

CHARACTERIZATION OF THE HERPES
SIMPLEX VIRUS RIBONUCLEOTIDE
REDUCTASE

By

DENNIS HUSZAR, B.Sc.

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 Ribonucleotide Reductase

AUTHOR: Dennis Huszar, B.Sc. (McGill University)

SUPERVISOR: Dr. Silvia Bacchetti

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ABSTRACT

Ribonucleotide reductase catalyzes the first unique step in DNA synthesis by reduction of all four ribonucleotides to the corresponding deoxyribonucleotides. Herpes simplex virus (HSV), which codes for at least three enzymes of DNA metabolism (thymidine kinase, DNA polymerase and DNAase) was found to induce a novel ribonucleotide reductase activity upon infection of mammalian cells. The HSV-2 induced reductase was purified essentially free of the endogenous cellular enzyme and found to differ from the cellular reductase in several of its biochemical properties, most notably in its resistance to allosteric inhibition by dTTP and dATP (Huszar and Bacchetti, 1981). In addition, a rabbit antiserum was prepared (R1 serum) which was capable of specifically immunoprecipitating the HSV-2 induced reductase, thus demonstrating that the induced and cellular enzymes could also be immunologically distinguished (Huszar *et al.*, 1983). Further experiments established that R1 serum cross-reacted with two monoclonal antibodies, both specific for HSV-2 polypeptides of approximately 144,000 and 38,000 daltons, which were capable of either immunoprecipitating the HSV-2 induced reductase (H11 antibodies) or directly neutralizing it in solution (Bq7 antibodies) (Huszar *et al.*, 1983).

These data demonstrate that either one or both of the HSV-2 144,000 and 38,000 dalton polypeptides are associated with

viral ribonucleotide reductase activity. Based on the mapping of these polypeptides (Anderson *et al.*, 1981; Docherty *et al.*, 1981; Galloway *et al.*, 1982a), these data also locate the coding sequences for at least a component of the enzyme between .56 - .60 map units on the viral genome within DNA sequences associated with cell transformation. The identification of viral DNA sequences coding for, and of viral polypeptides associated with, the HSV-2 ribonucleotide reductase will facilitate studies on the relevance of the enzyme to viral replication, latency and cell transformation.

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This thesis is dedicated to

Linda and Trevor Grant

and

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LIST OF ABBREVIATIONS

A,T,G,C,U	refer to adenosine, thymidine, guanosine, cytidine, and uridine, respectively; mono, di, and triphosphates are designated MP, DP, and TP, respectively. The prefix "d" refers to the reduced forms of the nucleotides.
BHK	baby hamster kidney
CaCl ₂	calcium chloride
Ca(NO ₃) ₂	calcium nitrate
Ci	Gurie(s)
cpm	counts per minute
dCD	deoxycytidine deaminase
dCMPD	deoxycytidylate deaminase
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(3 aminoethyl ether) N,N'-tetraacetic acid
FeCl ₃	ferric chloride
g	gravity
HCl	hydrochloric acid
HClO ₄	perchloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HSV	herpes simplex virus
IgG	immunoglobulin G
K	thousand
KCl	potassium chloride
KOH	potassium hydroxide
M	molar
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
MOI	multiplicity of infection
mol	mole
mRNA	messenger ribonucleic acid

ABBREVIATIONS (Continued)

MW	molecular weight
N	normal
NaCl	sodium chloride
NaF	sodium fluoride
NaHCO ₃	sodium bicarbonate
PBS	phosphate buffered saline
PFU	plaque forming unit
RNA	ribonucleic acid
RNAase	ribonuclease
RNR	ribonucleotide reductase
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TK	thymidine kinase
Tris	Tris(hydroxymethyl)aminomethane
v/v	volume/volume
w/v	weight/volume

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1. INTRODUCTION

This study is directed at characterizing the ribonucleotide reductase induced in mammalian cells infected with herpes simplex virus type 2 with the aim of determining whether the enzyme is virally coded. Both the biology of herpesviruses and the enzymology of ribonucleotide reductase are equally relevant to this research and are accordingly reviewed in sections 1.1 and 1.2 of the Introduction, respectively. The information available on the reductase induced by herpesviruses, at the time this study was initiated, is described in section 1.3. In addition, a brief description of deoxycytidine deaminase and deoxycytidylate deaminase, two enzymes included in our initial studies of herpes simplex virus infected cells, is given in section 1.4. Lastly, the relevance of studying virally induced enzymes, in relation to the biology of herpes simplex viruses, is discussed in section 1.5.

1.1. Herpes Simplex Viruses

1.1.1. General characteristics

The two serotypes of herpes simplex virus, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), are members of the herpesviruses, a large and diverse group of viruses which have been isolated from a wide variety of eucaryotic hosts. Herpesviruses replicate in the nuclei of infected cells where they can be morphologically identified by electron microscopy as icosahedral nucleocapsids approximately 100 nm in diameter. The capsids characteristically acquire a lipoprotein envelope by budding through the nuclear membrane. The genetic material of these

viruses is DNA, in the form of a linear double stranded molecule which ranges in size, among different members of the group, from approximately 80×10^6 to 150×10^6 daltons (for a general review, see Roizman and Furlong, 1974). At least five distinct herpesviruses replicate in man as their primary natural host: HSV-1 and HSV-2, Epstein-Barr virus, varicella-zoster virus, and cytomegalovirus. Except for HSV-1 and HSV-2, which are quite similar in their molecular and biological properties, the human herpesviruses vary considerably in their host range and clinical manifestations. They do, however, share one key pathogenetic property, and that is the ability to establish latency following a primary infection and to subsequently undergo reactivation (Hirsch, 1979).

Although the two herpes simplex virus serotypes are closely related, and for the purposes of much of the discussion which follows it is not necessary to distinguish them, it should be borne in mind that significant genetic divergence has occurred between the two viruses. Their genomes appear to be colinear with respect to gene order (Rapp, 1980) however the DNA's share only 47-50% homology (Kieff *et al.*, 1972), and differ in G+C content (67 and 69% G+C moles for type 1 and 2 respectively; Goodheart *et al.*, 1968; Kieff *et al.*, 1971) and restriction endonuclease cleavage sites (Morse *et al.*, 1977; Cortini and Wilkie, 1978). This latter feature has been useful both as a means of differentiating the two serotypes, as well as in the genetic mapping of HSV polypeptides by analysis of intertypic recombinants. In addition, although corresponding polypeptides of the two serotypes are functionally similar, they can often be differentiated on the basis of their molecular weight and/or antigenic properties.

1.1.2. Clinical aspects of HSV infection and association with human cancer

HSV has an extremely broad host range in experimental animals and cells grown in tissue culture, however man appears to be the only natural reservoir for the virus (Hirsch, 1979). Human infection with HSV is characterized by entry and replication of the virus at a mucocutaneous site (i.e. primary infection, which can be either asymptomatic or associated with virus induced cutaneous lesions), colonization of corresponding sensory ganglia and establishment of latency in the sensory neurons (Klein, 1982). The onset of latency, operationally defined by the inability to isolate free infectious virus from ganglion cells, signals the end of the acute phase of infection. It is not yet clear what mechanisms influence the transition from the acute to the latent stage, however it has been suggested that immune factors play a major role (Openshaw *et al.*, 1981). Latent virus is maintained in sensory neurons in a static state, with little or no expression of viral genetic information (Puga *et al.*, 1978; Galloway *et al.*, 1982c). Latent HSV can persist for the lifetime of the individual and is subject to periodic reactivation by as yet ill-defined stimuli. Reactivation is associated with the reappearance of infectious virus which is shed either asymptotically or with the reappearance of cutaneous lesions (Klein, 1982).

The pathogenetic mechanism just described underlies the most prevalent of the clinical syndromes associated with HSV infection, i.e. primary and recurrent oral, genital and ocular infections. In rare instances the viral infection can spread, apparently via neural routes, to the brain, giving rise to encephalitis (Hirsch, 1979). In addition

HSV can also give rise to life threatening infections of neonates and the immunologically compromised (Nahmias and Roizman, 1973; Rawls, 1973). Furthermore, a vast amount of seroepidemiological data has established a correlation between cervical cancer and genital infection with HSV-2 (reviewed by Rawls *et al.*, 1977). In general, women with a past history of HSV-2 infection have a higher incidence of cervical cancer, and cancer patients have higher titers of HSV-2 antibodies, than do matched controls. While this data is circumstantial, it is worth noting that of the DNA viruses, only members of the herpesvirus group have been shown to cause malignant disease in their natural hosts. For example Marek's disease virus and herpesvirus sylvilagus give rise to lymphomas in chickens and wild cottontail rabbits, respectively (Churchill and Biggs, 1967; Hinze, 1971), and Lucke frog virus to kidney adenocarcinomas in American leopard frogs (Lucke, 1952). In addition, Epstein-Barr virus has been associated with both Burkitt's lymphoma and nasopharyngeal carcinoma in man (reviewed by zur Hausen, 1981).

While HSV does not appear to induce tumors in experimental animals (Nahmias *et al.*, 1970), a role for the virus in human malignancy is supported by the observation that HSV can transform cells *in vitro*. DNA sequences capable of effecting either morphological or oncogenic transformation, or both, have been mapped to several locations in the viral genome, at .416-.580 (Jariwalla *et al.*, 1980, 1982) and .583-.596 map units (Galloway *et al.*, 1982b), and also possibly at .31-.42 map units (Reyes *et al.*, 1980). The mechanism of HSV mediated transformation appears to differ from that of the other DNA tumor viruses in two important respects: 1) the efficiency of HSV transformation is generally

much lower than that observed with other DNA viruses, including herpesviruses (Hampar, 1981), and 2) whereas with the latter viruses maintenance of the transformed state seems dependent on the retention and expression of specific viral DNA sequences, such is not the case with HSV transformants. Indeed a progressive, and often complete, loss of viral information has been observed in HSV transformed cells over time in culture; retention of viral DNA sequences, when it occurs, appears to be at random and not to necessarily involve the putative transforming sequences (Frenkel *et al.*, 1976; Minson *et al.*, 1976; Galloway *et al.*, 1980; Galloway and McDougall, 1983).

These observations have led to the hypothesis that HSV transforms via a "hit and run" mechanism in which viral genetic information is required for initiation, but not maintenance, of the transformed state (Skinner, 1976; Hampar, 1981). Such a mechanism could, for example, be mediated by the induction of mutations (including point mutations, deletions and translocations) in the host DNA. While it has not yet been satisfactorily determined whether HSV is mutagenic, it has been observed that the virus, like some chemical mutagens, can activate the expression of endogenous proviruses in mouse cells (Duff and Rapp, 1975; Hampar *et al.*, 1977). In addition, HSV has been shown to induce chromosomal breaks and rearrangements (Hampar and Elison, 1961; Stich *et al.*, 1964), phenomena which have been found to be associated with some human malignancies (Erikson *et al.*, 1983; Marcu *et al.*, 1983). Lastly, the observed lack of retention of viral information in HSV transformants is also compatible with mutagenesis by insertion of viral promoter sequences (too small to be detected by any

of the techniques used to date) near a potential cellular oncogene (Neel *et al.*, 1981).

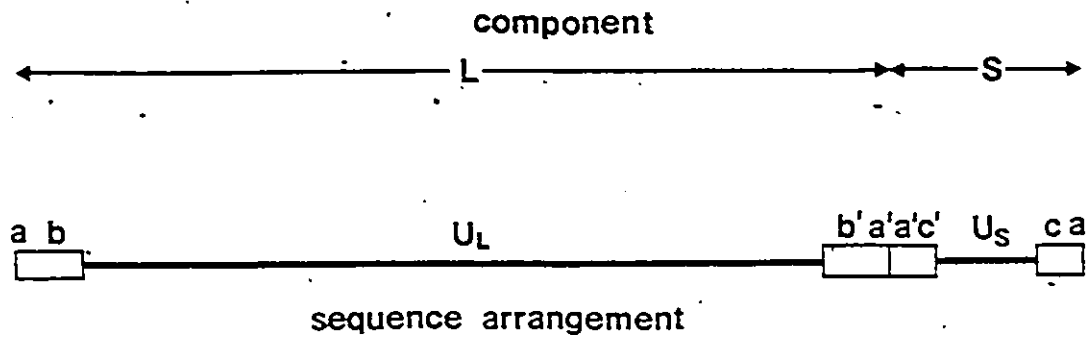
1.1.3. Virion morphology, genomic organization, and DNA replication

The HSV virion contains an electron dense core within which is packaged approximately 100×10^6 daltons of DNA. This core is enclosed within a capsid, consisting of 162 capsomers, which is approximately 100 nm in diameter. The capsid is surrounded by an amorphous electron dense material, called the tegument, which is in turn enclosed within an envelope consisting of a lipid bilayer and associated viral proteins.

The sequence organization of the HSV genome is somewhat unusual and bears a brief description. Like several other herpesviruses, but unlike any other DNA virus, the HSV genome consists of two covalently joined segments of DNA, the L and S segments, comprising 82% and 18% of the genome respectively. Both segments consist of unique sequences bracketed at either end by inverted terminal repeats (Sheldrick and Berthelot, 1974). As shown in Fig. 1, for the long unique segment (U_L) these terminal sequences are designated αb , and for the short unique segment (U_S) αc . The α sequences are thus present in the same orientation at the ends of the genome, and in an inverted orientation at the L-S junction. All four α sequences have, up until recently, been considered identical, however there is now data demonstrating that slight differences do exist between the terminal α_S and α_L sequences (Mocarski and Roizman, 1982). Because the termini of the HSV genome are inverted internally, Sheldrick and Berthelot (1974) suggested that recombination might occur between the ends of the molecule and the inverted repeats, giving rise to

7
Figure 1

Schematic diagram of the sequence arrangement of the HSV genome. Total genomic size is $\pm 10^8$ daltons or ± 150 kb. The genome is made up of two covalently joined segments of DNA, designated L and S, each of which consists of unique sequences (U_L and U_S , respectively) bracketed by inverted repeats. The repeats at the termini of U_L (ab and b'a'; the primed letters represent inverted sequences) each comprise 6% of the total DNA, those bracketing U_S (a'c' and ca) each make up 4.3% of the total DNA.



inversion of the L and S segments relative to each other. That such inversion indeed occurs was demonstrated by Hayward *et al.*, (1975) and Delius and Clements (1976) who found that HSV DNA preparations consist of four equimolar populations differing only in their genome segment orientation. Because of this, one particular arrangement of the genome has been chosen, by convention, as the prototypical configuration, however it appears that all four isomers are equally infectious and can each singly give rise to all four segment orientations (Hoggan *et al.*, 1960; Roizman, 1979).

There is at present no known functional role for HSV segment inversion, however it does seem likely that the process occurs during DNA replication. The current model of replication invokes circularization of viral DNA, perhaps by blunt end ligation of terminal α sequences (Mocarski and Roizman, 1982), to create a rolling circle structure which generates head to tail concatemers of the genome (Roizman, 1979). Concatemers are apparently processed to linear unit length molecules by cleavage within adjacent copies of the α sequence (Mocarski and Roizman, 1982), and are subsequently or simultaneously packaged into capsids. Many of the details of the replication and processing of HSV DNA remain yet to be elucidated, and thus many features of the present models are hypothetical.

1.1.4. RNA and protein synthesis

Approximately 50 virus specified polypeptides have been identified in HSV infected cells, ranging in size from 20,000 to >250,000 daltons (Hones and Roizman, 1973; Powell and Courtney, 1975). This value does

not include the numerous processing intermediates which have been detected for several of the polypeptides. The criteria for identification of viral proteins include 1) demonstration of increasing rates of synthesis of a polypeptide after infection, which is aided by the fact that HSV infection rapidly inhibits host protein synthesis (Sydiskis and Roizman, 1966, 1968; Ben-Porat *et al.*, 1971), 2) variation in the electrophoretic mobility of a given polypeptide in HSV-1 vs HSV-2 infected cells, and 3) immunoprecipitation of the polypeptide by HSV specific antisera. Definitive proof of the viral origin of a polypeptide requires its mapping to the viral genome by techniques such as *in vitro* translation of selected viral messages or identification of viral mutations which directly affect the polypeptide.

The expression of HSV genetic information occurs in at least three stages during the infectious cycle. Each successive stage is characterized by an increased complexity of RNA transcripts appearing in the cytoplasm (Jones and Roizman, 1979) which corresponds roughly to the synthesis of three coordinately regulated classes of HSV polypeptides (Hones and Roizman, 1974). The immediate early, or α , class of transcripts comprises a limited set of mRNA's transcribed predominantly from DNA sequences within or near the terminal repeats bracketing U_L and U_S (Clements *et al.*, 1977; Jones and Roizman, 1979). These mRNA's are synthesized in the absence of *de novo* protein synthesis, indicating that they are transcribed by an unmodified host RNA polymerase. Recent experiments, however, have demonstrated that α gene expression is enhanced by non- α gene products which may be structural components of the virion (Post *et al.*, 1981), indicating that the virus may carry into the

cell elements which regulate the expression of at least one α gene. The polypeptides specified by α genes reach maximal rates of synthesis at 2 to 4 hours post-infection and decline thereafter. At least one of these polypeptides, ICP4, plays a regulatory role in the modulation of viral transcription since viral mutants carrying a temperature-sensitive lesion in this function overproduce immediate early polypeptides and synthesize reduced or undetectable amounts of early and late gene products at the non-permissive temperature (Preston, 1979; Dixon and Schaffer, 1980; Watson and Clements, 1980).

Following the expression of functional α polypeptides, and prior to viral DNA synthesis, a relatively large number of early, or β mRNA's are synthesized. These mRNA's map throughout the HSV genome in non-contiguous segments (Stringer *et al.*, 1978; Jones and Roizman, 1979) and encode mostly non-structural polypeptides, which are synthesized maximally at 5-7 hours post-infection after which their rate of synthesis decreases. β polypeptides appear to be involved in switching off the synthesis of the α polypeptides (Hones and Roizman, 1974, 1975) and in the initiation and maintenance of viral DNA replication; several members of this group are enzymes involved in various facets of DNA metabolism.

Concomitant with the onset of viral DNA replication another large class of RNA transcripts is synthesized: the late, or γ , mRNA's. Late genes, like early genes, are interspersed throughout the viral genome (Swanstrom and Wagner, 1974; Clements *et al.*, 1977; Jones and Roizman, 1979). They code almost exclusively for viral structural proteins, large quantities of which accumulate in the cell from 12-17 hours post-infection.

The synthesis of γ polypeptides also coincides with a rapid decline in β polypeptide synthesis (Hones and Roizman, 1974, 1975), suggesting that one or more polypeptides from the former group inhibit the synthesis of the latter group of proteins.

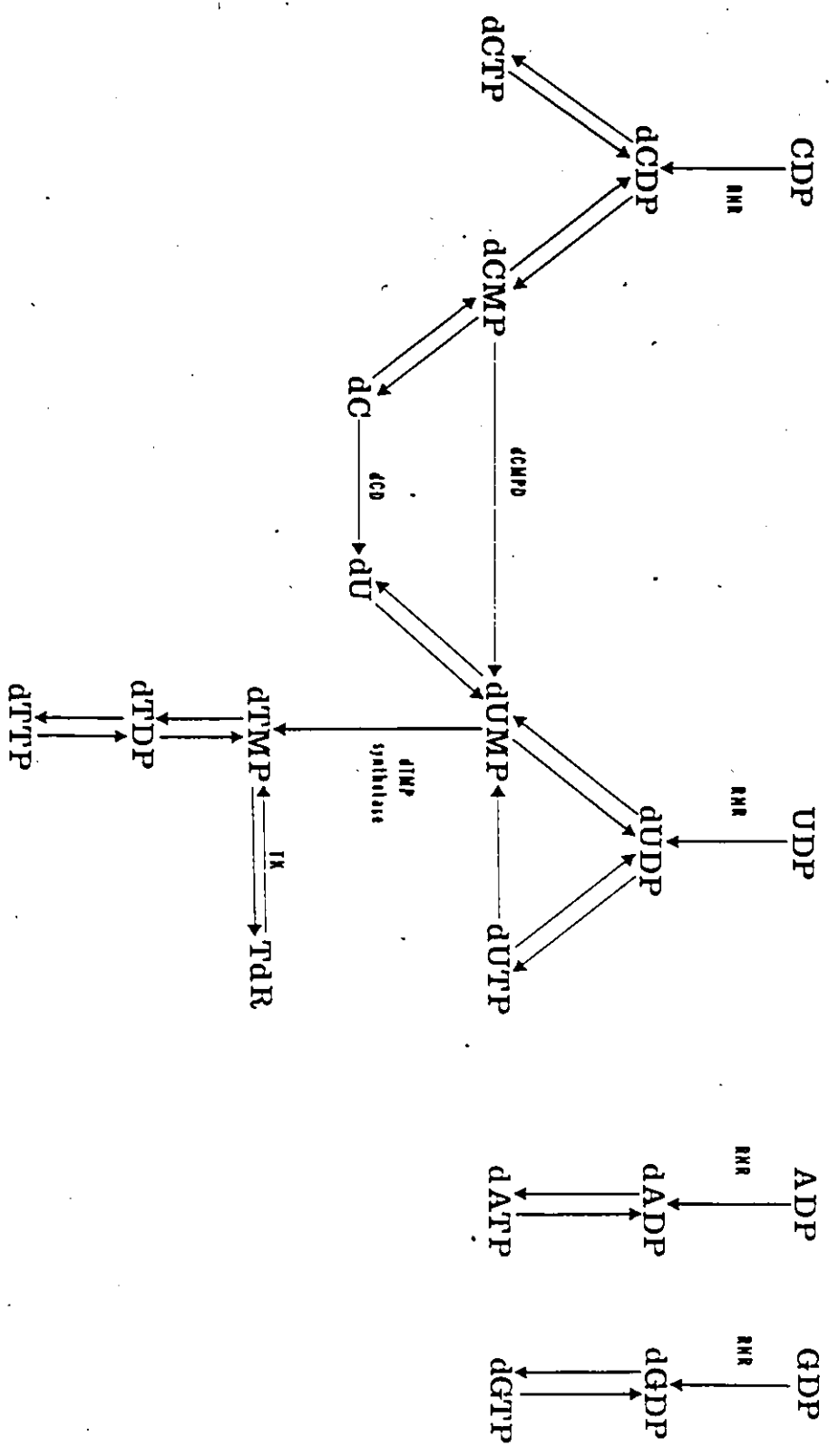
1.1.5. Viral enzymes of DNA metabolism

As already mentioned, the early, or β , class of polypeptides is synthesized maximally prior to viral DNA synthesis and includes several functions involved in DNA replication. At least three virally coded enzymes which participate in DNA metabolism belong to this group: thymidine kinase, DNA polymerase, and alkaline DNAase, all three of which differ biochemically and immunologically from their cellular counterparts.

Thymidine kinase (TK), an enzyme of the salvage pathway of thymidylate synthesis, phosphorylates thymidine to the monophosphate form (see Fig. 2). The viral enzyme has a broader substrate specificity than the cellular TK, and can phosphorylate deoxycytidine (Jamieson *et al.*, 1974) as well as several nucleoside analogues (Prusoff, 1981). In addition the viral TK is more resistant than the cellular enzyme to feedback inhibition by dTTP (Klemperer *et al.*, 1967). Under most conditions of cell culture TK is a non-essential viral function, i.e. TK⁻ viral mutants are viable, however in serum-starved cells the enzyme becomes essential for viral growth (Jamieson *et al.*, 1974). In addition TK appears to play a role in the establishment of viral latency, of which more will be discussed later. Lastly, the availability of a powerful selection system for both the TK⁺ (HAT medium (Littlefield, 1964)) and TK⁻ (BrdU (Dubbs and Kit, 1964)) phenotype, as well as the availability of TK⁻

Figure 2

Schematic outline of nucleotide metabolism. The diagram indicates those pathways and enzymes of nucleotide metabolism which are relevant to this study. Abbreviations: RNR, ribonucleotide reductase; dCMPD, deoxycytidylate deaminase; dCD, deoxycytidine deaminase; TK, thymidine kinase.



cell lines, has rendered this gene a very useful vector, and the enzyme an equally useful genetic marker, in a variety of studies on eucaryotic gene expression.

The HSV coded DNA polymerase can be readily differentiated from the cellular α polymerase by its stimulation at salt concentrations which inhibit the host isozyme (Keir *et al.*, 1966), and its sensitivity to inhibition by phosphonoacetic acid (PAA) and zinc ions (Hones and Watson, 1977; Purifoy and Powell, 1977; Knopf, 1979). In addition, unlike α , β or γ eucaryotic DNA polymerases, but similar to the procaryotic enzymes and to the mammalian polymerase δ (Kornberg, 1980), purified preparations of the HSV enzyme are tightly associated with a 3' to 5' exonuclease activity (Weissbach *et al.*, 1973; Knopf, 1979). It has not yet been determined whether this nuclease provides a proof reading function as in the procaryotic systems. The K_m values of deoxyribonucleotide triphosphates for the viral polymerase are approximately one log lower than that of the human enzyme (Ostrander and Cheng, 1980), indicating that the viral enzyme appears to have an advantage over the cellular one in securing DNA precursors. The HSV polymerase is an essential function (Aron *et al.*, 1975; Purifoy and Benyesh-Melnick, 1975) whose coding sequences have been located in the viral genome by correlating viral temperature sensitive mutants with *in vitro* thermolability of the purified enzyme (Purifoy *et al.*, 1977). Interestingly, although the enzyme is active *in vitro* as a monomer, temperature sensitive mutants in two distinct complementation groups of HSV-1 have been found to specify a thermolabile polymerase (Purifoy and Powell, 1981). The implications of this data for the *in vivo* structure and

function of the enzyme have not yet been elucidated.

The HSV alkaline DNAase, less well characterized than the other two viral enzymes, has a pH optimum of about 8.2 and exhibits both endonuclease and exonuclease activity (Morrison and Keir, 1968; Hoffmann and Cheng, 1978). A temperature sensitive viral mutant specifying a thermolabile DNAase activity has been identified and used to map the enzyme in the viral genome (Francke *et al.*, 1978; Moss *et al.*, 1979). The DNAase appears to be a non-essential viral function (Moss *et al.*, 1979) whose role in viral replication remains speculative. It has been suggested, by analogy with phages such as T7, that the nuclease may serve to degrade host DNA to provide an additional source of nucleotides for viral DNA synthesis; in addition, or alternatively, the enzyme could play a role in the processing of viral DNA during replication.

In addition to those described above, several other enzymes of DNA metabolism have been reported to be induced following HSV infection. Included in this group are ribonucleotide reductase, deoxycytidine deaminase, and deoxycytidylate deaminase. The reactions catalyzed by these enzymes are illustrated in Fig. 2, and discussed in greater detail in section 1.2. and 1.4. of the Introduction. At the time this project was initiated, little more was known about these enzymes than that their activities increased following infection and that they differed in some respects from the corresponding uninfected cell enzymes. As described in the Results, following an initial screening of infected cells we were able to detect only the induction of ribonucleotide reductase. We thus focussed our attention exclusively on this enzyme, to which a detailed introduction is provided in the following section.

1.2. Ribonucleotide Reductase

1.2.1. General characteristics

DNA synthesis requires a continuous and balanced supply of the four deoxyribonucleotide triphosphate (dNTP's) which originate from the reduction of ribonucleotides. Ribonucleotide reductase, found in all procaryotic and eucaryotic cells synthesizing DNA, is the sole enzyme responsible for the conversion. For the well characterized procaryotic enzymes it has been demonstrated that all four ribonucleotides are reduced at the same catalytic site of the enzyme. The specificity of this site for each substrate is modulated by the binding of the end products of the reaction (dNTP's) to allosteric sites on the enzyme. In mammalian cells the substrates for the reaction are ribonucleoside diphosphates. As shown in Fig. 2, following their conversion to deoxyribonucleotides only a single kinase step is required to convert them into dNTP's, with the exception of dTTP which arises from reduction of either CDP or UDP and introduction of a methyl group at the monophosphate level. The reduction itself occurs by replacement of the 2' OH group in the ribose moiety of the ribonucleotide by hydrogen. The ultimate hydrogen donor for the reaction is NADPH, and two small molecules have been identified which can independently act as the hydrogen carriers: thioredoxin and glutaredoxin. These molecules are regenerated to their reduced forms, following oxidation, by thioredoxin reductase and by glutathione and glutathione reductase, respectively. For enzymatic assays, the physiological hydrogen transport systems are usually substituted by dithiols, such as dithiothreitol, which act as direct hydrogen donors for the ribonucleotide reductase (Thelander and Reichard, 1979;

Holmgren, 1981).

A tight correlation exists between ribonucleotide reduction and DNA replication in many systems, suggesting a rate-limiting role for the enzyme in the regulation of DNA synthesis (Larsson, 1969; Elford, 1972; Milland, 1972; Noronha *et al.*, 1972; Peterson and Moore, 1976; Lewis *et al.*, 1981; Takeda and Weber, 1981). The activity of ribonucleotide reductase fluctuates in a cell cycle dependent manner, being virtually undetectable in resting cells and increasing dramatically just prior to S phase (Peterson and Moore, 1976; Lewis *et al.*, 1981). Concomitantly, dNTP's, the intracellular concentrations of which limit the capacity of the cell to undergo DNA replication (Holmgren, 1981), are extremely low in resting cells but increase significantly before each round of DNA synthesis and are continuously synthesized during S phase (Nordenskjold *et al.*, 1970; Skoog and Nordenskjold, 1971; Skoog *et al.*, 1973). Recent evidence from both procaryotic and eucaryotic systems indicate that ribonucleotide reductase may function as part of a multienzyme complex in functional as well as physical association with several other enzymes of nucleotide metabolism (Allen *et al.*, 1979; Prem Veer Reddy and Pardee, 1980). The data suggest that the complex might serve to channel dNTP's to replication forks in the DNA, thus creating high localized concentrations of DNA precursors at their sites of utilization.

Two distinct classes of ribonucleotide reductase have been identified. One class is represented by the enzyme from *Lactobacillus leichmannii*, a monomer of molecular weight 76,000 which requires adenosylcobalamin (coenzyme B12) as a cofactor, and uses ribonucleoside triphosphates as substrates (Blakely, 1965; Panagou *et al.*, 1972;

Chen *et al.*, 1974). Members of this class are common among the procaryotes but are rare in eucaryotes, having only been observed in the *Euglenophyta* and the fungus *Phytomyces chaptarium* (Thelander and Reichard, 1979). The other class of enzymes, represented by the reductase from *Escherichia coli*, is found in all organisms not containing the B12 dependent enzyme, i.e. some procaryotes, most eucaryotes and all mammalian cells examined to date. The *E. coli* enzyme is a 240,000 dalton dimer of two non-identical subunits, both of which are required for activity (Brown *et al.*, 1967; Brown *et al.*, 1969a; Thelander, 1973). Since the mammalian reductase is closely related to the *E. coli* enzyme, and since the HSV induced reductase has been shown to be sensitive to hydroxyurea (Langelier and Buttin, 1981), a phenomenon typical of the *E. coli* type of enzymes (Thelander and Reichard, 1979), further discussion on the structure and properties of the reductase will be restricted to this class of enzymes.

1.2.2. *E. coli* ribonucleotide reductase

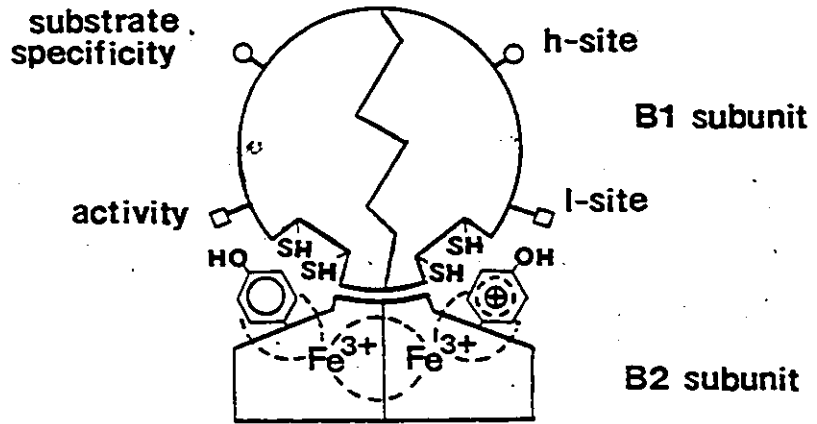
The two subunits of the *E. coli* enzyme, referred to as B1 (M.W. 160,000) and B2 (M.W. 78,000) each consist of two apparently identical polypeptide chains (Thelander, 1973; see Fig. 3a). The subunits are coded for by two closely linked genes (Fuchs *et al.*, 1973; Bachman *et al.*, 1976), and are weakly bound together by Mg^{2+} in a 1:1 stoichiometry (Brown and Reichard, 1969a). Both subunits have been purified to homogeneity and extensively characterized. Subunit B1 contains binding sites for the substrates (ribonucleoside diphosphates) as well as for the allosteric effectors (nucleoside

Figure 3

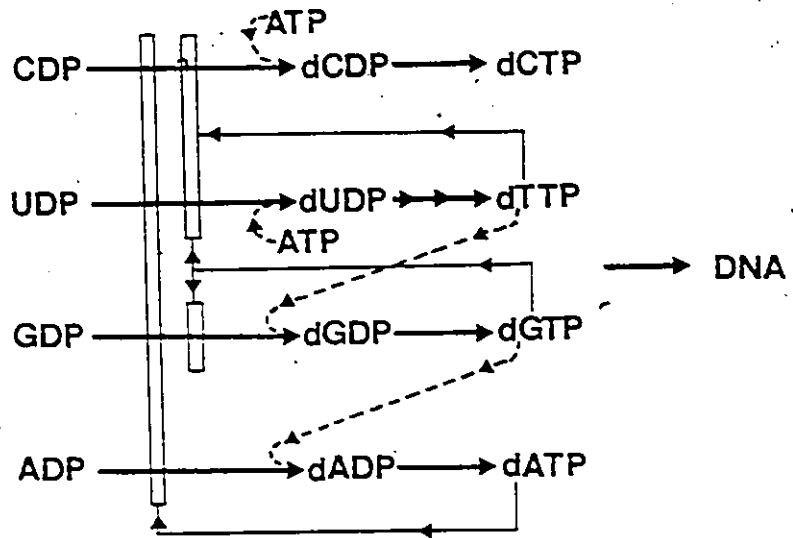
- (a) Schematic model of the structure of the *E. coli* ribonucleotide reductase. The enzyme consists of two subunits (B1 and B2) held together by Mg^{2+} and each consisting of two apparently identical polypeptide chains. B1 contains three classes of nucleotide binding sites. One class binds the nucleotide diphosphate substrates; there are two substrate binding sites per B1 subunit, each of which binds all four substrates. The other two classes of binding sites, designated h and l sites, bind the allosteric effectors and each consist of two sites. The h sites bind ATP, dATP, dTTP and dGTP which modulate the substrate specificity of the enzyme; binding of ATP or dATP to the l sites, on the other hand, regulates the overall level of enzyme activity. The B2 subunit contains 2 atoms of iron, in a binuclear complex, which apparently act to generate and stabilize a tyrosine free radical which is localized over the aromatic ring of the amino acid, and which forms part of the active site of the enzyme.
- (b) Scheme of the proposed physiological regulation of ribonucleotide reduction in mammalian cells. The broken arrows represent positive effects, the open bars negative effects.

Both diagrams taken from Thelander and Reichard (1979).

(a)



(b)



triphosphates) (Brown and Reichard, 1969b; van DobeIn and Reichard, 1976). These latter sites can be differentiated into two classes on the basis of their affinity for dATP (Brown and Reichard, 1969a, 1969b). The high affinity (h) sites bind the allosteric effectors ATP, dTTP, dGTP, and dATP which modulate the substrate specificity of the enzyme apparently by inducing conformational changes at the catalytic site which in turn results in the preferential binding of one substrate. The low affinity (l) sites bind only the effectors ATP and dATP which determine the overall activity of the enzyme in a manner somewhat analogous to an "on/off" switch (i.e. binding of ATP turns on enzyme activity whereas dATP is inhibitory). Although the diagram in Fig. 3a depicts a symmetrical distribution of effector binding sites, their actual location on the B1 subunit has not been determined. The B1 subunit also contains oxidation-reduction active sulfhydryls in the catalytic site (see Figure 3a) which can reduce stoichiometric amounts of substrate in the absence of an external hydrogen donor (Thelander, 1974). They are then oxidized to a disulfide which can be reversibly reduced by thioredoxin, glutaredoxin or an artificial hydrogen donor.

Subunit B2 contains two atoms of bound iron as well as a tyrosyl free radical (Brown *et al.*, 1969b; Ehrenberg and Reichard, 1972; Sjoberg *et al.*, 1977). It is apparently the function of the iron to generate and stabilize this free radical which is localized over the aromatic ring of the amino acid (as shown in Fig. 3a) and is involved in enzyme activity (Brown *et al.*, 1969b; Ehrenberg and Reichard, 1972). Ribonucleotide reductase inhibitors such as hydroxyurea, guanazole and hydroxylamine act

by selectively destroying the B2 free radical (Krakoff *et al.*, 1968; Brown *et al.*, 1969b).

1.2.3. T4 ribonucleotide reductase

Infection of *E. coli* with the bacteriophages T2, T4, T5 and T6 results in the synthesis of a virally coded ribonucleotide reductase (Biswas *et al.*, 1965; Berglund *et al.*, 1969; Eriksson and Berglund, 1974). The best characterized of these enzymes is specified by T4, and is very similar to the *E. coli* enzyme. T4 reductase is a 225,000 molecular weight dimer of non-identical subunits each composed of two polypeptide chains; the larger subunit contains binding sites for allosteric effectors and ribonucleoside diphosphate substrates, the smaller one contains iron and a free radical (Berglund, 1972a; Berglund, 1975). The allosteric regulation of T4 reductase activity by positive effectors is very similar to that of the *E. coli* enzyme. The most significant difference is that the activity of the T4 enzyme is not switched off by dATP (Berglund, 1972b). This difference has been interpreted as indicating that the regulatory subunit of the phage enzyme lacks activity, or 1; sites (see Fig. 3). Presumably this modification is geared towards the requirements of phage replication, where a single large burst of DNA replication (approximately 10 fold the host level (Kornberg, 1980)) is required for synthesis of progeny phage genomes. The other significant difference between the host and phage enzymes is that the T4 reductase does not require Mg^{2+} ions for activity (Berglund, 1972a), reflecting a tighter binding of its two subunits than in the *E. coli* enzyme.

The polypeptides constituting the subunits of the phage enzyme

are coded for by two closely linked genes on the T4 chromosome, nrd A and nrd B; mutation of these genes by hydroxylamine has demonstrated that the reductase is not an essential phage function (Yeh *et al.*, 1969). There is also a third, unlinked gene associated with phage reductase activity, nrd-C, which encodes a thioredoxin (Yeh and Tessman, 1972). The T4 reductase shows specificity for this thioredoxin and is unable to use the bacterial analogue as a hydrogen donor (Berglund and Sjoberg, 1970).

Lastly it is worth noting that T4 also codes for a number of other enzymes of DNA metabolism (including thymidine kinase, deoxycytidylate deaminase, DNA polymerase, and several DNAases) indicating that the phage is fairly self sufficient in this process. Indeed, as far as nucleotide metabolism is concerned, T4 is almost completely independent of host functions in synthesizing DNA precursors. Only two bacterial enzymes are required: the nucleoside diphosphate kinase and deoxyadenylate kinase (Kornberg, 1980).

1.2.4. Mammalian ribonucleotide reductase

The mammalian reductase has not been as well characterized as the procaryotic enzyme, but it resembles the *E. coli* reductase in both its structural and regulatory features. Molecular weight estimates of the mammalian enzyme vary between approximately 200,000 and 300,000 daltons, and the molecule consists of two subunits, usually called M1 and M2, neither of which is active alone (Moore, 1977; Cory *et al.*, 1978; Chang and Cheng, 1979; Thelander *et al.*, 1980; Youdale *et al.*, 1982). The M1 subunit, which has been purified to homogeneity, contains binding sites for allosteric effectors and the ribonucleoside diphosphate substrates

(Thelander *et al.*, 1980; Eriksson *et al.*, 1981; Eriksson *et al.*, 1982), and appears to consist of a dimer of polypeptides of approximately 90,000 daltons (Moore 1977; Thelander *et al.*, 1980). The M2 component has not yet been obtained in a pure form, but it too is a dimer, and contains iron as well as a tyrosine free radical (Graslund *et al.*, 1982).

Although characterization of the calf thymus ribonucleotide reductase has demonstrated that highly purified enzyme preparations are capable of reducing all four substrates (Engstrom *et al.*, 1979; Eriksson *et al.*, 1979), Youdale *et al.*, (1982) have recently purified from rat liver cells a 45,000 dalton polypeptide, analogous to the M1 subunit, which reduces only CDP when combined with the M2 subunit. On the basis of their data, Youdale *et al.*, (1982) postulate that mammalian cells may contain 4 different polypeptides of 45,000 daltons each specific for reduction of a single substrate, and that the M1 subunit may thus consist of various combinations of these 4 components. Further characterization of the rat liver reductase is required to substantiate this hypothesis, however it should be noted that some data from earlier studies in other systems have also indicated that there may be multiple forms of the mammalian enzyme (reviewed by Wright, 1983).

Nucleotide binding studies with the purified calf thymus M1 subunit (Eriksson *et al.*, 1979; Thelander *et al.*, 1980), and characterization of mutant mouse M1 proteins (Eriksson *et al.*, 1981), has demonstrated that as in the *E. coli* enzyme there are two classes of effector binding sites: one class binding ATP and dATP to regulate overall enzyme activity, the other binding ATP, dATP, dTTP and dGTP to modulate substrate specificity. In addition, kinetic studies of the purified calf

thymus enzyme have indicated that the general pattern of allosteric regulation is similar to that of the *E. coli* reductase, but is more refined in that the effectors tend to show a more specific effect in the mammalian system (Eriksson *et al.*, 1979; Thelander *et al.*, 1980). For example, in *E. coli* dTTP stimulates the reduction of GDP and ADP, and binding of dATP to high affinity sites stimulates CDP and UDP reduction (Thelander and Reichard, 1979), whereas for the mammalian reductase dTTP stimulates reduction of only GDP, and dATP is only a negative effector. In both systems the multiplicity of effectors and the presence of two effector binding sites allows the enzymes to assume a large number of conformations with differing activities. The conformational states of the mammalian enzyme have been integrated into the scheme shown in Fig. 3b, which assumes a sequential reduction of the four ribonucleotides *in vivo* (Thelander and Reichard, 1979). The ATP activated enzyme reduces CDP and UDP until the accumulation of dTTP inhibits reduction of the first two substrates and shifts the enzyme towards reduction of GDP. In turn dGTP inhibits the reduction of GDP, UDP and CDP and the dGTP activated enzyme reduces ADP. High concentrations of dATP completely inhibit enzyme activity, but this inhibition is reversible by binding of ATP.

Although the scheme depicted in Fig. 3b is based upon data derived from *in vitro* enzyme studies, it does appear to be consistent with *in vivo* observations. For example, cell lines containing an altered reductase with decreased sensitivity to inhibition by dATP are more resistant to high concentrations of deoxyadenosine and contain larger

than normal dNTP pools (Meuth *et al.*, 1976). In addition, treatment of cells with excess thymidine (i.e. "thymidine blockage" of DNA synthesis) results in an increase in dGTP and dATP pools but in a specific depletion of dCTP pools (Bjursell and Reichard, 1973).

1.2.5. Association of a mutator function with mammalian ribonucleotide reductase

Even though DNA synthesis requires an approximately equal supply of all four precursors, there are large differences in the sizes of the four dNTP pools in S phase cells (Skoog *et al.*, 1973). The concentration of the largest pool, usually dCTP, can exceed that of the smallest pool, dGTP, by as much as 100 fold. While the significance of these differences is not yet understood, the maintenance of these ratios is clearly important in preserving the fidelity of DNA replication. Perturbations in the relative sizes of the pools, induced either by experimental manipulation of the pools or by the presence of a mutant ribonucleotide reductase altered in its regulatory control, leads to an increase in the spontaneous mutation rate at several loci (Meuth *et al.*, 1979; Chan *et al.*, 1981; Weinberg *et al.*, 1981). In both cases, the mutator phenotype can be eliminated by experimentally normalizing the pool ratios (Weinberg *et al.*, 1981). The exact mechanism of mutagenesis is not yet known, however by analogy with more detailed data on the effect of nucleotide pool imbalances on the fidelity of procaryotic DNA replication (Weymouth and Loeb, 1978; Fersht, 1979; Hibner and Alberts, 1980), it likely involves an increased frequency of base misincorporation and/or induction of an error prone repair system. In addition, in

mammalian cells dCTP has been implicated as a positive effector of DNA synthesis (Bjursell and Reichard, 1973), and it has been suggested that high concentrations of the nucleotide may accelerate DNA polymerization at the expense of accurate base incorporation (Weinberg *et al.*, 1981).

1.3. Herpesvirus Induced Ribonucleotide Reductase

At the time this project was initiated only a few reports were available in the literature on the induction of ribonucleotide reductase by herpesviruses. Both Epstein-Barr virus (EBV) and equine herpesvirus types 1 and 3 (EHV-1 and EHV-3) had been shown capable of replicating in the presence of hydroxyurea, a potent inhibitor of mammalian ribonucleotide reductase (Mele *et al.*, 1974; Allen *et al.*, 1978). EBV and EHV-1 were subsequently shown to induce a ribonucleotide reductase activity which was resistant to hydroxyurea *in vitro* (Cohen *et al.*, 1977; Henry *et al.*, 1978). HSV replication, on the other hand, is sensitive to hydroxyurea (and more recent experiments have demonstrated that the virally induced reductase is sensitive to the drug *in vitro* (Langelier and Buttin, 1981)) however the virus was found to induce a reductase whose activity, in crude extracts, was refractory to allosteric inhibition by dTTP (Cohen, 1972). Ponce de Leon *et al.*, (1977) partially purified the reductase from uninfected, HSV-1 infected, and HSV-2 infected KB cells by fractionation of cell extracts with ammonium sulfate. The enzymes from all three sources copurified but were found to differ in two significant respects: 1) the virally induced enzymes were inhibited by only approximately 20% at concentrations of dTTP which inhibited the KB enzyme by 60-75%, and 2) the HSV induced enzymes retained 50-60% of their activity

in the absence of exogenous Mg^{2+} , whereas the cellular enzyme was completely inhibited in the absence of the ion. Interestingly, the properties of the HSV induced reductase thus appear to be similar to those of the T4 coded enzyme which is also altered in its allosteric properties and does not require exogenous Mg^{2+} for activity (see section 1.2.3.). Shortly after work on this project had begun, another report appeared in the literature confirming the induction of a dTTP resistant reductase in HSV infected hamster cells and demonstrating that the enzyme activity present in crude extracts of infected cells is also resistant to inhibition by dATP (Langelier *et al.*, 1978).

1.4. Herpes Simplex Virus Induced Deaminase Activity

As described in the Results, in our early studies we screened HSV infected cells also for the induction of deoxycytidine deaminase (dCD) and deoxycytidylate deaminase (dCMPD) activity, since both enzymes had been reported to be induced following infection. dCD, an enzyme of the salvage pathway of nucleotide synthesis, deaminates deoxycytidine to deoxyuridine, which can then be metabolized to dTTP (see Fig. 2). dCMPD performs an analogous deamination at the monophosphate level, converting dCMP to dUMP, and is part of the *de novo* pathway of thymidylate synthesis (see Fig. 2).

Chan (1977) reported the induction of a dCD activity in several different HSV-1 infected cell lines, including a dCD deficient mouse line. The induced enzyme was more thermolabile than the enzyme from a mouse line expressing dCD, and appeared to differ from the enzyme from Hep-2 cells

in its electrophoretic mobility. A more recent publication has also described an apparent increase in dCD activity following HSV-1 infection of mammalian cells (North and Matthews, 1981).

Rolton and Keir (1974b) described a two-fold enhancement of dCMPD activity in HSV-1 infected BHK-21 cl.13 cells; the induced enzyme differed only slightly from the uninfected cell enzyme, most notably in its heat stability and in its susceptibility to inhibition by HSV specific antiserum. To date this is the only report of dCMPD induction by HSV.

1.5. Rationale for the Study.

This study has been focussed on HSV-induced enzymes of nucleotide metabolism. The primary motivation for studying these enzymes is to gain a better understanding of HSV replication and consequently of the various biological properties of the virus which are dependent upon it.

As described earlier, replication of DNA, whether of cells or viruses, is intimately connected to the supply of dNTP precursors and is thus dependent on the activity of several enzymes of nucleotide metabolism. In the case of a virus such as HSV, which, as mentioned earlier, rapidly shuts off host protein synthesis, at least some of these enzymatic activities are likely to be virally coded, as is indeed the case for thymidine kinase. The requirement for viral enzymes may not be absolute when viral replication takes place in dividing cells, which may still contain significant levels of endogenous enzyme activity and dNTP's, but the ability of HSV to replicate in quiescent, non-dividing cells (Jamieson *et al.*, 1974), implies the synthesis of at least a few key viral enzymatic activities. In other words, a comprehensive understanding of HSV replication necessitates the identification and characterization of viral enzymes which play a role in this process.

Apart from the information to be derived, from such studies, on the basic biology of HSV replication, characterization of viral enzymes might also provide relevant data for the treatment of viral infections. In fact all of the available antiviral compounds which show specificity for HSV (e.g., the nucleoside analogues acycloguanosine, adenosine arabinoside, thymidine arabinoside and bromovinyldeoxyuridine) exploit differences in substrate specificity and/or sensitivity between viral and cellular enzymes to selectively inhibit HSV replication (North and Cohen, 1979; Schnipper and Crumpacher, 1980; Elion *et al.*, 1981). The identification of additional enzymes involved in viral DNA metabolism, and an elucidation of their properties, may allow the development of more effective strategies of antiviral chemotherapy, perhaps through combinations of drugs aimed at more than one viral enzyme.

As a result of their involvement in viral replication, enzymes of nucleotide metabolism are also likely to play a role in the process of HSV latency and reactivation. Indeed several studies, carried out in mice and guinea pigs, indicate that TK⁻ viral mutants are unable to replicate in neurons and that no induction of latent TK⁻ virus is observed in explants of ganglia cells from animals infected with the mutant virus (Tenser and Dunstan, 1979; Klein *et al.*, 1981; Price and Khan, 1981). These data have been interpreted as indicating that TK is essential for viral replication in neurons and that replication, in turn, is essential either for the establishment of latency (Tenser and Dunstan, 1979) or for the reactivation of latent virus (Price and Khan, 1981). The requirement for TK activity likely derives from the fact that the neurons of adult

animals are non-dividing cells which contain very low levels of DNA precursors and no detectable TK activity (Yamogami *et al.*, 1972; Price and Khan, 1981), and, as discussed earlier, TK is essential for viral replication in similarly quiescent serum-starved cells. These observations suggest that HSV may require other enzymatic activities, particularly an enzyme like ribonucleotide reductase, to provide the necessary DNA precursors for replication in neurons.

With regard to the HSV induced ribonucleotide reductase, the information available on the enzyme suggested that it is altered, relative to the cellular enzyme, in its regulatory control (see section 1.3. of the Introduction). As described in section 1.2.5. these alterations might render the virally induced enzyme a mutator function. The possibility that this property is relevant to HSV mediated cell transformation, with regard to a hit and run mechanism, is an appealing hypothesis which is discussed further in the Discussion.

Lastly, our decision to screen HSV infected cells for the induction of ribonucleotide reductase, dCD and dCMPD was based also on the possibility of devising a selection system for each enzyme which might allow transformation of cells and thus facilitate genomic mapping and genetic studies of the enzymes. For selection of dCD it was anticipated that cytidine arabinoside might be a useful selective agent, since the nucleoside analog is deaminated by the enzyme to a non-toxic form (Steuart and Burke, 1971; Meyers *et al.*, 1973). Transformation of cells lacking endogenous dCD activity, such as BHK21 cl.13 cells (Cullen and Bich, 1978), with viral DNA sequences encoding the enzyme should thus enhance the survival of these cells in the presence of ara-c. Selection for dCMPD was to be

carried out on the *La1* hamster cell line lacking both dCMPD and dCD activity (Langelier *et al.*, 1978) in the presence of HAM medium (containing hypoxanthine, aminopterin and 5-methyldeoxycytidine (Chan *et al.*, 1975). Aminopterin inhibits thymidylate synthetase (see Fig. 2), thus blocking the *de novo* pathway of thymidylate synthesis, and the scavenger pathway, via TK, can not provide sufficient dTTP to sustain cell growth (Chan *et al.*, 1975). Only cells which have acquired a dCMPD can bypass the aminopterin block by direct deamination of 5-methyl dCMP (derived from 5-methyl dC by deoxycytidine kinase mediated phosphorylation, see Fig. 2) to dTMP.

In the case of ribonucleotide reductase, it was expected that the differences in regulatory control between the virally induced and cellular enzymes could be exploited to prevent expression of the latter without affecting the former activity. In the presence of excess deoxyadenosine, or thymidine, or both, only cells containing the virally induced reductase should be capable of replicating since the endogenous enzyme would be inhibited by the phosphorylated derivatives of the nucleosides. This selection system has the inherent advantage that it could be used on virtually any kind of recipient cell bearing a wild type reductase activity, with no requirement for a mutated cell line, unlike the selection system for TK⁺ cells, or for the enzymes described above. This raised the possibility that the putative HSV reductase could be used as a universal vector for gene transfer into a wide variety of cell types.

2. MATERIALS AND METHODS

2.1. Cells

Baby hamster kidney 21 clone 13 cells (BHK21 cl. 13) and African green monkey kidney cells (Vero) were obtained from Flow Laboratories and grown in monolayer cultures in Dulbecco modified and F-15 modified minimal essential media, respectively (Grand Island Biological Company (GIBCO)). Human KB cells, obtained from Dr. F. Graham (McMaster University), mouse L cells, obtained from Dr. L. Prevec (McMaster University), and human 143 TK⁻ mutant cells (Bacchetti and Graham, 1977) were grown on monolayers in α -modified minimum essential medium (GIBCO). All cell lines were grown in plastic bottles (Corning) or plates (Lux Scientific Corp.) and subcultured by trypsinization with trypsin-EDTA (GIBCO).

BHK21 cl. 13 modified for suspension culture (Banerjee and Rhodes, 1973) were obtained from Dr. H. Ghosh (McMaster University) and grown in Joklik modified minimal essential medium (GIBCO). Suspension cultures were diluted to a cell density of 2×10^5 cells/ml and allowed to grow to a density of 1×10^6 cells/ml prior to subculturing.

All media were supplemented with 5% v/v heat-inactivated calf serum (GIBCO), 100 units/ml penicillin (GIBCO), 100 μ g/ml streptomycin (GIBCO), 0.03% w/v l-glutamine (GIBCO), 0.075% w/v

NaHCO₃ and 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid):

2.2. Virus.

For preparation of virus stocks, HSV-2 strains 219 and 333 (Seth *et al.*, 1974), HSV-1 cl. 101 and the B2006 TK⁻ mutant derived from it (Dubbs and Kit, 1964) and HSV-1 KOS (Seth *et al.*, 1974) were propagated in Vero cells. The cells were infected at a multiplicity of infection (M.O.I.) of 0.2 to 0.5 plaque forming units (PFU)/cell and harvested by scraping with a rubber policeman at a time (usually 24 hrs) corresponding to maximal viral cytopathic effect. The cells were pelleted by centrifugation, resuspended in medium and virus was released from the cells by freezing and thawing followed by sonication of the cell suspension. After removal of cell debris by centrifugation, the clarified supernatant was aliquoted and stored at -70°C. Virus titers were determined by plaque assay on Vero cell monolayers. Tenfold serial dilutions of the virus stocks were plated on the cells, allowed to adsorb for 1 hr, and were then overlaid with 2% agar noble (Difco Laboratories): 2 X F11 modified minimal essential medium (GIBCO) (1:1, v/v). The medium was supplemented with 20% v/v heat-inactivated fetal calf serum (GIBCO), 200 units/ml penicillin, 200 µgm/ml streptomycin, 0.06% w/v l-glutamine, 0.15% w/v NaHCO₃, 20 mM Hepes and 0.08% protamine sulfate (Sigma). Plaques were allowed to develop at 37°C until visible to the naked eye (approximately 3 days) at which point the cells were fixed with Carnoy's fixative

(methanol:glacial acetic acid, 3:1, v/v) and stained with crystal violet.

For experimental purposes, confluent monolayers, or suspension cultures at a density of 0.8 to 1×10^6 cells/ml, were infected at an M.O.I. of 7 to 12 PFU/cell, unless otherwise indicated. Virus was applied to the cells in ice cold medium containing 1% heat-inactivated calf serum and allowed to adsorb to the cells for 1 hr at 37°C , after which prewarmed medium containing 1% serum was added to the infected cultures. The time of addition of the prewarmed medium was taken as the beginning of the infectious cycle (i.e., 0 hrs post-infection).

2.3. Screening of Cells for Mycoplasma Contamination

BHK21 cl. 13 and Vero cells were screened for mycoplasma contamination by the Hoechst staining procedure of Chen (1977). Cells were grown on glass coverslips and fixed with Carnoy's fixative while subconfluent. Following air drying the cells were stained with $0.05 \mu\text{g}/\text{ml}$ Hoechst stain No. 33258 (Sigma) in Hanks' balanced salt solution (GIBCO) for 10 min. The coverslips were then washed 3 times with distilled water, mounted on microscope slides with citric acid mounting medium (22.2 ml 0.1 M citric acid, 27.8 ml 0.2 M Na_2HPO_4 , pH 5.5) and read under a UV fluorescent microscope with a UG-1 Zeiss filter. In uncontaminated cells only the nucleus of the cell was visible under fluorescent light; contaminated cells were identified by the presence of spotty cytoplasmic fluorescence. Reference samples of cells contaminated with mycoplasma were provided by Dr. S.K. Liao (McMaster University).

2.4. Preparation of Crude Extracts and Partial Purification of Enzymes

Infected or uninfected cells were washed twice with phosphate buffered saline (PBS, without Ca^{2+} and Mg^{2+} , GIBCO) and usually frozen as a dry pellet at -20°C . All subsequent steps were carried out at 4°C . The cells were resuspended in buffer A (20 mM HEPES, pH 7.2, 1 mM dithiothreitol (DTT)), sonicated (2 x 30 sec., on ice, at the maximum setting of a Bronwill Biosonik sonicator) and centrifuged at $70,000 \times g$ for 30 min. The supernatant was used as crude extract. In initial experiments the extracts were passed through a column (of volume equal to that of the extract) of the cation-exchange resin AG1X8 (Bio-Rad Laboratories) to remove deoxynucleotides (Peterson and Moore, 1976); this step was omitted in later experiments as it gave little or no increase in reductase activity. Similarly, dialysis of crude extracts did not appreciably enhance enzyme activity.

2.4.1. Ammonium sulfate fractionation of crude extracts

Prior to ammonium sulfate fractionation, nucleic acids were removed from crude extracts by addition of a 5% solution of streptomycin sulfate (dropwise and under continuous stirring) to a final concentration of 0.5%. The suspension was stirred for 20 min more and then centrifuged at $11,000 \times g$ for 10 min. The pellet was discarded, and the supernatant was brought to 27% saturation by the slow addition, under constant stirring, of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ in buffer A. After 45 min of further stirring, the precipitate was pelleted by centrifugation and then dissolved in

0.5 - 1 ml buffer A. The supernatant was then raised to 41% salt saturation, and subsequently to 74% saturation; each time the precipitates were collected and dissolved as described above. The resulting ammonium sulfate fractions were desalted by either 1) filtration through a Sephadex G-25 column (Pharmacia Fine Chemicals), in which case the eluates were concentrated by filtration through Minicon B15 concentrators (Amicon Corp.), or absorption with aquacide 11-A (Calbiochem) or 2) extensive dialysis against buffer A. The partially purified enzyme preparations were stored at -70°C at a protein concentration of approximately 7-13 mg/ml. Protein concentration was determined by the method of Lowry *et al.* (1951).

2.4.2. Sedimentation on glycerol gradients

Enzymatically active salt fractions were further purified by sedimentation through 5-25% glycerol gradients prepared in buffer A containing 6 mM magnesium acetate. Each gradient was loaded with 1-3 mg of protein corresponding to 1000-2500 units of ribonucleotide reductase activity and centrifugation was carried out in the Beckman SW40 Ti rotor at 4°C , initially at 130,000 x g (32,000 rpm) and subsequently at 81,000 x g (25,000 rpm), for 17 hrs. Gradient fractions of 700 μl were collected by pumping from the bottom of the centrifuge tube and were assayed for reductase activity as described in the Results.

2.5. Enzyme Assays

2.5.1. Ribonucleotide reductase

Ribonucleotide reductase was assayed by monitoring the conversion of CDP to dCDP, using a modified version of the assay described by Moore (1967). The standard reaction mixture (in a total volume of 400 μ l for assay of crude extracts and 160 μ l for partially purified extracts) contained 50 mM HEPES, pH 7.2, 0.06 mM FeCl_3 , 2.7 mM magnesium acetate, 8.3 mM NaF, 6.2 mM dithioerythritol (DTE) or dithiothreitol (DTT), 4.4 mM ATP, 0.1 mM CDP and 2.5 μ Ci of [^3H] CDP (specific activity 21-26 Ci/mmol, New England Nuclear Corp.). As assay conditions for partially purified enzymes were optimized in the course of the study, the concentrations of specific cofactors were altered as indicated in the Results. Unless otherwise indicated, the assays contained 1 - 1.5 mg of crude extract protein or 300 - 350 μ g of ammonium sulfate purified protein. After incubation at 37°C for 30 min, the reaction was terminated by addition of HClO_4 to a final concentration of 1 M, and the nucleotides were then converted to the monophosphate form by boiling for 15 min. For analysis of enzyme activity in radiolabelled enzyme samples the HClO_4 precipitated proteins were removed by centrifugation prior to boiling of the samples. Following boiling, the samples were cooled on ice and brought to a pH between 4 and 8 with KOH. The resulting precipitate was pelleted and unlabelled CMP and dCMP were added to the supernatant to final concentrations of 1 mM each as carriers and markers. Aliquots of the supernatant were analysed by descending chromatography

on Whatmann 3 MM paper using EDTA (250 mM): ammonium acetate (5 M): sodium tetraborate (saturated): ethanol (1:20:80:220, by volume) as solvent (Reichard, 1958). Following chromatography for approximately 40 hrs, the chromatograms were air dried and spots corresponding to dCMP were visualized by UV fluorescence, cut out, and the radioactivity quantitated by scintillation counting.

2.5.2. Deoxycytidine and deoxycytidylate deaminase

Deoxycytidine deaminase was assayed according to Chan *et al.* (1975). The reaction mixture contained, in a volume of 200 μ l, 100 mM tris HCl pH 8.1, 0.1 mM EDTA, 0.1 mM DTT, 0.25 mM dC, 1 μ Ci of [3 H] dC (specific activity 20 Ci/mmol, New England Nuclear Corp.) and 200 μ g of crude extract protein. The reaction was incubated for 60 min at 37°C.

Deoxycytidylate deaminase was assayed by a modification of the procedures of Kit *et al.* (1967) and Rolton and Keir (1974a). The reactions were carried out in a volume of 200 μ l, containing 80 mM tris HCl pH 8, 2 mM $MgCl_2$, 0.1 mM dCTP, 5 mM 2-mercaptoethanol, 2 mM dCMP, 1 μ Ci of [3 H] dCMP (specific activity 21 Ci/mmol, New England Nuclear Corp.) and 200 μ g of crude extract protein. Incubation was for 30 min at 37°C.

Following incubation of either assay, 100 μ l of 1N HCl was added to the reactions which were then applied to a 2.5 ml AG 50WX8 column equilibrated with 0.1N HCl. The cation exchange resin binds dC and dCMP, but not their deaminated counterparts, which were recovered and quantitated following elution with 9 ml 0.1N HCl.

2.5.3. Thymidine kinase

Thymidine kinase activity was assayed essentially by the procedure of Munyon *et al.* (1972). In a final volume of 100 μ l, the reaction mixture contained 100 mM tris maleate pH 6.5, 25 mM KCl, 20 mM MgCl₂, 1.4 mM 2-mercaptoethanol, 10 mM ATP, 1 μ Ci of [³H] thymidine (specific activity 20 Ci/mmol, New England Nuclear Corp.) and between 10-50 μ g of crude extract, or ammonium sulfate fractionated, protein. Following incubation for 60 min at 37°C, the reactions were placed on ice and 50 μ l aliquots were spotted on 1.6 cm squares of Whatman DEAE cellulose paper which had been previously wetted with 50 μ l of a 1.2 mg/ml solution of unlabelled thymidine, and then dried. DEAE cellulose is an anion exchanger which binds the negatively charged phosphorylated product of the kinase reaction (TMP) but not the substrate (thymidine). The papers were washed 3 times with 1 mM ammonium formate, twice with water, once with ethanol, and the amount of radioactivity retained was quantitated by scintillation counting.

2.5.4. Alkaline DNAase

Alkaline DNAase was assayed according to Morrison and Keir (1968), except in our experiments the amount of trichloroacetic acid (TCA) precipitable material remaining after enzymatic digestion of DNA was measured. Reactions, of 100 μ l, contained 50 mM tris HCl pH 9, 2 mM MgCl₂, 10 mM 2-mercaptoethanol, 6 μ g of Vero cell DNA (labelled with [³H] thymidine (New England Nuclear Corp.) to a specific activity of \pm 20,000 cpm/ μ g) and 10-100 μ g of protein from enzyme

preparations purified by ammonium sulfate fractionation. Following incubation at 37°C for 45 min, the reactions were placed on ice; calf thymus DNA (50 µg) and 4 ml of ice cold 10% TCA were added and after 15 min, the TCA precipitable material was collected on glass fibre filters and quantitated by scintillation counting.

2.5.5. DNA polymerase

HSV DNA polymerase was assayed according to Keir *et al.* (1966) in a 200 µl reaction containing 50 mM tris HCl pH 8, 3 mM MgCl₂, 1 mM 2-mercaptoethanol, 10 µM each of dATP, dGTP and dTTP, 5 µM dCTP, 1 µCi [³²P] dCTP (specific activity approximately 600 Ci/mmol, New England Nuclear Corp.), 100 µg of heat-denatured calf thymus DNA, 100 mM ammonium sulfate (to selectively stimulate the activity of the viral enzyme and inhibit that of the cellular αDNA polymerase) and 10-100 µg of protein from enzyme preparations purified by ammonium sulfate fractionation. The reactions were incubated at 37°C for 45 min, then transferred to ice and terminated by addition of 4 ml of ice cold 10% TCA. Samples were then processed as described for the DNAase assay.

2.6. Immunization of Rabbits

Two adult male New Zealand rabbits were each injected (subcutaneously and intramuscularly) with 150 µg of the HSV-2 induced enzyme preparation, partially purified by ammonium sulfate fractionation and glycerol gradient sedimentation, in complete Freund's adjuvant. Each animal received three subsequent intravenous boosts at 21 day

intervals with 70 μg of the same antigen, and was bled one week after each boost. Later boosts were delivered at monthly intervals using 120-150 μg of the 0-27% ammonium sulfate fraction from infected cells and the animals were bled at approximately 6, 10 and 14 days after each injection.

2.7. Indirect Immunofluorescence

Vero cells grown to confluence on glass coverslips were mock-infected or infected with HSV-2 at a multiplicity of 0.1 PFU/cell. Twenty-four hrs after infection the medium was removed and the coverslips washed with PBS, fixed with cold acetone for 10 min, washed again, and stored dry at -20°C .

For antibody screening, each coverslip was incubated with 150 μl of serial dilutions of immune serum for 30 min at 37°C , washed with PBS, and incubated again as above with 75 μl of fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories). After further washings with PBS, the coverslips were mounted with tris-glycerol pH 9.6 (1:9 v/v) and scored by microscopy under UV light. Optimal concentrations of reagents were 1:20 for the immune rabbit serum and 1:60 to 1:100 for the goat anti-rabbit IgG.

2.8. Antibody Purification

Purification of IgG was carried out on columns of protein A-sepharose CL-4B beads (Pharmacia Fine Chemicals) by the procedure of Hudson and Hay (1980). The protein A-sepharose beads were swollen in PBS for 1 hr at room temperature and then poured into a column.

Two ml of rabbit serum were diluted with an equal volume of PBS and loaded onto a 5 ml column of protein A-sepharose. The column was washed with 10 ml of PBS and the IgG was subsequently eluted with 4.5 ml of 0.1 M glycine pH 2.5. The glycine eluate was collected as 1 ml fractions in tubes containing 100 μ l of 1M tris-HCl pH 7.5 to neutralize the solution, dialyzed against PBS and concentrated by aquacide to a protein concentration of approximately 30 mg/ml.

2.9. Radiolabelling of Cells, Immunoprecipitation and Gel Electrophoresis

In this study, immunoprecipitations were performed under both denaturing and non-denaturing conditions. The former reactions assessed the specificity of antibodies for the polypeptides present in cell lysates, the latter conditions tested the ability of the antibodies to precipitate ribonucleotide reductase activity.

For the former experiments (shown in Figures 14,15,19,23 and part of 22), monolayers of BHK21 cl. 13 cells growing in 150 cm² tissue culture flasks were mock-infected or infected with 20 PFU/cell of HSV-1 (KOS) or HSV-2 (333). The cells were labelled between 0-7 hrs after infection with 100 μ Ci of L-[³⁵S] methionine (specific activity 900 to 1,100 Ci/mmol, New England Nuclear Corp.) in 5 ml of methionine-free 199 medium (GIBCO) supplemented with 5% dialyzed fetal calf serum. The cells were harvested by scraping, washed three times with PBS and resuspended in RIPA buffer (50 mM tris HCl, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 100 U of aprotinin (Sigma) per ml, 0.1 mM phenylmethylsulfonyl fluoride (Sigma) and 50 μ g of DNAase (Sigma) per ml). The suspension was

sonicated and centrifuged at 100,000 x g for 1 hr. For immunoprecipitation, 500 μ l of the supernatant was incubated with 30-50 μ l of rabbit serum or 10 μ l of ascitic fluid in the presence of approximately 19 mg of protein A-sepharose CL-4B beads for 2 hrs at 4°C under constant mixing. Immunoprecipitates were then collected by centrifugation, washed with RIPA buffer and solubilized in sample buffer (0.37 M tris HCl pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 10% SDS (Laemmli, 1970)). Electrophoresis was carried out on 9% polyacrylamide, 0.24% Bis, 0.1% SDS gels which were run at constant voltage. The gels were stained with Coomassie brilliant blue, destained, infused with 2,5-diphenyloxazole, dried on filter paper and exposed to Kodak X-Omat film at -70°C.

As mentioned above, immunoprecipitations were also performed under non-denaturing conditions to allow assaying of the reaction products for enzymatic activity. These experiments were carried out with both [³⁵S] methionine labelled and unlabelled enzyme preparations partially purified from HSV-2 (333) infected and mock-infected BHK21 cl. 13 cells. Radiolabelling was performed as described above, and cell lysates were prepared and fractionated with ammonium sulfate as described in section 2.4. Aliquots of the enzymatically active salt fractions were mixed with either buffer A or with purified IgG in buffer A, in the presence of approximately 6 mg of protein A-sepharose CL-4B beads. Within each experiment, the final volume of the reactions was the same, and varied from 50-150 μ l between experiments. Following incubation at 4°C for 1.5 - 2 hrs with constant mixing, the beads were removed by centrifugation and the supernatants from each reaction

were assayed for residual reductase activity under standard assay conditions, at the optimal Mg^{2+} and ATP concentrations for the viral and cellular enzymes (see Results). For enzymatic assay of immunoprecipitates the beads were washed twice with buffer A, incubated in the presence of 0.1 M DTT for 15 min at $4^{\circ}C$, and then made up to the enzyme reaction mixture, resulting in a final concentration of 67 mM DTT. Lastly, for electrophoretic analysis of the immunoprecipitates, beads were washed twice with buffer A, the bound polypeptides were solubilized in sample buffer and electrophoresed as described above.

2.10. Neutralization of Ribonucleotide Reductase Activity

Aliquots of the ammonium sulfate purified enzymes were incubated with either buffer A or purified IgG in buffer A for 30 min at room temperature with intermittent mixing. Immediately after incubation the reactions were assayed for reductase activity in the presence of 60 mM DTT and the optimal Mg^{2+} and ATP concentrations for the viral and cellular enzymes.

3. RESULTS

3.1. Characterization of Enzyme Induction

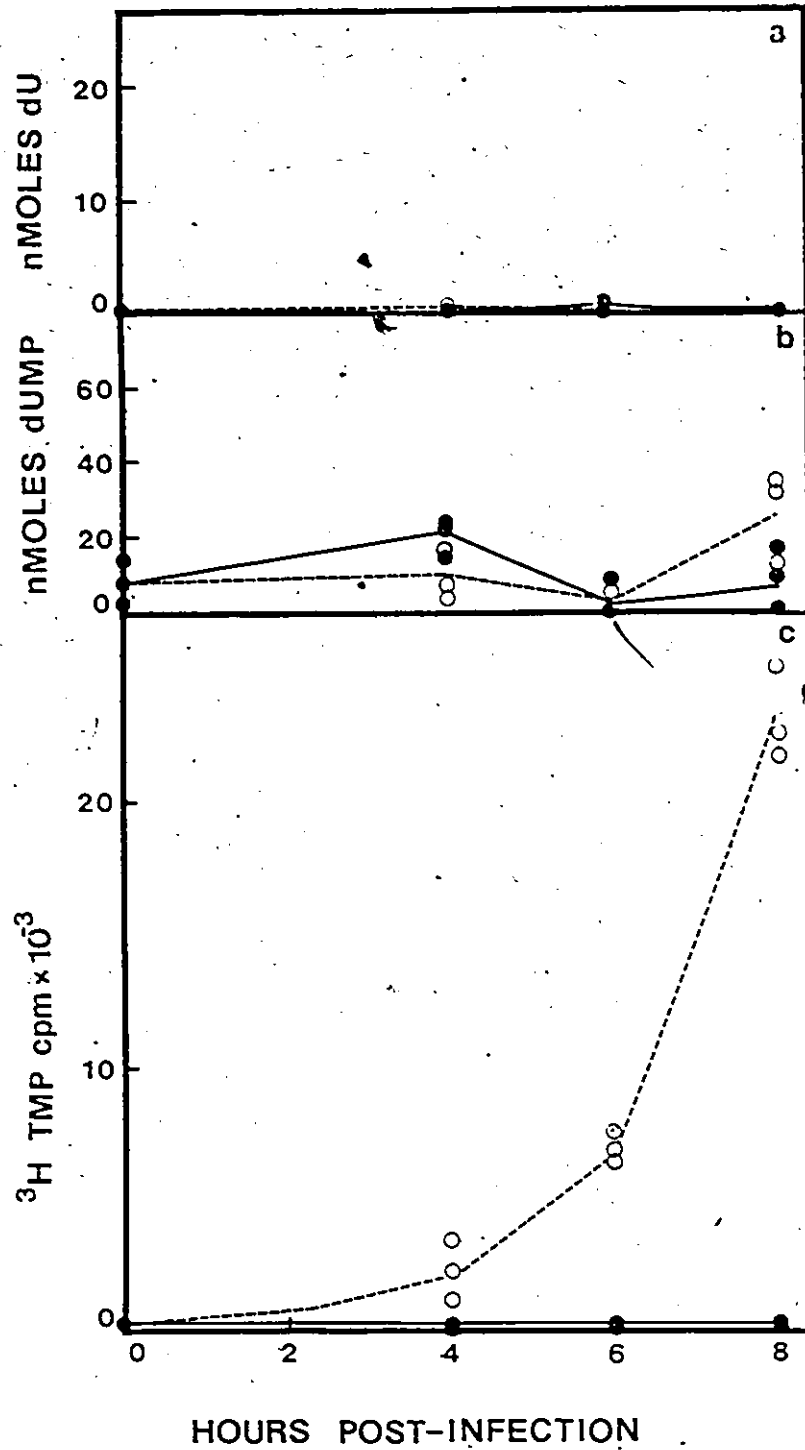
As mentioned in the Introduction, initial experiments were aimed at assessing whether infection of mammalian cells with HSV resulted in the synthesis of elevated levels of ribonucleotide reductase, deoxycytidine deaminase (dCD) or deoxycytidylate deaminase (dCMPD). To this end monolayers of BHK21 cl. 13 cells were infected with either HSV-1 or HSV-2, or mock-infected, harvested at various times from 0 to 10 hours post-infection, and crude extracts were prepared as described in Materials and Methods. Enzymatic activities were determined using assays which had been calibrated and optimized for cellular enzymes in extracts of exponentially growing cells. The 0-10 hour time span for cell harvesting was chosen because the early class of viral polypeptides, to which enzymes such as thymidine kinase, DNA polymerase, and DNAase belong, are synthesized maximally at approximately 5 to 7 hours post infection, prior to the onset of viral DNA synthesis. It thus seemed reasonable to assume that other enzymes involved in DNA metabolism would likely be synthesized at a similar time.

Fig. 4 shows the results of dCD (panel a) and dCMPD (panel b) assays of HSV-2 infected cells; neither deaminase activity was increased under conditions in which TK activity (panel c), assayed as a positive control, was clearly induced. Similar results have been observed in HSV-1 infected cells (not shown). In addition, others have reported a

Figure 4

Deoxycytidine deaminase (panel a), deoxycytidylate deaminase (panel b), and thymidine kinase (panel c) activity in HSV infected cells. Monolayers of BHK21 cl. 13 cells were either mock-infected or infected with HSV-2 at a multiplicity of infection of 8 plaque forming units per cell (M.O.I. of 8), and crude extracts were assayed for enzyme activity. The activity of each enzyme was determined in triplicate, from a single crude extract, for each of the time points indicated. Symbols: (○) enzyme activity in infected cells; (●) enzyme activity in mock-infected cells.

S.



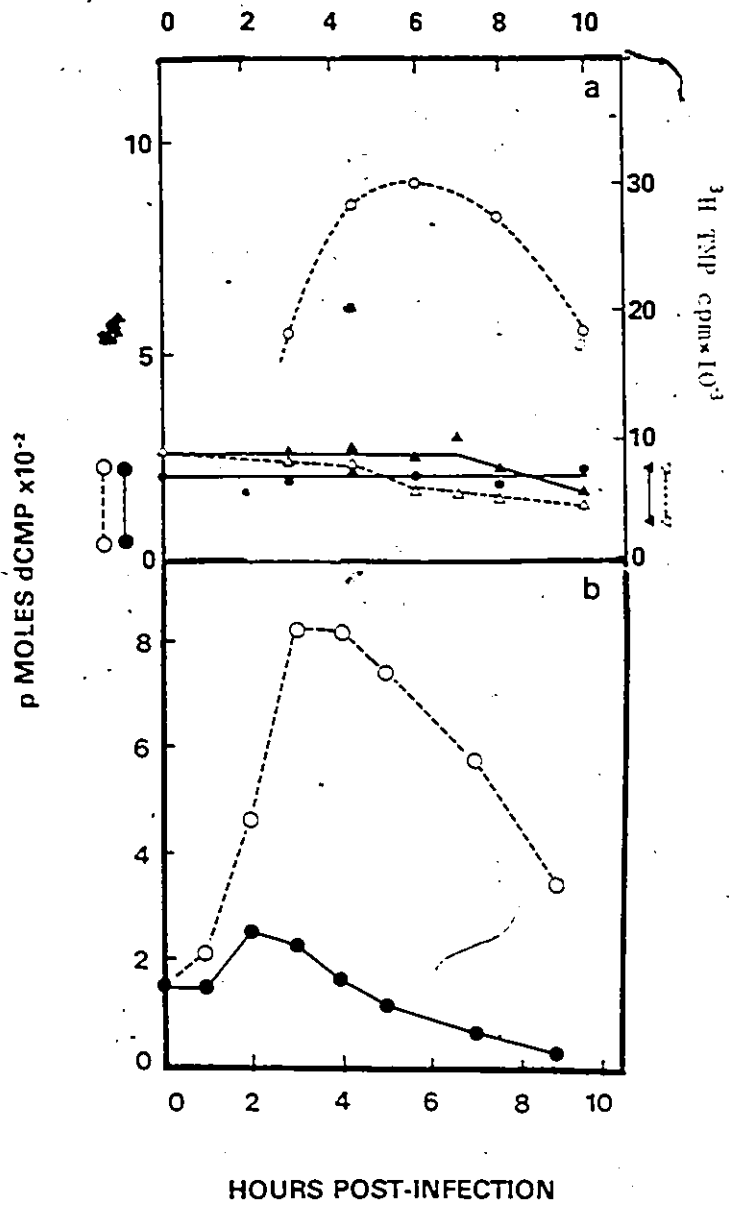
similar inability to detect induction of these enzymes (Langelier *et al.*, 1978; Dutia, personal communication); and the suggestion has been made that the reported induction of dCD (Chan, 1977) may be due to mycoplasma contamination of the virus stocks (Charron and Langelier, 1981).

In contrast to these results, induction of ribonucleotide reductase by both HSV serotypes was consistently observed in these experiments. Shown in Fig. 5a is the time course of induction in HSV-1 infected cells; essentially the same pattern was obtained in HSV-2 infected cells. Enzyme activity was enhanced by 2-4 hours post-infection, attained maximal levels at 6-8 hours post-infection and then gradually declined such that at 10 hours post infection approximately 30-60% of the maximal activity remained. Since large scale production of crude extracts of infected cells was to be carried out using BHK21 cl. 13 cells growing in suspension, the time course of enzyme induction under these conditions was also determined and found to be abbreviated relative to that observed in monolayer. Induction of both ribonucleotide reductase (Fig. 5b) and of TK (not shown) was detected by 1 hour post-infection, increased rapidly to a maximum at 3-4 hours post-infection and then gradually declined. The time course of reductase induction has not been determined in any cell types other than BHK, however it was established that an increase in reductase activity could be detected 7 hours after infection of human (KB and 143), monkey (Vero), and mouse (L) cell lines with HSV-2. In all subsequent experiments, unless otherwise indicated, BHK cells were used for infection: monolayers were harvested at 7 hours post-infection, and suspension cultures at 4 hours post-infection.

Figure 5

- (a) Ribonucleotide reductase and thymidine kinase activity after infection with the B2006 TK⁻ mutants of HSV-1. Monolayers of BHK21 cl. 13 cells were either mock-infected or infected with B2006 at an M.O.I. of 12 and crude extracts were assayed for enzyme activity. Symbols: reductase activity of infected (○) and mock-infected (●) cells; TK activity of infected (△) and mock-infected (▲) cells.
- (b) Ribonucleotide reductase activity following infection of suspension cultures of BHK21 cl.13 cells with HSV-2 at an M.O.I. of 10. Symbols: enzyme activity in infected (○) and mock-infected (●) cells.

Enzymes assays in both panel a and panel b were performed in duplicate, and the points shown represent the average value of the duplicates.



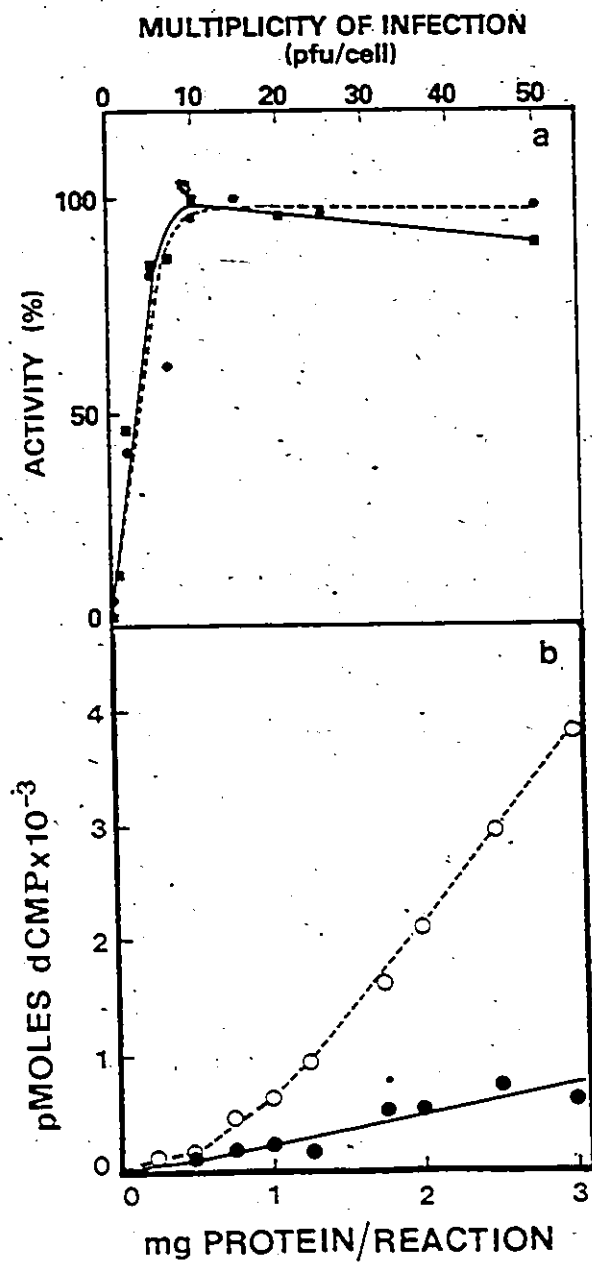
The induction of the reductase, as well as that of TK, was found to be dependent on the multiplicity of infection (M.O.I.). An increase in the activity of both enzymes could be detected upon infection at an M.O.I. of 1 plaque forming unit per cell, and the activity was proportional to the input of virus up to an M.O.I. of 10, after which the response persisted at a plateau level until at least an M.O.I. of 50 (Fig. 6a). Subsequent infections were carried out at an M.O.I. of 7-12 and routinely resulted in a 3-6 fold induction of enzyme activity, relative to the activity in mock infected cells, at 7 hours post-infection. However, the actual degree of induction was probably greater, since in the course of this study, and as described later, it became apparent that the conditions used for assaying enzymatic activity in crude extracts were suboptimal for assay of the induced enzyme.

The data shown in Fig. 5a also serve to rule out the possibility that the induction of the reductase resulted from nonspecific elevation of cellular enzymes by the high input of virus. Confluent monolayers were infected with the B2006 TK⁻ mutant of HSV-1 cl. 101 at an M.O.I. of 12 and assayed for both ribonucleotide reductase and TK activity. If high input of virus results in nonspecific enhancement of cellular enzymes, both reductase and TK should be induced by the TK⁻ mutant. However, only the induction of ribonucleotide reductase was observed; TK activity did not increase. As expected, wild-type HSV-1 cl. 101, included as a control, induced both enzymes (data not shown).

Fig. 6b illustrates the activity of the reductase from infected and uninfected cells as a function of protein concentration. In both cases, at concentrations lower than approximately 0.75 mg protein per

Figure 6

- (a) Kinetics of induction of ribonucleotide reductase and thymidine kinase as a function of M.O.I. Crude extracts of HSV-2 infected cells (harvested at 7 hours post-infection) and mock-infected cells were assayed for ribonucleotide reductase (●) and TK activity (■). Enzyme activity is expressed as the percentage of maximal activity for either enzyme; the data presented for both enzymes represents the average value of duplicates from two independent experiments.
- (b) Relationship between ribonucleotide reductase activity and protein concentration. Crude extracts were assayed at the indicated protein concentrations in a volume of 0.4 ml. Symbols: enzyme activity in HSV-2 infected (○) and uninfected (●) cells.



reaction the enzyme activity was not proportional to protein concentration, whereas a linear relationship was observed from approximately 0.75 to at least 3 mg protein per reaction. The non-linearity of the response is less apparent for the uninfected cell enzyme only because of the low levels of enzyme activity in that sample. This type of concentration curve has been observed by others in several different systems (Larsson, 1969; Hopper, 1972; Engström *et al.*, 1979) and appears to be a characteristic of the enzyme which likely reflects the kinetics of association of the two enzyme subunits, as well as typically low enzyme concentrations in crude extracts. All assays of crude extracts were carried out using 1 to 1.5 mg of protein per reaction.

3.2. Partial Purification of the HSV-2 Induced Ribonucleotide Reductase

To better characterize the HSV-2 induced ribonucleotide reductase, the enzyme was partially purified from BHK21 cl. 13 cells infected with HSV-2 (strain 219 or 333) as well as from uninfected exponentially growing BHK21 cl. 13 cells. Generally $2-3 \times 10^9$ cells were used for purification resulting in a yield of approximately 200-300 mg of protein in the crude extract (70,000 x g supernatant). Briefly, nucleic acids were removed by precipitation with streptomycin sulfate, and proteins fractionated by precipitation with ammonium sulfate.¹ As shown in Table 1, about 50% of the protein was recovered from both uninfected and infected cells in the three salt fractions; however whereas approximately 70 percent of the enzyme activity could be recovered from

¹

Contrary to previous reports (Huszar and Bacchetti, 1981) the actual percentage salt saturation used in these experiments was found to be 27% (fraction 1), 41% (fraction 2) and 74% (fraction 3), rather than 35, 55 and >95%.

Table 1

Partial Purification of Ribonucleotide Reductase^a

Fraction	Fraction no.	Uninfected cells			HSV-2 Infected cells				
		U(total)	Protein (mg)	Sp.act. (U/mg)	Recovery ^c (%)	U(total)	Protein (mg)	Sp.act. (U/mg)	Recovery ^c (%)
70,000 X g supernatant		90,000	300	300	100	120,000	200	600	100
Streptomycin sulfate supernatant		ND ^b	ND	ND	ND	ND	ND	ND	ND
(NH ₄) ₂ SO ₄ (0-27%)	1	800	16	50	1	23,250	17	1,370	19
(NH ₄) ₂ SO ₄ (27-41%)	2	58,500	45	1,300	65	22,000	24	917	18
(NH ₄) ₂ SO ₄ (41-74%)	3	6,000	80	75	7	2,400	55	44	2

^a Enzyme activity was assayed under standard conditions, as described in the text, using 1 mg of protein per reaction. One unit of ribonucleotide reductase activity is defined as the amount of enzyme which catalyzes the formation of 1 pmol of dCMP in 30 min at 37°C.

^b ND, Not done.

^c Percentage of enzyme activity recovered.

uninfected cells, only about 40% was recovered from infected cells. The distribution of enzyme activity among the ammonium sulfate fractions was also markedly different in the two extracts. Virtually all (85-95%) of the activity recovered from uninfected cells precipitated at 27-41% salt saturation (fraction 2) with the majority of the remaining activity precipitating above 41% saturation (fraction 3). In contrast, up to 55% of the activity from infected cells precipitated at 0-27% salt saturation (fraction 1) with the remainder precipitating at the same salt concentration as the enzyme from uninfected controls. The partial purification thus seemed to effect the physical separation of two enzymatic activities from infected cells; this separation, however, was not complete. As described below, fraction 1 from infected cells contained a reductase with properties different from those of the enzyme from uninfected cells. On the other hand, fraction 2 of infected cells appeared to contain a mixture of the two activities since the reductase present in this fraction exhibited properties intermediate between those of the enzyme in fraction 1 and the enzyme from uninfected cells. Attempts to maximize the degree of separation of the two enzymes by raising the salt saturation level of fraction 1 to 30% resulted in contamination of this fraction with the cellular enzyme. Accordingly, fraction 2 from uninfected cells was subsequently used as the source of cellular enzyme, and fraction 1 from infected cells as the source of the virally induced enzyme.

Partial purification resulted in an increase in specific activity of 4-5 fold for the cellular enzyme and of at least 2-3 fold for the

induced enzyme (Table 1). This latter value, however, is likely an underestimate since crude extracts of infected cells contain a mixture of cellular and induced enzymes, the relative proportions of which vary with the degree of enzyme induction and the levels of endogenous cellular reductase activity.

Enzyme concentration curves of the partially purified enzymes indicated that the activity of both the induced and cellular enzymes was proportional to protein concentration from 250 μ g to at least 1500 μ g. Accordingly, between 300-500 μ g of the partially purified enzymes was routinely assayed in the experiments described below.

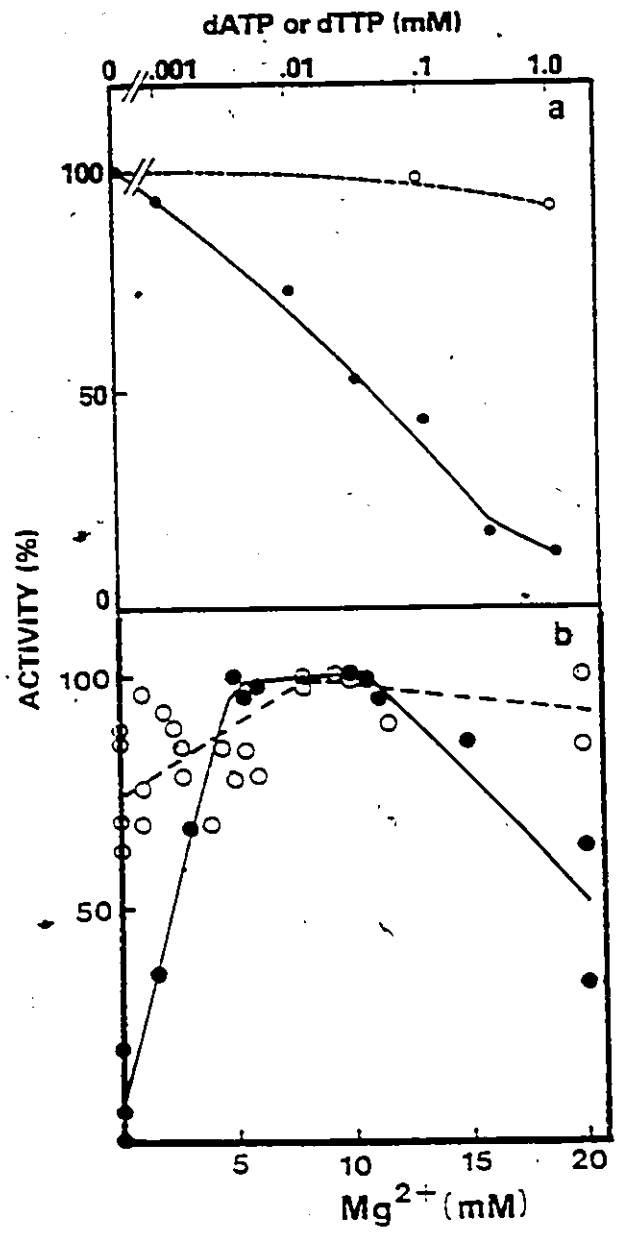
3.3. Characterization of the Partially Purified HSV-2 Induced Enzyme

3.3.1. Response to dTTP and dATP

As mentioned in the Introduction, previous reports had indicated that the HSV induced reductase was insensitive to allosteric inhibition by dTTP or dATP (Ponce de Leon *et al.*, 1977; Langelier *et al.*, 1978). In order to ascertain that fraction 1 of infected cells did indeed contain the virally induced reductase, this fraction and the cellular enzyme (i.e., fraction 2 from uninfected cells) were tested for their susceptibility to the two allosteric effectors. As shown in Fig. 7a the activity of the cellular enzyme was reduced by 50% when either deoxy nucleotide was present at a concentration of 0.05 mM, and was further inhibited by 80-95% when their concentration was raised to 1 mM. In contrast, the fraction 1 enzyme from infected cells behaved as the reported HSV induced activity and was only inhibited up to a maximum of 20% by 1 mM dTTP or

Figure 7

- (a) Response of the induced (○) and cellular (●) ribonucleotide reductase to the allosteric effectors dATP and dTTP. The partially purified enzymes were assayed under standard conditions in the presence of the indicated concentrations of dATP or dTTP. Enzyme activity is expressed as the percentage of activity in the absence of either nucleotide. Each point represents the average of two independent experiments.
- (b) Response of the induced (○) and cellular (●) ribonucleotide reductase to Mg^{2+} . The partially purified enzymes were assayed under standard conditions except the concentration of magnesium acetate was varied as indicated. Enzyme activity is expressed as the percentage of maximal activity for either enzyme. The experimental points for the cellular enzyme represent individual assays from two independent experiments; for the induced enzyme, data from four independent experiments are presented.



dATP. Fraction 2 from infected cells was inhibited by 60% at 1 mM dTTP (not shown), suggesting that this fraction contained a mixture of the induced and cellular enzymes.

3.3.2. Requirement for Mg²⁺

Since a lack of requirement for exogenous Mg²⁺ has also been reported to be characteristic of the HSV induced reductase (Ponce de Leon *et al.*, 1977), the effect of this ion was tested on the partially purified enzyme preparations. The cellular reductase displayed a broad peak of maximal activity between 5 and 10 mM Mg²⁺ and was inhibited by at least 80% in the absence of the ion (Fig. 7b). The induced enzyme, on the other hand, retained between 60-90% of its activity in the absence of exogenous Mg²⁺ and in addition did not exhibit a clear and reproducible dependence on Mg²⁺ concentration for maximal activity. Enzymatic activity appeared to fluctuate between 60 to 90% maximal levels at Mg²⁺ concentrations of 0 to 6 mM, and to attain 100% activity between 6 and 20 mM Mg²⁺. That this variability in the response to Mg²⁺ does not reflect a variability inherent in the enzyme assay is evidenced by the data obtained with the cellular enzyme, as well as by the data presented elsewhere in the Results. Despite various manipulations of the assay conditions (e.g., substitution of magnesium chloride for magnesium acetate, altering ATP concentration, etc.), the response of the induced enzyme to Mg²⁺ could not be standardized or clarified. Nevertheless, the salient feature of the data in Fig. 7b is that the induced enzyme does not require exogenous Mg²⁺ for its activity whereas the cellular enzyme does. This criterion can thus effectively be used to differentiate the two activities, but unless

otherwise noted, both enzymes were usually assayed in the presence of 6 mM Mg^{2+} .

3.3.3. Further analysis of the response to dTTP and Mg^{2+}

In order to rule out the possibility that the properties of the virally induced enzyme were artefactually generated by factors present in the 0-27% salt fraction of infected cells, the following reconstruction experiment was undertaken. Aliquots of the cellular and induced enzymes were assayed either separately or in combination in the presence or absence of either Mg^{2+} or 1 mM dTTP. As shown in Table 2, and in agreement with the data presented in Fig. 7, the absence of Mg^{2+} or presence of dTTP inhibited only or primarily the cellular enzyme and had no or limited effect on the induced enzyme. On the other hand, and as expected, the combined enzyme sample was affected by both conditions to an intermediate degree, indicating that the 0-27% salt fraction of infected cells does not in itself confer altered properties upon ribonucleotide reductase.

3.3.4. Effect of pyridoxal phosphate

In light of the differences observed in the regulatory properties of the induced and cellular enzymes, the effect of pyridoxal phosphate was tested. This compound inhibits a wide range of enzymes by reacting with lysyl residues in an active or allosteric site of the enzyme to form a Schiff base (Cory and Mansell, 1975). In particular, it has been shown to reversibly inhibit mammalian ribonucleotide reductase, possibly by interacting with the allosteric site of the enzyme (Cory and Mansell, 1975). Despite the altered regulatory control of the HSV induced enzyme,

Table 2

Effect of Mg^{2+} and dTTP on the Activity of the Induced and Cellular Ribonucleotide Reductase, and on a Reconstituted Mixture of the Two Enzymes

Enzyme sample ^a	Enzyme activity (% of control ^b)	
	- Mg^{2+} ^c	+1 mM dTTP ^d
Induced	100	82
Cellular	19	10
Induced + Cellular	51	30

^a Equal amounts of both partially purified enzymes in terms of protein concentration and activity, were used.

^b Control enzyme activity, taken as 100%, was assayed under standard conditions (i.e., in the presence of 2.7 mM Mg^{2+} and the absence of dTTP).

^c Enzyme activity was assayed under standard conditions in the absence of Mg^{2+} , and is expressed as the percentage of control activity (see ^b).

^d Enzyme activity was assayed under standard conditions in the presence of 1 mM dTTP, and is expressed as the percentage of control activity (see ^b).

however, both induced and cellular enzymes proved to be equally susceptible to inhibition by pyridoxal phosphate; in either case enzyme activity was reduced by 65% at 1 mM, and by approximately 75% at 2 mM concentration of the compound (Fig. 8a).

3.3.5. Effect of ATP

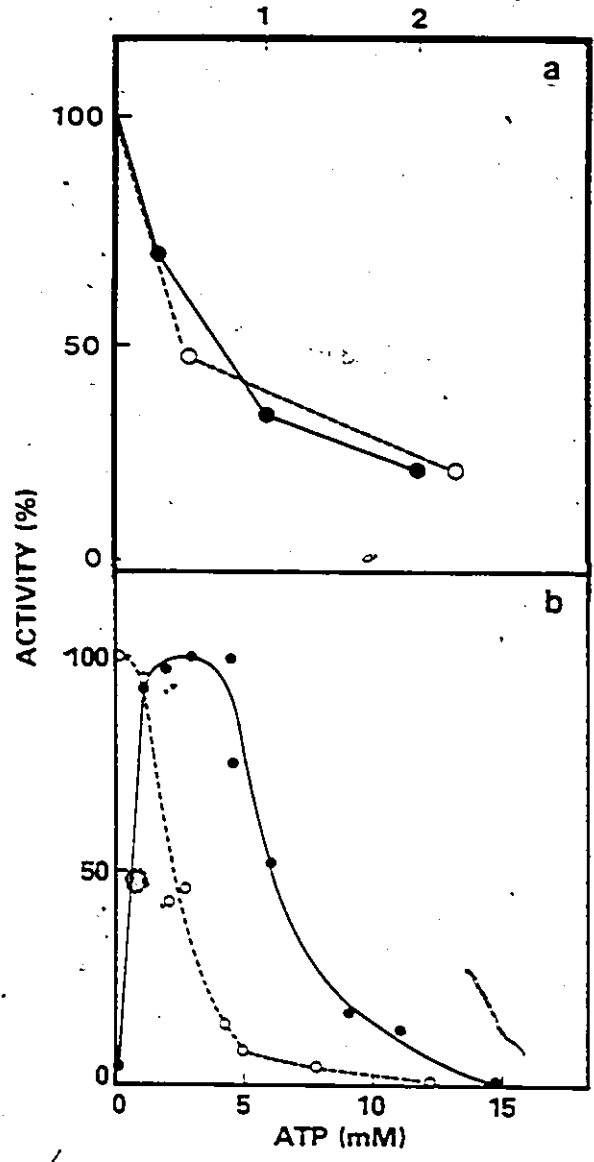
Since, as described earlier, ATP is a positive effector of mammalian reductase activity, the requirement of the partially purified enzymes for this nucleotide was tested. As shown in Fig. 8b, the cellular enzyme was found to exhibit maximal activity at 3 mM ATP and to be inhibited by 95% in the absence of the nucleotide. In contrast, the induced enzyme showed maximal activity only in the absence of exogenous ATP and was inhibited by 65% at 3 mM ATP. Although the ammonium sulfate fractions used in these experiments were filtered through a Sephadex G-25 column, and should thus have been relatively free of endogenous ATP, the possibility remained that removal of the nucleotide might have been incomplete or unequal in different samples, especially if endogenous levels of ATP were higher in infected than in uninfected cells. This possibility, however, was discounted by the fact that results similar to those of Fig. 8b were obtained even after extensive dialysis of the enzyme samples.

Other herpesvirus-induced reductases have been shown not to require ATP for activity. Cohen *et al.* (1977) found that the enzyme induced by equine herpesvirus type 1 retained maximal activity in the absence of exogenous ATP and Lankinen *et al.* (1982) reported that pseudorabies virus induces a reductase which does not require ATP for maximal activity. The latter group has suggested, however, (Lankinen *et al.*, 1982) that the

Figure 8

- (a) Effect of pyridoxal phosphate on ribonucleotide reductase activity. The induced (○) and cellular (●) enzymes were assayed under standard conditions in the presence of increasing concentrations of pyridoxal phosphate. Enzyme activity is expressed as the percentage of activity in the absence of pyridoxal phosphate.
- (b) Response of the induced (○) and cellular (●) enzymes to ATP. The cellular enzyme was assayed in the presence of 5 mM Mg^{2+} , and the induced enzyme in the absence of Mg^{2+} (see text); enzyme activity is expressed as the percentage of maximal activity for each enzyme. The experimental points represent individual assays from two independent experiments.

PYRIDOXAL PHOSPHATE (mM)



inhibition of the HSV-2 induced enzyme shown in Fig. 8b may be due to a lack of Mg^{2+} ions at high ATP concentrations since 1) ATP binds Mg^{2+} ions in a one-to-one stoichiometry, and 2) in the experiment shown in Fig. 8b the induced enzyme was assayed in the absence of exogenous Mg^{2+} , the cellular enzyme in the presence of 5 mM Mg^{2+} . In other words, they suggest that ATP acts to remove endogenous Mg^{2+} ions which are necessary for induced enzyme activity. To test this hypothesis, the effect of ATP on the induced reductase was retested in the presence of 8 mM Mg^{2+} . Essentially the same results were obtained as those shown in Fig. 8b, indicating that the inhibition of the induced enzyme does not appear to be an artefact.

These data indicate that the total amount of reductase activity detected in extracts of infected cells, as well as the observed degree of induction, were likely underestimated, since, as detailed in Materials and Methods, crude extracts were assayed in the presence of 4.4 mM ATP.

Lastly, a final feature of these results worthy of note is that in some experiments the absence of ATP from the reaction mixture resulted in the appearance of a new labelled reaction product, as detected by paper chromatography of acid hydrolysed reaction mixtures (as described in Materials and Methods, reductase assays were routinely terminated by acid hydrolysis and reaction products subsequently identified by paper chromatography). In the presence of ATP, 3H -dCMP was the only labelled product of the enzyme assay, in the absence of the nucleotide an additional labelled product, migrating slightly faster than dCMP, was sometimes observed. This new radioactive peak was found in assays of both the induced and cellular enzymes, and

was eliminated by concentrations of ATP equal to or greater than 1 mM ATP. The presence of approximately equal amounts of the unidentified product in assays of both the induced and cellular enzymes, coupled with the fact that it was only occasionally observed, indicate that whatever the reactions were which generated this nucleotide, it is extremely unlikely that they were contributing factors in the differential response of the induced and cellular enzymes to ATP. Nevertheless, in order to avoid metabolism of the substrate to this unidentified product, 1 mM ATP was routinely included in assays of the induced enzyme; the cellular enzyme was assayed in the presence of 3 mM ATP.

3.3.6. Properties of HSV-1 induced ribonucleotide reductase

Although the HSV-1 induced ribonucleotide reductase was not partially purified, a limited number of studies were carried out on crude extracts of HSV-1 (cl. 101) infected BHK21 cl. 13 cells. It was found that enzyme activity was inhibited by 10-15% in the presence of 1 mM dTTP or dATP, was unaffected by the absence of exogenous Mg^{2+} and enhanced by the absence of ATP. In contrast, cellular activity from crude extracts of uninfected cells was inhibited 95% by 1 mM dTTP or dATP, 85% in the absence of Mg^{2+} and 30% in the absence of ATP (the residual cellular activity in the absence of exogenous ATP likely reflects the presence of endogenous ATP in the crude extracts); (data not shown). Thus, the HSV-1 induced enzyme appears to be similar to the HSV-2 induced enzyme in its properties.

3.3.7. Effect of salt and detergent on enzyme activity

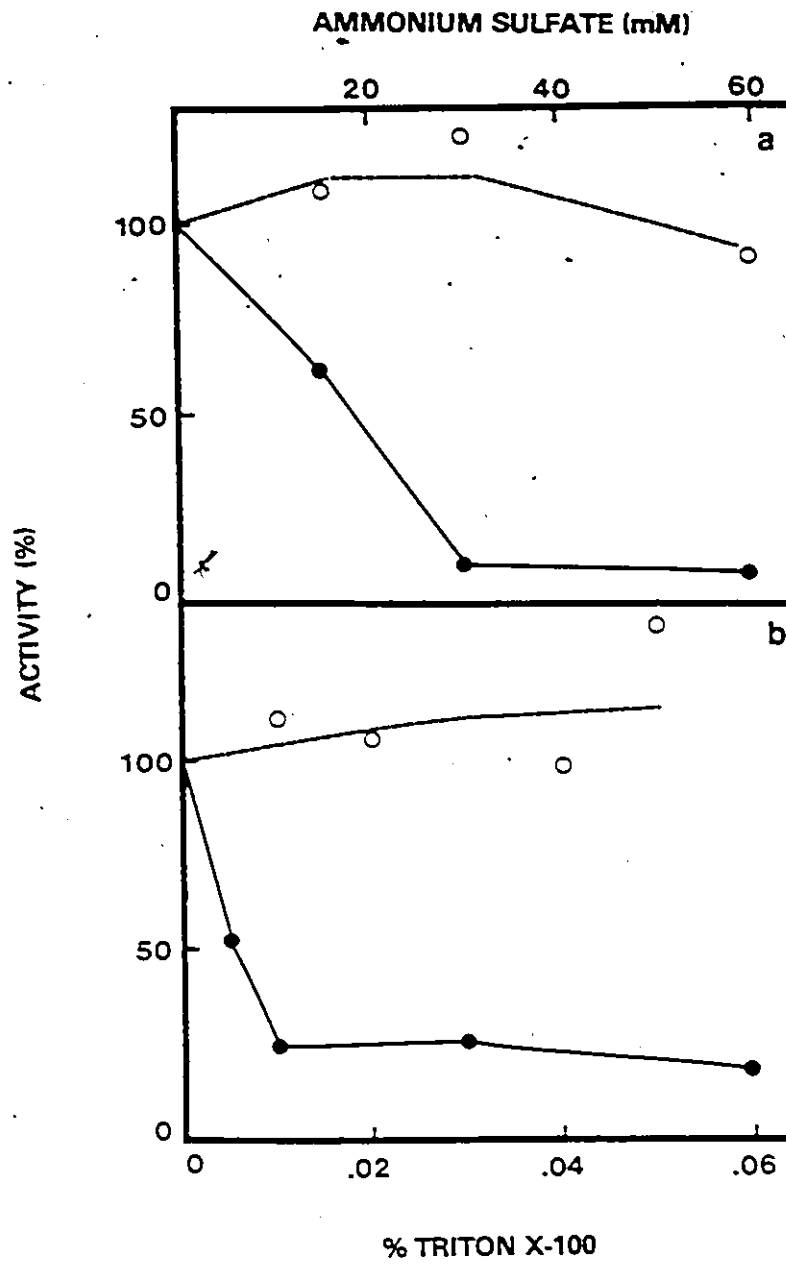
As mentioned earlier, the HSV DNA polymerase is stimulated by salt concentrations which almost completely inhibit cellular α DNA polymerase activity (Keir *et al.*, 1966). As shown in Fig. 9a, the partially purified HSV-2 induced and cellular ribonucleotide reductase also displayed a differential response to high salt concentrations; whereas the cellular enzyme was inhibited by 90% in the presence of 30 mM ammonium sulfate, the induced enzyme was uninhibited by concentrations of ammonium sulfate up to 60 mM. Similar differences were also observed when enzyme samples were assayed in the presence of detergent (Fig. 9b); 0.01% Triton X-100 inhibited cellular enzyme activity by 80% without reducing the activity of the induced enzyme. These two agents can thus serve as additional criteria for differentiating the two enzymes.

3.3.8. Enzyme stability

In view of the relatively low recovery obtained during purification of ribonucleotide reductase from HSV-2 infected cells ($\pm 40\%$), compared with that from uninfected cells ($\pm 70\%$), experiments were carried out to determine whether this resulted from differential stability of the two enzymes. Both enzymes, however, were found to be equally stable upon incubation at 4°C for at least 24 hours; indeed in both cases enzyme activity tended to increase over this time period, possibly due to increased solubilization (data not shown). In addition, both enzymes were equally stable upon storage at -70°C ; for up to at least 8 months no significant loss of activity was detected in either preparation. Lastly, the induced and cellular

Figure 9

Effect of ammonium sulfate (panel A) and triton X-100 (panel B) on ribonucleotide reductase activity. The induced enzyme (○) was assayed in the presence of 1 mM ATP and 6 mM Mg^{2+} ; the cellular enzyme (●) in the presence of 3 mM ATP and 6 mM Mg^{2+} . Enzyme activity is expressed as the percentage of activity in the absence of salt and detergent.



enzymes were inactivated at approximately the same rate and to the same extent by preincubation at 46°C, 50°C or 56°C (Fig. 10); neither activity was inhibited following a 20 minute preincubation at 42°C (not shown).

As mentioned earlier, bacteriophage T4 encodes a thioredoxin which acts as the specific hydrogen donor for the phage reductase. As a result of ammonium sulfate fractionation, the thioredoxin is purified away from the T4 reductase and must be recombined with the enzyme in order to restore maximal levels of enzyme activity (Berglund *et al.*, 1969; Berglund, 1972). This raised the possibility that the apparent loss of HSV-2 induced reductase activity following salt fractionation could be due to separation of the enzyme from a cofactor (or cofactors) required for optimal levels of enzyme activity. To examine this possibility the salt fractions from HSV-2 infected and uninfected cells (see Table 1) were pooled in different combinations and assayed for enzymatic activity. These experiments indicated that only pooling of fraction 3 (41-74% salt saturation) with the induced enzyme (0-27% salt saturation) resulted in a significant enhancement of enzyme activity (see Fig. 11). This increase in activity was not due to the contribution of residual amounts of cellular enzyme in fraction 3 since 1) fraction 3 contains very low levels of enzyme activity, and 2) the induced enzyme was assayed under conditions inhibitory for the cellular reductase. Although the enhancement of induced enzyme activity was clearly not mediated by a virally coded molecule, since fraction 3 from either infected or uninfected cells was equally active, it was nevertheless specific for the induced enzyme since pooling of fraction 3 with

Figure 10

Thermal stability of ribonucleotide reductase. Aliquots of the partially purified induced (open symbols) and cellular (closed symbols) enzyme containing equal concentrations of protein, were preincubated at 46°C (Δ , \blacktriangle), 50°C (\square , \blacksquare) and 56°C (\circ , \bullet), for the indicated periods of time and assayed immediately thereafter. The induced enzyme was assayed in the absence of Mg^{2+} , the cellular in the presence of 5 mM Mg^{2+} . As indicated in the figure, 100% activity was measured in the absence of any preincubation and the experimental points are presented as percentages of this value.

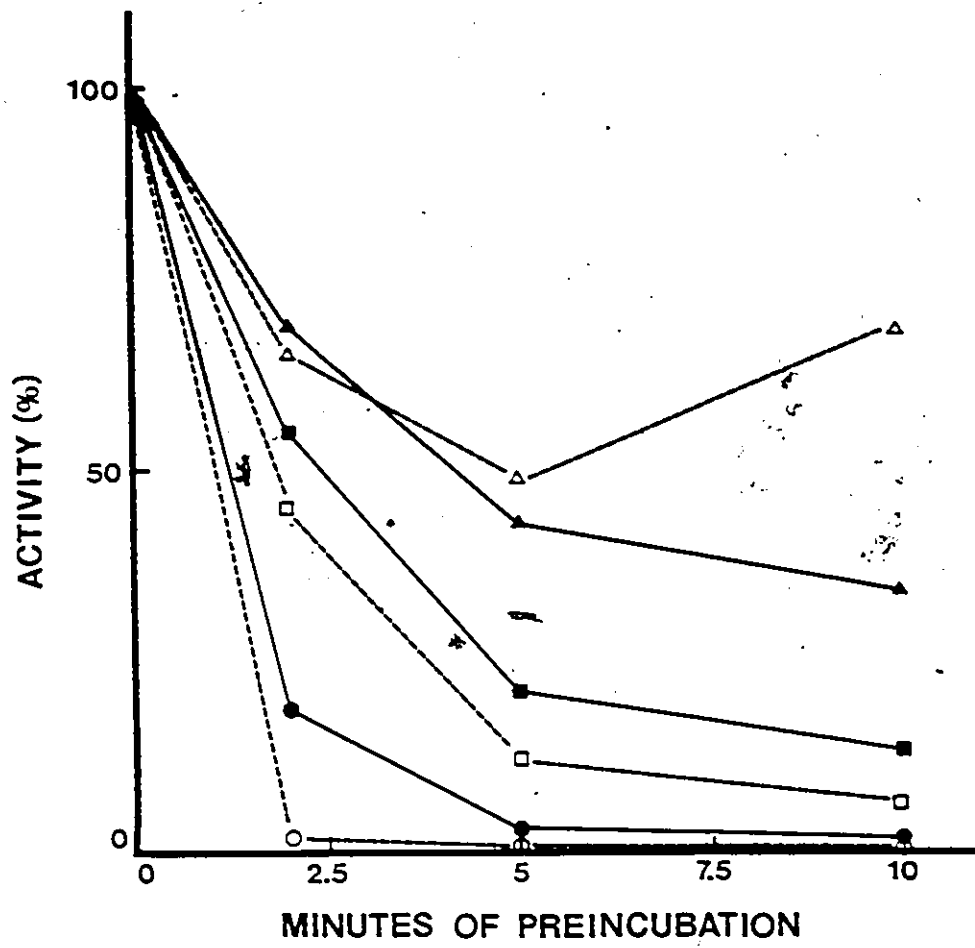
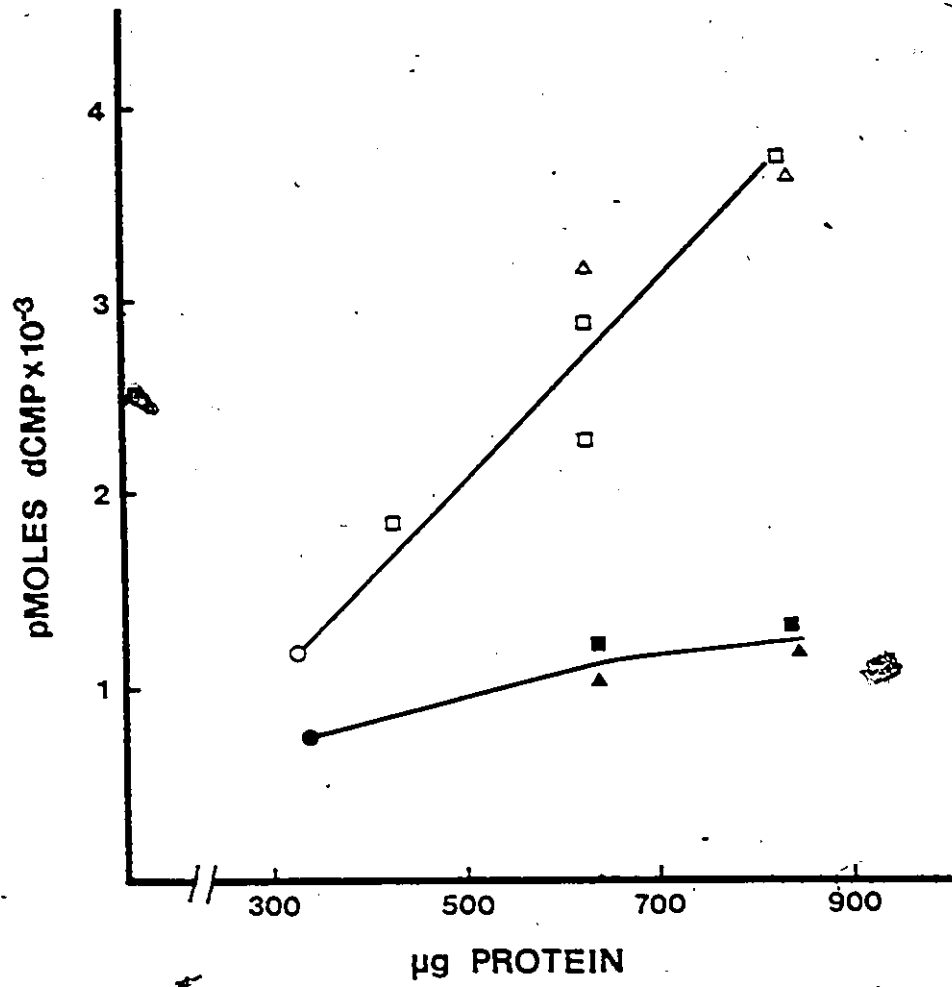


Figure 11

Reconstitution of partially purified ammonium sulfate fractions. Aliquots of the induced (0-27% salt saturation) and cellular (27-41% salt saturation) enzymes were mixed with increasing amounts (as indicated on the abscissa) of fraction 3 (41-74% salt saturation) from infected and uninfected cells, and assayed for activity. Induced enzyme activity (open symbols) was assayed in the absence of Mg^{2+} and ATP; similar results were however obtained when 4.4 mM ATP was added to the assay. Cellular enzyme activity was assayed at optimal concentrations of Mg^{2+} and ATP (i.e., 6 mM Mg^{2+} and 3 mM ATP). The experimental points for both enzymes represent individual assays from two independent experiments..

The symbols refer to the activity of the induced enzyme either prior to (○) or following addition of increasing amounts of fraction 3 from infected (□) or uninfected (△) cells; and similarly to the activity of the cellular enzyme either alone (●) or in combination with increasing amounts of fraction 3 from infected (■) or uninfected (▲) cells.



the cellular enzyme did not result in a similar increase in activity. These data indicate that there appears to be a cellular molecule (or molecules) which precipitates at 41-74% salt saturation and specifically enhances induced enzyme activity. Separation of this molecule from the induced enzyme may account, at least in part, for the low recovery of induced enzyme activity following partial purification. Characterization of this enhancing agent has not been pursued any further, nor have proteins precipitating at greater than 74% salt saturation been tested for their ability to enhance induced enzyme activity.

3.3.9. Sedimentation properties of the partially purified enzymes

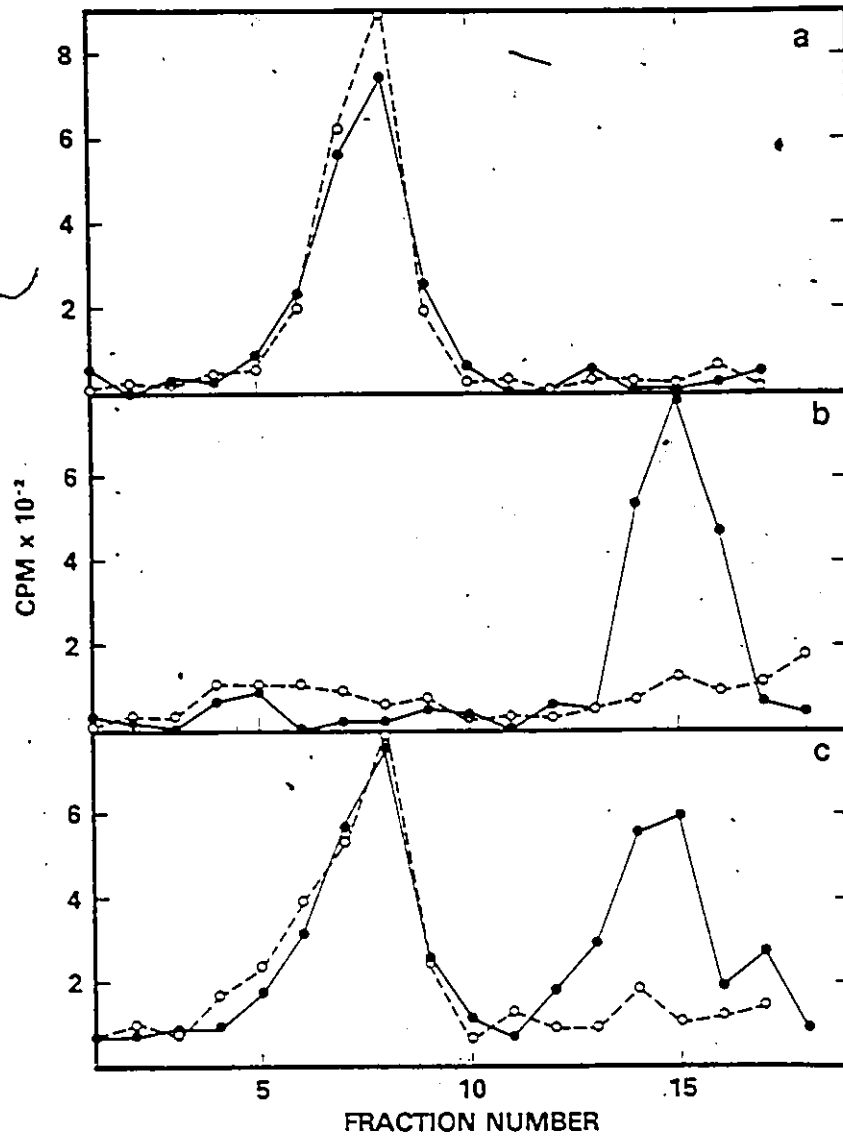
In order to further distinguish, and possibly further purify, the HSV-2 induced and cellular enzymes, their sedimentation properties in glycerol gradients were examined. Initial experiments demonstrated that upon centrifugation at 130,000 x g the cellular enzyme sedimented as a symmetrical peak whose recovery depended on the presence of Mg^{2+} in the gradient. Up to 50% of the input activity was recovered when 6 mM Mg^{2+} was present whereas only 20% could be recovered in the presence of 4 mM Mg^{2+} ; no activity was detected in the absence of the ion (not shown). The requirement for Mg^{2+} during sedimentation likely reflects the role the ion plays in binding together the two subunits of the enzyme. In contrast to these results, induced enzyme activity could not be recovered under similar conditions of centrifugation. After experimenting with a variety of sedimentation conditions, it was

found that the apparent loss of induced enzyme activity was due to pelleting of the enzyme during centrifugation. As shown in Fig. 12, by decreasing the sedimentation force to $81,000 \times g$, both the induced enzyme (panel a) as well as the cellular one (panel b), could be recovered as discrete peaks within the gradient. Enzyme activity was assayed both in the presence (open circles) and absence (closed circles) of 30 mM ammonium sulfate as a means of discriminating between the two enzymes (see section 3.3.7.).² In addition, a reconstruction experiment was carried out (Fig. 12c) in which partially purified enzymes were sedimented in the same gradient. The results of this experiment indicated that the induced reductase sedimented approximately 2.5 times faster than the cellular enzyme.

² Attempts were also made to differentiate the two enzymes on the basis of their response to dTTP and ATP (see section 3.3.1. and 3.3.5.). Contrary to the results obtained with the ammonium sulfate fractions, however, it was found that following sedimentation the induced enzyme was inhibited by approximately 60% in the presence of 1 mM dTTP and by approximately 40% in the absence of ATP. The response of the cellular enzyme appeared to be unchanged by sedimentation, its activity being almost completely inhibited in the presence of dTTP or absence of ATP. The variation in the induced enzyme response to the nucleotides could reflect real changes in enzyme properties as a result of additional purification. Alternatively, the different response might depend on the fact that gradient fractions were assayed at a 10-fold reduced concentration of unlabelled substrate (0.1 mM CDP) in order to lessen the dilution of the labelled substrate and thus maximize detection of the enzymes. It seems unlikely, however, that a decreased substrate concentration would result in alterations in the regulatory properties of the enzyme. This phenomenon was not explored in further detail.

Figure 12

Sedimentation of the partially purified induced and cellular ribonucleotide reductase on glycerol gradients. Aliquots of the induced and cellular enzymes were centrifuged on 5-25% glycerol gradients, containing 6 mM Mg^{2+} , in the Beckman SW40 Ti rotor at 4°C and 81,000 g for 17 hours. The gradients were then fractionated and assayed for reductase activity in the presence of 0.01 mM CDP, 3 mM ATP (see footnote 1) and 6 mM Mg^{2+} , with (○) or without (●) addition of 30 mM ammonium sulfate. Panel a: virus induced enzyme; panel b: cellular enzyme; panel c: a mixture of induced and cellular enzymes. Sedimentation is from right to left.



Since the mammalian reductase is itself a large protein of molecular weight at least 200,000 daltons (Chang and Cheng, 1979; Thelander *et al.*, 1980), it seems likely that the induced enzyme might sediment as part of a high molecular weight complex, either as a self-aggregate or in aggregation with other molecules. This putative complex does not appear to contain cell membranes since sedimentation in the presence of 0.05% Triton X-100 did not alter the mobility of the enzyme; nor was the mobility altered by extensive dialysis or solubilization of the enzyme in 5% ammonium sulfate/0.05% Triton X-100 prior to sedimentation. In addition, the aggregate is not likely to contain DNA, since nucleic acids were removed from enzyme extracts by precipitation with streptomycin sulfate. Since reports in the literature indicated that the reductase might be a member of a multienzyme complex (Allen *et al.*, 1980; Prem Veer Reddy and Pardee, 1980), the possibility was examined that the induced enzyme may be complexed with some or all of the known HSV coded enzymes involved in DNA metabolism (i.e., thymidine kinase, DNA polymerase, alkaline DNAase). However, none of these enzymes were found to copurify with the induced reductase in the 0-27% ammonium sulfate fraction, nor could their activity be detected in the gradient fractions which contained the reductase (data not shown).

3.4. Development of Antibodies Specific for the Virally Induced Reductase

The characterization of the partially purified enzymes, as described in section 3.3., clearly demonstrated that infection with HSV results in the appearance of a novel ribonucleotide reductase activity in the infected cells. In order to determine whether the enzyme is virally

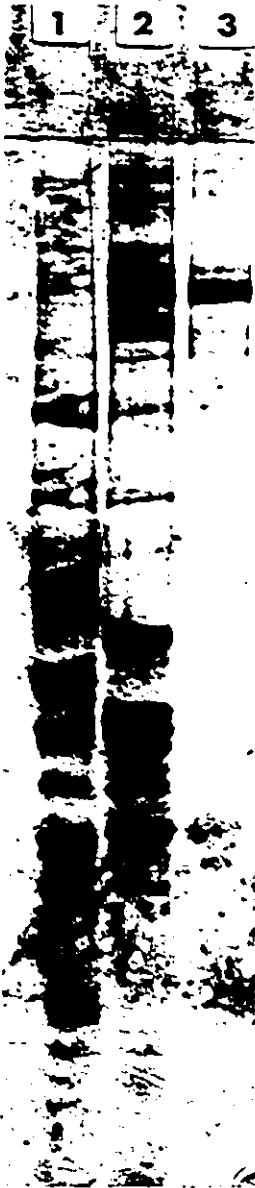
coded, two experimental approaches were followed. One approach involved determining whether purified viral nucleic acids could direct the synthesis of the induced reductase either *in vivo* (i.e., in *Xenopus laevis* oocytes), or *in vitro* (i.e., in rabbit reticulocyte lysates); these experiments are described in the Appendix. The second approach was to develop antibodies specific for the induced enzyme since such reagents would be useful in the identification and mapping of the reductase molecule. These studies are described in the sections which follow.

3.4.1. Characterization of the antigen

Sedimentation of the partially purified enzymes through glycerol gradients (described in section 3.3.9.), was carried out not only to further distinguish the two enzymes but also in an attempt to obtain an induced enzyme preparation of sufficient purity for immunization purposes. Indeed, following sedimentation of the induced enzyme, determination of the distribution of protein within the gradient demonstrated that the majority of the protein remained near the top of the gradient, suggesting that further purification had been obtained. This was confirmed by Coomassie blue staining of enzyme samples electrophoresed on SDS-polyacrylamide gels (Fig. 13) which indicated that significantly fewer polypeptides were present in the gradient fraction (lane 3) relative to the less purified fractions (lanes 1 and 2). Total enzyme activity recovered from the gradients appeared to be greater than the input activity, suggesting that sedimentation may have also separated the induced

Figure 13

Electrophoretic analysis of purified preparations of the virus induced ribonucleotide reductase. Lysates of infected cells were used as source of enzyme and purified as described in the text. Aliquots of each fraction (corresponding to equivalent amounts of protein) were then electrophoresed on 9% polyacrylamide-SDS gels. The gels were stained with Coomassie blue, destained and photographed. Lane 1: crude extract; lane 2: 0-27% ammonium sulfate fraction; lane 3: gradient fraction.

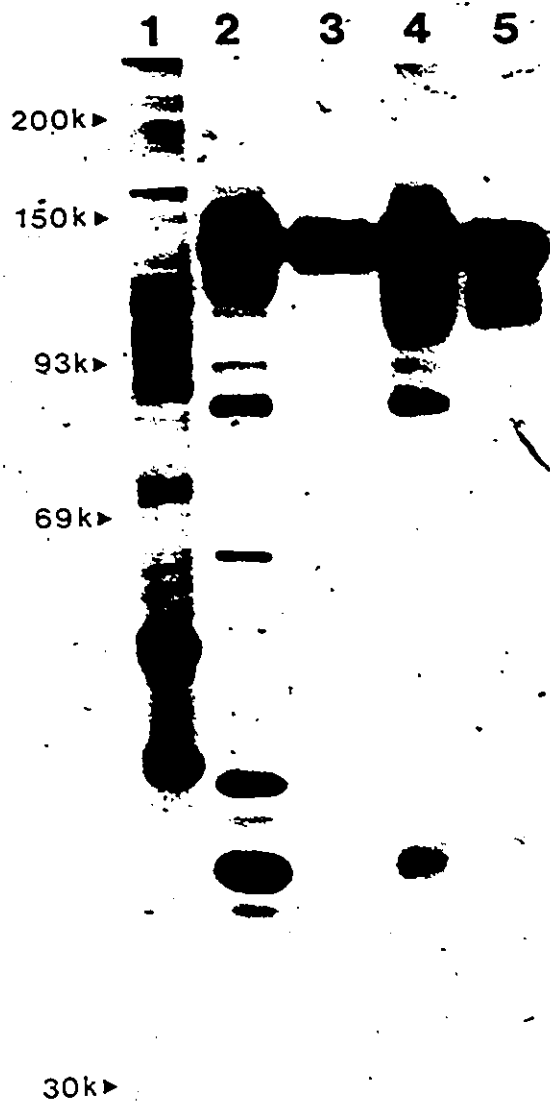


enzyme from an inhibitor of its activity. This complicated calculation of the degree of purification provided by the sedimentation step, but at least a 10-fold increase in specific activity of the enzyme was obtained relative to the ammonium sulfate fraction. To further analyse the composition of the sedimented enzyme preparation, extracts of radio-labelled HSV-2 infected and uninfected cells were partially purified and electrophoresed on SDS gels. As shown in Fig. 14, considerable quantitative and qualitative differences can be seen between the labelled polypeptides present in the induced enzyme salt fraction (0-27% salt saturation) (lane 2) and the 0-27% salt fraction from uninfected cells (lane 1); virtually all of the major labelled polypeptides present in the induced enzyme sample appear to be unique to infected cells. As expected from the data of Fig. 13, the sedimented induced enzyme preparation (lane 4) was quite pure and contained only one major labelled species of approximate molecular weight 140,000 as well as additional minor polypeptides of MW 152,000, 119,000, 112,000, 96,000, 87,000, 67,000, 45,000, 38,000 and less than 30,000. Of note is that in terms of newly synthesized labelled polypeptides, and relative to the ammonium sulfate fraction (lane 2), the increased purification obtained by sedimentation appeared much less striking than that observed on the basis of Coomassie blue stain (Fig. 13). Evidently, the majority of the species which sediment away from the induced enzyme are unlabelled molecules. In any case, the relative purity of the enzyme preparation recovered from gradients rendered it suitable for use as antigen to raise antisera in rabbits.

Figure 14

Electrophoretic analysis of radiolabelled polypeptides in purified preparations of the induced ribonucleotide reductase. BHK21 cl. 13 cells were labelled with [^{35}S] methionine from 0-7 hours following mock-infection or infection with HSV-2, and harvested at 7 hours; cell lysates were prepared and purified as described in the text and aliquots of the fractions (corresponding to equal amounts of radioactivity) were then electrophoresed on 9% SDS-polyacrylamide gels. In the case of the induced enzyme samples, aliquots were also immunoprecipitated with 30 μl of R1 serum and the reaction products electrophoresed together with the partially purified fractions. Molecular weight markers of 200,000 (200K), 150,000 (150K), 93,000 (93K), 69,000 (69K) and 30,000 (30K) daltons were included in the gel; polypeptides were visualized by fluorography.

Lanes 2 and 4 refer respectively to the 0-27% salt fraction of infected cells (containing the virally induced enzyme) before and after sedimentation in glycerol gradients. Lanes 3 and 5 refer to the same fractions following immunoprecipitation. The 0-27% fraction from uninfected cells is included for comparison in lane 1.



3.4.2. Preparation and screening of antisera

Each of two New Zealand rabbits was primed with 150 μ g of the sedimented HSV-2 induced enzyme preparation and subsequently challenged at approximately monthly intervals, initially with 70 μ g of the same antigen, then with 120-150 μ g of the less pure salt fraction. The animals were initially bled one week after each boost, but following the fourth boost, they were routinely bled at 6, 10 and 14 days after injection. Pre-immune serum was obtained from both rabbits prior to immunization. Screening of the rabbit sera was carried out by indirect immunofluorescence on mock-infected and HSV-2 infected confluent Vero cells. These experiments showed the presence of HSV specific antibodies by one week after the first boost; high background fluorescence was, however, obtained with normal serum from several rabbits when tested under a variety of experimental conditions.

3.5. Characterization of the Antiserum

3.5.1. Immunoprecipitation

A highly reactive bleed from one of the rabbits (designated R1 serum) was further tested by immunoprecipitation using as antigen partially purified enzyme preparations from labelled infected cells. These results are also shown in Fig. 14. When reacted with the ammonium sulfate or gradient purified fractions (lanes 3 and 5, respectively), the R1 serum precipitated primarily and very efficiently a polypeptide of 140,000 daltons corresponding to the major labelled species present in the sample used for immunization (lane 4). Additional species of lower molecular

weight (119,000, 112,000, 96,000, 87,000, and 38,000) were also immunoprecipitated by R1. Of these, the 119,000 and 112,000 dalton polypeptides were found only in the sedimented enzyme sample and may thus represent degradation of the 140,000 MW species during centrifugation.

A similar pattern was obtained, as expected, when the R1 serum was reacted with unfractionated lysates of infected cells. For these experiments HSV-1 and HSV-2 infected and mock-infected BHK21 cl. 13 cells were labelled with [³⁵S] methionine from 0-7 hours post-infection, or mock-infection, and harvested. Equal amounts of cell lysates, in terms of radioactivity, were then reacted with either R1 or pre-immune serum and the products of the reactions electrophoresed on SDS gels. The results, shown in Fig. 15, indicated that the R1 serum recognized predominantly a polypeptide of approximate MW 144,000 from HSV-2 infected cells (lane 1), as well as minor species of approximately 170,000, 114,000, 110,000, 97,000, 93,000, 86,000 and 38,000 daltons. It was also found that with different preparations of antigen the abundance and apparent MW of the polypeptides migrating between 86,000 and 114,000 daltons tended to vary, perhaps indicating that either these species undergo variable degrees of processing, or that they represent degradation of the 144,000 dalton polypeptide. As shown in lane 3, R1 serum also reacted with HSV-1 antigens, but with much lower affinity than in the case of HSV-2 antigens. No reaction was observed with mock-infected cells (lane 5). Similarly, pre-immune rabbit serum did not react with any of the antigens tested (lanes 2, 4 and 6). Serum from the second of the two immunized rabbits gave results similar to those obtained with the R1 serum (not shown).

Figure 15

Electrophoretic analysis of the antigens immunoprecipitated by R1 serum from crude lysates of radiolabelled cells. HSV-1, HSV-2 and mock-infected BHK21 cl. 13 cells were labelled with [^{35}S] methionine from 0-7 hours post-infection and harvested at 7 hours. Equal amounts, in terms of radioactivity, of HSV-2 infected (lanes 1 and 2), HSV-1 infected (lanes 3 and 4), and mock-infected (lanes 5 and 6) cell lysates were reacted with 30 μl of R1 serum (lanes 1,3 and 5) or pre-immune rabbit serum (lanes 2,4 and 6) and the reaction products electrophoresed on 9% SDS-polyacrylamide gels. Molecular weight markers, as in Figure 14, were included in the gel and polypeptides were visualized by fluorography.

200k▶

150k▶

93k▶

69k▶

30k▶



Lastly, it was observed that whereas the major polypeptide immunoprecipitated from whole cell lysates was of approximate MW 144,000 (see Fig. 15), the major species immunoprecipitated from partially purified enzyme fractions was estimated to be 140,000 daltons (Fig. 14). Electrophoresis of immunoprecipitates of whole cell lysates and of the partially purified samples on the same gel verified that the electrophoretic mobility of the major polypeptide, in both the ammonium sulfate and the gradient purified enzyme fractions, was slightly increased relative to that in whole cell lysates. The significance of this apparent change in molecular weight has not been determined, nor has it been ascertained whether this alteration is compatible with reductase activity. Nonetheless, it seems likely that the purified labelled fractions are enzymatically active, since an unlabelled enzyme preparation which was copurified, under identical conditions, with the labelled samples was found to contain reductase activity.

3.5.2. Effect of R1 antibodies on enzyme activity

Having established that HSV specific antibodies were present in the immune rabbit serum, the ability of these antibodies to react with the HSV-2 induced ribonucleotide reductase was tested. In order to detect the presence of both neutralizing and non-neutralizing antibodies, these experiments were carried out by mixing R1 serum and ammonium sulfate purified enzyme in the presence of protein A-sepharose beads; the beads were then removed by centrifugation and the supernatant tested for residual enzyme activity. Incubation with beads alone resulted in the loss of a

small percentage of the activity of both the induced and cellular enzymes, probably due to non-specific trapping of the enzymes by the beads. Addition of immune R1 serum to the reactions resulted in a preferential removal of induced enzyme activity from the reaction mixture at high serum concentrations; however the effect was not observed at serum dilutions greater than 1:4 or 1:5. A decrease of induced enzyme activity was also observed in the presence of pre-immune serum, but it was significantly less than that effected by R1 serum. In order to determine whether the reduction of induced enzyme activity by R1 serum was mediated by antibodies, the immunoglobulin G (IgG) fraction was purified from R1 serum by adsorption to columns of protein A-sepharose. As shown in Fig. 16, almost all of the induced enzyme activity was removed from solution by concentrations of IgG which reduced cellular enzyme activity by only approximately 35%. However, high concentrations of R1 IgG (approximately 1 mg) were required to mediate this effect; in the presence of 200 μ g of IgG no selective reduction of enzyme activity was observed. Similar results were obtained with IgG derived from sera collected after additional boosts.

The requirement for high concentrations of immune IgG may be due to the presence of very low levels of the antibodies specific for the induced enzyme in the IgG preparation. In other words, the antibody-antigen reaction may be in antigen excess at concentrations of IgG below 1 mg. If this were the case, a series of successive adsorptions, using less than 1 mg of R1 IgG, should effect a stepwise and eventually complete reduction of induced enzyme activity. The results of such an experiment are shown in Fig. 17. The induced and cellular enzymes were

Figure 16

Effect of IgG purified from R1 serum on ribonucleotide reductase activity. Aliquots of the induced (○) and cellular (●) enzymes, purified by salt fractionation, were incubated with increasing concentrations of R1 IgG in the presence of protein A-sepharose beads, as described in the text. Immune complexes were then removed by centrifugation and the supernatants assayed for residual enzyme activity at the optimal ATP and Mg^{2+} concentrations for either enzyme (i.e., 1 mM ATP and 6 mM Mg^{2+} for assay of the induced enzyme, 3 mM ATP and 6 mM Mg^{2+} for assay of the cellular enzyme). Enzyme activity is expressed as the percentage of activity measured following incubation of either enzyme with protein A-sepharose beads alone.

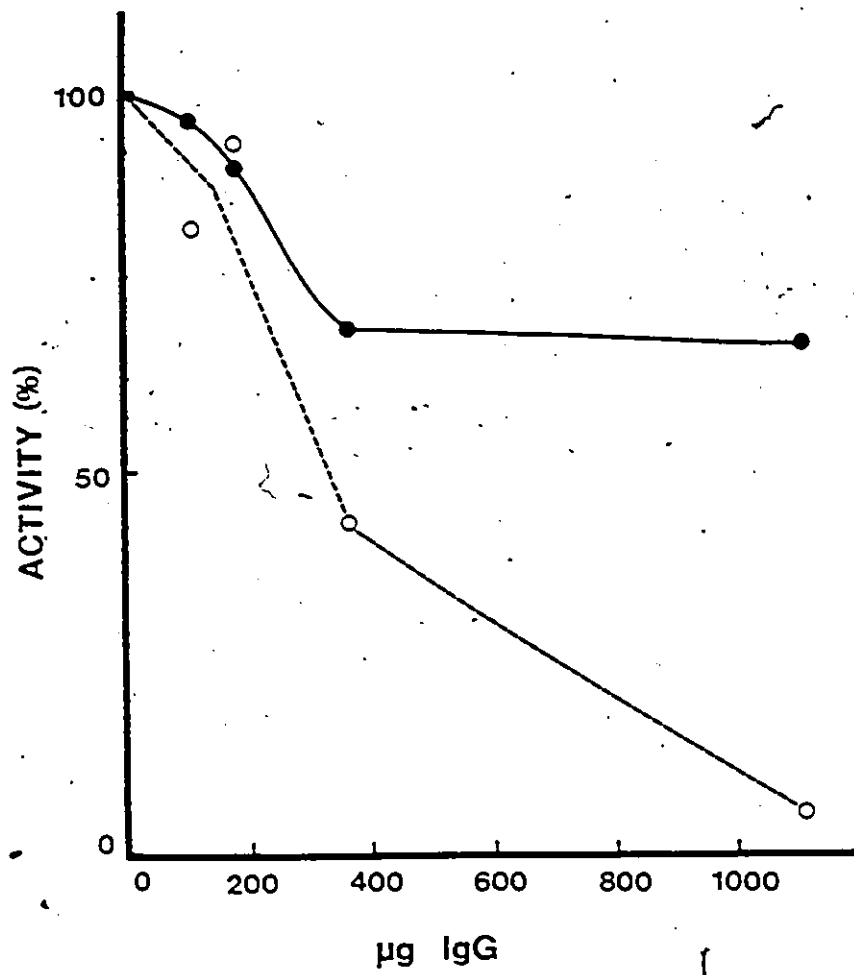
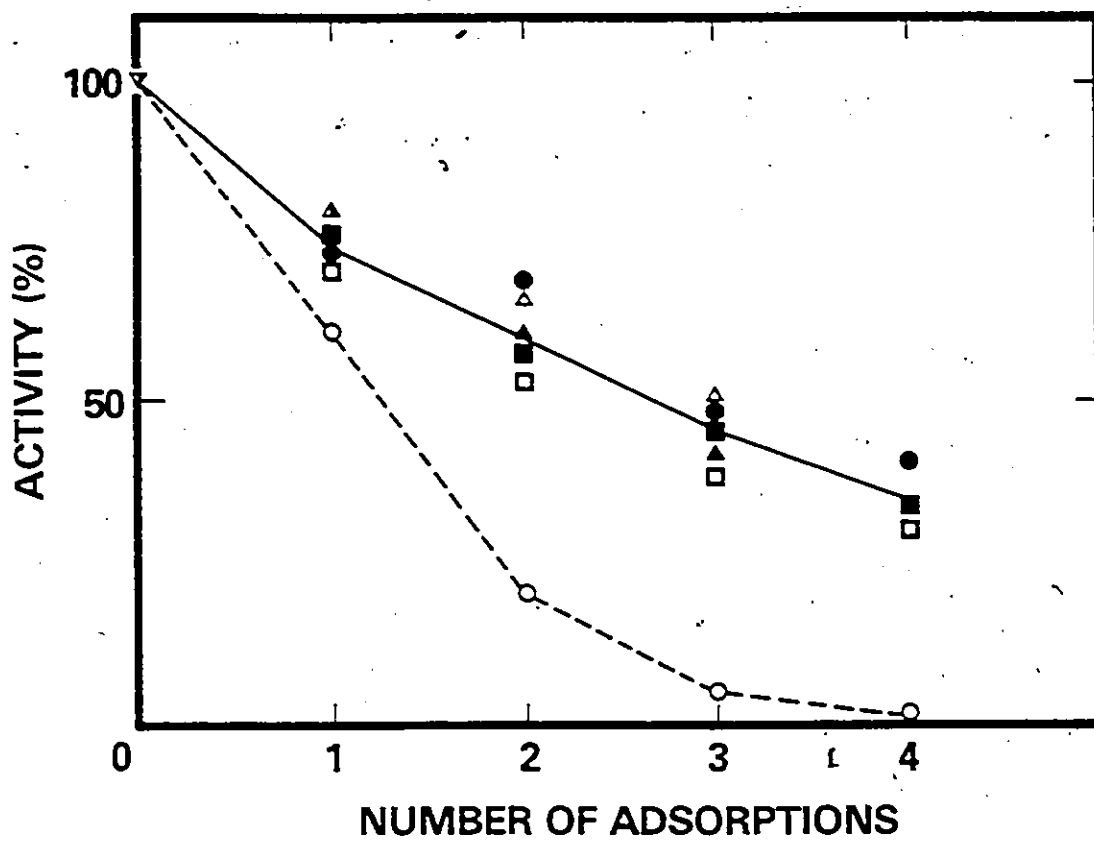


Figure 17

Effect of successive adsorptions with R1 IgG on ribonucleotide reductase activity. Aliquots of the ammonium sulfate purified induced (open symbols) and cellular (closed symbols) enzymes were reacted four times successively with protein A-sepharose beads, either alone (Δ, \blacktriangle) or in the presence of 200 μg of R1 IgG (\circ, \bullet) or 200 μg of pre-immune IgG (\square, \blacksquare). Following each reaction, immune complexes were removed by centrifugation and the supernatants assayed for enzyme activity at the optimal Mg^{2+} and ATP concentrations for the two enzymes. Enzyme activity is expressed as the percentage of activity measured in untreated controls ($\nabla, \blacktriangledown$).



adsorbed successively with 200 μ g of either RI or pre-immune IgG, or with buffer alone, in the presence of protein A-sepharose beads. The amount of enzyme activity remaining in the reaction mixture is expressed as a percentage of the activity of untreated enzyme samples (inverted triangles). As shown by the open circles, the activity of the induced enzyme was progressively decreased by successive adsorptions with immune IgG such that by the third or fourth reaction, no significant residual activity could be detected (this reduction seemed to be mediated by non-neutralizing antibodies, since no decrease in enzyme activity was observed in the absence of protein A-sepharose (not shown)). In contrast to the induced enzyme, the activity of the cellular enzyme was reduced to the same extent by incubation with IgG from immune (closed circles), or pre-immune rabbit serum (closed squares), or with buffer (closed triangles). A similar reduction of enzyme activity was also observed when the induced enzyme was incubated with IgG from pre-immune serum or with buffer (open squares and triangles; respectively). This non-specific reduction of enzyme activity was likely due to the dilution effect resulting from successive adsorptions, as well as to non-specific trapping of the enzymes by the beads (as mentioned previously).

3.6. Identification of Virally Coded Polypeptides with Reductase Activity

3.6.1. Immunoprecipitation of the HSV-2 induced reductase activity with H11 antibodies

Recent studies by Dutia (1982, 1983) have identified an HSV-1 temperature-sensitive (ts) mutant, designated ts_G, which induces a thermolabile ribonucleotide reductase activity. The ts lesion has been

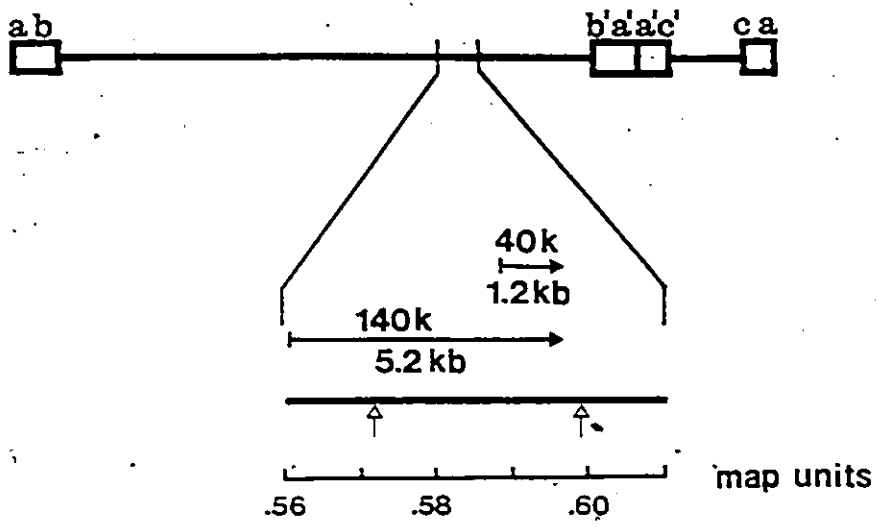
mapped in the Bam HI 0 fragment (.572 - .599 map units) of the viral genome, within DNA sequences encoding a 5.2 kb mRNA and close to or within sequences encoding a related 1.2 kb mRNA; both mRNA's are members of the β , or early, class of viral transcripts (Draper *et al.*, 1982). As shown in Fig. 18, the two mRNA species are 3' co-terminal and are unspliced, thus the nucleotide sequence of the smaller message is entirely contained within the larger one (Draper *et al.*, 1982; McLauchlan and Clements, 1982). *In vitro* translation of the mRNA's has indicated that the 5.2 kb species encodes a polypeptide of 140,000 daltons (140K) whereas a 40,000 (40K) dalton polypeptide is specified by the 1.2 kb mRNA (Anderson *et al.*, 1981). Dutia (1982) has also identified an HSV-2 ts mutant (ts 8) which seems to specify a thermolabile reductase activity. Ts 8 has not been mapped, however, it has been shown incapable of complementing the HSV-1 mutant.

Given that the HSV-1 and HSV-2 genomes are largely colinear with respect to gene order (Rapp, 1980), it seems likely that the ts 8 mutation will also map at .572 - .599 map units. RNA selected by this region of the HSV-2 genome also codes for two polypeptides of MW 140,000 and 35,000³ (Docherty *et al.*, 1981; Galloway *et al.*, 1982a) which appear to be specified by 2 overlapping 3' co-terminal mRNA species,

³ These are very likely the homologs of the HSV-1 140K and 40K polypeptides. As is often the case when molecular weight estimates are performed by different groups of investigators, there is some variability in the designation of the two polypeptides. In HSV-1 these polypeptides have been reported as 140K and 40K (Anderson *et al.*, 1981) and 136K and 38K (McLauchlan and Clements, 1982); in HSV-2 as 138 and 37.8K (Docherty *et al.*, 1981) and 140 and 35K (Galloway *et al.*, 1982).

Figure 18

Schematic diagram of mRNA species encoded by DNA sequences within .572-.599 map units of the HSV-1 genome. The open arrows indicate the location of the restriction endonuclease sites which define the BamHI 0 fragment; the closed arrows indicate the location and direction of transcription of the mRNA species homologous to the region. The size of the mRNA's is shown below the arrows and the size of the polypeptides encoded by each species is shown above. This data is based on S1 nuclease analysis and *in vitro* translational studies by Anderson *et al.* (1981), Draper *et al.* (1982) and McLauchlan and Clements (1982).

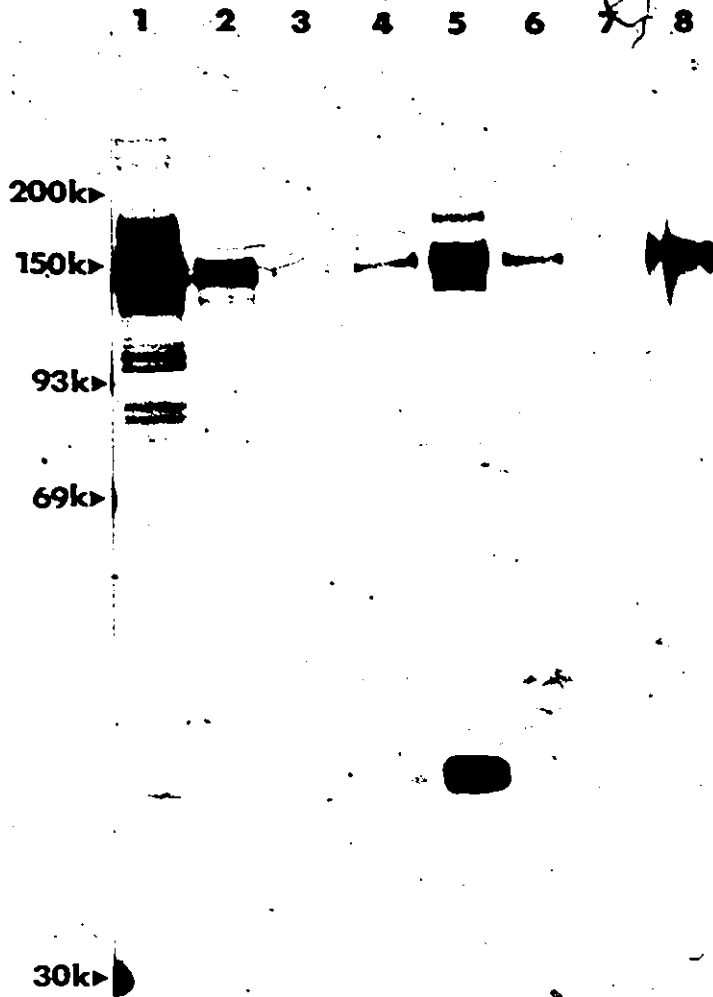


as in HSV-1 (Galloway *et al.*, 1982a; Galloway and McDougall, 1983). Interestingly, these HSV-2 DNA sequences also overlap the two regions of the HSV-2 genome (.42 - .58 map units (Jariwalla *et al.*, 1982), and .58 - .62 map units (Galloway *et al.*, 1982b)) which have been implicated in cell transformation (as discussed in greater detail in the Discussion; see Fig. 24).

As described in the previous section, results obtained with the R1 serum also indicated that the HSV-2 induced reductase activity might be associated with one or more polypeptides of MW 170,000, 144,000, 114,000, 110,000, 97,000, 93,000, 86,000 and 38,000 (see Fig. 15). As a result of the mapping data described above, attention was focussed on the 144K and 38K species precipitated by R1 serum. A recent report in the literature had described a monoclonal antibody (designated H11), used in the mapping of transformation related HSV-2 proteins, which reacted with both the 140K and 35K viral polypeptides (Galloway *et al.*, 1982a). The data presented in that report indicated that the two polypeptides share amino acid sequences, and antigenic sites, since H11 reacted with both molecules following transfer of infected cell proteins from a gel to nitrocellulose paper. In order to determine whether the 144K and 38K polypeptides recognized by R1 serum were identical to the virally coded 140K and 35K species, R1 serum was tested for cross reactivity with the H11 monoclonal antibodies. Lysates of HSV-2 infected radio-labelled BHK21 cl. 13 cells were exhaustively adsorbed with either R1 serum or H11 ascitic fluid and finally cross-adsorbed with H11 fluid or R1 serum, respectively. The products of the reactions were analysed on SDS gels. As shown in Fig. 19, lanes 1 and 5 respectively, in our

Figure 19

Cross-reactivity between R1 serum and H11 monoclonal antibodies. HSV-2 infected BHK21 cl. 13 cells were labelled with [^{35}S] methionine from 0-7 hours post-infection and harvested at 7 hours. Cell lysates were adsorbed four times in succession with either 50 μl of R1 serum or 10 μl of H11 ascitic fluid, and were then reacted with the heterologous antibody. The immunoprecipitates from each adsorption were electrophoresed on 9% SDS-polyacrylamide gels. Lanes 1-3 show the results of the first, third and fourth adsorption with R1 serum. Cross-adsorption of the final supernatant with H11 fluid is shown in lane 4. Lanes 5-8 refer respectively to the first, third and fourth adsorption with H11 fluid and cross-adsorption of the final supernatant with R1 serum. Molecular weight markers, as in Figure 14, were included in the gel, and polypeptides were visualized by fluorography.

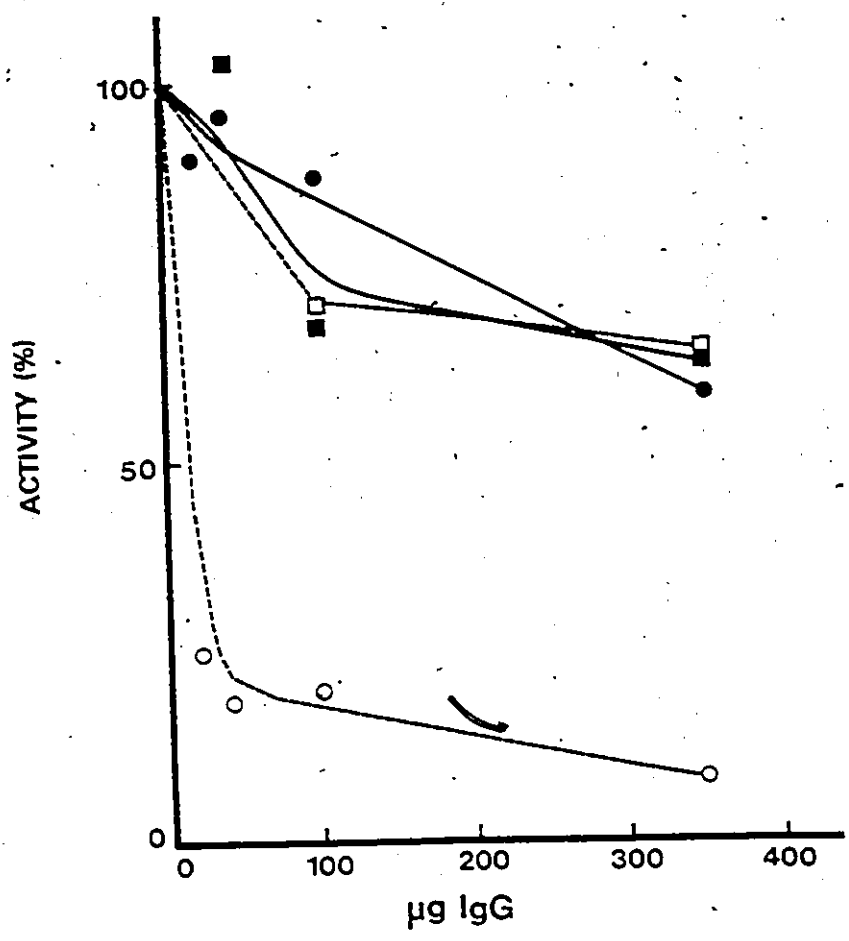


system both antibodies precipitated polypeptides of MW 144,000 and 38,000. However, R1 serum reacted preferentially with the 144K species whereas the H11 monoclonal antibodies were relatively more specific for the 38K polypeptide. In addition, as in Fig. 15, the polyvalent R1 serum precipitated other minor species which were not recognized by the H11 monoclonal antibodies. Successive adsorptions with R1 serum (lanes 2-3) removed almost all of the antigens recognized by H11 fluid (compare lane 4 to 5). In turn, H11 immunoprecipitated all of the 38K (lanes 5-7) but not all of the 144K molecule, residual amounts of which could still be immunoprecipitated by the R1 serum (compare lane 8 to lane 1). These data have been interpreted as indicating that R1 and H11 share specificity for the 144K and 38K polypeptides, and that their different affinities for the two species may reflect differential avidities of the antibodies and/or prevalence of one type of antibody in the polyvalent R1 serum.

In order to determine whether reactivity towards the 144K and 38K polypeptides was equivalent to reactivity with the virally induced reductase activity, the ability of H11 antibodies to inhibit the HSV-2 induced enzyme was tested. Preliminary experiments indicated that, as was the case with R1 antibodies, H11 antibodies did not directly neutralize enzyme activity (see Table 4). As shown in Fig. 20, however, when H11 IgG was incubated with enzyme in the presence of protein A-sepharose beads (as described in section 3.5.2. for R1 IgG) the induced enzyme activity was specifically and almost completely removed from the reaction mixture (open circles). Indeed, in this respect H11 IgG was much more efficient than R1 IgG, since induced enzyme activity was reduced by 90%

Figure 20

Effect of IgG purified from H11 ascitic fluid on ribonucleotide reductase activity. The ammonium sulfate purified induced (open symbols) and cellular (closed symbols) enzymes were incubated with IgG from H11 ascitic fluid (○, ●) or from normal rabbit serum (□, ■) in the presence of protein A-sepharose beads. Immunoprecipitates were removed by centrifugation and the supernatants assayed for enzyme activity at the optimal Mg^{2+} and ATP concentrations for either enzyme. Enzyme activity is expressed as the percentage of activity measured following incubation of each enzyme with protein A-sepharose beads alone.



in the presence of 300 μ g of H11 IgG compared to 1 mg of R1 IgG (see Fig. 16). As observed previously with R1 antibodies, the activity of the cellular enzyme was reduced to the same extent by treatment with either H11 (closed circles) or non-immune (closed squares) IgG; a similar reduction in activity was also observed following incubation of the induced enzyme with non-immune IgG (open squares).

These data demonstrate that removal of the 144K and 38K polypeptides from the enzyme assay is accompanied by removal of induced enzyme activity. In order to determine whether the enzyme was itself immunoprecipitated by the H11 antibodies the immunoprecipitates were tested for enzyme activity. Preliminary experiments indicated that only low and variable levels of enzyme activity could be detected in direct assays of the immunoprecipitates. This suggested that the enzyme might indeed be part of the immunoprecipitates but might be bound to the protein A-sepharose beads in a manner which impairs its activity. In an attempt to dissociate the antigen-antibody complexes, immunoprecipitates were incubated with 0.1 M DTT prior to enzymatic assay; this compound was chosen as it is known to reduce disulfide bridges and could thus alter the conformation of the antibody molecules. As shown in Table 3, no cellular enzyme activity was detected in immunoprecipitates. In contrast, high levels of induced enzyme activity were consistently detected in immunoprecipitates derived from reaction of the induced reductase with H11 IgG and protein A-sepharose beads. Similar results have been obtained with immunoprecipitates of the induced enzyme by R1 IgG (not shown). Significantly lower levels of induced enzyme activity

Table 3

Recovery of Ribonucleotide Reductase Activity
from Immunoprecipitates

µg IgG		Enzyme activity (pmoles dCMP/30 minutes)			
		Cellular enzyme ^b		Induced enzyme ^b	
H11	NRS ^a	supernatant ^c	beads ^d	supernatant ^c	beads ^d
-	-	350	4	189	15
20	-	314	0	47	576
40	-	338	0	35	573
100	-	308	0	38	416
350	-	208	4	16	623
-	100	239	0	134	78
-	350	223	2	122	156

^a Normal rabbit serum.

^b The enzymes used were partially purified by ammonium sulfate fractionation.

^c Aliquots of the enzymes were incubated with the indicated concentrations of NRS IgG or H11 IgG in the presence of protein-A sepharose beads. Immunoprecipitates were then removed by centrifugation and the supernatants assayed for enzyme activity under standard conditions at the optimal Mg^{2+} and ATP concentrations for the two enzymes.

^d Immunoprecipitates were washed twice with buffer, incubated with 0.1 M DTT for 15 minutes and then made up to the enzyme reaction mixture, including the optimal concentrations of Mg^{2+} and ATP for either enzyme. The final concentration of DTT in the assays was 67 mM.

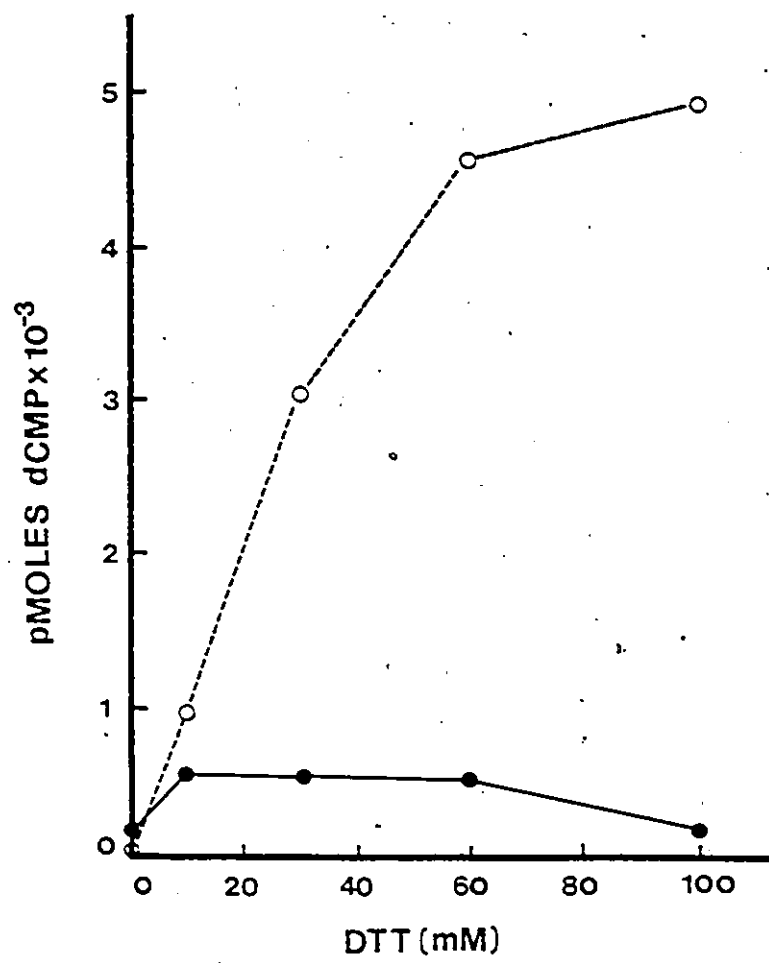
were recovered following reaction of the enzyme with non-immune IgG and beads. Quantitation of the recovery of enzymatic activity was complicated by the fact that well over 100% of the input activity was detected in the immunoprecipitates (this value varied between experiments, from 200-400% of the input activity). Further experiments showed the enhancement to be due to the increased concentration of DTT in the assay mixture following treatment with 0.1 M DTT. The final concentration of the reducing agent in these assays was 67 mM, whereas, as described in Materials and Methods, under standard conditions the enzymes were assayed in the presence of 6.2 mM DTT. As shown in Fig. 21, the activity of the induced enzyme (open circles) was found to be dramatically enhanced by increasing concentrations of DTT up to 100 mM; in particular at 67 mM DTT the enhancement was 7-8 fold relative to 6.2 mM DTT. The cellular enzyme (closed circles) attained a maximal but much lower level of activity between 10 and 60 mM DTT and was inhibited by higher concentrations of the reducing agent.

Experiments carried out in parallel with those shown in Table 3 demonstrated that treatment of immunoprecipitates with 0.1 M DTT did not result in solubilization of the induced enzyme (not shown). These data, together with those shown in Fig. 21, suggest that the role DTT plays in the recovery of enzyme activity from immunoprecipitates is to enhance the activity of the immunoprecipitated enzyme, rather than to dissociate enzyme-antibody complexes.

The low amount of induced enzyme activity in immunoprecipitates derived from reaction of the induced enzyme with non-immune IgG (Table 3) indicates that, as suggested earlier, non-specific trapping of the

Figure 21

Effect of DTT on ribonucleotide reductase activity. Aliquots of the induced (○) and cellular (●) enzymes purified by salt fractionation were assayed at their optimal Mg^{2+} and ATP concentrations. The abscissa indicates the concentration of exogenous DTT in the assays; in addition, each assay contained approximately 0.1 mM DTT due to the presence of the compound in the enzyme preparations. This experiment was performed by Dr. S. Bacchetti and B. Muirhead.

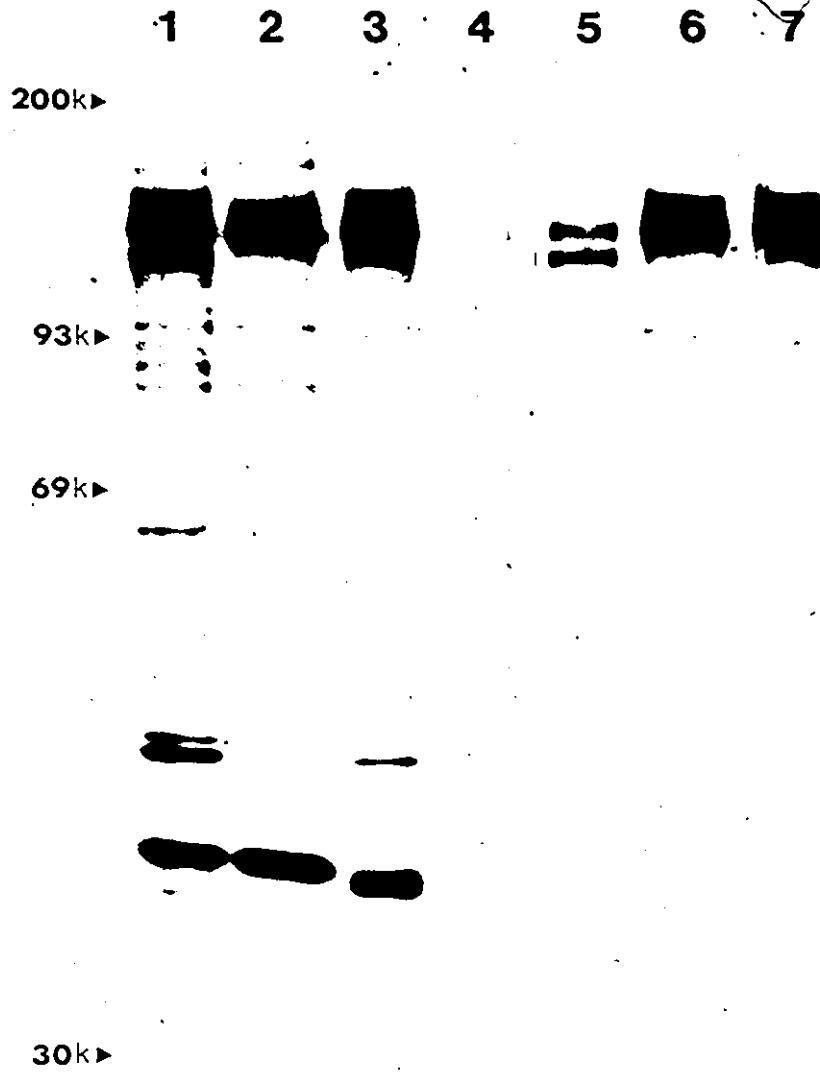


enzymes by the protein A-sepharose beads does occur. The apparent absence of a similar trapping of cellular enzyme activity in these experiments is likely due to the fact that unlike the induced enzyme, the activity of the cellular reductase is not enhanced by high concentrations of DTT (see Fig. 21) and thus remains below detectable levels in immunoprecipitates.

The results presented in Table 3 demonstrate that H11 antibodies are indeed precipitating the HSV-2 induced ribonucleotide reductase and suggest that either the 144K or 38K polypeptides, or both, might be associated with enzyme activity. On the other hand, the data do not rule out the possibility that enzyme activity might be associated with other polypeptides which may be non-specifically precipitated by H11 antibodies under the conditions of the enzyme precipitation reactions. The immunoprecipitations shown in Figures 14, 15 and 19 defined the specificity of R1 and H11 antibodies and were performed under denaturing conditions (i.e., in RIPA buffer, see Materials and Methods) to minimize aggregation of the polypeptides. For obvious reasons, immunoprecipitation of enzyme activity was carried out under non-denaturing conditions, and it is thus possible that some degree of non-specific precipitation occurred in those experiments. To control for this possibility, the induced enzyme was partially purified by ammonium sulfate fractionation of lysates of radiolabelled HSV-2 infected cells and then immunoprecipitated with H11, R1, or non-immune antibodies under both denaturing and non-denaturing conditions. The immunoprecipitates were then electrophoresed on 9% SDS-polyacrylamide gels, as shown in Figure 22. Similar to the results previously obtained with R1 antibodies (Fig. 14), under denaturing conditions

Figure 22

Electrophoretic analysis of the antigens immunoprecipitated by H11, R1 and non-immune antibodies from radiolabelled purified preparations of the induced enzyme, under denaturing and non-denaturing conditions. BHK21 cl. 13 cells were labelled with [^{35}S] methionine from 0-7 hours following infection with HSV-2, harvested at 7 hours, and the induced enzyme partially purified by ammonium sulfate fractionation of cell lysates. Aliquots of the induced enzyme preparation corresponding to 310 μg of protein were then immunoprecipitated under denaturing conditions (i.e., in the presence of RIPA buffer, see Materials and Methods) with either 10 μl of H11 ascitic fluid (lane 2), 150 μg of IgG from normal rabbit serum (lane 4), or 40 μl of R1 serum (lane 6), as well as under non-denaturing conditions with 150 μg of H11 IgG (lane 3), 150 μg of IgG from normal rabbit serum (lane 5), and 1 mg of R1 IgG (lane 7). Lane 1 shows the partially purified induced enzyme preparation prior to immunoprecipitation. Molecular weight markers, as in Figure 14, were included in the gel, and polypeptides were visualized by fluorography.



H11 fluid precipitated primarily two polypeptides of approximate MW 144,000 and 38,000 daltons (lane 2), corresponding to the major labelled species in the partially purified preparation of the induced enzyme (lane 1). A few minor species, not consistently obtained in every experiment and also seen under similar conditions with R1 serum (lane 6), but not with normal rabbit serum (lane 4), were also immunoprecipitated. Since H11 is a monoclonal antibody (Galloway *et al.*, 1982a) these polypeptides are likely related to the 144,000 dalton species and to originate by post-translational modification or degradation.

The results of immunoprecipitations with H11, normal rabbit serum, or R1 IgG's under non-denaturing conditions are shown in lanes 3,5, and 7 respectively. Both H11 and R1 IgG's recognized primarily the 144K and 38K polypeptides and immunoprecipitated both species in absolute and relative amounts comparable to those obtained under denaturing conditions. Significantly lower amounts of these two polypeptides were also immunoprecipitated by IgG from normal rabbit serum (lane 5). In addition, R1 and H11 antibodies precipitated minor species of which only a subset corresponded to those obtained under denaturing conditions. Of the remainder, some were precipitated with H11 but not with R1 IgG, the others, with the possible exception of the 155,000 dalton polypeptide, were present in approximately equal or greater amounts in immunoprecipitates obtained with IgG from normal rabbit serum. Enzymatic assays of the supernatants, following removal of immunoprecipitates from the reactions, demonstrated that the immune IgG's precipitated 96% (H11) and 78% (R1) of the input enzymatic activity, whereas IgG from normal rabbit serum removed only 40%. These values appear to correlate well with the amounts of 144K and 38K immunoprecipitated in each case; on the other hand, no such correlation seems

to exist for the minor species, an observation which renders it unlikely that these species are involved in enzymatic activity.

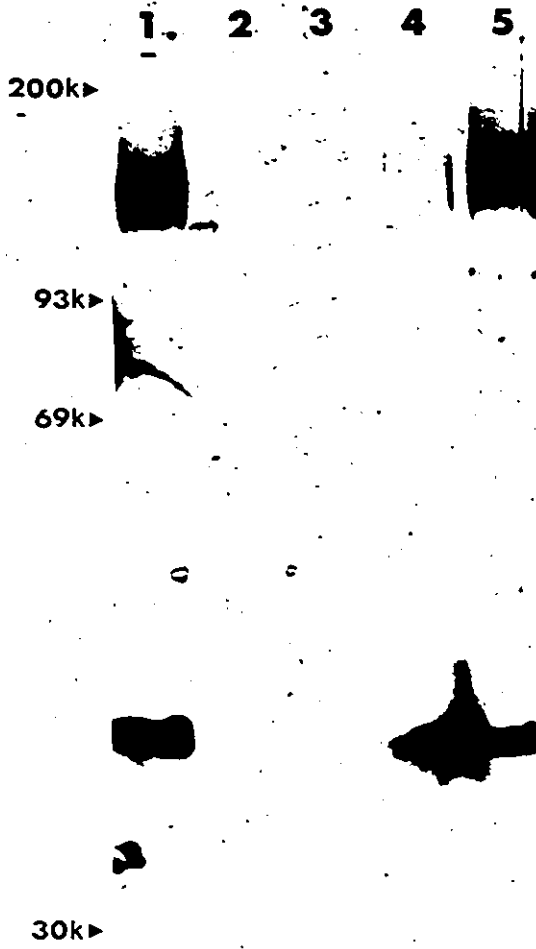
3.6.2. Neutralization of the HSV-2 induced reductase activity with Bg7 antibodies

The hypothesis that the 144K and/or the 38K polypeptides are constituents of the viral reductase is further supported by data obtained with an additional hybridoma antibody, Bg7, developed by M.J. Eveleigh in our laboratory. Bg7 was found to immunoprecipitate antigens of molecular weight 144,000 and 38,000; that these are the same antigens recognized by H11 antibodies was established by competition experiments, as previously described for H11 and R1 antibodies. The results of one such experiment are shown in Fig. 23. Successive adsorption with H11 ascitic fluid (only the 1st and 4th are shown in lanes 1 and 2 respectively) removed all of the 144K and 38K antigens, and cross-adsorption with Bg7 fluid (lane 3) did not result in additional immunoprecipitation. Lane 4 refers to the products of the reaction of Bg7 with a control antigen which had been incubated with protein A-sepharose beads alone while the H11 adsorptions were being carried out, and lane 5 refers to the reaction of Bg7 with fresh control antigen. Apparently, extensive degradation of the 144K species occurred during the experiment; nevertheless, both this and the 38K species persist in sufficient amounts to react with Bg7 antibodies (lane 4), whereas they are depleted after reaction with H11 antibodies (lane 3).

In addition to immunoprecipitating the 144K and 38K antigens, Bg7 was found capable of specifically neutralizing the HSV-2 induced

Figure 23

Cross reactivity between H11 and Bg7 antibodies. HSV-2 infected BHK21 cl. 13 cells were labelled with [³⁵S] methionine from 0-7 hours post-infection and harvested at 7 hours. Cell lysates were adsorbed 4 times in succession with 10 μ l of H11 ascitic fluid and then reacted with 10 μ l of Bg7 ascitic fluid. Lanes 1 and 2 show the first and fourth adsorption with H11 fluid respectively, and lane 3 shows cross-adsorption of the final supernatant with Bg7 fluid. The reaction of Bg7 fluid with control antigen which had been incubated with protein A-sepharose beads alone, for the time required to perform the H11 adsorptions, is shown in lane 4; lane 5 refers to the reaction of Bg7 with fresh control antigen. Molecular weight markers, as in Figure 14, were included in the gel, and polypeptides were visualized by fluorography.



ribonucleotide reductase, as shown in Table 4. The HSV-2 induced and cellular enzymes were incubated with IgG purified from Bg7 or H11 ascitic fluid, or normal rabbit serum, for 30 min at room temperature and then assayed for enzymatic activity. As little as 10 μ g of Bg7 IgG effected a greater than 50% neutralization of induced enzyme activity, and in the presence of 30 μ g of IgG the activity of the enzyme was almost completely inhibited. In contrast, similar concentrations of Bg7 IgG did not inhibit the activity of the cellular enzyme. As already mentioned, H11 IgG was incapable of neutralizing induced enzyme activity.

These data demonstrate that reaction of Bg7 with the 144K or 38K polypeptides specifically inhibits the activity of the HSV-2 induced reductase, indicating that at least one of these polypeptides is a constituent of the induced enzyme. Based on the mapping of the 144K and 38K polypeptides (Anderson *et al.*, 1981; Docherty *et al.*, 1981; Galloway *et al.*, 1982a) these data locate the coding sequences of at least a component of the HSV-2 ribonucleotide reductase between .56 and .60 map units on the viral genome, within DNA sequences associated with cell transformation. The mapping is in agreement with that obtained by Dutia (1982) for a *ts* lesion affecting the HSV-1 ribonucleotide reductase.

Table 4

Neutralization of Virus Induced
Reductase Activity

µg IgG		% Enzyme activity ^a	
Bg7	H11	Induced	Cellular
-	-	100	100
10	-	43	ND ^b
30	-	3	105
60	-	1	72
100	-	1	75
-	50	128	ND
-	100	76	ND

^a Aliquots of the ammonium sulfate purified induced and cellular enzymes were incubated either with buffer alone, or with IgG from Bg7 or H11 ascitic fluid, in a constant volume for 30 minutes at room temperature with intermittent mixing, and assayed immediately thereafter. Both enzymes were assayed in the presence of 60 mM DTT and at their optimal Mg²⁺ and ATP concentrations. As indicated, enzyme activity is expressed as the percentage of activity measured following incubation of each enzyme with buffer alone.

^b ND, not determined.

4. DISCUSSION

The results of the present study have shown that infection of mammalian cells with HSV-2 results in the synthesis of a novel ribonucleotide reductase which is biochemically and immunologically distinct from the uninfected cell enzyme. They have also shown that antibodies specific for the induced enzyme are equally specific for two HSV-2 polypeptides of 144,000 and 38,000 daltons; thus either one, or both, of these viral gene products appear to be constituents of the enzyme. In addition to establishing the viral origin of the induced reductase, the identification of the viral polypeptides involved in enzymatic activity has allowed the mapping of the enzyme (or part of it) within coordinates .56 - .60 of the viral genome. This location is consistent with that recently reported for a ts lesion affecting the HSV-1 induced reductase (Dutia 1982, 1983). To date, the only other viruses known to encode a ribonucleotide reductase are the bacteriophages T2, T4, T5 and T6 (Holmgren, 1981). Although several other herpesviruses (Epstein-Barr virus, equine herpesvirus and pseudorabies virus (Henry *et al.*, 1978; Cohen *et al.*, 1977; Lankinen *et al.*, 1982) are capable of inducing a novel ribonucleotide reductase upon infection of cells, the viral origin of these enzymes has yet to be established.

The induction of the HSV reductase is kinetically similar to the induction of the viral thymidine kinase, suggesting that the reductase belongs to the early, or β , class of viral polypeptides, as do other viral functions involved in DNA metabolism. Characterization of the viral reductase was carried out primarily on enzyme preparations which had been partially purified by ammonium sulfate fractionation of infected cell lysates. Although this procedure afforded only a limited degree of purification in terms of the specific activity of the enzyme, it resulted in a viral enzyme preparation which was essentially free of cellular reductase activity. Separation of the viral and cellular reductases by ammonium sulfate precipitation has since been reproduced by others, using both HSV-1 and HSV-2 infected cells (Dutia, personal communication; Cohen *et al.*, 1982).

The allosteric regulation of the HSV enzyme appears to be quite different from that of the cellular reductase. The data presented in Fig. 7a, as well as those reported by others (Ponce de Leon *et al.*, 1977; Langelier and Buttin, 1981), indicate that reduction of CDP by the viral enzyme is only slightly inhibited by concentrations of dATP and dTTP which almost completely inhibit cellular enzyme activity. In this respect, as well as in others, the HSV enzyme resembles the T4 reductase. Unlike the *E. coli* and mammalian enzymes, the overall activity of the T4 reductase is not inhibited by dATP since the enzyme lacks the I sites (see Fig. 3a) which bind the nucleotide to mediate its inhibitory effect (Berglund, 1972b). In addition, the T4 enzyme is virtually insensitive to inhibition by the other nucleotide effectors; binding of dCTP, dTTP, dGTP, dATP and ATP

to the single effector binding site (analogous to the bacterial h site; see Fig. 3a) results almost exclusively in stimulation of enzyme activity, albeit to varying degrees depending upon both the effector and the substrate (Berglund, 1972b). As mentioned in the Introduction, the virtual elimination of inhibitory effects is likely geared towards all out synthesis of phage DNA during which large amounts of the DNA precursors are required. By analogy with the regulation of T4 enzyme activity, the data of Fig. 7a may indicate that the HSV reductase is also insensitive to overall inhibition by dATP, as well as to inhibition by the other nucleotide effectors. An apparent difference between the HSV and T4 enzymes is that whereas the reduction of CDP by the latter enzyme is stimulated by both dATP and dTTP (Berglund, 1969; 1972b), the activity of the HSV reductase seems to be unaffected by the two nucleotides (Fig. 7a). This may indicate that the HSV enzyme does not respond to allosteric regulation, however an accurate assessment of this property will require testing of all the effectors on the reduction of all four substrates. Although recent data by Cohen *et al.* (1982) have indicated that the HSV enzyme is also capable of reducing ADP and GDP, all of the work published to date in this system has monitored only the reduction of CDP, likely because it is the most convenient of the assays to perform.

Although the preceding discussion has stressed the absence of regulatory effects on the reduction of CDP by the HSV enzyme, this reaction can apparently be inhibited by ATP (see Fig. 8b). As discussed in section 3.3.5., this effect was consistently observed under a variety of experimental conditions using enzyme preparations partially

purified by fractionation with salt. These data are in contrast to those of Ponce de Leon *et al.* (1977) who reported that both the HSV-1 and HSV-2 enzymes show an absolute requirement for ATP for activity. Indeed, for none of the other reductases so far described, including those induced by other herpesviruses, does ATP behave as a negative effector. This raises the possibility that the results shown in Fig. 8b might be an artefact of our system due to indirect effects of the nucleotide on enzyme activity. This possibility is supported by preliminary results, described in the footnote to section 3.3.9., which suggest that more purified enzyme preparations recovered from glycerol gradients appear to require ATP for maximal activity. These data indicate that proper assessment of the allosteric regulation of the HSV reductase will likely require homogeneously pure enzyme preparations for testing.

The data presented in Fig. 21, demonstrating that high concentrations of DTT are required for maximal activity of the HSV reductase, indicate that considerably higher levels of viral enzyme activity were present in partially purified enzyme preparations than originally estimated under standard assay conditions (i.e., in the presence of 6.2 mM DTT). This is also true of viral enzyme activity in crude extracts, which was also stimulated by DTT, albeit to 50% the level obtained with purified enzyme preparations (not shown). DTT is known to effect ribonucleotide reductase in several ways: firstly, low concentrations of the compound (1 mM) stabilize enzyme activity (Brown *et al.*, 1969; Ponce de Leon *et al.*, 1977); secondly, as mentioned in the Introduction, DTT can act as a direct hydrogen donor

for the enzyme; lastly, in the presence of the physiological hydrogen donor, DTT can serve to regenerate this molecule, thus acting as an indirect hydrogen donor (Brown *et al.*, 1969; Berglund, 1972a). It is not possible, on the basis of the experiment shown in Fig. 21, to discriminate between the latter two possibilities, and therefore these data could reflect differential affinities for DTT of either the enzymes themselves or of their physiological hydrogen donors. While the partitioning of the physiological hydrogen donors during partial purification has not been determined, it is interesting to note that fraction 3 from infected or uninfected cells is capable of specifically enhancing viral enzyme activity, indicating that this fraction may contain the physiological hydrogen donor for the enzyme.

The sedimentation properties of the viral reductase suggest that either it has an unusually large molecular weight (far in excess of 200,000 daltons) or more likely that it sediments as part of a high molecular weight complex. Although it has been reported that, *in vivo*, the reductase might form part of a multienzyme complex, such structures seem to be very labile and have only been detected in permeabilized mammalian cells (Prem Veer Reddy and Pardee, 1980) or gently lysed bacterial cells (Allen *et al.*, 1980). As described in Materials and Methods, sonication of cell suspensions was used in the preparation of the HSV enzyme, and multienzyme complexes were thus likely disrupted. Furthermore, the HSV reductase did not appear to cosediment with any of the known viral enzymes involved in DNA metabolism, or to be associated with cell membranes. The high molecular weight of the viral reductase thus likely results from *in vitro* aggregation with other polypeptides, or from self-aggregation. Indeed,

in some experiments in which crude cell extracts were used, the bulk of the viral enzyme activity was found to cosediment with the cellular enzyme (not shown). Although these results were not always reproducible, they suggest the possibility that the high molecular weight form of the reductase may result from aggregation during precipitation with ammonium sulfate. Whatever the nature of the aggregate, it appears to result from specific interactions, since the sedimentation rate of the partially purified viral enzyme was reproducible between experiments, and for different enzyme preparations.

Electrophoretic analysis of the glycerol gradient fractions containing viral enzyme activity revealed the presence of only a limited number of polypeptides, among which the major species appeared to be specific to virus infected cells. Immunization of rabbits with this material resulted in the production of antibodies (RI serum) which were capable of specifically immunoprecipitating, although not of neutralizing, the HSV-2 reductase activity. Subsequent experiments identified monoclonal antibodies to viral specified polypeptides which were even more efficient in immunoprecipitating the viral enzyme activity (H11 antibodies) or could directly neutralize it in solution (Bg7 antibodies). Although others have described immunological differences between cellular and herpesvirus induced reductases (Lankinen *et al.*, 1982), these data constitute the first report of antibodies with specificity for a herpesvirus induced reductase. Such antibodies might provide a means for rapid and efficient purification of the viral enzyme by immunoaffinity chromatography which in turn would allow precise characterization of the structure of the

molecule and its activity.

The ability of R1, H11 and Bg7 antibodies to react with the HSV-1 reductase has not been assessed, however as tested by immunoprecipitation all three antibodies appear to react preferentially with HSV-2 antigens (Fig. 15; Galloway *et al.*, 1982a; M. Eveleigh, unpublished) suggesting that the enzymes encoded by the two HSV serotypes are immunologically distinguishable. Similar differences have been observed for essentially all of the viral antigens tested to date (Spear and Roizman, 1981), including the viral enzymes thymidine kinase (Campione-Piccardo and Rawls, 1981) and DNAase (Hoffman and Cheng, 1978). This is the only significant difference thus far detected between the HSV-1 and HSV-2 reductases; the limited characterization of the HSV-1 enzyme carried out in this study, as well as work carried out by others (Ponce de Leon *et al.*, 1977; Langelier and Buttin, 1981), indicates that otherwise the HSV-1 reductase is quite similar to its HSV-2 counterpart.

In addition to their ability to react with the viral reductase, all three antibodies share specificity against two virally coded polypeptides of 144,000 and 38,000 daltons. This indicates that enzymatic activity must be associated with either one, or both, of these polypeptides and that at least a component of the reductase is virally coded. In addition, since the map location of the polypeptides is known (.56 -.60 map units), these data allow the identification of viral DNA sequences encoding part, or all, of the enzyme. The mapping is in agreement with results recently reported by Dutia (see section 3.6.6.) which locate a *ts* lesion affecting the HSV-1 reductase within

a region of the HSV-1 chromosome (.572 - .599 map units) which also encodes polypeptides of molecular weight 144K and 38K. These data, however do not rule out the possibility that the viral enzyme may be a hybrid molecule consisting of viral and cellular polypeptides. On the other hand, recent experiments by Langelier *et al.* (personal communication) have shown that viral enzyme activity is sensitive to inhibitors of the M1 and M2 subunits of the mammalian reductase and cannot be restored by addition of either one of these components. This suggests that the subunits of the mammalian enzyme do not participate in viral reductase activity and that the HSV enzyme might thus be entirely coded for by the virus.

Unless the HSV reductase is structurally different from the reductases characterized to date, it should resemble the *E. coli* class of enzymes by virtue of its sensitivity to hydroxyurea (see Introduction), and thus consist of two non-identical subunits. In the T4 and *E. coli* enzymes, polypeptides of approximately 85,000 and 80,000 daltons constitute the nucleotide-binding moiety, whereas the iron-containing (or hydroxyurea sensitive) moiety consists of polypeptides of approximately 35,000 and 39,000 daltons, respectively. The iron-containing subunit of the mammalian enzyme has not yet been purified to homogeneity, but several groups have estimated a molecular weight of approximately 90,000 daltons for the nucleotide binding subunit (Moore, 1977; Chang and Cheng, 1979; Thelander *et al.*, 1980). As mentioned in the Introduction, however, a recent study has suggested that the latter subunit may be formed by the association of various combinations of four polypeptides of molecular weight 45,000 daltons

each specific for reduction of a single substrate, giving rise to nucleotide-binding subunits with molecular weights of 45,000, 90,000, 135,000 and 180,000 units (Youdale *et al.*, 1982). Although a comparison on the basis of size might be meaningless, it is possible to speculate from these data that the HSV 38K and 144K polypeptides could represent, respectively, the iron-containing and nucleotide-binding subunits of the viral reductase. It should be noted, however, that enzymatically active immunoprecipitates contain, in addition to the 144K and 38K species (Fig. 22), other polypeptides. Although their presence does not appear to correlate with enzyme activity, the possibility that they are components of the viral enzyme has not been ruled out.

As described in section 3.6.1., in both HSV-1 and HSV-2 the 144K and 38K polypeptides appear to be encoded by overlapping, 3' co-terminal mRNA species located between .56 and .60 map units. DNA sequencing of the region encompassing the message for the 38K polypeptide has recently indicated that the two polypeptides of both serotypes appear to be translated in different reading frames and are thus unlikely to share amino acid sequences (Clements and McLaughlan, 1981; Draper *et al.*, 1982; Galloway, personal communication). This contradicts results previously reported by Galloway *et al.*, (1982a) claiming that H11 monoclonal antibodies are capable of recognizing both polypeptides even after their separation by electrophoresis. Although this point remains unsolved, the common specificity of several antibodies for both polypeptides indicates either the existence

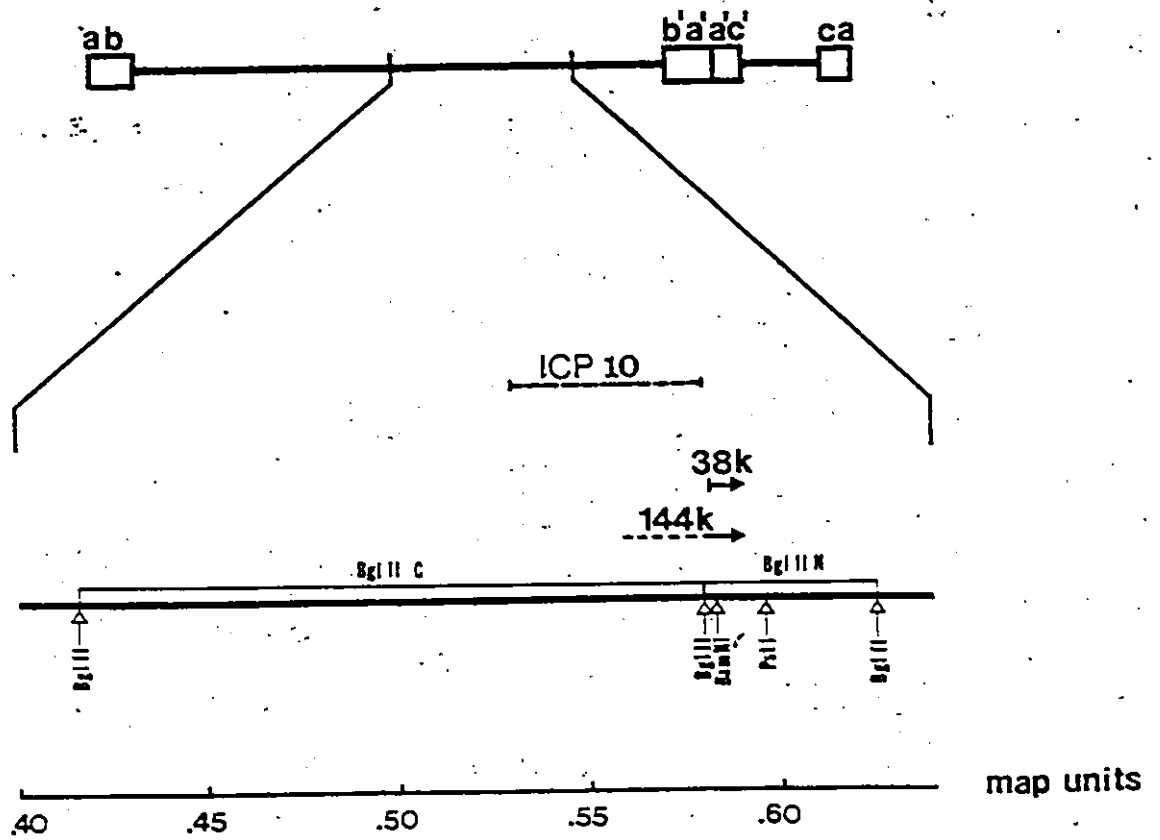
of common antigenic sites or a very specific and persistent association of the two polypeptides.

An apparent difference between the two serotypes in the relative abundance of the mRNA species encoding the 144K and 38K polypeptides has been reported. In HSV-1, the 5.2 kb mRNA specifying the 144K polypeptide is a major species, present in greater amounts than the 1.2 kb mRNA encoding the 38K polypeptide (Anderson *et al.*, 1981). The abundance of the analogous HSV-2 mRNA's has not been directly assessed, however it has been determined that the 38K polypeptide is the major *in vitro* translation product of this region of the genome, whereas only low levels of the 144K product can be detected (Docherty *et al.*, 1981; Galloway *et al.*, 1982a). At present, it is not clear whether these represent real differences between the serotypes, or whether they are artefacts of RNA extraction and/or *in vitro* translation. Indeed, our *in vivo* studies (see Fig. 14) indicate that both the 144K and 38K polypeptides appear to be major polypeptides in HSV-2 infected cells.

As mentioned earlier, the HSV-2 144K and 38K polypeptides are encoded by DNA sequences overlapping two regions of the HSV-2 genome which have been implicated in cell transformation. Studies by Jariwalla *et al.* (1980, 1982) have shown that the Bgl II C fragment (.416 - .580 map units, see Fig. 24) of HSV-2 can transform Syrian hamster embryo cells; DNA sequences from the left end of the fragment (.419 - .525 map units) being capable of immortalizing the cells, whereas sequences from the entire fragment are required for their acquisition of oncogenic potential. Expression of the latter property

Figure 24

Schematic diagram of HSV-2 DNA sequences associated with cell transformation. The closed arrows indicate the location and direction of transcription of the mRNA species encoding the 144K and 38K polypeptides. This data is based on *in vitro* translational studies and DNA sequencing, the mRNA species themselves have not been characterized or precisely mapped (Galloway *et al.*, 1982a; Galloway and McDougall, 1983). The broken line spans DNA sequences which reportedly contain the gene for the polypeptide ICP 10 (Jariwalla *et al.*, 1980).



was found to correlate with the expression of the viral protein ICP 10 (Aurelian *et al.*, 1981). Although the sequences coding for this polypeptide have not been precisely identified, they have been roughly mapped to the area of the genome between .53 and .58 map units (Fig. 24), and indeed it has been suggested that ICP 10 and the 144K polypeptide might be identical. In contrast to the results of Jariwalla *et al.*, studies by Reyes *et al.* (1980) and Galloway and McDougall (1981) have identified the Bgl II N fragment of HSV-2 (.58 - .62 map units, see Fig. 24) as capable of inducing morphological alterations and oncogenic potential in primary or cultured rodent cells. More recent studies have shown that a cloned 2.1 kb Bam HI-Pst I fragment from the left end of Bgl II N (see Fig. 24) is sufficient to effect this transformation (Galloway *et al.*, 1982b) although these sequences are not retained in the transformants (Galloway and McDougall, 1981). DNA sequencing has demonstrated that the 2.1 kb transforming region contains all the coding sequences for the 38K polypeptide, except for the two initial nucleotides, but lacks the promoter sequences which are just upstream of the Bam HI site (Galloway and McDougall, 1983). Although, as mentioned, the transforming fragment is not retained in the cells, and indeed it is not known whether the 38K polypeptide can be expressed by the recombinant plasmid, it is worth noting that a polypeptide of approximately 38,000 daltons has been immunoprecipitated with anti HSV-2 sera from HSV-2 transformed cells (Suh *et al.*, 1980). In addition, sera from cancer patients have higher titers of antibodies to a 38,000 dalton polypeptide than do sera from control patients (Gilman *et al.*, 1980).

The results of the present study and the data reviewed above thus suggest that polypeptides which are associated with HSV-2 reductase activity may also be associated with cell transformation. This is an intriguing possibility in light of the fact that as described in the Introduction, HSV may transform via a "hit and run" mechanism mediated by the transient expression of viral genes. According to this hypothesis, HSV-2 might transform cells by acting as a mutagen or carcinogen. It is tempting to speculate that such a mutagenic effect could be exerted by the transient expression of the viral reductase. As discussed in section 1.2.5., imbalances in intracellular pools of deoxyribonucleoside triphosphates are known to have a mutagenic effect, and ribonucleotide reductases which are altered in their regulatory control, can indeed act as mutators. The reductase induced by HSV-2 appears to be insensitive to allosteric inhibition by dTTP and dATP, and infection of cells with HSV has been shown to induce imbalances in intracellular levels of deoxyribonucleotides (Cheng *et al.*, 1975; Jamieson and Bjursell, 1976a). Taken together, these data support a role for the HSV-2 reductase in cell transformation via mutagenesis, however it is at present difficult to reconcile the mapping of both the 144K and 38K polypeptides with the location of the two transforming regions (see Fig. 24). This difficulty is in large part due to the uncertainty on the role of each polypeptide in enzymatic activity and to the absence of a more accurate mapping of the transforming function(s) within the Bgl II C fragment. In addition, expression of ribonucleotide reductase might only be one of several mutagenic processes occurring during viral infection. Indeed,

transformation of cells by HSV-1 appears to be associated with DNA sequences located within .31 - .42 map units (Reyes *et al.*, 1979) indicating that the reductase may not play a role in HSV-1 mediated transformation. In any case, the possible role of the viral reductase in transformation could be assessed by determining mutation frequencies of specific loci in cells infected with wild type virus and reductase mutants, or transfected with viral DNA sequences coding for the enzyme.

An understanding of the role of the HSV reductase in viral replication will require further characterization of the system. Preliminary studies by Dutia (personal communication) have shown that at the non-permissive temperature the titer of an HSV-1 ts mutant carrying a lesion affecting reductase activity was reduced approximately 10^4 fold relative to wild type virus. These data suggest that the viral reductase might be an essential function at least for efficient viral replication. The requirement for reductase activity likely reflects the need for large amounts of DNA precursors to sustain a high rate of DNA synthesis. Since the cellular reductase is only synthesized just prior to and during S phase and, in addition, infection with HSV results in rapid shut-off of host protein synthesis (Sydiskis and Roizman, 1966, 1968; Ben-Porat *et al.*, 1971), the cellular enzyme is probably not available in sufficient amounts in infected cells. Furthermore, its activity would likely be impaired by the elevated intracellular levels of dTTP resulting from HSV infection (Cheng *et al.*, 1975; Jamieson and Bjursell, 1976a, 1976b). Whether the reductase is a truly essential viral function (e.g.,

such as the DNA polymerase) will only be determined by further characterization of well defined viral mutants, however the enzyme should at least prove to be indispensable for viral replication in cells containing very low levels of dNTP's and endogenous reductase activity, such as neurons and serum-starved cells. Concomitantly, the viral reductase is also likely to be essential, as is the TK, for the establishment of, or reactivation from, latency in neurons.

The identification of viral gene products involved in ribonucleotide reductase activity has rendered it possible to assess the relevance of the enzyme in primary and recurrent infections. Specific mutations in the DNA sequences encoding these polypeptides can now be engineered *in vitro* and their effects assessed *in vivo* following rescue of the mutated fragment into the virus. If indeed viral mutants exhibit decreased virulence and are unable to either establish, or undergo reactivation from, latency, ribonucleotide reductase could provide an additional target for antiviral chemotherapy.

5. APPENDIX

5.1. Introduction

As mentioned previously, following characterization of the virally induced reductase (section 3.3.), two experimental approaches were taken to determine whether the enzyme was virally coded. One of the approaches was based on the development of antibodies specific for the induced enzyme and, as described in sections 3.4. - 3.6., was successful in establishing the viral origin of the enzyme. The other approach, described in this Appendix, was to determine whether viral nucleic acids could direct the synthesis of ribonucleotide reductase in either amphibian oocytes or in an *in vitro* mRNA translation system. These experiments were prompted by reports in the literature that 1) oocytes of the African clawed frog, *Xenopus laevis* were capable of synthesizing enzymatically active HSV thymidine kinase (TK) following microinjection of appropriate fragments of viral DNA into their nuclei (McKnight and Gavis, 1980), and 2) that mRNA from infected cells could also direct the synthesis of active TK *in vitro* (Preston, 1977). These studies suggested that a functional ribonucleotide reductase might also be synthesized from either injected DNA or selected mRNA, thus providing a convenient system for mapping the reductase gene to the viral genome. Furthermore, in the event that the ribonucleotide reductase was synthesized, but not in an enzymatically active form, it was anticipated that the availability of antibodies directed against the induced enzyme could prove useful in identifying the inactive polypeptide(s).

5.2. Materials and Methods

5.2.1. Preparation of DNA

The recombinant plasmids L3PK1, A273-2, A272-B1 and A272-B9, were obtained from Dr. S. Bacchetti (McMaster University); PTK173 was obtained from Mr. R. McKinnon (McMaster University). The plasmids were propagated in *E. coli* strains LE392 or HB101, which were grown in Luria broth (10 mM tris HCl pH 7.6, 0.4% w/v glucose, 0.5% w/v NaCl, 1% w/v bacto tryptone (Difco), 0.5% w/v yeast extract (Difco)) to A_{660} between 0.6 and 0.7. The plasmids were amplified by addition of chloramphenicol (50 μ g/ml; Sigma) to the cultures and purified by the alkaline extraction procedure of Birnboim and Doly (1979). Briefly, bacterial cells from a 100 ml culture were pelleted by centrifugation and resuspended in 2.5 ml of 25 mM tris HCl pH 8, 50 mM glucose, 10 mM EDTA, 2 mg/ml lysozyme (Sigma) and incubated for 30 min on ice. Five ml of alkaline SDS (0.2N NaOH, 1% SDS) were then added and following incubation for 5 min on ice, proteins and high molecular weight nucleic acids were precipitated by addition of 3.75 ml 3 M sodium acetate pH 4.8. After a further 60 min on ice, the precipitate was pelleted by centrifugation at 11,000 x g for 15 min and plasmids in the supernatant were precipitated with two volumes of ice cold ethanol. Precipitates were washed with ethanol, air dried and dissolved in 10 mM tris HCl pH 7.5, 1 mM EDTA.

For oocyte injection, plasmids were further purified by buoyant density centrifugation in cesium chloride gradients (final density = 1.54 grams/ml) containing 200 μ g/ml ethidium bromide.

Centrifugation was for 60 hrs at 35,000 rpm and 15°C in the Beckman 50 Ti rotor. Plasmids were visualized under UV light and collected from the side of the centrifuge tube with a syringe. Ethidium bromide was removed by extraction with iso-amyl alcohol and the DNA preparation was then dialyzed against water, brought to 0.3 M sodium acetate and precipitated with 2 volumes of ethanol. Prior to oocyte injection, the precipitates were pelleted by centrifugation, air dried, dissolved in 10 mM tris HCl pH 7.5, 88 mM NaCl, 0.5 mM EDTA and subsequently stored at -20°C. Plasmid concentrations were estimated on agarose gels by comparison with a standard plasmid of known concentration.

HSV-2 (219) DNA was purified from infected Vero cells by the procedure of Bacchetti and Graham (1977). Cell monolayers were infected at a multiplicity of 0.5 PFU/cell and harvested at approximately 24 hrs after infection. The cells were lysed and digested overnight in 10 mM tris HCl pH 8, 10 mM EDTA, 0.2% w/v SDS, 0.5 mg/ml pronase (Sigma), and the lysates were phenol-extracted and ethanol precipitated. Viral DNA was purified by two cycles of buoyant density centrifugation in sodium iodide gradients (final density = 1.53 grams/ml) containing 20 µg/ml ethidium bromide. Centrifugation and removal of ethidium bromide was as described above, and the DNA was stored in 10 mM tris HCl pH 7.5, 10 mM NaCl, 1 mM EDTA at -70°C. DNA concentration was determined by A_{260} (1 mg/ml = 20 A_{260} units) using DNA samples which had been treated with 50 µg/ml RNase (Sigma) for 1 hr at 37°C.

5.2.2. Preparation of RNA

All glassware used in these experiments was baked at 350°C for 2 hrs and all buffers and solutions were autoclaved. Cytoplasmic RNA was extracted from Vero or BHK21 cl. 13 cells by a modification of the procedures of Kumar and Lindberg (1972) and Berk and Sharp (1977). Monolayer cultures were mock-infected or infected with HSV-2 (333) at a multiplicity of 10-15 PFU/cell and harvested 4 hrs after infection by scraping with a rubber policeman. The cells were washed twice with PBS and resuspended in ice-cold 100 mM tris HCl pH 7.5, 250 mM sucrose, 25 mM NaCl, and 5 mM MgCl₂ (1 ml buffer/4 x 10⁷ cells); a solution of 2.5 mg/ml polyvinyl sulfate (Sigma) and 3.5 mg/ml spermidine (Sigma) was freshly prepared for each experiment, filtered through a Millex-GS 0.22 µm filter and diluted 100 fold into the buffer. The cell suspension was homogenized on ice in the presence of 0.1 volume of 10% NP-40, 10% sodium deoxycholate; the nuclei were pelleted at 11,000 x g for 5 min at 4°C and the supernatant mixed with an equal volume of 100 mM sodium acetate, 200 mM NaCl, 20 mM EDTA, 1% SDS and 500 µg/ml proteinase K (Boehringer Mannheim; proteinase K was made 25 mg/ml in sterile water, stored at -70°C and added to the buffer prior to use). Following incubation at 37°C for 25 min, the solution was extracted twice with phenol:chloroform (1:1, v/v), once with ether, and then precipitated with 2.5 volumes of ice cold ethanol for at least 15 hrs at -20°C. The precipitate was collected by centrifugation at 11,000 x g for 30 min at 4°C, dried with nitrogen gas, and dissolved in 0.3 M sodium acetate pH 6, reprecipitated with

ethanol and stored at -70°C as a precipitate. For oocyte injections, aliquots of the precipitate were pelleted, washed with ethanol, dried under vacuum in a Speed-Vac concentrator (Savant Instruments, Inc.) and dissolved in 15 mM tris HCl pH 7.6, 88 mM NaCl, 1 mM KCl.

Poly(A)-containing RNA was separated from total cytoplasmic RNA by oligo(dt)-cellulose chromatography (Aviv and Leder, 1972). The cytoplasmic RNA precipitate was pelleted, washed with ethanol, dried with nitrogen gas, and dissolved in 0.5 - 1 ml of low salt buffer (10 mM tris pH 7.5, 1 mM EDTA, 0.05% SDS). The RNA was heated at 80°C for 45 sec, cooled rapidly in ice water, made up to high salt buffer (10 mM tris pH 7.5, 1 mM EDTA, 0.2% SDS, 500 mM NaCl) and applied to a column containing 100-200 mg of oligo(dt)-cellulose (Sigma) which had been equilibrated with high salt buffer. Chromatography was performed at room temperature. The column was washed with 5 ml of high salt buffer to elute RNA lacking poly(A) sequences and polyadenylated RNA was then eluted with 1.5 ml of low salt buffer, brought to 0.2 M sodium acetate and precipitated with 2.5 volumes of ice cold ethanol for at least 15 hrs at -70°C . The precipitate was collected by centrifugation, dried with nitrogen gas, dissolved in 0.2 M potassium acetate, reprecipitated with ethanol and stored at -70°C . For *in vitro* translation, aliquots of the precipitate were pelleted, washed with ethanol, dried in a Speed-Vac concentrator, and dissolved in sterile water.

5.2.3. Oocyte injection

Xenopus laevis females were obtained from Carolina Biological Supply Company and fed weekly with calf liver. For initial experiments the frogs were injected in the dorsal lymph sac with 500 units of human chorionic gonadotropin (Sigma) to stimulate oogenesis, and oocytes were used between 35-60 days following hormone treatment. As mentioned in the Results, it was subsequently found that oocytes from mature females not primed with hormone gave consistently higher levels of gene expression following microinjection of plasmids, and were also less fragile than those from primed frogs. For removal of oocytes, frogs were anesthetized by hypothermia (i.e., placed in ice water until immobile) and then laid on ice while a small ventral incision was made and the ovarian lobes were removed. The lobes were placed in petri dishes containing modified Barth's solution (MBS: 15 mM tris HCl pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 10 mg/liter penicillin and streptomycin sulfate; Gurdon, 1968) and individual stage V and VI oocytes (Dumont, 1972) were removed from surrounding tissue with watchmaker's forceps and transferred to fresh MBS with a pasteur pipette. The oocytes were usually maintained at approximately 18°C and were used for microinjection within 30 hrs of their removal from the frogs.

Since DNA must be injected into the nucleus of the oocyte for transcription to occur (Mertz and Gurdon, 1977), and because the oocyte is opaque, the nuclei were localized within oocytes by the procedure of Kressmann *et al.* (1978). Individual oocytes were placed

in small wells formed on a wax surface in a petri dish. The wells contained MBS and were large enough to hold a single oocyte each. The oocytes were oriented with their animal (brown) pole facing upward and centrifuged at 1750 g for 10 min at 18°C. During centrifugation the nucleus rises towards the surface of the animal pole displacing pigment granules and creating a dark ring of pigment which marks its location. This ring was not observed in all centrifuged oocytes, indeed the percentage with rings varied considerably between experiments; nevertheless even in those oocytes where the pigment had not been noticeably displaced, the nucleus had apparently moved towards the animal pole since injection of DNA into this region of the oocyte resulted in expression of the injected sequences.

The oocytes were generally injected within 30-45 min of centrifugation. The microinjection equipment consisted of a glass injection needle mounted on a Brinkman MM33 micromanipulator and connected via a plastic tube filled with water to a 2 ml Gilmont syringe (Canlab). The syringe served to draw DNA or RNA solutions into, and expel them from, the needle, and was operated by hand. The injection needles were made from "Kwik fill" glass capillary tubes with an outer diameter of 1.2 mm (Hybrid Electronics) which were drawn on a model 700 C vertical pipette puller (David Kopf Instruments). Since this machine generated needles with extremely fine points, the tips were manually broken with watchmaker's forceps under a Zeiss-Jena dissecting microscope to yield a tip diameter which was wide enough to minimize blockage of the tip during microinjection

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without resulting in excessive leakage from the oocytes following injection. The centrifuged oocytes were aligned against a wax support and injected 15 at a time; generally a total of 50 oocytes were injected for each concentration of DNA or for each plasmid. Injections were carried out under a dissecting microscope. Using a 5 μ l capillary tube, .45 μ l of the DNA solution was roughly measured out and then drawn from the capillary tube into the injection needle. This volume was equally divided among 15 oocytes resulting in an injection volume of approximately 30 nl/oocyte. The oocytes were injected into the center of the pigment ring, or, in the case where no ring was visible, into the center of the animal pole; the tip of the needle was held just below the surface of the oocyte during injection. Injected oocytes were transferred to fresh MBS and incubated at 18°C for 20 hrs. The surviving oocytes (70-95%) were washed twice with PBS and either stored as a dry pellet at -20°C or immediately resuspended in 10 mM tris base pH 8, 1 mM ATP, 1.4 mM 2-mercaptoethanol and disrupted by dounce homogenization. The cell lysate was cleared by centrifugation at 70,000 x g for 30 min at 4°C and the resulting supernatant was used as crude extract for the enzyme assays.

For microinjection of RNA, the oocyte centrifugation step was omitted and the oocytes were injected with approximately 50 nl of 20 mg/ml or 10 mg/ml solutions of total cytoplasmic RNA just below the surface of the vegetal (light) pole. Incubation and processing of the oocytes was as described for the DNA injections.

5.2.4. In Vitro Translation

In vitro translation was carried out using the rabbit reticulocyte lysate system from Bethesda Research Laboratories. Since the lysate is sensitive to sodium ions, RNA preparations were precipitated with potassium acetate (rather than sodium acetate) prior to translation, as described in section 5.2.2. The reactions were carried out in a volume of 30 μ l containing rabbit reticulocyte lysate (1.16 mM $MgCl_2$, 0.016 mM EDTA, 8.33 mM KCl, 0.166 mM DTT, 8.3 μ M hemin, 16.6 μ g/ml creatine kinase, 0.33 mM $CaCl_2$, 0.66 mM EGTA, 23.3 mM NaCl), protein biosynthesis reaction mixture (25 mM HEPES, 40 mM KCl, 10 mM creatine phosphate, 19 amino acids, 50 μ M each), 86.6 mM potassium acetate and varying concentrations of polyadenylated RNA as indicated in the Results. Incubation was at 30°C for 60 min following which the reactions were placed in ice water, made up to the appropriate enzyme assay reaction mixture and assayed for thymidine kinase, DNA polymerase or ribonucleotide reductase activity as described in section 2.4..

5.3. Results and Discussion

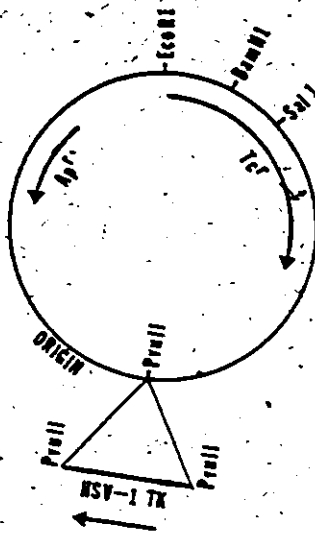
Preliminary experiments established that mature *Xenopus laevis* oocytes expressed very low, but detectable, levels of endogenous TK and

reductase activity. To establish and calibrate the microinjection technique, oocytes were injected with plasmid PTK173 (R. McKinnon, unpublished; see Fig. 25a) [which contains the HSV-1 TK gene (as a Pvu II fragment) inserted into the Pvu II site of PBR322 (Bolivar *et al.*, 1977)] and subsequently assayed for TK activity. Table 5 shows the levels of TK activity obtained at different concentrations of injected DNA. Enzyme activity was detected at what appeared to be maximal levels at concentrations of DNA as low as 5 $\mu\text{g/ml}$. This level of activity persisted until a DNA concentration of 100 $\mu\text{g/ml}$ and then declined to detectable but barely significant values. In this experiment no attempts were made to establish the minimum amount of PTK173 DNA required for detectable synthesis of TK. The inhibition of TK expression at high plasmid concentrations could be due to the presence of inhibitors of transcription in the DNA preparation (although plasmids were purified by banding in cesium chloride gradients); alternatively there may be an upper limit to the amount of DNA which can be injected into the nuclei of oocytes without impeding transcriptional activity.

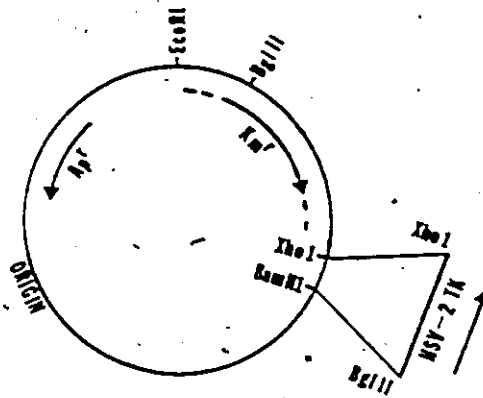
As a preliminary screening for viral DNA sequences encoding the ribonucleotide reductase, oocytes were to be injected with total HSV-2 DNA and assayed for reductase activity. Viral DNA was partially digested with Bam HI to eliminate infectivity, and because of reports in the literature claiming preferential transcription of circular DNA templates (Brown and Gurdon, 1978; DeFranco *et al.*, 1980), an aliquot of the partially digested DNA was subsequently ligated with T4 ligase under conditions favoring the formation of circular molecules. Both the partial digestion and ligation were monitored by electrophoresis of the reaction

Figure 25

Schematic structure of the plasmids used for microinjection. The arrows show the location and direction of transcription of the ampicillin resistance (Ap^r), tetracycline resistance (Tc^r) and kanamycin resistance (Km^r) genes, as well as the direction of transcription of the inserted TK genes. (a) PTK173 contains 6.3 kb of DNA, including the 2 kb PvuII fragment of HSV-1 TK; (b) L3PK1 contains 8.9 kb, including the 3.4 kb Bgl II-XhoI fragment of HSV-2 TK.



(a) PTK 173



(b) L3PK1

Table 5

Expression of Thymidine Kinase Activity in Oocytes Following

Injection of Varying Concentrations of PTK173

DNA concentration ($\mu\text{g/ml}$) ^a	0	5	10	30	50	100	200	500	1,000
TK activity (cpm/100 μg protein) ^b	780	9,500	8,900	9,300	9,000	9,100	4,100	1,500	1,500

^a As described in Materials and Methods, approximately 30 nl of each DNA concentration was injected into the oocyte nuclei. The concentrations given thus correspond approximately to the injection of 0, 0.15, 0.3, 0.9, 1.5, 3, 6, 15, and 30 ng of DNA.

^b Groups of 50 oocytes were injected with each concentration of DNA and incubated for 20 hours at 18°C. Crude extracts of each group of 50 oocytes were then prepared and assayed for TK activity.

products on agarose gels. As shown in Fig. 26, neither linear (open circles) nor ligated (closed circles) viral DNA synthesized detectable TK activity, in contrast to the result obtained with oocytes injected with plasmid PTK173 (squares). The inability of total viral DNA to express detectable TK activity was likely the result of a gene dilution effect since, unlike the situation with plasmid DNA, in total viral DNA the TK gene was co-injected with at least 50 other genes which compete for the oocyte transcriptional machinery.

On the basis of these data, it was decided to screen cloned fragments of viral DNA for their ability to synthesize ribonucleotide reductase. For these experiments plasmids containing Bgl II fragments of the HSV-2 genome cloned into the Bgl II site of L3PK1 were used (see Fig. 25b). L3PK1 (S. Bacchetti, unpublished) is a derivative of PKC7 (Rao and Rogers, 1979) in which the sequences between the Bam HI and Xho I sites have been replaced by the Bgl II-Xho I fragment of HSV-2 TK. The TK gene in these constructs was intended to serve as an internal positive control for the expression of the injected DNA sequences in the absence of ribonucleotide reductase activity. Preliminary experiments established that the HSV-2 TK gene in L3PK1 could indeed be expressed in oocytes, although at a level approximately 2.5 fold lower than that obtained with PTK173 (containing the HSV-1 TK gene) (see Table 6). Unexpectedly, when the L3PK1 derivatives, containing Bgl II inserts of the HSV-2 genome, were tested, virtually no TK activity was detected even though several concentrations of plasmid DNA were tested (see Table 6; the two L3PK1 derivatives tested were designated A272-B1 and A272-B9). Interestingly, similar data were obtained with a derivative of PTK173, designated A273-2

Figure 26

Expression of thymidine kinase activity in oocytes injected with either total viral DNA or the cloned viral TK gene. HSV-2 DNA was either partially digested, or partially digested and religated, as described in the text, brought to a final concentration of 100 $\mu\text{g/ml}$ and injected into oocytes. PTK173 was also used for injection at a concentration of 100 $\mu\text{g/ml}$. Fifty oocytes were injected with each sample of DNA, and incubated at 18°C for 20 hours. Crude extracts of each group of 50 oocytes were then prepared and assayed for TK activity as a function of increasing protein concentration. Symbols: (\square) TK activity in PTK173 injected oocytes; TK activity in oocytes injected with either partially digested (\circ) or partially digested and religated (\bullet) total HSV-2 DNA; endogenous TK activity of uninjected oocytes (\blacktriangle).

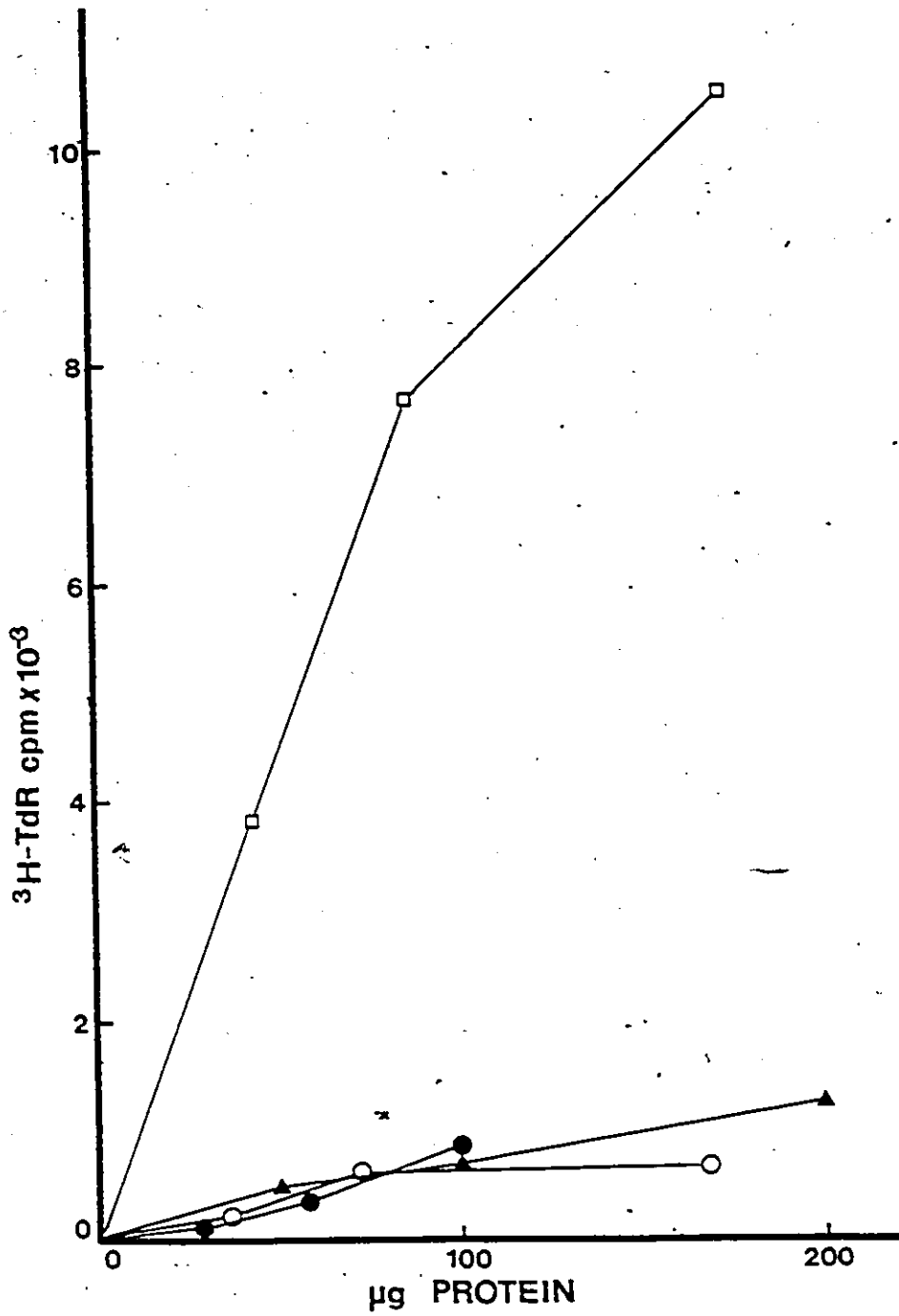


Table 6
 Expression of Thymidine Kinase Activity in Oocytes Injected
 with L3PK1, PTK173 and Derivative Plasmids^a

Plasmid	L3PK1	A272-B1 ^b	A272-B9 ^b	PTK173 ^a	A273-2 ^b
TK activity (cpm/100 µg protein)	5,900	1,000 1,850 1,000 700	550	14,200	1,200 1,800 2,200 1,350 1,600
DNA concentration (µg/ml)	60	30 60 90 180	17	50	9 30 60 100 130

^a For these experiments oocytes were collected from mature frogs which had not been primed with hormone to stimulate oogenesis, unlike the oocytes used in previous experiments. It was found that the untreated oocytes gave consistently higher levels of TK activity, following microinjection of PTK173, than did the treated ones. Each plasmid, or each of several concentrations of a plasmid, were injected into 50 oocytes, which were then incubated for 20 hours at 18°C. Crude extracts were then prepared and assayed for TK activity.

^b A272-B1 contains the HSV-2 Bgl II 0 and P fragments in L3PK1; A272-B9 contains the HSV-2 Bgl II Q and N fragments in L3PK1; A273-2 contains the XhoI C fragment of adenovirus 5 in PTK173.

^c PTK173 was always included in the microinjection experiments as a positive control.

which contains the Xho I C fragment of adenovirus 5 DNA inserted between the Bam HI and Sal I sites of PTK173 (S. Bacchetti, unpublished; see Fig. 25a). As also shown in Table 6, injection of A273-2 resulted in the expression of very low levels of TK under conditions in which high levels of activity were obtained with PTK173.

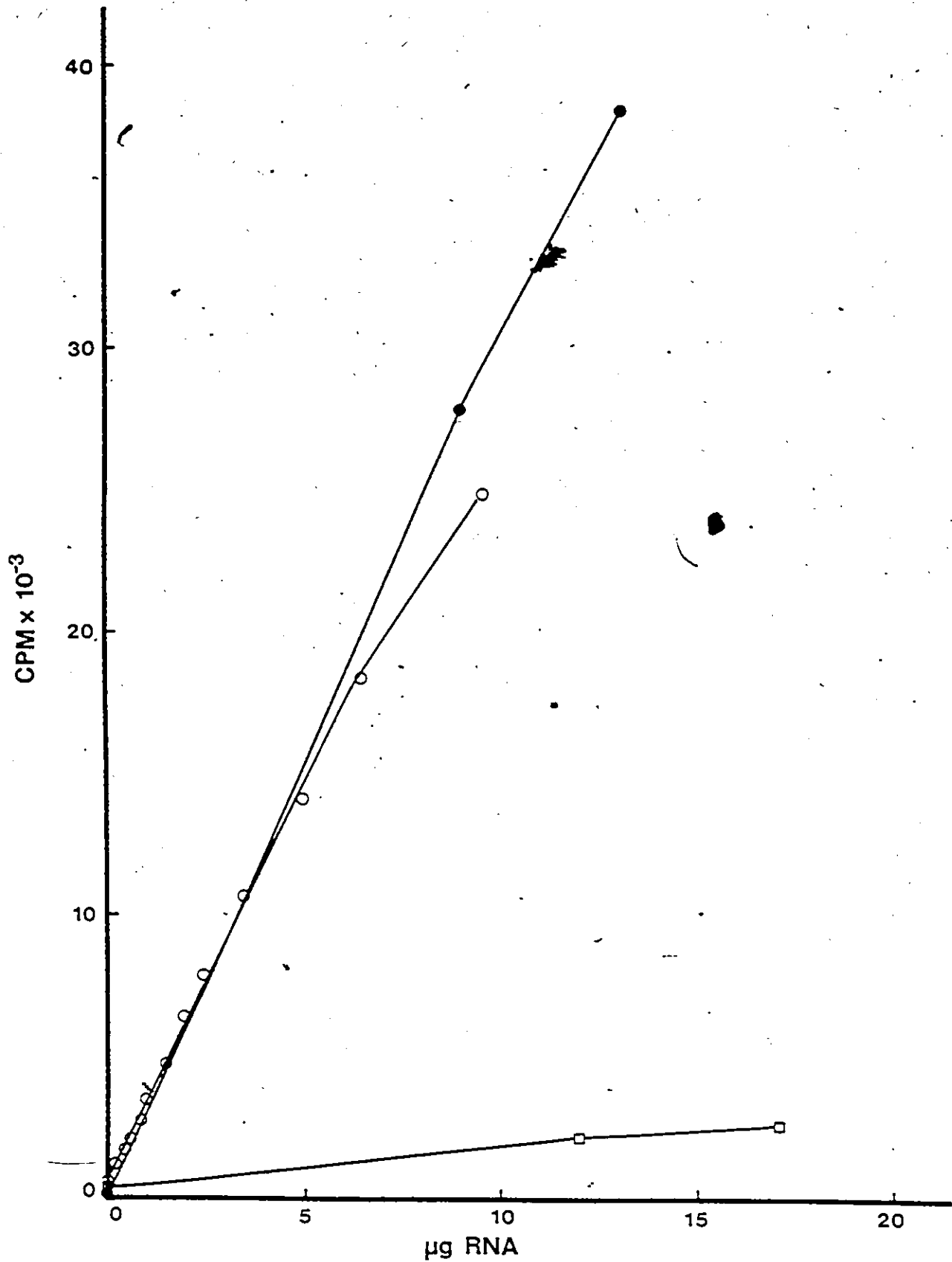
Electrophoresis of the A272-B1 and A273-2 plasmid preparations on agarose gels demonstrated that their inability to express significant levels of TK activity was not the result of degradation of these plasmids during storage. Nor did it seem likely that the inhibition of TK expression was due to the presence of inhibitors in the plasmid preparations, since 1) A273-2 was injected at a concentration (9 $\mu\text{g/ml}$) which represented a 160 fold dilution of the plasmid sample and 2) repurification of A273-2 and A272-B1 by banding in cesium chloride gradients followed by phenol extraction was also ineffective in recovering TK activity following microinjection. It thus appears that the abrogation of TK activity was in some fashion related to the structure of the recombinant plasmids derived from PTK173 and L3PK1. While experiments were underway to further examine this phenomenon, and to determine whether the inserted sequences themselves were expressed in A272-B1 or A273-2, technical difficulties were encountered with the oocyte microinjections. Fortunately, at this time, parallel experiments being carried out with antibodies specific for the induced enzyme were proceeding very well (see section 3.4. - 3.6.) and the oocyte injections were not pursued any further.

Concomitantly, with the oocyte injections, experiments were also undertaken to assess the ability of viral RNA to direct the synthesis of ribonucleotide reductase. The aim of these experiments was, firstly,

to determine whether the enzyme could be translated from cytoplasmic RNA from HSV-2 infected cells and secondly, to ascertain the viral origin and map location of the reductase gene by translation of viral mRNA selected by hybridization to fragments of viral DNA. Initial experiments were performed by injection of RNA into the cytoplasm of *Xenopus laevis* oocytes. No ribonucleotide reductase activity or viral DNA polymerase activity could be detected in this system. In contrast, TK activity was detected although at considerably variable levels in different experiments (not shown). Because of this variability, translations were subsequently carried out in commercially available rabbit reticulocyte lysates which gave highly reproducible results. As shown in Fig. 27, polyadenylated mRNA from infected cells directed the *in vitro* synthesis of high levels of TK (open circles) and DNA polymerase activity (closed circles). Low amounts of ribonucleotide reductase activity were also detected in these experiments (squares). However, it was subsequently determined that slightly higher levels of reductase synthesis could be obtained from polyadenylated mRNA extracted from uninfected cells, and that the cells used for RNA extraction were contaminated with mycoplasma. At the time this contamination was detected, the viral origin of the ribonucleotide reductase had been established by immunological means, as described in section 3.6., and the *in vitro* translation experiments were discontinued.

Figure 27

Enzyme activity in rabbit reticulocyte lysates following translation of polyadenylated infected cell mRNA. Cytoplasmic RNA was extracted from Vero cells 4 hours after infection with HSV-2 and passed over columns of oligo dT cellulose to select for polyadenylated mRNA. Increasing concentrations of mRNA were translated in rabbit reticulocyte lysates, as described in the text, and the translation mixtures were then made up to the appropriate enzyme reaction buffer and assayed for thymidine kinase (○), ribonucleotide reductase (□) and DNA polymerase (●) activity (DNA polymerase was assayed in the presence of 100 mM ammonium sulfate which stimulates viral DNA polymerase activity but inhibits the cellular α DNA polymerase).



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