir et al., 1966a).

(iv) The induced DNA polymerase appears to be antigenically distinct from the uninfected-cell enzyme(s) (Keir et al., 1966b). Antisera prepared in rabbits against antigens produced in rabbit kidney (RK) cells by HSV (Watson et al., 1966) inactivate the polymerase induced by HSV-infection of both BHK 21 (C13) cells and HEp-2 cells, but not the enzymes of the corresponding uninfected cells. It is unlikely that HSV would induce the same enzyme in BHK, HEp-2 and RK cells if these were usually-repressed host enzymes only induced on infection by HSV, since the genetic origins of the three cell types are so different.

Furthermore, the induction of the enhanced polymerase activity by HSV is dependent upon RNA and protein synthesis (Keir, 1968).

The overall evidence thus strongly suggests that the enhanced DNA polymerase activity is caused by the synthesis of a new enzyme specified by the HSV genome.

3. OBJECTIVES OF THE PRESENT PROJECT.

The early work on the HSV-induced DNA polymerase (Introduction section 2. 4. 3.) comprised characterisation studies using mainly unfractionated extracts of HSV-infected cells, and some preliminary fractionation studies. The latter suggested the possibility that the herpes-induced DNA polymerase and DNase activities might reside in the same protein molecule, whereas some

of the characterisation studies suggested that this was not the case.

The aims of the project : - Purification and characterisation of the enzymes of DNA synthesis and degradation in HSV-infected cells.

The first objective was to attempt to purify the induced DNA polymerase and DNase, if possible to homogeneity, but at least to free them from contaminating interfering activities - viz. host-cell DNA polymerase and DNase activities present in un-fractionated extracts. In addition these "contaminating" activities, separated from the virus-induced enzymes of the extract, would be further purified and characterised.

These procedures would then:

(1) Establish the identity or non-identity of the protein or proteins carrying virus-induced DNA polymerase and DNase activities;

(2) Allow an extensive more meaningful characterisation of the virus induced DNA polymerase and DNase to be carried out without interference from the DNA poly-merases and DNases of host origin present in the extracts used in the early work.

(3) Permit more valid comparisons to be made between host and viral DNA polymerases.

CHAPTER II - MATERIALS AND METHODS.

1. MATERIALS.

1. 1. Inorganic chemicals.

Inorganic chemicals were ANALAR grade where possible, and were purchased from The British Drug Houses Ltd., B.D.H. Laboratory Chemical Division, Poole, Dorset. Ammonium sulphate- "Enzyme Grade" - used for enzyme preparations - was obtained from Mann Research Laboratories, Inc., 136 Liberty St., New York. Caesium chloride (ANALAR) was purchased from Hopkins and Williams Ltd., Chadwell Heath, Essex. "Hyflo Super Cel" and "Celite 505" were acquired from Koch-Light Laboratories, Colnbrook, Buckingham-shire. ^{32}P-labelled inorganic orthophosphate was purchased from the Radiochemical Centre, Amersham, Buckinghamshire.

1. 2. Organic chemicals.

1. 2. 1. Constituents of buffers and media.

Tris base ("Trizma") and cacodylic acid were purchased from the Sigma (London) Chemical Co., Ltd., London. 2-mercaptoethanol was obtained from Koch-Light Laboratories, Colnbrook; sucrose ("Special Enzyme Grade") from Mann Research Laboratories Inc., New York; and Lyphogel (Gelman Instrument Company) from Hawksley and Sons, Ltd., Lancing, Surrey. Polyethylene glycol 6000 (NON-ANALAR) and glycerol (ANALAR grade) were from B.D.H., Ltd.

1. 2. 2. Stains.

Naphthalene black, orcein, fast green (F.C.F.) and crystal violet were all purchased from George T. Gurr, Ltd., London, S.W.6, while bromophenol blue was the product of B.D.H. Ltd.

1. 2. 3. Inhibitors.

Phenylmethanesulphonyl fluoride (PMSF) was obtained from the Sigma (London) Chemical Co., Ltd.

1. 2. 4. Polyacrylamide gel reagents.

Acrylamide, NN'-methylenebisacrylamide and NNN'N'-tetramethylethylenediamine (TEMED) were all products of Koch-Light Laboratories; riboflavin was from B.D.H. Ltd.

1. 3. Biochemicals.

1. 3. 1. Nucleic acids.

Calf thymus DNA, as the sodium salt, used for co-precipitation purposes and salmon sperm DNA as the "highly polymerised sodium salt (type III)", as used in priming studies, were purchased from the Sigma (London) Chemical Co., Ltd.

Bacteriophage T7 DNA was the generous gift of Mr. D.J. Jolly, who prepared it essentially by the method of Thomas and Abelson (1966) from phage purified and isolated by the method of Sargeant, Yeo, Lethbridge and Schooter (1968).

The RNA used in enzyme heat-stability studies was the "Highly polymerised A grade from yeast" of Calbiochem, Ltd., London.

1. 3. 2. Proteins.

Cytochrome-c was purchased from C.F. Boehringer und Soehne, GmbH, Mannheim, human-haemoglobin (crystallised twice) from Nutritional Biochemicals Corporation, Cleveland, Ohio, and transferrin from Miles Laboratories Inc., Kankakee, Illinois. Bovine serum albumin was either "Bovine albumin powder Fraction V from bovine plasma", as supplied by the Armour Pharmaceutical Co., Ltd., Eastbourne, or, "Albumin from bovine serum, crystallised and lyophilised" from the Sigma (London) Chemical Co., Ltd.

1. 3. 3. Enzymes.

Yeast alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1.), pancreatic deoxyribonuclease I (Deoxyribonucleate oligonucleotido-hydrolase, EC 3.1.4.5) (1x crystallised from bovine pancreas) and snake venom 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5.) (GradeII from *Crotalus adamanteus* venom) were all purchased from the Sigma (London) Chemical Co., Ltd., as was pancreatic ribonuclease [polyribonucleotide 2-oligonucleotide-transferase (cyclizing), EC 2.7.7.16.] (Ribonuclease-A from bovine pancreas *Type 1-A.*).

Micrococcal nuclease (EC 3.1.4.7.) was provided by

Worthington Biochemical Corporation, Freehold, New Jersey; spleen phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3. 1. 4. 1.) by Schwartz Bioresearch Inc., Orangeburg, New York; and Pronase by Calbiochem Ltd., London.

1. 3. 4. Nucleotides.

Non-radioactive deoxyribonucleoside 5'-triphosphates: dATP, dCTP, dGTP and dTTP, were supplied by PL-Biochemicals, Milwaukee, Wis. and were further purified by chromatography on DEAE-Sephadex A-25 using a linear gradient of triethylammonium bicarbonate buffer, pH7.5 (Weimann and Khorana, 1962) then checked for purity using paper chromatography with isobutyric acid-ammonia-water (66: 1: 33, by vol.).

[α-^{32}P] dTTP and [α-^{32}P] dCTP were obtained from the International Chemical and Nuclear Corporation (ICN), City of Industry, California, while [5-methyl-^{3}H] dTTP was supplied by the Radiochemical Centre, Amersham. Again, purity was checked by the paper chromatographic method outlined above and, if necessary, the nucleotides could be purified by the same technique on a preparative scale.

Nicotinamide-adenine dinucleotide (NAD^+) was purchased from C.F. Boeringer und Soehne, thymidine 5'-monophosphate (disodium salt) from the Sigma (London) Chemical Co., Ltd., thymidine 3', 5'-diphosphate from Calbiochem Ltd., London, while d(pT-T-T) was the gift of Professor H.M. Keir.

1. 4. Biological Materials.

1. 4. 1. Cells.

A continuous line of baby hamster kidney fibroblasts BHK21(C13) was used for all experiments (Macpherson and Stoker, 1962).

1. 4. 2. Virus.

Herpes simplex virus used was strain HFEM(α) in all experiments (Holmes and Watson, 1963; Russell et al., 1964).

1. 4. 3. Medium.

A modified Eagle's medium (Eagle, 1959; Vantsis and Wildy, 1962) containing 10% (^{v}/v) tryptose phosphate broth and 10% (^{v}/v) calf serum was used at all times. Tryptose phosphate broth consisted of a 2.95% (^{w}/v) solution of tryptose phosphate broth in distilled water. The tryptose phosphate was obtained from Difco Laboratories, Detroit, Michigan U.S.A.: Eagle's medium and calf serum were obtained from the Institute of Virology, University of Glasgow.

1. 4. 4. Standard tissue culture solutions.

Standard-saline-citrate solution (SSC).

This was a solution containing 0.15M-NaCl and 15mM-trisodium citrate, pH 7.0.

Phosphate buffered Saline solution (A) (PBS(A)).

This consisted of 0.17M-NaCl, 3.4mM-KCl, 10mM-NaH_2PO_4 and 2mM-KH_2PO_4 at pH 7.4 (Dulbecco and Vogt, 1954).

1. 5. Materials for chromatography and electrophoresis.

DEAE-cellulose (DE52) (Microgranular-preswollen) was obtained from H. Reeve Angel and Co., Ltd., London E.C.4. as was Phosphocellulose P11 and the Whatman Chromatography papers - No.1, 3MM and DE 81. Hydroxylapatite for column chromatography (Bio-Gel HTP) was purchased from Micro-Bio Laboratories Ltd., 46, Pembridge Road, London W.11, while Sephadex G-150, DEAE-Sephadex A-25 and Blue Dextran 2000 were obtained from Pharmacia (G.B.) Ltd., London.

1. 6. Materials for liquid scintillation counting.

2, 5-diphenyloxazole (PPO) was the product of Koch-Light Laboratories, Ltd., and "Hyamine" hydroxide 1M in methanol was from Nuclear Enterprises (G.B.) Ltd., Edinburgh. Toluene-based scintillation fluid consisted of 0.5% PPO in ANALAR toluene.

2. METHODS.

2. 1. Assays - proteins; nucleic acids; ions.

2. 1. 1. Assay for protein.

Protein was assayed by the method of Lowry, Rosebrough,

Farr and Randall (1951) using dry bovine serum albumin as a standard. Because of the presence of 2-mercaptoethanol and KCl (both of which interfere with the assay) in the protein solutions to be assayed, prior precipitation with ice-cold 5% ($^{w}/_{v}$) trichloroacetic acid was carried out and the precipitate taken up in distilled water.

2. 1. 2. Assay for DNA.

DNA was estimated either by its u.v. absorption, taking an extinction value of 1.00 at 260nm to be equivalent to a solution of 50µg DNA/ml, or by the method of Ceriotti (1952).

2. 1. 3. Assay for inorganic orthophosphate.

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

2. 1. 4. Estimation of gradients in general, and potassium ion concentration in particular.

Potassium ion concentration in gradients used in column chromatography was followed routinely by measuring the index of refraction of the solutions using an Abbé refractometer: this also proved a quick and reliable method for estimating all kinds of gradients. Occasionally, a flame photometer was employed for the absolute determination of K^{+} concentration in gradients.

2. 2. Enzyme assays.

2. 2. 1. Assay for DNA polymerase (replicative DNA nucleotidyltransferase).

DNA polymerase was assayed with a "high salt" assay for infected-cell DNA polymerase and with a "low salt" assay for uninfected-cell DNA polymerase (Morrison, 1967). The reaction mixtures, modified from Morrison (1967), consisted of the following components:- $MgSO_4$ at 8mM; tris-HCl, pH 7.5 at 50mM; KCl at 60mM for the "low salt" assay and 120mM for the "high salt" assay; EDTA at 0.4mM; 2-mercaptoethanol at 10mM; dATP, dCTP, dGTP and dTTP - all at 0.2mM, with one of these - usually the dTTP - as either [α-^{32}P] dTTP (2×10^4 dpm/nmol) or [5-Me-^{3}H] dTTP (10^6dpm/nmol); heat-denatured calf thymus DNA at 400µg/ml and protein carrying DNA polymerase in amounts such that the extent of the reaction was still linear with respect to the amount of protein added. The total reaction volume was 0.25ml in early work and later reduced to 0.125ml (half-scale assay) to conserve isotope and enzyme. The assays were carried out in 4.5x1.2cm assay tubes with caps and these were incubated usually for 30min at 37°C. The reaction was terminated at 0°C in ice and aliquots, normally 50µl, were then collected on 2.5cm Whatman No.1 filter paper discs (previously impregnated with 50µl of 2mg/ml bovine serum albumin and dried). The discs were then plunged into ice-cold 5% (w/v) trichloroacetic acid containing 0.05M-sodium pyrophosphate (15ml/disc) and left to steep for 10min. This washing was repeated three times and was

followed by two ethanol and one ether wash and the discs were then dried.

^{3}H - containing discs were then counted in a scintillation spectrometer in the presence of 0.5ml 1.0M-"Hyamine" hydroxide (10min at 60°C) and 10ml toluene-based scintillation fluid.

When the precursor was [α-^{32}P] dTTP, the discs were usually placed on stainless-steel planchettes and counted in a low-background Nuclear Chicago Mark II gas-flow counter.

1 unit of DNA polymerase was equivalent to 1nmol dTMP incorporated/1h at 37°C in the standard assay. In later work, "activated DNA" was used in place of denatured DNA in the standard assay.

The DNA polymerase assay thus measures the amount of dTMP incorporated into acid-insoluble material precipitated on to filter-paper discs; the presence of pyrophosphate in the trichloroacetic acid wash eliminates, almost completely, non-specific binding of triphosphates to the filter-disc-DNA-protein complex.

2.2.2. Assay for terminal DNA nucleotidyltransferase activity.

This was conducted with two distinct reaction mixtures.

(1) A reaction mixture of total volume 0.25ml containing sodium cacodylate buffer pH6.8 at 200mM, $CoCl_2$ at 1mM, dTTP [α-^{32}P] at 1mM, and d(pT-T-T) at 0.01mM together with enzyme-

containing solution (Kato et al., 1967). Reaction mixtures were incubated for 30min and 60min at 37°C then plated, washed and counted as in the normal DNA polymerase assays with [α-^{32}P] dTTP as label. This first assay estimated terminal DNA nucleotidyltransferase activity similar to the cytoplasmic type of terminal DNA nucleotidyltransferase such as that found in calf thymus gland (Bollum, 1966, 1968).

(2) The second type of terminal DNA nucleotidyltransferase assay was identical to the "low salt" DNA polymerase assay (Methods section 2. 2. 1.) except that only one triphosphate was used, usually dTTP (α-^{32}P). Denatured calf thymus DNA was used as primer.

Terminal DNA nucleotidyltransferase activity of the calf thymus cytoplasmic type is recorded in both types of assay: however, activity of the calf thymus nuclear terminal DNA nucleotidyltransferase type is registered only with the second type of assay using denatured DNA (Gottesman and Canellakis, 1966).

2. 2. 3. Assay for alkaline deoxyribonuclease activity. (Standard DNase assay).

This assay is based upon the acid-solubility of oligodeoxyribonucleotides (shorter than 7 nucleotide units long), formed by the action of DNase upon ^{32}P-DNA and was carried out as described by Morrison and Keir (1968a).

The assay mixture (0.20ml) contained the following

components:- tris-HCl, pH 9.0 at 50mM, $MgSO_4$ at 2mM, 2-mercapto-ethanol at 10mM and ^{32}P-labelled DNA (Native or denatured, $2x10^3$ c.p.m./µg) at 50µg/ml. Enzyme or buffer was then added and incubation carried out for 30min at 37°C. After cooling in an ice-bath to 0°C, 0.2ml of heat-denatured DNA (1mg/ml) was then added and the tubes vortexed. 0.6ml of 1.0M-perchloric acid containing "Hyflo Super Cel" or "Celite 505" (20g/litre) was then added and the mixture vortexed; then, after standing for 10min at 0°C over ice, the tubes were centrifuged at 800g for 10min at 0°C. 0.2 - 0.6ml aliquots of the supernatant from each tube were then plated out on to planchettes with one drop of 10M-KOH and the planchettes dried and counted in a gas-flow counter. Blanks with no enzyme and plated aliquots of the substrate DNA were then used to estimate the amount of activity in each tube.

1 unit of DNase activity was taken to be equivalent to 10nmol DNA(P) (3µg DNA) hydrolysed or rendered acid-soluble/30min at 37°C under these standard conditions (Morrison and Keir, 1968a).

2. 2. 4. Determination of the mode of action (exo- or endonucleolytic) of DNase.

Two methods were employed for such a determination.

2. 2. 4. 1. Method using ascending chromatography of the DNase digest on DEAE-paper.

In this technique, a typical DNase assay incubation

(Methods section 2. 2. 3.) was carried out and 0.05ml of the chilled reaction mixture was applied to the origin of a 23x28.5cm sheet of Whatman DE81 paper and dried. The chromatogram was developed (ascending) with 0.75M-NH_4HCO_3 buffer, pH 8.6 for 2-3h, dried, and then cut up into strips 3.8cm wide for scanning in a Nuclear Chicago "Actigraph III" chromatogram scanner. In this system, dPydMP and dPuoMP have R_F values of 0.70 and 0.50 respectively: a slight separation of dGMP and dAMP occurs accounting for the somewhat broader purine peak; material of more than 30 nucleotides long remains at the origin and oligonucleotides of intermediate length appear between the origin and R_F 0.5 (Furlong, 1966; Keir, 1968). Inspection of the "Actigraph" scan defined the products of the reaction and therefore the mode of action of the DNase.

If the 5'-mononucleotide products of digestion of DNA by an exonuclease are treated with snake venom 5'-nucleotidase before application to the DEAE-paper, the purine and pyrimidine mononucleotide peaks are replaced by an inorganic orthophosphate peak (R_F 0.8) and this allows identification of the dNMP products as 5' or 3' (Keir, 1968). The incubation conditions for 5'-nucleotidase digestion were as follows (essentially as according to the method of Heppel and Hilmol (1955)). To 0.10ml aliquots of the standard acid-soluble DNase assay (Methods section 2.2.3), the reaction mixtures having been boiled for 3min then cooled to stop the DNase reaction at the end of the standard DNase incubation,

0.002ml of 0.5M-$MgCl_2$ and 0.005ml of a solution of 0.10mg/ml 5'-nucleotidase enzyme powder in distilled water were added and incubation carried out for 1 hour at 37°C. The tubes were then chilled before spotting on to DE81 paper.

2. 2. 4. 2. Method using DEAE-paper-pulp fractionation of the DNase digests.

In this technique, the standard DNase assay incubation was carried out (Methods section 2. 2. 3.) and the reaction mixture chilled and made up to 1.0ml with 0.8ml of Buffer I (0.01M-NaH_2PO_4, pH7.0). This solution was then transferred to a punctured cellulose-nitrate tube which contained pulped DEAE-paper and which was held about 7.5cm from the bottom of a 15ml conical glass centrifuge tube. The Buffer I + DNase reaction mixture were allowed to adsorb to the DEAE-paper pulp for a few minutes then the tubes were centrifuged to drain the pulp; the liquid centrifuged down was retained and the pulp washed sequentially as follows.

Buffer II	0.01M-NaH_2PO_4 + 0.15M-NaCl	1x1.0ml wash to which was added the first wash with Buffer I + DNase reaction mixture.
Buffer III	0.01M-NaH_2PO_4 + 0.50M-NaCl	(2x1.0ml washes).
Buffer IV	0.01M-NaH_2PO_4 + 1.0M NaCl	(2x1.0ml washes).
Buffer V	0.2M-NH_4OH + 2.0M-NaCl	(2x0.5ml washes).
Buffer VI	1.0M-NaOH	(2x0.5ml washes).

The eluates were then plated, dried and counted essentially as in

Methods section 2. 2. 4. 1.

An estimate both of the amount of DNase activity and the mode of action of the DNase can be made since DNA exonuclease action causes increases in fractions II and III over the control, and endonuclease causes a shift of counts from fractions V and VI to lower fractions IV, V and also III.

It is best to assay with several different times of incubation to follow the course of product formation, in both this method and the previous one, so that advanced endonuclease activity is not mistaken for exonuclease activity (Davila, Charles and Ledoux, 1965a,b; Charles and Ledoux, 1966).

2. 2. 5. Assay for alcohol dehydrogenase.

This was carried out at pH 8.5 according to the method of Racker (1950) and enzyme activity was quoted simply as ΔE_{360} nm/min.

2. 3. Preparation and treatment of DNA from various sources.

2. 3. 1. Preparation of DNA from various sources.

DNA was prepared from calf thymus or BHK21(C13) cells by the method of Kay, Simmons and Dounce (1952).

$[^{32}P]$ DNA was prepared by growing *E. coli* in the presence of $[^{32}P]$ orthophosphate (Lehman, 1960), the isolation and purification of the DNA being carried out as described by Morrison

and Keir (1968a): non-radioactive DNA from *E. coli* was prepared in the same way.

HSV DNA was prepared as follows: herpes simplex virus was purified by cycles of low and high speed centrifugation from the medium over infected cells and from sonicates of these cells (Low, 1970). The virus was then incubated overnight with pronase at 500µg/ml and sodium dodecylsulphate at 1% (w/v) in SSC. A further 500µg/ml pronase was then added and incubation continued for 6h at 37°C. CsCl was added until a refractive index of 1.4005-1.4015 (1.70g/ml CsCl) was attained and protein aggregates were removed by centrifugation (800g/10min). The liquid was then centrifuged for 2 days at room temperature (20°C) in a Spinco Type 50Ti angle rotor at 33,000 rev./min. Fractions from each tube were collected by piercing the base with a hypodermic needle and the material appearing as a peak in the region expected for HSV DNA (refractive index of CsCl solution = 1.4015-1.4020) was pooled and dialysed against 0.02M-KCl. This preparative method gave a buoyant density value for the DNA and was a useful check on its identity.

2. 3. 2. Denaturation of DNA.

DNA solutions were incubated for 10min at 100°C at an ionic strength of 0.02 or less, followed by rapid cooling to 0°C in an ice bath.

2. 3. 3. "Activation" of DNA.

This was carried out essentially by the method of Aposhian and Kornberg (1962). For routine activation of calf thymus DNA, a solution of 1ml of 1mg/ml calf thymus DNA was incubated with 0.01ml of 0.5M-$MgCl_2$, 0.062ml of 0.8M-tris-HCl, pH 7.5, 0.005ml of 1µg/ml pancreatic DNase I in 10mg/ml bovine serum albumin and 0.01ml of 50mg/ml bovine serum albumin. The whole was mixed and incubation carried out for 15min at 37°C; then the solution was heated for 5min at 77°C and immediately cooled in an ice bath. Other sources of DNA were "activated" in an identical manner.

2. 4. Cell culture methods. Growth of cells; infection with virus; harvesting of cells.

BHK21 (C13) cells were grown in monolayer cultures in rotating 80oz. Winchester bottles (House and Wildy, 1965) in the modified Eagle's medium containing 10% (v/v) tryptose phosphate broth and 10% (v/v) calf serum described in Materials section 1. 4. 3.

Infection of cells with HSV was performed at an input multiplicity of 10-15 plaque-forming units/cell with an adsorption period of 30min.

Cells were harvested 18h post infection by scraping the cells from the glass with a rubber-tipped rod and washing twice by centrifugation (200g/10min) in cold PBS(A) solution after which the cell pellets were stored at -70°C until required.

2. 5. Preparation of buffers and other solutions.

Buffers were prepared by weighing out the required amount of solid, dissolving in less than the required volume of water, titrating the solution to the desired pH with the appropriate acid or base at 20°C and adjusting the final volume to the mark, checking the pH again.

Buffer stocks were normally made up at 5 or 10x the desired final concentration of the buffer to be used; they were then diluted for use, checking the pH and adjusting if necessary. The pH values of all buffers used were correct for 20°C.

2. 6. Preparation of cell-extracts of infected or uninfected cells.

Three different extracting techniques were employed. (1) The whole-cell extract. (2) The soluble extract with soluble (S_1) and nuclear (S_2) fractions. (3) Subcellular (nuclear-cytoplasmic) fractionated extracts.

2. 6. 1. The whole-cell extract of infected or uninfected cells.

Cell pellets were thawed quickly at 37°C and five volumes of "TEM buffer" (0.10M-tris-HCl(pH 8.0)-1mM-EDTA-10mM-2-mercapto-ethanol) were then added and the resulting suspension hand-homogenised at 0°C by 5 strokes in the Potter-Elvehjem homogeniser.

4 bursts of ultrasonic vibration of 15s duration from an MSE ultrasonic power unit were then applied to the suspension at $0^{o}C$ and samples stained (Kurnick and Ris, 1948) and checked microscopically for complete disruption of cells and nuclei. The homogenate was then centrifuged for 1 hour at 105,000g and the supernatant fluid stored at $0^{o}C$ over ice in a Dewar flask.

2. 6. 2. The Soluble extract.

Soluble extracts of cells were obtained after lysis in 10 volumes of hypotonic buffer (1mM-EDTA-10mM-2-mercaptoethanol-20mM-tris-HCl buffer, pH 8.0). The suspension was again hand-homogenised at $0^{o}C$ (Potter-Elvehjem) and nuclei pelleted by centrifuging at 800g for 10min. The concentration of tris-HCl, pH 8.0 in the supernatant fraction was raised to 0.10M and the extract centrifuged at 30,000 or 105,000g for 1 hour and the supernatant from this constituted the "soluble fraction" (S_1).

The nuclear pellet was resuspended in 5 times its volume of "TEM buffer" and disrupted ultrasonically (Methods section 2. 6. 1.). This solution was centrifuged at 30,000g for 1 hour to give the "nuclear fraction" (S_2).

2. 6. 3. Nuclear-cytoplasmic fractionation.

Nuclei were prepared from BHK 21 cells by homogenisation, using a Potter-Elvehjem homogeniser, in [0.25M-sucrose-1.5mM-$CaCl_2$-10mM-2-mercaptoethanol-10mM-tris-HCl buffer, pH 8.0] ("SCTM buffer")

after scraping the cells from the glass in the same medium. The nuclei were sedimented (10min at 800g), resuspended in "SCTM buffer" by homogenisation and re-centrifuged. The two supernatant fractions were pooled, dialysed against "TEM buffer" and centrifuged for 30min-1 hour at 30,000g to give the "cytoplasmic fraction".

The washed nuclei were then re-suspended in "TEM buffer", disrupted by ultrasonic vibration, dialysed against "TEM buffer", centrifuged for 30min-1 hour at 30,000g and the resultant supernate termed the "nuclear fraction". The washed nuclei were slightly contaminated with cytoplasmic material as revealed by aceto-orcein/fast green staining.

2. 7. Ammonium sulphate fractionation of enzymes. [$(NH_4)_2SO_4$ fractional precipitation].

This was carried out at pH8.0 and 0°C using "Enzyme Grade" $(NH_4)_2SO_4$ according to the method of Morrison (1967) as outlined by Morrison and Keir (1968a).

2. 8. Dialysis.

Dialysis tubing was always pretreated as follows. A solution of 0.10M-EDTA was heated in a beaker to 70°C and the dialysis tubing immersed in this for 15min at 70°C. The EDTA solution was then poured off and the tubing rinsed thrice with distilled water, then equilibrated with the buffer or other solution to be used.

2.9. Concentration of enzyme fractions and other solutions.

In early work, polyethylene glycol as a 30% (W/v) solution or crystalline sucrose was used to surround dialysis sacs containing the enzyme solutions to be concentrated. In later work, the polyacrylamide solid concentrating agent "Lyphogel" became available and was then routinely used according to the manufacturers' instructions.

2.10. Column chromatography.

2.10.1. Hydroxylapatite column chromatography.

Hydroxylapatite, as supplied, was suspended in the starter buffer: 0.04M-potassium phosphate buffer (pH 7.5)-10mM-2-mercapto-ethanol, in early work, and 0.01M-potassium phosphate buffer (pH 7.5)-10mM-2-mercaptoethanol-0.18M-KCl, in later work. The fines were decanted off and the material thoroughly equilibrated with starter buffer, checking the pH of the buffer in contact with the gel after each change of buffer. 1-1.5x10cm column bed volumes were routinely used in 'Whatman' columns and these were connected to an LKB "ULTRORAC" fraction collector with a "UVICORD" apparatus, the eluting buffer being pumped from the gradient-maker reservoir. Normally rates of flow of 20ml/hour were employed. After an initial wash of the column and adjustment of shrinkage, the sample (1-15ml) was pumped on to the gel and left to adsorb for 15-20min prior to the initial starter buffer wash (approx.100ml). The

gradient consisted of 100ml of starter buffer and either 100ml of 0.10M-potassium phosphate buffer, pH 7.5 with 10mM-2-mercaptoethanol, as in later work, or 100ml of 0.20M-potassium phosphate buffer, pH 7.5 with 10mM-2-mercaptoethanol, as in earlier work. Fractions were kept at 0°C and glycerol added to 30% (v/v) as the fractions were collected. The tubes were then stored at -70°C.

Occasionally other types of column and eluting conditions were used.

2.10. 2. DEAE-cellulose column chromatography.

This was carried out essentially as described for hydroxylapatite: DE52 gel was cleared of fines and equilibrated with "DEAE-starter buffer" (0.05M-tris-HCl buffer (pH 8.0)-10mM-2-mercaptoethanol-1mM-EDTA). Eluting gradients consisted of 100ml "DEAE-starter buffer" and 100ml of 0.05M-tris-HCl buffer(pH 8.0)-1mM-EDTA-10mM-2-mercaptoethanol-0.35M-KCl, giving a salt gradient of 0-0.35M-KCl.

From time to time, different columns and eluting conditions were employed.

2.10. 3. Sephadex G-150 gel-filtration.

A long glass column with fixed plastic ends (Pharmacia (G.B.) Ltd.,) and internal diameter 2.5cm was used in all experiments with a 67.5cm. gel bed height. The buffer used was 0.10M-tris-HCl

buffer (pH 8.0)-10mM-2-mercaptoethanol with 0.5mg/ml bovine serum albumin. Concentrated samples of enzyme (approx. 4ml) were applied with 10mg cytochrome c as marker. Separate runs with yeast alcohol dehydrogenase, transferrin, Blue Dextran and cytochrome c were carried out in order to calibrate the column for mol.wt. studies (Andrews, 1965). Samples were applied in sucrose solution to assist in the layering process in the sample applicator. When the sample had run into the gel, the applicator was removed and a constant head of pressure of buffer set up using a bottle reservoir. 4.5ml fractions were routinely collected. Transferrin was detected by its extinction at 280nm and cytochrome c by its extinction at 410nm.

2.10. 4. Phosphocellulose column chromatography.

Pre-cycled phosphocellulose P11 was equilibrated and screened as described for the other media. Two distinct starter-eluting buffer conditions were employed.

(1) Starter buffer 0.05M-potassium phosphate buffer (pH 6.5)-1mM-EDTA-10mM-2-mercaptoethanol-20% (^{v}/v)glycerol. Eluting gradient conditions consisted of equal volumes of this starter buffer and eluting buffer 0.30M-potassium phosphate buffer (pH 7.5)-1mM-EDTA-10mM-2-mercaptoethanol-20% (^{v}/v)glycerol. This gave a combined phosphate buffer and pH gradient. 1 hour was allowed for adsorption of the enzyme sample and a Whatman column with a bed

height of 10cm was used.

(2) Starter buffer 0.05M-potassium phosphate buffer (pH 7.3)-1mM-EDTA-10mM-2-mercaptoethanol and two stepwise washes, one with 0.10M-potassium phosphate buffer (pH 7.3), with the other components as before, and a second with 0.20M-potassium phosphate buffer (pH 7.3), with the other components as before. With this buffer system, a modified Pasteur pipette was used as a column. The sample was applied in a volume of 8ml and allowed to adsorb for 20min.

2.11. Polyacrylamide gel disc electrophoresis.

The theory of this technique was worked out by Ornstein (1964) and the general procedure used in this work was as described by Davis (1964).

7% (fine pore) gels were used with a larger pore spacer gel (2.5% gel) above to allow even application of the sample to the top of the fine-pore gel. Polymerisation of the gels was catalysed using the riboflavin-TEMED system rather than ammonium persulphate, and NN'-methylenebisacrylamide was used as cross-linking agent. The fine pore gels were poured to a height of 6cm in vertical 7cm long open ended cylindrical glass tubes of 1cm diameter. Spacer gels were poured to a depth of about 0.2cm and photopolymerised after the fine-pore gel was photopolymerised. The gel electrophoresis was carried out using a Shandon apparatus which was essentially as described by Davis (1964). The buffer used was

the tris-glycine buffer, pH 8.3 described by Davis (1964) but with 2-mercaptoethanol added to 10mM. Samples with added sucrose or glycerol were layered on to the spacer gels using a fine pipette. The apparatus was cooled to 4°C using a refrigerated ethylene glycol bath. Routinely, a total current of 30mA for 6 gel tubes was passed for 2 hours, bromophenol blue as a 0.001% (W/v) solution sometimes being added to one or more samples in order to follow the progress of the run. Protein staining. This was carried out using a 1% (W/v) solution of naphthalene black in 7% (V/v) acetic acid as described by Davis (1964). Destaining was carried out either by prolonged washing with 7% (V/v) acetic acid or by a faster electrophoretic removal using the same solution. The stained gels were then either scanned in a "Vitatron" gel scanner or placed over a fluorescent screen and scale drawings made of the protein bands. Enzyme assays. The gel was sliced at 4°C into discs of 0.2-0.5cm depth using a sharp safety-razor blade and one half of each disc was used in the enzyme assay. DNase assay. For this, each gel fraction was crushed in 0.1ml of [bovine serum albumin 1mg/ml-0.05M-tris-HCl (pH 8.0)-10mM-2-mercaptoethanol-1mM-EDTA] and was left overnight to extract at 0-4°C in the presence of the DNase assay mixture. A standard DNase assay at 37°C was then carried out for 1 hour. 0.4ml of the supernatant was plated out since solid gel residue had to be avoided. DNA polymerase assay. For this, the gel fragments were crushed and extracted overnight at 0-4°C in 0.2ml of the extracting buffer used in the DNase assay above. Then 0.05ml of the extract

was removed and assayed in a half-scale standard DNA polymerase assay.

2.12. Constant velocity sucrose gradient zone sedimentation.

This technique allows the fractionation of proteins of different sedimentation coefficients and the direct determination of $s_{20,w}$-values without recourse to using standards (Martin and Ames, 1961; Noll, 1967).

Gradients of 5.25ml volume (excluding sample) were constructed as follows. A special mixing device (Henderson, 1969) was employed and 5.68ml of 5% ($^{w}/_{w}$) sucrose in buffer (either 0.01M, 0.05M or 0.10M-tris-HCl buffer (pH 8.0)-5mM-2-mercaptoethanol-1mM-EDTA) were mixed at 0-4^{o}C with 27.1% ($^{w}/_{w}$) sucrose in the same buffer to give the required gradients (5.25ml in 5x1.2cm tubes). This gradient ensures a constant velocity of sedimentation for proteins at 5^{o}C and the method of calculating and constructing the gradient was as described by Noll (1967).

Enzyme samples of 0.25ml containing either 1mg human haemoglobin or 1mg human haemoglobin together with 100µg of yeast alcohol dehydrogenase were applied carefully to the gradients by a fine-tipped pipette holding the tip just under the surface and against the side of the tube.

The tubes were then fitted into a Spinco SW50L rotor at

5°C and centrifuged at 40,000 rev./min (130,576g)($r_{av.}$ 7.3cm) for 11-21 hours noting the entire run time precisely and adding on one third of the time of acceleration and deceleration to the time at full speed to obtain the effective total time 't' at full speed (Schachman, 1959).

The gradients were harvested essentially as described in Methods section 2. 3. 1. for HSV DNA. Usually, 3-drop fractions were collected at 4°C and samples of each fraction assayed for DNA polymerase, DNase and yeast alcohol dehydrogenase by the standard methods. Human haemoglobin was estimated by its extinction at 420nm. The volumes of the fractions were also measured and, from the known dimensions of the tubes, the distances migrated down the tube corresponding to each fraction were calculated.

$\underline{S}_{20,w}$ values were calculated from the following formula worked out by Dr. R. Eason from Noll's data.

$$\underline{S}_{20,w} = \frac{R}{\omega^2} \cdot \frac{A}{K} \cdot \frac{1}{t}$$

where R = distance migrated by the protein from the meniscus in cm. (R = 0 at the top of the tube).

A = 39.9373

K = 103.608

$$\omega^2 = \left(\frac{2 \text{ rev./min}}{60}\right)^2$$

t = time (in seconds) of the migration (effective total time).

The known $\underline{s}_{20,w}$ values of the alcohol dehydrogenase and

haemoglobin markers acted as checks on the fidelity of the gradients and, if this was satisfactory, the apparent $s_{20,w}$ values of enzymes could be calculated by simple proportion.

2.13. Heat stability studies.

In these, duplicate tubes of enzyme were incubated for periods of time over a range indicated in the figures at the temperature stated - usually 45°C . The tubes were then removed from the water bath and placed on ice at 0-4°C until all tubes had been incubated. Then the assay components for the standard DNase or DNA polymerase assays were added to each tube and standard assays carried out at 37°C.

2.14. Partial nearest-neighbour frequency analysis of DNA synthesised by the virus-induced DNA polymerase.

In this technique, a DNA template is copied by a replicative DNA polymerase in the presence of all four dNTP's, one of which is labelled with ^{32}P in its 5'-α-phosphate position. This DNA copy is then hydrolysed by nucleases which convert the total DNA finally to deoxyribonucleoside 3'-monophosphates, transferring the label to the 3'-position of the nucleotide which was the nearest neighbour on the 5' side of the incoming labelled dNTP in the synthesised DNA. Then separation and estimation of the % of the total radioactivity in each of the four possible deoxyribo-

nucleoside 3'-monophosphates gives a measure of the frequency of occurrence of the sequences ApN, CpN, GpN and TpN (Josse, Kaiser and Kornberg, 1961).

The practical procedure beyond the incubation stage was essentially that of McGeoch (1970).

Incubation.

Three separate incubations were carried out as follows.

(I) Contained 0.035ml of hydroxylapatite-purified enzyme in glycerol; 0.02ml of reaction mixture components (standard assay - "high salt"); 0.06ml of "activated" HSV DNA (49µg) and 0.01ml [α-^{32}P] dCTP (0.3µC).

(II) Contained 0.035ml enzyme as in I; 0.02ml reaction mixture components as in I; 0.06ml "activated" HSV DNA (67µg) and 0.01ml [α-^{32}P] dCTP (0.3µC).

(III) Contained 0.035ml enzyme as in I; 0.02ml reaction mixture components as in I; 0.06ml of "activated" calf thymus DNA (66µg) and 0.01ml of [α-^{32}P] dCTP (0.3µC).

(I) was incubated for 3 hours, (II) for 2 hours and (III) for 4 hours to achieve sufficient incorporation of label.

Isolation of the DNA.

Following the incubations, the DNA was precipitated by 0.5M-perchloric acid at 0°C in the presence of denatured DNA to give a total of 400µg DNA per tube. After standing for 5min, 2.5ml of cold water was added and mixed giving a total volume of 3.5ml and the precipitate collected by centrifugation (800g for 10min).

The DNA was washed twice by dissolving in NaOH and reprecipitating with perchloric acid and the DNA finally dissolved in 0.1ml of 0.05M-NaOH in 0.02M-tris base plus 0.3ml cold water (McGeoch, 1970).

Digestion of the DNA.

This was carried out using exhaustive digestion with micrococcal nuclease and spleen phosphodiesterase (McGeoch, 1970).

Electrophoretic separation of the mononucleotides.

The digests were centrifuged at 800g for 10min to sediment any protein which had come out of solution during the incubations and the supernatants blown to dryness with a compressed air stream at room temperature. The residue was then dissolved in 0.08ml of water and the whole sample applied as a 2.5cm streak on Whatman 3MM paper. The electrophoresis was carried out in 0.05M-ammonium formate buffer, pH 3.5 at 3-4kV for 2-5 hours after 300V for 30min. The paper was then dried and the nucleotides detected under u.v. light. The four nucleotide spots and the regions between them were cut out and counted in 10ml of toluene-based scintillation fluid in a Nuclear Chicago scintillation spectrometer. The radioactivity in each nucleotide spot was then expressed as a fraction of the total nucleotide counts (McGeoch, 1970).

CHAPTER III. RESULTS

1. MISCELLANEOUS PRELIMINARY STUDIES.

1. 1. The optimal reducing conditions for DNA polymerase of HSV-infected cells.

1. 1. 1. Extraction of DNA polymerase.

A suspension of HSV-infected cells was made in 1mM-EDTA-0.1M-tris-HCl buffer, pH 8.0 with no reducing agent present, and the suspension equally divided into five fractions. The reducing agents 2-mercaptoethanol and dithiothreitol were added as follows:-

Tube 1 :- No reducing agent.

Tube 2 :- 2-mercaptoethanol to 10mM.

Tube 3 :- dithiothreitol to 0.1mM.

Tube 4 :- dithiothreitol to 1.0mM.

Tube 5 :- dithiothreitol to 5.0mM.

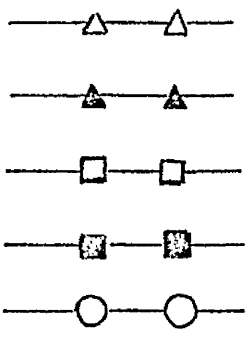
Each fraction was then disrupted by ultrasonic vibration (Methods section 2. 6. 1.) and the supernatant collected by centrifugation for 1 hour at 30,000g at 4^{o}C.

The pellet from tube 2 was resuspended and re-extracted as before and from DNA polymerase assays of the first and second supernatants it was demonstrated that only 2.8% of the soluble DNA polymerase activity in this standard whole-cell extract remained in the debris after centrifugation.

Fig. III. 1.

The effect of reducing agents on the extraction of DNA polymerase from infected cells.

Infected cells were extracted (Methods section 2. 6. 1.) in the presence of reducing agents as below and standard DNA polymerase assays carried out (Methods section 2. 2. 1.) at 5mM-2-mercaptoethanol.



No reducing agent

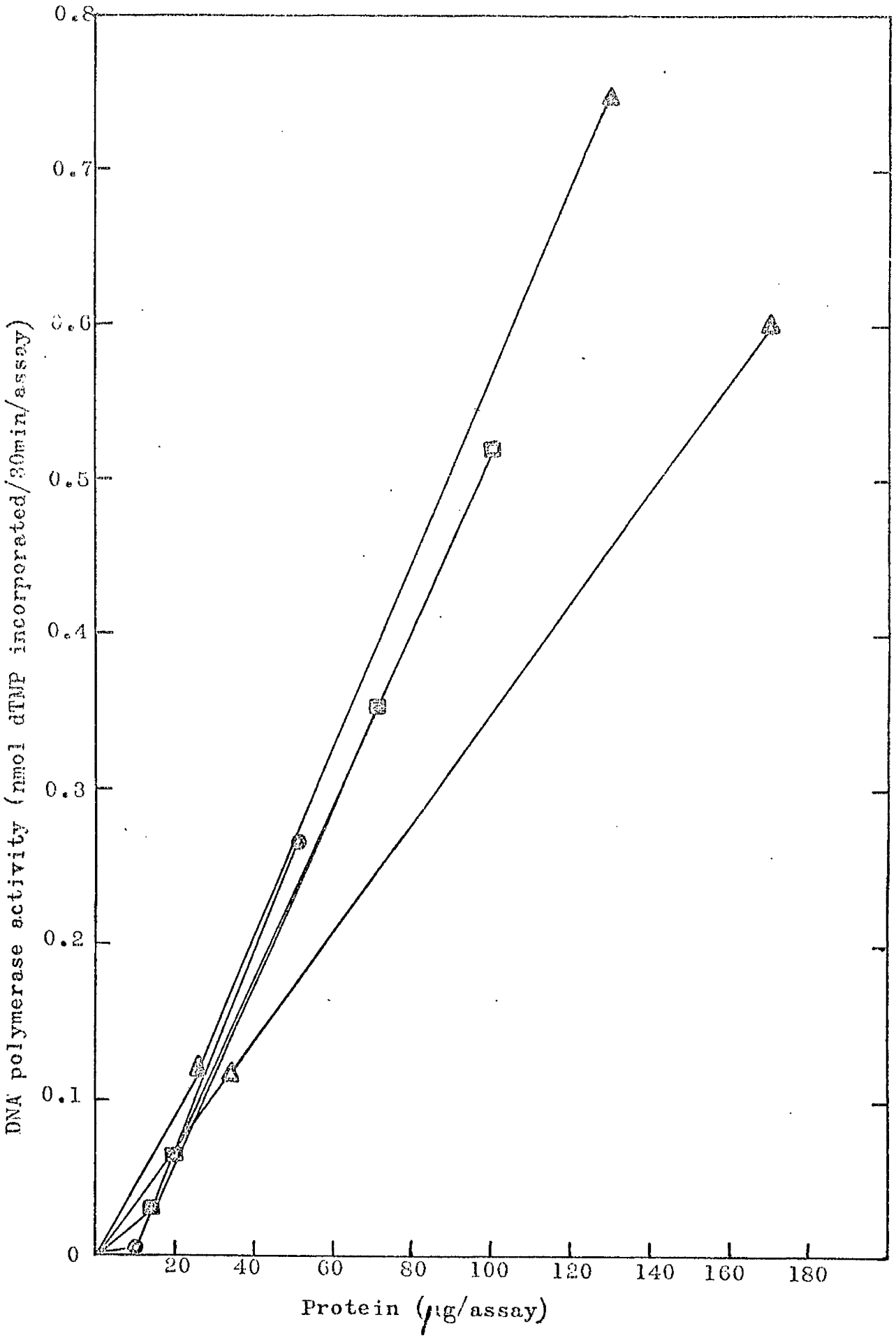
2-mercaptoethanol at 10mM

Dithiothreitol at 0.1mM

Dithiothreitol at 1.0mM

Dithiothreitol at 5.0mM

Fig. III. 1.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
20
40
60
80
100
120
140
160
180
DNA polymerase activity (nmol dTMP incorporated/30min/assay)
Protein (µg/assay)

Extracts 1 - 5 were assayed in the standard DNA polymerase assay (Methods section 2. 2. 1.) but with 5mM-2-mercaptoethanol. On the basis of these results (Fig. III. 1. and Fig. III. 2.) it was decided to use 2-mercaptoethanol at 10mM routinely in whole-cell extracts.

1. 1. 2. Assay of DNA polymerase.

Tube 1 extract from the previous experiment (no reducing agent) was utilised and increasing concentrations of 2-mercaptoethanol or dithiothreitol were added in the otherwise standard assay (Methods section 2. 2. 1.). From the result (Fig. III. 3.) it was seen that, above 5mM-2-mercaptoethanol, a plateau of activity existed and dithiothreitol was more effective than 2-mercaptoethanol as a reducing agent in the standard assay; however, the difference was small and, since 2-mercaptoethanol was employed in extraction (Results section 1. 1. 1.) it was decided to incorporate this agent at 10mM in the standard assay.

1. 1. 3. Stability of DNA polymerase.

Extracts made in the earlier experiment (Results section 1. 1. 1.) were used. Tube 1 (no reducing agent), tube 2 (10mM-2-mercaptoethanol) and tube 3 (0.1mM-dithiothreitol) were stored in ice in a Dewar flask and assayed at intervals with 10mM-2-mercaptoethanol. In addition, batches from tube 2, stored at -20°C or -70°C, were also assayed after certain times. The over-

Fig. III. 2.

Progress of the DNA polymerase reaction using enzyme extracted from infected cells with various reducing agents.

Extracts of infected cells were prepared and assayed as described in the Legend to Fig. III. 1.

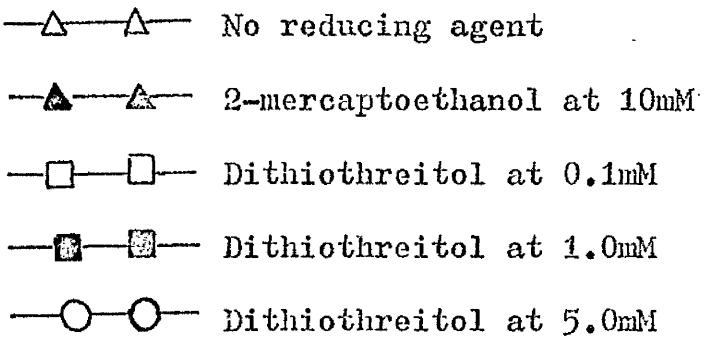


Fig. III. 2.

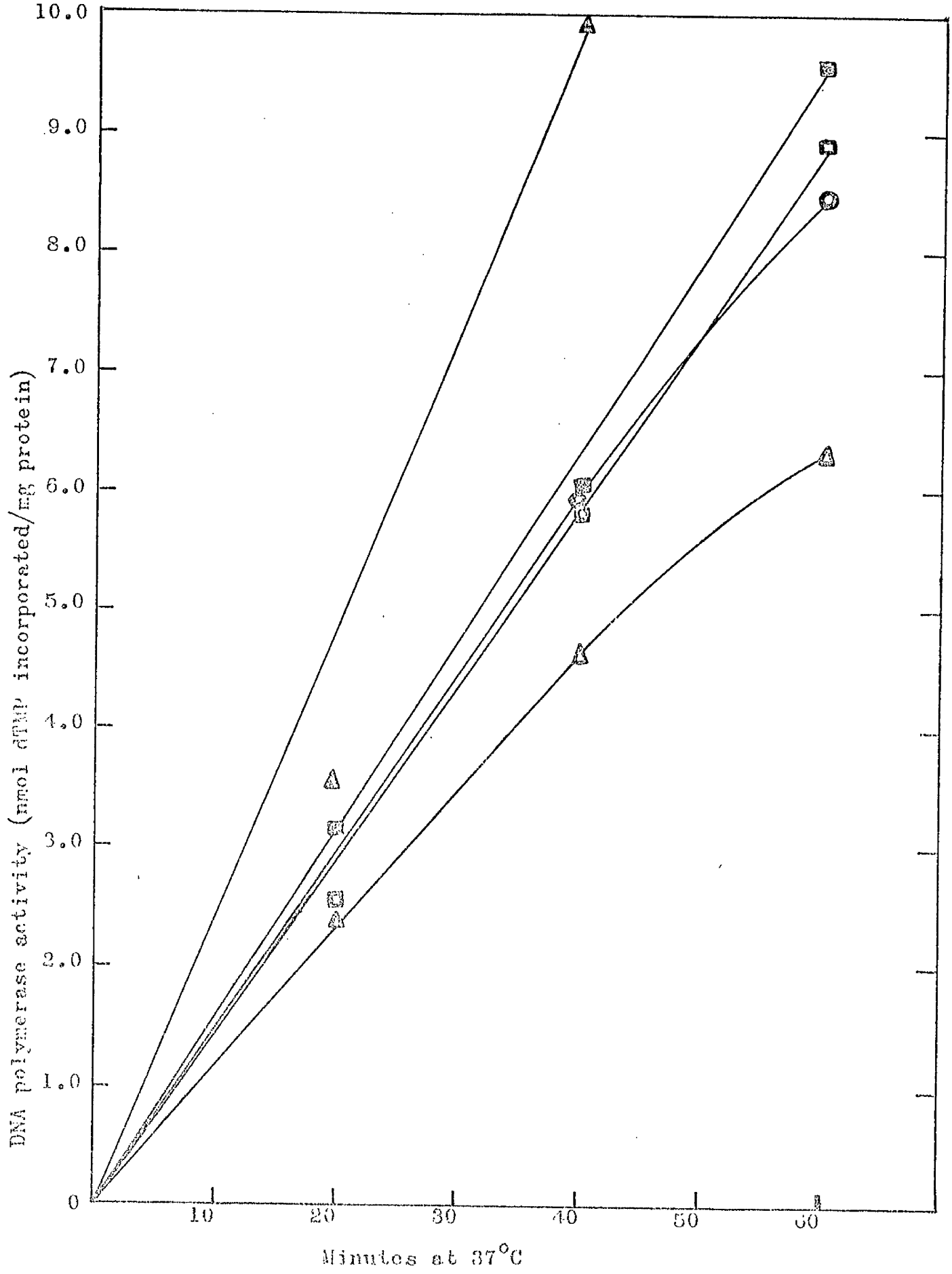

10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
DNA polymerase activity (nmol dTMP incorporated/mg protein)
10
20
30
40
50
60
Minutes at 37°C

Fig. III. 3.

The effect of reducing agents on the assay of DNA polymerase from infected cells.

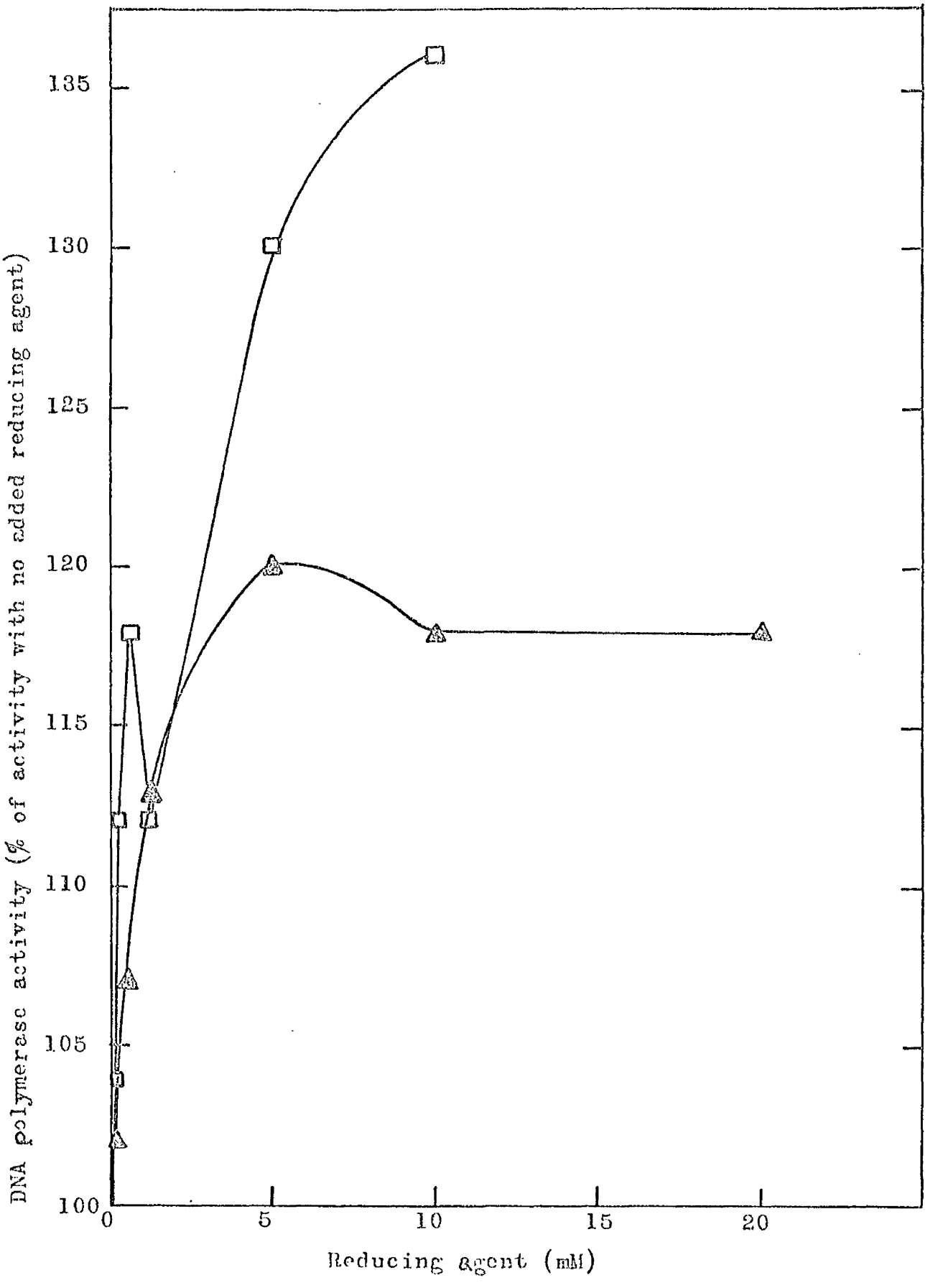
The whole-cell extract of HSV-infected cells made in the absence of reducing agent (Legend to Fig. III. 1.) was assayed in a standard DNA polymerase assay (Methods section 2. 2. 1.) with various concentrations of either 2-mercaptoethanol or dithiothreitol.

100% activity was = 0.785nmol dTMP incorporated/h/assay (170µg protein).

—△—△— 2-mercaptoethanol

—□—□— Dithiothreitol

Fig. III. 3.


DNA polymerase activity (% of activity with no added reducing agent)
135
130
125
120
115
110
105
100
0
5
10
15
20
Reducing agent (mM)

all results of the storage experiments at $0^{o}C$, $-20^{o}C$ and $-70^{o}C$ are shown in Fig. III. 4.

We conclude that storage at 0 - $4^{o}C$ is satisfactory in the presence of reducing agent for 7 days. After this, decay is rapid so that after 14 days' storage, a whole cell extract, with 10mM-2-mercaptoethanol present has decayed to 33% of its original value and, after 4 weeks, activity is negligible. Decay of such extracts at $-20^{o}C$ or - $70^{o}C$ is even more drastic so that, of the three temperatures of storage, 0 - $4^{o}C$ seems to be optimal for whole-cell extracts in the presence of reducing agent and in the absence of glycerol.

1. 2. The extraction of DNA polymerase from nuclei and cytoplasm of HSV-infected cells: effect of replacement of Ca^{2+} with Mg^{2+} on the leakage of DNA polymerase from nuclei.

Nuclear-cytoplasmic fractionation of HSV-infected cells was investigated as a possible first step in the purification of the virus-induced DNA polymerase. The presence of Ca^{2+} in the extracts, however, made dialysis necessary to remove this ion which is inhibitory to many DNA polymerases (Keir, 1965) and the possibility of using Mg^{2+} in place of Ca^{2+} was therefore of interest since this would allow the omission of dialysis, Mg^{2+} being a normal DNA polymerase assay component. (Dialysis was found to result in considerable losses of HSV-induced DNA

Fig. III. 4.

Storage of the DNA polymerase in whole-cell extracts of infected cells in the presence of different reducing agents at various temperatures.

Whole-cell extracts of infected cells extracted in the presence of 10mM-2-mercaptoethanol, 0.1mM-dithiothreitol or in the absence of reducing agent (Legend to Fig. III. 1.) were assayed for DNA polymerase then stored at $0^{\circ}C$ in a Dewar flask, and aliquots assayed after various time intervals. Aliquots of the extract with 10mM-2-mercaptoethanol were stored at $-20^{\circ}C$ or $-70^{\circ}C$ for a period of 24 days then assayed for DNA polymerase.

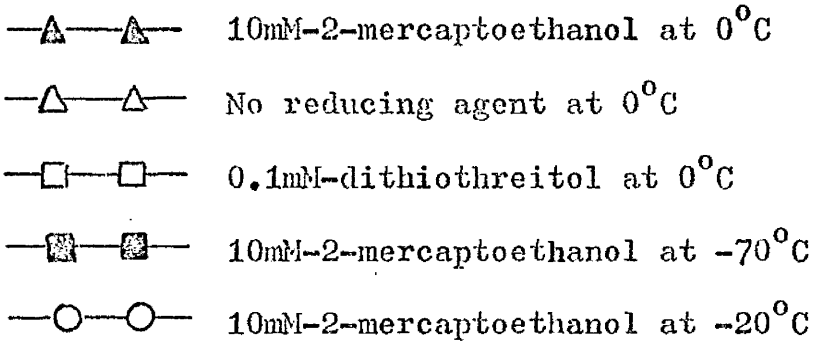
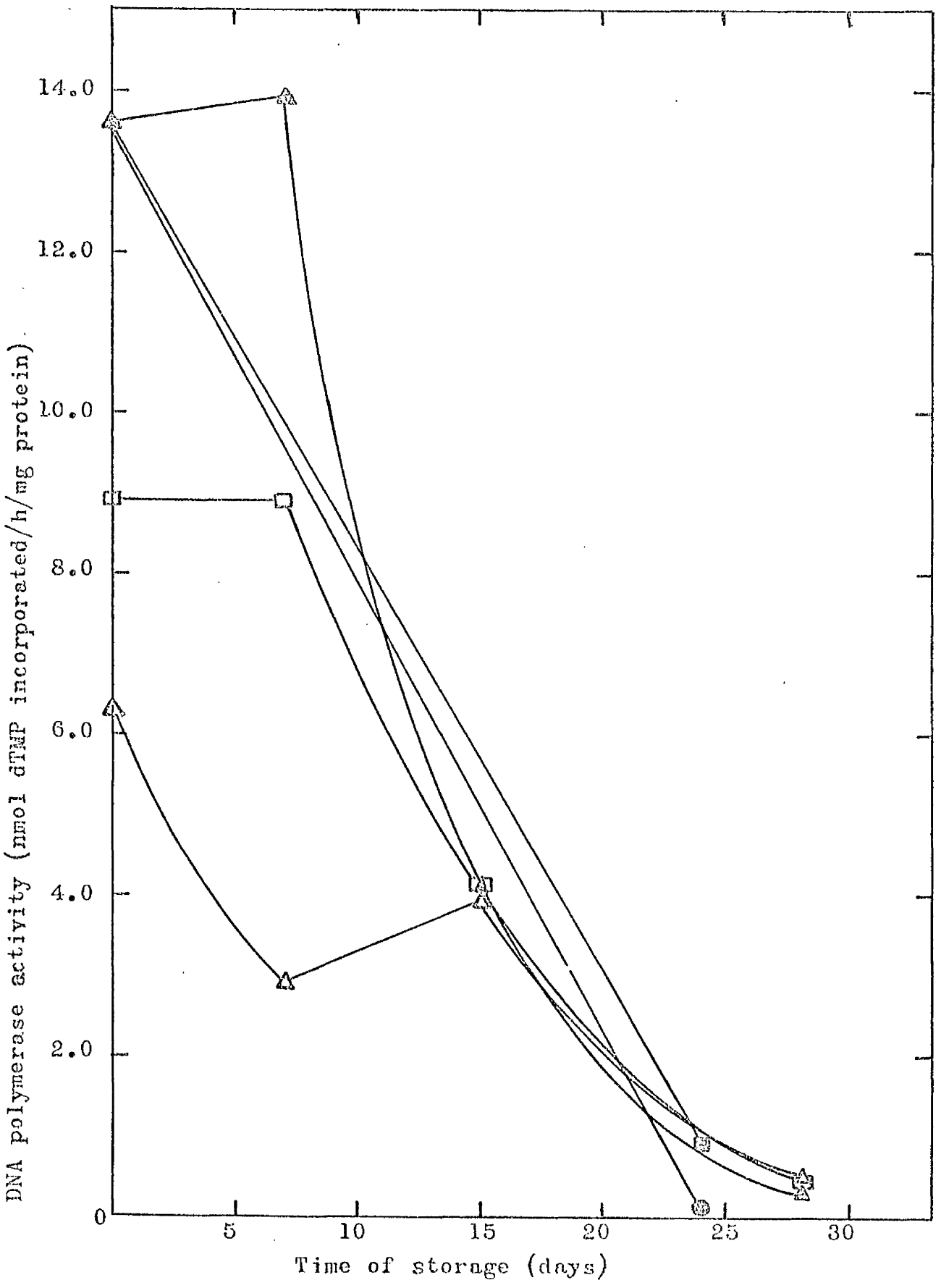


Fig. III. 4.


14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
5
10
15
20
25
30
DNA polymerase activity (nmol dTMP incorporated/h/mg protein)
Time of storage (days)

polymerase activity).

The experiment on the comparison of Mg^{2+} and Ca^{2+} was carried out essentially as described in Methods section 2. 6. 3. except that in one extract, Mg^{2+} was present at 1.5mM and, in the other Ca^{2+} at 1.5mM. The results of this experiment are presented in Table III. 1. from which it can be seen that Ca^{2+} appears to be more effective than Mg^{2+} in preventing enzyme-leaching from the nuclei. However, as found in this and in later experiments, low enzyme activities were recovered in this extracting technique compared with activities extracted from whole cells. This latter technique (Methods section 2. 6. 1.) was therefore adopted for routine enzyme preparation.

1. 3. The effect of KCl concentration on DNA polymerase activity in extracts of uninfected and HSV-infected cells.

The standard DNA polymerase assay (Methods section 2. 2. 1.) was used with 90mM-tris-HCl, pH 7.5 and varying KCl concentrations. The results using both native and heat-denatured DNA are presented in Fig. III. 5. They compare well with those of Morrison (1967) although the optima are at slightly different positions; the higher tris-HCl concentration employed in this present work probably accounts for the differences.

On the basis of these results, concentrations of 60mM and 120mM-KCl were adopted for uninfected-cell DNA polymerase and

Table III. 1.

The Effect of Replacement of Ca^{2+} by Mg^{2+} on the Leakage of DNA Polymerase from Nuclei during Nuclear-Cytoplasmic Fractionation of HSV-Infected Cells.

Fraction	Volume of Fraction (ml)	DNA Polymerase Activity (units/ml)	Total units/ fraction	Protein (mg/ml)	Total Protein (mg/ fraction)	Polymerase Specific Activity (units/ mg)
Nuclear Fraction (Ca^{2+})	14.5	0.317	4.60	0.30	4.35	1.057
Nuclear Fraction (Mg^{2+})	11.5	0.256	2.90	0.34	3.91	0.742
Cytoplasmic Fraction (Ca^{2+})	24.5	0.152	3.72	0.30	7.35	0.506
Cytoplasmic Fraction (Mg^{2+})	33.0	0.208	6.85	0.22	7.26	0.943

Fig. III. 5.

The effect of KCl concentration on DNA-polymerase activity extracted from uninfected and HSV- infected cells.

Nuclear (S2) fractions from soluble extracts of uninfected or HSV-infected cells (Methods section 2. 6. 2.) were assayed for DNA polymerase using the standard assay (Methods section 2. 2. 1.) but with varying KCl concentrations and assaying with either 400µg/ml denatured calf thymus DNA or 400µg/ml native calf thymus DNA as primer.

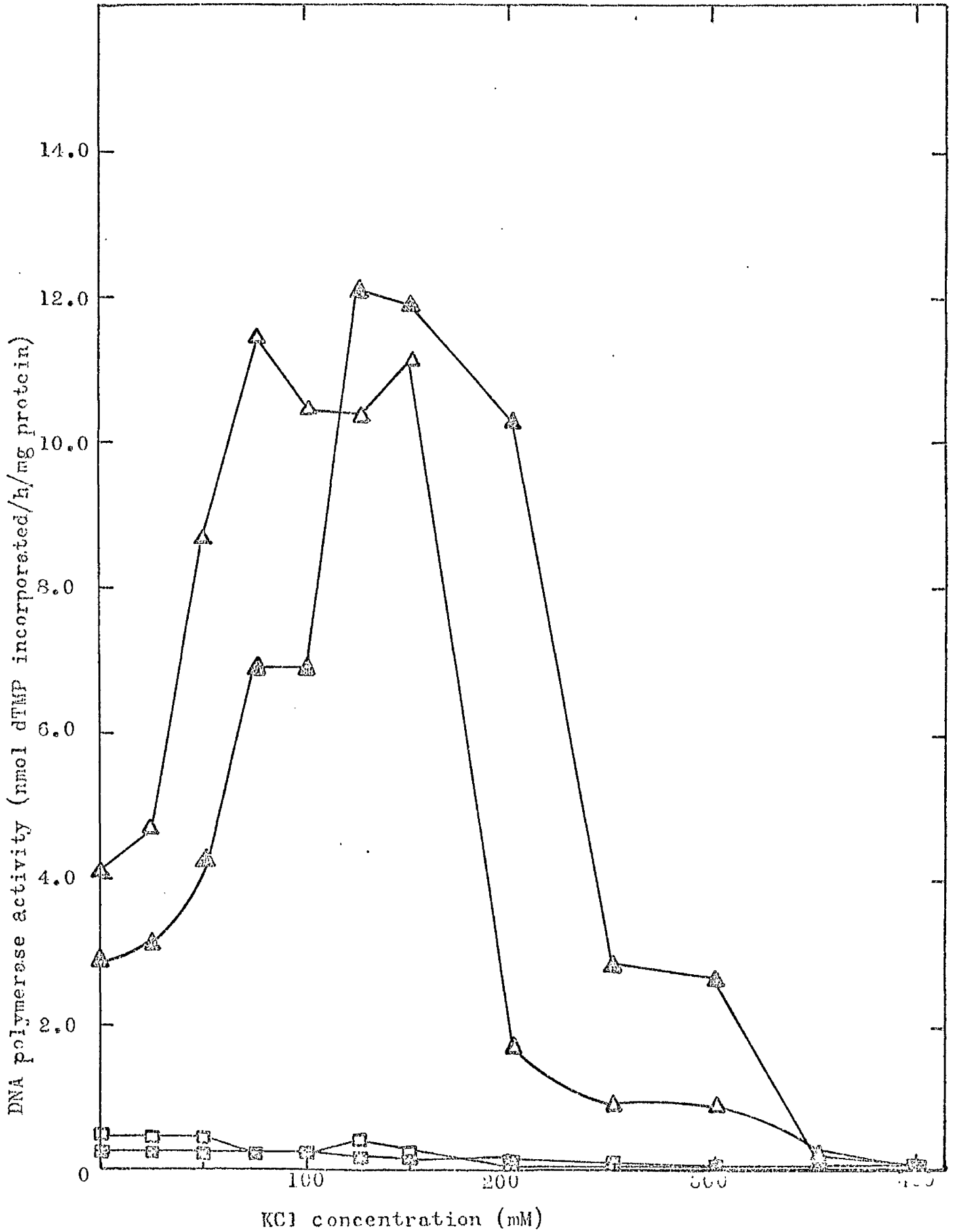
—△—△— Extract of infected cells : heat-denatured DNA

—△—△— Extract of infected cells : native DNA

—▣—▣— Extract of uninfected cells : heat-denatured DNA

—□—□— Extract of uninfected cells : native DNA

Fig. III. 5.


14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
100
200
DNA polymerase activity (nmol dTMP incorporated/h/mg protein)
KCl concentration (mM)

infected-cell DNA polymerase assays respectively, since these values appeared to give optimal enzyme activity in each case.

1. 4. The effect of the concentration of potassium phosphate buffer, pH 7.5, on the DNA polymerase activity of extracts of HSV-infected cells.

This experiment was designed to test the effect of potassium phosphate since it would be present in later experiments in the fractionations involving hydroxylapatite.

The enzyme used in this experiment was a $(NH_4)_2SO_4$ fraction (Methods section 2. 7.) of a whole-cell extract (Methods section 2. 6. 1.) and was dissolved in 0.05M-potassium phosphate buffer, pH 7.2-10mM-2-mercaptoethanol - 30% (v/v)glycerol. The standard DNA polymerase assay (Methods section 2. 2. 1.) was employed minus KCl and with several different concentrations of potassium phosphate buffer pH 7.5 in place of tris-HCl.

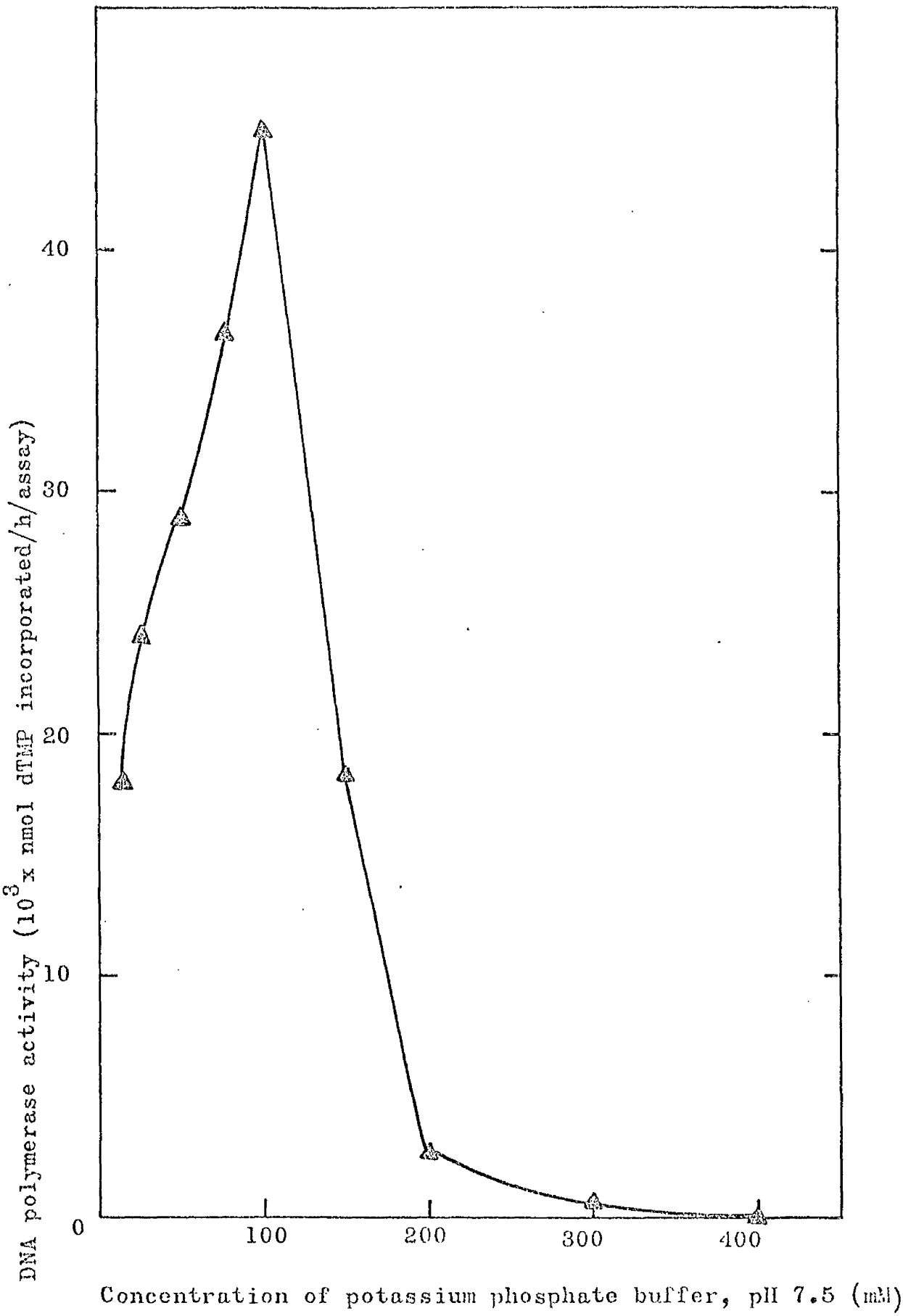
From the results (Fig. III. 6.) it can be seen that a sharp optimum exists at 100mM-potassium phosphate (166mM-K^+). It seems likely that we are observing the K^+ effect seen earlier (Results section 1. 3.). The existence of an Mg^{2+}-complexing effect with Pi is also possible and may account for the sharp fall-off of activity. Potassium phosphate buffer, pH 7.5, seems to be an acceptable substitute for tris-HCl in the standard assay.

Fig. III. 6.

The effect of the concentration of potassium phosphate buffer, pH 7.5 on the DNA polymerase activity of extracts of HSV-infected cells.

An ammonium sulphate fraction (Methods section 2. 7.) of a whole-cell extract (Methods section 2. 6. 1.) of HSV-infected cells was dissolved in 0.05M-potassium phosphate buffer, pH 7.2-10mM-2-mercaptoethanol-30%(v/v) glycerol and assayed for DNA polymerase using the standard assay (Methods section 2. 2. 1.) minus KCl and with several different concentrations of potassium phosphate buffer (pH 7.5) in place of tris-HCl.

Fig. III. 6.


DNA polymerase activity (10^3 x nmol dTMP incorporated/h/assay)
0
10
20
30
40
100
200
300
400
Concentration of potassium phosphate buffer, pH 7.5 (mM)

1. 5. The effect of tris-HCl pH 7.5 on the DNA polymerase activity in extracts of uninfected and HSV-infected cells.

Half-scale DNA polymerase assays (Methods section 2. 2. 1.) using activated DNA (Methods section 2. 3. 3.) and minus KCl were set up with a range of different concentrations of tris-HCl to give the results presented in Fig. III. 7. Again the profiles are reminiscent of those for KCl and potassium phosphate; the infected-cell DNA polymerase optimum is at 290mM-tris-HCl. (≡ 192mM cation added to the standard assay minus KCl).

1. 6. The effect of Ca^{2+} on DNase activity in extracts of HSV-infected cells.

This experiment was designed to explore the use of Ca^{2+} as a protease inhibitor throughout the purification of the virus-induced DNA polymerase, and DNA-exonuclease activity.

Standard DNase assays were set up (Methods section 2. 2. 3.) (2mM-Mg^{2+}) to which Ca^{2+} was added (2.5mM). 80 - 100% inhibition of activity was found at 0.3 - 0.5mM-EDTA and in the presence and absence of glycerol. Owing to this strongly inhibitory effect on the virus-induced DNase, Ca^{2+} was not used.

2. PURIFICATION STUDIES ON THE HSV-INDUCED DNA POLYMERASE AND DEOXYRIBONUCLEASE.

Fig. III. 7.

The effect of concentration of tris-HCl buffer (pH 7.5) on the DNA polymerase activity of extracts of uninfected and HSV-infected cells.

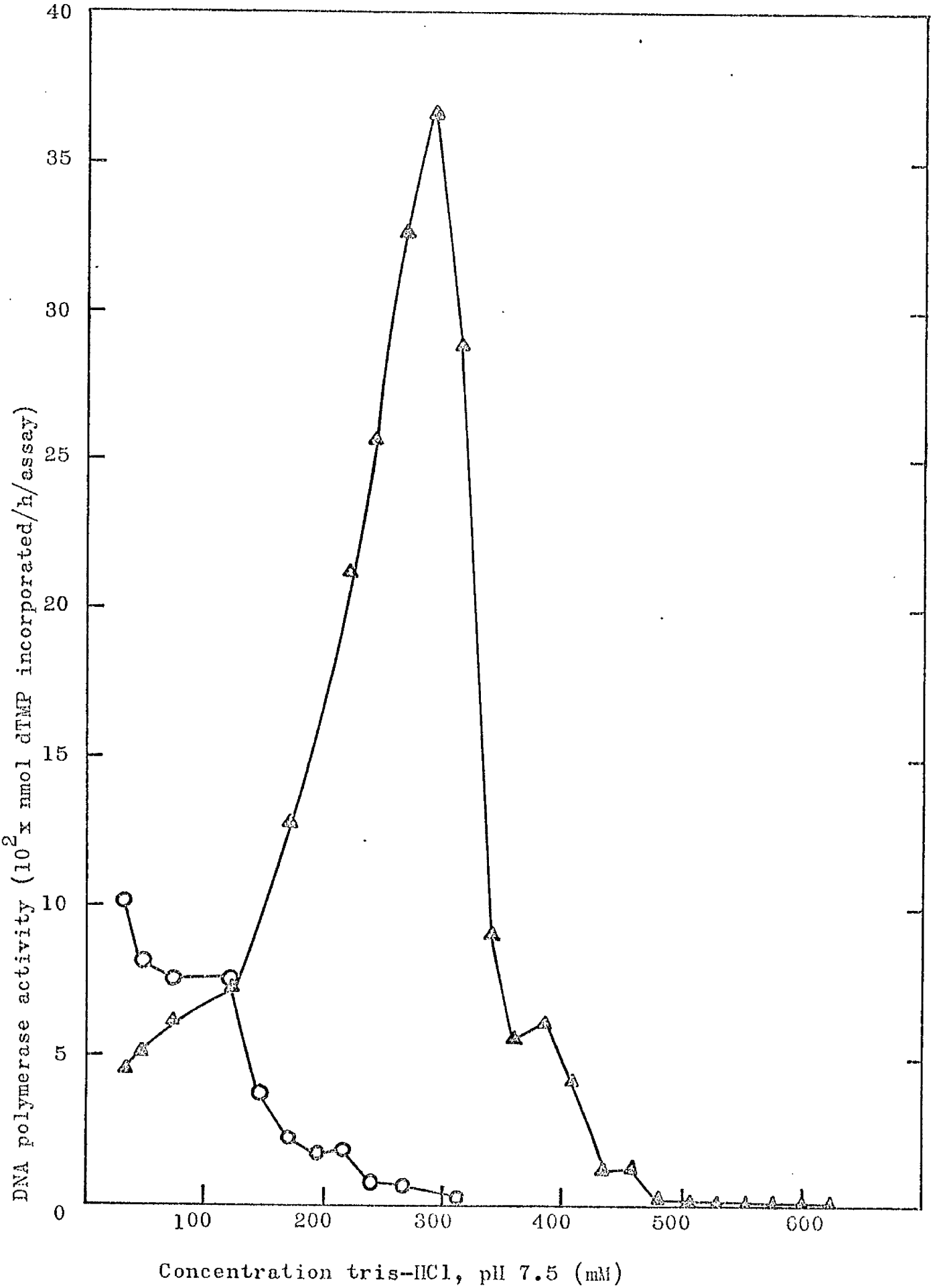
Half-scale DNA polymerase assays (Methods section 2. 2. 1.) using activated DNA at 400µg/ml (Methods section 2. 3. 3.) and minus KCl, were set up with a range of different concentrations of tris-HCl (pH 7.5) and using whole-cell extracts of uninfected or HSV-infected cells as enzyme source.

0.02ml aliquots of a 1 in 2 dilution of typical whole-cell extracts were used in each case.

—△—△— DNA polymerase from HSV-infected cells.

—○—○— DNA polymerase from uninfected cells.

Fig. III. 7.


40
35
30
25
20
15
10
5
0
100
200
300
400
500
600
DNA polymerase activity (10^2 x nmol dTMP incorporated/h/assay)
Concentration tris-HCl, pH 7.5 (mM)

2. 1. DEAE-cellulose column chromatography.

2. 1. 1. DEAE-cellulose column chromatography of extracts of uninfected cells.

It was considered desirable to establish the chromatographic behaviour of the DNA polymerase and DNase of extracts of the uninfected BHK 21 (C13) host cells before going on to consider the more complex situation with extracts of HSV-infected cells with the extra virus-induced enzymes.

The experiment was carried out essentially as described in Methods section 2. 10. 2. and the results of a typical fractionation are presented in Fig. III. 8. For this experiment, 3.0ml of uninfected BHK 21 (C13) cells were used to prepare a whole-cell extract and the extract diluted 1 in 2 in 10mM-2-mercaptoethanol and 1mM-EDTA, to give 32ml of sample in column starter buffer. 30 units of DNA polymerase ("low salt"-denatured DNA primer) and 860 units DNase (denatured DNA substrate) were applied to the column: 8.4 units DNA polymerase were recovered (26% recovery), and 773 units DNase (90% recovery). After the E_{280} of the fractions had been measured, bovine serum albumin was added to the fraction tubes to give a final concentration of 1mg/ml to stabilise the enzymes for storage.

From the results, it can be seen that we find a peak of endonuclease active against heat-denatured DNA appearing in the column wash. A small peak of DNA polymerase can be discerned co-chromatographing with this non-adsorbed DNase and, on gradient-

Fig. III. 8.

DEAE-cellulose column chromatography of a whole-cell extract of uninfected cells.

A whole-cell extract (Methods section 2. 6. 1.) of 3.0ml of uninfected BHK21 (C13) cells was diluted 1 in 2 with 10mM-2-mercapto-ethanol-1mM-EDTA to give 32ml of sample in DEAE-starter buffer. This contained 30 units DNA polymerase ("low salt") and 860 units DNase (denatured DNA as substrate).

The sample was applied and elution carried out as described in Methods section 2. 10. 2. Aliquots of the fractions were then assayed for DNA polymerase ("low salt"-denatured DNA as primer)(Methods section 2. 2. 1.) and DNase (denatured DNA as substrate). The E_{280nm} of the fractions was followed and the salt gradient checked (Methods section 2. 1. 4.).

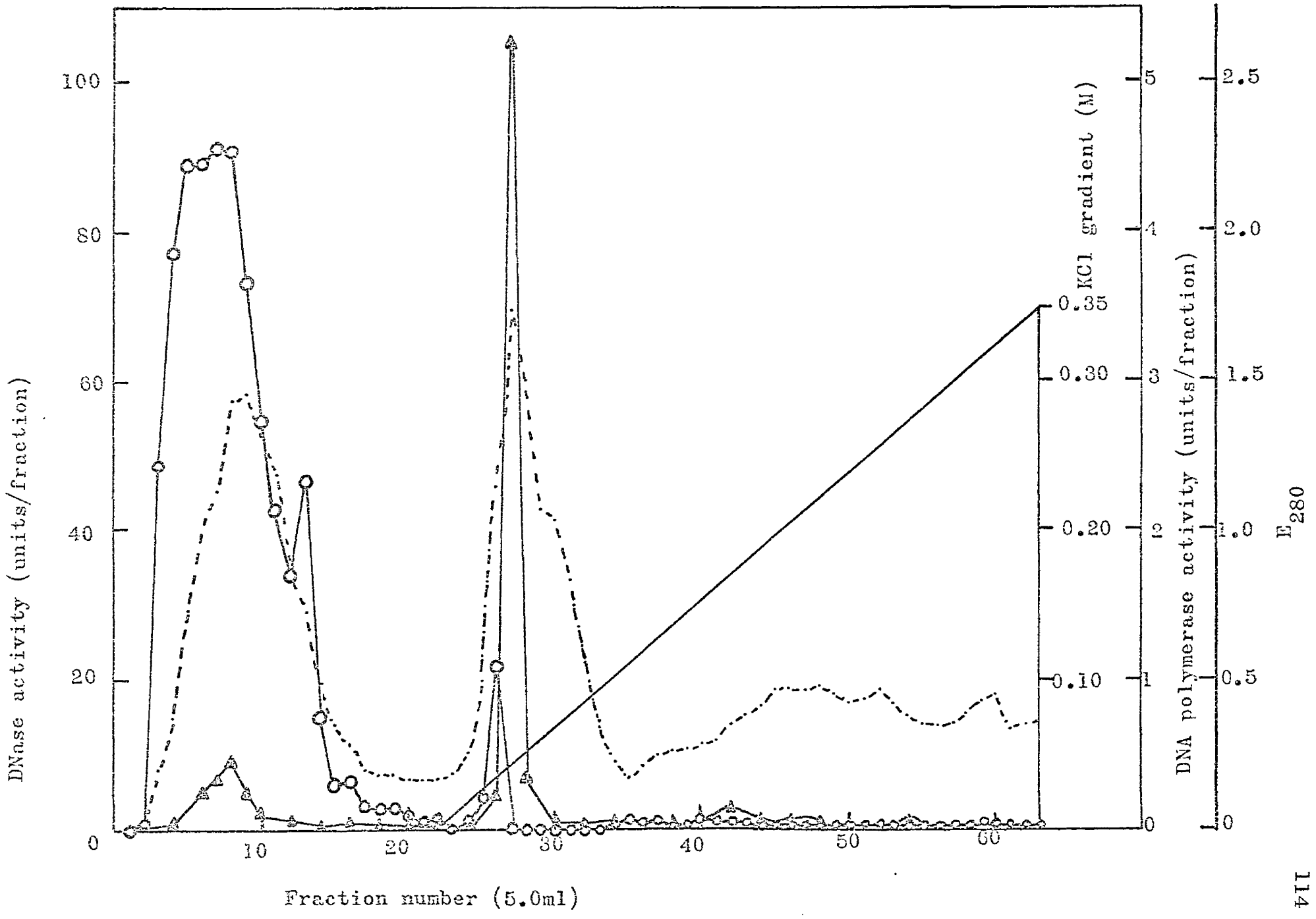
—○—○— DNase activity (denatured DNA as substrate)

—△—△— DNA polymerase activity

-·-·-·-·- E_{280nm}

———— KCl gradient

Fig. III. 8.


DNase activity (units/fraction)
DNA polymerase activity (units/fraction)
KCl gradient (M)
E_{280}
Fraction number (5.0ml)
0
20
40
60
80
100
10
20
30
40
50
60
0.10
0.20
0.30
0.35
0.5
1.0
1.5
2.0
2.5
1
2
3
4
5

elution, almost immediately, at low KCl concentrations, an adsorbed peak of DNA polymerase appears together with a small peak of DNase. Further smaller peaks of DNA polymerase activity then follow, the largest of these eluting at approx. 0.16M-KCl.

Mode-of-action studies on the DNase peaks (Methods sections 2. 2. 4. 1. and 2. 2. 4. 2.) revealed that both the large non-adsorbed peak and the small adsorbed peak represented DNA endonucleases active against heat-denatured DNA. It is likely that the second peak of DNase represents elution by salt of residual DNase of the non-adsorbed type.

The DNA polymerase peaks of another similar run were examined for terminal DNA nucleotidyltransferase activity by each of the assays described in Methods section 2. 2. 2.; however, no such activity was associated with the first peak and very little, if any, with the second. Characterisation of the DNA polymerase activities from the non-adsorbed and first adsorbed peak will be described in Results section 4. 1. 7.

2. 1. 2. DEAE-cellulose column chromatography of whole-cell extracts of HSV-infected cells.

The results of a typical column chromatography run (Methods section 2. 10. 2.) are presented in Fig. III. 9. A volume of 27ml enzyme solution, made by diluting 13.5ml of a whole-cell extract 1 in 2, as described in the previous section (2. 1. 1.), was applied to the column. This was equivalent to

Fig. III. 9.

DEAE-cellulose column chromatography of a whole-cell extract of HSV-infected cells.

27ml enzyme solution, made by diluting 13.5ml of a whole-cell extract of HSV-infected cells 1 in 2 as described in the legend to Fig. III. 8., was applied to a DEAE-cellulose column and fractionated as described in Methods section 2. 10. 2. This was equivalent to 270 units DNA polymerase ("high salt" assay - denatured DNA as primer) and 338 units DNase (denatured DNA as substrate). The fractions from the column were assayed for DNA polymerase ("high salt" assay)(Methods section 2. 2. 1.) and DNase activity (denatured or native DNA as substrate)(Methods section 2. 2. 3.). The KCl gradient was checked (Methods section 2. 1. 4.).

—△—△— DNA polymerase activity ("high salt" assay)

—○—○— DNase activity (denatured DNA as substrate)

—●—●— DNase activity (native DNA as substrate)

———— KCl gradient

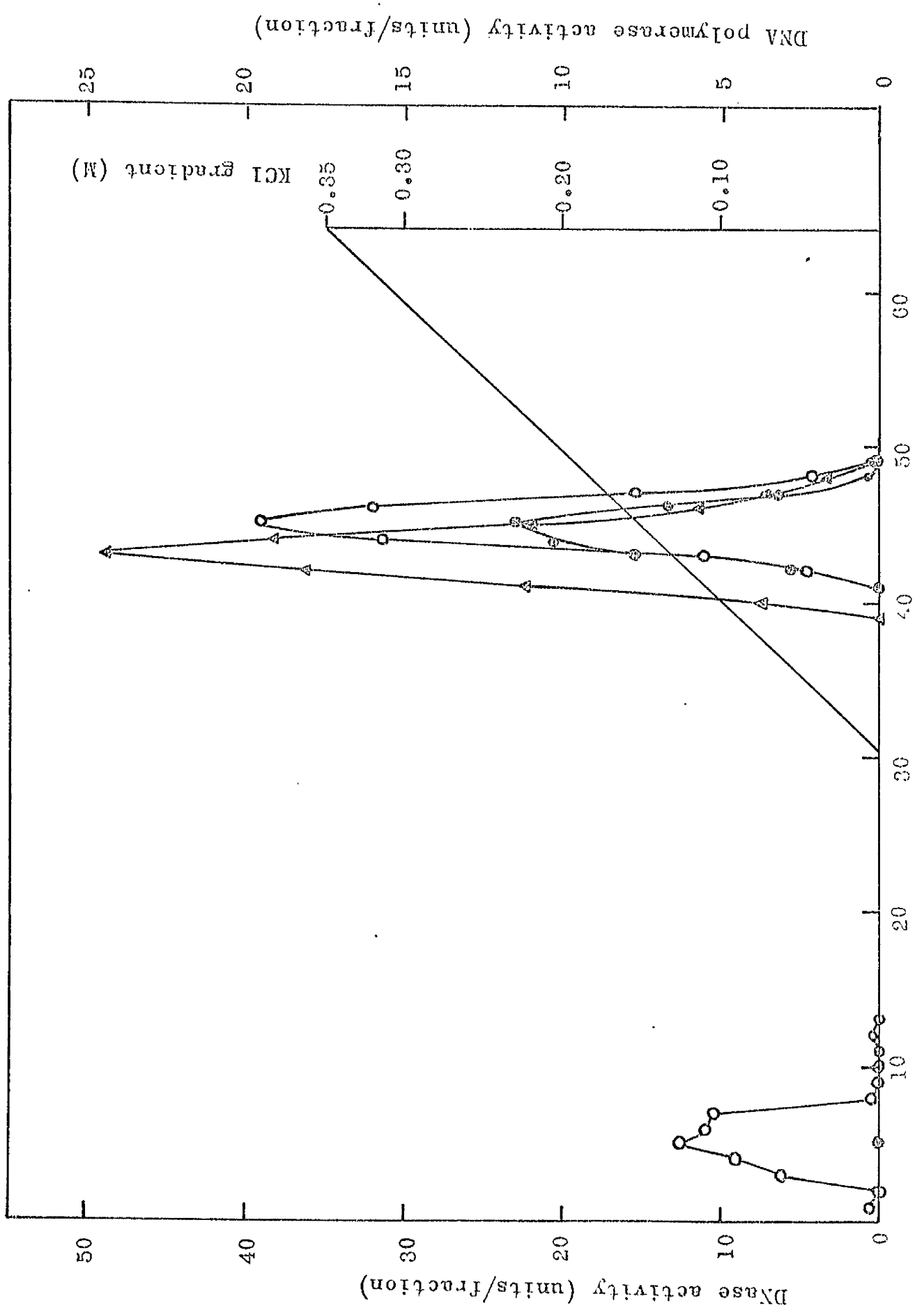

DNase activity (units/fraction)
DNA polymerase activity (units/fraction)
KCl gradient (M)
Fraction number (5.5ml)
0
10
20
30
40
50
0
5
10
15
20
25
0.10
0.20
0.30
0.35
10
20
30
40
50
60

Fig. III. 9.

270 units DNA polymerase ("high-salt" assay - denatured DNA primer)& 338 units DNase (denatured DNA as substrate). "High salt" and "low salt" DNA polymerase assays (Methods section 2. 2. 1.) were carried out on the fractions collected, together with DNase assays (Methods section 2. 2. 3.) with native or denatured DNA as substrate. Mode-of-action studies (Methods section 2. 2. 4.) were carried out with nuclease from the two DNase peaks. The E_{280} profile was followed using the LKB UVICORD apparatus.

It can be seen from the results that a DNase which has the properties of an endonuclease active against heat-denatured DNA but with little or no activity with native DNA, is not adsorbed to the column. It is likely that this enzyme corresponds to the nuclease described by Koh, Waddell and Aposhian (1970).

A second, adsorbed peak of DNase appears on gradient elution at approx. 0.1 - 0.2M-KCl: this activity has the properties of an exonuclease active on both heat-denatured and native DNA. These results correspond closely to those of Morrison and Keir (1968a). From the column 100 units of DNA polymerase (37% recovery) and 184 units DNase (54% recovery) were collected.

A peak of salt-stimulated DNA polymerase activity appears almost coincident with the adsorbed DNase; however, the polymerase peak is consistently found to elute slightly ahead of the peak of DNase activity.

2. 1. 3. DEAE-cellulose column chromatography of combined extracts of uninfected and HSV-infected cells.

Since, in the chromatographic fractionations with infected-cell extracts, there was little or no DNA polymerase activity in the positions of host DNA polymerase, it was decided to carry out a chromatographic run with a mixture of uninfected-cell and infected-cell extracts so that the distinct chromatographic behaviour of the host and virus-induced DNA polymerase activities in the presence of each other could be ascertained.

Accordingly, whole-cell extracts made from 2ml packed cell volume of uninfected cells and 2ml packed cell volume of HSV-infected cells were mixed, diluted 1 in 2, as in Results sections 2. 1. 1. and 2. 1. 2., and applied to a DEAE-cellulose column in a total volume of 40ml (37mg protein; 131 units DNA polymerase primed by heat-denatured DNA; 48 units DNA polymerase primed by native DNA and 688 units DNase active on heat-denatured DNA). The chromatographic run was carried out as before and the results are presented in Fig.III. 10.

There was a greater than 100% recovery of native DNA-primed DNA polymerase activity from the column and 56% recovery of heat-denatured DNA-primed DNA polymerase. A large peak of DNA polymerase was found in the position of the infected-cell enzyme (Results section 2. 1. 2.) and small peaks of activity

Fig. III. 10.

DEAE-cellulose column chromatography of combined extracts of uninfected and HSV-infected cells.

Whole-cell extracts (Methods section 2. 6. 1.) of 2ml of uninfected cells and 2ml of HSV-infected cells were mixed, diluted 1 in 2 (as in Legend to Fig. III. 8.) and applied to a DEAE-cellulose column in a total volume of 40ml (37mg protein; 131 units DNA polymerase ("low salt" assay - denatured DNA as primer); 688 units DNase (heat-denatured DNA as substrate)). 5.0ml fractions were collected and assayed for DNase (heat-denatured DNA as substrate), DNA polymerase (activated DNA, denatured DNA, or native DNA as primer, with the "low salt" assay condition - Methods section 2. 2. 1.). The KCl gradient was also checked and the E_{280nm} of the fraction read as before (Legend to Fig. III. 8.).

—○—○— DNase activity (denatured DNA as substrate)

—▲—▲— DNA polymerase activity (activated DNA as primer - "low salt")

—△—△— DNA polymerase activity (denatured DNA as primer - "low salt")

—·—·—·— E_{280nm}

———— KCl gradient

DNA polymerase activity with native DNA as primer not shown

Fig. III. 10.

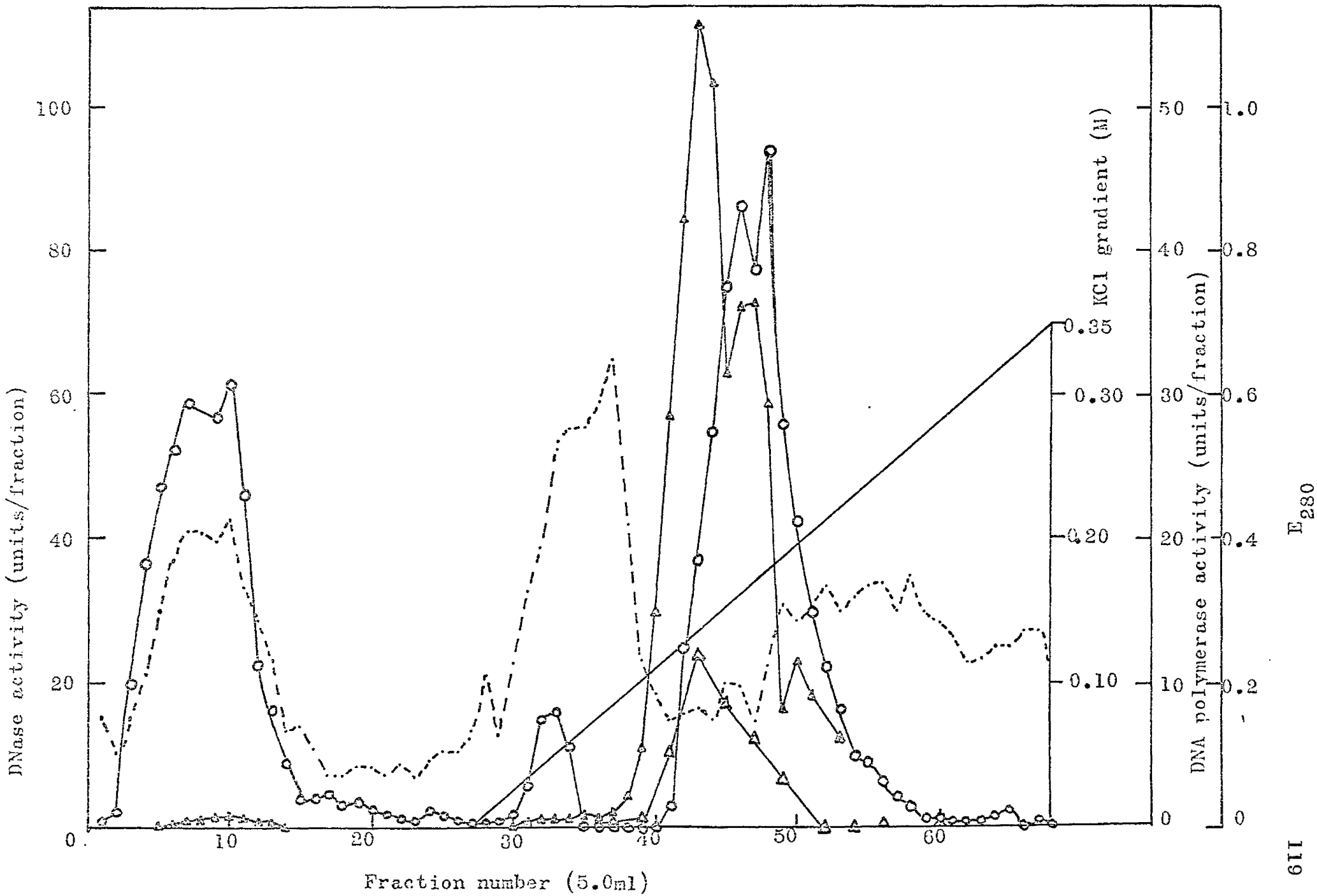

DNase activity (units/fraction)
DNA polymerase activity (units/fraction)
KCl gradient (M)
E_{230}
Fraction number (5.0ml)
0
10
20
30
40
50
60
80
100
0.10
0.20
0.30
0.35
0.4
0.6
0.8
1.0

Table III. 2.

Experiment involving assay of whole-cell extracts of uninfected and HSV-infected cells and mixtures of the two in various proportions to determine if there is an excess of an inhibitor or activator of DNA polymerase activity in these extracts.

Aliquots of whole-cell extracts (Methods section 2. 6. 1.) of uninfected and HSV-infected cells were assayed alone and as mixtures of various proportions of the two extracts in "low salt", half-scale DNA polymerase assays (Methods section 2. 2. 1.) making up the volume of samples for assay where necessary to 0.05ml with TEM buffer. The d.p.m. (^{3}H) incorporated into DNA per 0.05ml aliquots of reaction mixtures were compared, in the case of the mixtures, with the calculated values obtained by adding the activities obtained from assay of the separate components of the mixture.

Table III. 2.

Vol. extract of HSV-infected cells (ml)	Vol. extract of uninfected cells (ml)	Vol. buffer (TEM) (ml).	d.p.m. ([^{3}H] dTMP) incorporated into DNA/disc.	Calculated expected d.p.m. incorporated into DNA/disc.
0	0.01	0.04	1,612	
0	0.02	0.03	2,921	
0	0.03	0.02	3,283	
0	0.04	0.01	4,243	
0	0.05	0	4,670	
0.01	0	0.04	2,859	
0.02	0	0.03	5,996	
0.03	0	0.02	7,898	
0.04	0	0.01	11,581	
0.05	0	0	14,020	
0.03	0.02	0	11,041	10,819
0.02	0.03	0	9,204	9,279
0.01	0.04	0	7,487	7,102
0.01	0.02	0.02	6,127	5,780
0.01	0.03	0.01	5,832	6,142
0.02	0.02	0.01	8,648	8,917

were discerned in the region of the host-cell enzymes, but of interest was the finding that only 0.6 unit of heat-denatured DNA-primed DNA polymerase was recovered in the positions of host-cell DNA polymerase, representing a recovery of only 1%.

The reason for this apparently very low recovery was not salt-inhibition of the host-cell polymerase since "low salt" assays were used. "High salt" assays were used in the infected-cell extract chromatographic run described in Results section 2. 1. 1. and so salt inhibition together with a possible shut-off of synthesis and possibly decay of host-cell DNA polymerase following HSV-infection might account for the apparent absence of uninfected-cell enzyme in the case of column chromatography with the extract of HSV-infected cells (Fig. III. 9.). It still seemed possible that some inhibition of the host polymerase activity was occurring when the infected-cell extract was added to the host-cell extract.

The results of an investigation of this possibility are presented in table III. 2. from which it can be seen that activities of mixtures, made up in a wide variety of proportions, of control and infected-cell extracts show additive activities: there is neither mutual inhibition nor stimulation of activity.

These mixing experiments, however, do not completely rule out the possibility that some inhibition of host cell DNA polymerase occurs in the presence of HSV-infected cell extracts since host DNA polymerase levels are normally so much lower than those of the polymerase activity of extracts of HSV-infected cells.

Nevertheless, the reason for the apparently low activities of host-cell DNA polymerase in the DEAE-cellulose chromatogram shown in Fig. III. 10. is still uncertain.

2. 1. 4. DEAE-cellulose column chromatography of a whole-cell extract of HSV-infected cells using stepwise elution.

A whole-cell extract (Methods section 2. 6. 1.) of volume 6.1ml was made from 1.0ml of packed HSV-infected cells and 2.5ml of extract applied to an adapted Pasteur-pipette type of column with a 7cm bed-height of gel. A wash was carried out with DEAE-starter buffer then successive stepwise washes with starter buffer containing 0.10M-, 0.15M- and 0.20M-KCl were made and the fractions assayed for DNase and DNA polymerase activity.

Essentially the same results as for gradient-elution were obtained: a non-adsorbed peak of DNA endonuclease was observed and then coincident peaks of DNase and DNA polymerase occurred early in the 0.10M-KCl wash and a further smaller coincident peak of DNase and DNA polymerase appeared early in the 0.15M-KCl wash.

This double-peak effect appears to be common in stepwise gradients while only one peak is found in gradient-elution.

2. 2. Hydroxylapatite column chromatography.

2. 2. 1. Hydroxylapatite column chromatography of extracts of HSV-infected cells.

2. 2. 1. 1. Early elution conditions.

A whole-cell extract was made from 2.0ml packed HSV-infected cells (Methods section 2. 6. 1.) and an ammonium sulphate precipitate (Methods section 2. 7.) made from this and suspended in 2.0ml of 0.04M-potassium phosphate buffer, pH 7.5 - 10mM-2-mercaptoethanol. This sample was then applied to a 10x1cm bed of gel and column chromatography carried out as described in Methods section 2. 10. 1. The results are shown in Fig. III. 11. and represent a typical fractionation: from time to time rather more DNase appeared in the second peak than in the first.

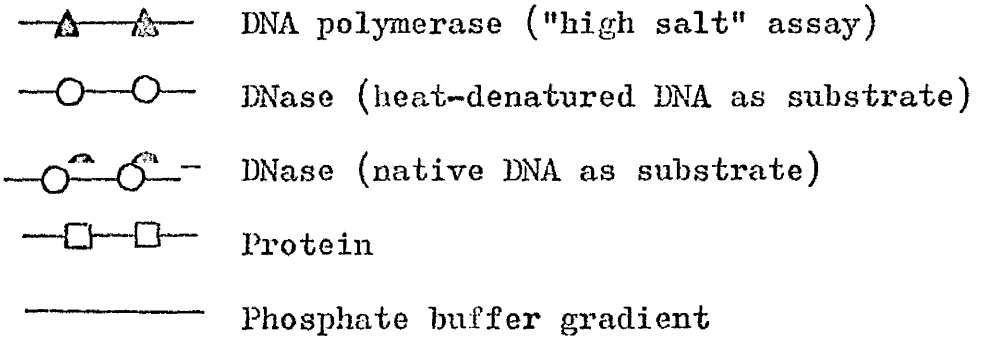
Mode-of-action studies (Methods section 2. 2. 4.) with enzyme from the DNase peaks revealed that the nucleases from both the non-adsorbed peak and the adsorbed and phosphate-eluted peak were exonucleases. The second peak was coincident with a salt-stimulated DNA polymerase peak and the ratio of DNase : DNA polymerase varied little over the peak region.

The recovery of "high salt" - denatured DNA primer - assayed DNA polymerase from the column was 33% while over 100% recoveries of DNase activity active against both native and denatured DNA were achieved. 14.5mg protein was applied and about 8mg recovered. Purifications of DNA polymerase of 7.3x in the pooled peak and 9.1x in the peak fraction were attained (denatured DNA as primer).

Fig. III. 11.

Hydroxylapatite column chromatography of an extract of HSV-infected cells (early elution conditions).

A whole-cell extract of 2ml HSV-infected cells (Methods section 2. 6. 1.) was made and an ammonium sulphate precipitate (Methods section 2. 7.) made from this and suspended in 2.0ml hydroxylapatite column starter buffer (early elution conditions - Methods section 2. 10. 1.). The sample (14.5mg protein; 392units DNA polymerase ("high salt" assay); 1066 units and 1083 units DNase with native and denatured DNA as substrate respectively) was applied to a 10x1cm bed of hydroxylapatite and fractionation carried out (Methods section 2. 10. 1.). The fractions collected were assayed for DNA polymerase ("high salt" assays except over the polymerase peak where "low salt" assays were also carried out - Methods section 2. 2. 1.), DNase (with native or denatured DNA as substrate - Methods section 2. 2. 3.) and protein (Methods section 2. 1. 1.). The salt gradient was also checked (Methods section 2. 1. 4.).



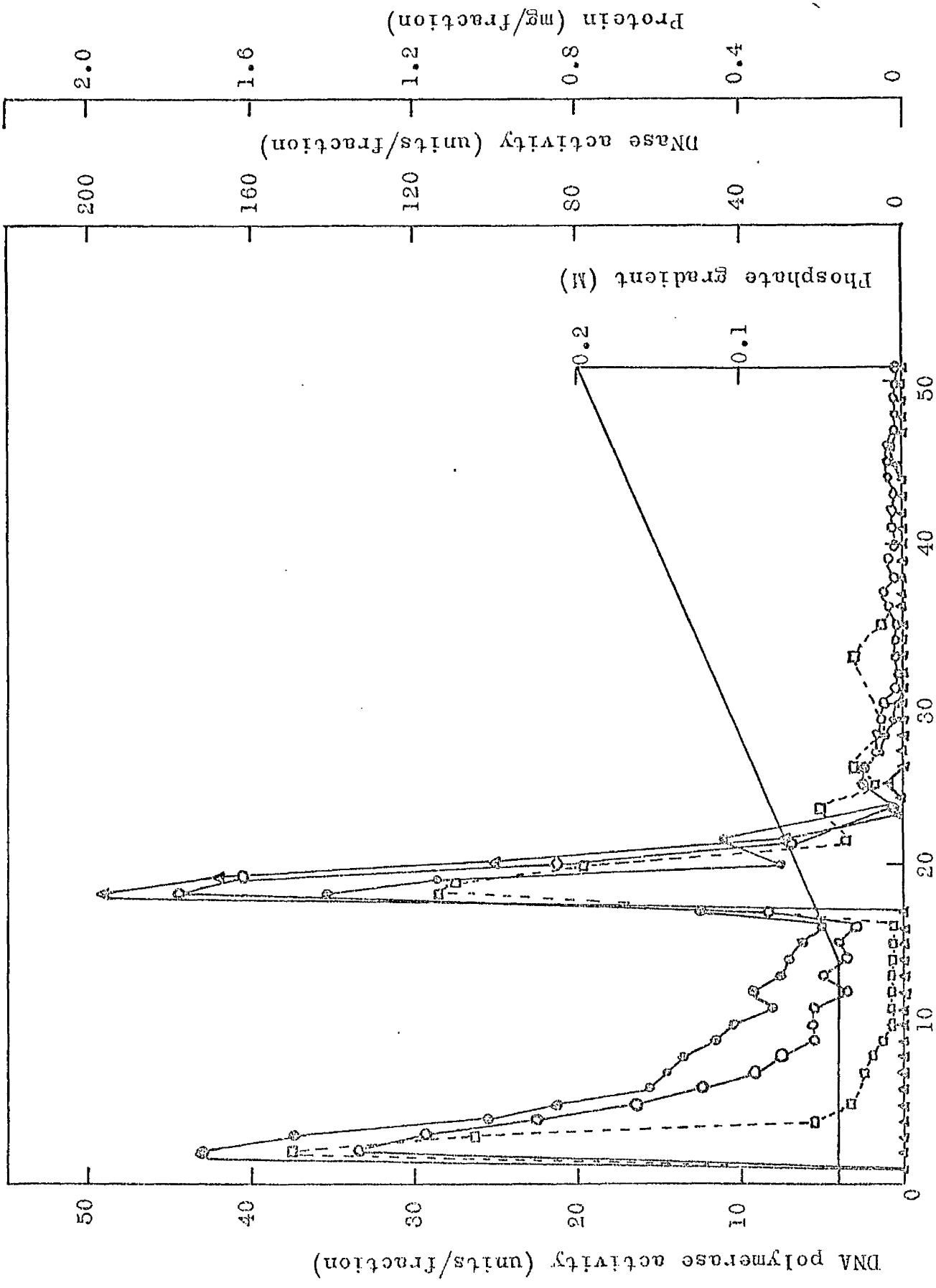

DNA polymerase activity (units/fraction)
0
10
20
30
40
50
Phosphate gradient (M)
0.1
0.2
DNase activity (units/fraction)
0
40
80
120
160
200
Protein (mg/fraction)
0
0.4
0.8
1.2
1.6
2.0
Fraction number (5.0ml)
10
20
30
40
50

Fig. III. 11.

2. 2. 1. 2. Later elution conditions: small scale analytical chromatographic studies.

Several column chromatographic fractionations were carried out as described in Methods section 2. 10. 1. using the later elution conditions. In these experiments, a 3ml packed-cell volume of HSV-infected cells was disrupted ultrasonically in starter buffer (later elution conditions) (Methods section 2. 10. 1.) and the 105,000g supernatant (1 hour) applied to the column. It was decided to adopt the new elution conditions partly in order to stabilise the enzymes with the high salt concentrations which exist throughout wash and elution, and partly because the nearly constant K^+ concentrations allow the assay of all fractions without interference from the varying K^+ concentrations which occur with the earlier eluting conditions. A further reason for employing the new conditions was to eliminate the possibility that the first non-adsorbed peak of DNA exonuclease activity (Fig. III. 11) might be caused by an activity which was only loosely adsorbed at a concentration of 0.04M-phosphate. If the first peak of nuclease is truly distinct from the activity in the second peak, it should still elute ahead of the second peak under the later elution conditions.

As can be seen from Fig. III. 12. this was found to be the case: a peak of non-adsorbed DNA exonuclease appears in the wash followed by a little more of this activity early in the gradient. Then, as the gradient develops, a peak of DNA exonuclease alone appears, followed by a further peak of DNA exonuclease associated with

Fig. III. 12.

Hydroxylapatite column chromatography (later elution conditions) of a whole-cell extract of HSV-infected cells - small scale analytical fractionation.

A whole-cell extract of 3ml HSV-infected cells (Methods section 2. 6. 1.) was made in column starter buffer (later elution conditions) and 7ml of the extract (332 units DNA polymerase-activated DNA as primer - "low salt" assay; 240 units DNase - denatured DNA as substrate) applied to a 10x1.5cm column and fractionation carried out (later elution conditions)(Methods section 2. 10. 1.). Aliquots of the 4.5ml fractions were assayed for DNA polymerase ("low salt" - activated DNA as primer), DNase (denatured DNA as substrate) and the extinction of the fractions at 280nm also recorded.

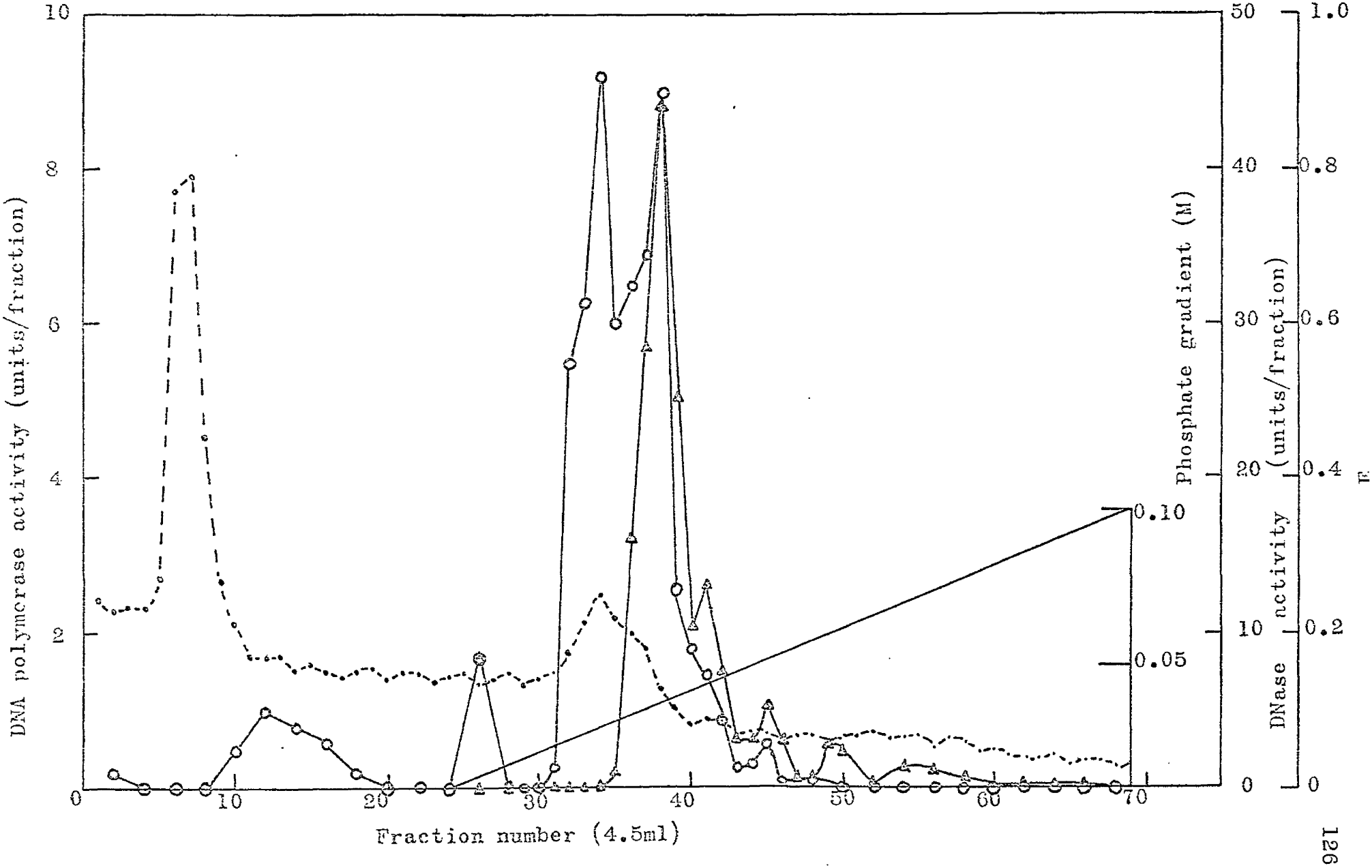
—△—△— DNA polymerase activity

—○—○— DNase activity

—·—·—·— Extinction at 280nm

———————— Phosphate gradient

Fig. III. 12.


DNA polymerase activity (units/fraction)
Phosphate gradient (M)
DNase activity (units/fraction)
Fraction number (4.5ml)
0
2
4
6
8
10
10
20
30
40
50
60
70
0.05
0.10
0
10
20
30
40
50
0
0.2
0.4
0.6
0.8
1.0

DNA polymerase activity. A total of 10% of the DNA polymerase activity was recovered.

2. 2. 1. 3. Later elution conditions: large scale preparative chromatography.

In this case, 6ml aliquots of packed HSV-infected cells were extracted in 14ml of starter buffer (later elution conditions) as described in the previous section (2. 2. 1. 2.) and applied to a 10x2.5cm bed of gel. The column was then washed with 300ml starter buffer and elution carried out with 600ml of gradient (concentration limits as before). 10.0ml fractions were collected and 4.3ml glycerol added to each to give a final concentration of 30% (^{v}/v) glycerol. The fractions were assayed, then stored at -70^{o}C. The results of a typical run are shown in Fig. III. 13. in which the wash fractions are not included. As can be seen, the profiles of DNase and DNA polymerase are similar to the small scale results (Fig. III. 12.).

By means of these preparative chromatographic runs, purified HSV-induced DNA polymerase could be stored for long periods and made available for characterisation studies.

The specific activity of DNA polymerase activity in whole-cell extracts of HSV-infected cells has routinely been found to be 16-17 units/mg with heat-denatured or activated DNA as primer and 8 units/mg with native DNA as primer in a "high salt" assay. The peak DNA polymerase fraction - fraction 26 of the large-scale preparative hydroxylapatite chromatographic run (Fig. III. 13.)-

Fig. III. 13.
phy (later elution conditions)

Hydroxylapatite column chromatography (later elution conditions) - large scale preparative fractionation of a whole-cell extract of HSV-infected cells.

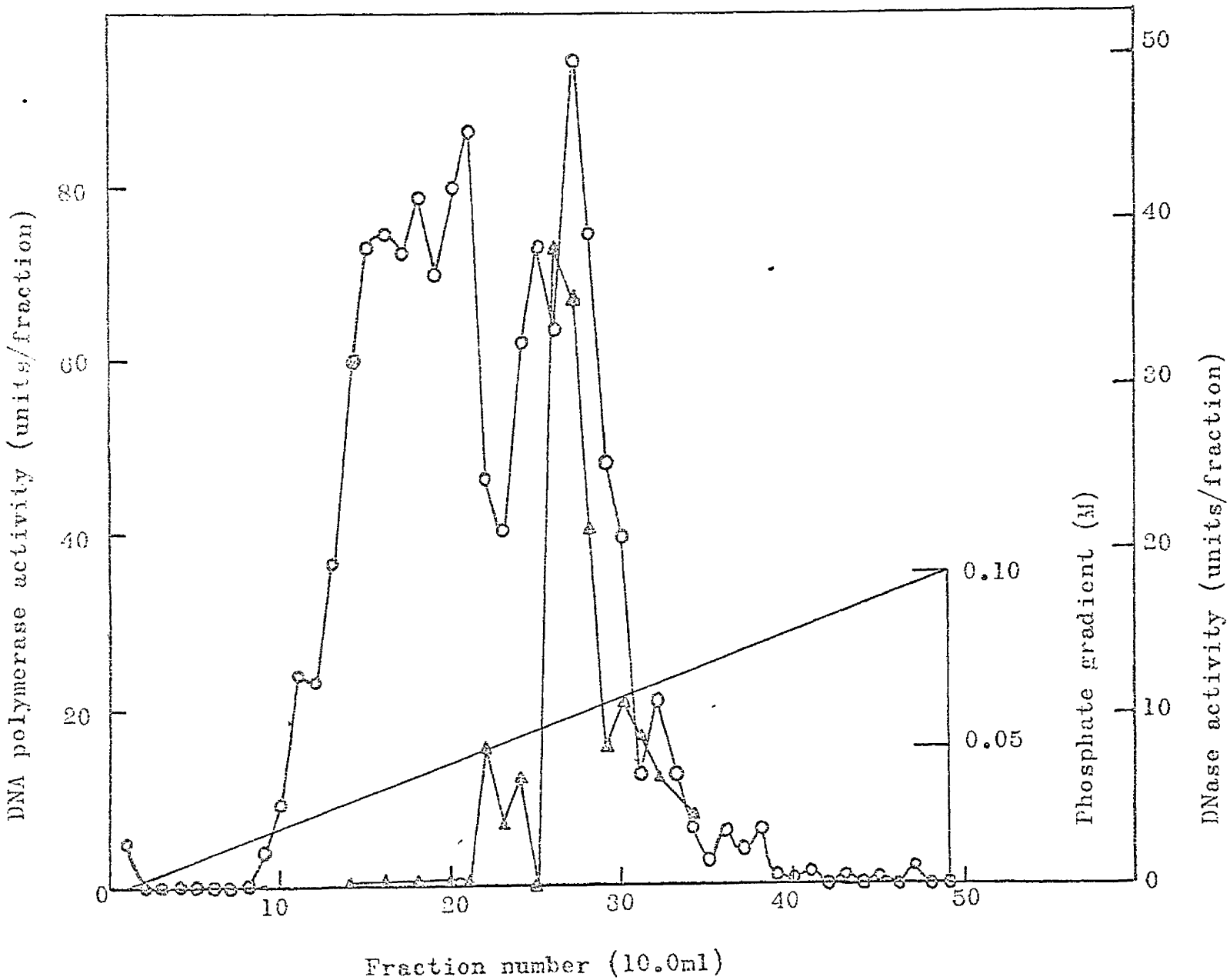
6ml of infected cells were extracted (Methods section 2. 6. 1.) in 14ml of column starter buffer (later elution conditions) and applied to a 10x2.5cm bed of hydroxylapatite. The column was then washed with 300ml starter buffer (wash fractions not shown in Fig. III. 13.) and elution carried out with 600ml of gradient. The 10ml fractions collected were mixed with 4.3ml glycerol to give a concentration of 30% (v/v) glycerol for storage. The fractions were assayed for DNA polymerase ("high salt" assay - activated DNA as primer)(Methods section 2. 2. 1.) and DNase (denatured DNA as substrate)(Methods section 2. 2. 3.).

—▲—▲— DNA polymerase activity

—○—○— DNase activity

———— Phosphate gradient

Fig. III. 13.


DNA polymerase activity (units/fraction)
DNase activity (units/fraction)
Phosphate gradient (M)
Fraction number (10.0ml)
0
20
40
60
80
10
20
30
40
50
0.05
0.10

dialysed against TEM buffer gave the following figures for specific activity:- 94 units/mg, 342 units/mg and 3372 units/mg in standard DNA polymerase assays ("high salt") (Methods section 2. 2. 1.) with heat-denatured, native and activated calf thymus DNA as primer respectively.

Hydroxylapatite therefore appears to give purifications of virus-induced DNA polymerase of 5.7x; 42x and 203x with heat-denatured, native and activated calf thymus DNA as primer respectively. It can be seen, therefore, that a great change in the primer requirement of the enzyme occurs after hydroxylapatite chromatography and this is confirmed and extended to other fractionation methods by the results of subsequent primer experiments (Results section 4. 1.).

This change may reflect the removal of DNA endonuclease and possibly some viral DNA exonuclease so that the apparent purification depends very much upon the type of primer used. This sort of effect has been noticed by Richardson et al., (1964) and Roychoudhury and Bloch (1969a,b) on other DNA polymerases with purification.

2. 2. 1. 4. Some additional observations on hydroxylapatite column chromatography.

While most chromatographic fractionations with extracts of HSV-infected cells have shown almost complete coincidence of DNA polymerase and the second peak of DNA exonuclease, occasionally, while there is yet considerable overlap of these activities, partial

separation has been observed. The results of one experiment in which this has happened are shown in Fig. III. 14. and the implications of the findings are discussed in the Discussion section.

2. 2. 1. 5. Hydroxylapatite chromatography of virus-induced DNA polymerase and DNase activities which elute together from DEAE-cellulose.

This was carried out in two ways: once with material from stepwise elution of a DEAE-cellulose column (0.10M and 0.15M-KCl wash fractions) (Results section 2. 1. 4.) and the second with material from gradient elution of DEAE-cellulose (fractions 40-48) (Results section 2. 1. 2.). These fractions were then applied to hydroxylapatite using earlier elution conditions (Methods section 2. 10. 1.) and in both cases the result was identical to that shown in Fig. III. 11: two peaks of DNA exonuclease appeared, one non-adsorbed and not associated with DNA polymerase, the other adsorbed and polymerase-associated. The result thus supports the hypothesis that the DEAE-cellulose salt-eluted peak of virus-induced DNA polymerase and DNase contains two DNA exonucleases - one adsorbed to hydroxylapatite and associated with DNA polymerase, the other not adsorbed to hydroxylapatite and not associated with DNA polymerase.

Fig. III. 14.

Hydroxylapatite column chromatography, (later elution conditions) small scale fractionation, of an extract of HSV-infected cells, showing partial separation of the virus-induced DNA polymerase from associated DNase.

A whole-cell extract of 2ml HSV-infected cells (Methods section 2. 6. 1.) was fractionated by ammonium sulphate (Methods section 2. 7.) and the active fractions' precipitate taken up in 3.5ml column starter buffer (later elution conditions). The sample (400 units DNA polymerase- activated DNA as primer - "high salt" assay; 339 units DNase - active against denatured DNA, and 7.18mg protein) was applied to a 10x1.5cm bed of hydroxylapatite and fractionation carried out (Methods section 2. 10. 1.). Aliquots of fractions were assayed for DNA polymerase (activated DNA primer - "low salt") and DNase (denatured DNA as substrate (Methods sections 2. 2. 1., 2. 2. 3.) and the extinction of the fractions at 280nm also recorded.

—△—△— DNA polymerase activity

—○—○— DNase activity

—·—·—·— E_{280}

———— Phosphate gradient

Fig. III. 14.

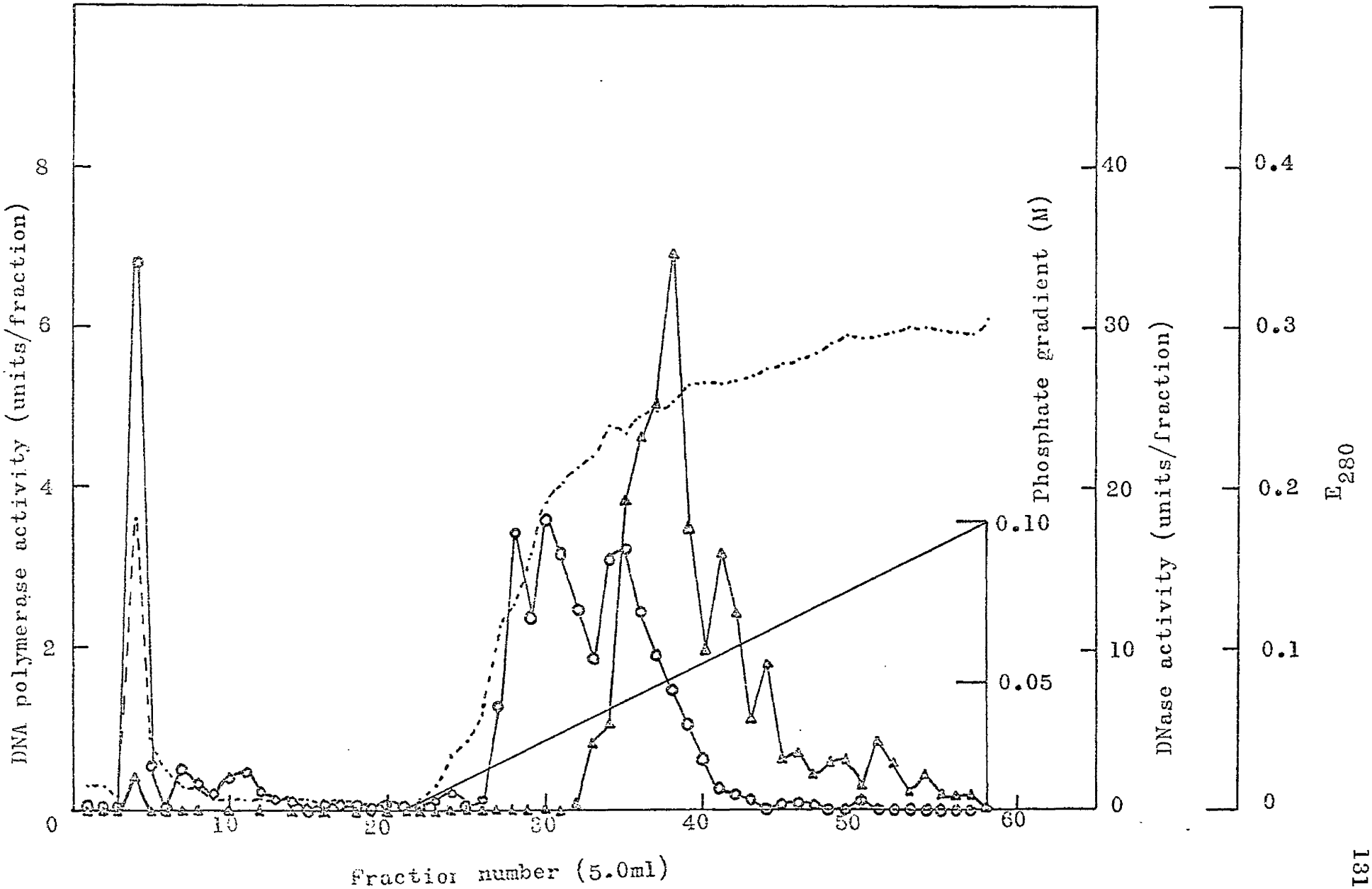

DNA polymerase activity (units/fraction)
DNase activity (units/fraction)
Phosphate gradient (M)
E_{280}
Fraction number (5.0ml)
0
2
4
6
8
10
20
30
40
50
60
0.05
0.10
0.1
0.2
0.3
0.4

Table III. 3.

Rechromatography on hydroxylapatite of the two DNA exonuclease activities from hydroxylapatite column chromatography (early elution conditions) of extracts of HSV-infected cells.

Nature of sample applied to hydroxylapatite column.	Fraction collected.	Enzyme Activity (units recovered/fraction).
First exonuclease peak (polymerase-free) from hydroxylapatite.	0.04M-phosphate wash	13. 1 (DNase)
	0.10M-phosphate eluate	5.6 (DNase)
	0.20M-phosphate eluate	0 (DNase)
Second exonuclease peak (polymerase-associated) from hydroxylapatite	0.04M-phosphate wash	7.3 (DNase)
	0.10M-phosphate eluate	13.4 (DNase)
	0.20M-phosphate eluate	1.03 (DNase)
Virus-induced DNA polymerase peak (associated with second exonuclease peak) from hydroxylapatite.	0.04M-phosphate wash	0.373 (DNA polymerase)
	0.10M-phosphate eluate	1.213 (DNA polymerase)
	0.20M-phosphate eluate	0.318 (DNA polymerase)

Pooled DNase activities from each of the two peaks of virus-induced DNA exonuclease from hydroxylapatite column chromatography of an ammonium sulphate fraction of an extract of HSV-infected cells (Fig. III. 11.) were separately re-chromatographed using stepwise elution in Pasteur-pipette columns. The E_{280} of the subfractions was followed to indicate when all protein was eluted and the sub-fractions from each stepwise wash, or fraction, pooled and arranged for DNase or DNA polymerase in the standard way (Methods section 2. 2. 3. and 2. 2. 1. respectively.)

2. 2. 1. 6. Re-chromatography on hydroxylapatite of the two DNA-exonuclease activities from hydroxylapatite column chromatography (early elution conditions) of extracts of HSV-infected cells.

Pooled DNase activities from each of the two peaks, equilibrated with starter buffer (early elution conditions) were applied to adapted Pasteur-pipette columns and eluted with stepwise increases of phosphate buffer concentration: the results are given in Table III. 3. It can be seen that the first and second exonuclease peaks from hydroxylapatite chromatograph largely as before with some 30 - 35% of the total activity overlapping into the next fraction: DNA polymerase is also seen to behave largely as before with even less overlap.

2. 2. 1. 7. Hydroxylapatite column chromatography of an extract of HSV-infected cells after the addition of a protease inhibitor.

The DNA polymerase I of E. coli is known to be capable of being cleaved on storage by proteolytic action, into two active fragments (Introduction section 2. 4. 4.). One of these retains DNA polymerase and some DNase activity and the other may have DNase activity alone (Brutlag et al., 1969; Klenow and Overgaard-Hansen, 1970). It seemed possible that the appearance after hydroxylapatite chromatography of a polymerase-free DNA exonuclease in addition to the polymerase-associated DNase might be the result

of similar cleavage of the HSV-induced DNA polymerase.

To investigate this possibility, a protease-inhibitor phenylmethylsulphonyl fluoride (PMSF) (Fahrney and Gold, 1963) (14mg in 0.4ml of 2-propanol) was added to 1.0ml of 5x concentrated column starter buffer (later eluting conditions). This, plus 3.6ml water was added to 3.0ml packed-cell volume of HSV-infected cells to give a final concentration of 10^{-2}M-PMSF. The cells were ultrasonically disrupted and centrifuged (Methods section 2. 6. 1.) and 5ml of the supernatant (105,000g/1 hour) was applied to a hydroxylapatite column (Methods section 2. 10. 1.). 0.25ml of PMSF (35mg/ml in 2-propanol) was added to each 5.0ml fraction as it was collected and the fractions assayed for DNase and DNA polymerase (Methods sections 2. 2. 1. and 2. 2. 3.).

From the results (Fig. III. 15.) it is clear that the polymerase-free exonuclease peak is present as before (as in Fig.III. 12.). The similarity of elution in the presence (Fig.III. 15.) and absence (Fig. III. 12.) of the inhibitor suggests that the polymerase-free exonuclease is not a breakdown product of proteolytic action on the intact HSV-induced DNA polymerase.

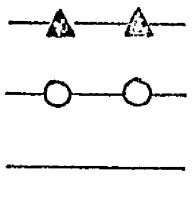
2. 2. 2. Hydroxylapatite column chromatography of whole-cell extracts of uninfected cells.

Owing to losses in host-cell DNA polymerase activity when a whole-cell extract was made with the usual TEM buffer (Methods section 2. 6. 1.) and fractionated by precipitation with $(NH_4)_2SO_4$

Fig. III. 15.

Hydroxylapatite column chromatography (later elution conditions) of a whole-cell extract of HSV-infected cells made in the presence of the protease inhibitor PMSF.

3.0ml HSV-infected cells were suspended in 5.0ml column starter buffer (later elution conditions) containing 10^{-2}M-PMSF and a whole-cell extract made (Methods section 2. 6. 1.). 5ml extracts were applied to a 10x1.5cm hydroxylapatite column and chromatography carried out as described in Methods section 2. 10. 1. (later elution conditions). As the fractions (5ml) were collected, 0.25ml of PMSF (35mg/ml in 2-propanol) was added to each and the fractions assayed for DNase (denatured DNA as substrate - Methods section 2. 2. 3.) and DNA polymerase ("high salt" assay - activated DNA as primer).



DNA polymerase activity

DNase activity

Phosphate gradient

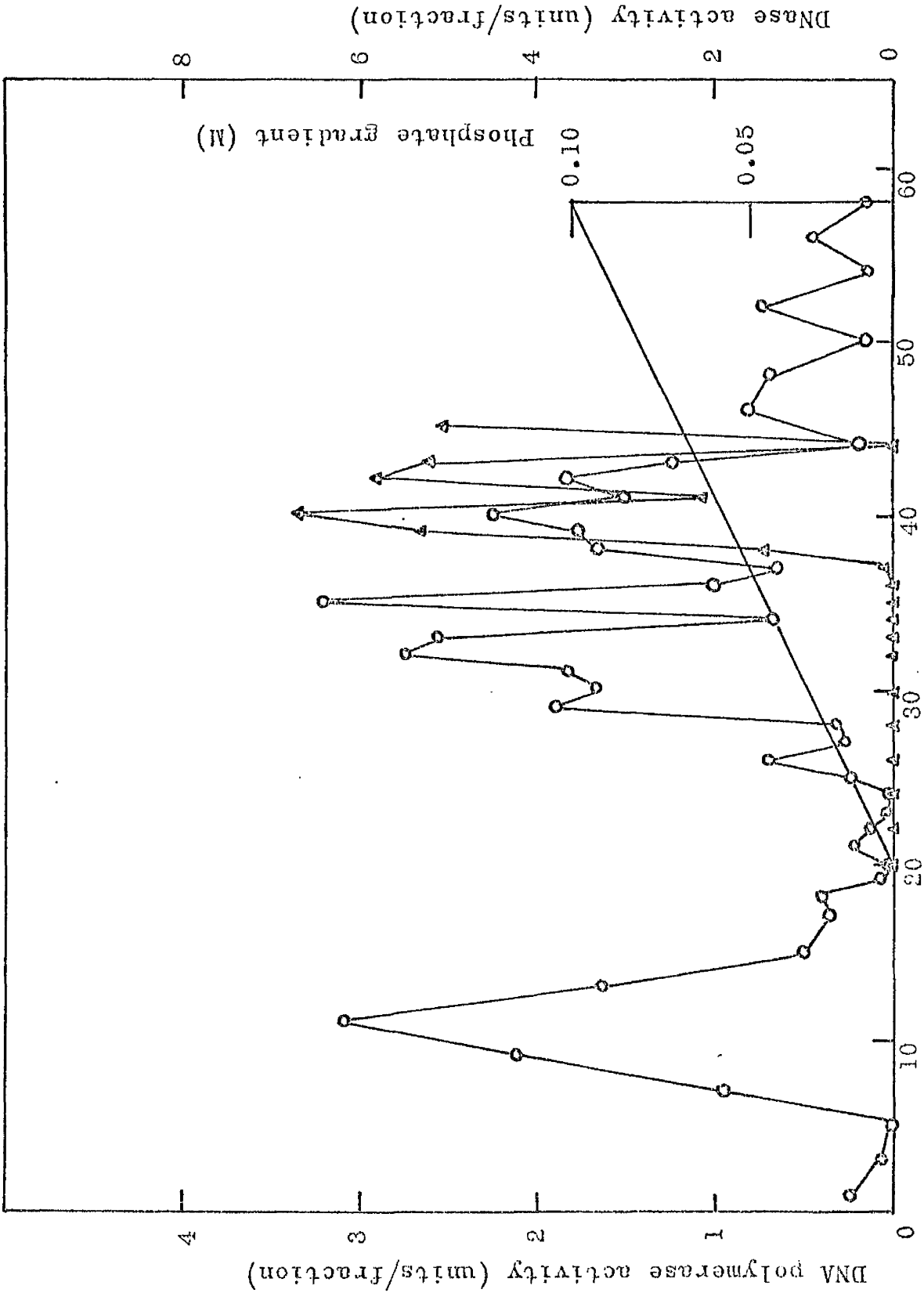

DNA polymerase activity (units/fraction)
DNase activity (units/fraction)
Phosphate gradient (M)
0.10
0.05
Fraction number (5.0ml)

Fig. III. 15.

(Methods section 2. 7.), extracts were made directly into hydroxylapatite column starter buffer (later conditions of elution) (Methods section 2. 10. 1.). 2ml packed-cell volume of host-cells was suspended in 4ml column starter buffer, ultrasonically disrupted and the supernatant from 1 hour at 105,000g applied to a hydroxylapatite column (10x1.5cm) in the usual way (later eluting conditions). A total of 31mg protein, 132 units DNase (denatured DNA as substrate) and 26 units DNA polymerase (activated DNA as primer, "low salt") was applied.

The results of this chromatographic fractionation showed that essentially all of the DNase activity appeared as a non-adsorbed peak of DNA endonuclease and of particular interest was the complete absence of host-cell DNA polymerase activity from the wash and eluate. 40% of the applied DNase activity was recovered.

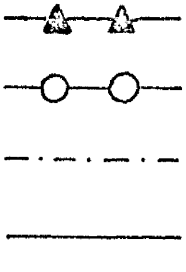
When this experiment was repeated in an extract of 3ml infected cells (Fig. III. 16.) it was observed that some of the DNA endonuclease activity appeared during gradient-elution at approx. 30mM-potassium phosphate; support for this finding was furnished by mode-of-action studies (Methods section 2. 2. 4.) which revealed some endonuclease in DNase from the same region of infected-cell extract runs (Fig. III. 12.). The effect seems to occur when extracts of 3ml rather than 2ml of packed-cells are applied.

Once more, however, DNA polymerase activity did not appear in significant amounts in the wash or in the region of the viral DNA polymerase; there did, however, appear to be a little activity

Fig. III. 16.

Hydroxylapatite column chromatography (later elution conditions) of a whole-cell extract of uninfected cells.

3.0ml of uninfected BHK21 (C13) cells were taken, 5ml column starter buffer added and a whole-cell extract made (Methods section 2. 6. 1.). 8ml of this extract (142mg protein, 44.2 units DNA polymerase ("high salt" assay - activated DNA primer) and 74 units DNase (heat-denatured DNA as substrate))were applied to a 10x1.5cm column and fractionation carried out (later elution conditions - Methods section 2. 10. 1.). 5.0ml fractions were collected and assayed for DNA polymerase ("high salt" assay - activated DNA primer) and DNase (denatured DNA as substrate - Methods section 2. 2. 3.).



DNA polymerase activity

DNase activity

Extinction at 280nm

Phosphate gradient

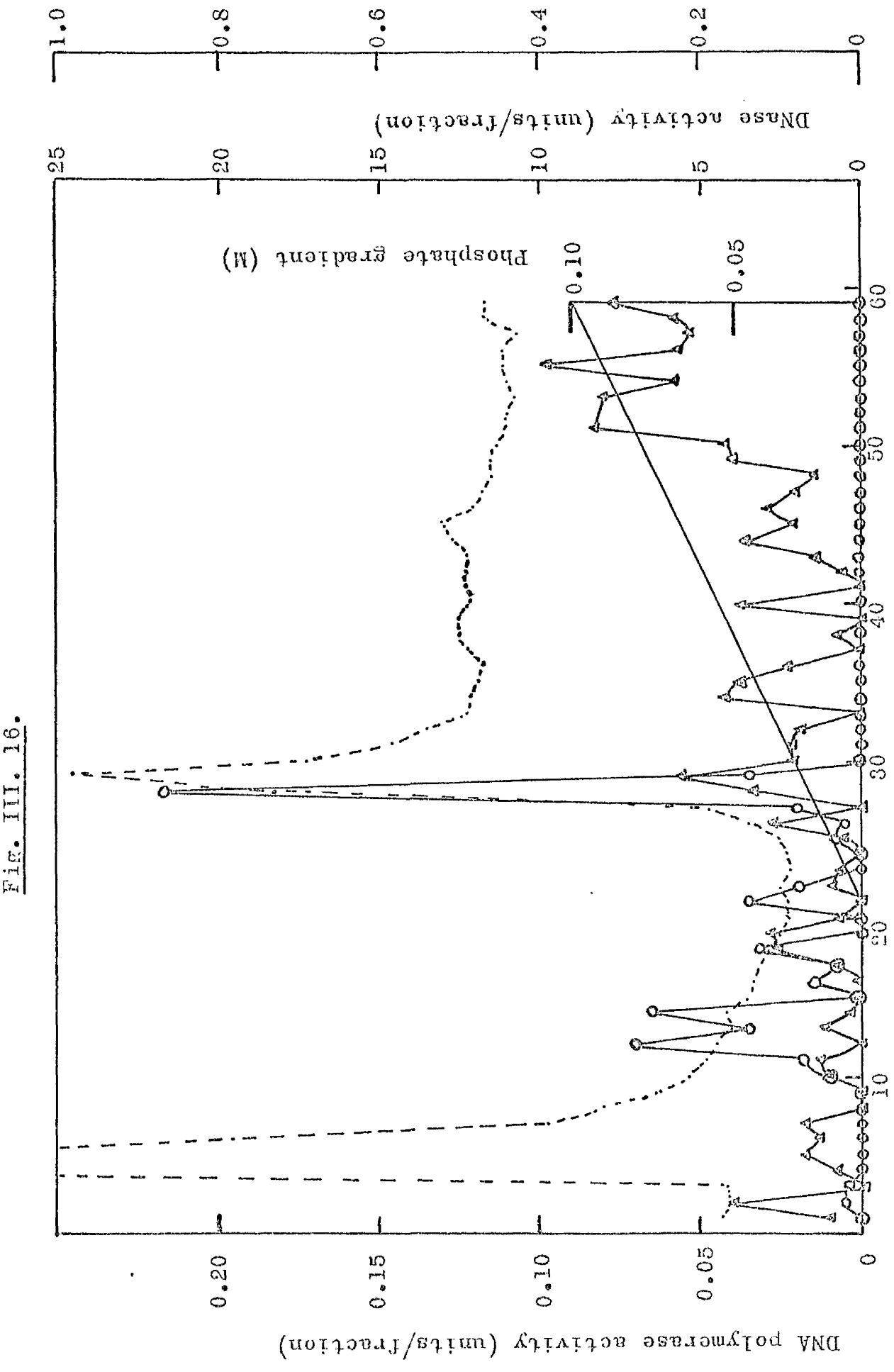

E_{280}
DNase activity (units/fraction)
Phosphate gradient (M)
DNA polymerase activity (units/fraction)
Fraction number (5.0ml)

Fig. III. 16.

eluting at 80-100mM potassium phosphate and the significance of this will be discussed later.

These results indicate that the infected-cell DNA polymerase and DNase activities are distinct from these activities in uninfected cells. The weight of evidence, at this point, seems to favour the existence of two distinct exonucleases induced by virus-infection.

2. 3. Phosphocellulose column chromatography.

2. 3. 1. Phosphocellulose column chromatography of extracts of HSV-infected cells.

The column and eluting conditions described in Methods section 2. 10. 4. 2. were used. 8.0ml of whole-cell extract containing 70 units DNase (heat-denatured DNA as substrate) and 50 units DNA polymerase were applied: 18 units DNase and 5-6 units DNA polymerase were recovered from the column. The results are shown in Fig. III. 17. Once again, association of the DNA polymerase and DNase was found in both the non-adsorbed and the 0.2M-potassium phosphate-eluted peaks of DNA polymerase. The high activity of the non-adsorbed peak of DNA polymerase is probably due to the interference of DNA with the phosphocellulose column chromatography since much of the DNA polymerase may be bound to DNA leaving only DNA-free enzyme adsorbed to the column.

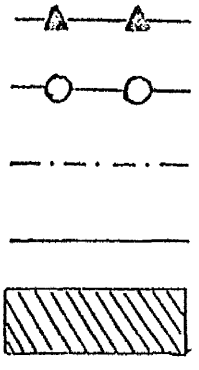
For this reason, phosphocellulose column chromatography of whole-cell extracts was abandoned and chromatography of a

Fig. III. 17.

Phosphocellulose column chromatography of a whole-cell extract of HSV-infected cells.

8ml of a whole-cell extract (Methods section 2. 6. 1.) of HSV-infected cells (≡ 70 units DNase and 50 units DNA polymerase assayed as below) were dialysed against column starter buffer and fractionated on a phosphocellulose column with stepwise elution as described in Methods section 2. 10. 4. 2.

Aliquots of the 2nd fraction were assayed for DNA polymerase ("high salt" assay - denatured DNA primer - Methods section 2. 2. 1.) and DNase (denatured DNA as substrate - Methods section 2. 2. 3.), and the extinction of the fractions at 280nm measured.



DNA polymerase activity

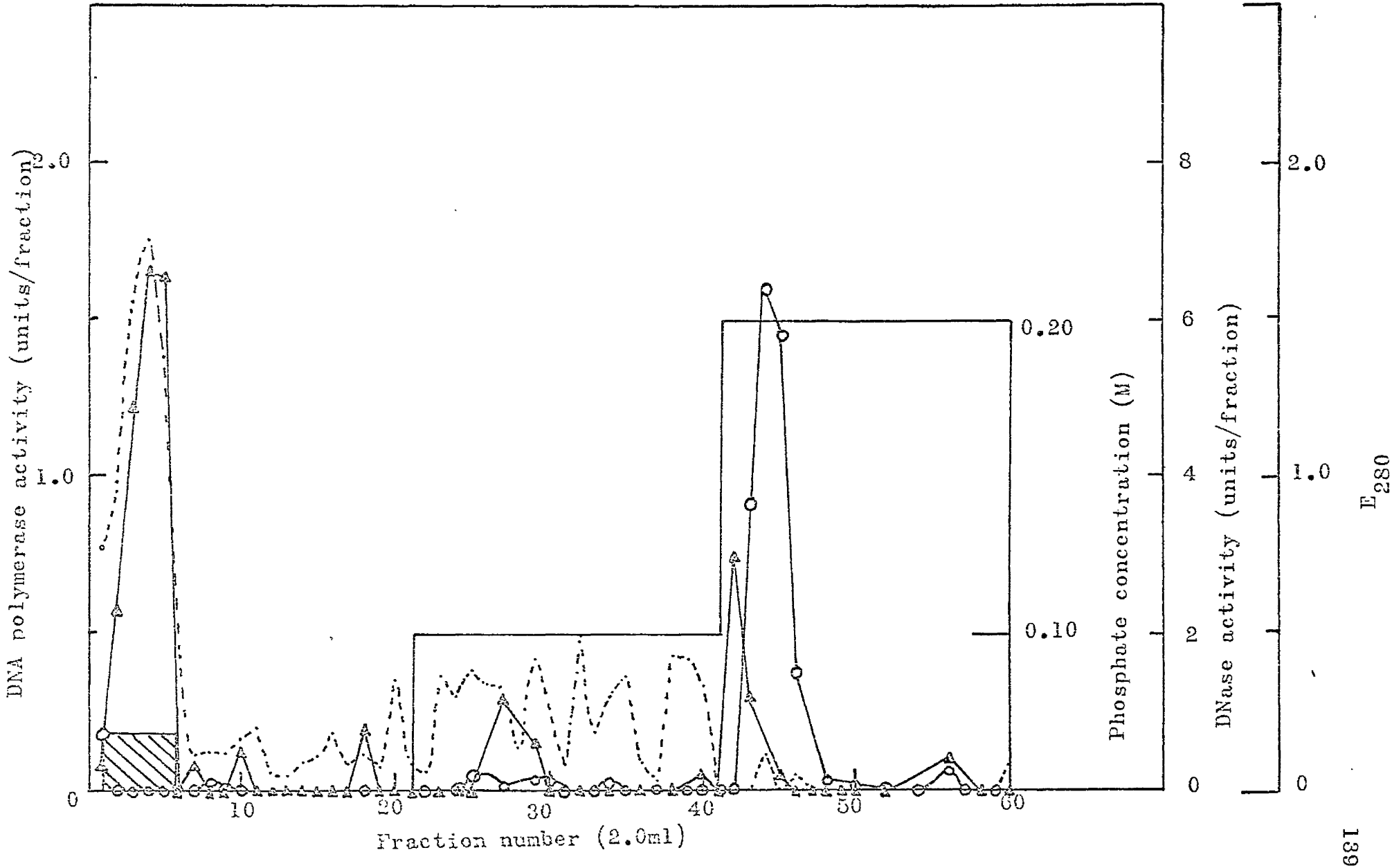
DNase activity

Extinction at 280nm

Phosphate buffer concentration

Cross-hatched region from fractions 1-6 represents DNase activity found in the pooled and concentrated non-adsorbed first peak of DNA polymerase - this activity was not apparent in fractions 2-6 (inclusive) on assay of aliquots as the fractions were collected.

Fig. III. 17.


DNA polymerase activity (units/fraction)
2.0
1.0
0
10
20
30
40
50
60
Fraction number (2.0ml)
0.20
0.10
Phosphate concentration (M)
8
6
4
2
0
DNase activity (units/fraction)
2.0
1.0
0
E_{280}

hydroxylapatite-purified induced DNA polymerase preparation was attempted (Methods section 2. 10. 4.). From this fractionation, small amounts of both non-adsorbed and gradient-eluted DNase activities were obtained and no DNA polymerase activity was recovered. The reason for these low activities may well be the low pH (6.5) of the buffer (Methods section 2. 10. 4. 1.) which was used by Yoneda and Bollum (1965) for purification of calf thymus DNA polymerase. Thus no further experiments were carried out using phosphocellulose particularly since recoveries of enzyme with either of the two buffer systems had given little promise.

2. 4. Constant velocity sucrose gradient zone sedimentation.

2. 4. 1. Sedimentation of a whole-cell extract of HSV-infected cells.

3.5ml of a whole-cell extract of HSV-infected cells containing 20% ($^{v}/v$) glycerol was centrifuged for 2h at 100,000g in a Spinco 50Al rotor at $4^{o}C$, the supernatant concentrated down to 0.45ml using polyethylene glycol (Methods section 2. 9.) and dialysed against DEAE-starter buffer (Methods section 2. 10. 2.). 0.25ml (2mg protein) of the non-diffusible material was taken, 1mg human haemoglobin added, the sample layered carefully on a sucrose gradient (Methods section 2. 12.) and centrifugation for 19h 33min at $129,560g_{av.}$ carried out at $5^{o}C$. The gradient was harvested by displacement with 40% $^{w}/w$ sucrose in the Isco apparatus and 0.05ml

of each 0.16ml fraction was assayed for DNA polymerase and DNase (Methods sections 2. 2. 1. and 2. 2. 3. respectively.). Mode-of-action studies (Methods section 2. 2. 4.) were carried out on the DNase assays after 1h. Haemoglobin was estimated by its extinction at 420nm.

The results (Fig. III. 18.) indicate that there is one peak of DNA polymerase activity and that DNase activity is spread over the gradient with two main peaks, one just ahead of the haemoglobin peak, the other around the polymerase peak. The mode of action of the nuclease from the top of the gradient to the first DNase peak is mainly endonucleolytic; at, and beyond this first peak, the mode of action is exonucleolytic.

$\underline{s}_{20,w}$ values for DNA polymerase and haemoglobin were calculated using the formula presented in Methods section 2.12. $\underline{s}_{20,w}$ values of (4.4-4.8) and (9.3-9.8) were obtained for haemoglobin and DNA polymerase respectively: The value for haemoglobin was close to published figures (Edsall, 1953).

The molecular weight of the DNA polymerase, assuming it to be a spherical molecule with a standard protein partial specific volume, was estimated to be 190,000 $\pm$ 20,000, using the relationship

$$\frac{\underline{s}1}{\underline{s}2} = \left(\frac{\text{mol.wt.}_1}{\text{mol.wt.}_2}\right)^{\frac{2}{3}} \qquad \text{(Martin and Ames, 1961.)}$$

This value may be inflated by possible binding of the enzyme to residual DNA in the whole-cell extract.

Fig. III. 18.

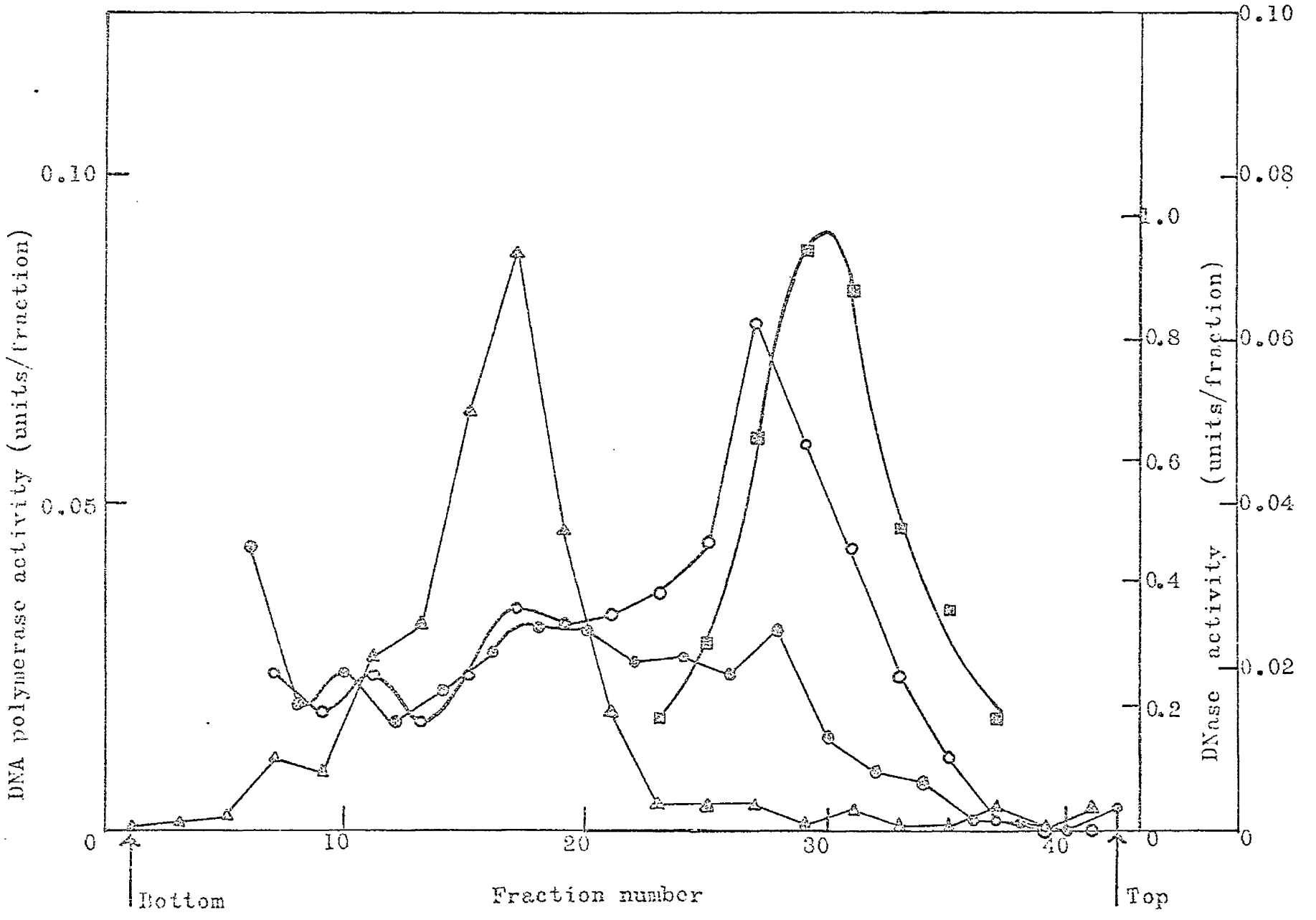
Constant velocity sucrose gradient zone sedimentation of a whole-cell extract of HSV-infected cells.

A concentrate of a whole-cell extract of HSV-infected cells (Methods section 2. 6. 1.) was dialysed against DEAE-starter buffer (the buffer of the gradient) and 0.25ml of the dialysis residue supplemented with 1mg human haemoglobin giving a total of 3mg protein, 5.5 units DNA polymerase ("high salt" assay) and 50 and 83 units DNase active against native and denatured DNA respectively. The 0.25ml sample was then layered on to a 5.25ml constant velocity sucrose gradient and centrifugation carried out for 19h 33min (allowing for acceleration and deceleration) at 40,000rev./min. (Methods section 2.12.) at 5°C in a Spinco SW50L rotor.

The gradient was then harvested with 40% (W/w) sucrose in the Isco apparatus to give 42 fractions of average volume 0.14ml. Aliquots of these fractions were assayed for DNA polymerase ("low salt" assay) and DNase (denatured or native DNA as substrate). 0.05ml aliquots of the DNase reaction mixture were taken off and used for Mode-of-action studies (Methods section 2. 2. 4. 1.). The extinction at 420nm of aliquots of tube contents showing visible traces of red or yellow colour (due to haemoglobin) was read at a 1 in 10 dilution.

—△—△— DNA polymerase activity
—○—○— DNase activity (denatured DNA as substrate)
—◎—◎— DNase activity (native DNA as substrate)
—▣—▣— E_{420} (due to haemoglobin)

Fig. III. 18.


DNA polymerase activity (units/fraction)
0.10
0.05
0
10
20
30
40
Bottom
Fraction number
Top
1.0
0.8
0.6
0.4
0.2
0
DNase activity (units/fraction)
0.10
0.08
0.06
0.04
0.02
0
E_{420}

2. 4. 2. Sedimentation at low ionic strength of a hydroxylapatite-purified sample of HSV-induced DNA polymerase.

To avoid the complications of residual DNA, a hydroxylapatite-purified sample of HSV-induced DNA polymerase was analysed. Centrifugation was carried out in low ionic strength buffer which was considered less likely to interfere with the isokinetic properties of the gradient than the buffer used in the previous experiment. 0.25ml of a pooled and polyethylene glycol-concentrated DNA polymerase peak from hydroxylapatite (early eluting conditions) was applied to a gradient with 1mg of human haemoglobin and centrifuged (129,560g for 20h 29min, as described in Methods section 2. 12.).

The results (Fig. III. 19.) show that DNA polymerase and DNase sediment together close to the bottom of the tube: in this case, however, little DNase activity appears in the region around and just ahead of the haemoglobin peak (cf. Fig. III. 18.). The $\underline{s}_{20,w}$ values calculated for haemoglobin (6.0S) and DNA polymerase (11.7S) were considerably higher than those estimated from the previous gradient but the ratio of $\underline{s}_{20,w}$ DNA polymerase : $\underline{s}_{20,w}$ haemoglobin was approximately as before. The reason for these high s-values was considered to be aggregation of the proteins at the low ionic strength of the buffer used in this experiment, (0.01M-tris-HCl, pH 8.0-1mM-EDTA-5mM-2-mercaptoethanol). Nevertheless, considerable association of DNA polymerase and DNase was

Fig. III. 19.

Constant velocity sucrose gradient zone sedimentation at low ionic strength of a hydroxylapatite-purified (early elution conditions) sample of HSV-induced DNA polymerase.

0.25ml of a pooled and polyethylene-glycol-concentrated HSV-induced DNA polymerase peak from hydroxylapatite column chromatography (early elution conditions) of an ammonium sulphate fractionated whole-cell extract of HSV-infected cells, with 1mg of human haemoglobin dissolved in the sample was layered on to a 5.25ml constant velocity sucrose gradient (with 0.1M-tris-HCl(pH8.0)-1mM-EDTA-5mM-2-mercaptoethanol as buffer) prepared as described in Methods section 2. 12. The gradient was then centrifuged at 40,000$^{rev.}$/min (130,576g ($r_{av.}$ 7.3cm) for a total effective time of 20h 34min at this speed (having added on the appropriate correction for acceleration and deceleration as described in Methods section 2. 12.).

The gradient was then harvested as described in the above Method section and 0.05ml aliquots of the fractions assayed for DNA polymerase in "high salt" half scale assays with heat denatured DNA as primer (Methods section 2. 2. 1.) and also for DNase (denatured DNA as substrate) in the standard DNase assay (Methods section 2. 2. 3.). 0.02ml samples of fractions were diluted to 0.42ml with distilled water and their extinction at 420nm read as a measure of haemoglobin.

—△—△— DNA polymerase activity

—○—○— DNase activity

—▣—▣— E_{420}

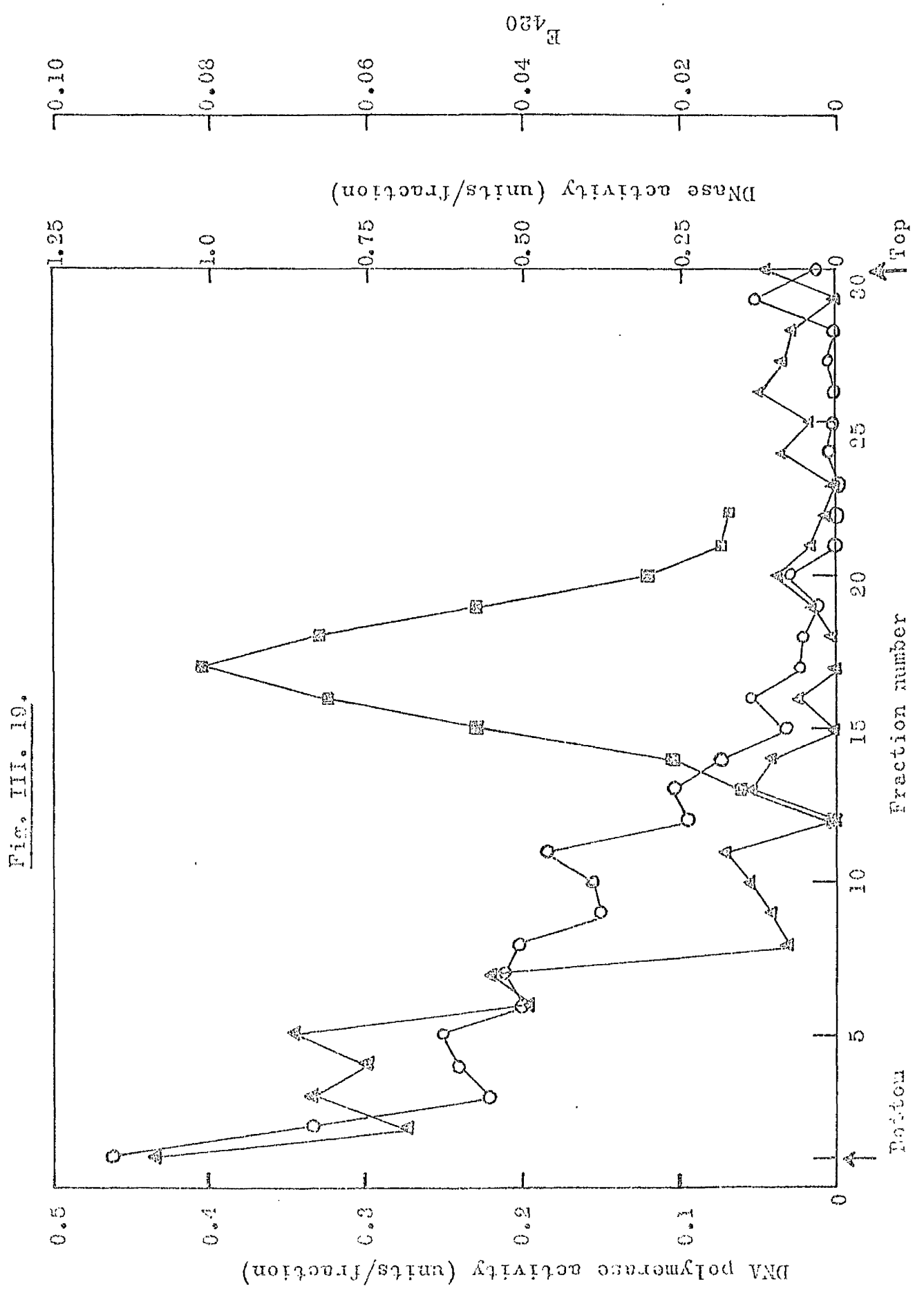

DNA polymerase activity (units/fraction)
0.5
0.4
0.3
0.2
0.1
0
Bottom
5
10
15
20
25
30
Top
Fraction number
DNase activity (units/fraction)
1.25
1.0
0.75
0.50
0.25
0
E_{420}
0.10
0.08
0.06
0.04
0.02
0

Fig. III. 19.

again seen with a ratio of DNase : DNA polymerase very close in value to that obtained from the DNA polymerase/DNase peak from hydroxylapatite column chromatography (Results section 2. 2. 1. 1. and Fig. III. 11.).

2. 4. 3. Sedimentation of hydroxylapatite-purified HSV-induced DNA polymerase and polymerase-free DNA exonuclease in TEM buffer.

Several gradients were now set up (Methods section 2. 12.) using the purified enzymes but with high ionic strength buffer (Martin and Ames, 1961) to lessen the possibility of aggregation.

A typical gradient in which a sample of hydroxylapatite-purified virus-induced DNA polymerase was analysed in 0.10M tris-HCl is shown in Fig. III. 20. Both DNA polymerase and DNase rise to a peak just behind haemoglobin and spread to the region of alcohol dehydrogenase; a second smaller peak of DNA polymerase and DNase occurs at the alcohol dehydrogenase peak. The $\underline{s}_{20,w}$ value for the HSV-induced DNA polymerase (3.5-7.5S) seems considerably lower than the earlier work suggested (Results section 2. 4. 1.) and gives a molecular weight of 46,300-151,000. This is the range of size of molecule within which all the DNA polymerases studied have been found to lie. The bimodal distribution of the enzyme may arise from aggregation.

When the virus-induced DNA polymerase-free DNA exonuclease

Fig. III. 20.

Constant velocity sucrose gradient zone sedimentation at high ionic strength of a hydroxylapatite-purified (later elution conditions) sample of HSV-induced DNA polymerase.

0.25ml of pooled and concentrated HSV-induced DNA polymerase and its concomitant induced DNA exonuclease from hydroxylapatite column chromatography of a whole-cell extract of HSV-infected cells, with 1mg of human haemoglobin and 0.1mg yeast alcohol dehydrogenase dissolved in the sample was layered on to a 5.25ml constant velocity sucrose gradient (Methods section 2. 12.) containing 0.10M-tris-HCl(pH8.0)-10mM-2-mercapto-ethanol-1mM-EDTA as buffer.

Centrifugation was carried out in a Spinco SW50L rotor at 5°C for a total effective time of 12h 18min at 39,000 rev./min (123,267g) ($r_{av.}$ 7.3cm) (Methods section 2. 12.).

The gradient was then harvested (Methods section 2. 12.) and gave rise to 22 fractions the volumes of which were checked (they averaged 0.24ml). 0.05ml samples of the fractions were then assayed for DNA polymerase ("low salt"-half scale-activated DNA as primer)(Methods section 2. 2. 1.), DNase (denatured DNA as substrate)(Methods section 2. 2. 3.) and alcohol dehydrogenase (Methods section 2. 2. 5.). In addition 0.05ml samples of fractions were diluted to 0.55ml with distilled water and their extinction at 420nm read as a measure of haemoglobin concentration.

—△—△— DNA polymerase activity
—○—○— DNase activity
—▣—▣— E_{420} (haemoglobin)
—□—□— (Yeast) alcohol dehydrogenase (ΔE_{360}/min)

DNA polymerase activity (10^3 x units/0.05ml sample of fraction)
DNase activity (10^3 x units/0.05ml sample of fraction)
Alcohol dehydrogenase activity (ΔE_{360}/min/0.05ml sample of fraction)
Volume (ml) from bottom of gradient
Bottom
Top

Fig. III. 20.

from hydroxylapatite column chromatography was analysed, a DNase profile very similar to the profile for the virus-induced DNA-polymerase-associated DNase was obtained (Fig. III. 21.), a double peak of DNase activity again being found. Assuming $s_{20,w}$ values of 4.3S for haemoglobin (Edsall, 1953) and 7.6S for alcohol dehydrogenase (Kuff, Hogeboom and Striebich 1955), the polymerase-free exonuclease has an $s_{20,w}$ value of 3.5-6.3S.

The size of the polymerase appears to be decreasing with increasing tris-HCl concentration in the gradients probably because of protein association's being reversed.

2. 4. 4. Sedimentation of HSV-induced DNA polymerase from a hydroxylapatite-purified extract of HSV-infected cells prepared in the presence of the protease inhibitor PMSF.

DNA polymerase was prepared as described in Results section 2. 2. 3. and from the peak of enzyme shown in Fig. III. 15; was concentrated and layered on sucrose gradients (0.10M-tris-HCl) with alcohol dehydrogenase and haemoglobin as markers. Three such gradients were centrifuged and the results from one of these is shown in Fig. III. 22. The results showed three main DNase peaks, one essentially co-sedimenting with haemoglobin, one co-sedimenting with alcohol dehydrogenase and the third running ahead of the alcohol dehydrogenase. The DNA polymerase results, however, were very irregular probably because the activity was low: the enzyme

Fig.III. 21.

Constant velocity sucrose gradient sedimentation at high ionic strength of a hydroxylapatite-purified (later elution conditions) sample of HSV-induced DNA-polymerase-free DNA exonuclease.

0.25ml of pooled and concentrated HSV-induced DNA-polymerase-free DNA edonuclease from hydroxylapatite chromatography (later elution conditions) of a whole-cell extract of HSV-infected cells with 1mg human haemoglobin and 0.1mg yeast alcohol dehydrogenase dissolved in the sample was layered on to a 5.25ml constant velocity sucrose gradient (Methods section 2. 12) containing 0.10M-tris-HCl(pH8.0)-10mM-2-mercapto-ethanol-1mM-EDTA as buffer.

Centrifugation was carried out in a Spinco SW50L rotor at 5°C for a total effective time of 12h 18min at 39,000 rev./min (123,267g) $r_{av.}$ 7.3cm)(Methods section 2. 12.).

The gradient was then harvested (Methods section 2. 12.) and gave rise to 24 fractions, the volumes of which were measured (approx. 0.24ml). 0.05ml samples of the fractions were then assayed for DNase (denatured DNA as substrate)(Methods section 2. 2. 3.) and alcohol dehydrogenase (Methods section 2. 2. 5.). In addition 0.05ml samples of fractions were diluted to 0.55ml with distilled water and their extinction at 420nm read as a measure of haemoglobin concentration.

—○—○— DNase activity

—▣—▣— E_{420} (haemoglobin)

—□—□— Yeast alcohol dehydrogenase activity (ΔE_{360}/min)

DNase activity (10^3 x units/0.05ml sample of fraction)
0
10
20
30
40
50
Alcohol dehydrogenase activity (ΔE_{360}/min/0.05ml sample of fraction)
0
0.04
0.08
0.12
0.16
0.20
E_{420}
Bottom
Top
Volume (ml) from bottom of gradient
1.0
2.0
3.0
4.0
5.0
5.5

Fig. III. 21.

Fig. III. 22.

Constant velocity sucrose gradient zone sedimentation of HSV-induced DNA polymerase and its associated DNase pooled and concentrated from hydroxylapatite chromatography (later elution conditions) of a whole-cell extract of HSV-infected cells prepared in the presence of PMSF.

0.25ml of pooled and concentrated HSV-induced DNA polymerase (containing 0.475 units associated DNase) from the hydroxylapatite column chromatographic fractionation shown in Fig. III. 15. was layered, together with 1mg human haemoglobin and 0.1mg yeast alcohol dehydrogenase, on to a 5.25ml constant velocity sucrose gradient (Methods section 2. 12.) made with "TEM buffer".

Centrifugation was carried out at 5°C in an SW50L rotor at 36,500 rev./min (107,950g)($r_{av.}$ 7.3cm) for a total effective time of 15h 16.6min at this speed, then the gradient was harvested as described in Methods section 2. 12. 26 fractions were collected and their volume measured (0.217ml). 0.05ml samples of the fractions were then assayed for DNA polymerase ("low salt", half scale "activated" DNA as primer), DNase (denatured DNA as substrate) and alcohol dehydrogenase (Methods sections 2. 2. 1., 2. 2. 3. and 2. 2. 5. respectively). The extinction at 420nm of samples of appropriate fractions diluted 1 in 12.5 was estimated as a measure of haemoglobin concentration.

—△—△— DNA polymerase activity
—○—○— DNase activity
—▨—▨— E_{420}
—□—□— Alcohol dehydrogenase activity

DNA polymerase activity (10^3 x units/0.05ml sample of fraction)
2.5
2.0
1.5
1.0
0.5
DNase activity (10^3 x units/0.05ml sample of fraction)
50
40
30
20
10
0
Alcohol dehydrogenase activity (ΔE_{360}/min/0.05ml sample of fraction)
0.5
0.4
0.3
0.2
0.1
0
E_{420}
Bottom
Top
Volume (ml) from bottom of gradient
0
1.0
2.0
3.0
4.0
5.0
5.5

Fig. III. 22.

seemed to be distributed over the gradient and some seemed to have remained in the position of the sample.

The DNase profile indicated a larger proportion of enzyme in the position of and beyond alcohol dehydrogenase than in the absence of PMSF. This finding may represent more enzyme undegraded by protease or the presence of a monomer-dimer-tetramer relationship between the three peaks of activity. A typical result gave apparent $s_{20,w}$ values of 10.6S; 7.1S; 3.85S: corresponding to molecular weight values of 243,000; 136,000 and 52,400. If we allow a broad margin of error of 10,000 for the calculated molecular weight values, these figures are not incompatible with this oligomer hypothesis.

2. 5. Sephadex G-150 Column Chromatography.

2. 5. 1. Fractionation of whole-cell extracts of HSV-infected cells.

A whole-cell extract of HSV-infected cells was fractionated on a Sephadex G-150 column as described in Methods section 2. 10. 3. A separate run was carried out with standard proteins and the combined data incorporated in Fig. III. 23.

The peak of DNA polymerase activity appears just behind the void volume as judged by the position of the Blue Dextran and the E_{280nm} peak. A large peak of DNase essentially co-chromatographs with this polymerase peak: the DNase, however trails to give a further two undulations - one large one, followed by a smaller one -

Fig. III. 23.

Sephadex G-150 column chromatography of the DNA polymerase and DNase activities of a whole-cell extract of HSV-infected cells and standardisation of the column using Blue Dextran, yeast alcohol dehydrogenase, transferrin and cytochrome c.

A whole-cell extract of HSV-infected cells was fractionated as described in Methods section 2. 10. 3., on a 67.5x2.5cm column of Sephadex G-150 using (0.10M-tris-HCL buffer (pH8.0)-10mM-2-mercapto-ethanol-0.5mg/ml bovine serum albumin) as column buffer. 4.5ml fractions were collected and aliquots assayed for DNA polymerase ("low salt"-half scale-denatured DNA primer)(Methods section 2. 2. 1.) and DNase (denatured or native DNA as substrate)(Methods section 2. 2. 3.). The extinction at 280nm of the fractions gave an estimate of the position of the void volume.

A separate fractionation using exactly the same column and conditions was carried out but with a sample containing Blue Dextran, yeast alcohol dehydrogenase, transferrin and cytochrome c. Again 4.5ml fractions were collected and aliquots of these assayed for Blue Dextran (colorimetric method carried out with the blue coloured fractions), yeast alcohol dehydrogenase (Methods section 2. 2. 5.), transferrin (by reading the extinction at 280nm) and cytochrome c (by reading the extinction at 410nm). The data was incorporated in one figure quoting enzyme activity simply in arbitrary units since only the positions of the activities were of interest.

—△—△— DNA polymerase activity

—○—○— DNase activity (denatured DNA as substrate)

—◎—◎— DNase activity (native DNA as substrate)

—▣—▣— Cytochrome c (E_{410nm})

—□—□— Alcohol dehydrogenase activity

—·—·—·— E_{280} (near void volume as estimate of position of this and also for transferrin estimation)

—△—△— Blue Dextran

DNA polymerase activity (arbitrary units/fraction)
DNase activity (arbitrary units/fraction) (native or denatured DNA substrate)
Alcohol dehydrogenase (arbitrary units/fraction)
E_{280} or E_{410}
Fraction number (4.5ml)
Void volume (110ml) (Blue Dextran)
E_{280}peak (at approx. void volume)
E_{280}peak (transferrin)
DNA endonuclease (denatured DNA)
peak

Fig. III. 23.

and all of this is eluted in front of the alcohol dehydrogenase peak's position. A further peak of DNase - the host cell DNA endonuclease - is found eluting between the cytochrome c and transferrin.

The above experiment was repeated using a whole-cell extract of HSV-infected cells after $(NH_4)_2SO_4$ fractionation (Methods section 2. 7.), and the resulting profile is presented in Fig. III. 24. This confirms the pattern of elution found in the previous experiment, except that the later peaks of DNase have more activity against heat-denatured DNA and less activity against native DNA.

2. 5. 2. Gel-filtration of induced DNA polymerase and DNase purified on DEAE-cellulose.

The elution of the DNA polymerase and DNase so close to the void volume in the two previous experiments implied a very high molecular weight for these proteins, possibly owing to association with DNA. Accordingly, a preparation of virus-induced DNA polymerase and DNase purified on DEAE-cellulose (Methods section 2. 10. 2.) was applied (4.0ml sample) to the Sephadex G-150 column used above.

The results (Fig. III. 25.) indicate that both the DNA polymerase peak and the peaks of DNase are more retarded than in the previous runs: these molecules, however, are still larger than was suggested by the sucrose gradient results (Results sections

Fig. III. 24.

Sephadex G-150 column chromatography of an ammonium sulphate whole-cell extract of HSV-infected cells.

4.0ml of an ammonium sulphate fractionated whole-cell extract (volume 16.0ml) of HSV-infected cells containing approx. 151 units DNA polymerase (denatured DNA primer) and 480 units DNase (native DNA as substrate) together with 10mg cytochrome c as standard was applied to the same Sephadex G-150 column (2.5x67.5cm) as before (Fig. III. 23.). The buffer used was as before and 4.5ml fractions were collected and aliquots assayed as follows:- DNA polymerase ("low salt"-half scale-activated DNA primer)(Methods section 2. 2. 1.), DNase (native or denatured DNA as substrate)(Methods section 2. 2. 3.) and the extinction of the fractions at 280nm (using a water blank) and 410nm was measured as a guide to protein (in general) and cytochrome c (in particular) respectively.

—△—△— DNA polymerase activity.
—○—○— DNase activity (denatured DNA as substrate)
—◎—◎— DNase activity (native DNA as substrate)
—▣—▣— E_{410}
—·—·—·— E_{280}

Fig. III. 24.

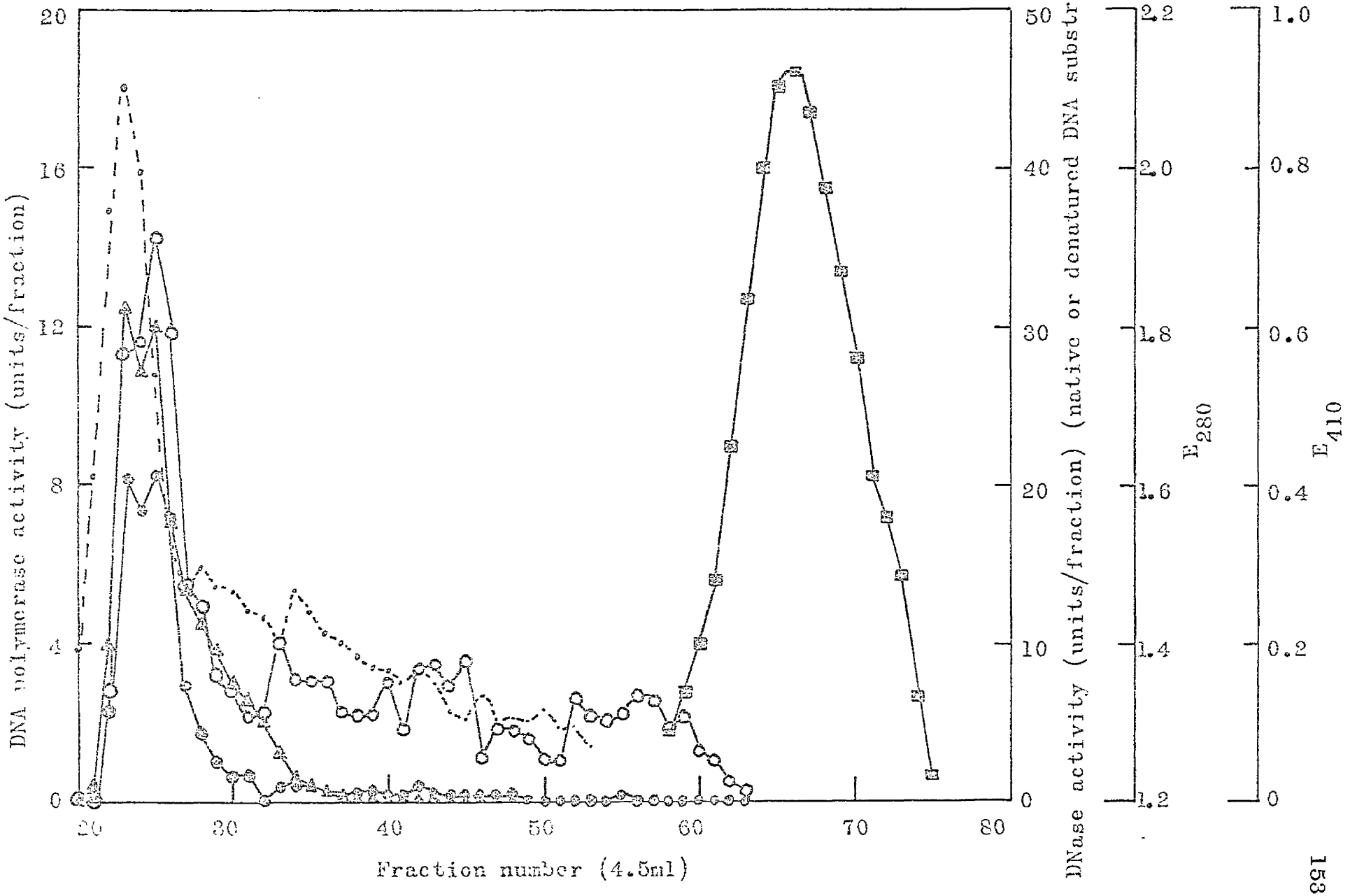

DNA polymerase activity (units/fraction)
DNase activity (units/fraction) (native or denatured DNA substrate)
E_{280}
E_{410}
Fraction number (4.5ml)
0
4
8
12
16
20
10
20
30
40
50
60
70
80
1.2
1.4
1.6
1.8
2.0
2.2
0.2
0.4
0.6
0.8
1.0

Fig. III. 25.

Sephadex G-150 column chromatography of a pooled and concentrated fraction of HSV-induced DNA polymerase and its associated DNase purified by DEAE-cellulose column chromatography.

The HSV-induced DNA polymerase and its associated DNase from DEAE-cellulose column chromatography of mixed whole-cell extracts of uninfected and HSV-infected cells (Fig. III. 10) was pooled and concentrated. 4.0ml of the concentrate containing approx. 5.6 units DNA polymerase (denatured DNA primer - "low salt" assay) and 65 units DNase (native DNA as substrate) and 10mg cytochrome c was applied to the 2.5x 67.5cm Sephadex G-150 column described in Legends to Figs. III. 23 and 24 and fractionation carried out using the same buffer as before. 4.5ml fractions were collected and aliquots assayed for DNA polymerase activity ("low salt"-half scale-activated DNA primer)(Methods section 2. 2. 1.),DNase (native or denatured DNA as substrate)(Methods section 2. 2. 3.) and the extinction at 280nm and 410nm of appropriate fractions monitored as a measure of protein and cytochrome c concentrations respectively.

—△—△— DNA polymerase activity
—○—○— DNase (denatured DNA substrate)
—◎—◉— DNase (native DNA substrate)
—·—·—·— E_{280} (protein generally)
—▣—▣— E_{410} (cytochrome c)

DNase activity (units/fraction) (native or denatured DNA substrate)
E_{280}
2.5
2.0
1.5
1.0
0.5
0
E_{410}
1.25
1.00
0.75
0.50
0.25
0
DNA polymerase activity (units/fraction)
0.5
0.4
0.3
0.2
0.1
0
Fraction number (4.5ml)
20
30
40
50
60
70
80

Fig. III. 25.

2. 4. 1. and 2. 4. 3.). The enzyme activities, however, were low as was routinely found for DEAE-cellulose-purified material (Morrison and Keir, 1968a).

DNA polymerase assays were carried out on activity from Sephadex and DEAE-cellulose fractionations of whole-cell extracts of HSV-infected cells in the absence of added DNA primer: These experiments indicated that little, if any, DNA was apparently associated with DNA polymerase under these conditions. These results agree with the conclusions of Morrison and Keir (1968a) for the virus-induced DNase.

2. 5. 3. Fractionation of a whole-cell extract of uninfected cells.

A whole-cell extract of uninfected cells was submitted to $(NH_4)_2SO_4$ precipitation (Methods section 2. 7.) and applied to a Sephadex G-150 column with cytochrome c marker as before.

The result is presented in Fig. III. 26. and it can be seen that two peaks of DNA polymerase appear with low activities and different priming requirements. The first peak is more active than the second with heat-denatured DNA primer. DNase is distributed over the eluate accompanying the polymerase peaks (cf. Fig III. 24.) and the host DNA endonuclease is localised in front of the cytochrome c marker as before. The observation of twin polymerase peaks corresponds to that of R.L.P. Adams and J.G. Lindsay (unpublished observations) for L-cell DNA polymerase,

Fig. III. 26.

Sephadex G-150 column chromatography of an ammonium sulphate fractionated whole-cell extract of uninfected BHK21 (C13) cells.

16ml of a whole-cell extract (Methods section 2. 6. 1.) of uninfected cells was fractionated by ammonium sulphate (Methods section 2. 7.) and the precipitate (0-50% saturation fraction) taken up in 4ml 0.1M-tris-HCl(pH8.0)-10mM-2-mercaptoethanol. 10mg of cytochrome c was added as a marker and the sample applied to the 2.5x67.5cm Sephadex column and fractionation carried out as before, (Legends to figs. III. 23, 24, 25) 4.5ml fractions being collected. Aliquots of the fractions were then assayed for DNA polymerase (half scale assay - "low salt" - activated or denatured DNA primer) (Methods sections 2. 2., 2. 2. 1.) and DNase (denatured DNA substrate) (Methods section 2. 2. 3.).

The extinctions of appropriate fractions were read at 280nm and 410nm as a guide to general protein and cytochrome c elution respectively.

—△—△— DNA polymerase activity (activated DNA primer)

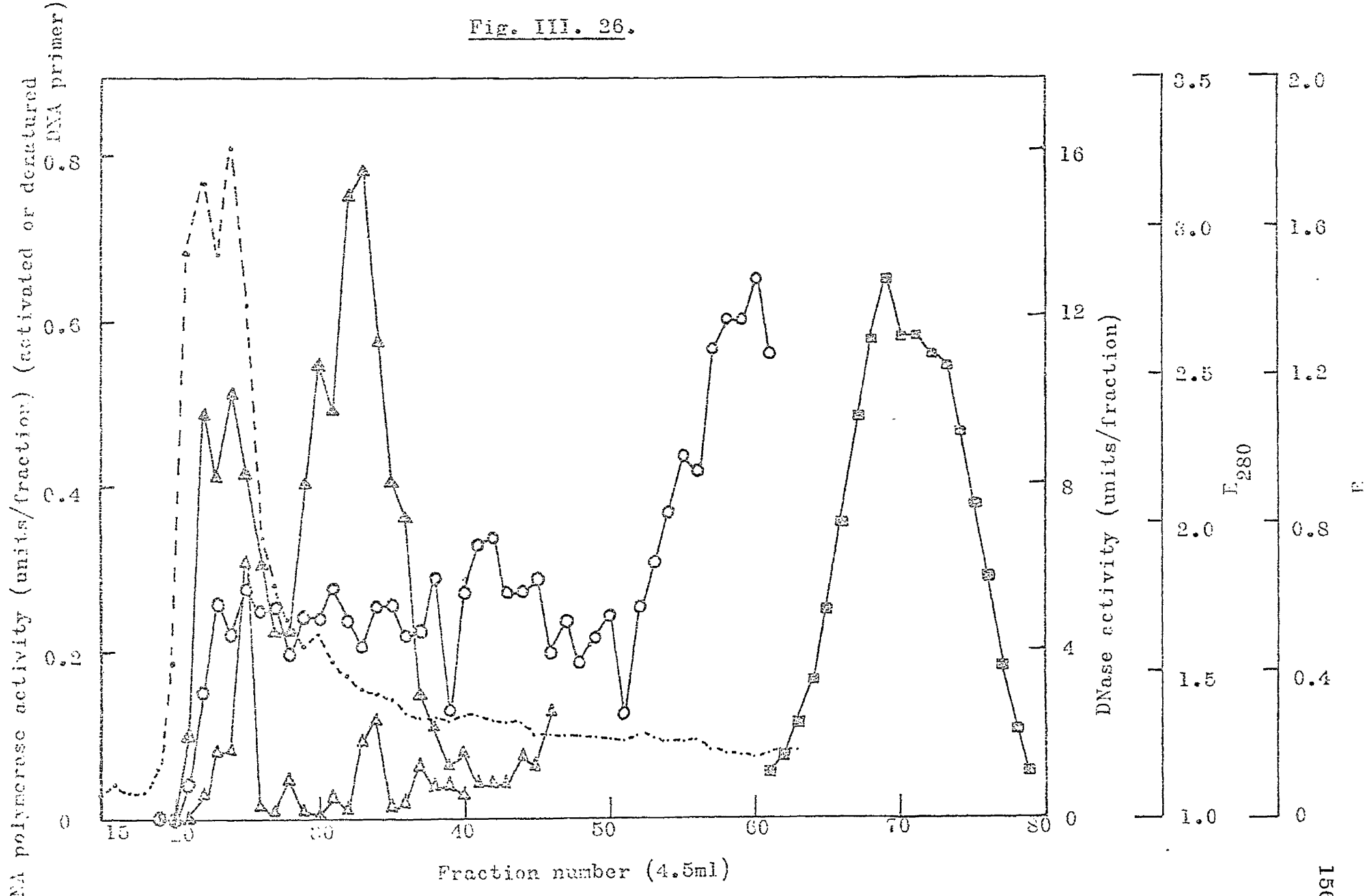
—△—△— DNA polymerase activity (denatured DNA primer)

—○—○— DNase activity (denatured DNA substrate)

—▣—▣— E_{410} (cytochrome c)

—·——·—·— E_{280} (general protein)

Fig. III. 26.


DNA polymerase activity (units/fraction) (activated or denatured DNA primer)
0
0.2
0.4
0.6
0.8
DNase activity (units/fraction)
0
4
8
12
16
E_{280}
1.0
1.5
2.0
2.5
3.0
3.5
0
0.4
0.8
1.2
1.6
2.0
10
20
30
40
50
60
70
80
Fraction number (4.5ml)

and to that of Bellair (1968), Iwamura et al.,(1968), and Ove et. al., (1969) for rat liver DNA polymerase.

2. 5. 4. Molecular weight estimations from Sephadex G-150 elution.

A plot of $\log_{10}$ (molecular weight of protein) against (Elution volume of protein/void volume) i.e. ($^{Ve}/Vo$) for the Sephadex column employed (Results sections 2. 5. 1. - 2. 5. 3.) is presented in Fig. III. 27.). It can be seen that the standard proteins all lie approximately on a straight line corresponding to the results of Andrews (1965, 1970).

The presence of bovine serum albumin in the column buffer not only protects the other proteins from denaturation but also causes the Sephadex G-150 results to resemble, more closely, results of Sephadex G-200 columns, i.e. the protein exclusion molecular size is raised above the 600,000 value described by Andrews (1970) for Sephadex G-150 for reasons explained by Hellsing (1968).

The extrapolation of the line beyond the yeast alcohol dehydrogenase position was carried out in two ways: in one, 600,000 was assumed to be the exclusion size for proteins, in the other, the straight line was continued to meet the $^{Ve}/Vo = 1$ base line which it met at a position corresponding to a molecular weight of approx. 900,000. The correct molecular size estimates in the region beyond mol.wt. 150,000 are uncertain but probably lie somewhere

Fig. III. 27.

Molecular weight estimations on the proteins of interest from the Sephadex G-150 fractionation results.

Protein or Dextran	Fractionation run Fig.No.	Peak Fraction Number	Ve (ml)	Ve / Vo	Log_{10} (mol. wt.)	Estimated or known Molecular weight protein
Blue Dextran 2000	III.23.	24	108	1.00	> 6	$2x10^6$
Early E_{280} protein peak	III.24,26.	24	108	1.00	>5.78	$> 6x10^5$
Yeast alcohol dehydrogenase	III.23.	43-44	195.8	1.81	5.18	$1.5x10^5$
Transferrin	III.23.	49	220.5	2.04	4.90	$8x10^4$
Cytochrome c	III.23,24, 25,26.	69.5,66, 68,69.5	312.8 297	2.90 2.75	4.11	$1.3x10^4$
Crude extract HSV-induced DNA polymerase + associated DNase	III.23. and 24.	23, 25, 26, 27.	121.5	1.13	5.70 5.84	$5x10^5$ $7x10^5$
DEAE-purified HSV-induced DNA polymerase	III.25.	34	153	1.42	5.48 5.56	$3x10^5$ $3.7x10^5$
Control cell DNA polymerase 1st peak	III.26.	22 24	99 108	<1.00 1.00	5.78	$> 6x10^5$
Control cell DNA polymerase 2nd peak	III.26.	32 34	144 153	1.33 1.42	5.54 5.47	$3x10^5$ $3.5x10^5$
2nd peak of DNase	III.23,24, 26.	33 36	148.5 162	1.38 1.50	5.25 5.48	$1.8x10^5$ $3x10^5$
3rd peak of DNase	III.23,24, 26.	39 44	175.5 198	1.62 1.86	5.35 5.16	$2.25x10^5$ $1.45 x10^5$
Host-cell DNA endonuclease	III.23,24 a nd 26	56-60	252 270	2.33 2.50	4.67 4.50	$4.7x10^4$ $3.2 x10^4$
2nd DNase peak (polymerase-free) from DEAE-cellulose	III.25	44.5	200.3	1.85	5.12	$1.38x10^5$

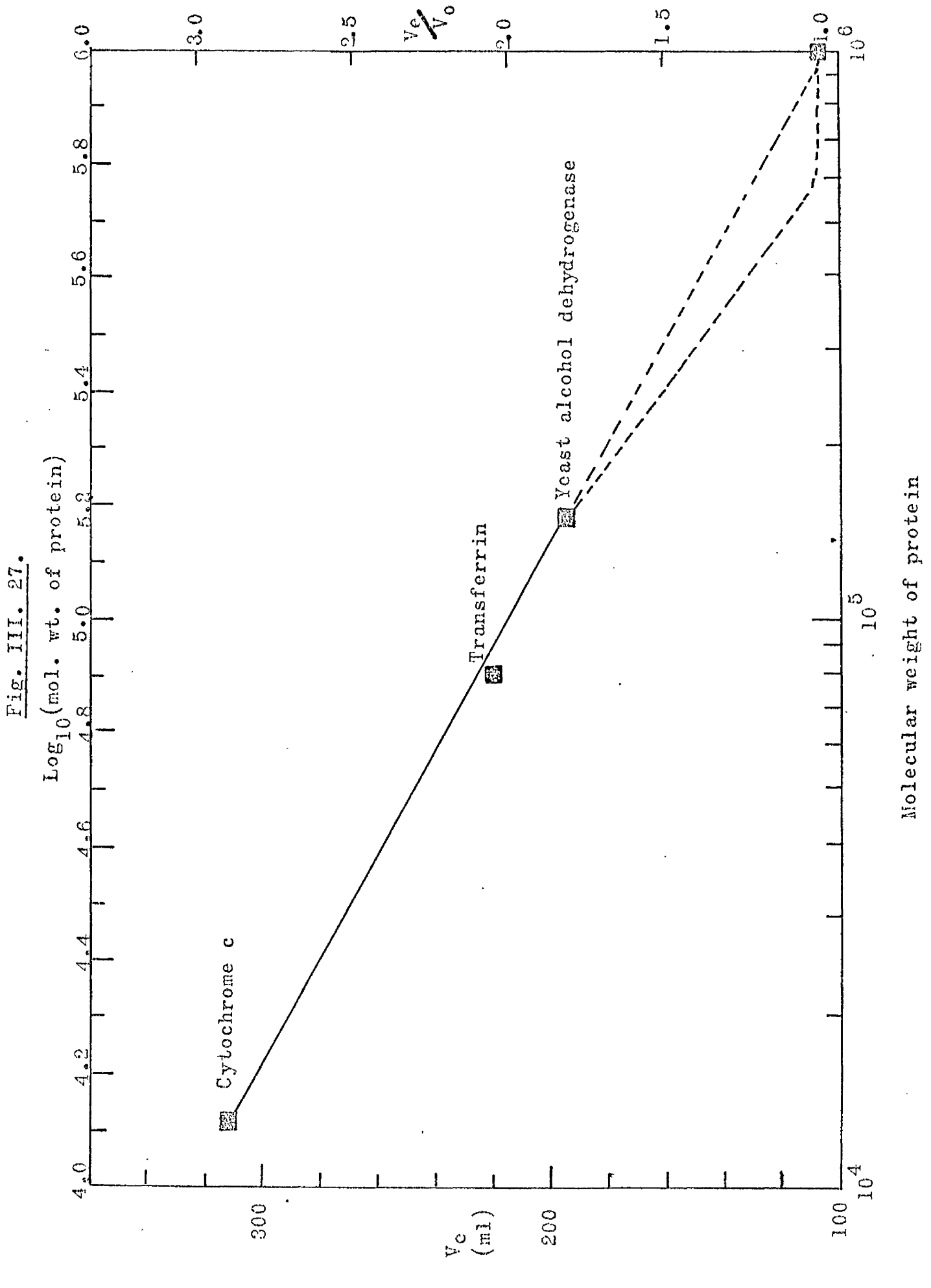

Ve/Vo
3.0
2.5
2.0
1.5
1.0
Log10(mol. wt. of protein)
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
Ve (ml)
300
200
100
Cytochrome c
Transferrin
Yeast alcohol dehydrogenase
10^6
10^5
10^4
Molecular weight of protein

Fig. III. 27.

between the estimates given by these two extrapolated lines and they are quoted as such: they might however possibly be still larger.

The virus-induced DNA polymerase and DNase from this plot appear to be of very high molecular weight (apparently well over 150,000) and estimates of the molecular size of these and the other molecules of interest are tabulated in the legend to Fig. III. 27.

The reasons for the large discrepancies between these values for the HSV-induced DNA polymerase and its associated DNase and the values suggested by sucrose gradient sedimentation are obscure but may reflect different degrees of aggregation in the two fractionation processes both of which involve considerably different chemical environments.

2. 6. Polyacrylamide gel electrophoresis of extracts of HSV-infected cells.

2. 6. 1. Electrophoresis of whole-cell extracts of HSV-infected cells.

This was carried out as described in Methods section 2. 11.

Three gels were run and to the first, 0.1ml of a whole-cell extract with 50% $^{v}/v$ glycerol was added and this gel used for protein-staining at the end of the run. To the second and third gels, 0.3ml of whole-cell extract with 50% ($^{v}/v$) glycerol was applied and the gels sliced and assayed for DNase and DNA polymerase

respectively, at the end of the run.

The results are presented in Fig. III. 28a. A peak of DNA polymerase and DNase appears close to the origin and a second small peak of DNase which has moved further into the gel also runs with a peak of DNA polymerase.

2. 6. 2. Electrophoresis of hydroxylapatite-purified HSV-induced DNA polymerase and its associated DNase.

This was carried out as described in Methods section 2. 11.

Three identical 0.3ml samples of concentrated pooled virus-induced DNA polymerase and its associated DNase from a hydroxylapatite column run (early elution conditions - Methods section 2. 10. 1.) were applied and electrophoresis carried out. One gel was then stained for protein, the other two were sliced and assayed for DNase and DNA polymerase activity.

The results are presented in Fig. III. 28b. It can be seen that the DNase activity profile is similar to that in the previous experiment (Fig. III. 28a.); on the other hand DNA polymerase is localised this time with DNase near the origin. A reduced number of protein bands is seen in this run but it is impossible to say if any one of these corresponds to DNA polymerase.

Earlier work with hydroxylapatite-purified virus-induced DNA polymerase correlates well with this work and a tube at the

Fig. III. 28.

Polyacrylamide gel electrophoresis of DNA polymerase, DNase and protein from (a) a whole-cell extract of HSV-infected cells, and (b) a hydroxylapatite-purified preparation of virus-induced DNA polymerase and DNase.

(a) Whole-cell extracts of HSV-infected cells.

Three gels were run as follows:-

(i.) 0.1ml of a whole-cell extract with 50% (^{v}/v) glycerol was applied and after electrophoresis the gel stained for protein with naphthalene black.

(ii.) 0.3ml of the same kind of extract was applied and the gel sliced into 0.3cm slices for DNase assay.

(iii.) 0.3ml of the above type of extract was applied and gel sliced into 0.4cm slices for DNA polymerase assay.

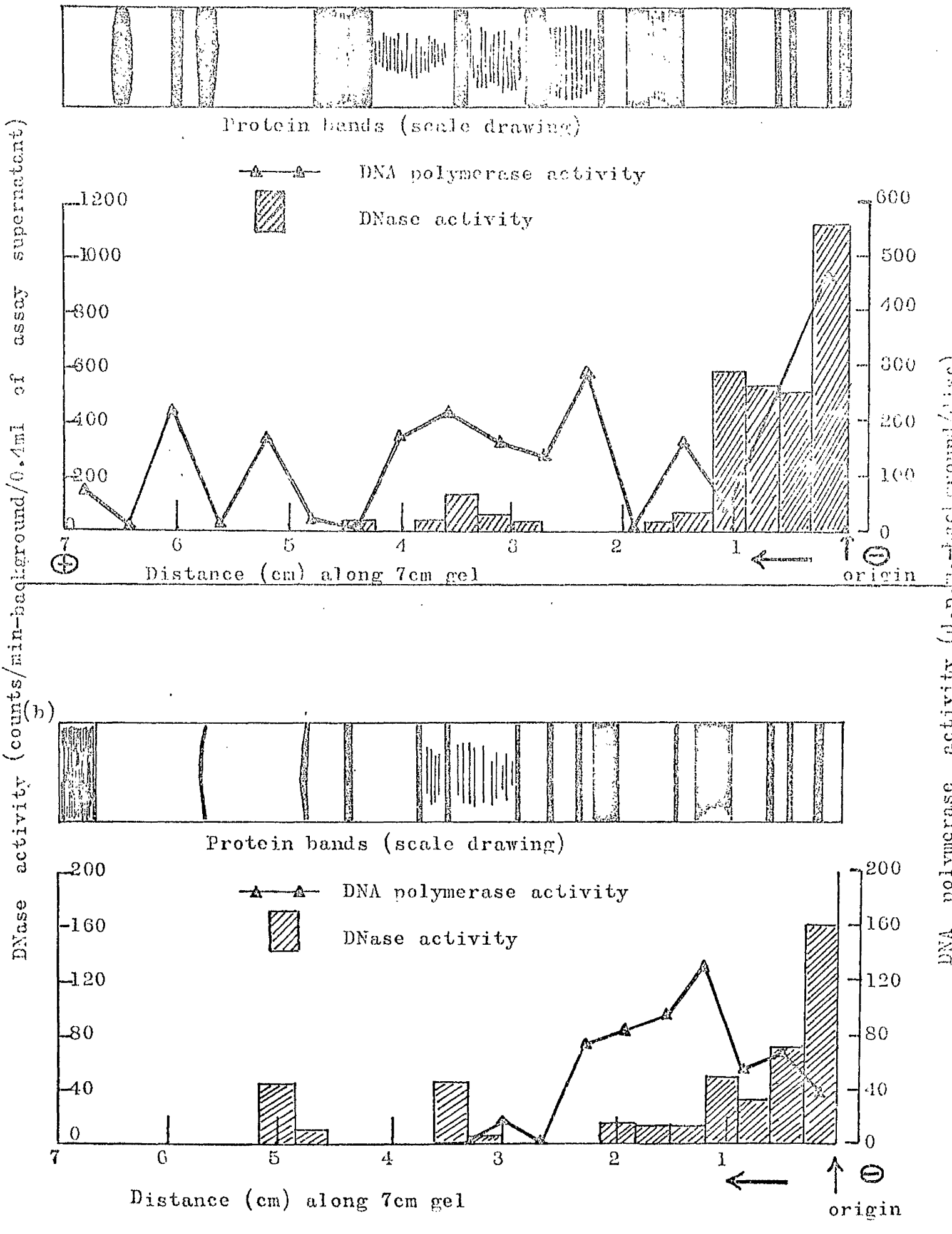
(b) Hydroxylapatite-purified HSV-induced DNA polymerase and associated DNase from chromatography of a whole-cell extract of HSV-infected cells (earlier elution conditions).

Three gels were run as follows:- 0.3ml of concentrated polymerase-DNase peak was applied to each and the first gel stained for protein, the second cut into 0.30cm slices and assayed for DNase and the third sliced into 0.35cm slices and assayed for DNA polymerase.

The method of electrophoresis and protein staining was as described in Methods section 2.11. The polymerase and DNase assays were carried out essentially as described in that section except that, instead of half of the same gel slice being used for the enzyme assays, separate gels were run under identical conditions and whole slices used. This required scaling up the polymerase assay extracts to 0.3ml, using 0.1ml for full scale polymerase assay after the overnight extraction.

The conditions described for enzyme assays in Methods section 2. 11. were applied strictly to later gel runs using hydroxylapatite-purified enzyme (later elution conditions).

Fig. III. 28.


(a)
Protein bands (scale drawing)
DNA polymerase activity
DNase activity
DNase activity (counts/min-background/0.4ml of assay supernatant)
DNA polymerase activity (d.p.m.-background/assay)
1200
1000
800
600
400
200
0
600
500
400
300
200
100
0
7
6
5
4
3
2
1
Distance (cm) along 7cm gel
origin
(b)
Protein bands (scale drawing)
DNA polymerase activity
DNase activity
200
160
120
80
40
0
200
160
120
80
40
0
7
6
5
4
3
2
1
Distance (cm) along 7cm gel
origin

peak of the polymerase activity from a hydroxylapatite column gave only 8 bands - 3 in the region of the DNA polymerase and DNase of the later gels.

Later work with hydroxylapatite-purified material (Methods section 2. 10. 1. - later elution conditions) indicated that rather more of the DNase activity moved from the origin than was found in the earlier work.

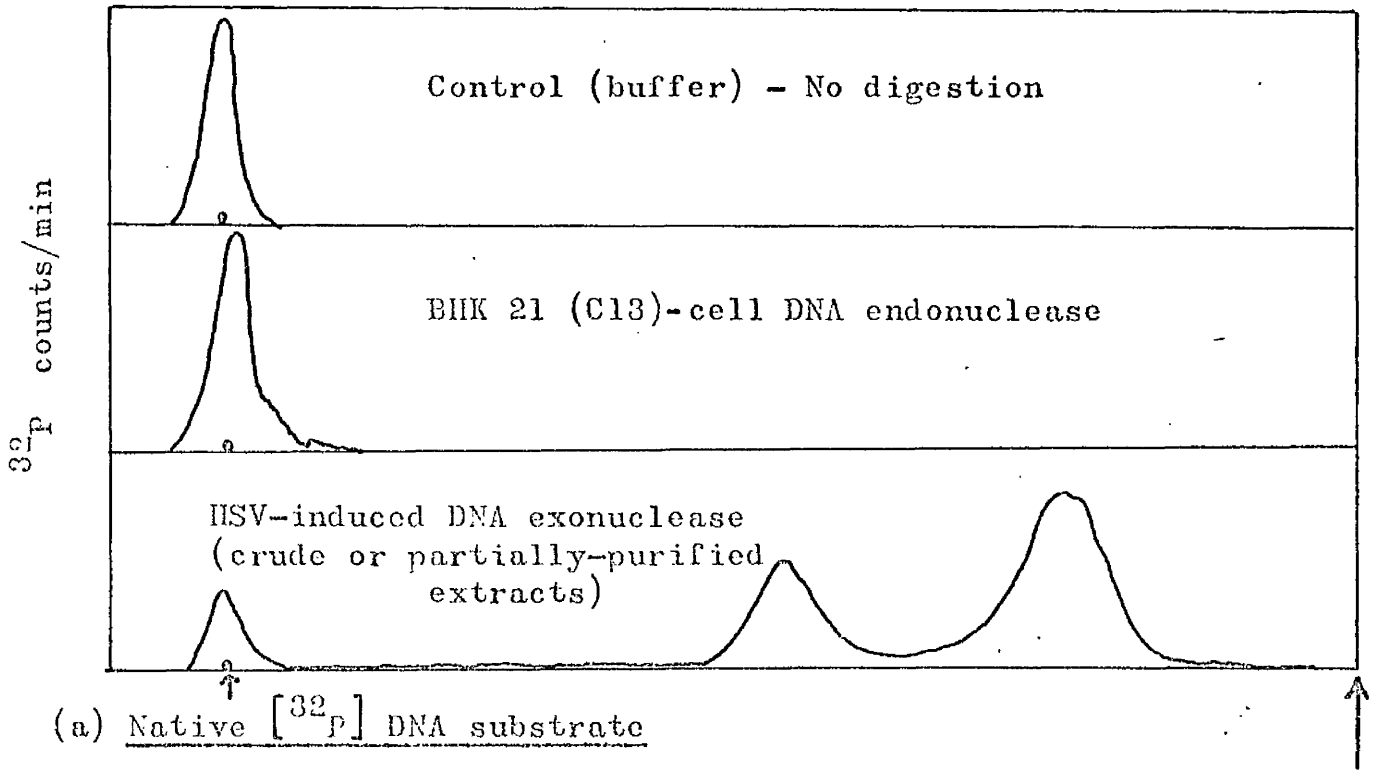
3. CHARACTERISATION STUDIES ON THE TWO DNA EXONUCLEASE ACTIVITIES FROM HYDROXYLAPATITE COLUMN CHROMATOGRAPHY OF EXTRACTS OF HSV-INFECTED CELLS.

3. 1. Studies on their mode of action.

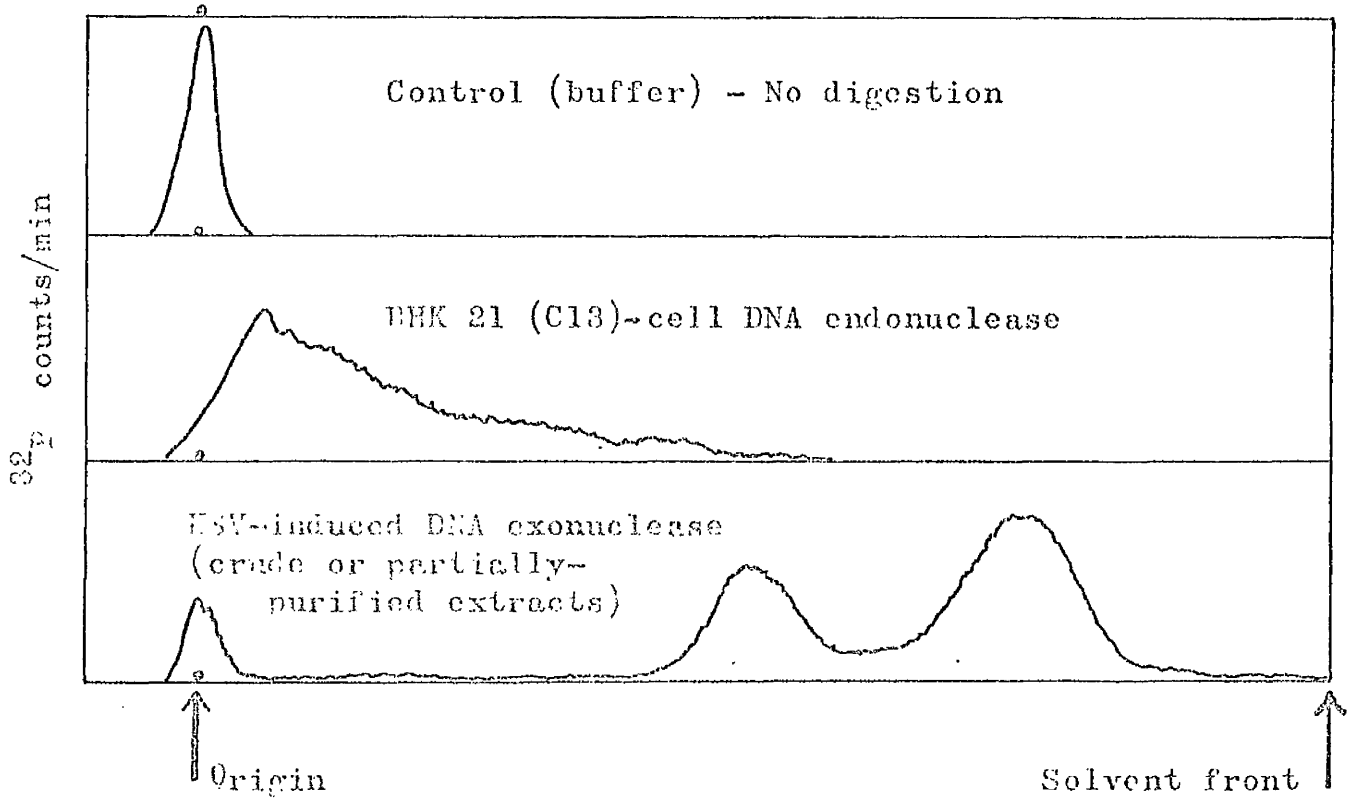
Mode-of-action studies, using both the techniques described in Methods section 2. 2. 4., have clearly shown that both peaks represent DNA exonucleases. Fig. III. 29. shows a diagram of a typical Actigraph trace of a DEAE-paper chromatogram of DNA hydrolysed by either DNase activity, together with a diagram of a trace typical of endonuclease action and a control trace where the DNA is unaffected by DNase. Fig. III. 30. shows typical DEAE-paper-pulp assay results for DNA incubated with either DNA exonuclease, DNA endonuclease or no DNase activity.

Fig. III. 29.

Determination of the mode of action (exonucleolytic or endonucleolytic) of deoxyribonucleases. Typical results from the method using ascending chromatography, on DEAE-paper, of the DNase digests.



(a) Native [^{32}P] DNA substrate

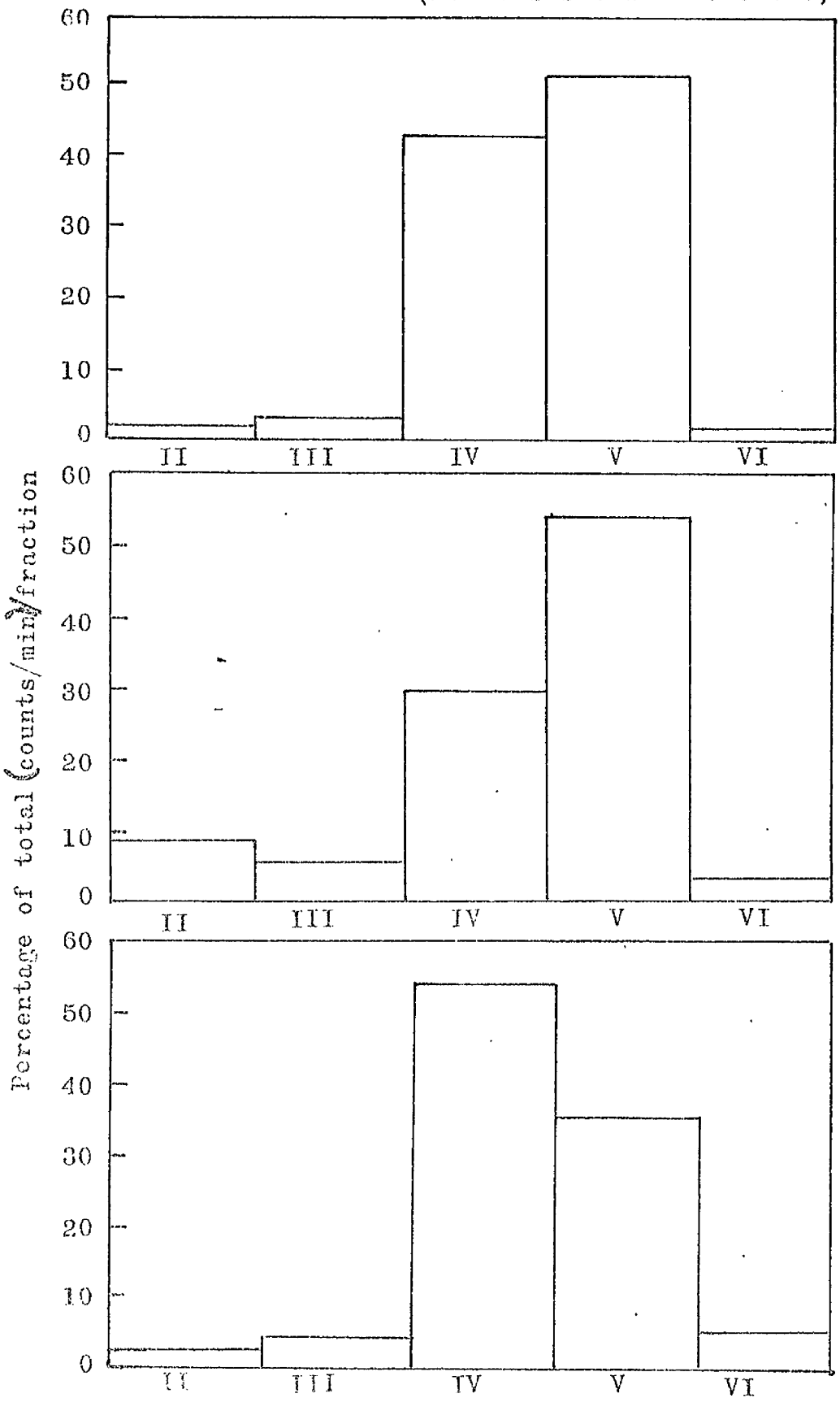


(b) Denatured [^{32}P] DNA substrate

Fig. III. 30.

Determination of the mode of action (exonucleolytic or endonucleolytic) of deoxyribonucleases. Typical results from the method using DEAE-paper-pulp fractionation of the DNase digests.

(Methods section 2.2.4.2.)



(a) No digestion (buffer only in DNase assay incubation)

(b) Exonucleolytic digestion (hydroxylapatite-purified DNA-exonuclease, free or associated with HSV-induced DNA polymerase)

(c) Endonucleolytic digestion (host-cell DNA endonuclease purified on hydroxylapatite)

3. 2. Further studies on the nature of product of the two DNase activities.

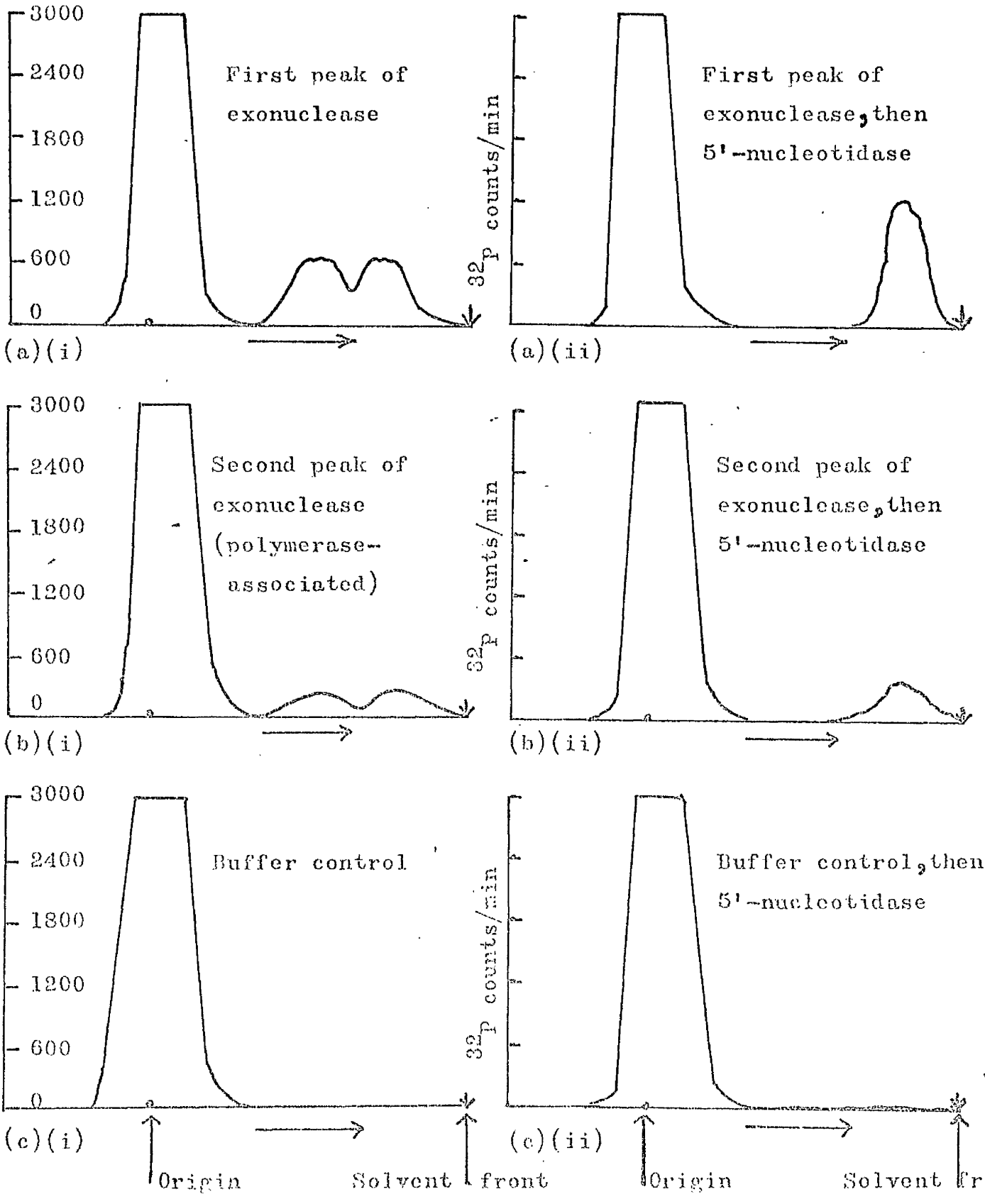
Standard DNase assays (Methods section 2. 2. 3.) using DNase from either of the two peaks were incubated with 5'-nucleotidase and the resulting digests chromatographed as described in Methods section 2. 2. 4. 1. This revealed in both cases a disappearance of the peaks of deoxyribonucleoside monophosphate and the appearance of $[^{32}P]$ Pi (Fig. III. 31.). The conclusion is that the exonuclease of both peaks produces deoxyribonucleoside 5'-monophosphates.

3. 3. Comparative studies on the substrate specificities of the two virus-induced DNA-exonuclease activities from hydroxylapatite.

5ml aliquots of fractions 28 and 19 from the large-scale hydroxylapatite column fractionation described in Results section 2. 2. 1. 3. were dialysed extensively against TEM buffer. 0.1ml aliquots of the dialysis residues of these were then assayed separately with heat-denatured DNA, native DNA, native DNA partially digested with pancreatic DNase I, and heat-denatured DNA partially digested with low and high concentrations of pancreatic DNase I. In the assays with native and heat-denatured DNA, incubation was carried out for 30min. In the experiments with prior digested DNA, DNase I (0.01ml) was added in bovine serum albumin to 0.1ml aliquots of the appropriate mixtures of DNase assay components to give final

Fig. III. 31.

Nature of product studies on the two HSV-induced DNA exonuclease peaks purified from hydroxylapatite column chromatography of extracts of infected cells - to determine whether the product of hydrolysis is deoxyribonucleoside 3'- or 5'-monophosphate.



concentrations of DNase I of 45 or 90 (low or high) ng/ml in 0.9mg/ml bovine serum albumin and incubation carried out for 2 hours at 37°C. After 5min at 77°C to destroy the DNase I, incubation was continued for 30min at 37°C in the presence of virus-induced DNase. All assays were carried out in triplicate and the results are presented in Table III. 4.

It can be seen that the substrate specificities of the DNase activities from the two exonuclease peaks are remarkably similar.

3. 4. Comparison of the heat-inactivation profiles of the DNase activities of the two peaks of DNA exonuclease from hydroxylapatite.

Duplicate tubes were set up with 0.05ml of either exonuclease in TEM buffer and 0.05ml of (4mg/ml) bovine serum albumin. The tubes were then prior incubated at 45°C for various times and assayed as described in Methods sections 2. 13. and 2. 2. 3.

The results are presented in Fig. III. 32. and it can be seen that the profiles are similar although the DNase of the polymerase-associated DNA exonuclease peak from hydroxylapatite appears to show a transient heat-stimulation between 3 and 5min at 45°C. This effect is reminiscent of earlier results of Keir et al. (1966a) for the virus-induced DNA polymerase.

Table III. 4.

Substrate specificities of the two virus-induced DNA exonucleases from hydroxylapatite column chromatography (later elution conditions) of an extract of HSV-infected cells (Results section 3. 3.).

(a) Comparison of the activities with denatured or native DNA in the standard assay.

Nature and amount of enzyme	Activity(units/assay)with Native DNA	Denatured DNA	Conclusions
First exonuclease peak (2.5µg protein/ 0.1ml)	0.2775	0.3050	Enzyme 10% more active with denatured than native DNA
Second exonuclease peak (polymerase associated) 0.1µg protein/0.1ml	0.2721	0.2963	Enzyme 9% more active with denatured than with native DNA

(b) Comparison of the effect of prior DNase I digestion of native DNA (to 25.3% acid solubility) on the two exonuclease activities.

Nature and amount of enzyme	Activity(units/assay)with Native DNA	Denatured DNA	Conclusions
First exonuclease peak (2.5µg protein/ 0.1ml)	0.3629	0.7746	114% increase over native
Second exonuclease peak (polymerase associated) 0.1µg protein/0.1ml	0.4704	1,0959	133% increase over native

(c) Comparison of the effect of prior DNase I digestion of denatured DNA to 5.9% and 9.0% acid solubility) on the two exonuclease activities.

Nature and amount of enzyme		Activity(units/assay) with			
	Denatured DNA	DNase I-treated DNA(5.9%)	% increase	DNase I-treated DNA(9.0%)	% increase
First exonuclease peak (1.5µg protein/ 0.1ml)	0.541	0.678	25%	0.756	38%
Second exonuclease peak (polymerase associated) 0.1µg protein/0.1ml	0.879	1.064	21%	1.135	29%

Fig. III. 32.

Comparative heat-stabilities of the two virus-induced DNA-exonuclease activities from hydroxylapatite column chromatography of extracts of HSV-infected cells.

0.05ml aliquots of polymerase-free and polymerase-associated DNA exonuclease concentrated from the peaks of the hydroxylapatite run displayed in Fig. III. 13. were dialysed against TEM buffer and 0.05ml of 4mg/ml bovine serum albumin added. The enzymes were incubated at 45°C for the times shown in the Figure opposite and then chilled to 0°C, DNase assay components added, and a standard DNase assay carried out (Methods sections 2. 13. and 2. 2. 3.).

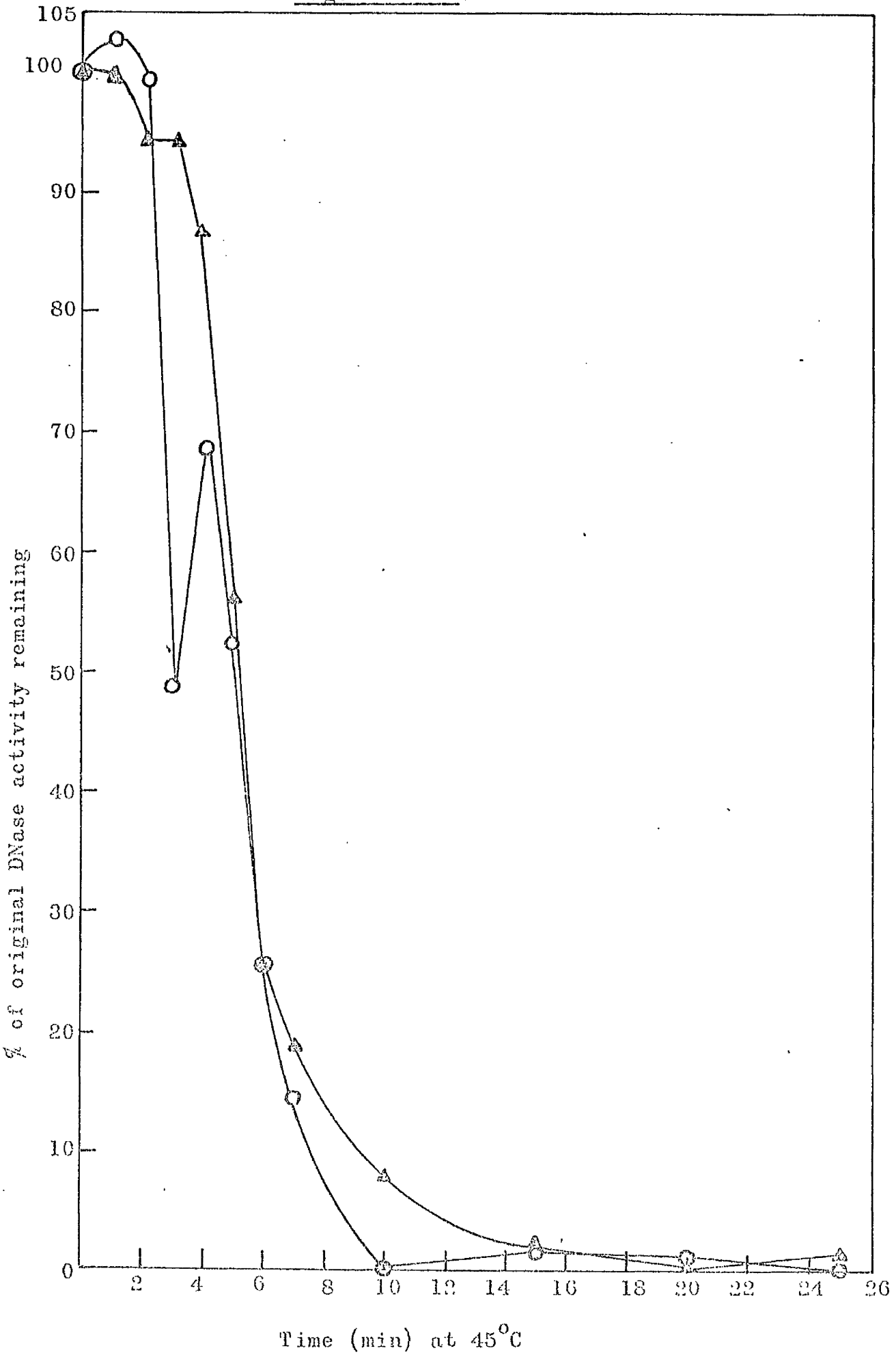
100% for the 1st peak of exonuclease = 0.531 units DNase/assay.

100% for the 2nd peak of exonuclease = 0.321 units DNase/assay.

—▲—▲— First peak of DNA exonuclease (polymerase-free)

—○—○— Second peak of DNA exonuclease (polymerase associated)

Fig. III. 32.


105
100
90
80
70
60
50
40
30
20
10
0
% of original DNase activity remaining
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (min) at 45°C

3. 5. Chromatography of the two exonuclease peaks from hydroxylapatite on DEAE-cellulose, using stepwise elution.

Material from hydroxylapatite elution (Methods section 2. 10. 1.) was applied to an adapted Pasteur-pipette column of DEAE-cellulose (Methods section 2. 10. 2.) and stepwise elution carried out with DEAE-starter buffer then successively with starter buffer containing 0.1M, 0.2M and 0.5M-KCl. Both exonuclease activities eluted at the same salt concentration (0.20M-KCl) in this study and the finding is consistent with previous observations that only one exonuclease peak is found on DEAE-cellulose column chromatography of extracts of HSV-infected cells whereas two are found with hydroxylapatite columns.

4. CHARACTERISATION STUDIES ON THE HSV-INDUCED DNA POLYMERASE AND ITS ASSOCIATED DEOXYRIBONUCLEASE AND ON THE UNINFECTED-CELL DNA POLYMERASE.

4. 1. Primer studies with DNA polymerase at various stages of purification.

4. 1. 1. Studies with Soluble Extracts (Soluble Fractions (S1)).

"Low salt" DNA polymerase assays (Methods section 2. 2. 1.) were set up with varying concentrations of primer and aliquots of

infected- or uninfected-cell soluble extracts (Methods section 2. 6. 2.). The reaction had been shown to be linear for 30min and this incubation time was employed.

The results are presented in Fig. III. 33. It can be seen that no endogenous priming activity exists in the S1 extracts and that, at low DNA concentrations (0-160µg/ml) native DNA is the more effective template. Above this DNA concentration, denatured DNA is more effective - especially with the uninfected-cell extract. Double reciprocal plots (Lineweaver and Burk, 1934) suggest that uninfected- and infected-cell DNA polymerases (S_1 fractions) have identical Km values towards native DNA and also towards denatured DNA and that these values are distinct.

4. 1. 2. Studies with soluble extracts (nuclear fractions - S2).

"Low salt" DNA polymerase assays were set up as above except that S2 fractions (Methods section 2. 6. 2.) were employed with an incubation time of 1 hour at 37°C. The reaction was linear up to this time.

It may be seen (Fig. III. 34.) that a considerable amount of endogenous priming activity is present in these S2-fractions and that, in this case, native DNA is, at all concentrations, more effective than heat-denatured DNA as a primer for the host-cell enzyme, wherease heat-denatured DNA is the preferred primer for the HSV-infected-cell activity - especially at high concentrations.

Fig. III. 33.

Effect of a range of concentrations of native or denatured DNA primer on the DNA polymerase activities of soluble extracts (soluble fractions S1) of uninfected and HSV-infected cells ("low salt" conditions).

0.10ml aliquots of soluble fractions (S1) (Methods section 2. 6. 2.) of uninfected and HSV-infected cells (containing 451 and 112µg protein/aliquot respectively) were incubated for 30min using the standard full scale "low salt" DNA polymerase assay (Methods section 2. 2. 1.) except that a range of concentrations (0-150µg/assay) of native or heat-denatured calf-thymus DNA was added as primer.

—△—△— DNA polymerase of HSV-infected cells (denatured DNA primer)

—▨—▨— DNA polymerase of HSV-infected cells (native DNA primer)

—△—△— DNA polymerase of uninfected cells (denatured DNA primer)

—□—□— DNA polymerase of uninfected cells (native DNA primer)

Fig. III. 33.

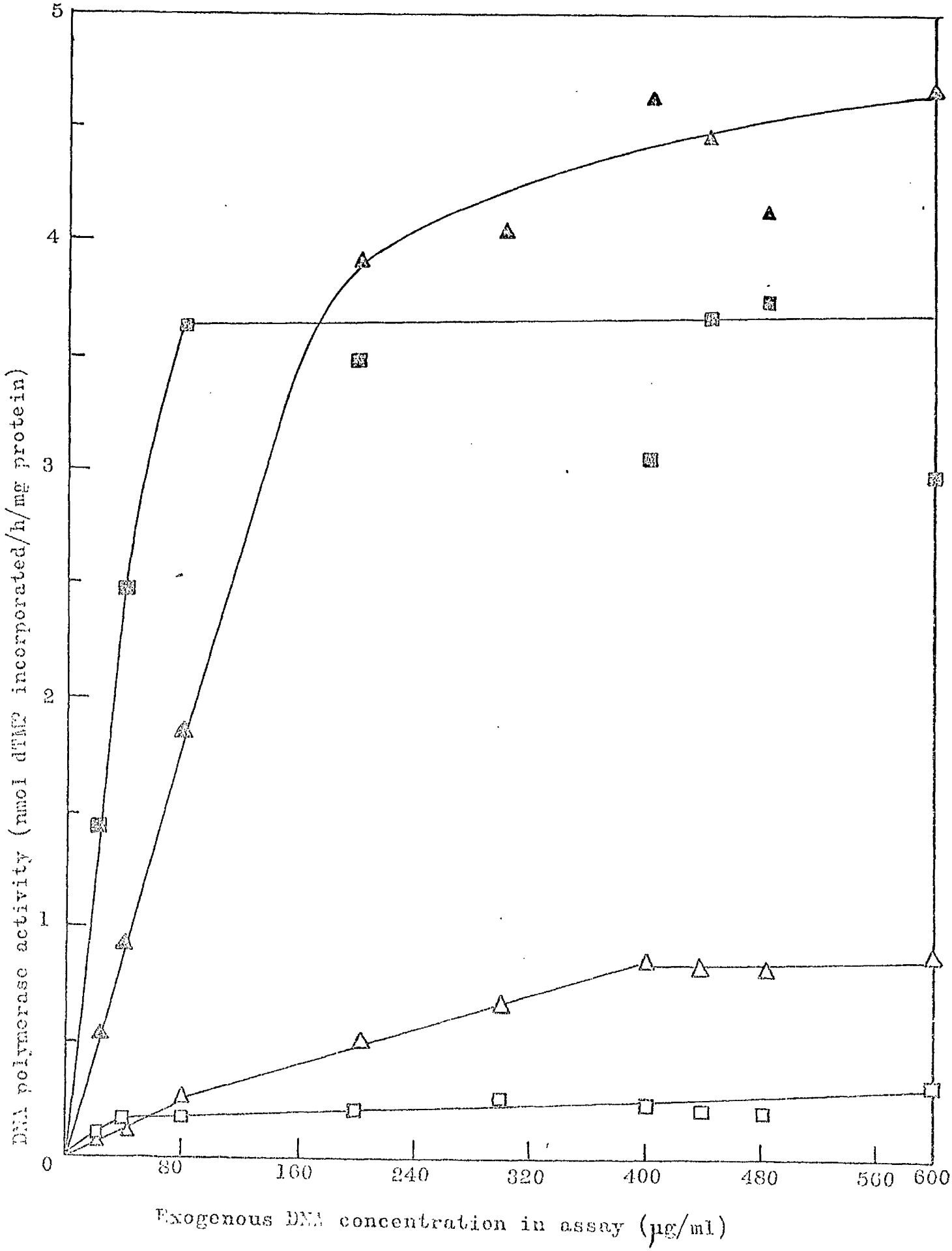

DNA polymerase activity (nmol dTMP incorporated/h/mg protein)
0
1
2
3
4
5
80
160
240
320
400
480
560
600
Exogenous DNA concentration in assay (µg/ml)

Fig. III. 34.

Effect of a range of concentrations of native or denatured DNA primer on the DNA polymerase activities of soluble extracts (nuclear fractions S2) of uninfected and HSV-infected cells ("low salt" conditions).

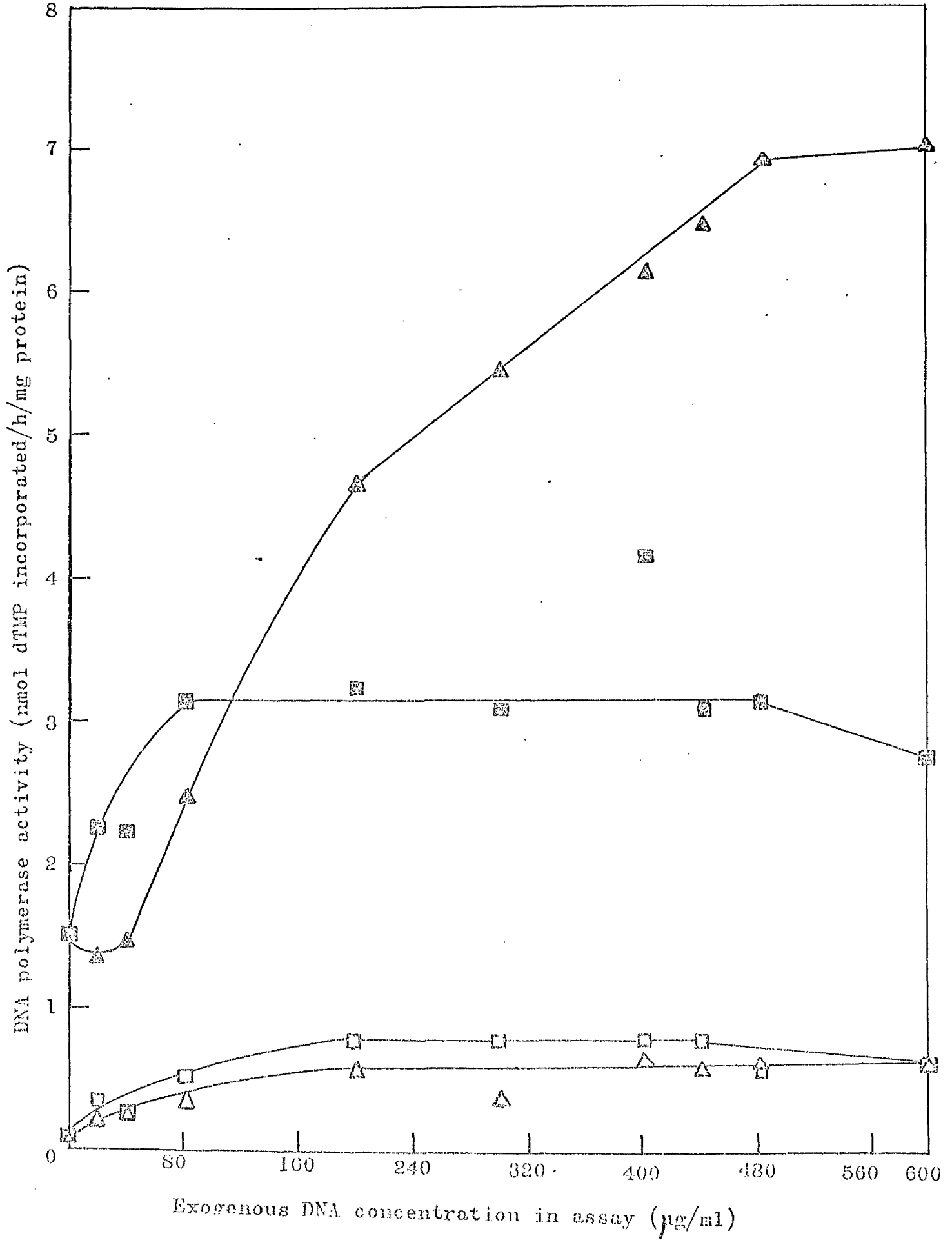
0.10ml aliquots (212µg protein) and 0.05ml aliquots (90µg protein) of soluble extracts (nuclear fractions S2) (Methods section 2. 6. 2.) of uninfected and HSV-infected cells were incubated in full scale "low salt" DNA polymerase assays - standard as described in Methods section 2. 2. 1., except that a range (0-150µg/assay) of native or heat-denatured calf-thymus DNA was added as primer. The incubation time utilised was 60min.

—△—△— Nuclear fraction S2 (HSV-infected) denatured DNA

—▣—▣— Nuclear fraction S2 (HSV-infected) native DNA

—△—△— Nuclear fraction S2 (uninfected) denatured DNA

—□—□— Nuclear fraction S2 (uninfected) native DNA


DNA polymerase activity (nmol dTMP incorporated/h/mg protein)
0
1
2
3
4
5
6
7
8
80
160
240
320
400
480
560
600
Exogenous DNA concentration in assay (µg/ml)

The denatured DNA profile for infected-cell extracts if extrapolated back passes through the origin.

4. 1. 3. Study with S1- fractions at 0.20M-KCl.

An experiment similar to that described in Results section 4. 1. 1. was set up with 0.20M-KCl present to stimulate the virus-induced DNA polymerase and to inhibit both host and virus-induced DNase activities.

The results (Fig. III. 35.) suggest differences between the "low salt" and "high salt" activities of both preparations. There is a marked inhibition of infected-cell DNA polymerase activity at concentrations of heat-denatured DNA above 400µg/ml. A similar, less marked effect occurs with the control-cell extract.

4. 1. 4. Studies with whole-cell extracts of HSV-infected cells at varying DNA concentrations.

The results of the "low salt" DNA polymerase assays (Methods section 2. 2. 1.) are presented in Fig. III. 36. Some endogenous priming (≡ 72µg native DNA/ml in the sonic extract) occurs and the activity is saturated by lower concentrations of native than of heat-denatured DNA. At saturation, there appears to be slighly more activity with heat-denatured DNA than with native DNA.

Fig. III. 35.

Effect of a range of concentrations of native or denatured DNA primer on the DNA polymerase activities of soluble extracts (soluble fractions S1) of uninfected and HSV-infected cells ("high salt" conditions).

0.10ml and 0.05ml aliquots of soluble fractions (S1) (Methods section 2. 6. 2.) of uninfected and HSV-infected cells (containing 451 and 112µg of protein/aliquot respectively) were incubated for 1 hour using the standard full scale DNA polymerase assay (Methods section 2. 2. 1.) except that the concentration of KCl was 0.20M in the assays and a range of concentrations (0-150µg/assay) of native or heat-denatured calf-thymus DNA was added as primer.

—▲—▲— DNA polymerase of HSV-infected cells (denatured DNA primer)

—■—■— DNA polymerase of HSV-infected cells (native DNA primer)

—△—△— DNA polymerase of uninfected cells (denatured DNA primer)

—□—□— DNA polymerase of uninfected cells (native DNA primer)

Fig. III. 35.

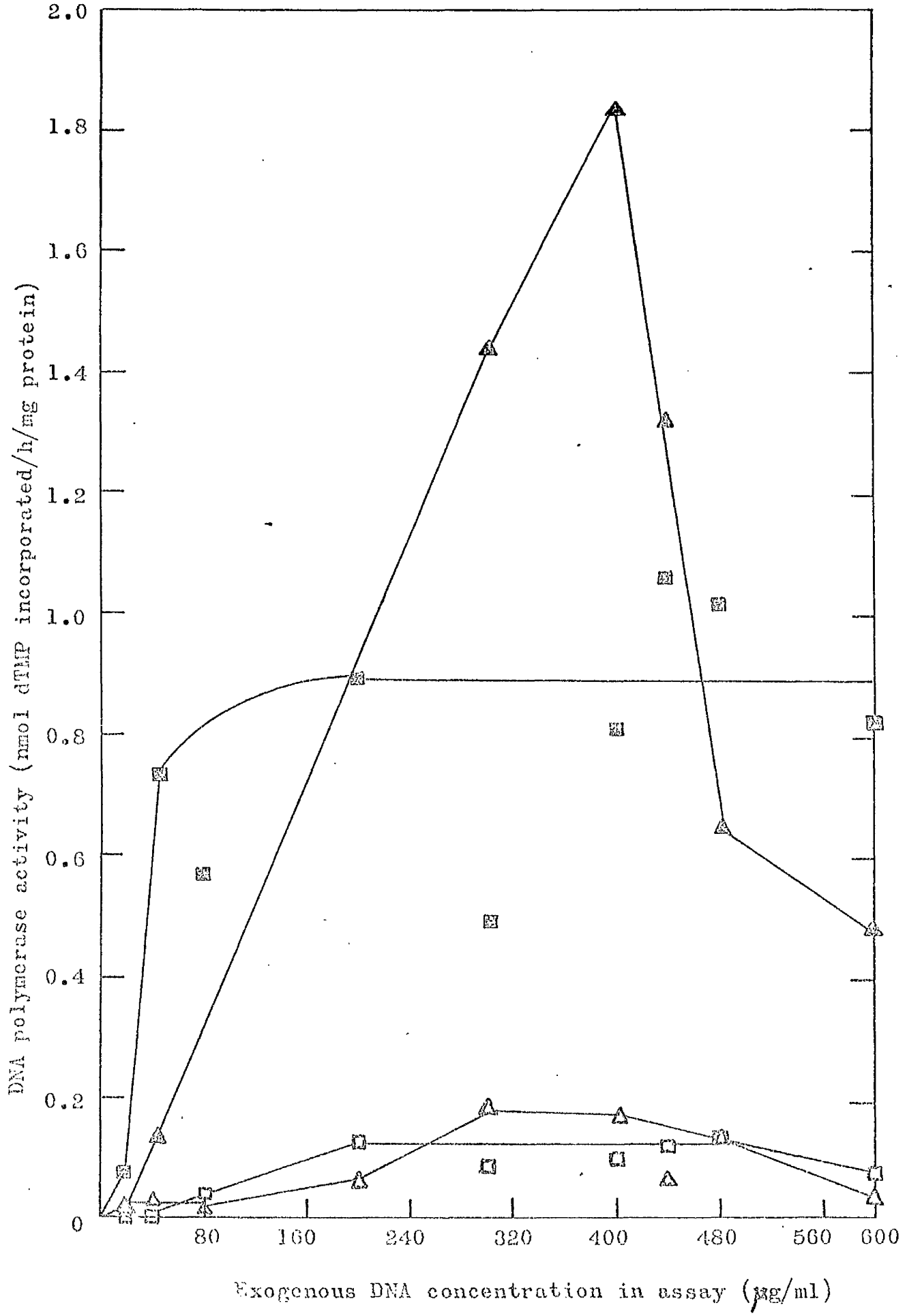

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
DNA polymerase activity (nmol dTMP incorporated/h/mg protein)
80
160
240
320
400
480
560
600
Exogenous DNA concentration in assay (µg/ml)

Fig. III. 36.

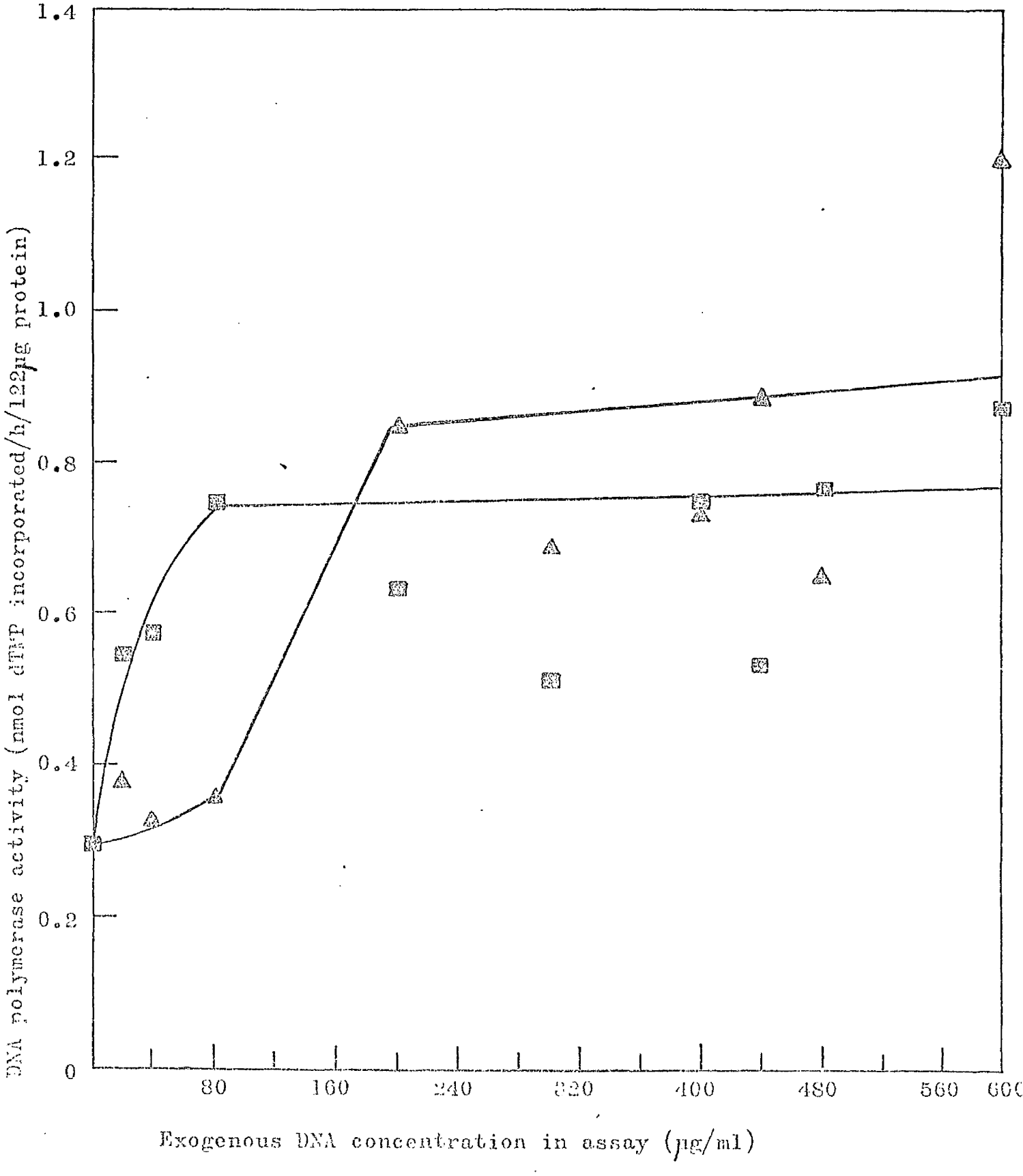
Effect of a range of concentrations of native and denatured DNA primer on the DNA polymerase activity of a whole-cell extract of HSV-infected cells.

0.05ml aliquots (122µg protein) of a whole-cell extract of HSV-infected cells (Methods section 2. 6. 1.) were assayed for DNA polymerase in "low salt" full scale assays - standard as described in Methods section 2. 2. 1. except that a range of concentrations (0-150µg/assay) of native or heat-denatured calf-thymus DNA was used as primer.

—△——△— DNA polymerase activity with heat-denatured DNA as primer

—▣——□— DNA polymerase activity with native DNA as primer

Fig. III. 36.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
DNA polymerase activity (nmol dTMP incorporated/h/122μg protein)
80
160
240
320
400
480
560
600
Exogenous DNA concentration in assay (μg/ml)

4. 1. 5. Studies with native, heat-denatured and activated calf thymus DNA and whole-cell extracts of uninfected and infected cells.

The DNA's were present at a final concentration of 400µg/ml in the standard assays (Methods section 2. 2. 1.). The ratios of activity of the DNA polymerase from uninfected cells and infected cells were respectively 4.40 : 2.45 : 1.00 and 1.71 : 2.25 : 1 (activated DNA : denatured DNA : native DNA).

4. 1. 6. Study with an $(NH_4)_2SO_4$ -purified fraction of infected-cell DNA polymerase.

The ratio of the activity of the DNA polymerase of this preparation (Methods section 2. 7.) (activated DNA : heat-denatured DNA) (400µg DNA/ml) was 2.2 : 1.

4. 1. 7. Study with DEAE-cellulose-purified infected- and uninfected-cell DNA polymerase activity.

DNA polymerase assays were set up as previously (Results section 4. 1. 5.) but with a range of KCl concentrations. The results are given in Table III. 5.

Assays with enzyme (host, second peak, low salt) fresh from the column gave the following result (3 : 3.8 : 1) (activated DNA : heat-denatured : native DNA). On storage, activity against denatured DNA seems to decline until the template characteristics of the DNA polymerase of the second peak are very similar to those

Table III. 5.

Priming specificities of DEAE-cellulose purified HSV-induced and uninfected-cell DNA polymerase activities at various KCl concentrations.

The volumes and natures of the enzyme fractions used were as below.

DEAE-cellulose-purified HSV-induced DNA polymerase:-

0.04ml aliquots of fraction 43 fresh from the fractionation shown in Fig. III. 10 (with bovine serum albumin added to 1mg/ml).

First and second peaks of host-cell DNA polymerase from DEAE-cellulose:-

0.04ml aliquots of fractions 10 and 27 respectively from the fractionation shown in Fig. III. 8. These fractions had been stored for approx. 7 months frozen at -70°C in the presence of 1mg/ml bovine serum albumin before use in this experiment.

The aliquots of each fraction were assayed for DNA polymerase in $\frac{1}{2}$ scale assays - with or without added KCl, to give the concentrations of that salt shown in the table opposite. For each fraction and salt concentration, "activated", denatured, or native DNA at 400µg/ml was used as primer; otherwise the assays and units of polymerase were as described in Methods section 2. 2. 1.

Table III. 5.

Nature of enzyme fraction.	KCl concentration in the DNA polymerase assay. (mM).	DNA polymerase activity (units/0.04ml of fraction with the primers specified below.)			Ratio of activities with different primers. (activated: denatured: native DNA).
		"Activated" DNA	Denatured DNA	Native DNA	
DEAE-cellulose-purified HSV-induced DNA polymerase.	108	0.33480	0.10590	0.02831	3.2 : 1.0 : 0.27
	188	0.59120	0.10260	0.03734	5.8 : 1.0 : 0.36
First peak of host-cell DNA polymerase from DEAE-cellulose	60	0.01085	0.00174	0.00371	6.25: 1.0 : 2.14
	140	0.00775	0.00051	0.00341	13.3 : 1.0 : 6.7
Second peak of host-cell DNA polymerase from DEAE-cellulose	60	0.03029	0.00204	0.00613	14.8 : 1.0 : 3.0
	140	0.01335	0.00133	0.00550	10.0 : 1.0 : 4.5

of the polymerase of the first peak of host enzyme (Table III. 5.). High salt concentration was seen to inhibit the host-cell DNA polymerase with all three templates.

4. 1. 8. Study with hydroxylapatite-purified HSV-induced DNA polymerase activity.

A range of concentrations of native, heat-denatured and activated forms of calf thymus DNA, E. coli DNA, Bacteriophage T7 DNA, salmon sperm DNA, BHK21 (C13) DNA and HSV DNA was incubated for 30 and 60min in half-scale "high salt" assays (Methods section 2. 2. 1.). The total DNA synthesised was calculated from the base composition of the primer.

The results are presented in Fig. III. 37.

It can be seen that, in all cases except for salmon sperm DNA, activated DNA is the optimal primer, native DNA is less effective, and heat-denatured DNA is a very poor primer. New HSV DNA appears to be the best native DNA primer and activated HSV shows an unusual almost linear response to DNA concentration. All other forms appear to plateau at concentrations of DNA above 192µg/ml. Neither endogenous priming activity nor unprimed DNA synthesis was observed.

4. 1. 9. The effect of chemical methylation on priming activity of BHK21 (C13) cell DNA using hydroxylapatite-purified HSV-induced DNA polymerase.

Fig. III. 37a and III. 37b.

Priming studies using DNA from several sources and HSV-induced DNA polymerase purified by chromatography on hydroxylapatite (later elution conditions).

The experiment was conducted as described in Results section 4. 1. 8. and the results presented in the figures opposite and following. The ordinate in both cases expresses nmol DNA nucleotide polymerised in the times stated ((a) 30min (b) 60min).

The enzyme used consisted of 0.04ml (0.44µg protein carrying DNA polymerase). 30µg bovine serum albumin was added to each tube in order to standardise conditions with all DNA samples.

—○—○— Denatured DNA as primer

—△—△— Native DNA primer

—□—□— Activated DNA primer

—◎—◎— 2x activated calf thymus DNA

—◬—◬— 5x activated calf thymus DNA

—▣—▣— 10x activated calf thymus DNA

} DNA activated by twice, five times or ten times the usual concentration of DNase I (Methods section 2. 3. 3.)

Fig. III. 37a.

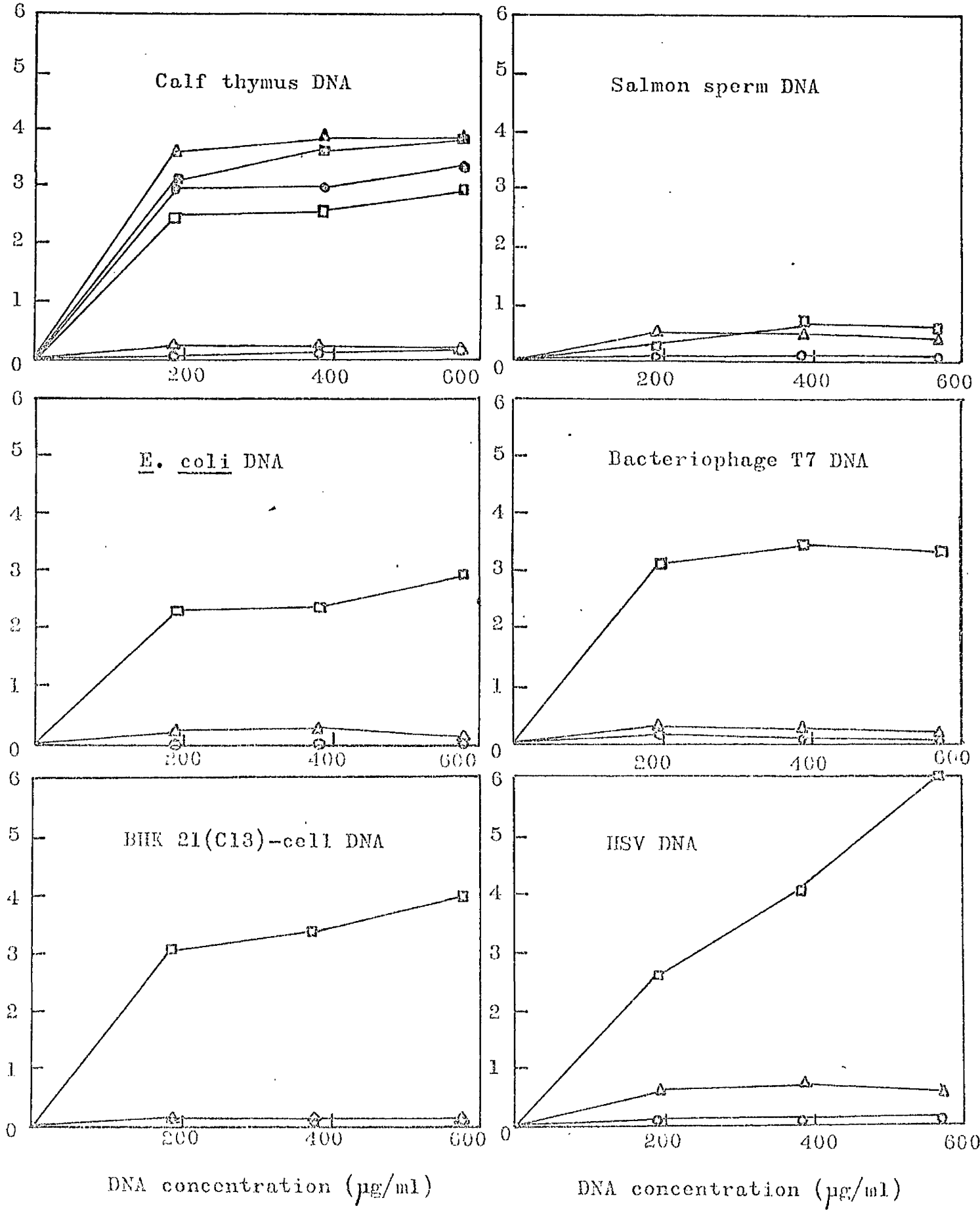
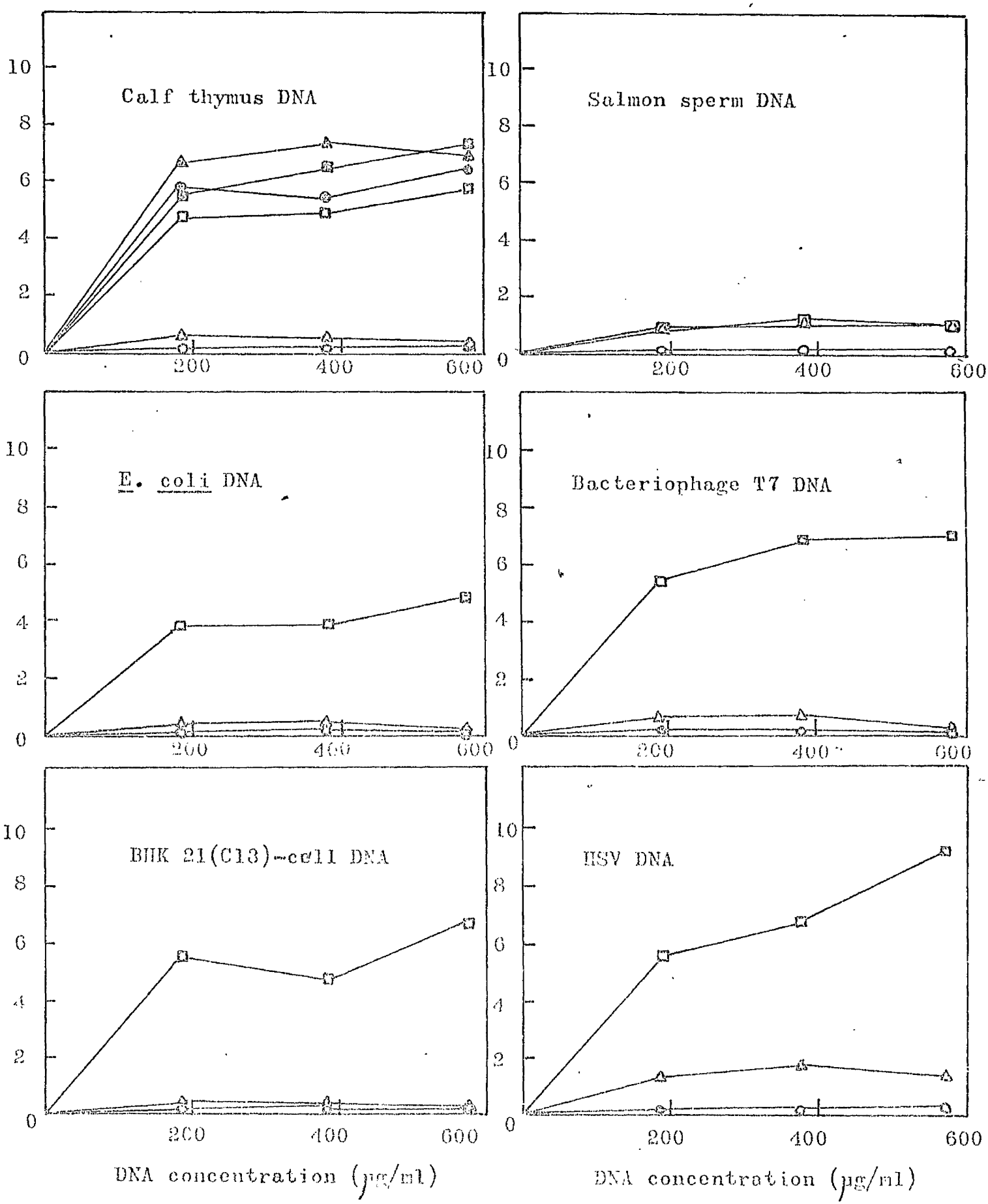

Calf thymus DNA
Salmon sperm DNA
E. coli DNA
Bacteriophage T7 DNA
BHK 21(C13)-cell DNA
HSV DNA
0
1
2
3
4
5
6
200
400
600
DNA concentration (µg/ml)
DNA concentration (µg/ml)

Fig. III. 37b.


Calf thymus DNA
Salmon sperm DNA
E. coli DNA
Bacteriophage T7 DNA
BHK 21(C13)-cell DNA
HSV DNA
0
2
4
6
8
10
200
400
600
DNA concentration (µg/ml)
DNA concentration (µg/ml)

This study was carried out to investigate whether the preference of the HSV-induced DNA polymerase for HSV DNA (Results section 4. 1. 8.) was connected with the lack of methylation of the viral DNA (Introduction section 3. 5.).

A series of half-scale assays containing 11µg of either non-methylated BHK21 (C13) cell DNA or BHK cell DNA which had been previously treated with methyl methane sulphonate (Strauss and Robbins, 1968) (kindly provided by Dr. M. Low) was set up and incubated for up to 8h (Methods section 2. 2. 1.).

The results are presented in Fig. III. 38, indicating that an increasing extent of chemical methylation is associated with a decrease in the priming activity of DNA. The non-methylated DNA which had not been treated in any way was less effective as a primer than the least methylated DNA.

It is possible therefore that some effect, such as single-strand "nicking", occurred in the DNA methylation process and was responsible for the effect on priming activity.

It is of interest that DNA synthesis with unmethylated (i.e. native) BHK21 DNA is linear for up to 4 hours then tends to flatten off towards 8 hours. In audition, no unprimed synthesis of polymers appeared to occur even after 8 hours' incubation.

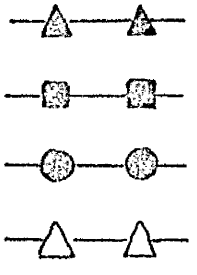
Fig. III. 38.

Effect of chemical methylation on BHK DNA as primer for the hydroxylapatite-purified HSV-induced DNA polymerase.

0.04ml aliquots of hydroxylapatite-purified DNA polymerase from the peak of the large-scale chromatography run shown in Fig. III. 13. were incubated in half-scale "high salt" polymerase conditions with 11µg aliquots of BHK21 (C13) cell DNA which were chemically methylated to a low, intermediate or high degree. A control (no DNA) and 11µg of BHK DNA, not chemically methylated, were also employed.

The incubation (37°C) was carried out for 8 hours and 0.03ml aliquots of enzyme removed at the times shown for plating and washing as in the standard polymerase assay (Methods section 2. 2. 1.). Controls showed no evidence of endogenous priming activity in the polymerase fraction and no non-specific binding of [^{3}H]dTTP to the DNA's used as primer.

The ordinate shows DNA polymerase activity in units of d.p.m. above background due to [^{3}H]dTMP incorporated into DNA/0.03ml aliquot of the assay mixture.



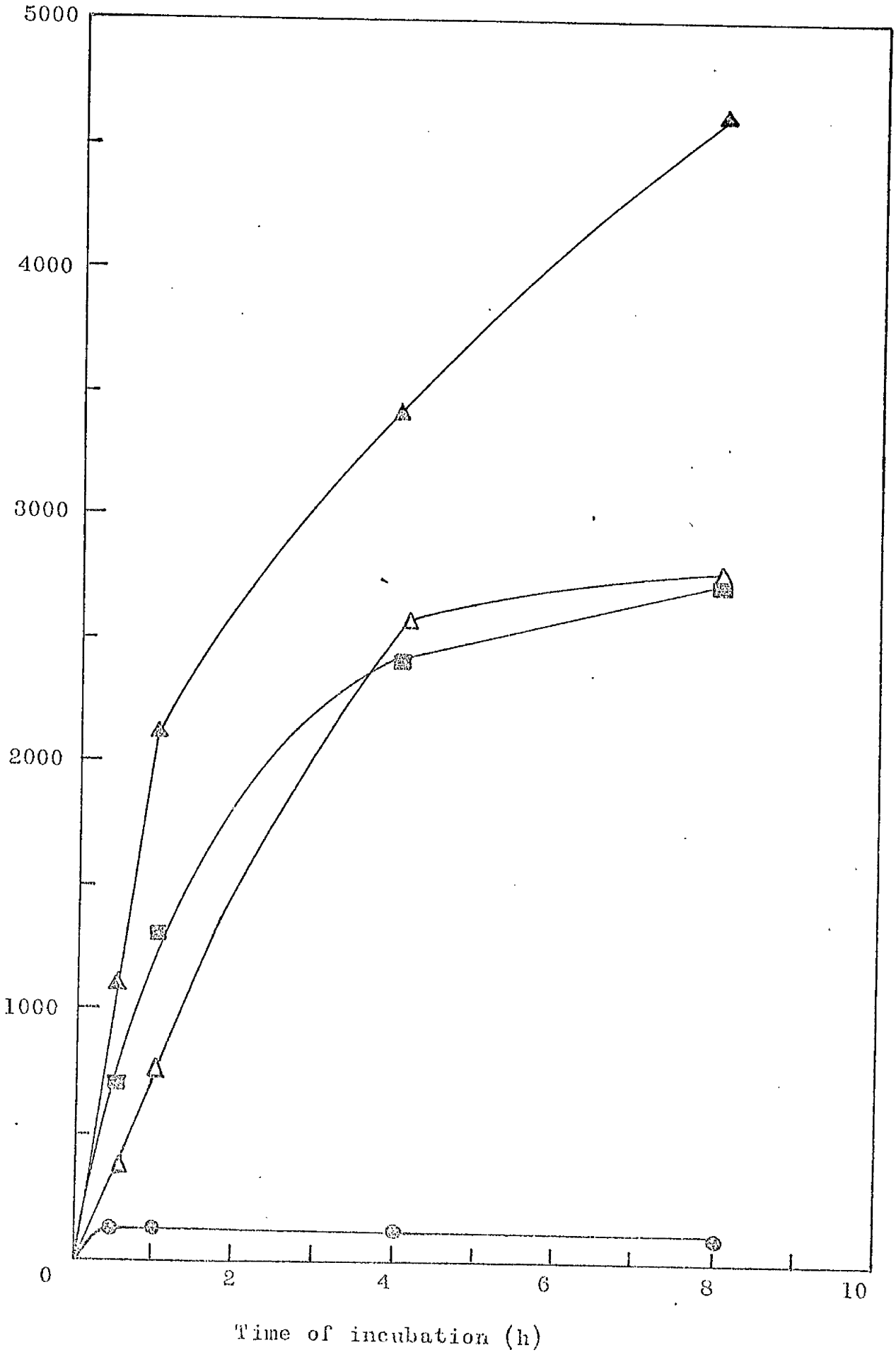
Least chemically-methylated DNA

Intermediately chemically-methylated DNA

Most chemically-methylated DNA

DNA not methylated chemically

Fig. III. 38.



4. 2. The effect of omitting one or three of the deoxyribonucleoside 5'-triphosphates in the DNA polymerase reaction with a hydroxylapatite-purified fraction of HSV-induced DNA polymerase (Methods section 2. 11. 1. early elution conditions).

The effects of using (1) dTTP as the sole triphosphate (2) dTTP, dCTP and dGTP but no dATP (3) all four triphosphates (4) All four triphosphates + added DNA were examined in "high salt" assays where components other than triphosphates and DNA were standard (Methods section 2. 2. 1.).

The results are given in Fig. III. 39. It is clear that the enzyme is significantly active only when all four deoxyribonucleoside 5'-triphosphates are present: the enzyme is thus, like the two species of host DNA polymerase from DEAE-cellulose, a replicative type of DNA polymerase. The results of the subsequent nearest-neighbour experiment (Results section 4. 5.) support this finding.

It will be noted that in this polymerase fraction (obtained using early elution conditions for hydroxylapatite) some endogenous priming activity was present.

4. 3. The extent of the DNA synthesis reaction.

The findings of the prolonged incubation utilised to study the effect of chemical methylation (Results section 4. 1. 9.)

Fig. III. 39.

The effect of omitting one or three deoxyribonucleoside 5'-triphosphates on a partially-purified preparation of HSV-induced DNA polymerase.

0.05ml aliquots of dialysed HSV-induced DNA polymerase partially purified by ammonium sulphate precipitation then hydroxylapatite column chromatography were incubated in a standard "high salt" DNA polymerase assay for 3 hours, 0.04ml samples being taken off, treated and counted at the intervals shown.

Endogenous DNA was known to be present in the polymerase fraction used as indicated by the assay for DNA and by the finding of DNA polymerase activity without added DNA. The effect of adding 60µg DNA is noted to be small so that there appears to be almost saturating levels of DNA primer present. The hydroxylapatite column buffer conditions were atypical, in this case similar to the early elution conditions (Methods section 2. 10. 1.) but differing in that 0.05M-phosphate was used for the wash and the gradient was between 0.05M and 0.15M-phosphate, the DNA polymerase coming off as a broad peak which was pooled and concentrated for use. The unusual conditions may account for the presence of endogenous DNA primer.

The triphosphates were at all times present at twice the standard polymerase assay concentrations.

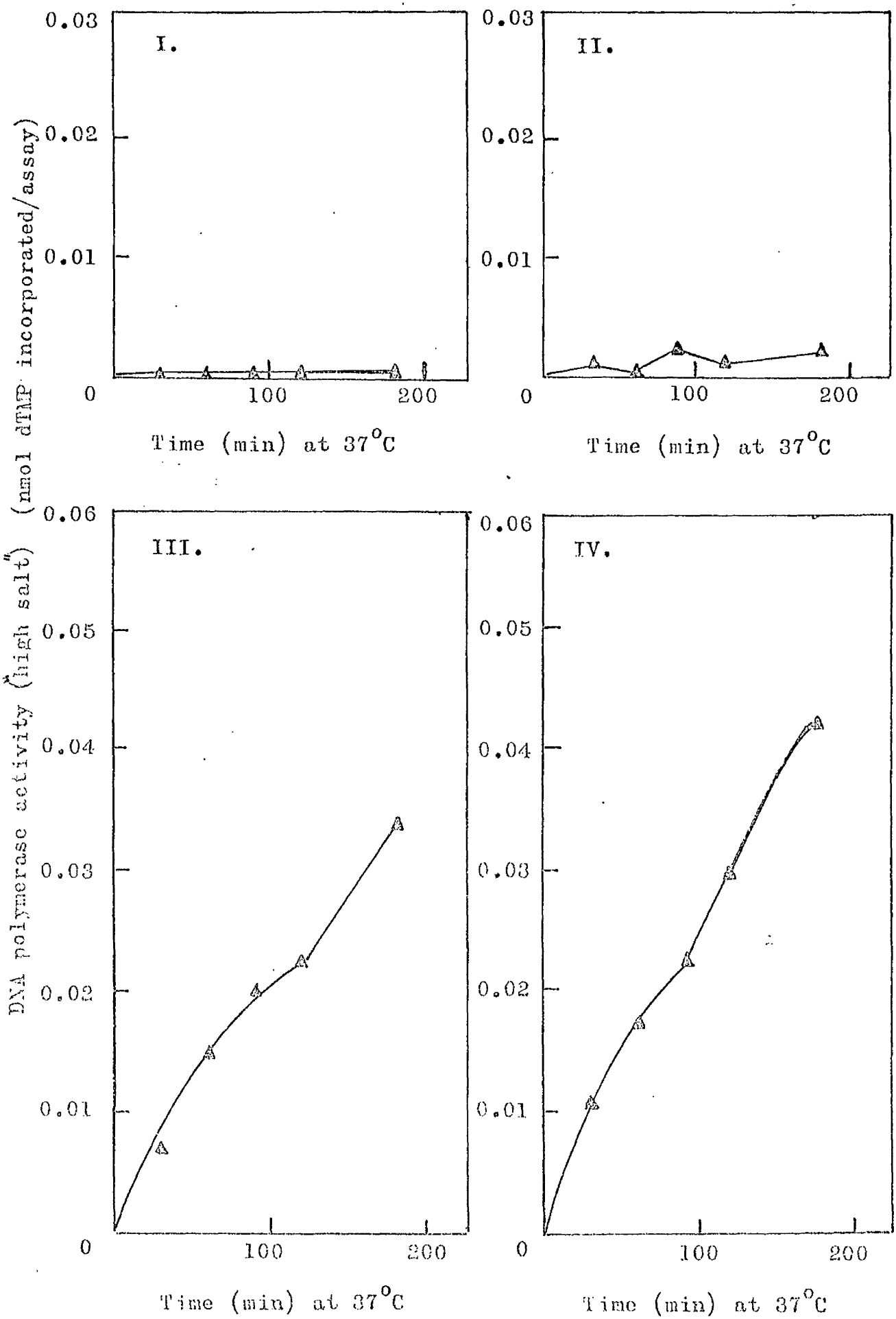
I dTTP the sole triphosphate, no extra DNA added.

II No dATP present but the other three triphosphates present - no extra DNA added.

III All four triphosphates present, no added DNA.

IV All four triphosphates present together with 60µg added calf thymus heat-denatured DNA primer.

Fig. III. 39.


I.
II.
III.
IV.
0.03
0.02
0.01
0
0.06
0.05
0.04
0.03
0.02
0.01
0
100
200
DNA polymerase activity ("high salt") (nmol dTMP incorporated/assay)
Time (min) at 37°C
Time (min) at 37°C
Time (min) at 37°C
Time (min) at 37°C

were confirmed using a hydroxylapatite-purified (early elution conditions) fraction of HSV-induced DNA polymerase (Methods section 2. 10. 1.).

The results of this experiment are shown in Fig. III. 40. The profile shows a linear incorporation up to at least 3 hours' incubation, a plateau between about 8 and 17 hours and a second rise in incorporation beyond 17 hours. This later rise almost certainly represents bacterial growth and incorporation of radioactive label into the acid-insoluble components of bacterial cells.

The overall results yield figures of up to 7% replication with activated HSV DNA in a 1h incubation and 8.3% replication in the same time with calf thymus DNA activated with 5x the usual concentrations of DNase I (Methods section 2. 3. 3.). The highest figure for native DNA appears to be 1.5% replication in 1 hour (native HSV DNA) and about 0.3% replication occurred in 1 hour with heat-denatured DNA (BHK DNA).

In the nearest-neighbour experiment (Results section 4. 5.) 0.25% replication occurred in a 2 hour incubation. If we assume synthesis to continue in a linear manner for up to 4 hours' incubation as suggested by the extent of synthesis experiments then up to 33% replication might be expected with activated DNA as primer.

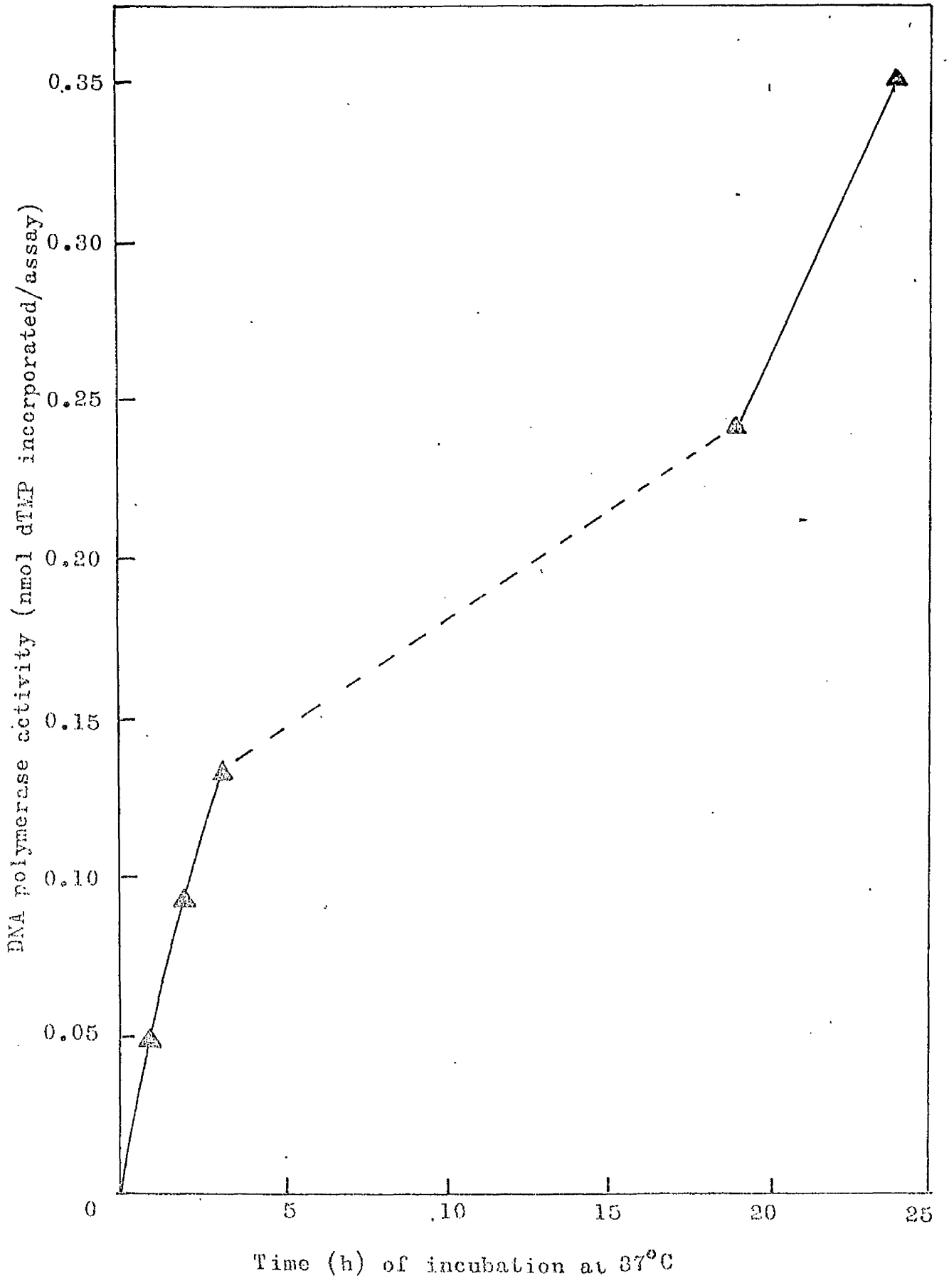
4. 4. Heat-stability of the HSV-induced DNA polymerase and DNA exonuclease.

Fig. III. 40.

Extent of synthesis experiment using partially purified HSV-induced DNA polymerase.

0.1ml aliquots of the pooled polymerase preparation described in the legend to Fig. III. 39. were incubated in 2mg/ml bovine serum albumin in otherwise standard DNA polymerase assays ("high salt"). 0.05ml aliquots of the reaction mixture were taken off at 1h, 2h, 3h, 19h and 24h and plated and washed as usual. The polymerase results are expressed as nmol dTMP incorporated/total assay (0.25ml).

Fig. III. 40.


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
5
10
15
20
25
DNA polymerase activity (nmol dTMP incorporated/assay)
Time (h) of incubation at 37°C

4. 4. 1. HSV-induced DNA polymerase and the polymerase-associated DNA exonuclease from hydroxylapatite chromatography.

To 0.05ml aliquots of the undialysed peak DNA polymerase fraction from the hydroxylapatite fractionation described in Results section 2. 2. 1. 1. (Fig. III. 11.), 0.05ml amounts of 4mg/ml bovine serum albumin were added and the tubes incubated for various times at 45°C (Methods section 2. 13.). Then either DNase with heat-denatured DNA as substrate (Methods section 2. 2. 3.) or "high-salt" DNA polymerase (Methods section 2. 2. 1.) was assayed with 30min incubations at 37°C. The results are shown in Fig. III. 41.

It can be seen that the temperature-sensitivities of the two enzymes are rather different and there is evidence of heat-activation of the DNA polymerase as found by Keir et al. (1966a).

A second experiment was designed in which the enzyme fractions were dialysed against DEAE-starter buffer and the results are presented in Fig. III. 42. It can be seen that the profiles are of the general form of those obtained earlier (Fig. III. 41.) and do not support the hypothesis that the DNA polymerase and DNase are inseparable activities of a single enzyme protein.

Fig. III. 41.

Heat-inactivation of HSV-induced DNA polymerase and its associated DNA exonuclease as eluted from a hydroxylapatite column fractionation:- early experiment without dialysis of the enzyme before inactivation studies began.

0.05ml aliquots of material from the peak fraction of the hydroxylapatite column chromatography run shown in Fig. III. 11. was taken without dialysis and incubated in the presence of an equal volume of 4mg/ml bovine serum albumin for various periods of time at 45°C then chilled and the reaction components for either DNA polymerase or DNase assays added and standard DNase and DNA polymerase ("high salt") assays carried out (Methods sections 2. 2. 3., 2. 2. 1., and 2. 13.).

100% DNA polymerase activity was equivalent to 207d.p.m. above a background of 9d.p.m. (ie. ≡ 0.15 units DNA polymerase).

100% DNase activity was equivalent to 35,455 counts/min above a background of 1,582 counts/min (ie. ≡ 2.2 units DNase).

—▲———▲— DNA polymerase activity

—○———○— DNase activity

Fig. III. 41.

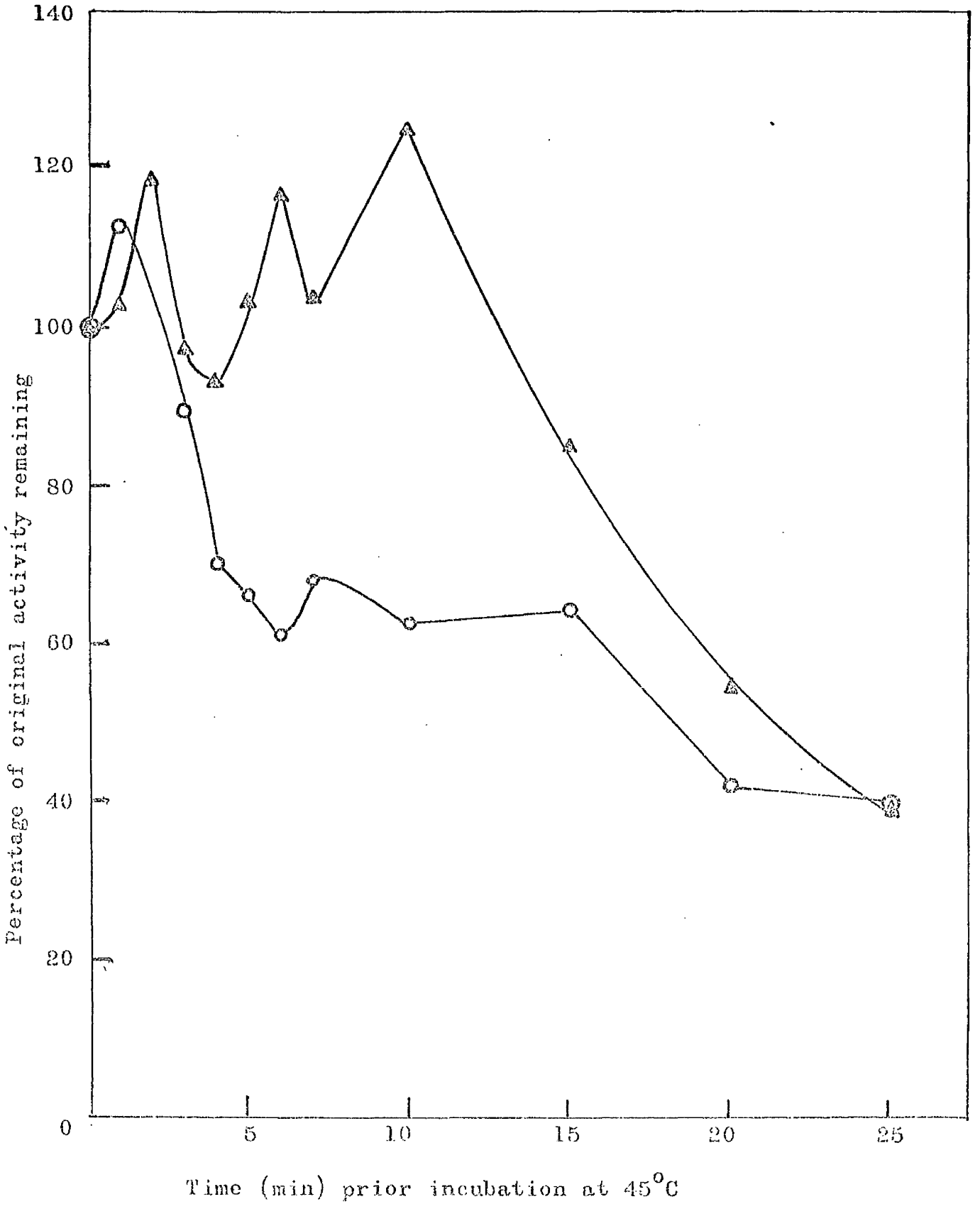

140
120
100
80
60
40
20
0
Percentage of original activity remaining
5
10
15
20
25
Time (min) prior incubation at 45°C

Fig. III. 42.

Heat-inactivation of HSV-induced DNA polymerase and its associated DNA exonuclease as eluted from a hydroxylapatite fractionation:- later experiment with dialysis of the enzyme into DEAE - column starter buffer before inactivation studies began.

0.05ml aliquots of pooled and concentrated polymerase and DNA exonuclease material from the associated peak fractions from a hydroxylapatite column run, were (after dialysis against DEAE-column starter buffer (Methods section 2. 10. 2.)) prior incubated for various times at 45°C in the presence of an equal volume of 4mg/ml bovine serum albumin then chilled and assayed for DNase or DNA polymerase ("high salt") in standard assays.

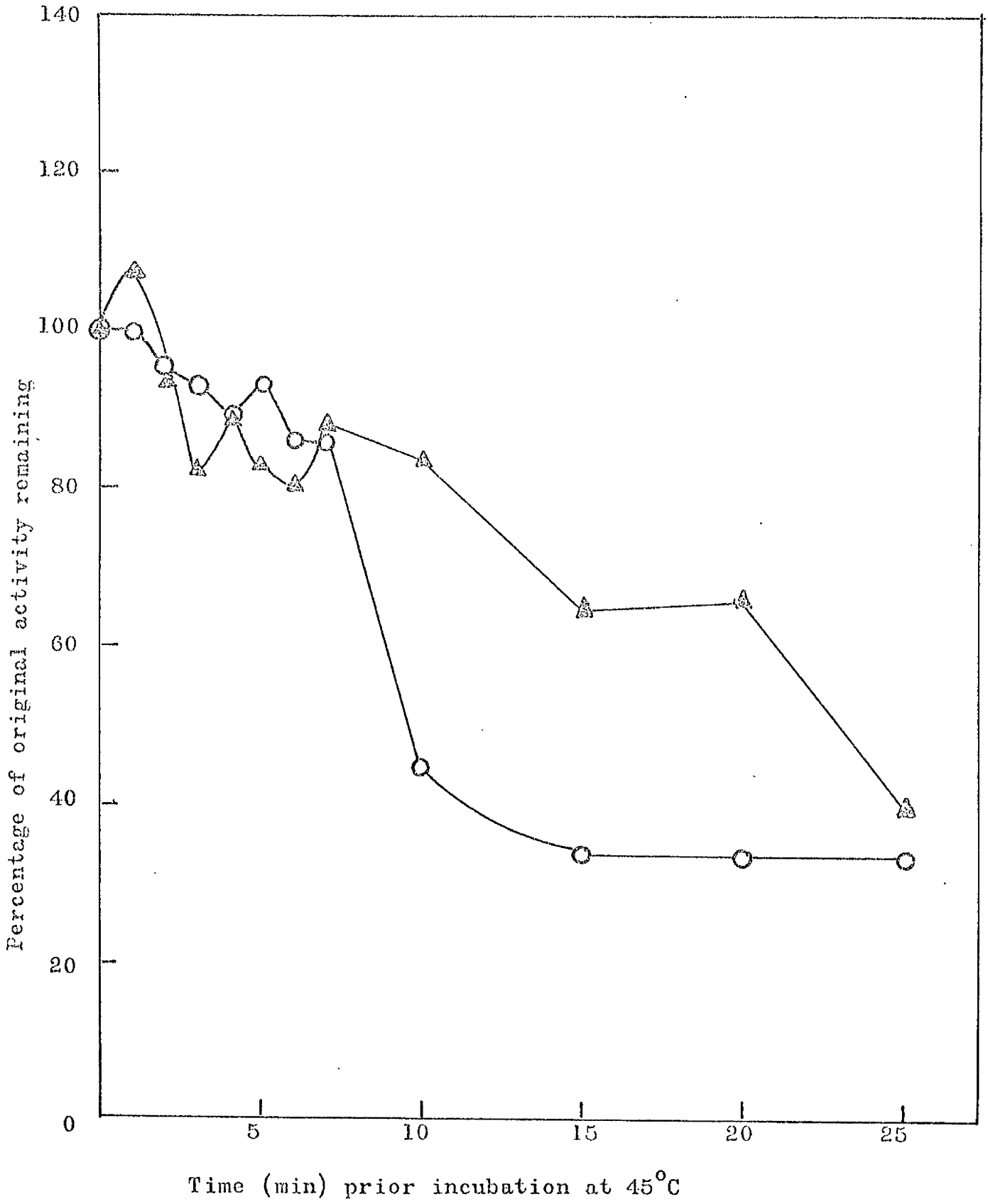
100% DNA polymerase activity was 130d.p.m. above a background of 56d.p.m. (ie. ≡ 0.017 units DNA polymerase/assay).

100% DNase activity was 310 counts/min above a background of 177c.p.m. (ie. ≡ 0.50 units DNase/assay).

—Δ——Δ— DNA polymerase

—○——○— DNase activity

Fig. III. 42.


140
120
100
80
60
40
20
0
Percentage of original activity remaining
5
10
15
20
25
Time (min) prior incubation at 45°C

4. 4. 2. Heat-stability of hydroxylapatite-purified HSV-induced DNA polymerase-associated DNA exonuclease in the presence of potential protecting agents.

In this experiment, the effects of a range of different agents including RNA, DNA, bovine serum albumin, 30% and 50% (^{v}/v) glycerol, polyethylene glycol, dTTP, dTMP and deoxythymidine 3', 5'-diphosphate were tested by incubating enzyme at 45°C in their presence (Methods section 2. 13.) before a standard DNase assay (Methods section 2. 2. 3.).

The results of this study are displayed in Fig. III. 43. A wide range of effects was observed but it was noticed that glycerol at 30% or 50% (^{v}/v) was the most satisfactory protecting agent.

4. 4. 3. Storage of the HSV-induced DNA polymerase and the associated DNase.

It was found that virus-induced DNA polymerase activity from a large-scale hydroxylapatite purification lost no activity in 30% glycerol at -70°C for 11 weeks: in fact, an increase in activity of 80% was found. Storage for 1 month at 0°C or -20°C also seemed to be satisfactory with various kinds of extracts (some stimulation of activity occurred) provided that glycerol was present to 30 or 50% (^{v}/v).

As already indicated (Results section 1. 1. 3.), storage

Fig. III. 43.

Effect of protecting agents on the heat-inactivation of the HSV-induced DNA polymerase-associated DNA exonuclease (hydroxylapatite purified enzyme).

0.05ml aliquots of hydroxylapatite-purified DNA exonuclease dialysed against "DEAE-starter buffer" were incubated with an equal volume of the various protecting agents, shown below, for various times at 45°C then chilled and assayed in a standard DNase assay. The DNase activity is expressed in counts/min above background (272) per 0.6ml of the standard DNase assay supernatant (Methods section 2. 2. 3.) (Incubation was for 1h however at 37°C). The agents and their concentrations during the prior incubation at 45°C are shown below. All agents were in DEAE-starter buffer.

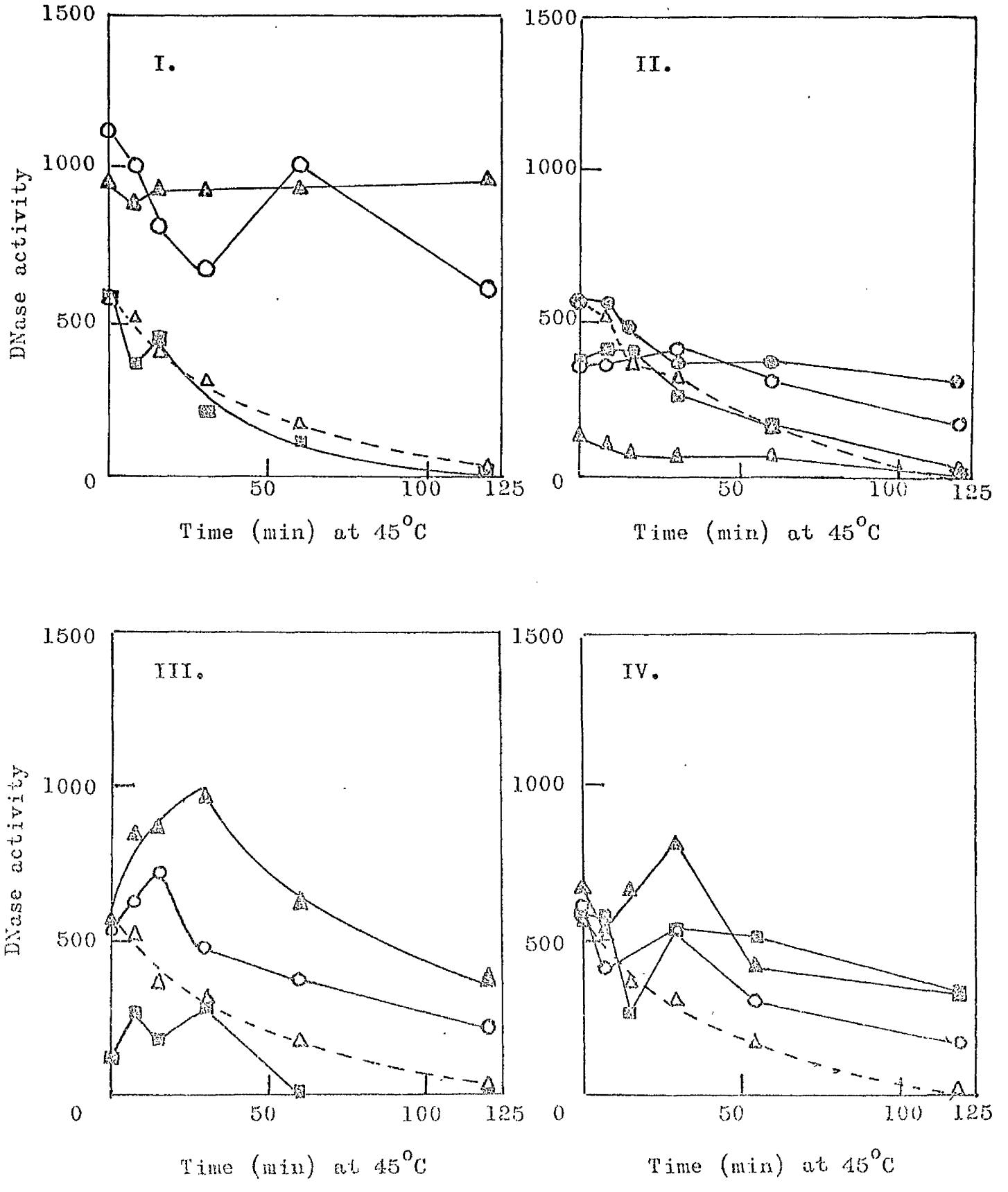
I. —△—△— 50% (v/v) glycerol
—○—○— 30% (v/v) glycerol
—▣—▣— 30% (v/v) polyethylene glycol
-△- -△— Control (DEAE-starter buffer)

II. —△—△— DEAE-starter buffer + 0.10M-KCl
—○—○— 0.10M-tris-HCl(pH8.0)-1mM-EDTA-10mM-2-mercaptoethanol
—▣—▣— 0.15M-tris-HCl(pH8.0)-1mM-EDTA-10mM-2-mercaptoethanol
—◉—◉— DEAE-starter buffer + 8mM-$MgCl_2$
-△- -△- Control (DEAE-starter buffer)

III. —△—△— Bovine serum albumin (1mg/ml)
—○—○— RNA (200µg/ml)
—▣—▣— DNA (200µg/ml)
-△- -△— Control (DEAE-starter Buffer)

IV. —△—△— dTTP (0.2mM)
—○—○— dTMP (0.2mM)
—▣—▣— dTDP (3',5')(0.2mM)
—△- -△— Control (DEAE-starter buffer)

Fig. III. 43.


I.
II.
III.
IV.
DNase activity
1500
1000
500
0
50
100
125
Time (min) at 45°C

of whole-cell extracts without added glycerol is unsatisfactory at 0°C, -20°C and -70°C.

4. 5. Partial nearest-neighbour sequence analysis of the DNA synthesized by hydroxylapatite-purified HSV-induced DNA polymerase.

This analysis was carried out using HSV and calf thymus DNA as primers as described in Methods section 2. 14.

The results are presented in Table III. 6.

It is of interest to compare these results for HSV DNA with those of Subak-Sharpe et al. (1966b) and those of Mr. G. Russell (personal communication) using E. coli DNA polymerase I. The analagous results of Josse et al. (1961) with calf thymus DNA are also presented for comparison.

The results suggest that the HSV-induced DNA polymerase is a replicative enzyme which copies its homologous DNA and other DNA's faithfully in an in vitro system. The findings also support the hypothesis that the virus-induced DNA polymerase functions in the replication of HSV DNA.

Table III. 6.

Partial Nearest-neighbour sequence analysis on HSV- and calf thymus DNA using hydroxylapatite-purified HSV-induced DNA polymerase instead of *E. coli* DNA polymerase I.

I. Results with HSV DNA using [α-^{32}P] dCTP as labelled precursor.

Sequence	Result of present study(tube I)	Result of present study(tube II - 1st sample	Result of present study(tube II - 2nd sample	Present study range of results	Results of Subak-Sharpe *et al.*(1966b) (*E. coli* DNA polymerase)	Results of G. Russell (*E. coli* DNA polymerase)	Least range between present and other studies
ApC	0.207	0.214	0.212	0.007	0.184	0.158 (0.156-0.160)	0.023
TpC	0.213	0.216	0.216	0.003	0.156	0.171 (0.170-0.171)	0.042
GpC	0.292	0.289	0.286	0.006	0.343	0.314 (0.311-0.317)	0.019
CpC	0.287	0.280	0.286	0.007	0.317	0.357 (0.356-0.358)	0.030

II. Results with calf thymus DNA using [α-^{32}P] dCTP as labelled precursor.

Sequence	Results of present study (frequency expressed as a part of unity)	Results of Josse *et al.* (1961)(frequency expressed as a part of unity)	Difference between the two
ApC	0.2747	0.2396	0.0351
TpC	0.3301	0.3088	0.0213
GpC	0.1840	0.2028	0.0188
CpC	0.2113	0.2488	0.0375

CHAPTER IV - DISCUSSION.

1. Purification of the HSV-induced DNA polymerase.

1. 1. DEAE-cellulose chromatography.

Several conclusions may be drawn from the result of chromatography on DEAE-cellulose.

(i) A clear difference is seen between both host-cell activities and the single HSV-induced activity in their chromatographic behaviour on DEAE-cellulose. Thus we may add this to the list of differences between host-cell and virus-induced DNA polymerases.

(ii) The host-cell DNA endonuclease chromatographs quite distinctly from the virus-induced DNA-exonuclease activity which co-chromatographs with the virus-induced DNA polymerase. Some experiments, however, showed only partial coincidence between exonuclease and polymerase and this suggests that at least part, and perhaps all, of the exonuclease and polymerase activities may represent separate proteins.

(iii) The separation of nucleic acids from fractions of virus-induced DNA polymerase (Results section 2. 5. 1. 3.) possibly accounts for the instability of the enzyme which follows DEAE-cellulose column chromatography, for it has been shown that the virus-induced DNA polymerase is more heat-stable in the presence of DNA than in its absence (Keir, 1968: Morrison and Keir, 1968a).

(iv) Essentially no host-cell DNA-polymerase activity could be detected during DEAE-cellulose fractionation of extracts of infected cells. Mutual inhibition or stimulation of DNA polymerase between extracts of uninfected and virus-infected cells had not been revealed by mixing experiments but application of mixed control and infected cell extracts to DEAE-cellulose resulted in only about 5% of the expected recovery of host DNA polymerase in the position of elution characteristic of uninfected cell activities. At this stage, this finding cannot be explained but further investigation seems worth while.

Lack of host DNA polymerase in infected cells may be caused by decay of the activity in the absence of its synthesis (Keir and Gold, 1963).

1. 2. Hydroxylapatite column chromatography.

Hydroxylapatite separates proteins which may be of similar size, shape and even net charge, on the basis of the carboxyl group and/or phosphoryl group content of the proteins' surfaces. Inorganic phosphate acts as the competing eluting ion (Bernardi and Kawasaki, 1968). Furthermore, under the conditions of adsorption and elution used in the chromatography of extracts of host-cells and virus-infected cells, macromolecular nucleic acids should be completely removed from the enzyme activities (Bernardi, 1965; Bernardi, 1969a,b,c). The absence of endogenous priming activity from the virus-induced polymerase

(Results section 4. 1. 8.) confirms this.

The DNA polymerases of L cells (R.L.P. Adams and K. Evans, personal communication) and Landschütz ascites tumour cells (Shepherd and Keir, 1966) elute from hydroxylapatite above 100mM-phosphate: the BHK enzyme appears to behave similarly (Results section 2. 2. 2.).

Thus it is clear that the host and virus-induced enzymes have once more been shown to be chromatographically distinct.

Hydroxylapatite chromatography is a useful purification technique for virus-induced DNA polymerase. It separates nucleic acid, host-cell DNA polymerase and DNA endonuclease from the enzyme and gives 200-fold purification of the induced polymerase when activated DNA is used as template (Results section 2. 2. 1. 3.).

Host DNA-endonuclease activity appears to be distinct from the two peaks of DNase which are eluted during chromatography of extracts of HSV-infected cells. The components of this double peak represent virus-induced DNase activity in the form of either one or two DNA exonucleases and, since only one of the peaks is clearly associated with DNA polymerase, this fractionation confirms the presence in infected cells of a DNA exonuclease separable from DNA polymerase. The fact that each of the two DNA-exonuclease peaks re-chromatographs on hydroxylapatite largely in its original position supports the concept that these peaks represent distinct enzymes.

1. 3. Constant velocity sucrose gradient zone sedimentation.

This technique separates proteins on the basis of their size, shape and density. The latter two variables are assumed to be constant in the design of the gradients: The shape is assumed to be spherical and the density assumed to be 1.4g/ml. These assumptions are valid for most globular proteins so that fractionation occurs on the basis of molecular weight.

From the results, the following conclusions may be drawn.

(i) In all cases, there was evidence for association between virus-induced DNA polymerase and DNase whether the enzymes were present in whole-cell extracts or in hydroxylapatite-purified fractions. In each of the three concentrations of tris-HCl buffer used in the gradients, this firm association was observed. The implication is that the polymerase and DNase either have the same size and shape or that they represent two functions of a single protein.

(ii) There was evidence in whole-cell extracts of HSV-infected cells for the presence of host-cell DNA endonuclease and two peaks of DNA exonuclease distributed over the gradient.

(iii) In the sedimentation of the hydroxylapatite-purified virus-induced polymerase, only polymerase-associated DNase could be detected.

(iv) $S_{20,w}$ values estimated for the virus-induced DNA polymerase

ranged from 3.5 - 9.3S, from which molecular weight values were estimated to be 46,000 - 210,000. This is the range into which the molecular weight values of the well-characterised DNA polymerases fall (Introduction section 1. 3. 3.).

(v) The apparent $s_{20,w}$ value of the virus-induced DNA polymerase rises with decreasing buffer concentration possibly owing to protein-protein interaction as discussed earlier (Results section 2. 4. 3.). Alternatively, the low $s_{20,w}$ values for the virus-induced DNA polymerase might represent some kind of artifact, perhaps owing to interaction among the components of the gradient. In this connection, Andrews (1965) quotes the case of caeruloplasmin which he could not study using the routine tris buffer in his gel-filtration work. He had to use KCl alone since Kasper and Deutsch (1963) had reported changes in the colour and sedimentation behaviour of this protein in the presence of tris. Such possibilities of artifact must be investigated in further controlled experiments.

(vi) The sedimentation behaviour in 100mM-tris of the hydroxylapatite-purified virus-induced polymerase-free DNA exonuclease (Results section 2. 4. 3., Fig. III. 21.) is almost identical to that of the DNA polymerase and polymerase-associated DNA exonuclease (Fig. III. 20.). This finding does not appear to fit the earlier data (Fig. III. 18. with a whole-cell extract at 50mM tris, or Fig. III. 19. with hydroxylapatite-purified virus-induced DNA polymerase (early elution conditions at 10mM-tris),

and even if we consider the possibility of association - dissociation between the DNA polymerase and DNA exonuclease we should not predict an identical pattern for the polymerase-free and polymerase-associated exonucleases. The possibility that the haemoglobin or alcohol dehydrogenase standards contained contaminating DNase activity was eliminated but interference by the artifacts already discussed (section (v)) must be considered: however, the similar sedimentation patterns of the two DNA exonucleases suggests that they may be identical and that they at least have the same size and shape. If, however, there is only one virus-induced DNA exonuclease, which exists partly associated with DNA polymerase, then we should have expected that the association with DNA polymerase would have increased the size of the exonuclease and that the enzyme would not then give a sedimentation pattern identical to that of the unassociated enzyme.

1. 4. Sephadex G-150 chromatography.

The results from this technique, which fractionates on the basis of differences in molecular size and shape, indicate the following.

(i) That the bulk of the virus-induced DNase and DNA polymerase elute together;

(ii) That the host DNA polymerase is fractionated as two peaks of activity with different template specificities: the first peak shows high activity with heat-denatured DNA as primer while the

the second strongly prefers activated DNA. Small amounts of DNase accompany the two host-cell polymerase peaks;

(iii) A second peak of DNA exonuclease in gel filtration with unfractionated extracts of HSV-infected cells, which is separated from DNA polymerase and appears to be of lower molecular weight than the polymerase-associated DNase.

(iv) That the molecular weight of the virus-induced DNA polymerase and its associated DNase seems to be very large - much greater than suggested by the sedimentation studies - and always greater in size than yeast alcohol dehydrogenase (150,000 daltons) strongly suggesting the occurrence of aggregation, either protein-protein or protein-nucleic acid or both. The last possibility is supported by an experiment (Fig. III. 25.) which showed some retardation of polymerase after DEAE-cellulose treatment. Even then, the estimated size is greater than that obtained from sedimentation studies.

In summary, there is clearly contradiction between the sedimentation and gel-filtration results. The lower apparent molecular weights given by the former technique are more reliable due to probable aggregations occurring during gel-filtration.

1. 5. Miscellaneous other fractionation studies.

(a) Phosphocellulose column chromatography.

The essential conclusions which emerged from the studies using phosphocellulose were (i) that DNA should be removed

prior to the use of phosphocellulose as a purification technique and (ii) that the DNA polymerase and DNase of infected cells still seemed to be associated in this medium, although the peaks, as in DEAE-cellulose column chromatography, were not quite coincident.

The possibility still exists that other pH values and concentrations of buffers might give satisfactory results in the absence of DNA.

(b) Polyacrylamide gel electrophoresis.

Although studies with this technique were not extensive, several conclusions emerged.

(i) Again, there was evidence for association of DNA polymerase and DNase in electrophoresis of both unfractionated and hydroxylapatite-purified extracts of DNA polymerase and DNase from infected cells.

(ii) The enzymes seemed to have a low mobility compared with most of the protein of the sample, the enzymes being localised in the first 0-2cm of the 7cm gel during a normal fractionation: again aggregation might explain this finding.

(iii) The purest enzyme preparations still showed several protein bands after electrophoresis, although these bands were fewer in number than those from whole-cell extracts. No single protein band could be unambiguously associated or identified with the polymerase activity.

(iv) DNase activity was more efficiently recovered than was polymerase.

The technique promises to be a useful one for testing the purity or homogeneity of polymerase fractions with respect to protein at various stages in purification.

1. 2. General discussion.

The results of the purification studies suggest that the virus-induced DNA polymerase exists in association with virus-induced DNA-exonuclease activity, and that there may be two rather than one virus-induced DNase activity. The association of DNA polymerase and exonuclease seems to survive five separate fractionation methods and various combinations thereof.

Since the fractionation techniques separate proteins on the basis of a wide number of variable aspects of protein structure, it seems most unlikely that co-fractionation would occur with so many techniques if there were not a firm association of the two activities involved, either as a very tightly associated pair of proteins or as a single protein with two enzymic activities.

The evidence for two virus-induced DNA exonucleases in HSV-infected cells comes from hydroxylapatite column chromatography, from Sephadex G-150 gel-filtration studies and also from sucrose gradient zone sedimentation of a whole-cell extract of HSV-infected cells. The latter two techniques provide rather less certain evidence than the first.

Four main hypotheses exist to explain the available evidence .

(I) HSV-infected cells contain a virus-induced DNA polymerase - DNA exonuclease protein together with a further polymerase-free DNA exonuclease.

(II) Herpes-infected cells contain a virus-induced DNA polymerase and a virus-induced DNA exonuclease both of which are separate and distinct proteins which tend to associate. There may be an *in vivo* significance in the association. The virus-induced DNase must exist in excess of the DNA polymerase and so two DNA exonucleases are seen: one free, the other polymerase-associated. Probably polymerase is inactive in the absence of exonuclease.

(III) HSV-infected cells contain a single virus-induced DNA polymerase - DNA exonuclease enzyme protein with both activities residing in the same protein molecule and, in the course of enzyme extraction, lysosomal or other proteases cleave the enzyme to produce free DNA exonuclease and DNA polymerase with residual exonuclease. From this hypothesis we should expect to find two peaks of DNA polymerase after fractionation. This is not found, and the lack of a definite effect of the protease inhibitor PMSF (although this agent may not be effective in this system) coupled with the constant finding of the polymerase-free exonuclease in all later work in the same sort of proportions to the polymerase-associated enzyme, suggests that proteolysis is unlikely to be the explanation for the findings and that one of the earlier hypotheses is more likely to be the correct one. Nevertheless, the possibility of a proteolytic origin for the two virus-induced nuclease peaks cannot be excluded

by our negative findings at the present stage.

(IV) There is one virus-induced DNA polymerase and one virus-induced DNA exonuclease and the DNA exonuclease forms dimers or other oligomers which give the appearance of co-chromatography with the virus-induced DNA polymerase, although there is no association with the polymerase. This hypothesis is very similar to hypothesis (II), but much less likely than (II) in view of the large number of variable properties of the proteins already discussed which would have to concur between the enzymes for their co-fractionation in so many techniques.

In view of these considerations, it seems that we should be justified in considering further only the two first-mentioned hypotheses (although (III) is still a possibility).

The evidence in favour of hypothesis (I).

(1) Purification studies, often involving sequential fractionation techniques, largely support the existence of a firm DNA polymerase-DNase association often giving constant ratios of activity over peaks of the associated enzymes (Paton and Morrison, 1969).

(2) A number of lines of evidence (see above) indicate the existence of a DNA-exonuclease activity fractionating free from DNA polymerase.

(3) Characterisation of the DNase activities from the two DNA exonuclease peaks from hydroxylapatite, although they show the enzymes to be very similar in many respects reveal some differences between the two activities. (i) Their heat stability behaviour

at 45°C although broadly similar shows slightly different patterns - especially a heat-stimulation effect for only the polymerase-associated exonuclease. (ii) Re-chromatography of the two activities on hydroxylapatite shows that the enzymes behave largely as before, i.e. their distinct chromatographic behaviour is confirmed.

(4) Studies on the heat-stabilisation of the virus-induced DNA polymerase-associated DNA exonuclease from hydroxylapatite show that dTTP, dTMP and thymidine 3', 5'-diphosphate all protect the DNase activity although there is neither significant stimulation nor inhibition of the DNase by these nucleotides. The lack of stimulation or inhibition by deoxyribonucleoside triphosphates was also shown by Morrison and Keir (1968b) and they considered their findings to represent a suggestion of the non-identity of the DNA polymerase and DNA exonuclease by analogy with the T4 phage DNA polymerase-exonuclease (Goulian et al., 1968). This showed strong inhibition of the exonuclease by the triphosphates, while the E. coli DNA polymerase I showed stimulation of the associated exonuclease II activity by triphosphates. The heat-stabilisation effect of the nucleotides, however, might be regarded as supporting the view that the two activities in the HSV system reside in a single protein molecule.

There is support for the hypothesis too from the literature in that several examples of association of DNA polymerase and DNA exonuclease have been described and in some cases both

activities have been clearly shown to reside in a single protein molecule (Introduction section 1. 3. 3.).

In addition, the two T-even bacteriophages T2 and T4 both appear to induce the synthesis of two DNA exonucleases with very similar properties: one is DNA polymerase-associated, the other is polymerase-free and both are optimally active with oligonucleotides as substrate. The two T-even phage DNases are separable on DEAE-cellulose columns (Short and Koerner, 1965).

The evidence for hypothesis (II).

(1) In an overall survey of the purification studies, occasional instances were found of poor coincidence between DNA polymerase and DNA exonuclease with variable ratios of polymerase/DNase over the peaks. DEAE-cellulose column chromatography appears to give an incomplete coincidence of the two activities, the peaks being regularly a few fractions apart: this might be due to the existence of two species of DNA exonuclease in the single DNase peak although the symmetry of the DNase peak argues against this.

Even with the hydroxylapatite second peak of polymerase and DNA exonuclease, poor coincidence was sometimes found (Fig. III. 14).

(2) Heat-stability studies by Morrison and Keir (1968a) on the virus-induced DNase purified by DEAE-cellulose chromatography suggested that a single DNase was present in this peak as the heat-stability curve was unimodal.

(3) Early work on the induction of DNA polymerase and DNase

synthesis suggested that the initiation of synthesis of the virus-induced DNA polymerase occurred before that of the virus-induced DNase (Russell et al., 1964; Keir et al., 1966a), and this fact was utilised for optimal extraction of DNA polymerase with minimal levels of DNase (Introduction section 2. 4. 3.).

(4) Studies with actinomycin D confirmed the different times of initiation of synthesis of the two enzymes (Morrison and Keir, 1968a).

Belief in the existence of two separate DNA exonucleases would imply that the findings described in (3) and (4) reflect composite or combined curves for the two exonucleases appearing as one. There is, however, no evidence of bimodality in the actinomycin D inhibition or normal induction curves, suggesting that there is only one induced DNA exonuclease.

(5) Subcellular fractionation experiments (Morrison and Keir, 1968a) suggest a different intracellular distribution for the virus-induced DNA polymerase and DNase, the DNase having a mainly cytoplasmic distribution and the DNA polymerase a mainly nuclear one, although such studies must be affected by existing host-cell DNase activities.

(6) Comparative studies between the two DNase activities from the two peaks of DNA exonuclease from hydroxylapatite show that the two enzyme activities are very similar in most respects.

(i) Their mode of action appears to be similar: both enzymes are exonucleases producing deoxyribonucleoside 5'-monophosphates.

(ii) Morrison and Keir (1968b) concluded that the DEAE-cellulose-purified virus-induced DNA exonuclease(s) attacked predominantly from a 3'-OH group liberating deoxyribonucleoside 5'-monophosphates. Since this peak of DNase from DEAE-cellulose would contain both virus-induced DNA exonucleases, if there are indeed two such enzymes, both enzymes would have the same direction of attack. At the present stage such studies are not completely conclusive in view of the analogous studies on the DNase activities associated with DNA polymerase I of E. coli.

(iii) Comparison of the heat-stability profiles of the two activities shows that they are closely similar with only one point of difference: a brief stimulation in the case of the polymerase-associated exonuclease.

(iv) The substrate specificities of the two activities are almost identical. In both cases, heat-denatured DNA is marginally preferred to native DNA and partly degraded (by pancreatic DNase I) DNA is favoured by both activities: the greater the degree of prior digestion the more active the exonuclease activities appear to be.

(v) The $s_{20,w}$ values of the two activities from hydroxylapatite sedimented in 0.10M-tris-HCl are the same and the enzyme-activity profiles virtually identical in this system.

(7) The heat-stability studies comparing the virus-induced DNA polymerase and its associated DNase from hydroxylapatite column chromatography of extracts of HSV-infected cells show definite

differences between the DNA polymerase and DNase heat-inactivation profiles at 45°C. The differences suggest, but do not prove, that the two enzyme activities reside in different proteins: it is possible that on a single protein, one active site is more heat-labile than the other.

(8) Finally, DNase appears to be more efficiently recovered from many of the fractionation techniques than is DNA polymerase and this tends to suggest that polymerase and DNase are separate molecules. The association of polymerase and exonuclease observed in the fractionation experiments may be partly physiological, reflecting the in vivo state and partly due to a fortuitous association of both with DNA, although association has been observed in cases where DNA has been removed. Thus the results obtained with the herpes-induced DNA polymerase may be the reflection of an interaction of virus-induced DNA polymerase and DNA exonuclease.

Incontrovertible proof of the association of the HSV-induced DNA polymerase and DNA exonuclease in a single protein molecule rests upon further purification of the enzymes to homogeneity.

Consideration of the evidence for the two hypotheses leads us to conclude that there is probably only one HSV-induced DNA exonuclease and that this associates at least partly with the HSV-induced DNA polymerase i.e. that hypothesis II is the correct one. If this is so, it should be possible on further purification to separate virus-induced DNA polymerase and DNA exonuclease.

3. Characterisation of the HSV-induced DNA polymerase, its associated DNase and host-cell DNA polymerase.

3. 1. The effects of salts upon the DNA polymerase activity of extracts of uninfected and HSV-infected cells.

The effects of these buffers and salts are probably attributable to a single factor - the monovalent cation concentration whether it be K^+, NH_4^+ or tris-H^+.

The potassium phosphate and tris-HCl profiles both show a rise to a sharp optimum and a steep fall beyond this; the KCl curves show a broader optimum but again a steep fall beyond this. The reason for the different responses of the host and virus-induced enzymes to high concentrations of salts is unknown: but mammalian nuclear DNA polymerases generally are inhibited by high salt (above 60mM) (Keir 1965).

It is of interest to note that rat liver mitochondrial DNA polymerase is stimulated by high salt concentration (0.15-0.20M) (Meyer and Simpson) as are also the DNA polymerases of M. luteus (Litman, 1968) and bacteriophage T-5 (Steuart, Anand and Bessman, 1968a): this may be a reflection on a common ancestry of these organisms and mitochondria (Watson, 1970). Herpes simplex virus has, on the basis of nearest-neighbour analysis results been postulated to be evolved from bacteria: the fact that the HSV-induced DNA polymerase is also stimulated by high salt concentration would tend to support this hypothesis.

3. 2. Priming studies.

3. 2. 1. The virus-induced DNA polymerase.

Purification of the virus-induced DNA polymerase gives rise to a marked change in its priming specificity.

In whole-cell extracts, activated DNA is slightly less effective than denatured DNA, and native DNA is only half as effective as denatured DNA. Furthermore, native DNA appears to saturate the enzyme at considerably lower concentrations than denatured DNA.

With soluble nuclear fractions, these effects are again noted but a higher level of denatured DNA is required for saturation.

With soluble cytoplasmic fractions the results are similar to those obtained with whole-cell extracts.

With DEAE-cellulose-purified virus-induced DNA polymerase, at both 108mM and 188mM-KCl, activated DNA is approximately 3 and 5 times, respectively, more effective than denatured DNA as primer and, in both cases, native DNA is about 3 times less effective than denatured DNA.

On the other hand, the hydroxylapatite-purified preparation of virus-induced DNA polymerase has dramatically altered priming specificities compared to crude extracts e.g. when HSV DNA is used as primer it is some 30 times more effective than denatured DNA and some 10 times more effective than native DNA. With calf thymus DNA, however, the changes in properties on purification of the enzyme are slightly less impressive.

The change in priming characteristics between the hydroxylapatite-purified fraction and crude extracts may be due to removal of some virus-induced DNA exonuclease or some other protein, or the combination of these with the host endonuclease. The differences after DEAE-cellulose fractionation are probably simply the result of host endonuclease removal. The detection of such changes in priming specificity is of the utmost importance in assaying for the enzyme after purification. Apparent losses of activity can often be ascribed to these changes and satisfactory activities and recoveries may be obtained only if the optimal primer is used (Roychoudhury and Bloch, 1969b).

Template or primer.

The question now arises: Do these primers act as true templates or are they merely initiators? The evidence from nearest-neighbour analysis and the requirement for all four deoxyribonucleoside 5'-triphosphates by the enzyme strongly suggests that the DNA is being utilised as a template.

On the other hand, it seems likely from the stimulatory effect of pancreatic DNase I that, at the very early stages of the reaction, 3'-OH groups produced on the primer act as initiators to which the first deoxyribonucleoside triphosphates are covalently joined in a priming type of reaction.

Source of primer.

The preference of the virus-induced DNA polymerase for DNA's of certain species is of considerable interest. Probable

variation in the physical state of these DNA's, however, should be borne in mind e.g. BHK-cell DNA and calf thymus DNA, prepared by similar methods, have rather similar characteristics as templates as do E. coli and Bacteriophage T7 DNA also prepared by related techniques.

The native DNA's form a group of primers with similar characteristics. The 3-4 fold preference of the purest enzyme preparation for native DNA over denatured DNA may have in vivo significance, especially since HSV DNA is by far the most effective primer, in particular being about 7 times as effective as the host-cell DNA. The methods of preparation of these two DNA's differed, however, and so the physical state of the DNA's may not be identical. Salmon sperm DNA (a commercial preparation) behaves anomalously, being less effective when activated than when native. This, and the fact that it is a very effective native DNA primer seems to imply that the salmon sperm native DNA is already considerably degraded and, when further activated it becomes less effective as a primer: this over-activation above the optimum degree is known finally to cause inhibition of priming (Roychoudhury and Bloch, 1969b).

The heat-denatured DNA's also form a set of results which are all remarkably similar in characteristics. It seems that, in this case, the loss of secondary structure causes loss of the species individuality of the DNA's.

The activated DNA's also form a group with rather similar

properties as primers.

HSV DNA appears, thus, to act more efficiently as a primer for HSV DNA polymerase than several other DNA's including that of the host-(C13) cell. Clearly, as discussed above, the physical states of the DNA's alone may be the important factor, but if there is a specific recognition by the virus-induced DNA polymerase of HSV DNA which reflects the in vivo situation, then this might partly explain the synthesis of an HSV-specific DNA polymerase and synthesis of HSV DNA to the exclusion of host DNA in HSV-infected cells.

Previous work (Keir et al., 1966a; Keir, 1968) has suggested an apparent preference for HSV DNA by the HSV-induced DNA polymerase at an earlier stage of purification than in the present study. Furthermore, an apparent specificity exists between the rat liver mitochondrial DNA polymerase and its homologous DNA and, although reservations similar to those raised above were advanced in this case, the possibility should be considered that at least some degree of specificity exists between certain DNA polymerases and their presumed in vivo substrates even if these degrees of specificity are far from the levels found with the RNA phage RNA replicases and their specific RNA's. It should be borne in mind that fractionation, of both the DNA and the DNA polymerase, may cause partial or complete loss of such specificities just as they might cause apparent specificities to occur.

Although it seems reasonable to predict that a homologous DNA would be preferred by its DNA polymerase, bacteriophage T2

DNA polymerase has been shown to require denatured DNA and, of the DNA's of various different species tested, salmon sperm DNA is preferred; the phage's "own" T2 DNA being a very poor primer indeed (Aposhian and Kornberg, 1962). Since T2 DNA polymerase is definitely involved in T2 phage DNA replication, the results of this priming experiment appear to caution us that correlation between *in vivo* and *in vitro* findings is not necessarily to be expected.

Studies with chemically methylated BHK DNA.

The possibility that the degree and pattern of methylation of DNA might have an effect on DNA synthesis has gained support from, firstly, the fact that DNA methylation appears to control the processes of DNA modification and restriction (Arber and Linn, 1969) - both of which processes involve "recognition" of DNA; and secondly, the fact that *E. coli* DNA synthesis does not continue in the absence of methylation of the primer (Lark, 1969).

As discussed earlier (Results section 4. 1. 9.) there appears to be a reduction in priming activity of BHK DNA with the HSV-induced DNA polymerase as the extent of chemical methylation of the DNA increases. Since HSV DNA is not methylated while BHK DNA contains approximately 1mol% methylated base, it is possible that the increased methylation in cell DNA is responsible for its poor priming efficiency relative to HSV DNA - as found in the experiment above.

Further experiments with chemically, or preferably,

enzymically methylated HSV DNA in which other physical differences are minimised are required to test this hypothesis.

Template studies comparing pseudorabies virus DNA and HSV DNA may also be of interest since, in this case, we should have two unmethylated DNA's (Low et al., 1969; Low, Mechie and Hay, 1971) allowing other aspects of specificity between the HSV-induced DNA polymerase and its homologous DNA to be examined.

3. 2. 2. Comparative priming studies with host-cell DNA polymerase.

(i) The studies with soluble extracts (nuclear fraction (S2)) of uninfected cells revealed differences in saturation levels and activities with denatured and native DNA between the DNA polymerase of extracts of uninfected cells and the DNA polymerase of extracts of HSV-infected cells (Fig. III. 34), confirming reports of distinct priming preferences of the two activities (Keir et al., 1966a; Morrison, 1967).

(ii) The studies with soluble cytoplasmic fractions of uninfected cells also reveal differences from the corresponding extracts of infected cells, (Fig. III. 33). The DNA polymerase activity of the soluble cytoplasmic fraction of uninfected cells prefers denatured DNA to native DNA, unlike the polymerase activity of the soluble nuclear fraction and this may reflect an active state of the nuclear enzyme and the possibly altered state of cytoplasmic enzyme activity (Introduction section 1. 3. 3.).

(iii) With 0.20M-KCl present in the assays to minimise DNase activity, the effectiveness of denatured DNA relative to native DNA decreased in uninfected-cell soluble fractions possibly owing to a decrease in the activating effect of the host-cell endonuclease on denatured DNA; under the same conditions, however, the denatured DNA appeared to be a more effective primer relative to native DNA for the virus-induced enzyme than in the "low salt" conditions.

(iv) The DEAE-cellulose-purified peaks of host-cell DNA polymerase co-chromatograph with host-cell DNA endonuclease activity and this may influence the observed priming properties of the DNA polymerase from these peaks. The polymerase activities, however, gave similar results in assays at two concentrations of salt, the higher of which should have largely eliminated the host-cell DNase activity (Morrison and Keir, 1968a).

Using the second (salt-eluted) peak of host-cell DNA polymerase, denatured DNA was the optimal primer, followed by activated then native DNA. On storage, however, the priming properties became similar to those of the first peak of polymerase (unaltered by storage): i.e. activated DNA being the best primer, then native, then denatured DNA at both 60mM- and 140mM-K^+. This effect is analogous to that found by J.G. Lindsay (personal communication) with the peaks of denatured DNA-preferring DNA polymerase obtained by gel-filtration of L-cell DNA polymerase activity: with this fraction, four freezing and thawing steps changed the ratio of activity with denatured DNA : activity with native DNA from

4 : 1 to 1 : 9.

The priming properties of the uninfected-cell DNA polymerase differ from these of the DEAE-cellulose-purified HSV-induced DNA polymerase, the latter showing activity in decreasing order of magnitude with activated DNA, denatured DNA and native DNA at both 108mM-and 188mM-KCl. This may represent a true difference between the host and virus-induced DNA polymerases but the differences in their accompanying nucleases, minimised by high salt, should be remembered.

The results with DEAE-cellulose column chromatography and Sephadex gel-filtration of extracts of uninfected BHK21(C13) cells are very similar with respect to the chromatographic profiles and priming properties of DNA polymerase obtained from the corresponding fractionations of rat liver DNA polymerase extracts on DEAE-cellulose (De Recondo and Fichot, 1969) and on Sephadex G-200 (Bellair, 1968).

Because of the apparently dual nature of the host-cell DNA polymerase and the accompanying nuclease activities, experiments investigating possible specificities between host-cell DNA polymerase and host-cell DNA as compared to HSV and other DNA's seem less likely to be valid than the corresponding experiments with the virus-induced DNA polymerase and so have not been carried out.

3. 3. The requirements for the HSV-induced DNA polymerase reaction and the extent of replication.

The fact that the omission of one or more deoxyribonucleoside triphosphates causes very poor incorporation of nucleotides into DNA, whereas the presence of all four deoxyribonucleoside 5'-triphosphates gives a linear incorporation of nucleotides into DNA indicates that the virus-induced DNA polymerase is a replicative enzyme and not a terminal DNA nucleotidyltransferase type of DNA polymerase which does not show true use of a template (Keir, 1965).

The experiments on the priming requirements of the enzyme have also indicated an absolute requirement for added DNA, so that both a full complement of four deoxyribonucleoside triphosphates and an added DNA are necessary for the polymerase reaction.

The partial nearest-neighbour sequence-analysis of DNA synthesised using calf thymus DNA or HSV DNA as primer indicates the fidelity of the replication reaction, for, in both cases, the enzyme achieved the synthesis of a DNA product which closely resembled the template in nearest-neighbour pattern.

These three lines of evidence taken together constitute a compelling indication that the HSV-induced DNA polymerase is a replicative enzyme capable of synthesising a faithful copy of a template DNA *in vitro* and possibly also *in vivo*.

The extent of replication achieved by the virus-induced DNA polymerase has not exceeded 9% of the input DNA in one hour's incubation in the present study. Incorporation, however, has been

shown to be linear for up to 4 hours and so over 30% replication might possibly be achieved. It seems likely too, that the addition of further enzyme or deoxyribonucleoside 5'-triphosphates might allow up to 100% replication (or more) to occur. Since the supply of triphosphates in a standard assay is only sufficient for the synthesis of a maximum of 60µg DNA, it seems likely that this should be a limiting factor in the reaction.

3. 4. Studies on heat-stability, possible protecting agents and enzyme storage with the virus-induced DNA polymerase and its associated DNase from hydroxylapatite column chromatography.

The results of the comparative heat-stability studies with the HSV-induced DNA polymerase and its associated DNA exonuclease from hydroxylapatite need little further comment: there were clear differences between the two profiles regardless of the buffer used for prior incubation at 45°C. The surprising feature of the results, however, was that the DNase seemed to be more heat-labile than the polymerase whereas previous findings had indicated the reverse (Morrison and Keir, 1968b) and recoveries from columns, sucrose gradients and polyacrylamide gel seemed to suggest that the polymerase was more labile than the DNase. Nevertheless, degrees of lability are often specific for certain definite conditions and the conclusion of importance remains that the proteins carrying the two enzyme activities appear to be distinct.

The heat-stabilisation and protection studies with the virus-induced polymerase-associated DNase from hydroxylapatite revealed some interesting and useful effects of several agents: the stabilising effects of dTTP, dTMP and dThd-3', 5'-P_2 have already been mentioned; the stimulatory and protective effects of glycerol are of great potential use.

Bovine serum albumin was a good protector without giving stimulation. RNA seemed to give some protection too and, of the buffers and ions, 0.10M-tris-HCl(pH8.0) inhibited but protected, 0.15M-tris-HCl(pH8.0) and 0.05M-tris-HCl(pH8.0)- 0.10M-KCl both inhibited and did not protect, and added Mg^{2+} gave some protection. Of the agents tested, 30 - 50% glycerol and bovine serum albumin were utilised as protecting agents in fractionation work and for storage of enzyme. Studies on DNA polymerase storage indicated that these agents were useful at all temperatures. The satisfactory storage of the hydroxylapatite-purified fraction of HSV-induced DNA polymerase in 30% (^{v}/v) glycerol at -70^{o}C should be helpful in future purification work.

It appears, from the studies with whole-cell extracts, that freezing without glycerol, no matter the temperature, leads to enzyme inactivation; the presence of glycerol at 30 - 50% (^{v}/v) seems to overcome this difficulty in some way, partly, it is considered, by lowering the freezing point of the solutions and possibly by altering the structure of the frozen solution so that less mechanical trauma is brought to bear on the proteins.

In a preliminary experiment, results suggested that agents which stimulate and/or protect DNA polymerase are not necessarily those which act on DNase: glycerol is an exception.

3. 5. Nearest-neighbour sequence analysis using HSV-induced DNA polymerase.

The results for HSV DNA with the virus-induced DNA polymerase and for HSV DNA using the E. coli DNA polymerase resemble each other closely (Table III. 6.). The ApC and CpC values obtained with the HSV-induced DNA polymerase deviate 12.5% and 9.5% respectively from the nearest estimates using the E. coli DNA polymerase I (Subak-Sharpe et al., 1966b): similarly the TpC and GpC values deviate 24.5% and 6.0% respectively from the closest estimates - these of Mr. G. Russell (personal communication).

Comparisons of the values obtained for calf thymus DNA using the two distinct polymerases reveal that the CpC, ApC, GpC and TpC estimates obtained using the HSV-induced DNA polymerase deviate 15%, 14%, 9% and 13% respectively from the corresponding values obtained with the E. coli DNA polymerase I.

These deviations compare quite favourably with deviations of 9 - 17% between the results of the other two groups using the E. coli DNA polymerase for HSV DNA analysis.

With both types of DNA it was noticed that the results with the HSV-induced DNA polymerase showed higher values for the A- and T-containing sequences and lower values for the CpC and

GpC sequences than did the estimates obtained with the E. coli DNA polymerase.

Possible reasons for these apparent discrepancies are as follows: -

(i) Incomplete digestion of the DNA in the HSV-induced DNA polymerase work, so that dinucleotides containing C and G were appearing in the A and T regions of the electrophoretogram.

(ii) A specific DNA-polymerase effect with the HSV-induced DNA polymerase.

(iii) An effect of hydrolysis by the associated DNase over the long incubation.

Of the possibilities raised (i) seemed most likely, but no evidence of streaking or spreading of the A and T spots was found and about 98% of the total radioactivity was localised in these nucleotide spots. This indicates satisfactory digestion (McGeoch, 1970).

Possibility (ii) is supported by the findings of Mr. G. Russell (personal communication) that M. luteus DNA polymerase gives consistently high %A and %T compositions for the DNA's being analysed by the nearest-neighbour technique. It may be of significance that the natural substrates of both M. luteus and HSV DNA polymerases are DNA's of high (G + C) content.

Possibility (iii) seemed unlikely owing to the high salt content of the incubation mixture which inhibits DNase activity.

Explanation (ii) seems thus the most likely of the three. The main inference of the results, however, seems clear: that the HSV-induced DNA polymerase is a replicative type of enzyme capable of synthesising a faithful copy of a template DNA offered whether this be its homologous DNA (HSV DNA) or an unrelated DNA (calf thymus. The results support the view that this enzyme may be capable of replicating HSV DNA *in vivo* and that it may be, in fact, a true *in vivo* HSV DNA replicating enzyme.

3. 6. DNA binding experiments.

The results of the priming studies which indicated a marked preference for native HSV DNA as primer for the HSV-induced DNA polymerase made it seem likely that a preferential binding of the enzyme to HSV DNA rather than to host-cell BHK21(C13) DNA might occur. Preliminary results of binding studies carried out essentially as described by Pitts and Fried (1969) seem to support this idea but the low activities of DNA polymerase recovered from the gradients make this result only a tentative pointer.

4. DNA metabolism in uninfected and in HSV-infected cells.

4. 1. Host-cell DNA polymerase and DNase.

DNA polymerase.

The results of the present and previous studies indicate

that uninfected BHK21(C13) cells have at least one, and probably two, distinct DNA polymerase species which differ by a large number of criteria from the enzyme induced in HSV-infected cells. There are apparent differences between the template specificities of the two species of polymerase from host-cells: it is possible that the one which has relatively greater activity with heat-denatured DNA may correspond to the "cytoplasmic" DNA polymerase of L-cells described by Lindsay et al., (1970), while the other species of DNA polymerase may take part in replication of the nuclear DNA. It is, of course, possible that neither of these enzymes is involved in in vivo DNA replication: the true host-cell DNA replicase might be a labile or membrane-associated enzyme which has not yet been detected and one, or both, of the apparently twin species of DNA polymerase studied in this work might have a role in DNA repair (Kelly et al., 1969), or in DNA recombination.

It would thus be of interest to irradiate the BHK cells to search for an increase in one or both species of DNA polymerase following this treatment as occurs in Tetrahymena sp. (Westergaard, 1970; Keiding and Westergaard, 1971). Studies with radiation - sensitive mutant cells would also be of interest.

At present, little evidence for the in vivo role of the host-cell DNA polymerase activities exists although, being replicative DNA polymerases (Keir, 1965), it seems likely that they may be involved in DNA replication.

Host DNase activity.

The host-cell has also been shown to possess at least two species of DNase. Cellular DNA endonuclease (Morrison and Keir, 1968a) seems likely to be identical to the nuclease with combined RNase and DNase activities described in normal and polyoma-transformed BHK21(C13) cells by Koh et al., (1970).

The phosphodiesterase I, associated mainly with the plasma membrane and detected and studied in BHK21(C13) cells by Braidwood and Morrison (Braidwood, 1970) is active on oligonucleotides. Although it is probably not involved in DNA metabolism in vivo, its removal by high-speed centrifugation is required to prevent interference with studies on herpes-induced and host DNases.

There exists also evidence for low levels of endonuclease and exonuclease activity, active on native DNA and for exonuclease active against denatured DNA.

To this list should also be added lysosomal "acid" DNase (Keir, 1968; Morrison and Keir, 1968a).

The functions of DNases of animal cells are unknown and we can only speculate at present upon their possible roles in replication, recombination, repair and breakdown of DNA.

This then is the present state of knowledge of the uninfected cell: there are at least two species of enzyme with DNA polymerising capability, and a number of enzymes capable of DNA degradation. The question of whether any one of these is

truly an enzyme of DNA synthesis or DNA degradation in vivo is as yet unanswered.

4. 2. HSV-induced DNA polymerase and DNase.

Upon infection with HSV, these cells appear, from past and present work, to allow synthesis of a new DNA polymerase and at least one new DNA exonuclease activity. These enzymes have been shown to differ in many respects from the corresponding cellular enzymes and immunological studies have indicated that the virus-induced DNA polymerase and DNase are similar in cells of widely different genetic origins so that the enzymes appear to be specified by viral genes.

Virus-induced DNA polymerase.

Why should the virus induce the synthesis of a new DNA polymerase? In partial answer to this question it is recalled that several viruses appear to induce enzymes which apparently duplicate activities already present in the uninfected cells, and the virus-induced enzyme seems to be sometimes actually dispensable e.g. the new thymidine kinase of HSV-infected cells (Keir, 1968). Nevertheless, it can often be shown that the new enzyme is required for optimal viral growth or growth under certain conditions and this may be a result of one or more of several causes.

The new enzyme may be required because enzyme levels may be normally low in the natural host-cells of the virus (e.g. nerve cells) or because shut-off of protein synthesis, which often occurs

on virus-infection, can result in insufficient levels of enzyme as the activity decays with time. In addition, the new enzyme may be more active in conditions of virus-infection, or it may be less sensitive to feed back inhibition, or it may have a new, much more specific action on either host or viral metabolism.

Experiments with the HSV-induced DNA polymerase seem to indicate that the enzyme synthesises HSV DNA more rapidly than host-cell DNA and it may be speculated that the virus-induced DNA polymerase is required for its specificity towards HSV DNA. Additionally, host-cell DNA polymerase may not be accessible to the virus DNA.

It is interesting to note that intracellular fluid is quoted as containing 177m.equiv./litre of cations; in this environment, the virus-induced DNA polymerase is optimally active, while the host-cell activity is inhibited. Virus-specific "shut-off" of host DNA synthesis may occur partially by a specific factor and partially by decay of host polymerase following virus-induced termination of host protein synthesis (Keir and Gold, 1963), the viral DNA polymerase being unable to effect host DNA replication.

There therefore seem to be several plausible reasons why the new HSV-induced DNA polymerase might be required:-

(i) HSV DNA might be an unacceptable or inaccessible template for host-cell DNA polymerase;

(ii) host DNA polymerase may naturally exist at, or may decay to levels insufficiently high for the synthesis of HSV DNA following "shut off" of host-cell protein synthesis;

(iii) the new DNA polymerase may also show adaptations to make it specifically suitable for HSV DNA synthesis showing *in vivo* either absolute or partial specificity towards viral DNA, host-cell DNA being either unavailable or less favoured as template.

It is possible that a combination of these reasons make the new enzyme's synthesis desirable: it is not yet known if it is indispensable for HSV DNA synthesis.

It is tempting to assume that this DNA polymerase activity is a true replicative enzyme for viral DNA and indeed, the results of the nearest-neighbour experiment suggest that this is so, but it must be remembered that the *E. coli* DNA polymerase I which was utilised in the bulk of the original nearest-neighbour studies is not now believed to be an *E. coli* DNA replicating activity, but rather an enzyme of DNA repair. The same may be true for the HSV DNA polymerase: the true HSV-DNA replicating activity may not yet have been detected: it may be an extremely labile, membrane-associated activity like the *E. coli* system described by Smith *et al.*, (1970) and Knippers (1970). The virus-induced DNA polymerase that we have been studying may also turn out to be an enzyme involved in some kind of repair or editing function.

Further biochemical experimentation in combination with genetic studies is likely to provide a solution to these problems.

Virus-induced DNA exonuclease.

We now turn to the question of the HSV-induced DNA exonuclease activity. As we have already concluded, it is likely that there is only one such enzyme and, if this is so, there seems to be a fairly strong tendency for association between the HSV-induced DNA polymerase and the DNA exonuclease - a finding which may have some physiological significance - for example, they may act together in a multienzyme complex. If, however, there are two rather than one virus-induced DNA exonuclease, then the polymerase-associated exonuclease may fulfil an "editing" or "nick-translation" function as has been postulated in the case of the E. coli DNA polymerase I (Kelly et al., 1969).

There remains then the question of the function of the virus-induced polymerase-free DNA exonuclease, if we consider two induced exonucleases to exist, or simply the function of the virus-induced DNA exonuclease, if we consider only one induced exonuclease to exist: again, the function is not known and we can only speculate from the available evidence.

The most likely answer proposed was that the virus-induced enzyme causes the hydrolysis of host DNA and so causes "shut-off" of cellular DNA synthesis and provides nucleotides for HSV DNA synthesis. It soon became obvious, however, that the termination

of host-cell DNA synthesis was an early event which occurred long before significant synthesis of the virus-induced DNase occurred. It was therefore clear that the function of the DNase was not "shut-off" of host-DNA synthesis; was it then to provide nucleotides for viral DNA synthesis? The answers to this question have been conflicting.

Early work carried out by Morrison (1967), in collaboration with Dr. J. Hay suggested that the contribution of nucleotides from host DNA to viral DNA was very small if indeed it existed at all. Then work by Perera and Morrison (1970) using different conditions of cell culture and virus-infection suggested that degradation of host-cell DNA did occur following virus-infection and led to incorporation of nucleotides from host-cell DNA into HSV DNA to give the same specific activity as the host DNA: this occurred also in the presence of hydroxyurea and FdUrd which block de novo synthesis of deoxyribonucleotides.

Sensitive studies in which uninfected or virus-infected cells were layered directly on to alkaline sucrose gradients and lysed spontaneously to release their DNA on top of the gradients, have revealed differences between uninfected - and infected-cell DNA indicative of virus-induced endonucleolytic breakage of cellular DNA (M. Low and J. Hay, unpublished observations).

It thus seems that, in at least some circumstances, the virus can carry out degradation of host-cell DNA thus providing nucleotides for HSV DNA synthesis. It is possible that herpes-

induced DNA exonuclease can provide this function.

There are then two further related questions: -

(i) For what other function could the new DNA exonuclease be synthesised?

(ii) What other source could exist for the nucleotides for HSV DNA synthesis?

In answer to the first question, the same arguments obtain as for the polymerase-associated exonuclease. In answer to the second, if cellular DNA is a source of nucleotides for viral DNA synthesis, then we should have expected to find a virus-induced DNA endonuclease in preference to or in addition to, the virus-induced DNA exonuclease: for exonuclease action alone would probably provide slow and inefficient breakdown of host-cell DNA. A search for a new endonuclease following virus-infection has, until very recently, been fruitless but recent results of M. Low and J. Hay in virus-infected mouse-embryo and BHK cells show evidence of cleavage of DNA into fragments of a size compatible with the induction of an endonuclease activity after virus infection.

Again, on the question of the origin of the nucleotides for viral DNA synthesis, as yet no final answer exists but there are items of evidence providing some indications.

In the absence of host DNA breakdown, de novo synthesis of deoxyribonucleotides would have to occur as existing intracellular pools of nucleotides are insufficient for continued viral

DNA synthesis.

Although there is good evidence for increases in deoxythymidine kinase and deoxycytidine kinase levels together and possible increases in dCMP deaminase and dTMP kinase after HSV infection, no increases in dTMP synthetase and dihydrofolate reductase have been found to occur in several host-cell types (Introduction section 2. 4. 3.). There thus appear to be deficiencies in the induced enzyme complement for the purposes of de novo deoxyribonucleoside 5'-triphosphate synthesis but these may be adequately compensated by host-cell enzymes. In resting cells, however, (Bürk, 1966) which probably most closely resemble the state of cells normally infected by HSV in life; some of the enzyme activities for such de novo synthesis are normally very low, and would require supplementation.

The deoxyribonucleoside kinases could be useful for phosphorylation of nucleosides derived from: (i) host-cell DNA breakdown, (ii) endogenous sources and (iii) hydrolysis of the products of de novo deoxyribonucleotide synthesis, so maintaining endogenous phosphorylation of nucleotide pools depleted by phosphomonoesterase (Ecker, 1965).

It seems likely, from all the results, that the source of nucleotide for HSV DNA is partly from de novo synthesis of deoxyribonucleotides, partly from exogenous nucleosides, and partly from host-cell DNA breakdown, the proportion of the total contribution from each source possibly varying widely, depending

on the conditions of cellular growth and virus infection.

It is interesting to note that the amount of HSV DNA provided by a cell never exceeds 10% of the total host-cell DNA at the end of infection so that the % of host-cell DNA that would have to be broken down to provide nucleotides for viral DNA is 10% (as a maximum) of the host DNA. Therefore, the extensive breakdown of host DNA that occurs in T-even phage-infected E. coli, can be seen to be of value to the phage, but is not apparently necessary for HSV DNA synthesis. Comparison, however, between T-phage and herpes systems may not be valid in view of colonial interactions between herpes-infected cells in which dead cells may contribute nucleosides or nucleotides to viable cells for HSV DNA synthesis.

It can be seen that since 10% is the maximum of the total host DNA requiring to be broken down, a situation where there was an equal contribution from host-cell DNA and from de novo synthesis would require only 5% of the host-cell DNA to be utilised. Thus, such small amounts of host-cell DNA hydrolysis might be achieved by the action of the virus-induced DNA exonuclease and there would be little evidence of host-cell DNA breakdown shown by alkaline sucrose gradient sedimentation. This may be the role of the viral DNA exonuclease: to cause small amounts of host-cell DNA breakdown to provide nucleotides for viral DNA synthesis. The recent evidence for the possible induction of DNA endonuclease by the virus, however, makes it

seem more likely that a combined endo- and exonucleolytic attack on the host DNA may be the actual pathway involved. The apparent preference of the virus-induced exonuclease for partially digested DNA supports this view also. Experiments on the specificity of the induced DNA exonuclease would be of great interest to see if it preferentially hydrolyses host-cell DNA rather than HSV DNA and although preliminary studies suggest no absolute specificity, it would be most interesting if the enzyme were found to have no action on HSV DNA and if this were so, to determine if methylation had any effect on HSV DNA as a substrate for the enzyme.

FUTURE POSSIBILITIES.

Further work is required to continue the purification of the virus-induced DNA-polymerase and DNA-exonuclease activities. This would ultimately answer unequivocally the question of whether there were one or two virus-induced exonucleases.

Hydroxylapatite fractionation would be a useful first step and rechromatography on this medium might also be of value. A DNA-cellulose column chromatography fractionation (Litman, 1968) might be useful as a later step.

The use of 30-50% (^{v}/v) glycerol in all buffers, and storage at -70^{o}C should allow satisfactory recoveries and storage.

With homogeneous virus-induced DNA polymerase a large number of valuable characterisation studies could be attempted. Those of particular interest would be:-

(i) Molecular weight studies using Sephadex G-200, Sepharose 4B and constant velocity sucrose gradients with a variety of concentrations of KCl to study aggregation or disaggregation.

(ii) Specificity studies comparing priming by HSV DNA, pseudorabies virus DNA and BHK21(C13) cell DNA - prepared by a similar method in all cases and the DNA's being physically characterised.

Studies on the effect of chemical and enzymic methylation on the HSV and pseudorabies virus DNA would also be of interest. Binding studies (Pitts and Fried, 1969) between the purified, virus-induced

DNA polymerase and these three types of DNA could also be fruitful.

Other investigations of value would be competitive studies using a mixture of host-cell and HSV DNA as template for nearest-neighbour analyses using the induced DNA polymerase, to see if the pattern were a hybrid or close to HSV DNA. A full nearest-neighbour analysis with the pure HSV-induced polymerase and HSV DNA at both "high" and "low salt" concentrations would also be desirable.

Similar priming and binding studies with purified BHK 21(C13) host-cell DNA polymerase and the DNA's mentioned above, including methylated HSV DNA, would also be of interest, as would further extensive physical characterisation studies.

Homogeneous virus-induced exonuclease(s), which might also be derived from the purification scheme outlined for the induced polymerase, could be physically characterised and substrate specificity studies using HSV DNA, pseudorabies DNA, BHK DNA and methylated HSV and pseudorabies DNA would be of interest.

Studies involving the isolation of mutants of the virus, temperature-sensitive in such functions as DNA replication, host DNA breakdown and HSV DNA recombination, and the examination of the induced DNA-polymerase and DNA-exonuclease activities, isolated and purified from cells infected with these mutant viruses could give an indication of the genetic origin of the enzymes.

Additionally, <u>in vitro</u> protein synthesis experiments

using HSV DNA either transcribed *in vitro* from HSV or isolated as natural viral m DNA from HSV-infected cells and the comparison of the *in vitro* synthesised proteins with the homogeneous enzymes purified from HSV-infected cells would give an unequivocal answer regarding the genetic origin of the virus-induced enzymes.

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S U M M A R Y.

Enzymes of DNA Synthesis and Degradation in Cells Infected with Herpes Simplex Virus.

by

Robert D. Paton

Summary of the thesis presented for the degree of Doctor of Philosophy, University of Glasgow, November 1971.

The main aim of the project was to purify the virus-induced DNA polymerase from herpes simplex virus-infected baby hamster kidney cells grown in culture. If this could not be carried to homogeneity, then the aim would be at least to separate host DNA polymerase and DNase from the enzyme in order to clarify the relationship of the induced DNA polymerase and DNase and eliminate the interfering effects of the other enzymes.

In the course of the present purification studies involving column chromatography on DEAE-cellulose, hydroxylapatite, Sephadex G-150, and phosphocellulose, and fractionations using sucrose gradient sedimentation and polyacrylamide gel electrophoresis, evidence was obtained that at least part of the virus-induced DNase activity could be separated from DNA polymerase especially on hydroxylapatite columns. The question arose whether there were one

or two new virus-induced DNA exonuclease(s) because, while one of the peaks of DNase from hydroxylapatite was polymerase-free, the other was closely associated with polymerase and remained so throughout several purification steps.

The two virus-induced exonuclease peaks were compared using several criteria including: sedimentation coefficients, substrate specificities, heat stability and rechromatography. The results showed that the enzymes were in the main similar by these criteria although differences detected by the last two criteria may be significant. On the whole these and other characterisation studies coupled with consideration of earlier work suggested that there was probably only one virus-induced DNA exonuclease, and that this was distinct from the DNA polymerase purified protein. Nevertheless, there is also substantial evidence in favour of there being two distinct virus-induced exonucleases, one polymerase-free, the other polymerase-associated. A further possibility that the polymerase-free exonuclease is a breakdown product of a single virus-induced DNA polymerase-DNA exonuclease protein has not been eliminated completely by studies carried out with a protease inhibitor. The final answer rests upon further purification studies.

Using hydroxylapatite column chromatography, enzyme purification of some 200-fold was obtained using activated DNA which was a particularly effective primer after this step. Further purification was hampered by instability of the enzyme but

studies on protecting agents revealed that storage at -70°C in high glycerol concentrations allowed retention of activity over long periods, opening the way to purification to homogeneity.

Studies on the molecular weight of the herpes DNA polymerase indicated values in the range 50,000 - 200,000, probably due to the occurrence of aggregation. The true value probably lies in the lower part of this range.

Characterisation studies on the hydroxylapatite-purified virus-induced polymerase revealed that the enzyme was of the replicative type, requiring all four deoxyribonucleoside 5'-triphosphates and DNA for activity. Nearest-neighbour frequency analysis using the herpes polymerase gave results consistent with faithful copying of template DNA's. Heat inactivation revealed different profiles for the polymerase and exonuclease activities but this does not necessarily preclude their being in the same protein. Evidence was also obtained showing a preference for native or activated herpes DNA as primer over DNA's of corresponding secondary structures from other sources including the host cell.

Chromatographic studies carried out using extracts of host cells revealed that the host DNA polymerase is of the replicative type and appears to exist in at least two separable forms concurring with the results obtained from other mammalian cell sources.

Finally, comparison of virus-induced DNA polymerase and DNase with the corresponding host activities using a wide range of

criteria clearly indicates their non-identity and supports a viral origin for the new DNA polymerase and DNA exonuclease(s). The results from the project confirm and amplify earlier research, and have achieved the aim of separating the host and virus-induced activities and laid a substantial base for continued purification and characterisation studies on the viral enzymes.