INHIBITION OF HERPESVIRUS RIBONUCLEOTIDE REDUCTASE BY THE SYNTHETIC NONAPEPTIDE YAGAVVNDL

by

Elizabeth Anne Reid Telford

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Church Street

Glasgow

G11 5JR

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
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TABLE OF CONTENTS

METHODS

SECTION 1 GROWTH OF CELLS AND VIRUS

SECTION II CHROMATOGRAPHY

SECTION III EXTRACTION AND ASSAY OF RIBONUCLEOTIDE REDUCTASE

SECTION IV PREPARATION OF CARRIER FREE [^{127}I]YAGAVVNDL.

SECTION V PURIFICATION OF TRITIATED YAGAVVNDL.

RESULTS

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SUMMARY

Ribonucleotide reductase catalyses the reduction of all four ribonucleotides and is a key enzyme in the de novo synthesis of DNA precursors. A novel ribonucleotide reductase activity is induced in cells infected with herpes simplex virus. The virus-induced ribonucleotide reductase is encoded by the virus and has biochemical properties which are distinct from that of the host cell enzyme and therefore is a potential target for antiviral chemotherapy. The HSV-1-encoded ribonucleotide reductase is composed of two non-identical subunits, termed RR1 and RR2, which are dimers of Vmw136 and Vmw38 polypeptides respectively. Previously it has been shown that a synthetic nonapeptide (YAGAVVNDL) corresponding to the carboxy-terminal nine amino acids of the HSV-1-encoded RR2 subunit inhibits the HSV-1-induced enzyme. The peptide prevents the association of RR2 with RR1 by competing with RR2 for a site of interaction on RR1.

The inhibitory activity of the nonapeptide was found to decrease upon prolonged incubation with enzyme extract and this observation prompted studies to determine the fate of the nonapeptide in cellular extracts. The synthetic nonapeptide was iodinated with non-radioactive iodine, purified by high pressure liquid chromatography on a reverse phase column and the iodinated peptide was then tritiated by reduction with tritium gas (Amersham International PLC). In a linear gradient increasing from 5% to 95% acetonitrile + 0.1% TFA between 0 and 10 minutes the tritiated peptide eluted from the column at 7 minutes. However, when the tritiated peptide was mixed with HSV-1-infected cell

extract, a new species was identified which eluted from the column at 5 minutes. This new species was thought to be a product of cleavage of the nonapeptide by proteases present in the cellular extract. The modified product was purified by HPLC on a reverse phase preparative column. A second modification product was observed using this column and was also purified. The purified modification products were characterised by analysis of amino acid content and relative molecular mass. The results suggested that the synthetic nonapeptide is cleaved into the octamer AGAVVNDL and free tyrosine. This was verified by the synthesis of the octapeptide and the demonstration that it and authentic tyrosine coeluted with the modification products. The octapeptide has a markedly reduced inhibitory potency and therefore a means of protecting the tyrosine-alanine bond from such degradation would be necessary in any compound based on the nonapeptide for it to be useful as an antiviral agent.

A range of protease inhibitors were tested to determine whether any could protect the nonapeptide from degradation. Of those tested, only bacitracin offered substantial protection from breakdown of the nonapeptide.

Serological evidence for conservation of the carboxy-terminal region of HSV-1 in HSV-2, VZV, PRV and EHV-1 made it likely that the nonapeptide might inhibit a broad range of herpesvirus ribonucleotide reductase activities. This proposal was tested on the novel ribonucleotide reductase activity induced in cells infected with one of two different strains of abortogenic EHV.

Rabbit kidney cells were infected with EHV-1 strain Vol939 or strain Kentucky A and virus-induced ribonucleotide reductase activity was partially purified by ammonium sulphate precipitation. The EHV-1-induced ribonucleotide reductase activity was assayed in the presence of various concentrations of the nonapeptide. The results showed the peptide to be inhibitory. Parallel experiments with the HSV-1-induced ribonucleotide reductase established that the concentration of peptide required to inhibit 50% of reductase activity was the same for both enzymes. This data and the finding that the PRV-induced enzyme is inhibited by the nonapeptide support the proposition that an antiviral drug based on the nonapeptide might have activity against a broad range of herpesviruses.

The large subunit of ribonucleotide reductase encoded by HSV (RR1) possesses an amino-terminal domain not found in the large subunit of other herpesvirus reductases or reductases from other organisms. It has previously been shown by other workers that truncated forms of the HSV RR1 exist which can form a functional enzyme. Oligopeptide-induced antisera were raised against the unique amino domain and the carboxy-terminal domain of HSV-1-induced RR1 and these antisera were used by Dr H. Lankinen in experiments to demonstrate that at least part of the unique amino-terminal domain is not necessary for enzyme activity.

The antisera which were raised against the HSV-1-induced RR1 were used in immunoblotting experiments with EHV-1-infected cell extracts. Antisera directed against the carboxy-domain of RR1 reacted specifically with a viral

protein M_r 90K. Antisera raised against peptides corresponding to sequences in the unique amino-terminal domain of the HSV-1 RR1 failed to react with any viral protein. These results suggest that the EHV-1-induced RR1 does not contain the amino-terminal region which is so far unique to HSV-1 and HSV-2.

ABBREVIATIONS

A	adenine
AdoCbl	adenosyl cobalamin
ATP	adenosine triphosphate
B1	large subunit of *E.coli* ribonucleotide reductase
B2	small subunit of *E.coli* ribonucleotide reductase
BDB	bis diazobenzidine
BHK	baby hamster kidney
BMV	bovine mammillitis virus
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
CDP	cytidine diphosphate
Ci	Curie(s)
cm	centimetre(s)
cpm	counts per minute
dAMP	deoxyadenosine monophosphate
dATP	deoxyadenosine triphosphate
dCMP	deoxycytidine monophosphate
dCDP	deoxycytidine diphosphate
dGMP	deoxyguanosine monophosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUDP	deoxyuridine diphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
EBV	Epstein-Barr virus
EHV	equine herpes virus
EPR	electron paramagnetic resonance
E.coli	*Escherichia coli*
Fc	crystallisable fragment of immunoglobulin
FPLC	fast protein liquid chromatography
G	guanine

g	gram(s)
g(D)	glycoprotein (D)
G+C	moles per cent guanosine + cytidine moieties
^{3}H	tritium
h	hour(s)
HCMV	human cytomegalovirus .
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HHV-6	human herpes virus 6
HPLC	high pressure liquid chromatography
HPR	horseradish peroxidase
HSV	herpes simplex virus
^{125}I	radiolabelled iodine
^{127}I	non-radiolabelled iodine
IC_{50}	concentration of peptide required to inhibit 50% of enzyme activity
IE	immediate-early
Ig	immunoglobulin
K	kilodalton(s)
kb	kilobase pair(s)
l	litre(s)
L.leichmannii	Lactobacillus leichmannii
M	molar
M1	large subunit of mammalian ribonucleotide reductase
M2	small subunit of mammalian ribonucleotide reductase
mA	milli amps
mCi	milliCurie(s)
MCMV	murine cytomegalovirus
MDV	Marek's disease virus
ml	millilitre(s)
min	minute(s)
mm	millimetre(s)
mM	millimolar
moi	multiplicity of infection
M_r	molecular weight
mRNA	messenger ribonucleic acid
mu	map units
NADPH	nicotinamide adenine dinucleotide phosphate

nm	nanometre(s)
O.D	optical density
ori	origin of DNA replication
oz	ounce
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming units
PMSF	phenylmethylsulphonylfluoride
PRV	pseudorabies virus
R	purine moiety
RK	rabbit kidney
rpm	revolutions per minute
RR1	large subunit of herpes virus ribonucleotide reductase
RR2	small subunit of herpes virus ribonucleotide reductase
s	second
^{35}S	radiolabelled sulphur
SDS	sodium dodecyl sulphate
T	thymine
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethylethylene diamine
TFA	trifluoroacetic acid
TK	thymidine kinase
TPCK	tosylphenylchloromethylketone
ts	temperature sensitive
TTBS	tris buffered saline + 0.05% Tween 20
Tween 20	polyoxyethylene sorbiton monolaurate
UV	ultraviolet
v/v	volume per volume
Vmw	molecular weight of viral polypeptide (kilodaltons)
VP	viral polypeptide
vol	volume
VZV	varicella-zoster virus
w/v	weight per volume
uCi	microCurie(s)
ug	microgram(s)
ul	microlitre(s)
uM	micromolar

One and Three Letter Abbreviations for Amino Acids

amino acid	one letter code	three letter code
alanine	A	ala
arginine	R	arg
asparagine	N	asn
aspartic acid	D	asp
cysteine	C	cys
glutamic acid	E	glu
glutamine	Q	gln
glycine	G	gly
histidine	H	his
isoleucine	I	ile
leucine	L	leu
lysine	K	lys
methionine	M	met
phenylalanine	F	phe
proline	P	pro
serine	S	ser
threonine	T	thr
tryptophan	W	trp
tyrosine	Y	tyr
valine	V	val

INTRODUCTION

SECTION I THE FAMILY HERPETOVIRIDAE

I.1 The structure of the virion.

In the last 50 years, more than 80 distinct herpesviruses have been isolated from a wide variety of eukaryotic species. Herpesviruses are defined as having DNA as their genetic material and as sharing distinctive structural features of their virions (reviewed by Roizman and Furlong 1974; Roizman 1982; Dargan 1986). The virion comprises four structural components, namely, the core, the capsid, the tegument and the envelope.

(i) The electron-dense core lies at the centre of the virion and appears as a cylindrical protein plug around which the double stranded viral DNA genome is wound (Furlong et al. 1972; Nazerian 1974; Irmiere and Gibson 1983). The attachment of this protein cylinder to the inner surface of nucleocapsids has been shown by Smid et al. (1977).

(ii) The capsid exhibits 2-, 3- and 5-fold symmetry and is composed of 162 capsomeres of which 150 are hexameric prisms and 12 are pentameric prisms arranged in the form of an icosahedron (Wildy et al. 1960; Schrag et al. 1989).

(iii) The tegument is defined as the amorphous structure between the capsid and the envelope. The thickness of the

tegument is variable among members of the herpes virus group (Roizman and Furlong 1974; Schrag et al. 1989).

(iv) The envelope is the outermost structure of the herpes virion consisting of a trilaminar membrane with glycoprotein spikes projecting from its outer surface (Wildy et al. 1960). The virus envelope is believed to contain most of the virus glycoproteins (Spear and Roizman 1972; Stannard et al. 1987), cell lipids (Asher et al. 1969) and spermidine (Gibson and Roizman 1971).

Based on this identification, herpesviruses have been reported in almost every eukaryotic species examined in detail including man, birds, amphibians, fish, a variety of domestic and wild animals and even fungi. However, to date only a few of these viruses have been studied in detail.

I.2 Classification of the herpesviruses.

Although the virions of various herpesviruses cannot be differentiated by electron microscopy, those herpesviruses that have been studied in some detail have been grouped into three subfamilies on the basis of host range, duration of the reproductive cycle, cytopathology and characteristics of latent infection (Roizman 1981).

(i) The Alphaherpesvirinae include herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), equine herpes virus (EHV), pseudorabies virus (PRV) and varicella zoster virus (VZV) among others. These viruses have a variable host range in vitro and a short reproductive cycle (less

than 24 hours). The infection spreads rapidly in tissue culture resulting in mass destruction of susceptible cells. Latency is usually established in the ganglia.

(ii) The Betaherpesvirinae have a very narrow host range *in vivo* which is usually restricted to the species or genus to which the natural host belongs. *In vitro,* most of these viruses replicate best in fibroblast cells. The reproductive cycle is relatively long (more than 24 hours) and in tissue culture the virus produces slowly progressing lytic foci and infected cells frequently become enlarged (cytomegalia). Latency may occur in secretory glands, lymphoreticular cells, kidney and other tissues. Members of this subfamily of herpesvirus include human and murine cytomegalovirus (HCMV and MCMV).

(iii) The Gammaherpesvirinae have a narrow host range *in vivo* usually restricted to the same family or order as the natural host. *In vitro*, all members of this subfamily can replicate in lymphoblastoid cells and lytic infection may occur in epithelioid cells and fibroblasts but the cytopathology is variable. Latent virus is frequently demonstrated in lymphoid tissue. Members of this subfamily include Epstein-Barr virus (EBV) and Marek's disease virus (MDV).

I.3 Pathogenicity.

I.3(a)Diseases caused by human herpesviruses.

Man is the natural host of well characterised herpesviruses, namely, HSV-1, HSV-2, VZV, EBV and HCMV. The epidemiology of HSV-1 and HSV-2 has been reviewed by Whitley (1985). A sixth human herpes virus, originally called human B-lymphotropic virus and now designated human herpes virus 6 (HHV-6), has been isolated from patients with acquired immune deficiency syndrome (Salahuddin et al. 1986) but as yet no disease has been definitely associated with it.

The mouth and lips are the most common sites of HSV-1 infection causing vesicular lesions known as 'cold sores'. Children, particularly those under the age of five years are the most often affected, although primary infection can occur in older individuals. HSV-1 is widespread in the human population and although infection is usually mild or asymptomatic, it can give rise to systemic illness and encephalitis particularly in immunosupressed individuals. Sexual transmission is the primary route of the spread of HSV-2 causing mucocutaneous lesions around the genital mucosae. Infection of the newborn with HSV-2 can occur by contact of the child with infected genital secretions during delivery although in utero infection has been reported (Whitley 1985). Infection of the newborn with HSV may result in a broad spectrum of symptoms ranging from skin vesicles to severe disseminated disease.

Other diseases caused by human herpesviruses include chickenpox, a primary infection and the recurrent disease, shingles, both caused by VZV. HCMV infects the

salivary glands resulting in mild disease and EBV is the causative agent of infectious mononucleosis.

A property of herpesviruses is their ability to establish a latent infection. This interesting biological phenomenon is characterised by lesions which reappear at a site of prior occurrence and by intervals of clinical normality during which it is difficult to isolate virus from the peripheral tissue where the lesions had occurred. Similarly, VZV, the causative agent of chickenpox in children, can reactivate in the adult to cause shingles while organ transplant or blood transfusion in immunosuppressed individuals frequently results in an outbreak of HCMV infection. The mechanisms by which latency is established and reactivation occurs are poorly understood.

I.3(b)Diseases caused by equine herpesviruses.

The equine herpesviruses comprise a group of antigenically distinct biological agents which cause a variety of infections in the horse ranging from subclinical to fatal disease. The epidemiology of these viruses has been reviewed by O'Callaghan et al. (1983). One of the most important viruses of the horse from an economic standpoint is equine herpes virus type 1 (EHV-1) which is a major cause of abortion and respiratory disease. It is also associated with neurological disease and, more rarely, with coital exanthema. Infection by EHV-1 is evidenced in young horses, which have little or no immunity, by fever, viraemia, pharyngitis and tracheabronchitis. Nasal discharge is

conspicuous during the febrile period and large amounts of virus are shed. The primary route of transmission from one horse to another is by inhalation of airborne secretions from the respiratory tract but it may also be disseminated by contaminated water, feed and the products of conception in viral abortion. Past exposure to EHV-1 usually confers sufficient immunity to the pregnant mare such that infection is not observable but may become evident by abortion of a virus-infected foetus or birth of an infected foal which dies within hours to several days of severe bronchial pneumonia and pulmonary oedema. Abortion usually occurs late in pregnancy, between the ninth month and full term (eleven months) without untoward results to the mare.

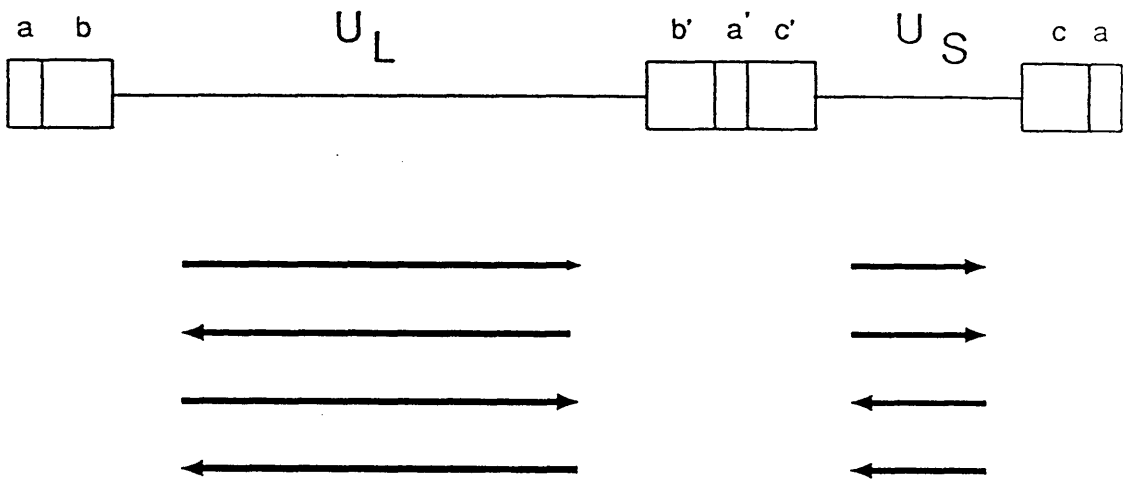
The existence of two subtypes of EHV-1 has been demonstrated at the genomic level by restriction endonuclease analysis (Studdert et al. 1981; Cullinane et al. 1988). EHV-1 subtype 2 (also known as EHV-4), like EHV-1 subtype 1, causes respiratory disease in young horses but is rarely the cause of abortion or neurological syndrome. Further discussion of EHV-1 will be restricted to EHV-1 subtype 1.

Equine herpes virus type 2 (EHV-2) is a slow growing virus usually requiring up to 28 days to produce maximal cytopathic effect in cell culture. Slow growth of the virus, a tendency to be cell-associated and its involvement in formation of intranuclear inclusions led to these agents being termed equine cytomegalovirus (Wharton et al. 1981). Infection occurs early in life and the primary route of transmission is by inhalation of contagious material from the respiratory tracts of other animals. As far as is

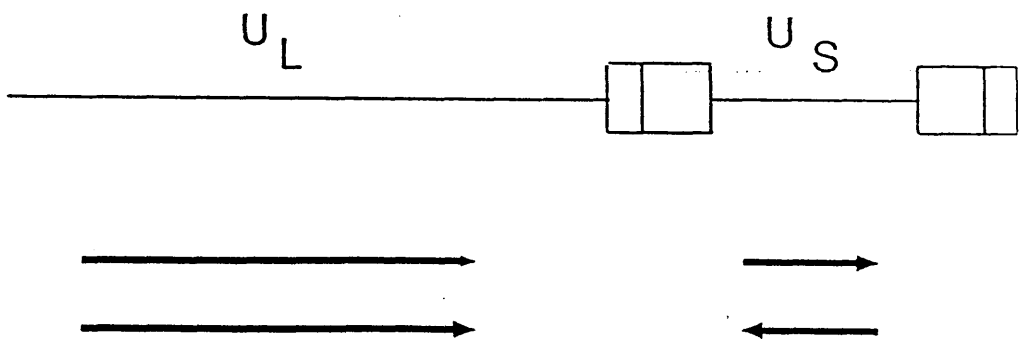
Figure 1 **Genome structure of HSV-1 and EHV-1.**

The structure of the herpes simplex virus and equine herpes virus genomes are illustrated. U_L and U_S indicate long and short unique sequences. Repeat sequences are represented by open boxes and a, b and c indicate repeat sequences with a', b' and c' their complement. The four possible isomers of the HSV genome and the two possible isomers of the EHV genome are represented by thick arrowed lines.

HSV-1



EHV-1



known, this group of herpesviruses does not cause disease in horses.

Equine herpesvirus type 3 (EHV-3) is the etiological agent of an acute but mild genital disease known as coital exanthema. The virus is usually venereally transmitted, however, transmission without venereal spread has been reported (reviewed by O'Callaghan et al. 1983).

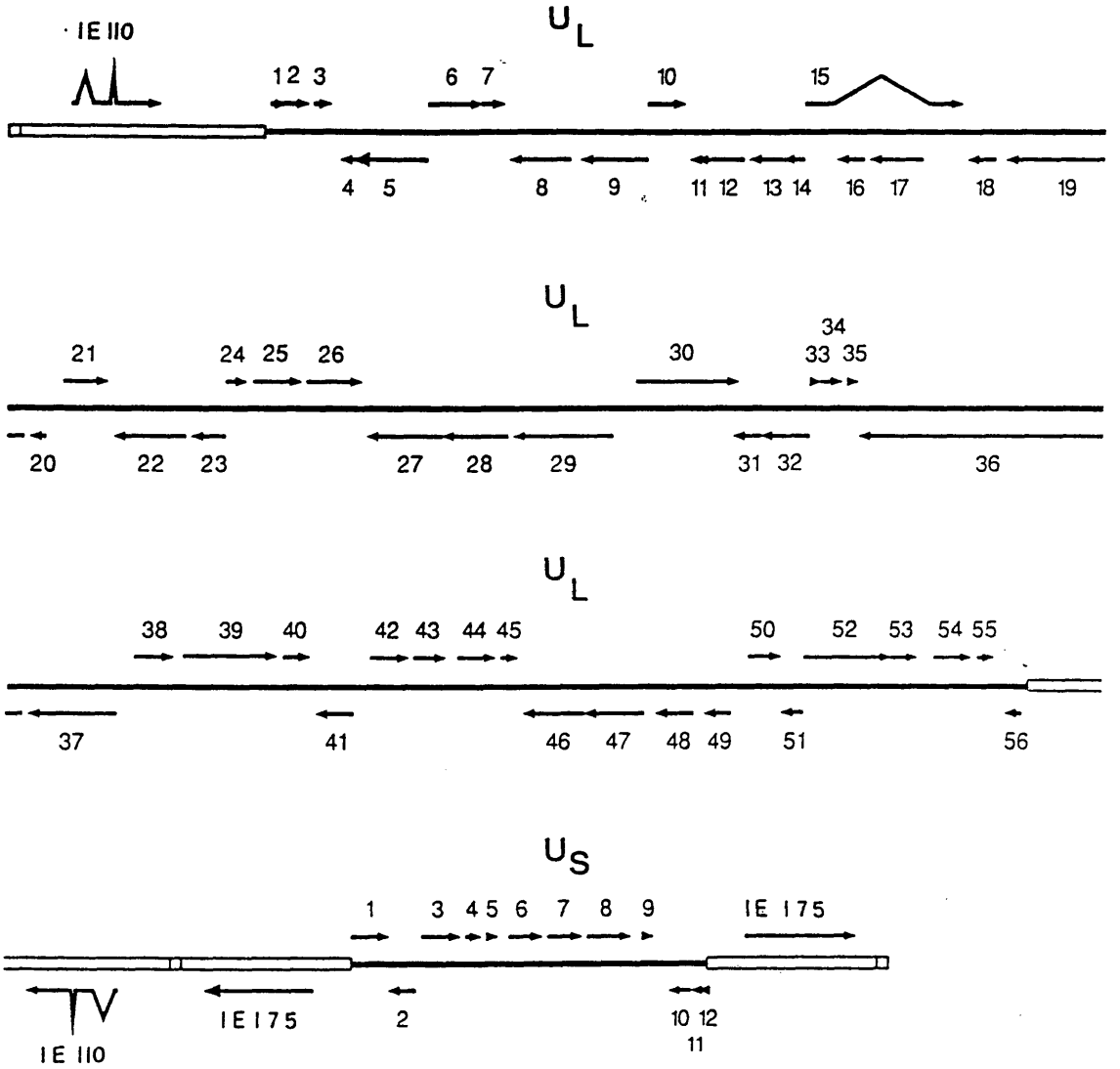
I.4 Structure and organisation of the herpesvirus genome.

I.4(a)Structure of the herpes simplex virus DNA.

The herpes simplex virus genome is a linear double stranded DNA molecule. The complete DNA sequence for HSV-1 strain 17 has been determined: the DNA contains 152,260 residues in each strand although the size of the genome varies due to the variation in copy numbers of repeat families and of the 'a' sequence. The genome has a base composition of 68.3% guanosine plus cytosine (McGeoch et al. 1988). The layout of the genome is shown in Figure 1 and can be envisaged as two covalently linked components, designated long (L) and short (S) comprising 82% and 18% of the DNA respectively. Both L and S consist of unique regions, U_L and U_S respectively, each flanked by inverted repeat sequences (Sheldrick and Berthelot 1974). The repeated sequences flanking U_L are designated ab and b'a' and the U_S component separates the right terminal ca from its inverted repeat a'c' (Wadsworth et al. 1975). The 'a' sequences, common to both the L and S components, are arranged in the same orientation at the ends of the molecule and one or more additional copies of this sequence are

Figure 2. Schematic representation of the arrangement of the genes in the HSV-1 genome.

The locations of HSV-1 λ which map within the unique U_L and U_S sequences are depicted. Genes which encode proteins with known functions or properties are listed in Table 1. Arrowed lines above or below the genome represent genes transcribed rightward or leftward respectively. Repeat sequences are represented as open boxes.


IE 110
U_L
1 2 3
6 7
10
15
4 5
8
9
11 12
13 14
16
17
18
19
U_L
21
24 25 26
30
34
33 35
20
22
23
27
28
29
31 32
36
U_L
38
39
40
42 43
44 45
50
52
53
54 55
37
41
46
47
48
49
51
56
U_S
1
3 4 5 6 7 8 9
IE 175
IE 110
IE 175
2
10 12
11

located internally at the junction between the L and S segments, in the opposite orientation to the terminal 'a' sequence (Sheldrick and Berthelot 1974; Wadsworth et al. 1975; Wagner and Summers 1978). Preparations of HSV DNA consist of a mixture of four sequence orientation isomers (Figure 1) which differ in the relative orientations of the L and S segments (Delius and Clements 1976; Wilkie 1976). It has been shown that the 'a' sequence plays a key role in the inversion of the L and S components (Mocarski et al. 1980; Mocarski and Roizman 1982; Chou and Roizman 1985).

The arrangement of the genes in the genome of HSV-1 is shown in Figure 2. Analysis of the HSV-1 DNA sequence has revealed the presence of 56 genes in the U_L segment, 12 genes in the U_S segment and one in each of the flanking repeat sequences amounting to 70 distinct polypeptides encoded by the viral genome. The functions of all of these polypeptides are not known but the properties of the HSV-1-encoded polypeptides which have been characterised are described in Table 1.

I.4(b)Structure of the EHV-1 DNA.

The genome of EHV-1 is similar to that of HSV in that it is a linear duplex molecule with an average M_r 100 x 10^6 and is comprised of two covalently linked long (111kbp) and short (39kbp) unique sequences (Henry et al. 1981; Whalley et al. 1981). Restriction endonuclease mapping of the EHV-1 genome has shown that there is only one set of inverted repeats which flank the short unique component thus permitting inversion of the S component

TABLE 1 Properties of HSV-1 - encoded polypeptides

Gene	Function or properties
IE110	immediate-early transcription regulator (Perry et al. 1986).
UL2	uracil-DNA glycosylase (Mullaney et al. 1989)
UL5	DNA replication (Challberg 1986; Wu et al. 1988)
UL8	DNA replication (Challberg 1986; Wu et al. 1988)
UL9	DNA replication; origin binding protein (Olivo et al. 1988; Weir et al. 1989)
UL12	deoxyribonuclease (Preston and Cordingley 1982)
UL13	putative protein kinase (Smith and Smith 1989)
UL19	major capsid protein (Costa et al. 1984)
UL22	glycoprotein H (McGeoch and Davison 1986b)
UL23	thymidine kinase (McKnight 1980; Wagner et al. 1981)
UL26	protein associated transiently with the capsid (Rixon et al. 1988)
UL27	glycoprotein B (Bzik et al. 1984; Pellet et al. 1985)
UL29	DNA replication; major DNA binding protein (Conley et al 1981; Quinn and McGeoch 1985)
UL30	DNA replication; DNA polymerase (Chartrand et al 1979; Quinn and McGeoch 1985)
UL39	large subunit of ribonucleotide reductase (Preston et al. 1984; Nikas et al. 1986)
UL40	small subunit of ribonucleotide reductase (McLauchlan and Clements 1983a; Preston et al. 1988)
UL41	virion protein causing host shut-off (Kwong et al. 1988)
UL42	DNA replication; 65K DNA binding protein (Parris et al. 1988)

TABLE 1 (continued)

UL44	glycoprotein C (Frink et al. 1983)
UL48	major tegument protein; activator of immediate genes (Campbell et al. 1984; Dalrymple et al. 1985)
UL50	deoxyuridine triphosphatase (Preston and Fisher 1984)
UL52	DNA replication (Challberg 1986; Wu et al. 1988)
UL54	immediate-early transcriptional regulator (IE63) (Everett 1986)
IE175	immediate-early transcriptional regulator (Murchie and McGeoch 1982; Rixon et al. 1982)
US1	immediate-early protein (IE68) (McGeoch et al. 1985)
US3	protein kinase (McGeoch and Davison 1986a; Frame et al. 1987)
US4	glycoprotein G (McGeoch et al 1985; Frame et al. 1986)
US5	putative glycoprotein (McGeoch et al. 1988)
US6	glycoprotein D (Watson et al. 1982)
US7	glycoprotein I (McGeoch et al. 1985)
US8	glycoprotein E (McGeoch et al. 1985)
US12	immediate-early protein (IE12) (Murchie and McGeoch 1982)

relative to the L component. The long unique region exists in only one orientation and therefore only two structural isomers of the EHV-1 genome exist (Figure 1). In support of restriction endonuclease analysis, electron microscopy studies of EHV-1 DNA revealed that single strands that were allowed to reanneal formed single stranded loops with double stranded stems only at one end of the molecule (Ruyechan et al. 1982).

A transcriptional map of the EHV-1 genome is not yet available, however, the mapping of some EHV-1 genes has been possible by comparison of sequence data with that of HSV-1. Homologues of HSV-1 glycoproteins gB (Allen and Yeargen 1987; Bonass et al. 1988; Robertson and Whalley 1988), gC (Allen and Yeargen 1987; Allen and Coogle 1988), gE (Yeargen and Allen 1987), gH and thymidine kinase (Robertson and Whalley 1988) have been identified and mapped to the EHV-1 genome at positions consistent with those reported for HSV-1. The relative positions of these EHV-1 homologues confirms the colinearity with HSV-1 at the level of individual genes.

SECTION II REPLICATION OF THE HERPESVIRUSES

II.1 Regulation of gene expression.

II.1(a) Temporal regulation of gene expression.

The HSV lytic cycle can be temporally divided into three broad stages: immediate-early, early and late. The virus specific infected cell polypeptides were initially classified as forming three groups whose synthesis is co-ordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman 1974). This closely parallels the regulation of viral transcription (reviewed by Wagner 1985). Upon infection of the host cell, a limited portion of the viral genome is transcribed by RNA polymerase II to produce immediate-early mRNAs (Kozak and Roizman 1974; Clements et al. 1977; Jones and Roizman 1979). The immediate-early genes are transcribed in the presence of inhibitors of protein synthesis such as cycloheximide (Honess and Roizman 1974; Jones and Roizman 1979) and their transcription is stimulated by a component of the HSV virion (Post et al. 1981; Batterson et al. 1983). This component has been identified as Vmw65 (Campbell et al. 1984) and acts in conjunction with cellular factor(s) (Preston et al. 1988). Immediate-early polypeptide synthesis reaches maximal rates two to four hours after infection. Translation of immediate-early mRNAs yields the infected cell polypeptides Vmw175, Vmw110, Vmw68, Vmw63 and Vmw12 which are encoded by genes IE 175, IE 110, US1, UL54 and US12 respectively (Figure 2).

Functional immediate-early polypeptides are required for the transcription of λ the genes encoding early polypeptides (Kozak and Roizman 1974; Honess and Roizman 1975). The expression of early genes occurs at maximal rates, following the initiation of DNA replication, approximately five to seven hours after infection. Many early gene products are involved in DNA replication such as DNA polymerase, ribonucleotide reductase and thymidine kinase. The inhibition of DNA replication causes a reduction in the accumulation of late mRNAs which include mRNAs encoding the virion structural polypeptides and are made at maximal rates between twelve and seventeen hours post infection (Honess and Roizman 1974; 1975).

Several polypeptides display kinetics of synthesis that are intermediate between these groups. For example, the large subunit of ribonucleotide reductase (RR1), although classified as an early gene often appears under immediate-early conditions (Watson and Clements 1978; Watson et al. 1979; MacDonald, Ph.D Thesis 1980) and glycoprotein D, another early protein, although synthesised in significant levels in the absence of DNA replication, requires DNA replication for optimal expression and is therefore sometimes termed an early-late gene (Gibson and Spear 1983; Johnson et al. 1986). Two sub-classes of late mRNA, termed late and true-late, are also distinguishable. In the absence of DNA synthesis late mRNAs are present whereas only very low amounts of true-late mRNA can be detected (Holland et al. 1980; Wagner 1985; Johnson et al. 1986).

Vmw175 is an essential factor in the transition from immediate-early to early and late stages of gene expression. The regulatory role of Vmw175 has been demonstrated by the use of ts mutants which fail to induce the expression of early and late mRNAs (Preston 1979a; Dixon and Schaffer 1980) reflecting their failure to induce early and late proteins (Benyesh-Melnick et al. 1974; Marsden et al. 1976). This pattern of transcription was also observed for mutants carrying deletions in Vmw175 (DeLuca et al. 1985). Vmw175 also exerts negative effects on HSV gene expression as evidenced by the fact that mutants with a ts lesion in this gene overproduce immediate-early mRNAs and polypeptides at the non-permissive temperature (Preston 1979a, 1979b; Dixon and Schaffer 1980; Watson and Clements 1980). Godowski and Knipe (1983, 1985, 1986) proposed that the major DNA binding protein, an early protein, is involved in the repression of gene expression at all stages of infection on the basis that a ts mutant in this gene accumulated mRNAs of the immediate-early, early and late classes at the non-permissive temperature.

EHV-1 gene expression in lytic infection has also been shown to be sequentially ordered and temporally regulated in an immediate - early / early / late fashion (Caughman et al. 1985). Four immediate-early polypeptides, termed IE1, IE2, IE3 and IE4, have been identified with M_rs 203K, 176K, 151K and 129K respectively (Caughman et al. 1985). Transcriptional analyses have mapped the immediate-early mRNA to a single 6kb message which maps within the inverted repeat sequences at 0.73-0.83 and 0.95-1.0 map units (Gray et al. 1987). This data raised questions as to whether the

four immediate-early proteins were each unique gene products or related products of the same gene. Characterisation of the immediate-early proteins with respect to limited proteolytic digestion profiles, antigenic cross reactivity and pulse-chase patterns have shown them to share similar peptide profiles and to be antigenically related (Caughman et al. 1988). In vitro translation of the 6kb immediate-early transcript produced a family of polypeptides that comigrated with immediate-early polypeptides produced in vivo and which were antigenically cross reactive (Robertson et al. 1988). No data is yet available on functional analyses of the EHV-1 immediate-early proteins but it is likely that one or more of the four polypeptides is an important regulatory protein in virus gene expression.

II.1(b) Regulation of immediate-early genes.

HSV-1 genes are transcribed throughout the reproductive cycle by RNA polymerase II (Constanzo et al. 1977). A central question concerning the regulation of viral gene expression is the nature of cis-acting sequences and viral trans-acting elements which permit the cellular transcriptional machinery to differentiate between immediate-early, early and late genes.

Immediate-early genes do not require de novo protein synthesis or the presence of functional Vmw175 to be efficiently transcribed and in this respect the regulation of immediate-early genes is unique within the viral genome. It is well established that control is primarily at the level of transcription (Preston 1979b; Everett 1984; O'Hare

and Hayward 1985a, 1985b; Coen et al. 1986; Gelman and Silverstein 1986). The regulation of these genes is mediated by strong promoters upstream of the mRNA 5' termini and, in the case of Vmw175, an enhancer-like element (Post et al. 1981; Mackem and Roizman 1982a; Cordingley et al. 1983; Lang et al. 1984; Preston and Tannahill 1984; Bzik and Preston 1986). An element far upstream of the mRNA 5' termini of all immediate-early genes has been found to increase transcription in response to the virion associated trans-inducing factor identified as Vmw65 (Post et al. 1981; Mackem and Roizman 1982b; Batterson and Roizman 1983; Cordingley et al. 1983; Preston and Tannahill 1984; Kristie and Roizman 1984; Campbell et al. 1984). The target sequences for stimulation by Vmw65 have been localised to an AT rich motif found upstream of all immediate-early genes, but not early or late genes, in HSV-1 and HSV-2. The consensus of this cis-acting sequence is TAATGARATTC (Mackem and Roizman 1982c; Whitton et al. 1983; Whitton and Clements 1984). Functional analyses have shown that the TAATGARATTC element is essential in mediating a response to the virion trans-inducing factor (Preston et al. 1984; Gaffney et al. 1985) however, full responsiveness required the presence of a GA rich element which itself was unable to confer a strong response (Bzik and Preston 1986). The gene product of Vmw65 does not bind to calf thymus DNA nor to DNA containing the TAATGARATTC element (Muller 1987; Marsden et al. 1987; Preston et al. 1988) but its interaction with DNA via a cellular factor has been demonstrated (Preston et al. 1988).

It has been suggested that cellular transcription factors can play a role in HSV transcription. The cellular

transcription factor Sp1 has been shown to bind to GC rich sequences within the promotor region of immediate-early genes and stimulate transcription of these genes *in vitro* (Jones and Tjian 1985).

II.1(c) Regulation of early gene expression.

In lytically infected cells, there is an absolute requirement for functional Vmw175 as temperature sensitive mutants with a lesion in the gene encoding this polypeptide fail to produce early and late gene products at the non-permissive temperature (Benyesh-Melnick *et al*. 1974; Marsden *et al*. 1976; Preston 1979a; Knipe *et al*. 1981). Transient expression assays in HeLa cells have been analysed for transcriptional activation of the promotor for HSV-1 thymidine kinase, a prototype early gene, by the products of individual immediate-early gene clones. Thymidine kinase is chosen as the target gene as its promotor is only minimally transcribed in the absence of immediate-early gene products. From these studies, it has been concluded that Vmw175 and Vmw110 can both independently stimulate early gene expression and that in combination they act synergistically on early promotors (Everett 1984; O'Hare and Hayward 1985a, 1985b; Quinlan and Knipe 1985; Gelman and Silverstein 1986). No transcriptional stimulatory function was demonstrated for Vmw63 or Vmw68 proteins although O'Hare and Hayward (1985a) suggested that Vmw12 may augment the stimulatory activity of Vmw175 and Vmw110 proteins. Unlike other immediate-early polypeptides, Vmw12 is non-phosphorylated, does not appear to bind to DNA and is located predominantly in the cytoplasm

(Preston et al. 1979a; Marsden et al. 1982; Palfreyman et al. 1984). It is therefore likely that if Vmw12 plays a role in augmenting the transcriptional effects of Vmw175 and Vmw110 then it does so at the post-transcriptional level. A viable mutant has been identified which lacks the gene encoding Vmw12, showing it to be a non-essential function in infected cell culture (Longnecker and Roizman 1986; Brown and Harland 1987).

The ability of Vmw110 to transactivate early gene promotors in transient transcription assays contrasts with the observation that ts mutants in Vmw175 fail to induce early gene expression (Watson and Clements 1979, 1980; Dixon and Schaffer 1980) which suggested that during normal infection Vmw110 cannot functionally substitute for Vmw175. A deletion mutant specifying a truncated form of the polypeptide Vmw110 was shown to be inactive in transfection assays. The growth of this mutant exhibited a multiplicity dependence, indicating that Vmw110 is not essential in tissue culture at high multiplicities of infection (Stow and Stow 1986).

II.1(d) Regulation of late gene expression.

Late gene expression during virus infection has been studied using the true-late promotors of gC (Johnson and Spear 1984; Godowski and Knipe 1985) and US11 (Johnson et al. 1986). Under conditions of DNA synthesis inhibition, the gene products of US11 and gC are expressed at extremely low levels (less than 5% of normal levels) (Godowski and Knipe 1985; Johnson et al. 1986). In these experiments which used chemical inhibitors, it was not possible to be

certain that absolutely no DNA replication has taken place. Therefore, to determine whether DNA synthesis is obligatory for late gene expression, the level of transcription from a plasmid-borne US11 promotor was studied in the presence or absence of a functional HSV-1 origin of replication (Johnson and Everett 1986). It was concluded from these studies that DNA replication is not an absolute requirement for activation of the plasmid-borne US11 promotor but is very important for achieving abundant expression.

Sequences involved in late gene expression were investigated using plasmids containing a functional HSV-1 origin of replication (ori_s). All the DNA elements necessary for fully efficient regulated expression of US11 and gC were found to lie within 33bp of the RNA cap sites (Johnson and Everett 1986; Shapira et al. 1987) indicating that a late gene promotor consists only of a proximal 'TATA' box and cap site region. In order to test the hypothesis that a proximal 'TATA' box is sufficient for late gene expression, the distal upstream region of the gD promotor required for its normal regulation as an early promotor, was removed and the truncated promotor was linked to ori_s. As predicted, this resulted in the conversion of gD promotor regulation to late gene kinetics during virus super-infection (Johnson and Everett 1986).

The promotors of late genes, like those of early genes, respond to trans-activation by Vmw175 and Vmw110 in transient expression systems (DeLuca et al. 1985; Mavromara-Nazos et al. 1986). Sacks et al. (1985) have shown that mutants with ts lesions in Vmw63 synthesized drastically reduced levels of several late polypeptides at

the non-permissive temperature. In addition, certain immediate-early polypeptides were overproduced at the non-permissive temperature whereas normal levels of early polypeptides were synthesized. It was therefore concluded that Vmw63 is an essential regulatory protein which is required after the onset of DNA replication.

No data is yet available on the role of Vmw68 in HSV transcription. All of the immediate-early polypeptides, with the exception of Vmw12, can bind calf thymus DNA (Hay and Hay 1980), however, whether the interaction with DNA occurs directly or indirectly remains to be established. Freeman and Powell(1982) reported that purified Vmw175 polypeptide was unable to bind to DNA even though the protein was capable of this activity in crude cell extract. Addition of infected or uninfected cell extract to the purified protein restored its DNA binding activity. These results suggested that Vmw175 binds to DNA only via a component of the uninfected cell. Alternatively, it is possible that Vmw175 becomes destabilised during the purification procedure and the addition of cellular proteins may stabilise Vmw175 restoring its ability to bind DNA. More recently, DNA sequences have been identified which bind protein complexes containing Vmw175 (Faber and Wilcox 1986, 1988; Kristie and Roizman 1986a, 1986b). However, it is not yet clear whether the association between Vmw175 and DNA is mediated by Vmw175 itself or another protein.

II.2 Herpesvirus polypeptides.

Analysis of the DNA sequence of the HSV-1 genome has revealed the existence of 70 distinct potentially polypeptide encoding regions (McGeoch et al. 1988). Functions have not yet been assigned to all of these genes, however, a number of enzymes and structural and regulatory polypeptides have been mapped and characterised by the use of mutants, by the expression of genes encoding an assayable function, by the analysis of species present in preparations of purified virions and by antisera directed against peptides corresponding to amino acid sequences in the predicted gene product. A transcriptional map of the EHV-1 genome is as yet unavailable, however, the mapping of some EHV-1 genes has been possible by comparison of DNA sequence with that of HSV-1.

II.2(a) HSV genes associated with DNA replication.

A method for identifying viral genes required for HSV DNA replication has been reported by Challberg (1986) based on the observation that a plasmid encoding the HSV origin of replication can be replicated by superinfecting HSV (Stow 1982). HSV-1 DNA digested to completion with Xba 1 was found to be as efficient as intact DNA in supporting in vivo replication of cotransfected plasmids encoding ori_s. Several Xba 1 fragments of HSV-1 DNA were therefore cloned into a plasmid vector and combinations of cloned DNA were tested for their ability to supply trans-acting functions required for HSV-1 origin dependent replication. In this way, seven genes were shown to be essential for plasmid DNA

replication which include the gene encoding DNA polymerase (UL29), the major DNA binding protein (UL30), the 65K M_r DNA binding protein (UL42), the origin binding protein (UL9) and UL5, UL8 and UL52 (Wu et al. 1988; McGeoch et al. 1988).

DNA polymerase. HSV-1-encoded DNA polymerase differs from cellular DNA polymerase in its requirement for high salt (Keir et al. 1966) and sensitivity to phosphonoacetic acid (Mao et al. 1975). In addition to DNA polymerisation, the enzyme has 3' to 5' exonuclease activity which is believed to serve as a proof reading function.

Major DNA binding protein. The major DNA binding protein shows preferential binding to single stranded DNA (Bayliss et al. 1975; Knipe et al. 1982). It may be involved in the dissociation of double stranded DNA as it reduces the melting temperature of poly A/T helices in vitro (Powell et al. 1981). It also stabilises single stranded DNA (Ruyechan 1983) and plays a role in the regulation of gene expression (Godowski and Knipe 1983; see section II.1(a)).

65K M_r DNA binding protein. The 65K M_r HSV-1 protein, $65K_{DBP}$, is distinct from the 65K M_r trans-inducing factor (Marsden et al. 1987). Studies with intertypic recombinants (Parris et al. 1988) indicated that the $65K_{DBP}$ was the serotype equivalent of the previously described ICSP 34/35 (Vaughan 1984; 1985). The protein is encoded by gene UL42 (Parris et al. 1988), binds to double stranded DNA in the absence of other proteins (Gallo et al. 1988) and is regulated as an early protein (Schenk et al. 1988; Goodrich et al. 1989).

Origin binding protein. Elias et al. (1986) described a protein which binds specifically to the viral origin of replication. This protein has been purified to apparent homogeneity and DNase 1 footprint analysis showed that the 82K M_r origin binding protein interacts with two related sites within ori_s located on each arm of a near perfect palindrome (Elias et al. 1988). The origin binding protein has recently been identified as the product of the UL9 gene (Olivo et al. 1988; Weir et al. 1989).

The product of the UL5 gene has been proposed to be associated with DNA helicase activity (Zhu and Weller 1988) whereas the products of genes UL8 and UL52 are, as yet, uncharacterised.

II.2(b) **HSV enzymes associated with nucleotide metabolism.**

Deoxypyrimidine kinase (thymidine kinase). The virus specifies a novel deoxypyrimidine kinase (Kit and Dubbs 1963) which phosphorylates both thymidine and deoxycytidine using the same active site (Jamieson and Subak-Sharpe 1974). This enzyme is not essential for virus growth in growing cells but is essential for growth in resting cells (Jamieson and Subak-Sharpe 1974).

Deoxyuridine triphosphate nucleotidyl hydrolase (dUTPase). Herpes simplex virus encodes a deoxyuridine triphosphate nucleotidyl hydrolase (Wohlrab et al. 1982; Preston and Fisher 1984) which catalyses the hydrolysis of dUTP to dUMP thus reducing the amount of dUTP incorporated into DNA. The

virus-induced dUTPase activity has been shown to be non-essential in tissue culture (Fisher and Preston 1986).

Ribonucleotide reductase. A key enzyme in DNA synthesis in all eukaryotic and prokaryotic organisms is ribonucleotide reductase which catalyses the conversion of ribonucleotides to deoxyribonucleotides. Herpes simplex virus type 1 encodes a novel ribonucleotide reductase activity (Averett et al. 1983; Dutia 1983) and this enzyme is discussed in detail in section III.

II.2(c) Other non-structural polypeptides encoded by HSV.

Alkaline exonuclease. Herpes simplex virus-induced alkaline exonuclease activity was first reported in HSV-1-infected cells (Keir and Gold 1963) and later in HSV-2-infected cells (Hay et al. 1971). A mutant of HSV-2 which is ts for the induction of alkaline exonuclease activity was reported to be incapable of inducing viral DNA synthesis at the non-permissive temperature and it was therefore proposed that alkaline exonuclease provides an essential function for HSV replication (Moss 1986). However, recent studies with a mutant containing a lac Z insertion in the gene encoding alkaline exonuclease showed that the mutant could synthesize almost wild type levels of viral DNA and late proteins suggesting that there is no defect in viral DNA synthesis (Weller et al. 1988). The reason for the discrepancy between these two sets of results is unclear.

Weller et al. 1988 observed large numbers of empty capsids in cells infected with the insertion mutant and very

few mature extracellular virions and therefore proposed that the alkaline exonuclease may be required for the efficient packaging of infectious progeny.

Uracil-DNA glycosylase. Uracil-DNA glycosylase is a DNA repair enzyme which removes uracil residues from DNA that arise either by misincorporation or by deamination of cytosine residues. The structural gene encoding an HSV-induced uracil-DNA glycosylase has been mapped to a location of 0.065-0.08 m.u (Caradonna et al. 1987) which corresponds to open reading frame UL2 (Mullaney et al. 1989).

Protein kinase. A novel protein kinase is induced after infection of cultured cells with HSV-1 (Blue and Stobbs 1981; Purves et al. 1986). DNA sequencing studies revealed that the US3 gene of HSV-1 and HSV-2 (McGeoch et al. 1985; 1987) and the corresponding gene of VZV (Davison 1983) encode proteins that are homologous with members of the protein kinase family of eukaryotes (McGeoch and Davison 1986). Frame et al. (1987) used an oligopeptide-induced antiserum specific for the HSV-1 US3 gene product combined with purification of protein kinase from infected cells to assign the protein kinase function to the US3 gene product. This activity is not essential for growth in tissue culture (Frame et al. 1987; Purves et al. 1987). Recently, a second protein kinase function has been suggested for the product of the gene UL13 (Smith and Smith 1989) but the putative gene product has not been identified.

II.2(d) HSV-encoded structural proteins.

Estimates of the number of polypeptides present in purified HSV virions have ranged from 15 to 33 (Spear and Roizman 1972; Marsden et al. 1976; Powell and Purifoy 1976; Heine et al. 1979) which include glycoproteins, capsid proteins and tegument proteins. HSV-1 encodes at least seven glycoproteins, gB, gC, gD, gE, gG, gH and gI (Spear 1976; Marsden et al. 1978, 1984; Bauke and Spear 1979; Roizman et al. 1984; Frame et al. 1986; Longnecker et al. 1987; Johnson and Feenstra 1987; McGeoch 1987), encoded by the genes UL22, UL27, UL44, US4, US6, US7 and US8 respectively (McGeoch et al. 1988). All have been shown to be present in the virion except gI, however, it is likely that gI is also present on virions since they possess an affinity for the Fc end of IgG (Bauke and Spear 1979) and this activity depends on a complex of both gE and gI (Johnson et al. 1988). An additional glycoprotein is potentially encoded by gene US5 of the HSV-1 genome (McGeoch et al. 1985) but this remains to be identified.

Only three of these glycoproteins, gB (Sarmiento et al. 1979; Little et al. 1981), gD (Ligas and Johnson 1988) and gH (Weller et al. 1983; Gompels and Minson 1986; McGeoch and Davison 1986b; Desai et al. 1988) are essential for infectivity in tissue culture, however, their precise role in virus entry is not yet fully understood. Virus mutants lacking gB (Cai et al. 1988) and gD (Ligas and Johnson 1988) have been shown to bind to the cell surface and are therefore not essential for adsorption. However, these mutants fail to form plaques and to synthesize

virus-specific polypeptides upon infection. The plating efficiency of these mutants was enhanced by polyethylene glycol, a membrane fusion agent, and therefore gB and gD are essential at a stage after viral attachment when the viral envelope fuses with the cell membrane. Monoclonal antibodies to gH inhibit the transfer of virus from infected cells to uninfected cells during plaque formation (Buckmaster et al. 1984; Gompels and Minson 1986) implying roles for gH in virus entry, and in virus release, or in the formation of intercellular junctions between infected and uninfected cells. More recently the defect in tsQ26, a ts mutant in gH, was shown to be due to loss of this glycoprotein from virions during their transport to the cell surface (Desai et al. 1988).

Mutations in gC do not affect viability (Heine et al. 1974; Holland et al. 1984) and, more recently, it has been shown that gE, gG gI and the product of the US5 gene are also dispensable in tissue culture (Longnecker and Roizman 1986, 1987; Longnecker et al. 1987; Weber et al. 1987; Harland and Brown 1988).

Full HSV-1 capsids, containing DNA, and empty capsids share five structural proteins, VP5 (Vmw155), VP19 (Vmw53), VP23 (Vmw36), VP24 (Vmw24) and a 12K component VP12. Additionally full, but not empty, nucleocapsids contain polypeptide VP21, a major component of the cylindrical protein plug which, with viral DNA, forms the core of the virion (Gibson and Roizman 1972).

Some controversy surrounds the nucleocapsid polypeptides VP22a or p40 which are products of the UL26 gene (McGeoch et al. 1988). In early studies these were identified using

purification procedures which gave different capsid forms as major components of full capsids but were not present in nucleocapsids lacking DNA (Gibson and Roizman 1972; Heilman et al. 1979). Polypeptide VP22a did not appear to be a constituent of mature virus particles (Gibson and Roizman 1972) and it was therefore proposed that VP22a undergoes some modification during virion maturation to form VP22, a virion polypeptide that is loosely bound to the nucleocapsid (Gibson and Roizman 1974).

Ts1201 has a defect in the processing of VP22a at the non-permissive temperature such that viral DNA is not packaged and large numbers of partially cored capsids accumulate in the nuclei (Preston et al. 1983). When cells infected with ts1201 were shifted to the permissive temperature, in the presence of cycloheximide, processing of VP22a and packaging of viral DNA took place suggesting that VP22a is closely involved in the process of viral DNA packaging.

More recently, Rixon et al. (1988) used immunoelectron microscopy and two mutants, ts1201 and ts1203, to determine the relationship between VP22a and different capsid forms. Ts1203 processes VP22a normally at the non-permissive temperature but fails to package viral DNA (Matz et al. 1983). The studies of Rixon et al. (1988) revealed, in contrast to earlier findings, that VP22a is present in empty capsids and that it is not present as a major component of full capsids. Their observations also contradict the proposal of Gibson and Roizman (1974) that the abundant virion protein VP22 might be a processed form of capsid protein VP22a. On the basis of results reported by Rixon et

al. (1988), it is likely that VP22a becomes transiently associated with capsids at an early stage of their assembly and that its removal from capsids is linked with the process of packaging DNA.

Proteins that reside in the tegument include VP1 to 3, Vmws273, 248 and 239, which are high molecular weight polypeptides and the 65K M_r virion trans-activating factor (Batterson and Roizman 1983; Campbell et al. 1984).

II.2(e) EHV-1-encoded polypeptides.

Changes in several enzymatic activities have been described following the infection of cells with EHV-1. The virus has been shown to induce a novel DNA polymerase (Cohen et al. 1975; Allen et al. 1977), thymidine kinase (Gentry and Aswell 1975; Allen et al. 1978a, 1978b) and ribonucleotide reductase (Cohen et al. 1977) activities. A protein kinase activity has also been described (Randall et al. 1972) but there is as yet no evidence that this is virus coded.

A common antigen of EHV-1, PRV and BMV was shown to be antigenically related to the major DNA binding protein of HSV-1 and HSV-2 (Killington et al. 1977). The purified cross-reacting polypeptide from cells infected with EHV-1 has been shown to have DNA binding properties (Littler et al. 1981).

EHV-1 virions contain 28 structural proteins ranging from M_r 270K to 16K (Kemp et al. 1974; Perdue et al. 1974). Analysis of purified EHV-1 virions revealed the presence of at least eleven glycoproteins (Perdue et al. 1974; Turtinen

and Allen 1982). The genes encoding six major glycoproteins of EHV-1 have been mapped by the use of EHV-1 specific monoclonal antibodies and the lamda gII expression vector system (Allen and Yeargen 1987). Five of these EHV-1 glycoproteins, gp2, gp10, gp13, gp14 and gp21/22, mapped to the genome L component and only one, gp17/18 was expressed from the unique S region of the genome. Glycoproteins 13 and 14 mapped to positions colinear with genes of the major glycoproteins gC and gB identified in HSV-1. Glycoprotein 17/18 mapped to a position in the U_S region colinear with that for HSV glycoprotein E. Further evidence that gp14 is homologous to gB of HSV-1 has been provided by hybridisation experiments using the HSV-1 gB gene to probe enzyme restricted EHV-1 DNA (Bonass et al. 1988). Robertson and Whalley (1988) reported that the open reading frame downstream of the EHV-1 TK gene has a hydrophobic signal peptide and potential N-glycosylation sites in addition to homology with the characterised gH glycoprotein of HSV-1 (Gompels and Minson 1986) which is also located downstream of the TK gene.

Purified nucleocapsids contain five major structural proteins with molecular weight 148K, 59K, 46K, 36K and 18K (Perdue et al. 1974) which comprise more than 96% of the total nucleocapsid protein. The 148K protein accounted for approximately 65% of the total nucleocapsid protein and was the major structural protein of both nucleocapsids and the intact virion. The remaining 4% comprised eight structural proteins of sizes ranging from 140K to 30K.

II.3 Maturation of virus.

II.3(a) Replication of viral DNA.

Analyses by electron microscopy shows that viral DNA molecules circularise after infection (Friedmann et al. 1977; Hirsch et al. 1977; Jacob and Roizman 1977). At the onset of replication, molecules showing 'eye' and 'D' loops at or near one end of the DNA were observed while later in infection larger than unit length molecules are found in 'head-to-tail' configuration (Jacob and Roizman 1977). The formation of 'head-to-tail' linkages does not require de novo protein synthesis (Poffenberger and Roizman 1985). The prevailing model for HSV DNA replication proposes that the viral genome replicates as 'head-to-tail' concatamers possibly generated by a rolling circle mechanism from which unit length genomes are cleaved and packaged (Jacob et al. 1979; Kaerner et al. 1981; Vlazney and Frenkel 1981).

II.3(b) Origins of DNA replication.

Electron microscopy studies of replicating DNA suggested that the HSV genome contains two origins of replication, one near the middle of U_L and the other near one molecular terminus (Friedmann et al. 1977; Hirsch et al. 1977). Indirect evidence supporting the existence of two origins of replication comes from studies with defective molecules of HSV-1 which are generated during serial passage of the virus at high multiplicities of infection. Defective DNA molecules consist of tandem duplications of viral DNA molecules containing sequences from either the 'c' repeats

which bracket U_S (Frenkel et al. 1975, 1976; Kaerner et al. 1979, 1981; Locker and Frenkel 1979) or sequences from U_L (Kaerner 1979). Both classes of defective DNA molecules contain the 'a' sequence which specifies a site for the cleavage of viral DNA concatamers during encapsidation. The HSV-1 genome, therefore, contains three origins of DNA replication; two in the diploid 'c' sequences (ori_s) and one in the unique long region (ori_L).

Direct evidence that the repeat units of defective genomes contained origins of replication was demonstrated by the amplification of defective DNA by cotransfection with wild type DNA to generate tandemly repeated structures (Vlazney and Frenkel 1981; Spaete and Frenkel 1982). It is believed that the wild type HSV DNA provides essential helper functions in trans.

The localisation of ori_s to the 'c' repeats flanking U_S was accomplished using cloned HSV-1 fragments in the presence or absence of super-infecting wild type helper virus (Mocarski and Roizman 1982b, Stow 1982). In this way Stow and M^{C}Monagle (1982) mapped ori_s to a 100bp fragment which lies between the 5' ends of two divergently transcribed immediate-early mRNAs. A prominent feature of the origin region is an almost perfect palindromic sequence 45bp long containing 18 consecutive A or T residues at its centre. Ori_L was inferred to lie within a 3.4kb segment of viral DNA spanning map co-ordinates 0.360 - 0.413 (Spaete and Frenkel 1982). The precise localisation and characterisation of ori_L has been difficult due to problems encountered in cloning the DNA fragment containing this region. However, it has been achieved using a yeast cloning

vector (Weller et al. 1985). Sequence analysis of a 425bp fragment spanning the deletion prone region revealed a perfect 144bp palindrome with homology to ori_s. In particular, the ori_s and ori_L sequences of HSV-1 and HSV-2 and ori_s of VZV all contain a conserved 11 base pair motif CGTTCGCACTT (Stow and Davison 1986) shown in recent nuclease protection studies of HSV-1 ori_s to be bound by the product of the UL9 gene (Elias et al. 1986, 1988; Olivo et al. 1988). Recent mutagenesis studies have suggested that the left arm of the ori_s palindrome, containing the conserved motif, is essential for origin activity whereas the right arm can be deleted without significantly inhibiting replication (Deb and Doelberg 1988). These results suggest that the ability to form a cruciform structure is not essential for ori_s activity.

Baumann et al. (1989) have recently reported the mapping and DNA sequencing of an EHV-1 origin of replication within a central portion of the internal inverted repeat sequence. Origin activity was shown to be contained within a 200 base pair fragment located at 0.840 to 0.841 map units on the EHV-1 genome. Comparison of the DNA sequence of this 200 base pair fragment to those containing the origins of replication of human herpes viruses demonstrated that substantial homology exists between them including the conserved 9 base pair motif CGTTCGCAC which was previously noted to be conserved between the human herpes virus origins of replication (Stow and Davison 1986). However, unlike the origins of other herpes viruses, the EHV-1 origin does not contain a near perfect palindromic sequence.

II.3(c) Cleavage and packaging of viral DNA.

Mature HSV genomes are cleaved from concatameric precursors by a site specific mechanism and these cleavage events are probably coupled to the encapsidation process. The presence of the 'a' sequence in defective genomes indicated that the 'a' sequence may contain necessary signals for both cleavage and encapsidation of DNA (Kaerner et al. 1981; Vlazney and Frenkel 1981). Stow et al. (1983) showed that plasmid molecules containing an HSV origin of replication were replicated and packaged in the presence of helper virus when the 'a' sequence was located on the same plasmid molecule. Varmuza and Smiley (1985) constructed recombinant HSV genomes in which various subfragments of the 'a' sequence were assayed for their ability to direct cleavage and packaging following insertion into the TK locus. A single 'a' sequence was processed to generate two new termini, each bearing a copy of the packaging signal. It was proposed that the termini of mature DNA are generated by a process involving two separate cleavages, one specifying the location of the new S terminus and the other, that of the new L terminus and that the sequences between the two cleavage sites are duplicated by the DNA maturation system.

Packaging of viral DNA has been shown to be linked to the processing of VP22a, a major structural protein (Preston et al. 1983). Studies involving a ts mutant with a defect in the gene encoding VP22a indicated that processing of this polypeptide was essential for the encapsidation of viral DNA as a revertant, TS^+ for growth , processed the polypeptide

normally at the non-permissive temperature. Dalziel and Marsden (1984) reported that the 'a' sequence of HSV-1 contained DNA sequences that interact specifically with the structural proteins VP21 and VP22. However, the functional significance of this is unknown.

II.4 The effect of HSV infection on host cell metabolism.

The infection of cells with HSV-1 and HSV-2 is characterised by the cessation of mitosis (Wildy et al. 1961) and of cellular polypeptide synthesis and the concomitant selective translation of viral mRNA (Roizman and Roane 1964; Roizman et al. 1965). The suppression of host polypeptide synthesis coincides with rapid disaggregation of infected cell polyribosomes and subsequent virus specific polypeptide synthesis is accompanied by the formation of new polyribosomes (Sydiskis and Roizman 1966; 1967). The majority, but not all, the mRNA species associated with polyribosomes late in infection were found to be virally encoded.

Shut-off of host cell polypeptide synthesis is generally more rapid in HSV-2-infected cells than in HSV-1-infected cells (Powell and Courtney 1975; Pereira 1977; Fenwick et al. 1979; Schek and Bachenheimer 1985) although this generalisation does not hold true for strain 17 of HSV-1 and strain HG52 of HSV-2 (Marsden et al. 1978). Initial shut-off of host cell polypeptide synthesis is mediated by a structural component of the HSV virion (Fenwick and Walker 1968; Nishioka and Silverstein 1978). The cessation of host polypeptide synthesis was found to occur in the presence of

actinomycin D and after infection with UV-irradiated viruses. A secondary shut-off function reduces the remaining levels of host protein synthesis and requires the expression of viral genes (Fenwick and Clark 1982; Nishioka and Silverstein 1978; Read and Frenkel 1983).

The abundance of host mRNA is reduced by HSV infection in a variety of cell types. It has recently been proposed that HSV encodes a function that indiscriminately shortens the half-life of host as well as viral mRNAs (Kwong and Frenkel 1987). Analysis of globin mRNA in infected Friend erythroleukaemia cells showed that after initial disaggregation of host polyribosomes, mediated by a virion component, the host mRNA was degraded by a process that required viral gene expression (Nishioka and Silverstein 1977; 1978). Schek and Bachenheimer (1985) reported that the degradation of specific cellular mRNAs (actin, tubulin, histone 3 and histone 4) induced by HSV-1 infection of Vero cells occurred under conditions in which de novo virus RNA and protein synthesis were inhibited. This result suggests that degradation of mRNA is induced by a factor associated with the infecting virion. In addition, a mutant virus, vhs 1, defective for the virion-associated shut-off function was apparently also defective for cellular mRNA degradation (Read and Frenkel 1983). Recently, the vhs 1 mutation has been mapped to a 265 base pair fragment spanning map co-ordinates 0.604 to 0.606 of the HSV-1 genome (Kwong and Frenkel 1988) which corresponds to the product of the UL41 gene (McGeoch et al. 1988). The protein encoded by this gene has not yet been identified.

Evidence that the virion-associated shutoff factor is encoded by the UL41 gene has been provided by Fenwick and Everett (manuscript in preparation). The UL41 gene from HSV-2 strain G, which has a strong shutoff function, was cloned into HSV-1 strain 17, which has a weak shutoff function and the recombinant virus was shown to have acquired a strong shutoff function. The mutation(s) affecting the functional half-lives of host mRNAs as well as viral mRNAs were also mapped within this fragment. These results suggest that the virion-associated factor is responsible for both cessation of host cell polypeptide synthesis and degradation of cellular and viral mRNA. Protein synthesis may be a secondary requirement of particular cell types for mRNA degradation and viral gene expression is required for full shut-off of cellular metabolism.

SECTION III RIBONUCLEOTIDE REDUCTASE

Ribonucleotide reductase catalyses the reduction of all four ribonucleotides to deoxyribonucleotides utilizing nucleoside diphosphates as substrates and is an essential enzyme for DNA synthesis in all prokaryotic and eukaryotic cells. Several herpesviruses, namely, HSV-1, HSV-2, EHV-1, EBV, PRV, and VZV have been shown to induce novel ribonucleotide reductase activities upon infection of the host cell (Cohen 1972; Cohen et al. 1974; Cohen et al. 1977; Henry et al. 1978; Lankinen et al. 1982; Averett et al. 1983; Dutia 1983; Huszar et al. 1983; Spector et al. 1987). In each case, the ribonucleotide reductase activity induced upon virus infection was found to be biochemically and immunologically distinct from the cellular enzyme making ribonucleotide reductase a possible target for antiviral chemotherapy.

III.1 Structure of the ribonucleotide reductase enzyme.

The ribonucleotide reductases isolated from E.coli and L.leichmannii have been intensely studied and although, from a mechanistic point of view, they share a number of common features (see section III.2), the two proteins are structurally quite distinct and represent two classes of ribonucleotide reductase.

III.1(a) Monomeric ribonucleotide reductase.

The enzyme encoded by L.leichmannii comprises a single 76K M_r polypeptide chain (Panagou et al. 1972; Chen et al.

1974; Blakely 1978). This enzyme utilises only nucleoside triphosphates as substrates and its activity is totally dependent on the coenzyme B12 adenosylcobalamin (AdoCbl) which acts as a dissociable cofactor in the reduction reaction (Singh et al. 1977). Ribonucleotide reductase isolated from L.leichmannii has been shown to catalyse a specific exchange of tritium between the hydrogen atoms of the deoxyadenosyl moiety of AdoCbl and water (Hogenkamp 1968). In this ability to catalyse the exchange of hydrogen between water and the cobalt-bound methylene group of the coenzyme, ribonucleotide reductase is quite unique as other B12 dependent enzymes catalyse transfer from the same position in the cobamide but to a carbon atom in the product molecule and not to water. The tritium exchange assay has been used to determine the activity of other B12-dependent reductases which are found to be common among prokaryotes.

III.1(b) Oligomeric ribonucleotide reductase.

The ribonucleotide reductase isolated from E.coli separates during purification into two catalytically inactive subunits, designated as B1 and B2 (Brown and Reichard 1969). Protein B1, M_r 160K, is composed of two polypeptide chains of similar size having isoleucine as a carboxy-terminal residue , however, differences in amino acid residue composition occur at the amino terminal end of the chain and the subunit structure is therefore described as $\alpha\alpha'$ (Thelander 1973). The B2 protein also comprises two polypeptide chains of similar or identical size, M_r 40K (Ehrenberg and Reichard 1972; Thelander 1973), which had the

same amino-terminal alanine and carboxy-terminal leucine and the subunit structure is therefore described as β_2 (Thelander 1973). Protein B2 also contains two atoms of iron which can be removed reversibly resulting in a reversible loss of enzyme activity (Ehrenberg and Reichard 1972). In the presence of magnesium ions, B1 and B2 form a complex, M_r 245K, which indicates that the active form of ribonucleotide reductase exists as a 1:1 complex between B1 and B2 of the type $\alpha_2\beta_2$ (Brown and Reichard 1969; Thelander 1973). The adjacent genes encoding the B1 and B2 polypeptides have been cloned and sequenced (Carlson et al. 1984; Nillson et al. 1988). Ribonucleotide reductases isolated from several sources have been found to be composed of the same two non-identical subunit structure as that isolated from E.coli. The cDNAs for the corresponding mouse subunits (M1 and M2) have been cloned and sequenced (Caras et al. 1985; Thelander and Berg 1986) as well as those encoding both subunits of the T4 bacteriophage enzyme (Sjoberg et al. 1986; Tseng et al. 1988), the gene encoding the small subunit of the yeast enzyme (Elledge et al. 1987; Hurd et al. 1987) and part of the gene encoding the small subunit of the clam enzyme (Standart et al. 1985). Considerable homology has been shown to exist between the small subunits from different organisms. For example, the small subunit of bacteriophage T4 shares 47% homology with the B2 subunit of E.coli (Sjoberg et al. 1986) and the yeast gene shows 63% exact homology to the clam and 60% homology to the mouse (Elledge and Davis 1987).

III.1(c) Structure of the HSV-encoded ribonucleotide reductase.

Herpes simplex virus type 1 and type 2 have also been shown to specify two subunits of ribonucleotide reductase, RR1 and RR2, with M_rs 136K and 38K for HSV-1 and 138K and 36K for the HSV-2 enzyme (Dutia 1983; McLauchlan and Clements 1983a; Preston et al. 1984; Cohen et al. 1985; Frame et al. 1985; Bacchetti et al. 1986; Ingemarson and Lankinen 1987; Darling et al. 1988). The HSV-1 RR1 and RR2 polypeptides are translated from two mRNAs of 5kb and 1.2kb which share a common 3' terminus (McLauchlan and Clements 1982) and are encoded by genes UL39 and UL40 respectively (McGeoch et al. 1988). Moreover, the 5' terminus of the 1.2kb mRNA is located within the carboxy-terminal region of RR1 (McLauchlan and Clements 1983a) and the ribonucleotide reductase locus on the HSV-2 genome is similarly arranged (McLauchlan and Clements 1983b; Galloway and Swain 1984). DNA sequences of HSV-2 RR1 and RR2 (McLauchlan and Clements 1983b; Swain and Galloway 1986), HSV-1 RR2 (Draper et al. 1982; McLauchlan and Clements 1983a) and HSV-1 RR1 (Nikas et al. 1986) have previously been obtained. The genes encoding the large and small subunits of EBV (Gibson et al. 1984) and VZV (Davison and Scott 1986) have also been identified and sequenced. Sequence comparisons have shown that the RR1 and RR2 subunits are highly conserved between HSV-1 and HSV-2 and show distinct amino acid homology with equivalent proteins from EBV (Gibson et al. 1984), VZV (Davison and Scott 1986; Nikas et al. 1986), mouse (Caras et al. 1985), E.coli (Carlson et al. 1984; Sjoberg et al. 1985), surf clam (Standart et al. 1985), T4 bacteriophage (Sjoberg et al.

1986), vaccinia virus (Slabaugh et al. 1988) and yeast (Elledge and Davis 1987; Hurd et al. 1987).

Amino acid comparisons with RR1 polypeptides from other organisms indicate that the HSV-1-encoded RR1 contains a unique amino-terminal domain which is absent from other RR1 polypeptides apart from HSV-2 (Nikas et al. 1986). This is consistent with the larger apparent M_r of the HSV-1-encoded RR1 (136K) (Preston et al. 1984) compared with that of RR1 from other species (approximately 80K to 90K). Ingemarson and Lankinen (1987) reported that in extracts of HSV-1-infected Vero cells, RR1 can be proteolytically degraded to a 93K form which retains enzymatic activity raising the possibility that the unique amino-terminal domain of the HSV RR1 may not be required for ribonucleotide reduction. Across the remainder of RR1 and RR2 Nikas et al. (1986) identified 15 blocks of conserved amino acid homology in RR1 and 6 blocks in RR2. These conserved blocks are believed to have functional importance for enzyme activity.

The existence of a complex between RR1 and RR2 has been proposed by Frame et al. (1985) on the basis of immunoprecipitation experiments with an anti-oligopeptide serum raised against the carboxy-terminal seven amino acids of the HSV-1-induced RR2. The antiserum precipitated RR2 from both HSV-1 and HSV-2 infected cells as expected, however, the inclusion of 0.5% SDS in the precipitation buffer resulted in the coprecipitation of RR1 with RR2. It was proposed that RR2 is present both as free polypeptide and complexed with RR1 and that SDS facilitates the binding of antibody to RR2 by partially denaturing the complex without allowing the complete dissociation of the complex.

A ts mutant, ts1207, with a lesion in the sequence encoding RR1, was used to show that the coprecipitation of RR1 with RR2 was not due to the antibody recognising common epitopes. In the presence of 0.5% SDS, the oligopeptide-induced serum precipitated both RR1 and RR2 from cells infected with ts1207 at the permissive temperature but only RR2 was precipitated at the non-permissive temperature. Similarly, an anti-RR1 monoclonal antibody precipitated both subunits from cells infected with wild type or mutant virus at the permissive temperature but only RR1 was precipitated from ts1207-infected cells at the non-permissive temperature. This result indicates that although ts1207 induced the synthesis of both subunits at the non-permissive temperature, the subunits failed to form a complex (Frame et al. 1985).

The anti-oligopeptide serum has also been shown to precipitate the large and small subunits from PRV-infected cells and the small subunit from EHV-1 and VZV-infected cells indicating that the carboxy-terminal region of RR2 is highly conserved among the herpesviruses (Dutia et al. 1986).

Bacchetti et al. (1986) have also shown that monoclonal antibodies against RR1 or RR2 polypeptides precipitate both polypeptides from HSV-1-infected cell extracts. The observed reactivity of these antibodies with both RR1 and RR2 was shown to be a result of their association in a complex by assay of the reactivity of anti-reductase antibodies with each of the polypeptides separated by gel filtration or selective expression in transformed or infected cells. Ingemarson and Lankinen (1987) demonstrated

by sedimentation analysis of the enzyme that both subunits are homodimers of the corresponding polypeptides and that RR1 comigrated with the RR2 polypeptide in a glycerol gradient. This position in the gradient corresponded to enzyme activity indicating further that the active form of the enzyme consists of a complex between RR1 and RR2 of the type $\alpha_2\beta_2$ and therefore has the same basic structure as the E.coli and mammalian reductases.

Biological or biochemical separation of the RR1 and RR2 subunits has been shown to result in a loss of activity (Bacchetti et al. 1986) and recently two reports have described the reconstitution of enzyme activity from separate subunits in vitro and in vivo (Darling et al. 1988; Huang et al. 1988).

III.1(d) Structure at the active site of ribonucleotide reductase.

The structure at the active site and the reaction mechanism of ribonucleotide reductase isolated from E.coli, which has been studied extensively, is believed to be a relevant model for eukaryotic and virus-induced enzymes. The catalytic site of the E.coli enzyme has been shown to comprise parts of both B1 and B2. The B2 subunit, in addition to two iron atoms, has been shown to contain a stable tyrosine radical (Sjoberg et al. 1977) which is essential for enzyme activity (Ehrenberg and Reichard 1972). The presence of the radical is closely linked to the presence of iron and is lost on the removal of iron and reformed upon the reconstitution of the iron centre. The function of iron as it binds to the protein is both to

generate the tyrosine radical and to stabilise the radical (Petersson et al. 1980; Sjoberg et al. 1982). An alignment of different primary structures of the small subunit of E.coli, clam, EBV and HSV showed three regions of amino acid sequence to be strikingly similar and therefore likely to be functionally important (Sjoberg et al. 1985). A conserved tyrosine residue (Tyr-122 in E.coli numbering) was proposed to be responsible for radical stabilisation. This was later confirmed by site-directed mutagenesis studies in which Tyr-122 was substituted by a phenylalanine residue (Larsson and Sjoberg 1986). The purified wild type and mutant B2 proteins were shown to be of the same size, iron content and iron-related absorption spectrum, however, the mutant lacked the tyrosyl radical and enzyme activity. It is probably not coincidental that the ribonucleotide reductase isolated from L.leichmannii also forms a free radical during catalysis (Stubbe et al. 1981).

Experiments with substrate analogues acting as inhibitors demonstrated that the catalytic site of the E.coli enzyme includes parts from both B1 and B2 subunits (Thelander et al. 1976). 2'-azido-substituted analogues caused an irreversible inactivation of the B2 subunit without affecting B1. On reduction by ribonucleotide reductase, the azido derivatives function as radical scavengers and selectively destroy the free radical of B2 indicating that this radical participates in ribonucleotide reduction directly. The azido derivative also inactivates the B12-dependent ribonucleotide reductase of L.leichmannii indicating a general involvement of free radicals in ribonucleotide reduction. Conversely, the addition of

2'-chloro-substituted nucleotides to the E.coli enzyme irreversibly inactivated B1 without affecting B2. The inactivation of B1 is caused by the modification of oxidation-reduction thiols present on the B1 molecule (see section III.2).

III.2 Mechanism of the reaction.

Ribonucleotide reductase catalyses the substitution of the hydroxyl group at the 2' position of ribose by a hydrogen atom. NADPH is the ultimate reducing agent but several proteins participate in the reaction as hydrogen carriers. The electrons required for the reduction of ribonucleotides are transported from NADPH via a flavoprotein thioredoxin reductase to the low molecular weight protein thioredoxin (Thelander 1974; Holmgren et al. 1975). This small acidic protein was purified and characterised as a physiological hydrogen donor for the E.coli ribonucleotide reductase by Laurent et al. (1964). Both thioredoxin and thioredoxin reductase contain reduction-oxidation active disulphides participating as electron carriers (Thelander 1974; Holmgren et al. 1975). In the absence of a hydrogen donor, ribonucleotide reductase was shown to reduce a limited amount of ribonucleotides (3 moles substrate per mole B1) at the expense of the sulfhydryls of B1 (Thelander 1974). Addition of reduced thioredoxin to the B1/B2 complex led to the regeneration of active sulfhydryl groups on B1 and further reduction of ribonucleotides. It was therefore proposed that in the reduction of ribonucleotides, electrons flow from thioredoxin to oxidation-reduction active disulphides of

subunit B1 and catalytic activity depends on the continued reduction of redox active S-S groups on B1 by an external hydrogen donor. There is no evidence for the existence of a major HSV-encoded thioredoxin and a recent report provided evidence that the principal hydrogen donor for the HSV-1-encoded ribonucleotide reductase in infected cells is the host cell thioredoxin (Darling 1988).

A second hydrogen donor protein for ribonucleotide reductase, glutaredoxin, has been identified in E.coli (Holmgren 1976) and mammalian cells (Luthman and Holmgren 1982). This protein also contains an oxidation-reduction disulphide which is reduced by NADPH via glutathione and glutathione reductase (Holmgren 1979).

The proposed mechanism of the reduction reaction is shown in Figure 3 and may be summarised as follows. The protein radical mediates hydrogen atom abstraction from the 3' position of the nucleotide. The release of the 2' hydroxyl gives rise to an intermediate substrate cation radical. Subsequent reduction of this intermediate and return of the hydrogen atom from the protein residue results in product formation. The predictions made by this model have been examined experimentally by studying the interactions of both monomeric and oligomeric enzymes with carbon-2 substituted nucleotide analogues (Stubbe and Kozarich 1980; Sjoberg et al. 1983; Stubbe et al. 1983; Harris et al. 1984; Harris et al. 1987).

Figure 3. Mechanism of the reaction of ribonucleotide reduction.

The proposed mechanism as typified for ribonucleotide reductase from E.coli is depicted. The balance of electrons (e) is shown and the radical is represented as (•).

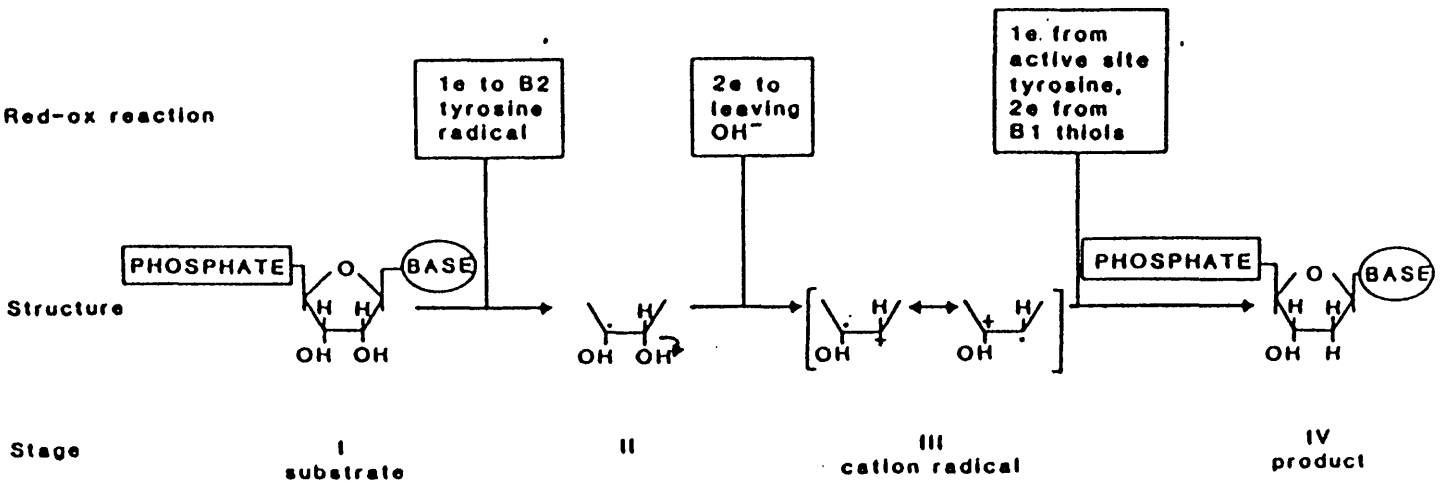
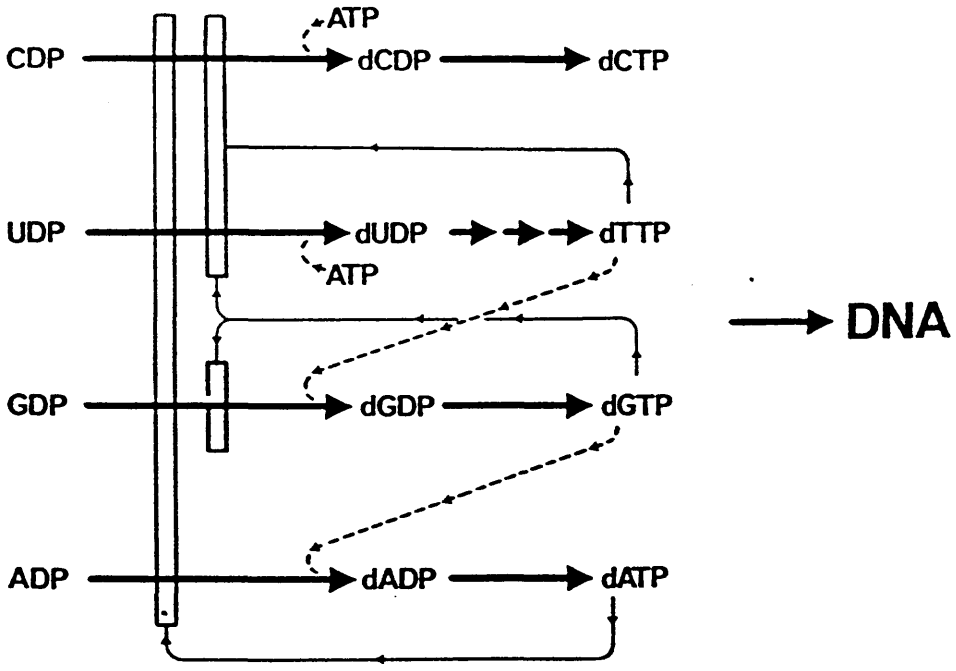

Red-ox reaction
1e to B2 tyrosine radical
2e to leaving OH⁻
1e from active site tyrosine, 2e from B1 thiols
Structure
PHOSPHATE
BASE
OH OH
Stage
I substrate
II
III cation radical
IV product

Figure 4. A scheme of the regulation of *E.coli* deoxyribonucleotide synthesis.

Substrates and reaction products are shown with the direction of the reaction pathways indicated by thick arrowed lines. The broken arrows represent positive effects and the open bars represent negative effects.

Deoxyribonucleotide synthesis begins with the reduction of CDP and UDP by an ATP-activated enzyme, proceeds to GDP reduction via a dTTP-enzyme and reaches ADP reduction by a dGTP-activated enzyme. Ribonucleotide reductase activity is turned off by an accumulation of dATP. Accumulation of dTTP shuts off the reduction of pyrimidine substrates and accumulation of dGTP also turns off GDP reduction.


ATP
CDP
dCDP
dCTP
UDP
dUDP
dTTP
ATP
DNA
GDP
dGDP
dGTP
ADP
dADP
dATP

III.3 Regulation of ribonucleotide reductase.

III.3(a) Allosteric regulation.

The ribonucleotide reductase isolated from E.coli is allosterically regulated to produce a balanced supply of deoxyribonucleoside triphosphates required for DNA synthesis (reviewed by Thelander and Reichard 1979). Protein B1 binds both substrates and effectors and two classes of allosteric binding sites have been demonstrated to exist on B1 (Brown and Reichard 1969; Thelander and Reichard 1979). It is believed that each B1 polypeptide contains only one binding site of each class. The two classes of binding site, designated as 'h' and 'l', are distinguished by their affinities for dATP (Brown and Reichard 1969). The 'h' sites have a high affinity for dATP and also interact with the effectors dTTP, dGTP and ATP and governs the substrate specificity of the enzyme. Binding of effectors to the 'h' sites results in conformational changes at the active site which induces preferential binding of one or other substrate. The 'l' sites have a low affinity for dATP and bind only ATP in addition to dATP. The 'l' sites regulate the overall activity of the enzyme; when ATP is bound at the 'l' site the enzyme is active and when dATP is bound at the 'l' site the enzyme is inactive. The large number of effectors and the existence of two classes of binding site therefore allow the enzyme to assume a multitude of conformational states with different activities. A scheme of the regulation of deoxyribonucleotide synthesis is outlined in Figure 4.

The mammalian ribonucleotide reductase is allosterically regulated in a similar manner to that of E.coli. The mammalian enzyme is also comprised of two non-identical dimeric subunits called proteins M1, an 80,000-M_r polypeptide, and M2, a 44,000-M_r polypeptide (Thelander and Reichard 1979). There is evidence to suggest that the M1 subunit contains two binding sites which are analogous to the 'h' and 'l' sites of E.coli (Thelander et al. 1980). The enzymes isolated from bacteriophage T4 and L.leichmannii are also allosterically regulated (reviewed by Thelander and Reichard 1979). In contrast, the catalysis of reduction of ribonucleotide diphosphates by the HSV- and PRV-induced enzyme are not inhibited by dATP and dTTP (Cohen et al. 1972; Ponce de Leon et al. 1977; Langelier et al. 1978; Huszar and Bacchetti 1981; Langelier and Buttin 1981; Lankinen et al. 1982; Averett et al. 1983). The apparent lack of allosteric regulation of the herpes virus-induced ribonucleotide reductase therefore differentiates the enzyme from other ribonucleotide reductases.

III.3(b) Regulation of ribonucleotide reductase expression.

The B1 and B2 polypeptides of E.coli ribonucleotide reductase are encoded by two adjacent genes, nrdA and nrdB, located at 48 min. on the E.coli map (Fuchs et al. 1972). The genes are part of an operon in which nrdA is the promotor proximal gene (Tuggle and Fuchs 1986) and the two genes have been cloned and sequenced (Carlson et al. 1984; Nilsson et al. 1988). The synthesis of B1 and B2 in E.coli is co-ordinately regulated (Fuchs 1977). Inhibition of DNA

synthesis by thymine starvation results in the increased synthesis of ribonucleotide reductase (Filpula and Fuchs 1977) and the increased level of ribonucleotide reductase activity in thymine starved cells was shown to be due to increased levels of nrd mRNA (Hanke and Fuchs 1983). The half life of nrd mRNA was unchanged in these cells and it was therefore proposed that induction was due to an increased transcriptional rate of the nrd operon. This increased rate of transcription was found to be dependent upon concomitant protein synthesis (Hanke and Fuchs 1984).

Deletion analysis of sequences upstream of the cloned nrd genes have been used to identify regulatory regions for expression (Tuggle and Fuchs 1986). The transcription start site was mapped 110bp upstream of nrdA, the first structural gene. A site required for positive regulation was mapped 135bp upstream of the transcription start site in a region with two direct repeat sequences as well as secondary structure. Two other sites, one upstream of the transcription start site and the other downstream, were identified as sequences whose deletion markedly increased expression. The regulation of transcription of the operon encoding ribonucleotide reductase in E.coli would therefore appear to be under both positive and negative control.

The tyrosyl free radical of the B2 subunit may also be a target for a regulatory mechanism. The radical is a prerequisite for enzyme activity and after treatment with hydroxyurea a radical-free form of B2 is generated which is inactive. Crude extracts from E.coli transform the inactive B2 subunit into the active form in the presence of magnesium ions, dithiothreitol and oxygen (Eliasson et al. 1986).

Upon purification of the activating crude E.coli extract, it was demonstrated that radical introduction required four proteins; a superoxide dismutase, flavin oxidoreductase and catalase (Eliasson et al. 1986; Fontecave et al. 1987a, 1987b). The identity of the fourth protein is unknown. This tyrosyl radical generating enzyme system may participate in the regulation of the E.coli ribonucleotide reductase at the post-transcriptional level via the B2 subunit.

Consistent with its specialised role in DNA synthesis, the mammalian ribonucleotide reductase is regulated in a cell cycle dependent manner (Eriksson and Martin 1981; Eriksson et al. 1984; Engstrom et al. 1985). The subunits M1 and M2 were isolated and separated from mouse lymphoma cells and each used to determine the activity of the complementary subunits in extracts of cells in different phases in the cell cycle (Eriksson and Martin 1981). The specific catalytic activity of the M1 subunit was the same in G1, G1/ early S phase and S phase/ G2 cell populations while that of M2 was decreased by 60% in the G1 population. Direct quantification of the M2 subunit by electron paramagnetic resonance (EPR) demonstrated that a 3 to 7 fold increase in the concentration of tyrosyl radical containing M2 subunit was obtained when mouse mammary tumour cells passed from G1 to the S phase (Eriksson et al. 1984). Addition of deuterated tyrosine to the cells gave rise to a different EPR signal in the newly synthesised M2 subunit. Pulse chase experiments with deuterated tyrosine showed that the S phase-correlated increase in radical containing M2 was due to de novo protein synthesis. The labelled M2 molecules disappeared with a half life of three hours indicating that

new molecules of M2 subunit must be synthesised at a high rate during S phase (Eriksson et al. 1984). In contrast, the level of the M1 subunit remains constant throughout the cell cycle and has a half life of 15 hours (Engstrom et al. 1985). The levels of M1 and M2 therefore vary independently of one another and ribonucleotide reductase is controlled during the cell cycle by the level of M2. In contrast, after hydroxyurea inactivation, mouse mammary tumour cells can rapidly generate the tyrosyl radical in already existing M2 molecules via an enzyme activation mechanism that is clearly different from the one responsible for the regulation of M2 during the cell cycle (Eriksson et al. 1984). Recent results (Bjorklund-unpublished data) show that both M1 and M2 mRNA are induced during S phase and regulated in the same manner and that differences in the M1 and M2 protein levels must be due to post-translational modifications.

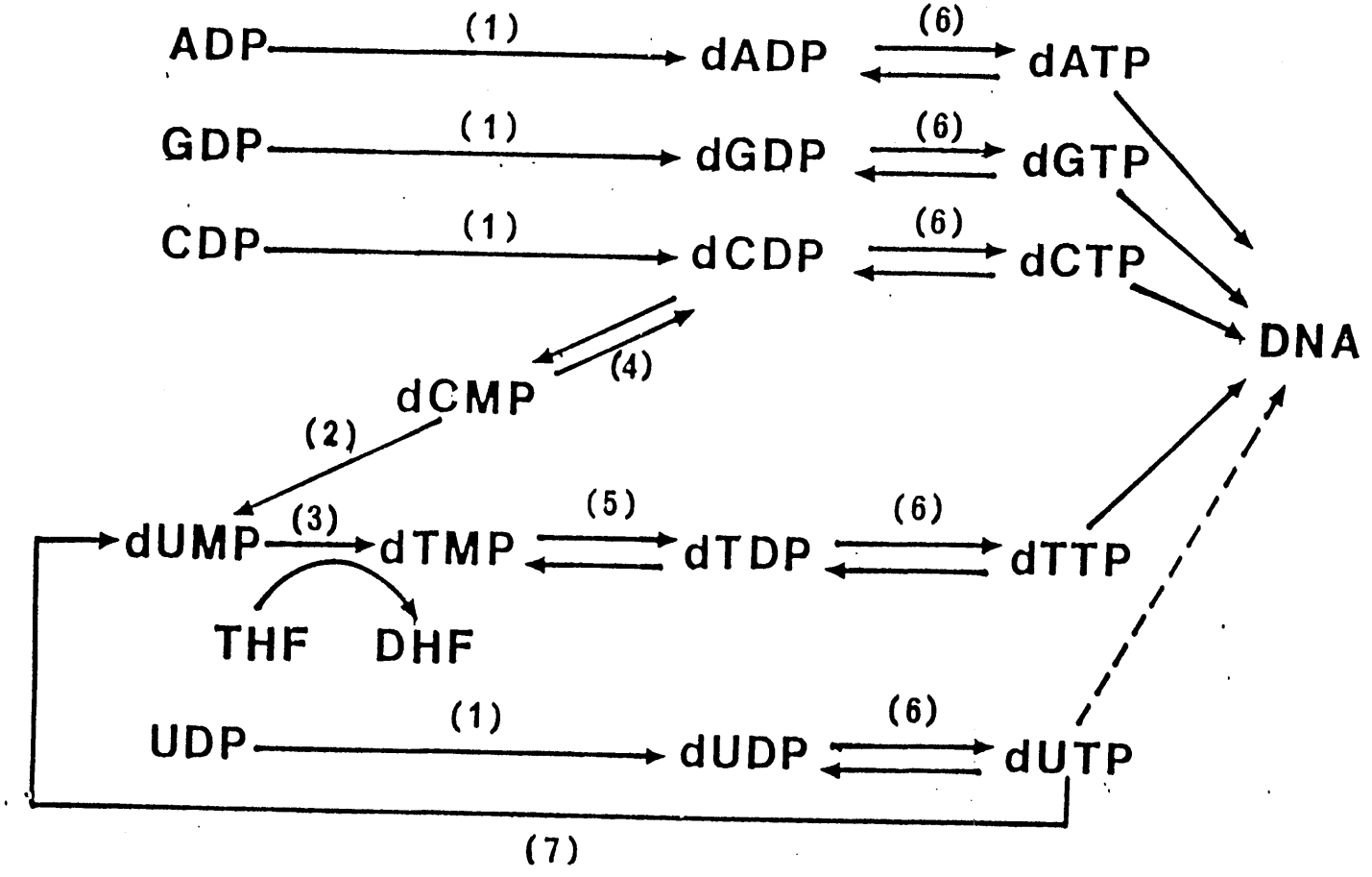
III.4 Other enzymes involved in the de novo synthesis of deoxyribonucleotides.

Reactions leading to the de novo synthesis of dNTPs in mammalian cells are shown schematically in Figure 5. This subject has recently been reviewed by Reichard (1988). The cell requires each of the four doeoxyribonucleoside triphosphates, dATP, dTTP, dCTP and dGTP, to replicate its DNA. Three deoxyribonucleoside diphosphates, dADP, dCDP and dGDP, are obtained through the action of ribonucleotide reductase, the first enzyme in the pathway of de novo synthesis of dNTPs. The fourth deoxyribonucleotide is also formed by reductase but via a more complex pathway. The

Figure 5. **Schematic representation of reactions leading to the de novo synthesis of dNTPs in mammalian cells.**

Allosterically regulated enzymes: (1) ribonucleotide reductase, (2) dCMP deaminase. Non-regulated enzymes: (3) dTMP synthase, (4) dCMP kinase, (5) thymidylate kinase, (6) nucleoside diphosphate kinase, (7) dUTPase.

THF,tetrahydrofolate, DHF, dihydrofolate


ADP
(1)
dADP
(6)
dATP
GDP
(1)
dGDP
(6)
dGTP
CDP
(1)
dCDP
(6)
dCTP
DNA
(4)
dCMP
(2)
dUMP
(3)
dTMP
(5)
dTDP
(6)
dTTP
THF
DHF
UDP
(1)
dUDP
(6)
dUTP
(7)

second enzyme in the pathway, dCMP deaminase, controls the amount of pyrimidine triphosphates (dTTP and dCTP) available for DNA synthesis. This enzyme, like ribonucleotide reductase, is allosterically regulated and is activated by dCTP and inhibited by dTTP. Upon activation by dCTP, dCMP deaminase provides dUMP which is required for the synthesis of dTTP. Thymidylate synthase catalyses the conversion of dUMP into dTMP and links dNTP synthesis to folate metabolism. The enzyme is therefore dependent on active dihydrofolate reductase. Four separate monophosphate kinases catalyse the phosphorylation of dAMP, dCMP, dTMP and dGMP in mammalian cells. The enzymes which phosphorylate dAMP, dCMP and dGMP accept ribonucleoside or deoxyribonucleoside monophosphates whereas dTMP kinase only phosphorylates dUMP in addition to dTMP. Finally, nucleoside diphosphate kinase, a ubiquitous and unspecific enzyme, is responsible for the phosphorylation of all natural ribose and deoxyribose diphosphates.

III.5 Ribonucleotide reduction and DNA synthesis.

A close correlation of ribonucleotide reduction and DNA synthesis has been observed in many systems. For example, the mammalian ribonucleotide reductase activity increases as the cells enter the S phase of the cell cycle (Eriksson and Martin 1981; Eriksson et al. 1984; Engstrom et al. 1985). The small subunit of ribonucleotide reductase isolated from yeast and its transcript has been shown to be induced approximately 10 - 20 fold by DNA damaging agents, a result which suggests that the enzyme is required for DNA repair in

yeast (Elledge and Davis 1987; Hurd et al. 1987). However, there is no evidence that the supply of deoxyribonucleotides by itself regulates DNA synthesis. It has been proposed that a physically associated complex of enzymes , named replitase, catalyses the production of deoxyribonucleotides and their incorporation into DNA in the S phase of the mammalian cell cycle (Reddy and Pardee 1980, 1982, 1983). Upon entering the S phase, it was suggested that dNTPs were synthesised within the complex and channelled directly into replicating DNA. However, the results of immunocytochemical experiments using two different monoclonal antibodies against the M1 subunit of mammalian reductase demonstrated an exclusively cytoplasmic localisation of this subunit in several cultured cell lines (Engstrom et al. 1984). These results indicate that, in mammalian cells, ribonucleotide reduction takes place in the cytoplasm and from there the deoxyribonucleotides are transported into the nucleus to serve in DNA synthesis. This is in contrast to the replitase model which states that most of the M1 protein in a cell should be located in the nucleus during S phase.

III.6 Inhibition of virus-induced ribonucleotide reductase activity.

There is evidence that the HSV-1-induced ribonucleotide reductase activity is virus-encoded (Dutia 1983; Preston et al. 1984) and essential for virus replication in tissue culture at high temperature (39.5^{o}C) and in resting cells (Goldstein and Weller 1988a, 1988b; Preston et al. 1988). Although herpesviruses have similar biochemical properties to the host cell enzyme eg. sensitivity to hydroxyurea and a

stable tyrosine free radical (Langelier and Buttin 1981; Lankinen et al. 1982) there are differences in Mg^{2+} and ATP requirements and in allosteric controls (Cohen 1972; Ponce de Leon et al. 1977; Langelier et al. 1978; Huszar and Bacchetti 1981; Averett et al. 1983). The differential behaviour of the virus and host enzymes suggest the possibility of using the viral reductase as a target for the design of antiviral agents.

A number of derivatives of the thiosemicarbazone, 2-acetyl pyridine, have been shown to be active against HSV-1 and HSV-2 reductase both in vitro and in a cutaneous herpes guinea pig model (Shipman et al. 1981, 1986; Turk et al. 1986) whereas the mammalian enzyme is not affected by these compounds. In contrast, some thiosemicarbazones, eg. guanozole and 1-formyl isoquinoline, preferentially inhibit the mammalian reductase (Spector and Jones 1985). The finding of Thelander and Graslund (1983) that the iron chelate of 1-formyl isoquinoline destroyed the tyrosine free radical at the active site of the M2 subunit of the mammalian reductase suggests RR2 as the probable site of action of these compounds.

Spector et al. (1985) demonstrated that a thiosemicarbazone derivative which inhibits HSV-1 reductase was tolerated by host cells and could significantly potentiate the antiviral activity of acyclovir. Acyclovir is an antiherpetic agent which is selectively phosphorylated by the HSV-encoded thymidine kinase but not by the host enzyme (Elion et al. 1977; Fyfe et al. 1978). Acyclovir-monophosphate is converted to a triphosphate form by cellular enzymes (Miller and Miller 1980) which serves as an alternative substrate for the viral

polymerase and is incorporated into a DNA primer template by this enzyme (Furman et al. 1979). It is not excised from the primer template by the 3',5'-exonuclease activity associated with viral DNA polymerase (Derse et al. 1981) and therefore prevents further chain elongation because of the absence of a 3'-hydroxyl moiety (Furman et al. 1980). In addition, acyclovir-triphosphate selectively inactivates the HSV-encoded DNA polymerase thereby inhibiting viral DNA synthesis (Furman et al. 1984).

Binding of acyclovir-triphosphate to HSV DNA polymerase can be competitively inhibited by dGTP and therefore this deoxyribonucleotide triphosphate has the potential to protect DNA polymerase from inactivation (Derse et al. 1981; Furman et al. 1984; St Clair et al. 1984). The inhibitor of viral reductase potentiates the antiviral activity of acyclovir by decreasing dGTP pools and in addition increases the level of acyclovir-triphosphate (Spector et al. 1985). The net increase in ratio of acyclovir-triphosphate to dGTP therefore facilitates the binding of acyclovir-triphosphate to HSV-1 DNA polymerase and promotes inactivation of the enzyme.

The HSV-induced ribonucleotide reductase activity can also be inhibited by the synthetic nonapeptide, YAGAVVNDL, which corresponds to the carboxy-terminal nine amino acids of RR2 (Cohen et al. 1986; Dutia et al. 1986). The cellular reductase was unaffected by the peptide. It was proposed (Dutia et al. 1986) that the peptide acted by competing for an RR2 binding site on RR1 thus interfering with subunit association. Direct evidence to support this model was obtained by McClements et al. (1988) who used an anti-RR2

monoclonal antibody which specifically precipitates RR2 and RR1 from HSV-1-infected cells. In the presence of increasing concentrations of YAGAVVNDL, the amount of RR2 precipitated remained constant while the amount of RR1 that was coprecipitated decreased. It was therefore proposed by McClements et al. (1988) that the inhibition of HSV-induced ribonucleotide reductase by this oligopeptide is due to induced subunit dissociation. However, Darling et al. (manuscript submitted for publication) have recently provided evidence that RR1 and RR2 can exist both associated and dissociated and that an equilibrium exists between these two states. From this data, a more likely explanation for peptide inhibition is proposed whereby subunit dissociation occurs during normal enzyme function and the peptide can then compete with RR2 for a binding site on RR1 consequently preventing the reassociation of the active holoenzyme.

Direct evidence that the inhibitory nonapeptide competes with RR2 for a binding site on RR1 has been reported by Paradis et al. (1988). A photoreactive analogue of the nonapeptide was shown to bind specifically to the HSV-1-induced RR1 polypeptide. Furthermore, this covalent association of the photoaffinity peptide with RR1 produced an irreversible inhibition of ribonucleotide reductase activity.

Gaudreau et al. (1987) studied the relationship between chemical requirements and inhibitory potencies of a series of peptides including fragments and analogues of YAGAVVNDL. Deletion of the amino-terminal tyrosine generated an octapeptide with a markedly reduced inhibitory potency (IC_{50} = 283uM) compared with the nonapeptide (IC_{50} = 36uM). In

contrast, deletion of the alanine and glycine residues, neighbouring the amino-terminal tyrosine, did not substantially modify the IC_{50} obtained with the octapeptide. The minimum active core was assigned to the sequence VVNDL which has an increased IC_{50} of 760uM. Monosubstitution of any of these residues by alanine completely abolished activity therefore stressing the importance of each amino acid contained in the minimum core. Peptides extended at the amino terminus, STSYAGAVVNDL and ECRSTSYAGAVVNDL respectively, have inhibitory potencies 2.1- and 1.4-fold greater than the nonapeptide. N^{α}-deamidation or acetylation also increased the inhibitory potency of YAGAVVNDL by 1.8- and 3.0-fold respectively indicating that the amino group is not necessary for maximum potency and removal of its associated charge increases potency. However, that a free carboxylic function is required is suggested by the observation that amidation of the α-carboxylic function diminishes its activity 3.2-fold.

Serological evidence for the conservation of the carboxy-terminal amino acid sequence of HSV-1 in HSV-2, PRV, VZV and EHV-1 implies that this nonapeptide may inhibit a broad range of herpesviruses (Dutia et al. 1986) and the recent finding that the PRV-induced enzyme is in inhibited by the nonapeptide supports this proposition (Cohen et al. 1987).

III.7 Ribonucleotide reductase as a target for antiviral chemotherapy.

In mammalian cells, the synthesis of DNA is dependent on the formation of dNTPs through the de novo pathway (section

III.5) or utilisation of preformed deoxynucleotides through the salvage pathway. It is not clear which source of DNA precursor is used for virus DNA synthesis during HSV-infection but the finding that a ts mutation, ts1207, in the HSV-1 RR1 polypeptide results in plaque reduction at the non-permissive temperature (Preston et al. 1984) implies that deoxyribonucleotide synthesis via the de novo pathway is important to the virus. The importance of this route in providing DNA precursors has, however, been questioned as HSV induces a DNase activity that breaks down host DNA and thus supplies an additional source of deoxyribonucleotides. Nutter et al. (1985a) showed that 95% inhibition of ribonucleotide reductase activity by hydroxyurea had no effect on dATP and dTTP pools in HSV-2 infected cells. The origin of dTTP in HSV-infected cells was investigated by examining the dTTP pool levels of acid-soluble dTTP and dTMP in virus DNA after HSV-2 infection of radio-labelled HeLa cells (Nutter et al. 1985b). When ribonucleotide reductase activity was inhibited by hydroxyurea, the dTTP pools were maintained by a compensatory increase in dTTP derived from the host DNA. These results suggested that at least 95% of the available ribonucleotide reductase activity may not be essential for virus replication and thus ribonucleotide reductase may not be a useful target against which to raise antiviral agents.

Goldstein and Weller (1988a) have shown that a lac Z insertion mutant which contained only the amino-terminal region of RR1 fused to β-galactosidase was competent for growth and viral DNA synthesis in actively dividing cells further demonstrating that an alternative pathway for

obtaining deoxyribonucleotides is operating which can compensate for the defect in viral ribonucleotide reductase. This demonstration that the virus-encoded ribonucleotide reductase is dispensable for virus growth and DNA synthesis in tissue culture is in apparent contradiction to the conclusion drawn from studies with ts1207 which, at the non-permissive temperature, failed to induce reductase activity with a concomitant decrease in virus production (Preston et al. 1984). Ts1207 has a single amino acid substitution in RR1 (Nikas et al. manuscript in preparation) which results in the inability of RR1 to form a complex with RR2 at the non-permissive temperature (Frame et al. 1985). Furthermore, in a plasmid replication assay designed to identify genes essential for virus DNA synthesis, a significant amount of DNA replication occurred in the absence of the virus-coded ribonucleotide reductase genes (Wu et al. 1988).

This apparent controversy has recently been resolved by the characterisation of a ts mutant, ts1222, which does not induce detectable amounts of ribonucleotide reductase activity at the permissive (31^0C) or the non-permissive temperature (39.5^0C) but viral replication is inhibited only at the higher temperature (Preston et al. 1988). This mutant contains a single base pair deletion at the 3' end of RR2 which alters the translational reading frame resulting in a change of all but one of the last 15 amino acids and in removal of the termination codon. Although these results indicate that the viral ribonucleotide reductase is dispensable for the replication of HSV in tissue culture cells at 31^0C, the ts mutant failed to grow in serum starved

cells. These results therefore demonstrate that HSV ribonucleotide reductase is essential for virus growth in non-replicating serum starved culture cells and in actively dividing cells at 39.5^0C but not at 31^0C.

Similar conclusions have been independently reached by Goldstein and Weller (1988b) using a deletion mutant lacking 90% of the coding sequences of the RR1 gene. A strong correlation of the mammalian ribonucleotide reductase activity to the rate of DNA synthesis has been discussed in section III.5. The dependence of the deletion mutant on the state of the cell for survival therefore probably reflects the requirement for cellular ribonucleotide reductase to compensate for the lack of the viral-induced enzyme.

Taken together, these results imply that if primary or acute infection by HSV occurs in cells which are dividing and contain adequate precursor pools then virus replication may not be dependent on viral ribonucleotide reductase activity. However, the induction of a viral reductase may be directly essential for the infection of terminally differentiated cells such as nerve cells. The introduction of RR-deficient viruses into animal models of both acute and latent infections has helped to elucidate the role of viral ribonucleotide reductase in these aspects of the HSV life cycle.

Cameron et al. (1988) have studied the role of HSV-encoded ribonucleotide reductase in the pathogenicity of the virus by the use of mutants with lesions in either RR1 or RR2. The virulence of the mutants in mice was reduced by approximately 10^6 fold when compared with wild type virus while the virulence of the revertant of one of these mutants

was restored to within 60-fold of that of the parent virus. These experiments show that although the virus-induced ribonucleotide reductase may be dispensable for growth of the virus in tissue culture, the enzyme is necessary for infection *in vivo* and is therefore a valid target for antiviral chemotherapy. These studies have been extended by the use of an epithelial zosteriform lesion model and similar conclusions reached (M. Ryan-unpublished data).

Similar conclusions were reached by Jacobsen *et al*. (in press) who used a mouse eye model to show that a deletion mutant of RR1 was severely impaired for replication in the eye for both acute and ganglionic infection. In contrast, a recombinant virus, in which the deleted sequences were restored, behaved similarly to wild type. Although these data show that the HSV ribonucleotide reductase is critical for the infection of mice, caution must be exercised in extrapolating the results to human infections. However, the same cautionary statement for virus-induced thymidine kinase and DNA polymerase, both of which are determinants of pathogenicity in mice (Field and Wildy 1978; Tenser *et al*. 1979; Field and Cohen 1986; Larder *et al*. 1986), would have applied but these enzymes are effective targets for antiviral chemotherapy as evidenced by the success of acyclovir therapy.

SECTION IV. PROJECT AIMS.

(1) To determine whether the inhibitory nonapeptide is stable in infected cell extracts and to identify the nature of any modifications which may occur.

(2) To test the proposal that the inhibitory nonapeptide might inhibit a broad range of herpesviruses (Dutia et al 1986) by determining whether the nonapeptide inhibited novel ribonucleotide reductase activity induced in cells infected with an abortogenic strain of equine herpes virus type 1.

(3) To investigate whether the unique amino - terminal domain of HSV-1 RR1 is required for enzymatic activity by means of raising oligopeptide - induced antisera against sequences representing the amino - terminal domain and the carboxy - terminal domain of the HSV-1 - induced RR1. This part of the project was performed in collaboration with Dr. H. Lankinen.

(4) To investigate the structure of the EHV-1 - induced ribonucleotide reductase by means of the same oligopeptide - induced antisera.

MATERIALS

SECTION I CHEMICALS AND REAGENTS

Tissue culture

All tissue culture reagents were supplied by Gibco-Biocult (Paisley, Scotland). RK13 cells were purchased from Flow Laboratories, Irvine, Scotland.

Electrophoresis

Acrylamide was supplied by Koch-Light Laboratories Ltd, Haverhill, Suffolk, England and N,N'-methylene bis acrylamide was supplied by BDH Chemicals Ltd, Poole, Dorset, England. Ammonium persulphate and TEMED were obtained from Biorad Laboratories, Richmond, California.

Autoradiography

Kodak X-OMAT XS-1 film was obtained from Kodak (London) Ltd. En^{3}hance was supplied from New England Nuclear, Boston, Mass, USA.

Immunoblotting

Nitrocellulose sheets were supplied by Schleicher and Schull, Dassel, West Germany. Horseradish peroxidase colour developer and gelatin were obtained from Biorad Laboratories, Richmond, California. Non-immune rabbit serum was from the Scottish Antibody Production Unit (SAPU), Scotland.

Affinity chromatography

CNBr-activated sepharose 4B, G10 sepharose, G75 sepharose, and G25 PD10 columns were all purchased from Pharmacia, Uppsalla, Sweden.

High pressure liquid chromatography

uBondapak C_{18} columns were purchased from Millipore UK Ltd, Waters Chromatography Division, Harrow, Middlesex, UK. HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals Ltd, Walkerburn, Scotland. Monofluor scintillation fluid was supplied by National Diagnostics, Highland Park, New Jersey, USA.

Protease inhibitors

All protease inhibitors used were purchased from Sigma (London) UK except alpha 2 macroglobulin which was obtained from BCL Boehringer Mannheim, East Sussex, UK.

All other reagents used were of analytical or reagent grade and were supplied by BDH Chemicals Ltd, Poole, Dorset, England or Sigma (London) Ltd.

SECTION II SOLUTIONS

Phosphate buffered saline

170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 pH 7.2 supplemented with 6.8mM $CaCl_2$ and 4.9mM $MgCl_2$.

Versene

6mM EDTA dissolved in PBS containing 0.0015% (w/v) phenol red.

Trypsin-versene

One volume 0.25% (w/v) Difco Trypsin (dissolved in 25mM Tris, 140mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 1mg/ml dextrose, 0,0015% phenol red pH 7.4 supplemented with 100 units/ml penicillin and 100 microgrammes / ml streptomycin plus 4 volumes versene.

Giemsa

1.5% (v/v) suspension of Giemsa in glycerol, heated at 56°C for 90-120 minutes and diluted with an equal volume of methanol.

Detergent lysis buffer

0.1M Tris-HCl pH 8.0, 10% glycerol, 0.5% NP40, 0.5% sodium deoxycholate, 2mM phenylmethylsulphonylfluoride (PMSF).

Denaturing buffer

50mM Tris-HCl pH 6.7, 2% SDS, 700mM 2-mercaptoethanol, 10% glycerol, bromophenol blue.

Electrophoresis buffer

52mM Tris, 53mM glycine, 0.1% SDS

Transfer buffer

25mM Tris pH 8.3, 192mM glycine, 20% (v/v) methanol.

Tris buffered saline (TBS)

20mM Tris pH 7.5, 500mM NaCl

Blocking buffer

1x TBS plus 3% gelatin

Antibody buffer

1x TTBS (1x TBS plus 0.05% Tween 20) plus 1% gelatin.

0.015% H_2O_2 development solution

Solution A

60mg HRP colour development reagent dissolved in 20ml ice cold methanol.

Solution B

60 microlitres ice cold 30% H_2O_2 added to 100ml room temperature TBS.

Solutions A and B were mixed to produce a 0.0015% H_2O_2 development solution which was used immediately.

SECTION III RADIOCHEMICALS

All radiochemicals were purchased from Amersham International PLC, Buckinghamshire, England.

L-(3,5-^{3}H)tyrosine	specific activity 40-60Ci/mmol
L-(^{35}S)methionine	specific activity >1000Ci/mmol
(5-^{3}H)cytidine 5'diphosphate	specific activity 10-30Ci/mmol
^{125}INa	carrier free

The synthetic nonapeptide [^{125}I]YAGAVVNDL was prepared as described in Results section I and was then tritiated by exchange with tritium gas by the Tritium Labelling Service, Amersham International, Forrest Park, Whitchurch, Cardiff.

SECTION IV. SYNTHETIC OLIGOPEPTIDES

Synthetic oligopeptides were purchased from Cambridge Research Biochemicals (CRB), Cambridge, UK with the exception of the nonapeptide YAGAVVNDL which was supplied by LKB Products Ltd, Cambridge, UK and the octapeptide AGAVVNDL which was synthesised by Miss Ania Owsianka. The molecular mass of synthetic peptides was determined by mass spectrometry (M-Scan, Berkshire, England) while purity was checked by reverse phase HPLC. All peptides were at least 95% pure.

METHODS

SECTION I GROWTH OF CELLS AND VIRUS.

I.1 Cell culture

BHK 21 clone 13 cells (MacPherson and Stoker 1962) were grown in the Glasgow modification of minimal essential medium (MEM, Stoker and MacPherson 1961) which was supplied as a 10x liquid concentrate. The concentrate was diluted in distilled water and supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 5mM glutamine and 2.75g/l sodium bicarbonate. Calf serum was added to a final concentration of 10% (v/v).

RK13 cells (Flow Laboratories Ltd) were grown in the same medium as described above except that it was additionally supplemented with 1% non-essential amino acids and foetal calf serum was added to a final concentration of 10% (v/v).

Cells were maintained in a humidified atmosphere comprising 95% air and 5% CO_2. The cells were harvested from roller bottles by pouring off the medium and washing the monolayer with 20ml versene. They were then washed twice with 20ml trypsin plus versene (1 in 4 dilution) and resuspended in fresh medium at a concentration of approximately 10^7 cells/ml.

I.2 Growth of virus

Strain 17 of herpes simplex virus type 1 (HSV-1) (Brown et al. 1973) was used throughout. BHK-C13 cells were grown to 80% confluency in 80 oz roller bottles and infected with HSV-1 at a multiplicity of 1 plaque forming unit per 300

cells in 40ml medium. The roller bottles were incubated at 31°C until maximum cytopathic effect was observed 4-5 days post infection. The infected cells were then shaken into the medium which was centrifuged at 2000 r.p.m for 10 minutes. The cell pellet was resuspended in 5ml supernatant, checked for sterility and frozen at -70°C. The supernatant was centrifuged at 12000 r.p.m for 2 hours on a Sorvall GSA rotor and the virus which was pelleted was resuspended in 5ml supernatant, sonicated in a bath sonicator until homogeneous, checked for sterility and stored at -70°C. This was referred to as the supernatant virus stock.

The cell suspension was thawed, treated in a bath sonicator until homogeneous and frozen at -70°C. It was then thawed, resonicated and centrifuged again at 2000 r.p.m for 10 minutes. The supernatant was decanted and stored at 4°C (supernatant 1). The pellet was resuspended in 5ml fresh medium, frozen at -70°C then thawed, resonicated and centrifuged at 2000 r.p.m for 10 minutes. The supernatant was decanted (supernatant 2) and added to supernatant 1. This was referred to as the cell associated stock. It was stored in 1ml aliquots at -70°C until required.

Four different isolates of equine herpes virus type 1 (EHV-1) were used during this study.

Strain Vol1939, supplied by Dr J. Mumford (The Animal Health Trust, Newmarket, UK).

Strain A.C, supplied by Dr A. Cullinane (The Irish Equine Centre, Johnstown, Naas, Co. Kildare, Ireland).

Strain Kentucky A, supplied by Prof. D. O'Callaghan (Louisiana State University Medical Centre, Shreveport, LA71130, USA).

Strain AB1, supplied by Dr R.A. Killington (University of Leeds, Leeds, UK).

RK13 cells were grown to 80% confluency in 80 oz roller bottles and infected with EHV-1 at a multiplicity of 1 plaque forming unit per 100 cells in 40ml medium. The roller bottles were incubated at 31°C until the cells were all rounded and detached from the bottle usually 4-7 days post infection. Both cell associated and supernatant virus were harvested as described for HSV except that the freeze-thawing step was omitted.

I.3 Sterility checks

Virus stocks were checked for sterility on brain heart infusion agar (BHI) and brain heart infusion agar plus 10% horse blood (BHI blood agar). 26g Difco Bacto BHI agar was dissolved in 500ml deionised water and sterilised by autoclaving for 20 minutes. The solution was cooled to approximately 40°C and poured into 50mm petri dishes (BHI plates) or 50ml horse blood (Biocult) was added just before pouring (blood agar). Plates were stored at 4°C before use. As required the virus stock was streaked onto the agar plates, using duplicate plates for each stock, which were then sealed with parafilm to prevent moisture loss during incubation. BHI agar plates were incubated at room temperature for seven days (to test for fungal contamination) and BHI blood agar plates were incubated at 37°C for seven days (to test for bacterial contamination). The plates were checked occasionally over the seven days and if no contamination was apparent, the virus stocks were assumed to be sterile.

I.4 Titration of virus stocks

EHV-1 stocks were titrated on RK13 cells, HSV-1 strain 17 was titrated on BHK-C13 cells. The titration procedure was the same for both. Monolayers of BHK-C13 or RK13 cells were inoculated with serial ten-fold dilutions of virus in PBS containing 10% calf serum or 10% foetal calf serum. Absorption was carried out at 37°C for 1 hour. The inoculum was removed and 5ml agar overlay (310ml Eagle's A, 60ml Eagle's B without phenol red, 125ml 3.6% Difco agar, 7ml calf serum) was added. The plates were incubated at 37°C for 2 days or at 31°C for 3 days. The agar overlay was removed and the cells were fixed with 50% Cidex for 2 hours. Clear plaques were counted after staining with Giemsa for 10 minutes and the concentration of virus was calculated.

SECTION II CHROMATOGRAPHY

II.1 Size exclusion chromatography

Sephadex G25 (PD10 columns)

Prepacked PD10 columns were obtained from Pharmacia. Each column contains 9.1ml swollen gel in 0.9% NaCl containing 0.01% thimerosol. The columns were pre-equilibrated with the appropriate buffer before use.

Sephadex G75

Sephadex G75 (Pharmacia) was supplied as a dry powder. The beads were swollen in 50mM Tris-HCl pH 8.0 for 24 hours at 20°C or 3 hours at 90°C before use. A 10ml column was packed with the G75 gel and the column was equilibrated with the appropriate buffer before use.

II.2 Affinity chromatography

Coupling of bovine serum albumin (BSA) to CNBr-activated Sepharose 4B as described by the manufacturer.

Sepharose beads were swollen in approximately 200ml 1mM HCl for 10 minutes and then washed in a sintered glass funnel with 1 litre of the same solution using a vacuum pump. 1g freeze dried material gave approximately 3.5ml final volume. The beads were then washed with 200ml coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl pH8.0). 0.5g BSA was dissolved in 2.5ml coupling buffer and the BSA solution was mixed with the swollen gel end over end for 2 hours at room temperature. The beads were then poured back into the sintered glass funnel, the coupling buffer was removed and the volume and optical density of the buffer was determined. The product of the $O.D_{(280nm)}$ and the volume of the coupling buffer was equal to the weight of BSA bound to the Sepharose. The coupling efficiency was then determined using the following equation:

$$\text{coupling efficiency} = \frac{\text{mg BSA bound}}{\text{mg BSA unbound}} \times 100$$

The coupling efficiency was usually 80-90%.

The remaining active groups on the gel were then blocked by incubation end over end with 100ml 1M ethanolamine for 2 hours at room temperature. Non-covalently bound proteins were removed by 3 cycles of low pH followed by high pH washes using 0.1M acetate buffer pH4.0 containing 0.5M NaCl and coupling buffer pH8.0. The beads were finally washed

with phosphate buffered saline and stored in PBS + 0.01% NaN_3 at 4°C until required.

II.3 Purification of IgG from mouse ascites fluid by FPLC.

1ml ascites fluid was diluted with 9ml 50mM Tris pH8.0. An 8ml mono Q anion exchange column was used on a Pharmacia FPLC system and was equilibrated with 50mM Tris pH8.0. The sample was applied to the column and eluted in a linear gradient increasing from 0 to 400mM NaCl over 120ml and remaining proteins were then eluted using 2M NaCl. The column flow rate was 5ml per minute and 6ml fractions were collected. Each fraction was assayed for IgG by immunoblotting (see section IX.7).

II.4 Reverse phase high pressure liquid chromatography

Analysis of samples by HPLC was carried out using a Waters HPLC system incorporating a WISP 710B automatic injection system all under the control of a Waters system controller model 720. Reverse phase chromatography was carried out on a uBondapak C18 analytical cartridge column (8mm x 10cm) fitted inside a Waters Z module (radial compression separation system). Peptides were purified using a uBondapak C18 preparative column (19mm x30cm).

All buffers used were dissolved in highly purified deionised water and both aqueous and organic buffers were filtered and degassed prior to use. The eluant from the column was monitored continuously by a Waters UV monitor model 441 and ^3H was measured continuously using a Ramona Raytest flow through scintillation counter. Data from the

UV monitor and from the flow through scintillation counter were collected by an IBM PC running under the 'Ramona Radio-Chromatography System' programme version 10.3 (Raytest Instruments, Sheffield, UK).

Samples were loaded into the Wisp automatic injection system and the run time, equilibration period and injection volume were programmed into this machine. The first sample was injected onto the column, sample injection and gradient formation being controlled by the system controller. The column was equilibrated automatically with starting buffer before subsequent injections. The data was analysed automatically by the computer after each sample was run and the results were either stored or printed out directly.

SECTION III EXTRACTION AND ASSAY OF RIBONUCLEOTIDE REDUCTASE

III.1 Partial purification of ribonucleotide reductase from HSV-1-infected cells.

Ribonucleotide reductase induced by HSV-1 was partially purified from infected cells by the method described by Dutia (1983). Roller bottles were infected with 10 p.f.u virus and extracts were made 15 hours post infection at 31°C. Dutia (1983). Infected cells were harvested into the medium by shaking with sterile glass beads. The cells were pelleted by centrifugation at 2000 r.p.m for 10 minutes, washed twice with PBS and frozen in 50mM HEPES pH7.6, 2mM DTT at -70°C. The cells were thawed and disrupted by sonication with a soniprobe and centrifuged at 15,000 r.p.m for 30 minutes on a Sorvall SS34 rotor. Nucleic acids were precipitated from the extract by adding a 5% solution of streptomycin sulphate

to the supernatant (0.3ml/ml) dropwise while stirring in an ice bath. Stirring was continued at 4°C for 20 minutes and then the extract was centrifuged at 7500 r.p.m for 15 minutes to pellet the nucleic acids. Viral ribonucleotide reductase was precipitated with 50% ammonium sulphate (either solid or a saturated solution) which was slowly added to the extract and the resulting mixture stirred on ice for 30 minutes. The precipitate was collected by centrifugation at 7500 r.p.m for 15 minutes and resuspended in 0.5ml 50mM HEPES pH7.6, 2mM DTT. The extract was desalted on a G75 column pre-equilibrated with 50mM HEPES pH7.6, 2mM DTT and stored in 100ul aliquots at -70°C.

III.2 Partial purification of ribonucleotide reductase from EHV-1-infected cells

The same procedure was followed as above except that the cells were sonicated without prior freezing and thawing as the EHV-1 induced ribonucleotide reductase rapidly loses activity with repeated freeze/thaw cycles.

III.3 Assay for HSV-1- and EHV-1- induced ribonucleotide reductase.

Ribonucleotide reductase was assayed by the method of Darling et al. (1987). The assay involves determining the amount of cytidine diphosphate (CDP) converted into deoxycytidine diphosphate (dCDP). The assay mixture contained, in addition to enzyme, in a final reaction mixture of 90ul, 200mM HEPES pH8.0; 10mM DTT; 100uM CDP; 1uCi ^{3}H CDP (specific activity 37.7mCi/mmol). To assay for EHV-1 induced reductase, the concentration of 'cold'

substrate was reduced to 10uM to increase the sensitivity of the assay. The reaction was incubated at 37°C for 30 minutes and then stopped by heating at 100°C for 2 minutes. After cooling, nucleotides were converted to their corresponding nucleosides by the addition of 2mg of Crotalux adamentus venom (10ul of a 200mgml^{-1} solution) and $MgCl_2$ to a final concentration of 15mM followed by incubation at 37°C for 2 hours. To stop this reaction and to precipitate proteins, samples were heated at 100°C for 2 minutes and the precipitate was removed by centrifugation at 2000 r.p.m for 5 minutes. The supernatants were removed and analysed by HPLC. The host enzyme requires Mg^{2+} and ATP for activity and therefore under the conditions of this assay is inactive.

III.4 Separation of dephosphorylated [^{3}H]CDP and [^{3}H]dCDP by HPLC.

The separation procedure was carried out as described by Darling et al. (1987). A uBondapak C18 column was equilibrated with 0.1M KH_2PO_4 pH5.4 (buffer A) and elution was accomplished in a linear gradient of methanol:water (80:20 buffer B). After an initial 3 minute elution in buffer A, flowing at 3ml min^{-1}, a linear gradient increasing from 0 to 55% buffer B between 3 and 8 minutes was applied. The column was then re-equilibrated for the next run by linearly reducing the concentration of buffer B to 0% between 8 and 11 minutes, followed by an 8 minute wash in buffer A. Under these conditions cytidine eluted as a broad peak at 4 minutes 32 seconds whereas deoxycytidine eluted as a sharp peak at 6 minutes 25 seconds. Radioactivity was detected by flow through scintillation counting as described using 3 parts scintillation fluid (Monofluor, National Diagnostics) to 1 part eluate.

SECTION IV PREPARATION OF CARRIER FREE [^{127}I]YAGAVVNDL

IV.1 Iodination of YAGAVVNDL with non-radioactive iodine.

The oligopeptide was iodinated as described by Hunter and Greenwood (1962). 2.5mg of YAGAVVNDL were dissolved in 500ul H_2O and 100ul NaI and 100ul chloramine T were added. A series of iodination reactions were carried out in which the concentrations of sodium iodide and chloramine T were varied to determine conditions under which the nonapeptide was best iodinated. Optimum iodination of the nonapeptide was achieved using a 1mg/ml solution of sodium iodide (dissolved in water) and a 2.5mg/ml solution of chloramine T (dissolved in 1M potassium phosphate buffer pH7.5). After 20 seconds at room temperature, the reaction was stopped by adding 100ul sodium metabisulphite (1mg/ml in 1M potassium phosphate buffer). Ten iodination reactions were carried out following this procedure.

IV.2 Separation of the iodinated and non-iodinated nonapeptide by HPLC.

When these experiments were initiated no preparative column was available, therefore nine consecutive 750ul injections were applied to a uBondapak C18 column (8mm x 10cm). The column was equilibrated with 95% buffer A (0.1% TFA in H_2O) and 5% buffer B (0.1% TFA in acetonitrile). After initial elution with 95% buffer A and 5% buffer B, a linear gradient increasing to 95% buffer B between 0 and 10 minutes was applied. The column was then re-equilibrated for the next run by linearly reducing the concentration of buffer B to 5% between 15 and 20 minutes. The column was

then washed for 20 minutes with 95% buffer A, 5% buffer B. Under these conditions, the nonapeptide was eluted at 8 minutes 10 seconds, the iodinated peptide eluted at 8 minutes 44 seconds and chloramine T eluted at 9 minutes 8 seconds.

0.1ml fractions were collected automatically every 0.1 minutes between 8 and 10 minutes and 25ul of each fraction was then assayed by HPLC using the same column and run parameters for the presence of the peak at 8 minutes 44 seconds. Fractions containing this material were pooled, the peptide lyophilised and an aliquot was sent for analysis by fast atom bombardment mass spectroscopy (M-Scan). Other peptides were purified by this same method but conditions vary from one experiment to another as will be discussed in the results section.

SECTION V PURIFICATION OF TRITIATED YAGAVVNDL

V.1 Reduction of [^{127}I]YAGAVVNDL with tritium gas.

4mg [^{127}I]YAGAVVNDL were sent to be tritium-labelled by reduction with tritium gas (^{3}H labelling service, Amersham Int. PLC). 80mCi of ^{3}H labelled nonapeptide was received in a 24ml volume. 2ul of this solution was mixed with 198ul of a solution of non-iodinated YAGAVVNDL (1mg/ml) as a marker and 5ul of this mixture was analysed by HPLC. The eluant was monitored at 214nm by the UV monitor and tritium was measured using the flow through scintillation counter. Two peaks of radioactivity were present, one of which eluted from the column at the same time as the YAGAVVNDL marker (8 minutes 4 seconds) and the second peak was eluted at 8 minutes 25 seconds.

V.2 Purification of [^{3}H]-labelled YAGAVVNDL by HPLC.

2ml [^{3}H]-labelled material was injected onto a uBondapak C18 column (8mm x 10cm) and the peptide was eluted in acetonitrile plus 0.1% TFA as described in section IV.2. 0.1ml fractions were collected automatically every 6 seconds between 8 and 10 minutes. The eluant was not monitored by the flow through scintillation counter as the amount of tritium present was too high. After the first run, 10ul of each fraction was counted in 3ml Ecoscint to ensure that both peaks of radioactivity were being collected. The remainder of the tritium-labelled nonapeptide was collected in the same way.

When all the runs were complete, 10ul of each fraction was mixed with 40ul of a 1mg/ml solution of YAGAVVNDL. 25ul of each fraction was then analysed by reverse phase HPLC and fractions containing a peak of tritium at 8 minutes 4 seconds were pooled and acetonitrile was removed by rotary evaporation. The solution was then divided into 100ul aliquots in 1.5ml 'V' vials and each aliquot was frozen in liquid nitrogen and lyophilised. The tritiated peptide was stored either as dry powder or resuspended in 250ul of 2% or 50% ethanol at -70^{o}C. The yield of [^{3}H]-YAGAVVNDL was 20mCi with a specific activity of 10Ci/mmol.

SECTION VI PRODUCTION OF OLIGOPEPTIDE-INDUCED ANTISERA

VI.1 Iodination of synthetic oligopeptides using ^{125}INa.

In order that the amount of oligopeptide coupled to carrier proteins could be determined, the peptides were first labelled via the NH_2 tyrosine residue with trace

amounts of ^{125}I. Iodinations were carried out as described by Hunter and Greenwood (1962) by Mrs M. Murphy. 10mg of oligopeptide were resuspended in 100ul 1M potassium phosphate buffer pH7.5 and 100uCi ^{125}I and 50ul chloramine T (0.5mg/ml) were added. After 20 minutes at room temperature, 50ul sodium metabisulphite (1mg/ml) was added followed 20 seconds later by 1ml PBS. To separate free ^{125}INa from ^{125}I covalently linked to peptide, the sample was applied to a Sephadex G10 column which had been equilibrated with 0.16M borate pH9.0, 0.13M NaCl. 0.5ml fractions were collected and assayed for radioactivity in a gamma counter. Iodinated peptide eluted in the void volume while free ^{125}INa was retarded on the column.

VI.2 Attachment of oligopeptides to BSA.

Peptides were coupled to bovine serum albumin (BSA) either with bis-diazobenzidine for tyrosine to tyrosine coupling or with gluteraldehyde for amino to amino coupling.

VI.2(a) Attachment of peptide to BSA with bis-diazobenzidine.

The preparation of bis-diazobenzidine (BDB) was carried out by Dr H. S. Marsden by the method described by Likhite and Seka (1967). 230mg benzidine hydrochloride were dissolved in 45ml 0.2M HCl. 5ml 0.5M sodium nitrite was added and the solution was slowly stirred at 4°C for 1 hour. The BDB was stored in 2ml aliquots at -70°C.

Peptides were coupled to BSA with BDB by the method described by Bassiri et al. (1979). 25mg BSA were dissolved in 5ml 0.16M borate, 0.14M NaCl pH9.0 at 0°C and the peptide

was added at 30 fold molar excess. 2ml BDB was added to the BSA/peptide solution dropwise while stirring in an ice bath; the pH was adjusted to 9.0 by the addition of 0.5M NaOH and stirring was continued at 4°C for 2 hours. Free oligopeptide was separated from oligopeptide linked to BSA by fractionation on a Sephadex G25 column. The column was pre-equilibrated in PBS + 0.1% BSA and the sample was eluted in PBS. 0.5ml fractions were collected and assayed for radioactivity in a gamma counter. Oligopeptide linked to BSA eluted in the void volume and fractions containing this material were pooled, dialysed extensively against PBS to remove residual BDB and stored at -70°C. The extent of linkage was determined by use of the equation:

$$\% \text{ linked} = \frac{\text{c.p.m in pooled fraction}}{\text{total c.p.m loaded on column}} \times 100$$

For most peptides this was between 25 and 30%.

VI.2(b) **Attachment of peptide to BSA with gluteraldehyde.**

Peptides were coupled to BSA with gluteraldehyde by the method described by Kagan and Glick (1979). Peptide was added at a 30 fold molar excess to 25mg BSA dissolved in 0.4M sodium phosphate buffer pH7.5 to give a final volume of 2ml. Gluteraldehyde (1ml, 20mM) was added dropwise with stirring and the reaction was allowed to continue for 30 minutes at room temperature. 1M glycine (1/10 volume) was added to block unreacted gluteraldehyde and the solution was stirred for a further 30 minutes at room temperature. The solution was dialysed extensively against PBS to remove

residual gluteraldehyde and stored at -70°C. As the peptides coupled by this method did not contain an amino terminal tyrosine residue to facilitate iodination, the extent of linkage of these peptides to BSA was not determined.

VI.3 Immunization of rabbits with synthetic oligopeptides.

New Zealand white female rabbits 6 to 10 months old were used. Synthetic oligopeptides linked to BSA were emulsified with an equal volume of Freunds complete adjuvant for the first injection and for subsequent injections, the conjugate was emulsified with an equal volume of Freunds incomplete adjuvant. Each rabbit received approximately 300ug oligopeptide in a 2ml volume for each subcutaneous immunization.

Rabbits were initially bled to provide pre-immune serum and one week later (week 1) were given their first injection. They were subsequently boosted after one week (week 2) and then 4 weeks later (week 5). Six weeks after the initial injection 20ml of blood from the ear was obtained and the serum was tested for the presence of specific antibodies against the oligopeptide. The rabbits received their final injection in week 8 and were bled 2 weeks later (week 10) by heart puncture.

VI.4 Preparation of serum.

To clot the blood, it was incubated at 37°C for 30 minutes and then at 4°C for up to 2 hours until it was processed. The blood was centrifuged at 2000 r.p.m for 10 minutes to pellet the red blood cells. The supernatant was decanted and centrifuged again at 2000 r.p.m for 10 minutes to separate any red blood cells remaining in the serum.

Antibodies against BSA were removed by absorption of the serum on a column of CNBr-activated Sepharose linked to BSA (section II.2). The beads, giving a bed volume of approximately 100 to 150ml, were poured into a 50mm x 330cm K50 column (Pharmacia) and washed slowly with PBS from a peristaltic pump for several hours thus allowing the column to pack slowly overnight. A small 2ml bed volume pre-column was set up as a sieve so that large particles would be retarded before entering the K50 column. This pre-column was periodically renewed. Serum was loaded onto the column and incubated on the column for at least 1 hour at room temperature. Coloured fractions (previously shown to be antibody containing, M.Murphy personal communication) were collected, pooled and stored in 1ml or 5ml aliquots at 20°C.

SECTION VII IMMUNOPRECIPITATIONS.

VII.1 Preparation of [^{35}S]methionine-labelled cell extracts.

Infected cell polypeptides were labelled with [^{35}S]methionine by the method described by Marsden et al. (1976). Cell monolayers were infected with virus at a multiplicity of 20 p.f.u per cell and incubated at 37°C for 1 hour. The cells were then washed with Eagle's medium containing one fifth the normal concentration of methionine plus 2% calf serum (Emet/5 C2). The cells were incubated with 100uCi [^{35}S]methionine per ml in Emet/5 C2 at 31°C for 15 hours.

The infected cells were harvested in detergent lysis buffer (Zweig et al. 1980). The infected monolayers were washed twice with cold PBS and incubated with 1ml detergent

lysis buffer for 1 hour at 4°C. Cells were then disrupted by sonication in a bath sonicator and centrifuged for 5 minutes on a bench centrifuge. The supernatant was removed and stored at -70°C.

VII.2 Immunoprecipitations.

Immunoprecipitations were carried out as described by Showalter et al. (1981). 50ul oligopeptide-induced antiserum was mixed with 30ul [^{35}S]methionine-labelled infected cell extract, which usually contained approximately 10^6 cpm in 1.5ml 'V' vials and incubated on ice for 3 hours. 50ul Protein-A-Sepharose (diluted 1:1 in detergent lysis buffer) was added to each vial and the reaction continued for 1 hour at 4°C. 600ul lithium chloride wash buffer was added to each vial and the samples were centrifuged for 20 seconds on a bench centrifuge. The Protein-A-Sepharose pellet was then washed 3 times in lithium chloride buffer and eluted in 50ul buffer comprising 2% SDS, 20% glycerol, 5% mercaptoethanol, 0.125M Tris-HCl pH6.8, bromophenol blue. The samples were heated at 100°C for 5 minutes then centrifuged as above and the supernatant was applied to a gel.

SECTION VIII GEL ELECTROPHORESIS.

VIII.1 SDS polyacrylamide gel electrophoresis

Gels were cast vertically between two glass plates separated by 1.5mm thick perspex spacers and sealed with teflon tape. Gradient gels containing a 5-12.5% linear gradient of acrylamide crosslinked with 1 part in 20 (w/w)

N,N' methylene bis-acrylamidein 1.5M Tris pH8.9, 0.4% SDS were polymerised with 0.006% ammonium persulphate and 0.004% TEMED. The gels were overlaid with butan-2-ol to ensure a smooth surface on polymerisation. Following polymerisation, a stacking gel comprising 5% acrylamide in 0.49M Tris-HCL pH6.7, 0.4% SDS was added into which was placed a perspex comb around which the stacking gel was allowed to polymerise using 0.006% ammonium persulphate and 0.004% TEMED to form wells. Samples were heated at 100°C in buffer containing 50mM Tris-HCl pH6.7, 2% SDS, 700mM 2-mercaptoethanol, 10% glycerol and sufficient bromophenol blue to allow vizualisation of the dye front. Electrophoresis was performed in 52mM Tris, 53mM glycine, 0.1% SDS at 12mA per gel for approximately 18 hours at room temperature.

VIII.2 Preparation of SDS-PAGE gels for autoradiography

The gels were fixed for 1 hour in methanol:acetic acid:water (50:7:50) and then washed several times in 5% methanol, 7% acetic acid. The fixed gels were then washed in 3 volumes of En^{3}hance for 1 hour with gentle agitation and washed with 10 volumes of H_2O which was changed twice over 30 minutes. Gels were dried under vacuum onto Whatman grade 182 filter paper and exposed to Kodak X-OMAT XS-1 film at -70°C.

SECTION IX IMMUNOBLOTTING

IX.1 Preparation of [^{35}S]methionine-labelled cell extracts.

IX.1(a) From a 90mm petri dish

Cell monolayers were either mock-infected or infected with 10 p.f.u virus and labelled overnight at 31^{o}C with [^{35}S]methionine as described in section VII.1. After 15 hours, the radioactive medium was removed and the cell monolayer was washed 3 times with PBS. The cells were harvested into 0.5ml denaturing buffer (diluted 1:3 with H_2O) by heating the plate at 80^{o}C for 10 minutes. The contents of the plate were then transferred to a black cap vial which was heated at 100^{o}C for 10 minutes. Extracts were stored at -70^{o}C until required.

IX.I(b) From a roller bottle

Cell monolayers were either mock-infected or infected with 10 p.f.u virus and labelled overnight at 31^{o}C with [^{35}S]methionine. After 15 hours, the radioactive medium was removed and the cell monolayer was washed 3 times with PBS. The cells were harvested into 25ml denaturing buffer (diluted 1:3 in H_2O) by rotating the roller bottle in boiling water for 10 minutes. The contents were then poured into a 5ml conical centrifuge tube and heated at 100^{o}C for 10 minutes. The solution was aliquoted into 1ml amounts and stored at -70^{o}C.

Cell extracts were loaded onto SDS polyacrylamide gels at approximately 10^6 cell equivalents per cm of gel and separated by electrophoresis as described.

IX.2 Transfer of proteins to nitrocellulose.

The method described by Towbin et al. (1979) was employed with several modifications. Dry nitrocellulose was cut into 15cm x 0.6cm strips, each of which was labelled in ink at the top and bottom with a number. The strips were soaked in transfer buffer (25mM Tris-HCl pH8.3, 192mM glycine,20% (v/v) methanol) for 10 minutes before blotting. This helps to prevent air bubbles and take off excess ink. An opened gel holder was placed in a shallow basin containing transfer buffer with the cathode side flat on the bottom of the basin. A fibre pad was placed on the cathode panel and a piece of Whatman no. 182 filter paper saturated in transfer buffer was placed on top of the pad. The gel, which had also been pre-equilibrated in transfer buffer was then placed on top of the filter paper and the nitrocellulose strips were laid face down on top of the gel. The sandwich was completed by placing another sheet of filter paper and a fibre pad on top of the nitrocellulose strips. The gel holder was closed and placed in a Biorad trans blot tank which was filled with transfer buffer. Proteins were transferred by electrophoresis at 250 mA for 3 hours.

IX.3 Blocking of free protein binding sites on the nitrocellulose membrane.

Nitrocellulose strips were transferred from the gel into plastic dishes containing blocking buffer (TBS + 3% gelatin) which had been preheated to 37^{o}C. The strips were incubated in blocking buffer for 1 hour at 37^{o}C with a change of buffer after 30 minutes. The strips were then washed twice

in TTBS (TBS + 0.05% Tween 20) for 5 minutes at room temperature.

IX.4 Incubation of nitrocellulose strips with antisera.

Antisera were diluted to the appropriate concentration with antibody buffer (TTBS + 1% gelatin). The nitrocellulose strips were placed protein side up in wells (5mm deep) cut in perspex blocks and each strip was incubated with 3ml of antiserum for 2 hours at room temperature. Excess antiserum was removed by washing the strips in TTBS.

IX.5 Incubation of the nitrocellulose strips with Protein-A-peroxidase.

Each nitrocellulose strip was incubated with 3ml Protein-A conjugated with horseradish peroxidase (1ug/ml TTBS + 1% gelatin) for 1 hour at room temperature in wells (5mm deep) cut in perspex blocks. The strips were washed twice in TTBS to remove excess Protein-A-peroxidase and then washed several times in TBS to remove excess Tween 20 before colour development.

IX.6 Staining of proteins with horseradish peroxidase colour developer.

Nitrocellulose strips were immersed in a 0.015% H_2O_2 development solution. Bands representing bound antiserum became apparent within approximately 30 minutes and the colour development reaction was stopped by rinsing the strips in deionised water. The strips were allowed to dry and were then taped to a piece of card. The card was marked

at each corner with spots of radioactive ink. The strips were exposed to Kodak X-OMAT XS1 film at -70°C to obtain a [^{35}S]methionine-labelled cell polypeptide profile. By alignment of the radioactive spots on the autoradiograph with the ink spots on the card, proteins stained with horseradish peroxidase could be identified from the labelled polypeptide profile.

IX.7 Immunoassay for IgG.

IgG was purified from ascites fluid as described in section II.3. 3ul of each fraction was spotted onto a sheet of nitrocellulose and allowed to dry. Free protein binding sites on the nitrocellulose membrane were blocked by incubating the nitrocellulose with 3% BSA in PBS at room temperature for 1 hour. Excess BSA was removed by washing twice with PBS + 0.05% Tween 20. The nitrocellulose was then incubated with anti-mouse antibody conjugated to horseradish peroxidase (Biorad Laboratories) which was diluted 1 part in 500 with a solution of 1% BSA in PBS. After 1 hour at room temperature, excess antibody was removed by washing twice with PBS. Fractions containing mouse antibody bound to IgG were identified by staining with horseradish peroxidase colour developer as described in section IX.6. Fractions containing IgG were pooled and concentrated to a minimum volume using an Amicon ultrafiltration cell fitted with a YM-5 membrane (M_r 5000 cut off) under nitrogen.

RESULTS

SECTION I PREPARATION OF THE TRITIATED NONAPEPTIDE YAGAVVNDL.

I.1 Preparation of iodo-YAGAVVNDL

To facilitate labelling of the tyrosine residue of the nonapeptide by reduction with tritium gas, it was first necessary to prepare iodo-tyrosyl labelled YAGAVVNDL.

I.1(a) Reverse phase chromatography of the components of the iodination reaction.

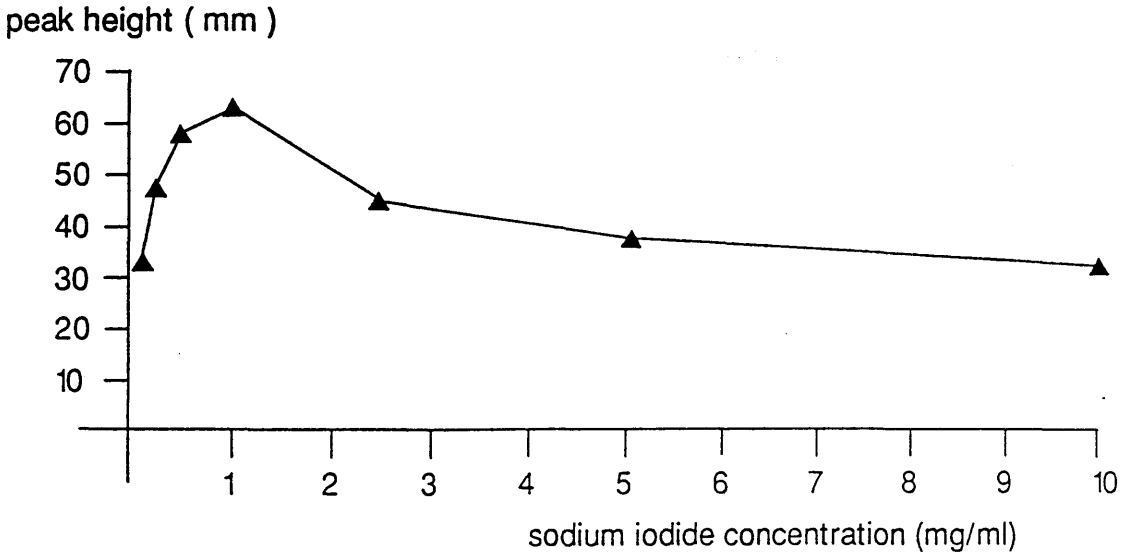
The nonapeptide YAGAVVNDL was iodinated with non-radioactive iodine by the method described in Methods section IV.1 and iodinated peptide was separated from the non-iodinated peptide by high pressure liquid chromatography under the conditions described in Methods section IV.2. Initially, solutions of nonapeptide, sodium iodide, chloramine T and sodium metabisulphite were each analysed by reverse phase HPLC to determine their retention times. Sodium iodide and sodium metabisulphite did not absorb UV at 214nm. The nonapeptide was eluted from the column as a single peak at 8 minutes 15 seconds and chloramine T eluted as a single peak at 9 minutes 10 seconds though these times varied slightly with each column and with amount loaded onto the column.

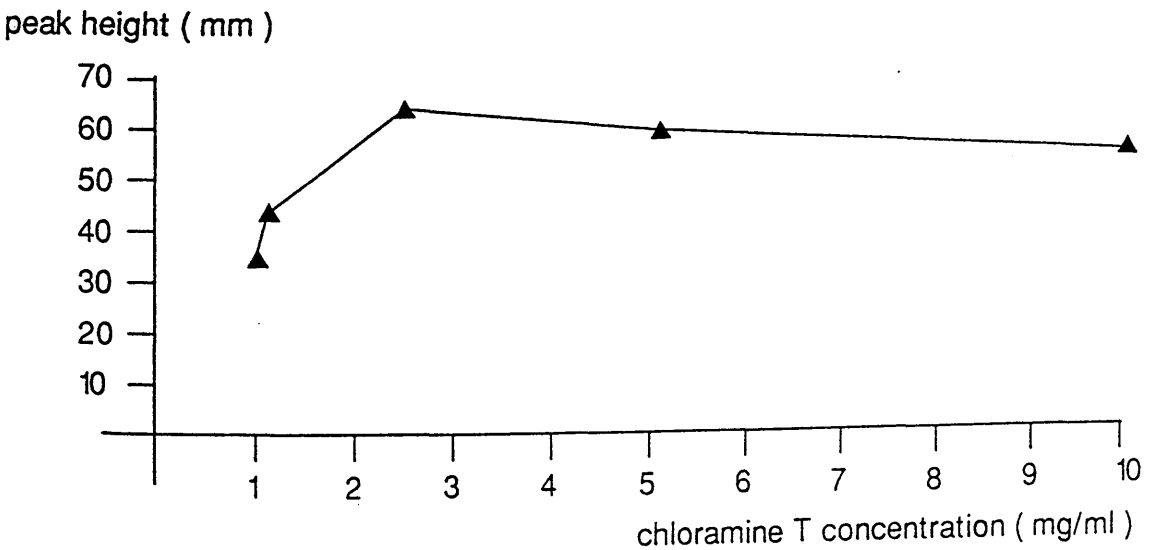
I.1(b) Optimisation of iodination conditions.

Iodination of the nonapeptide was at first carried out using a 200 molar excess of sodium iodide (815mg/ml w/v).

Figure 6. Optimisation of iodination conditions

The iodination reactions were carried out as described in the text at varying concentrations of sodium iodide (Figure 6a) or chloramine T (Figure 6b) and iodinated peptide was separated from non-iodinated peptide by reverse phase HPLC. The amount of peptide iodinated was estimated by measuring the height (mm) of the UV absorbing peak which was eluted from the column at 8 minutes 44 seconds.


peak height (mm)
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
sodium iodide concentration (mg/ml)


peak height (mm)
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
chloramine T concentration (mg/ml)

However, iodinated nonapeptide was never detected when such a high concentration of sodium iodide was present in the reaction mixture. Figure 6a shows that the best iodination was achieved when a 1mg/ml (w/v) solution of sodium iodide was used. The iodinated nonapeptide was eluted from the column at 8 minutes 44 seconds and the height (mm) of this UV absorbing peak was taken as a measure of the amount of peptide that had been iodinated. The iodination reactions just described were carried out using a 0.5mg/ml solution of chloramine T. Figure 6b shows the result of varying the concentration of chloramine T while maintaining the concentration of sodium iodide at 1mg/ml. The best iodination was obtained when chloramine T was used at 2.5mg/ml (w/v). Subsequent iodinations were therefore carried out by adding 100ul chloramine T (2.5mg/ml) and 100ul sodium iodide (1mg/ml) to 500ul nonapeptide (5mg/ml). The reaction was stopped by adding 100ul sodium metabisulphite (1mg/ml).

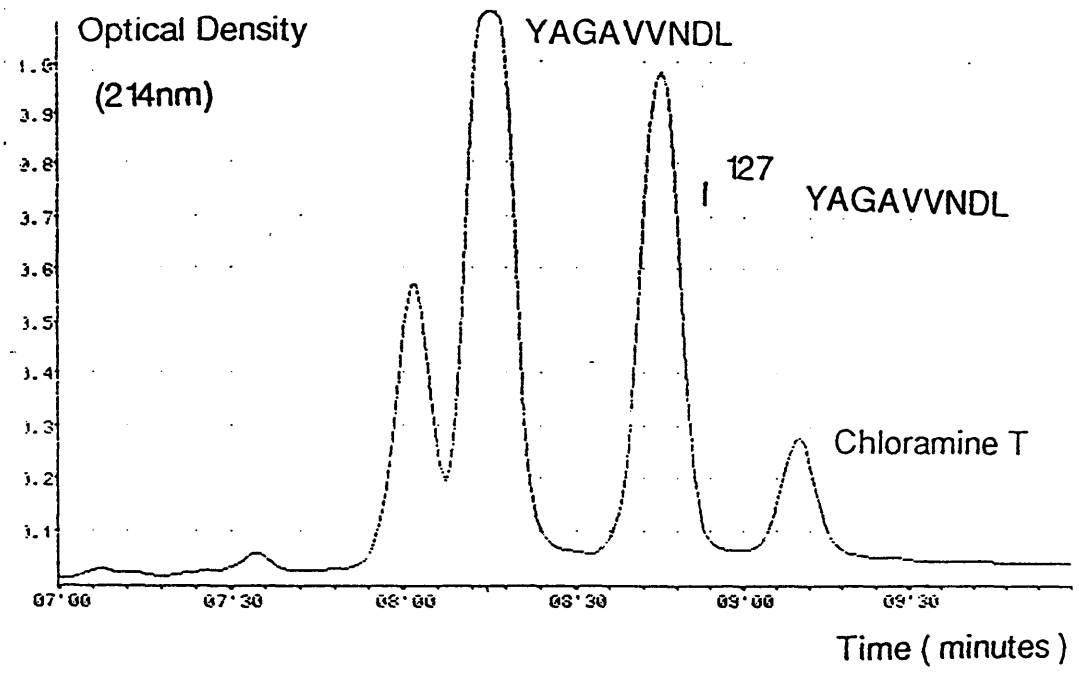
I.1(c) Separation of iodinated peptide, non-iodinated peptide and chloramine T by HPLC.

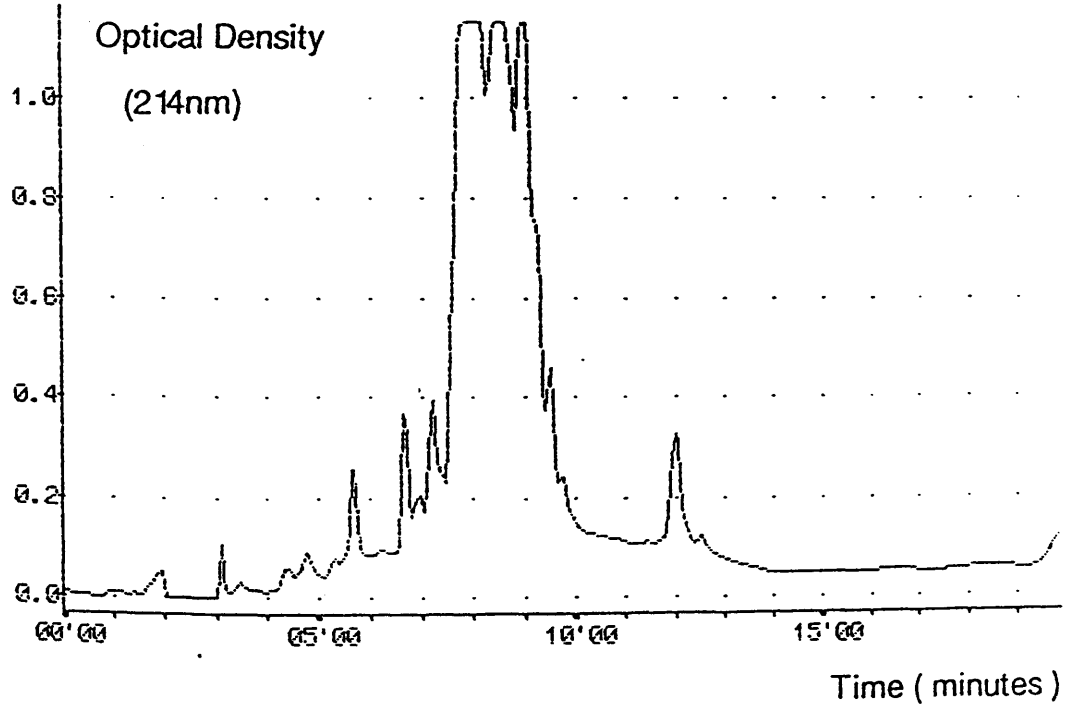
The separation of iodinated peptide from non-iodinated peptide and chloramine T achieved by reverse phase HPLC (Methods section IV.2) when 25ul of the iodination reaction mixture was applied to the column is shown in Figure 7a. The species which was eluted from the column at 8.00 minutes was not identified but was presumed to be the reduced form of chloramine T since chloramine T acts as an oxidising agent in the iodination reaction and the species was never present under conditions were the peptide was not iodinated.

Figure 7. Separation of iodinated peptide, non-iodinated peptide and chloramine T by reverse phase high performance liquid chromatography.

(a) Separation of 25ul of the iodination reaction mixture on a uBondapak C18 analytical column in a linear gradient increasing from 5% to 95% acetonitrile + 0.1% TFA between 0 and 20 minutes. The eluant was monitored by a UV monitor at 214nm. The time scale is from 7 minutes to 10 minutes.

(b) Separation of 750ul of the iodination reaction mixture as described above. The time scale is from 0 minutes to 20 minutes.


Optical Density
(214nm)
YAGAVVNDL
127
I
YAGAVVNDL
Chloramine T
07'00
07'30
08'00
08'30
09'00
09'30
Time (minutes)


Optical Density
(214nm)
1.0
0.8
0.6
0.4
0.2
0.0
00'00
05'00
10'00
15'00
Time (minutes)

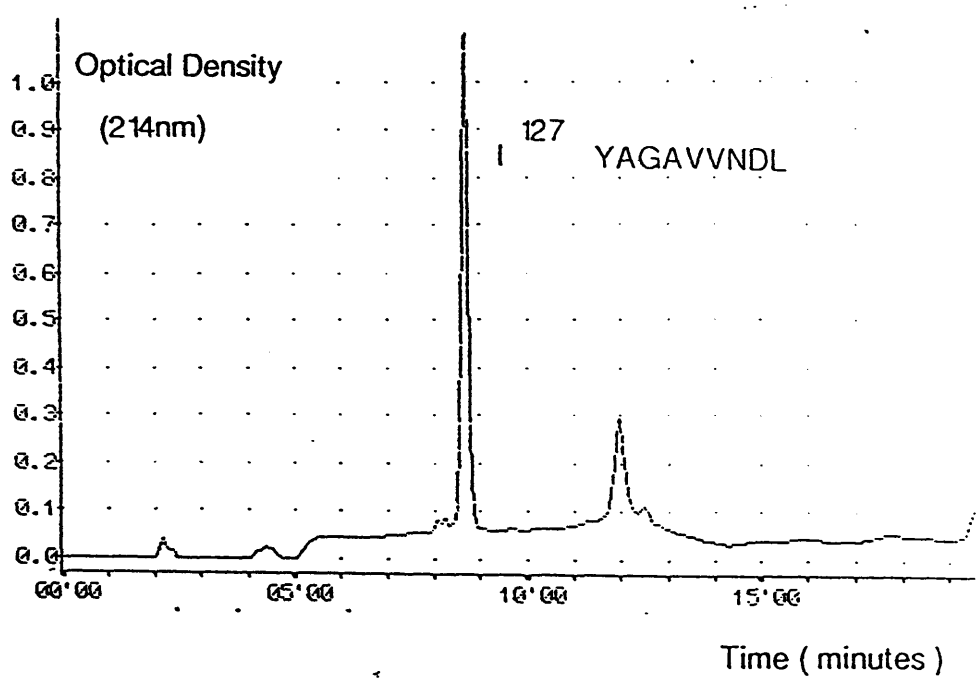


Figure 8. Purification of the iodinated YAGAVVNDL.

The figure shows the elution profile of 25ul of a fraction containing the iodinated peptide in the same gradient as described for Figure 7. The iodinated peptide eluted from the column as a sharp peak at 8 minutes 44 seconds.

To label the iodinated nonapeptide with with tritium, the iodinated peptide was first purified. This was achieved by injecting 10 x 750ul volumes of reaction mixture consecutively onto an 8mm x 10cm uBondapak C18 column and eluting the peptide under the same conditions as previously described. Figure 7b shows the separation achieved when 750ul of the reaction mixture was applied to the column. The iodinated peptide, non-iodinated peptide and chloramine T were eluted as 3 broad peaks with retention times 8 minutes 10 seconds, 8 minutes 44 seconds and 9 minutes 8 seconds respectively. Resolution between the species eluting at 8.00 minutes and unlabelled YAGAVVNDL was lost.

I.1(d) **Purification of iodinated peptide.**

0.1ml fractions were collected automatically every 6 seconds between 8 minutes and 10 minutes during each run. 25ul of each fraction was then analysed by HPLC for the presence of the peak which eluted from the column at 8 minutes 44 seconds. Figure 8 shows the elution profile of one fraction which contained the iodinated peptide. The peak which eluted at approximately 12 minutes was present in all the fractions and was a contaminant commonly present in the water used for the HPLC as it was also present when the HPLC grade water was applied to the column. Five fractions containing the iodinated peptide were pooled, resulting in a total volume of 5ml, which was evaporated to remove acetonitrile, frozen in liquid nitrogen and lyophilised overnight. 4.7mg of iodinated nonapeptide was thus obtained which appeared as a fluffy white powder.

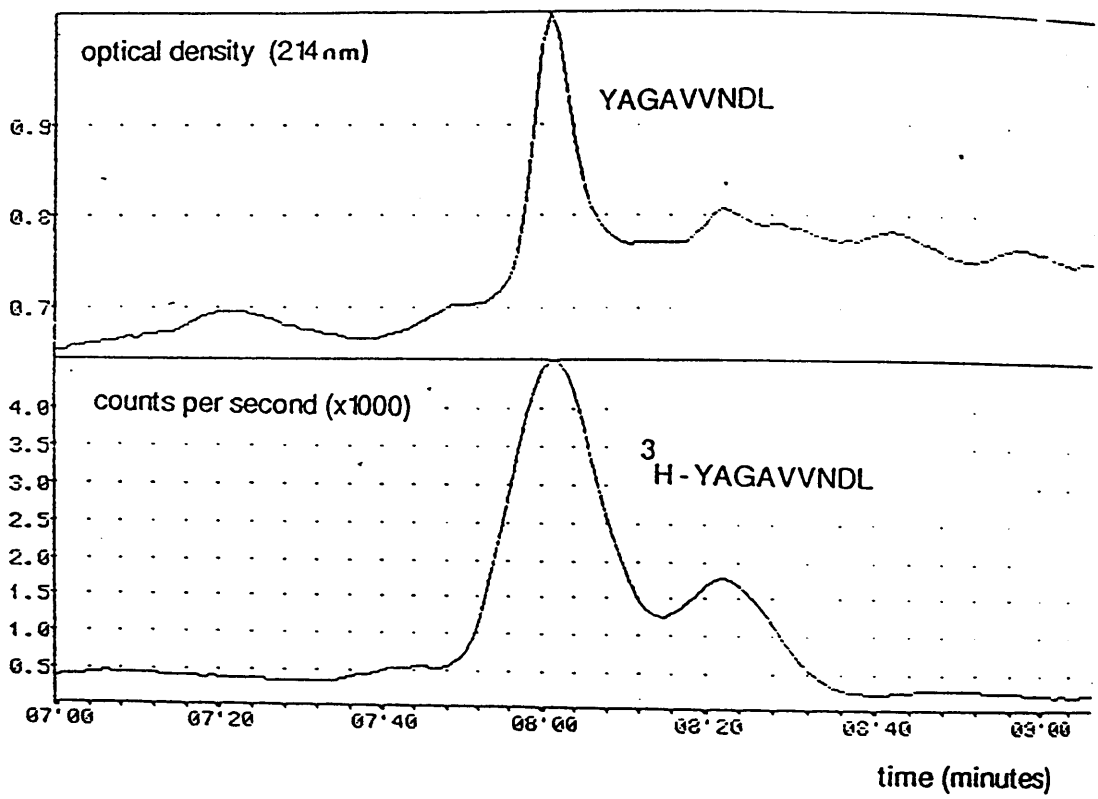


Figure 9. Reverse phase chromatography of tritium-labelled YAGAVVNDL.

2ul of a tritium containing solution (received from Amersham Int. PLC) was mixed with 198ul of a 1mg/ml (w/v) solution of YAGAVVNDL. 5ul of the solution was analysed by reverse phase HPLC on a uBondapak C18 analytical column in a linear gradient increasing from 5% to 95% acetonitrile + 0.1% TFA. The top trace shows UV absorbance at 214nm and the bottom trace shows the [^{3}H] trace as measured by the flow through scintillation counter.

I.1(e) Determination of the relative molecular mass of purified peptide.

The identity of the freeze-dried material was checked by determination of its molecular mass by fast atom bombardment mass spectroscopy analysis (M-Scan, Ascot,Berkshire,UK). Two species were identified, one of which produced a weak signal with M_r 919. This corresponds to non-iodinated YAGAVVNDL. The M_r of the major species was 1045 which is the expected molecular weight of YAGAVVNDL if a tyrosyl hydrogen is replaced by an iodine molecule. 4mg of freeze-dried material was therefore sent to the [^{3}H] Labelling Service (Amersham International PLC) to be tritiated by reduction with tritium gas.

I.2 Purification of tritium-labelled YAGAVVNDL

80mCi of tritium-labelled material in a 24ml volume was received from Amersham. 2ul of this solution was mixed with 198ul of a 1mg/ml (w/v) solution of YAGAVVNDL and 5ul of this mixture was analysed by HPLC. The eluant was monitored at 214nm as before and ^{3}H was monitored using a flow through scintillation counter. Figure 9 shows that two peaks of radioactivity were present, one of which was eluted from the column at the same time as the YAGAVVNDL marker as would be expected of [^{3}H]-YAGAVVNDL. The second peak was eluted slightly later at 8 minutes 25 seconds and probably corresponds to reduction of only one of two ^{127}I atoms on di-iodotyrosine to give [^{3}H, ^{127}I] YAGAVVNDL.

Tritium-labelled YAGAVVNDL was purified as described in Methods section V.2. Only 2ug of peptide was recovered

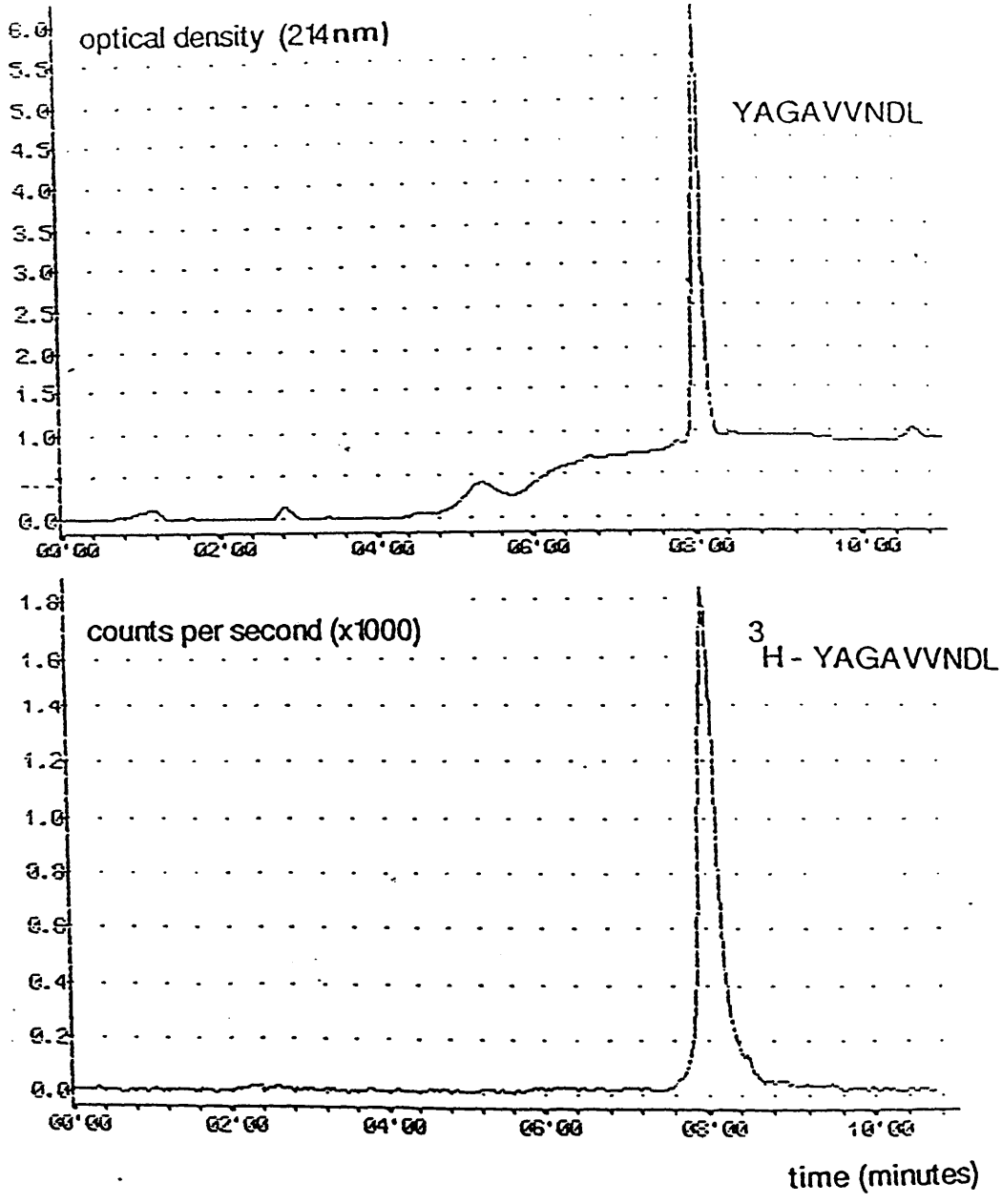


Figure 10. Purification of the [^{3}H]-labelled YAGAVVNDL by reverse phase HPLC.

Approximately 2uCi [^{3}H]-YAGAVVNDL was mixed with a 1mg/ml (w/v) solution of YAGAVVNDL. 5ul of the sample was analysed by HPLC/flow through scintillation counting.

which contained 18.4mCi of radioactivity. This was divided into 80 aliquots therefore each aliquot of peptide contains approximately 200uCi of radioactivty. An aliquot of [^{3}H]-YAGAVVNDL was resuspended in 100ul water and then diluted 1 part in 100 with a 1mg/ml (w/v) solution of YAGAVVNDL. Figure 10 shows that the tritium-labelled peptide was eluted from the column as a single peak at the same time as YAGAVVNDL. The [^{3}H] peptide was stored at -70^{o}C either as dry powder, in water + 2% ethanol or in water + 50% ethanol. The latter condition afforded the greatest stability yielding less than 5% radiochemical decomposition over one year.

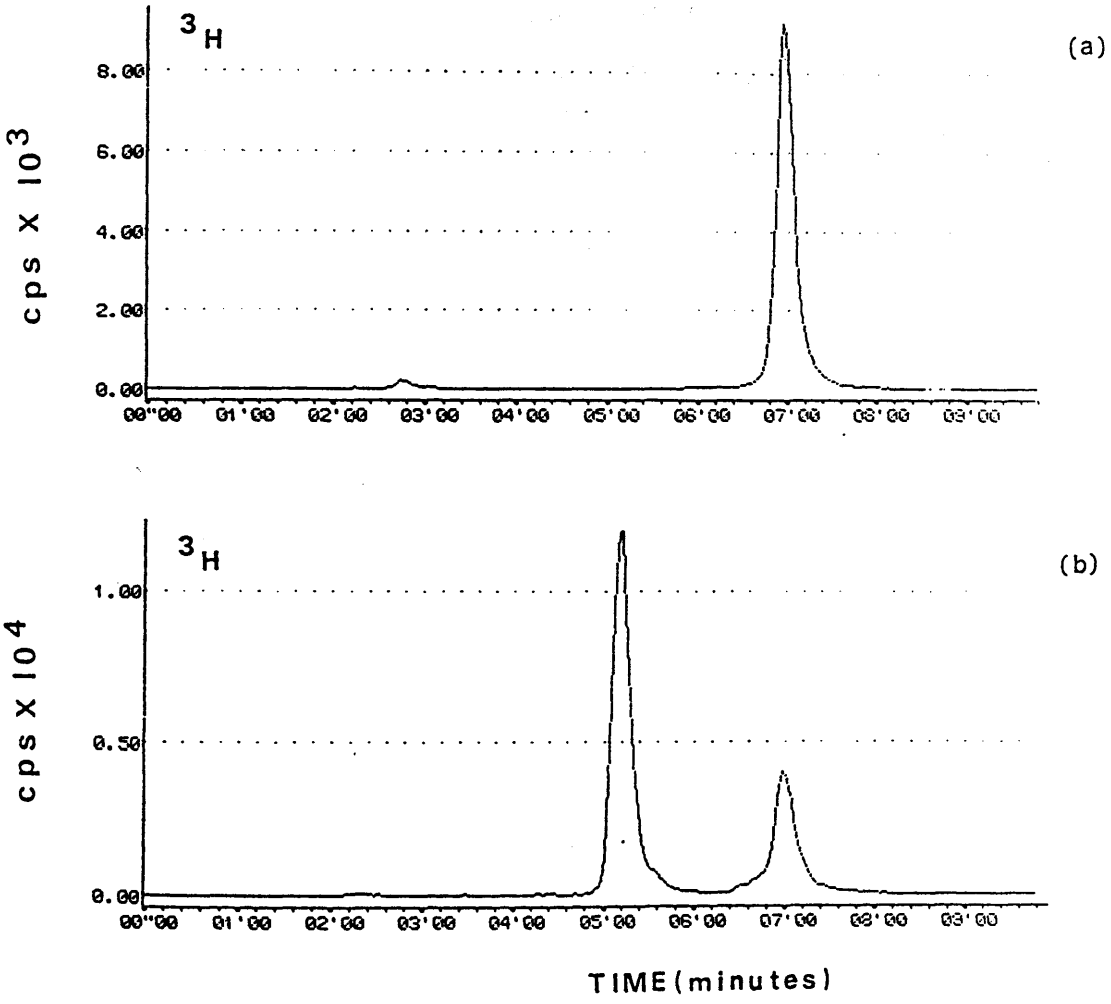
SECTION II STABILITY OF YAGAVVNDL IN HSV-1-INFECTED CELL EXTRACTS.

II.1 The effect of infected cell proteases on the stability of YAGAVVNDL.

An aliquot of [^{3}H]-YAGAVVNDL, which had been stored at -70^{o}C in 50% ethanol, was lyophilised and the peptide was resuspended in 100ul deionised water. 2ul of this solution (4uCi) was then mixed with either 98ul water or with 98ul HSV-1-infected cell extract which had been partially purified by ammonium sulphate precipitation (Methods section III.2). 25ul of each sample was applied to a uBondapak C18 column (8mm x 10cm) which had been equilibrated in 95% buffer A (0.1% TFA in H_2O) and 5% buffer B (0.1% TFA in acetonitrile). A linear gradient increasing to 95% buffer B was applied between 0 and 10 minutes. The column was then re-equilibrated by linearly reducing the concentration of buffer B to 5% between 10 and 15 minutes followed by a 5

Figure 11. Stability of YAGAVVNDL in HSV-1 infected cell extracts.

4uCi [^{3}H]-YAGAVVNDL was mixed with either 98ul H_2O (Figure 11a) or with 98ul of partially purified HSV-1-infected cell extract (Figure 11b). Each sample (25ul) was analysed by reverse phase HPLC in a linear gradient increasing from 5% to 95% acetonitrile + 0.1% TFA between 0 and 10 minutes and the [^{3}H] was measured using a flow through scintillation counter.


3 H
(a)
cps X 10 3
8.00
6.00
4.00
2.00
0.00
00"00
01'00
02'00
03'00
04'00
05'00
06'00
07'00
08'00
09'00
3 H
(b)
cps X 10 4
1.00
0.50
0.00
00"00
01'00
02'00
03'00
04'00
05'00
06'00
07'00
08'00
09'00
TIME (minutes)

minute wash with 95% buffer A, 5% buffer B. Hence the run time and equilibration period was half of that described previously. The eluant was monitored using the flow through scintillation counter.

Figure 11a shows that tritium-labelled nonapeptide mixed with water was eluted as a single peak at 7 minutes under these conditions. When tritium-labelled nonapeptide was mixed with HSV-1-infected cell extract, two peaks of activity were eluted from the column as shown in Figure 11b. One of these peaks eluted at the same time as [^{3}H]-YAGAVVNDL and the other eluted at 5 minutes 12 seconds. This new species was thought to be a cleavage product of the nonapeptide. Each sample was injected onto the HPLC immediately after mixing with water or infected cell extract. Therefore, as shown in Figure 11b, there was approximately 70% conversion to this new species within 2 minutes, the time taken for injection.

II.2 Effect of protease inhibitors on the stability of YAGAVVNDL.

4uCi [^{3}H]-YAGAVVNDL was mixed with 100ul of an HSV-1-infected cell extract containing either PMSF (1mM), TPCK (0.5mM), bacitracin (1mM), leupeptin (10^{-7}M), pepstatin A (10^{-7}M) or alpha 2 macrogloblin (0.3 inh. units/ml). Each sample was prepared immediately prior to analysis by HPLC. 25ul of each sample was applied to a uBondapak C18 column (8mm x 10cm) and peptide was eluted in a linear gradient as described in section III.1. Figure 12 shows that three of the six protease inhibitors used were able to protect the nonapeptide from modification to some extent. When PMSF

Figure 12. Effect of protease inhibitors on the stability of YAGAVVNDL.

4uCi [^{3}H]-YAGAVVNDL was mixed with 98ul partially purified HSV-1-infected cell extract containing either 10^{-7}M pepstatin A (Figure 12a), 10^{-7}M leupeptin (Figure 12b), 0.3 inh.units/ml alpha 2 macroglobulin (Figure 12c), 1mM PMSF (Figure 12d), 0.5mM TPCK (Figure 12e) or 1mM bacitracin (Figure 12f). Each sample (25ul) was analysed by reverse phase HPLC and [^{3}H] was monitored on a flow through scintillation counter.

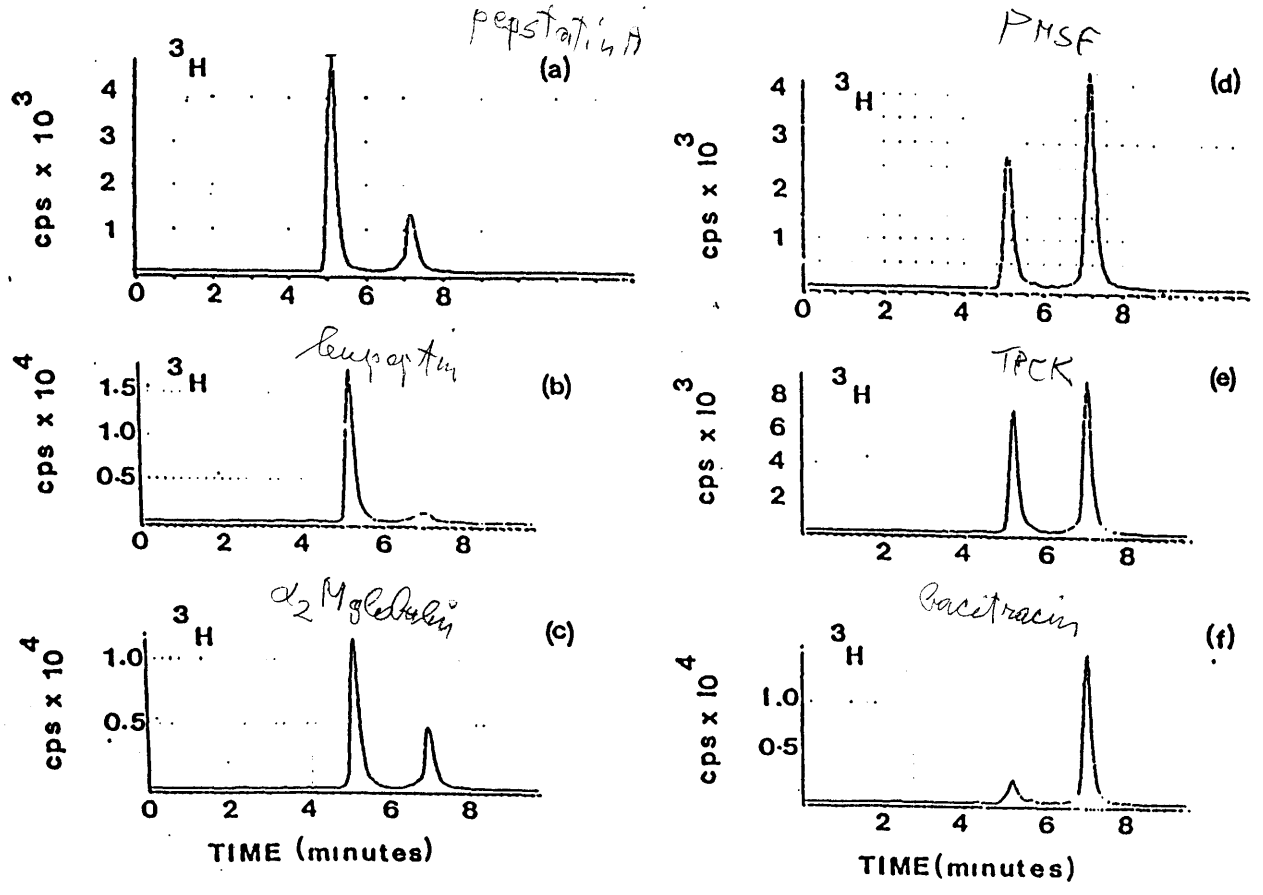
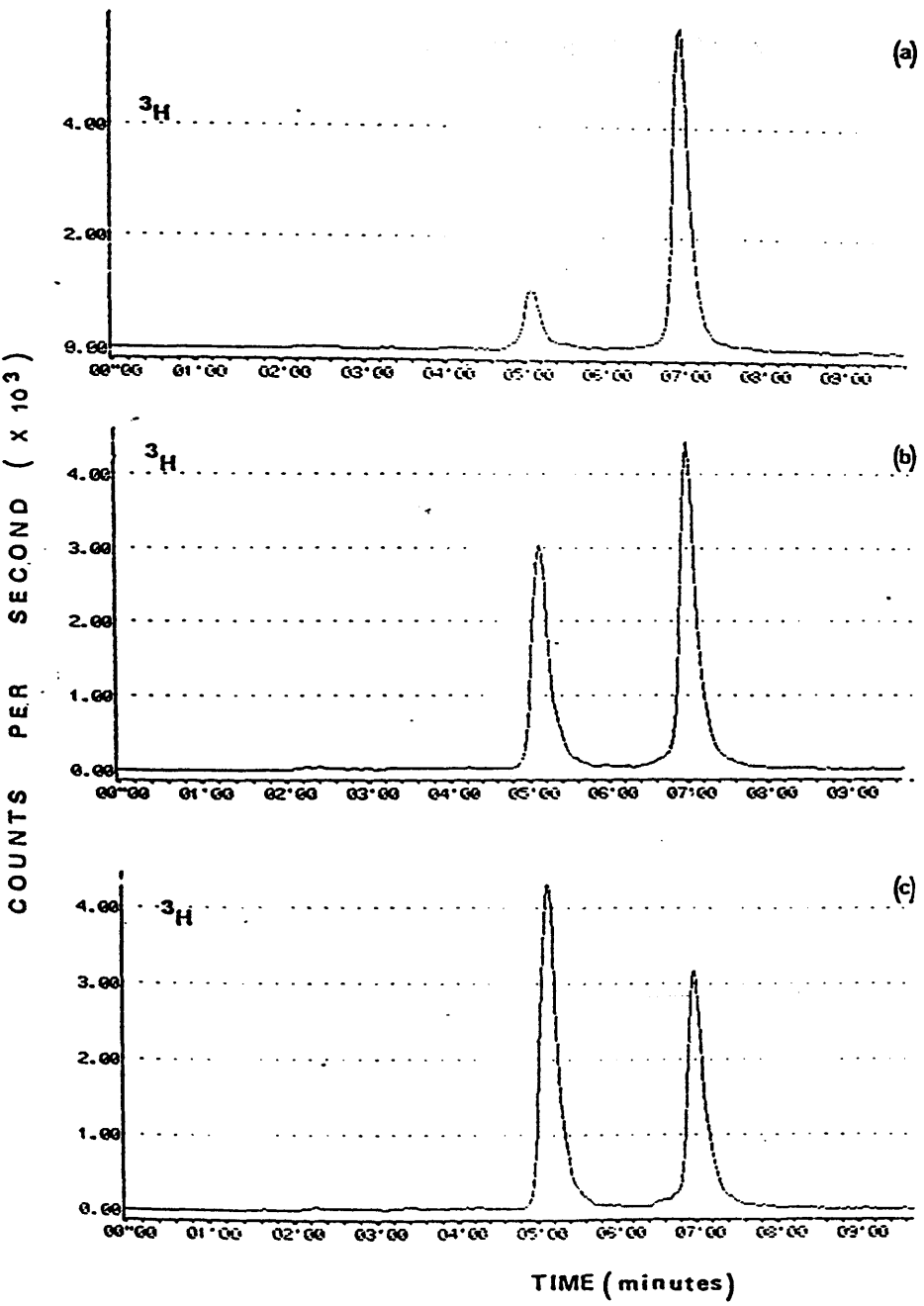

pepstatin A
PMSF
(a)
(b)
(c)
(d)
(e)
(f)
^{3}H
cps x 10^3
cps x 10^4
TIME (minutes)

Figure 13. Time course of the modification of YAGAVVNDL in the presence of bacitracin.

2uCi [^{3}H]-YAGAVVNDL was mixed with 50ul partially purified HSV-1-infected cell extract containing 1mM bacitracin. Samples (10ul) were analysed by HPLC/flow through scintillation counting at 2 min (Figure 13a), 22 min (Figure 13b) and 42 min (Figure 13c) after mixing.


(a)
^3H
4.00
2.00
0.00
00'00 01'00 02'00 03'00 04'00 05'00 06'00 07'00 08'00 09'00
(b)
^3H
4.00
3.00
2.00
1.00
0.00
00'00 01'00 02'00 03'00 04'00 05'00 06'00 07'00 08'00 09'00
(c)
^3H
4.00
3.00
2.00
1.00
0.00
00'00 01'00 02'00 03'00 04'00 05'00 06'00 07'00 08'00 09'00
COUNTS PER SECOND (X 10^3)
TIME (minutes)

(Fig.12d) or TPCK (Fig.12e) were present in the cell extract only approximately 40% of the nonapeptide was converted compared to the effect of pepstatin A (Fig.12a), leupeptin (Fig.12b) and alpha 2 macroglobulin (Fig.12c) were approximately 80% of the nonapeptide was converted. The nonapeptide was best protected by bacitracin (Fig.12f), a cyclic polypeptide, in the presence of which less than 20% of the peptide was modified. This observation that protease inhibitors could stabilise the peptide suggests that the modification is a proteolytic cleavage. Figure 13 shows however that the nonapeptide was progressively modified even in the presence of 1mM bacitracin. Three 10ul volumes of [^{3}H]-YAGAVVNDL mixed with infected cell extract plus 1mM bacitracin were analysed by HPLC at 2 min, 22 min and 42 min after mixing. Within 45 minutes, more than 50% of the nonapeptide had been converted.

II.3 The effect of protease inhibitors on ribonucleotide reductase activity.

Since protease inhibitors stabilised the nonapeptide in infected cell extracts an experiment was performed to determine whether the activity of the HSV-1-induced ribonucleotide reductase could also be stabilised by protease inhibitors. Extracts of HSV-1-infected cells were assayed for ribonucleotide reductase activity in the presence of one of each of the protease inhibitors at the concentrations described above at 37^{o}C for 15 min, 30 min, 1h, 2h or 3h. As is shown in Table 2, none of the protease inhibitors significantly increased the activity of the enzyme as compared to control assays. Enzyme activity was

Table 2 Effect of protease inhibitors on HSV-1-induced ribonucleotide reductase activity.

The enzyme (100ug of partially purified extract) was assayed in the presence of 1mM bacitracin, 10^{-7}M leupeptin, 10^{-7}M pepstatin A or 0.3 inh. units alpha 2 macroglobulin at 37^{o}C for 15 min, 30 min, 1h, 2h or 3h. The assay measures the conversion of [^{3}H]CDP to [^{3}H]dCDP and the rates of conversion are given in nmol h^{-1}.

INHIBITOR \ TIME(hr)	0.25	0.50	1	2	3
BACITRACIN	ND	0.5	1.0	1.1	1.0
LEUPEPTIN	ND	0.7	0.6	0.9	0.9
PEPSTATIN A	0.4	0.6	0.6	1.0	1.1
ALPHA 2 MACROGLOBULIN	ND	0.7	0.9	1.1	1.3
CONTROL	ND	0.5	0.6	0.8	1.1

(ND = not determined)

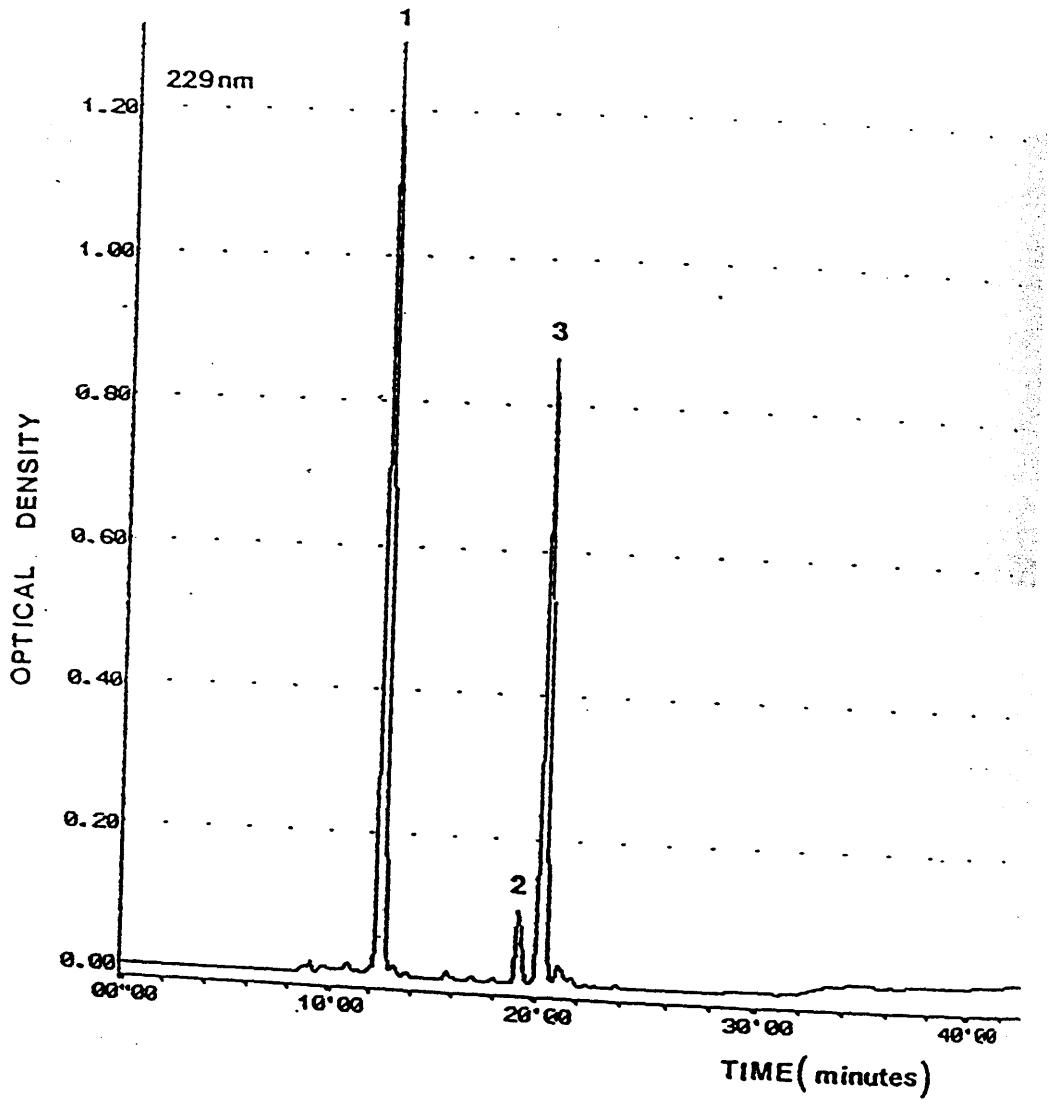


Figure 14. Separation of the modified products of YAGAVVNDL by reverse phase HPLC.

The modified products of YAGAVVNDL were separated by reverse phase HPLC on a uBondapak C18 preparative column in a linear gradient increasing from 0% to 100% acetonitrile + 0.05% TFA between 0 and 50 minutes. The eluant was monitored using a UV monitor at 229nm.

decreased in the presence of TPCK and PMSF (results not shown). This was probably due to the fact that both these inhibitors were dissolved in methanol and methanol decreased the activity of the enzyme.

SECTION III PURIFICATION OF MODIFIED PRODUCTS OF YAGAVVNDL.

III.1 Separation of the modified products by HPLC.

The modified products of the nonapeptide were purified by reverse phase HPLC using a uBondapak C18 column (19mm x 30cm). This column was first equilibrated with buffer A (0.1% TFA in H_2O) and a linear gradient increasing to 100% buffer B (0.05% TFA in acetonitrile) between 0 and 50 minutes was applied with buffer flowing at a rate of 10ml min^{-1}. The column was re-equilibrated by linearly reducing the concentration of buffer B to 0% between 75 and 90 minutes followed by a 2 hour wash with buffer A. The eluant was monitored at 229nm. Figure 14 shows that under these conditions, three major species were eluted from the column. The first, peak(1), was eluted at 12 minutes 32 seconds, the second, peak(2), was eluted at 19 minutes 13 seconds and the third, peak (3), was eluted at 20 minutes 21 seconds.

III.2 Purification of modified products by HPLC.

To generate sufficient quantities of the products for analysis, 3ml of a 4mg/ml (w/v) solution of YAGAVVNDL was mixed with 260ul HSV-1-infected cell extract and the mixture was incubated at room temperature for 3 hours. Initially, to check the reproducibility of the chromatography, three

Figure 15. **Reverse phase chromatography of the purified modification products.**

One aliquot of each of the purified modification products was resuspended in 50ul H_2O. 5ul of each sample was then analysed by reverse phase HPLC on a C18 uBondapak analytical column in a linear gradient increasing from 0% to 100% acetonitrile + 0.05% TFA between 0 and 20 minutes. The eluant was monitored at 229nm.

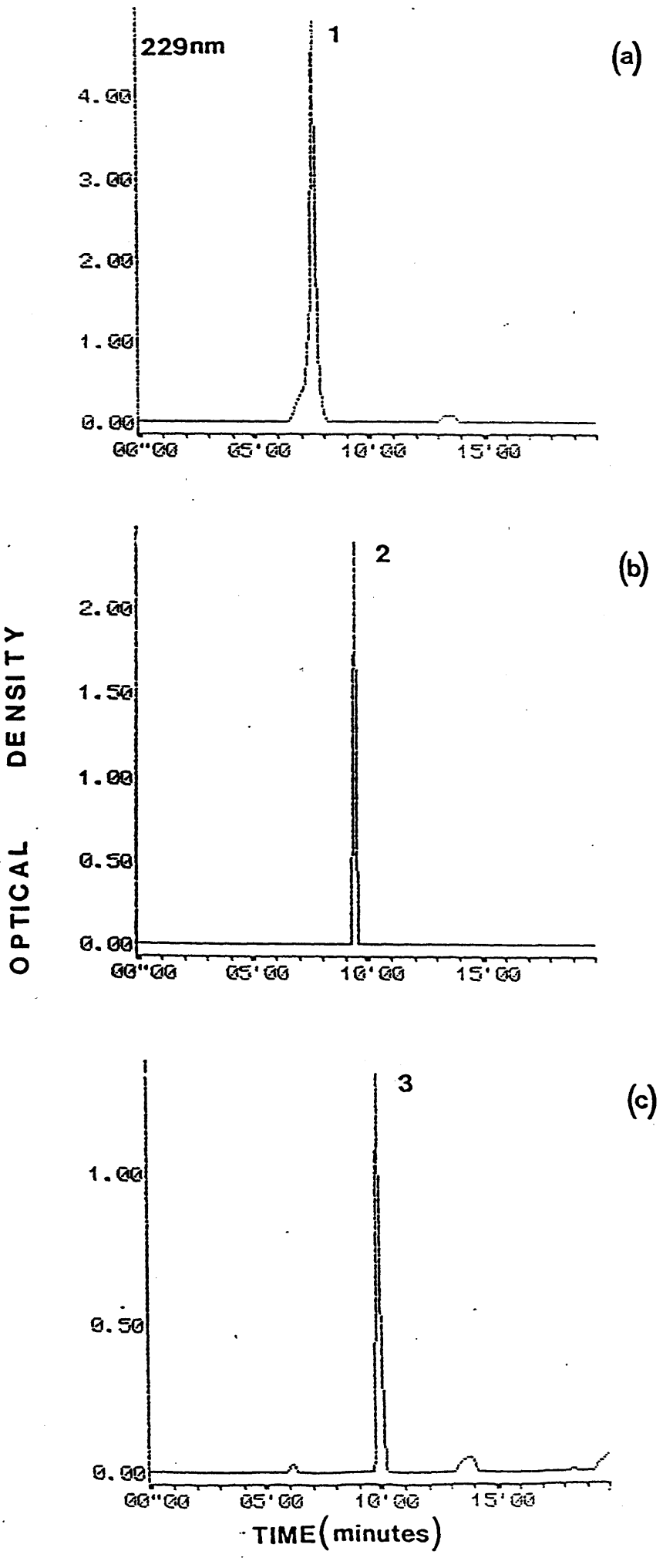

229nm
1
(a)
4.00
3.00
2.00
1.00
0.00
00"00
05'00
10'00
15'00
2
(b)
2.00
1.50
1.00
0.50
0.00
00"00
05'00
10'00
15'00
OPTICAL DENSITY
3
(c)
1.00
0.50
0.00
00"00
05'00
10'00
15'00
TIME(minutes)

Table 3. Identification of modified products of YAGAVVNDL by determination of relative molecular mass and amino acid composition.

PEAK	AMINO ACID COMPOSITION	M_r	PROBABLE SPECIES
1	Y	181	Y
2	A(2),G(1),V(2),N(1),D(1),L(1)	738	AGAVVNDL
3	Y(1),A(2),G(1),V(2),N(1),D(1),L(1)	919	YAGAVVNDL

100ul volumes were applied to the column then 800ul followed by a final injection of 1ml. During each run 2ml fractions were collected automatically between 12 and 14 minutes and also between 19 and 21 minutes. When all the samples had been run, 50ul of each fraction was applied to a uBondapak C18 analytical column (8mm x 10cm). This time the column was equilibrated with buffer A (0.1% TFA in H^2O) and a linear gradient increasing to 100% buffer B (0.05% TFA in acetonitrile) between 0 and 20 minutes was applied with buffer flowing at 2ml min^{-1}. The column was re-equilibrated by linearly reducing the concentration of buffer B to 0% between 30 and 36 minutes followed by a 9 minute wash in buffer A. Figure 15 shows that under these conditions, peak (1) was eluted from the column at 7 minutes 30 seconds, peak (2) was eluted at 9 minutes 32 seconds and peak (3) was eluted at 10 minutes. Fractions containing each peak were pooled and rotary evaporated to remove acetonitrile. The three samples were then shell frozen in liquid nitrogen and lyophilised.

III.3 Identification of the modified products.

A small amount of each of the three peptides was analysed for amino acid composition and molecular mass by fast atom bombardment mass spectroscopy (Glaxo Group Research Ltd., Greenford, UK). The results are shown in Table 3 and suggest that peak (1) was free tyrosine, peak (2) was the octapeptide AGAVVNDL and peak (3) was the nonapeptide YAGAVVNDL. The simplest interpretation of these data and the observation that formation of the products can be inhibited by protease inhibitors is that the modification

Figure 16. **Reverse phase chromatography of the synthetic octapeptide AGAVVNDL and the product purified from peak (2).**

40ul of a 1mg/ml (w/v) solution of authentic AGAVVNDL (Figure 16a), 40ul of product purified from peak (2) (Figure 16b) or a 1:2 mixture of authentic AGAVVNDL and the peak (2) product (Figure 16c) were analysed by reverse phase HPLC. The eluant was monitored at 229nm.

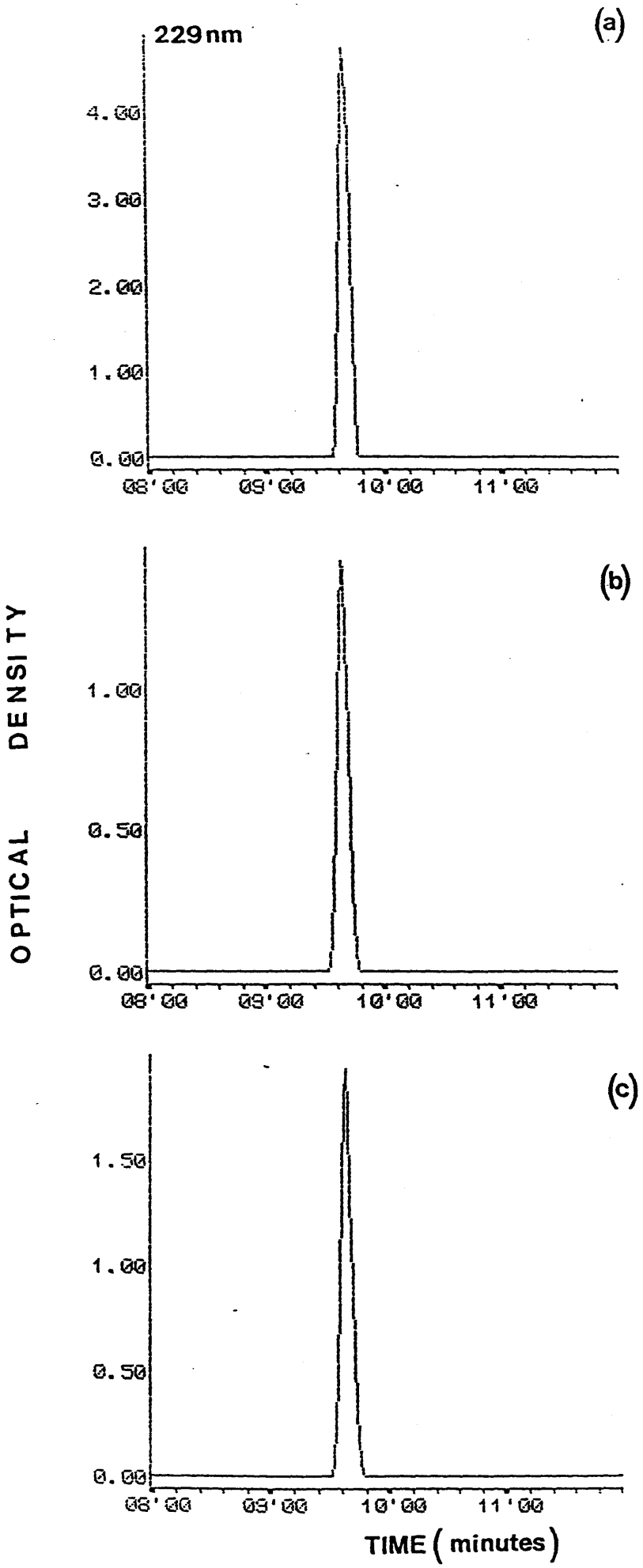
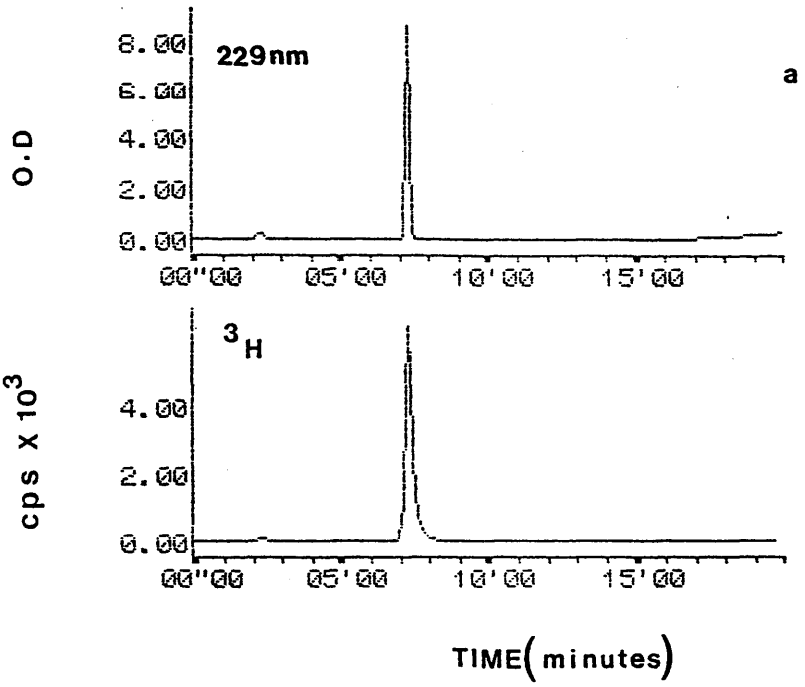
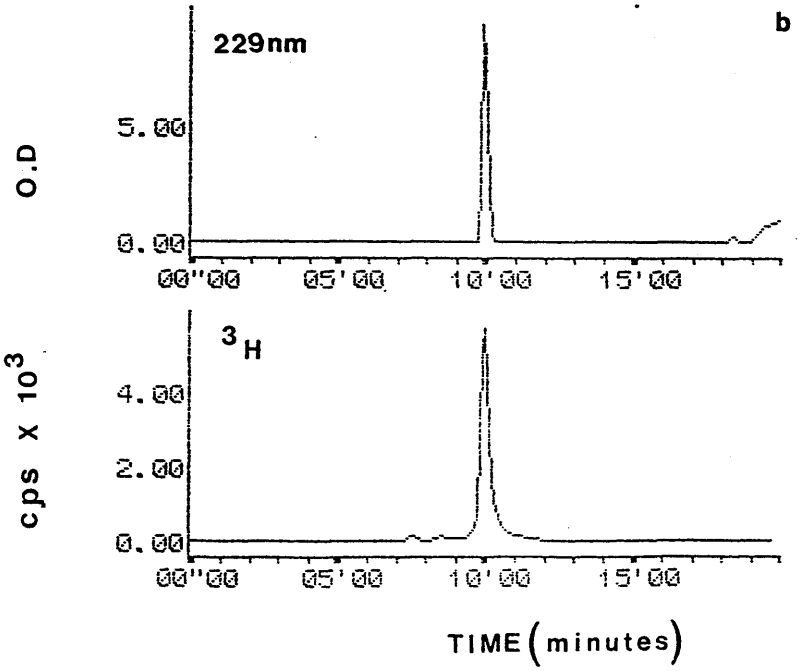

229nm
(a)
4.00
3.00
2.00
1.00
0.00
08'00
09'00
10'00
11'00
(b)
1.00
0.50
0.00
08'00
09'00
10'00
11'00
(c)
1.50
1.00
0.50
0.00
08'00
09'00
10'00
11'00
OPTICAL DENSITY
TIME (minutes)

Figure 17. Reverse phase chromatography of authentic [^{3}H]-tyrosine and [^{3}H]-YAGAVVNDL and the purified products of peaks (1) and (3).

(a) An aliquot of product purified from peak (1) was resuspended in 50ul H_2O and mixed with 3uCi authentic [^{3}H]-tyrosine. 40ul of the sample was analysed by HPLC/flow through scintillation counting.

(b) An aliquot of product purified from peak (3) was resuspended in 50ul H_2O and mixed with 10uCi [^{3}H]-YAGAVVNDL. 5ul of the sample was analysed by HPLC/flow through scintillation counting.


a
229nm
O.D
8.00
6.00
4.00
2.00
0.00
00''00
05'00
10'00
15'00
^{3}H
cps X 10^3
4.00
2.00
0.00
00''00
05'00
10'00
15'00
TIME(minutes)


b
229nm
O.D
5.00
0.00
00''00
05'00
10'00
15'00
^{3}H
cps X 10^3
4.00
2.00
0.00
00''00
05'00
10'00
15'00
TIME(minutes)

of YAGAVVNDL occurs by cleavage of the peptide bond between Y and AGAVVNDL.

Confirmation that peak (2) contains the octapeptide AGAVVNDL was obtained by a comparison of the retention times on analytical reverse phase HPLC of peak (2) and authentic AGAVVNDL (Figure 16). A single peak was eluted from the column at 9 minutes 32 seconds when 40ul of peptide 2 or 40ul of a 1mg/ml (w/v) solution of AGAVVNDL was analysed. A single peak was also eluted at the same time when 25ul of each of these solutions were mixed and 40ul of the mixture was analysed.

Confirmation that peak (1) was tyrosine was obtained by comparison of the retention times on analytical reverse phase HPLC of peak (1) and authentic [^{3}H] tyrosine by mixing 30ul peak (1) with 3uCi [^{3}H] tyrosine. The eluant was monitored at 229nm and [^{3}H] was monitored using the flow through scintillation counter. Figure 17a shows that both peak (1) and the tritium-labelled tyrosine marker were eluted from the column at the same time.

Finally, 40ul of peak (3) was mixed with 10uCi [^{3}H]-YAGAVVNDL as a marker and 5ul of this solution was analysed by HPLC. Figure 17b shows that peak (3) and [^{3}H]-YAGAVVNDL were eluted from the column at 10 minutes.

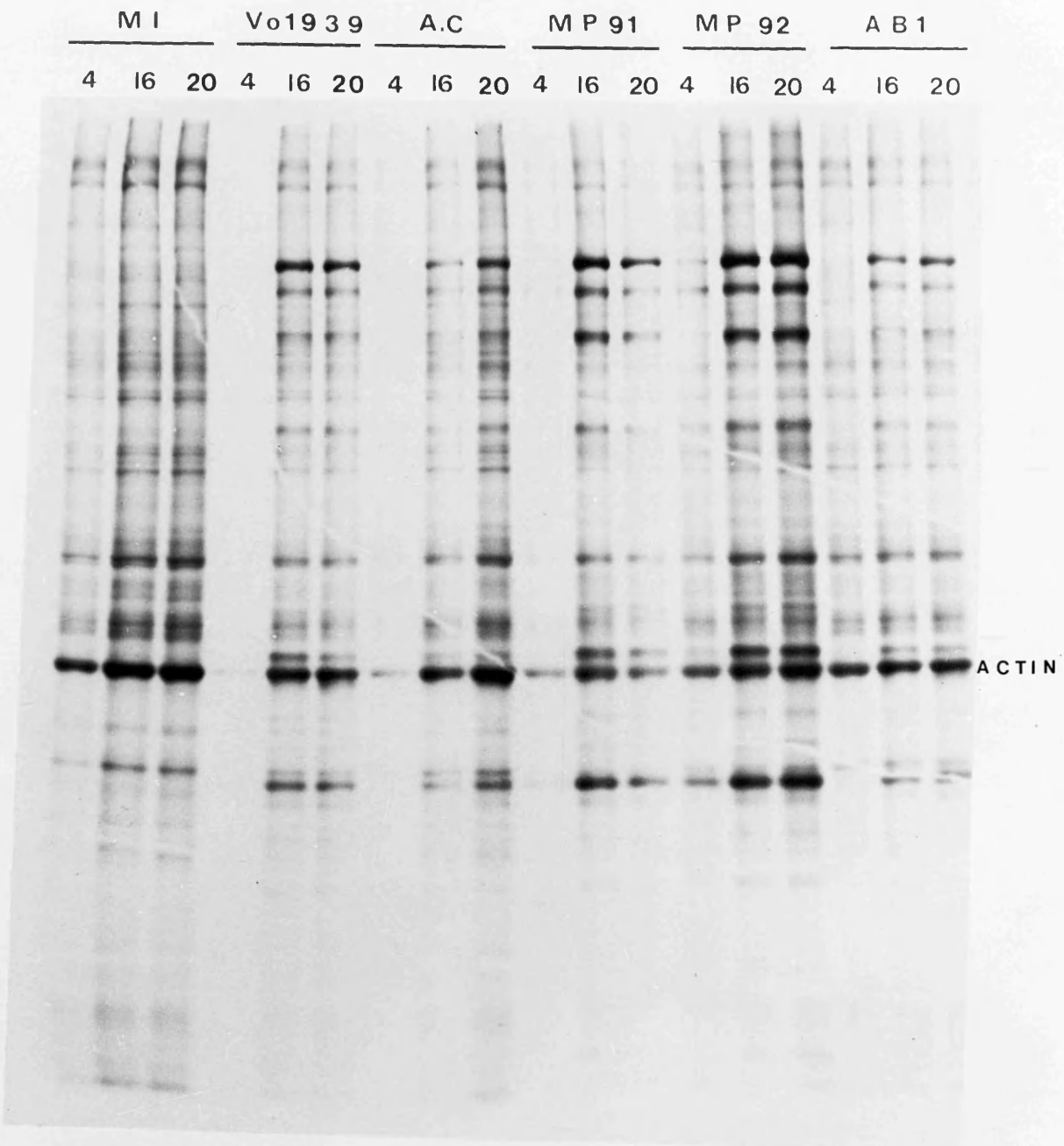
SECTION IV INHIBITION OF EQUINE HERPES TYPE 1-INDUCED RIBONUCLEOTIDE REDUCTASE ACTIVITY BY THE NONAPEPTIDE YAGAVVNDL.

IV.1 Comparison of proteins induced by different EHV-1 isolates.

Initially, rabbit kidney cells were infected with EHV-1 strain Vol939 and virus-induced ribonucleotide reductase was

Figure 18. Comparison of polypeptides produced upon infection with various isolates of EHV-1.

50mm plates of BHK-C13 cells were either mock-infected (MI) or infected with 10pfu EHV-1 strain Vol939, A.C, Kentucky A subclones MP91 or MP92 or strain AB1. The virus was adsorbed at 37°C for 1 hour and the plates were then labelled with [^{35}S]methionine for 4h, 16h or 20h at 37°C before harvesting. The infected cell polypeptides were resolved by electrophoresis on a 5% to 12.5% SDS polyacrylamide gel.


M I
Vo1939
A.C
MP 91
MP 92
AB1
4 16 20 4 16 20 4 16 20 4 16 20 4 16 20 4 16 20
ACTIN

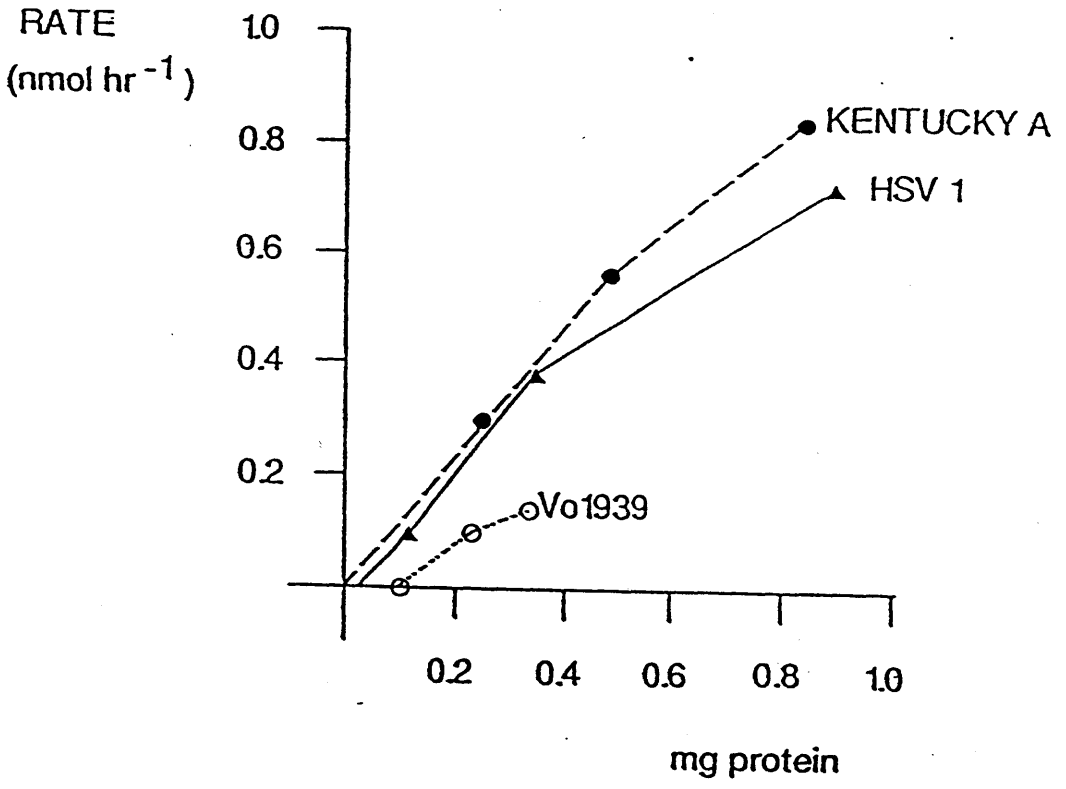


Figure 19. Standard curves of virus-induced ribonucleotide reductase activities.

BHK-C13 cells were infected with HSV-1 and RK13 cells were infected with EHV-1 strain Vol939 or Kentucky A. Virus-induced ribonucleotide reductase was partially purified by ammonium sulphate precipitation and extracts were assayed for ribonucleotide reductase activity at various concentrations of protein. The rates of conversion of CDP to dCDP are given in nmol h^{-1}.

partially purified by precipitation with ammonium sulphate. In RK13 cells this virus grows to only 5 x 10^8 pfu/ml which is 10 fold less than the usual titre of HSV-1 stocks. Large volumes of this virus stock were therefore required to infect roller bottles with 10pfu of this virus. It was also observed that RK13 and BHK infected cell extracts labelled with [^{35}S]methionine contained very few identifiable viral polypeptides and these were present in only low amounts. It was therefore decided to infect BHK cells with several EHV-1 isolates and to compare the [^{35}S]methionine-labelled infected cell polypeptides produced so that an isolate yielding the most virus-induced polypeptides could be selected. Figure 18 shows that the best infection was obtained when BHK cells were infected with strain Kentucky A.

IV.2 Standard curves of virus-induced ribonucleotide reductase activities.

BHK cells were infected with HSV-1 and RK13 cells were infected with EHV-1 strain Vol939 or Kentucky A and virus-induced ribonucleotide reductase was partially purified by ammonium sulphate precipitation. The virus-induced ribonucleotide reductase activities were assayed (as described in Methods section III.2) at various concentrations of enzyme. Figure 19 shows the amount of product formed versus the enzyme concentration for each virus. Higher amounts of Vol939 enzyme could not be assayed because a maximum volume of 70ul enzyme extract could be included in each assay. The enzyme extract from Vol939-infected cells had a much lower activity than enzyme prepared from either HSV-1- or EHV-1 strain Kentucky A-infected cells.

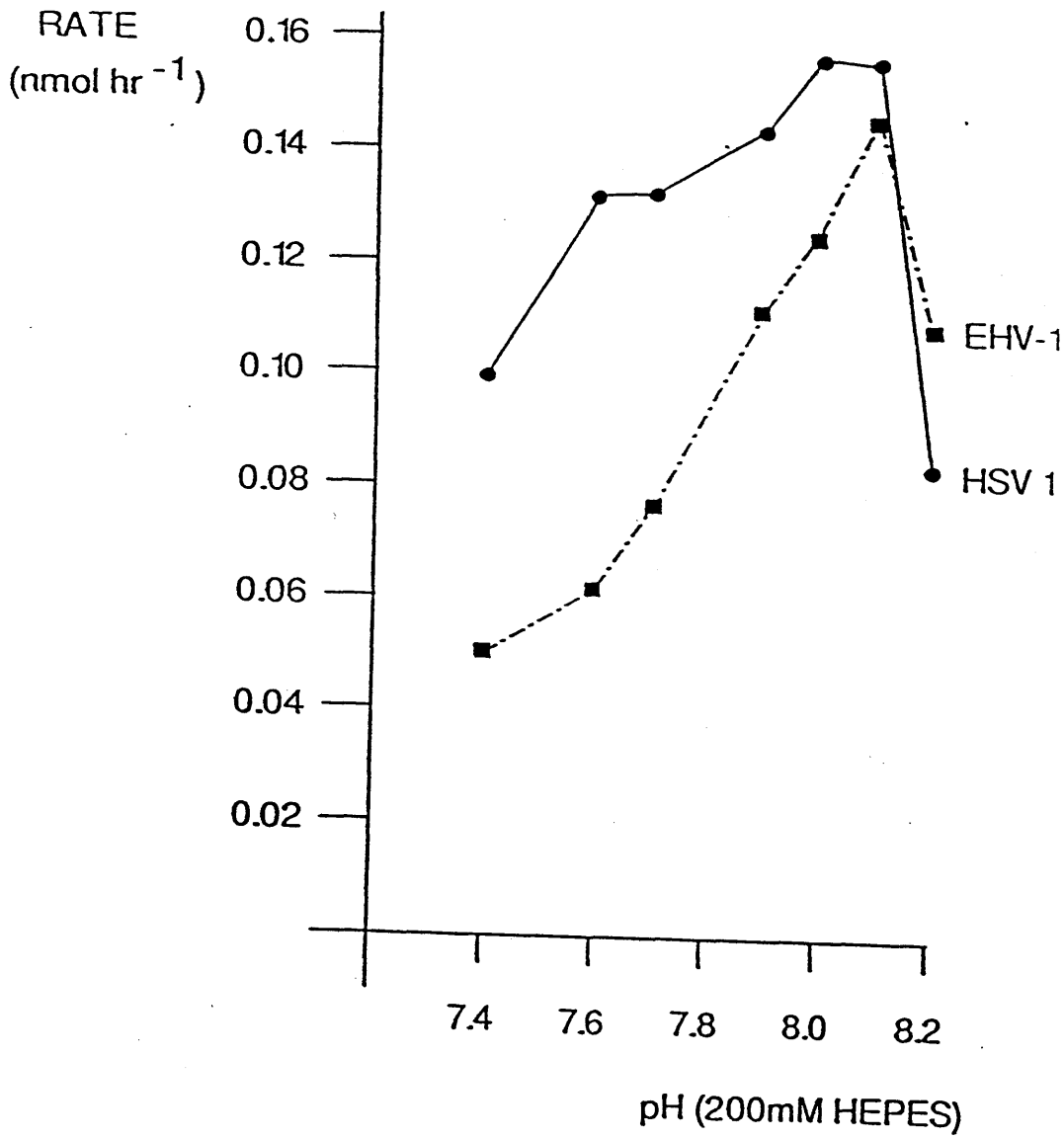


Figure 20. Effect of pH on EHV-1 and HSV-1-induced ribonucleotide reductase activities.

Ribonucleotide reductase partially purified from HSV-1- or EHV-1 strain Kentucky A-infected cells were assayed in 200mM HEPES at a range of pH. Each assay contained approximately 100ug partially purified extract.

IV.3 The effect of pH on EHV-1- and HSV-1-induced ribonucleotide reductase activities.

The effect of pH on the rate of CDP reduction by HSV-1-and EHV-1-induced ribonucleotide reductase was determined by varying the pH of 200mM HEPES buffer in the assay mixture between 7.4 and 8.2. Figure 20 shows that the optimum pH was approximately the same for both the EHV-1- and HSV-1-induced enzyme.

IV.4 Inhibition of EHV-1-induced ribonucleotide reductase by YAGAVVNDL.

IV.4(a) Inhibition of strain Vol939 ribonucleotide reductase activity.

Rabbit kidney cells were infected with EHV-1 strain Vol939 and virus-induced ribonucleotide reductase activity was partially purified by ammonium sulphate precipitation. EHV-1-induced ribonucleotide reductase activity was assayed in the presence of various concentrations of the nonapeptide. The peptide was found to be inhibitory as is shown in Figure 21. The concentration of peptide required to inhibit 50% of the EHV-1-induced ribonucleotide reductase activity (IC_{50}) was 110uM. In a parallel experiment, the IC_{50} of the HSV-1-induced ribonucleotide reductase was 150uM. Concentrations of peptide greater than 0.625mM could not be achieved because of volume limitations due to the low specific activity of the Vol939-induced enzyme.

IV.4(b) Inhibition of strain Kentucky A ribonucleotide reductase activity.

Rabbit kidney cells were infected with strain Kentucky A and virus-induced ribonucleotide reductase was partially

Figure 21. Inhibition of EHV-1 strain Vol939-induced ribonucleotide reductase by YAGAVVNDL.

The figure shows the effect of increasing concentrations of YAGAVVNDL on ribonucleotide reductase activity in a partially purified extract of HSV-1-infected BHK-C13 cells or EHV-1 strain Vol939-infected RK13 cells. Each assay contained approximately 100ug partially purified extract from HSV-1-infected cells or 300ug partially purified extract from EHV-1-infected cells.

100% enzyme activity is equivalent to 0.1 nmol h^{-1}.

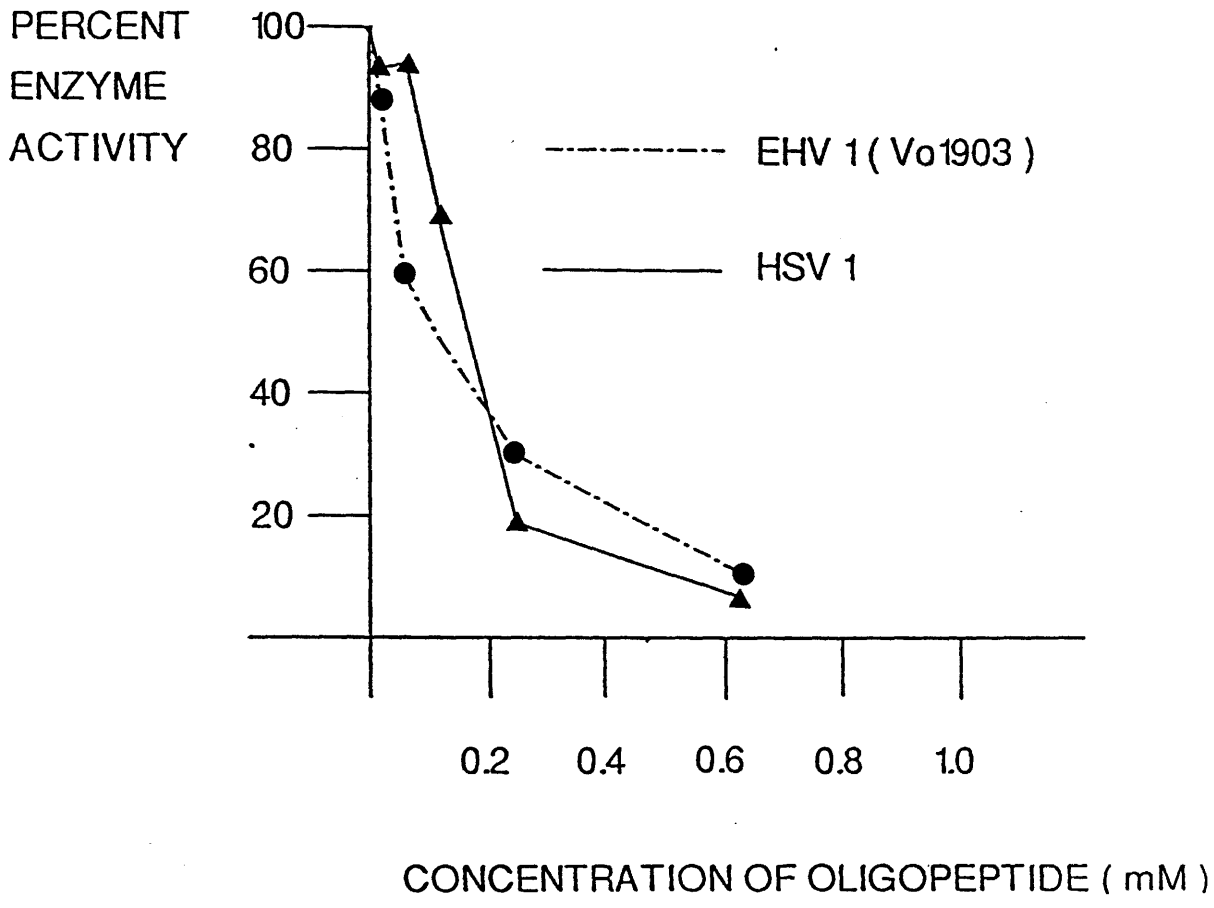
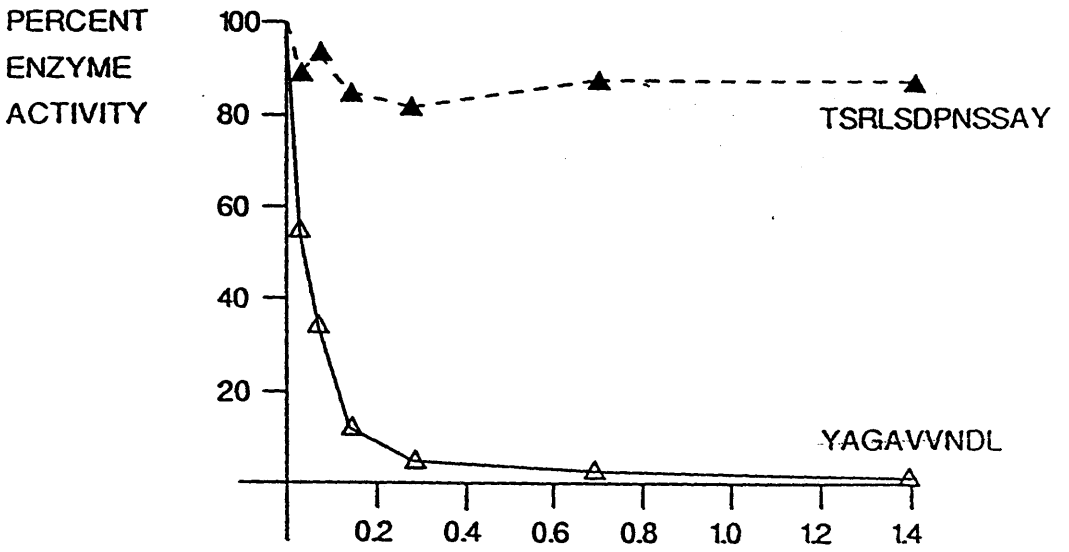

PERCENT
ENZYME
ACTIVITY
100
80
60
40
20
EHV 1 (Vo1903)
HSV 1
0.2
0.4
0.6
0.8
1.0
CONCENTRATION OF OLIGOPEPTIDE (mM)

Figure 22. **Inhibition of EHV-1 strain Kentucky A-induced ribonucleotide reductase activity by YAGAVVNDL.**

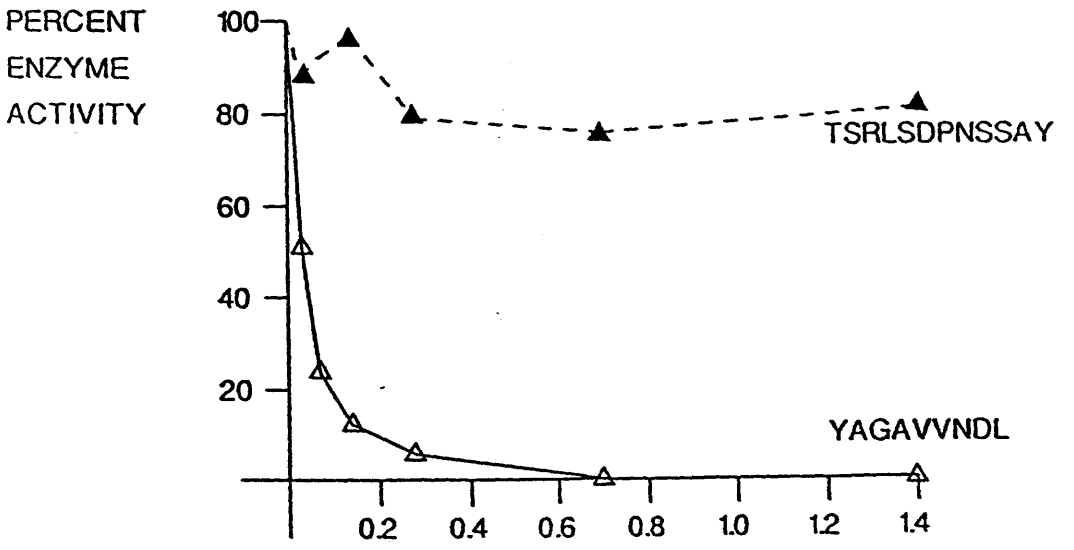
The figure shows the effect of increasing concentrations of YAGAVVNDL or the control peptide TSRLSDPNSSAY on ribonucleotide reductase activity in a partially purified extract of HSV-1-infected BHK-C13 cells (Figure 22a) or EHV-1 strain Kentucky A-infected RK13 cells (Figure 22b). Each assay contained approximately 100ug partially purified extract. Enzyme assays were carried out in the presence of 1mM bacitracin.

HSV 1



CONCENTRATION OF OLIGOPEPTIDE (mM)

EHV-1 (KENTUCKY A)



CONCENTRATION OF OLIGOPEPTIDE (mM)

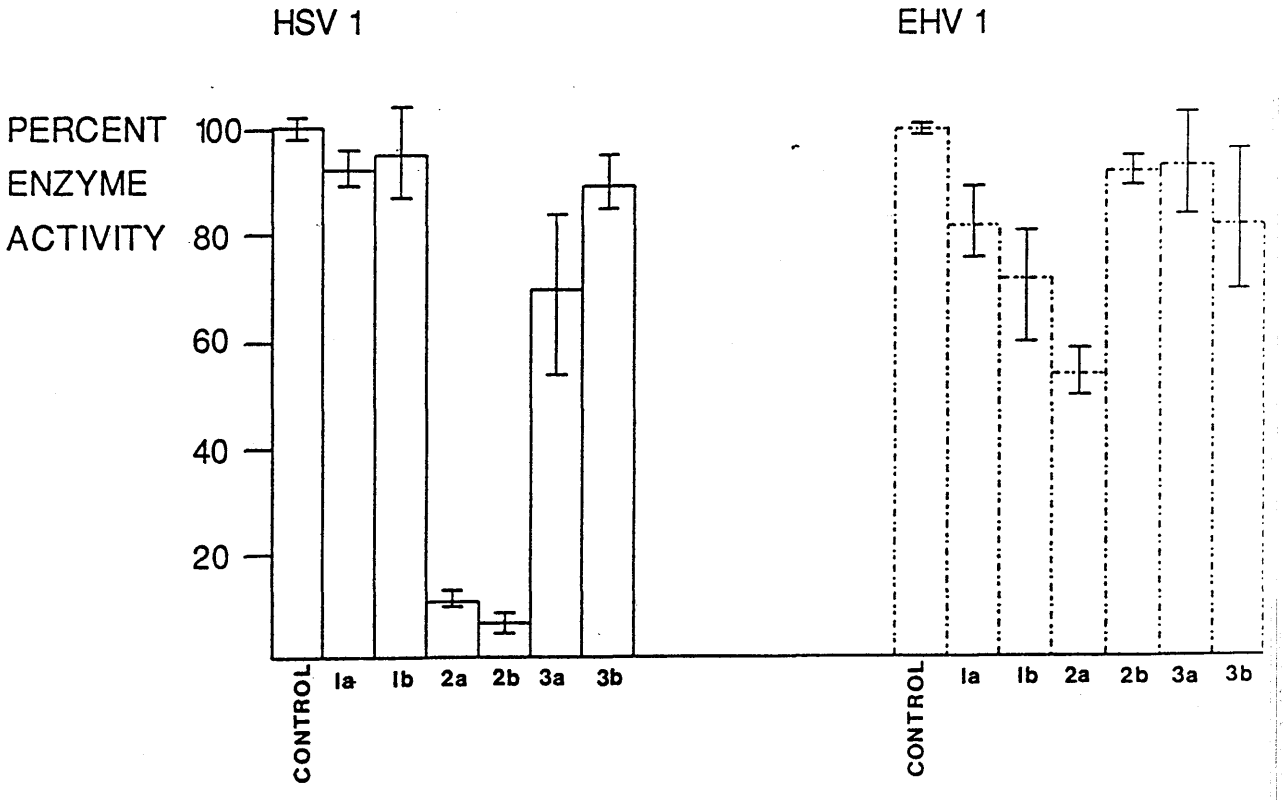
purified by ammonium sulphate precipitation. When EHV-1-induced ribonucleotide reductase activity was assayed in the presence of various concentrations of the nonapeptide, the peptide was found to be inhibitory. This result is shown in Figure 22. Parallel experiments with the HSV-1-induced ribonucleotide reductase established that the concentration of peptide required to inhibit 50% of the ribonucleotide reductase activity was 28uM for both enzymes. This concentration is much less than that required in the previous experiment because 1mM bacitracin was included in the assays which helps prevent the degradation of the nonapeptide by cellular proteases. Both the HSV-1- and the EHV-1-induced enzyme activities were relatively unaffected by the presence of various concentrations of a control peptide (TSRLSDPNSSAY) in the assay mixture.

IV.5 The effect of monoclonal antibody 7602 on EHV-1-induced ribonucleotide reductase.

Monoclonal antibody 7602 (provided by Dr A. Cross) reacts specifically with HSV-1 RR1 in immune precipitations and also cross reacts with HSV-2 RR1 (Dr A. Cross, unpublished results). IgG which was purified from this monoclonal antibody (Methods section II.3) was found to neutralise HSV-1- and HSV-2-induced ribonucleotide reductase activity when included in the assay mixture (Dr A. Darling, unpublished results). To determine whether monoclonal antibody 7602 would cross react with the RR1 of EHV-1, immune precipitations were carried out with EHV-1 (strain Kentucky A) infected cell extracts. No viral proteins were precipitated by monoclonal antibody 7602. EHV-1-induced

Figure 23. The effect of monoclonal antibody 7602 on EHV-1-induced ribonucleotide reductase.

IgG was purified from control ascites (1), monoclonal antibodies 7602 (2) or 1100 (3) by FPLC. Ribonucleotide reductase activity in an HSV-1- or an EHV-1 (Kentucky A)-infected extract was assayed in the presence of IgG purified from each antibody at either a 1/10 dilution (a) or a 1/100 dilution (b). The control was assayed in the absence of any IgG.


HSV 1
EHV 1
PERCENT ENZYME ACTIVITY
100
80
60
40
20
CONTROL
1a
1b
2a
2b
3a
3b
CONTROL
1a
1b
2a
2b
3a
3b

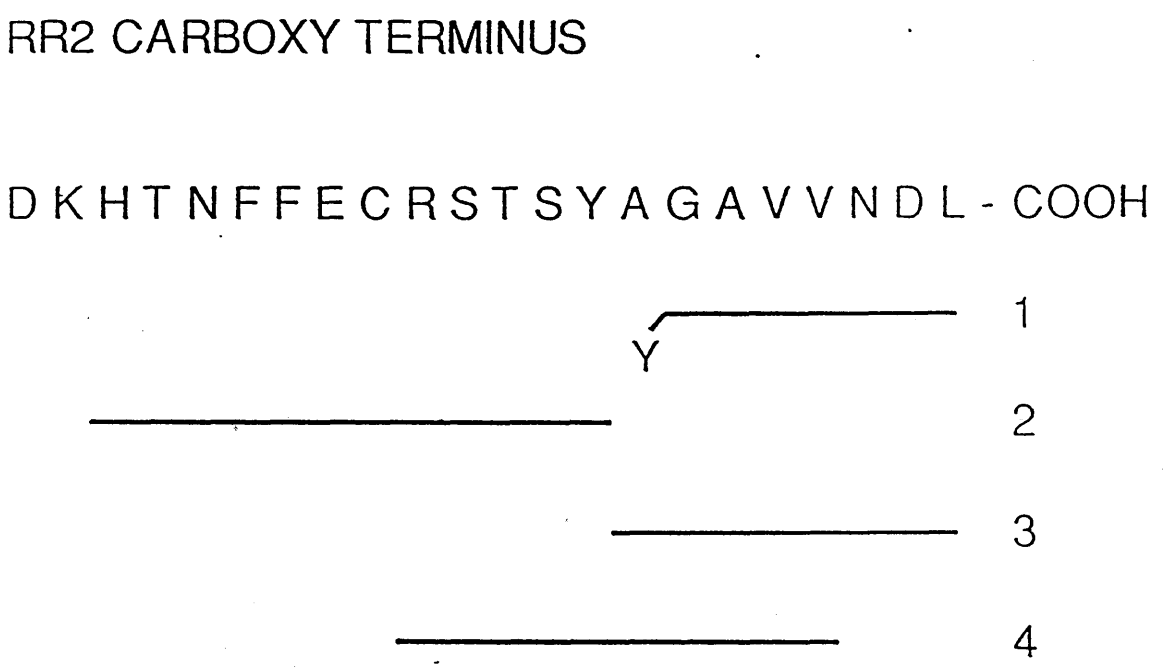


Figure 24. **Oligopeptide-induced antisera raised against the HSV-1-induced RR2.**

The figure shows amino acid sequences of the carboxy-terminal region of RR2 against which antisera were raised in rabbits. Antisera against peptide 1 were raised by Dr. M Frame.

ribonucleotide reductase was assayed in the presence of IgG purified from monoclonal antibody 7602 which was diluted either 1 part in 10 or 1 part in 100 in the assay mixture. The enzyme was also assayed in the presence of monoclonal antibody 1100, which does not neutralise the activity of the HSV-1-induced reductase and in the presence of IgG purified from control ascites fluid. Parallel experiments were also carried out on HSV-1-induced ribonucleotide reductase. These results are shown in Figure 23 and indicate some weak neutralisation of EHV-1 reductase by monoclonal antibody 7602.

SECTION V STRUCTURAL FEATURES OF THE EHV-1-INDUCED RIBONUCLEOTIDE REDUCTASE.

V.1 Production of anti-oligopeptide sera against HSV-1 RR2.

Figure 24 shows sequences of the carboxy-terminal region of RR2 against which anti-oligopeptide sera were raised in rabbits. Antisera against peptide 1 were raised by Dr M. Frame. This sequence represents the carboxy-terminal seven amino acids of RR2 plus an amino-terminal tyrosine to facilitate coupling of the oligopeptide to BSA. Peptide 3 represents the sequence of the inhibitory nonapeptide. To determine whether other carboxy sequences were involved in the association of RR2 with RR1, antisera were raised against peptides 2 and 4.

Immunoprecipitations were carried out using extraction buffer containing 0.1M Tris pH8.0, 10% glycerol, 0.5% NP40, 0.5% sodium deoxycholate. Frame et al. (1985) have shown that under these conditions, anti-oligopeptide serum against

Figure 25. Immunoprecipitations with anti-oligopeptide induced sera against RR2.

Immunoprecipitations of polypeptides from [^{35}S]methionine-labelled extracts of cells infected with HSV-1 were carried out in extraction buffer containing 0.5% SDS. Two rabbits were immunised with peptides 3 and 4 (3a, 3b, 4a and 4b) but only one rabbit was immunised against peptide 2. Rabbits were bled 6 weeks after the initial injection and the second bleed was taken three weeks later after the animals had been given a boost injection. Polypeptides labelled with [^{35}S] were eluted from Protein A-Sepharose-antigen-antibody complexes and separated on a 5% to 12.5% polyacrylamide gel.

NRS normal rabbit serum; 1 antiserum against YGAVVNDL; 2 antiserum against HTNFFECRSTS; 3 antiserum against YAGAVVNDL; 4 antiserum against RSTSYAGAVV; EXT HSV-1 infected cell extract; MI mock infected cell extract.

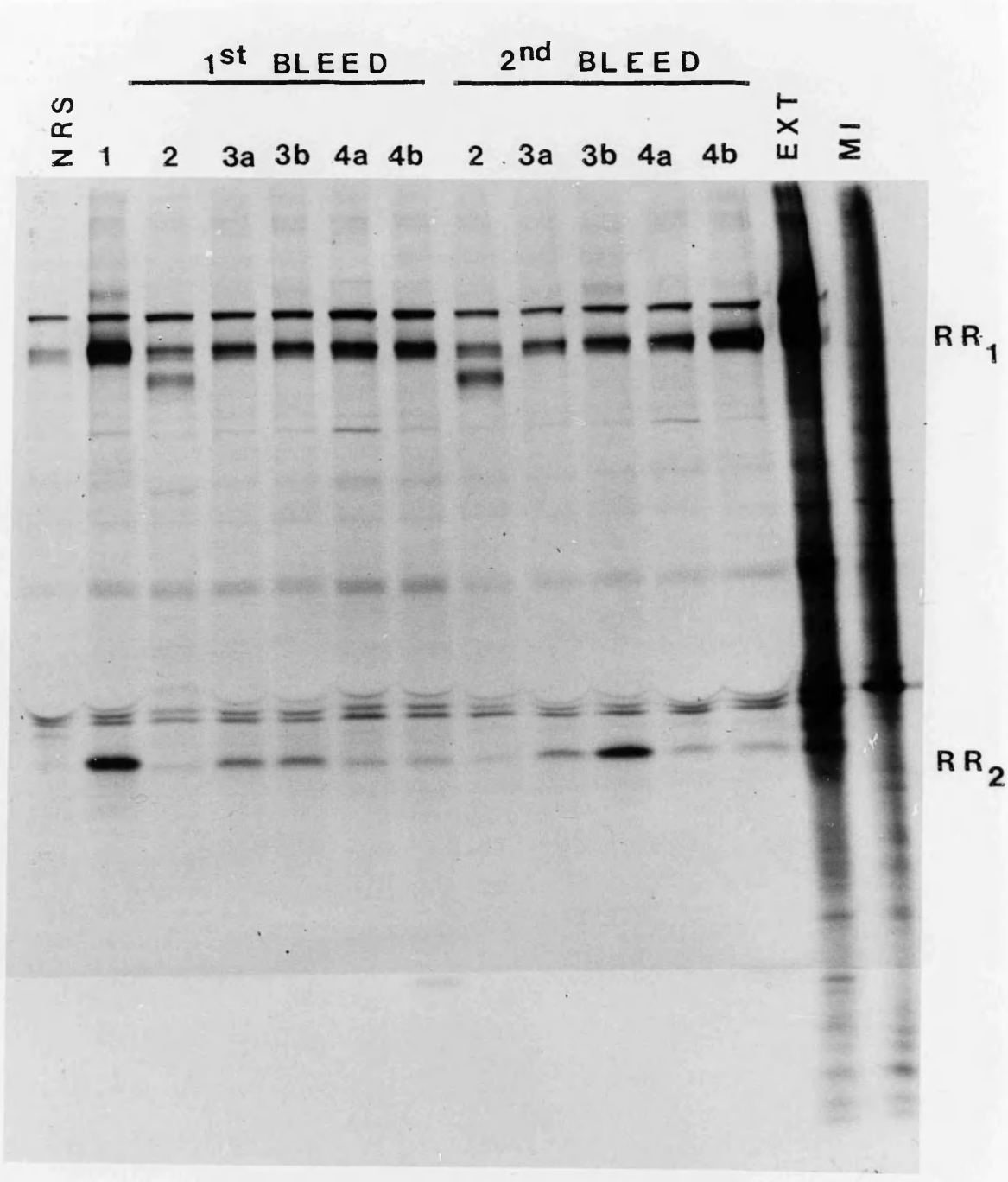

1st BLEED
2nd BLEED
NRS
1
2
3a
3b
4a
4b
2
3a
3b
4a
4b
EXT
MI
RR1
RR2

Figure 26. Immunoprecipitation with IgG purified from antiserum 2.

IgG was purified from antiserum 2 on an ion exchange column and used to immunoprecipitate polypeptides from HSV-1-infected cell extracts labelled with [^{35}S]methionine to determine whether the fast migrating band precipitated by antiserum 2 (Figure 25) is a product of proteolysis of RR1. Immunoprecipitations with monoclonal antibody 1100 and IgG purified from antiserum against peptide 1 were included as a control. The immunoprecipitations were carried out in the presence of 0.5% SDS. Polypeptides labelled with [^{35}S] were separated on a 5% to 12.5% polyacrylamide gel.

MA1100 monoclonal antibody 1100; CA control ascites; NRS normal rabbit serum; 1 IgG against YGAVVNDL; 2 IgG against HTNFFECRSTS; EXT HSV-1 infected cell extract; MI mock infected cell extract.

MA1100
CA
NRS
1
2
EXT
MI
RR1
RR2

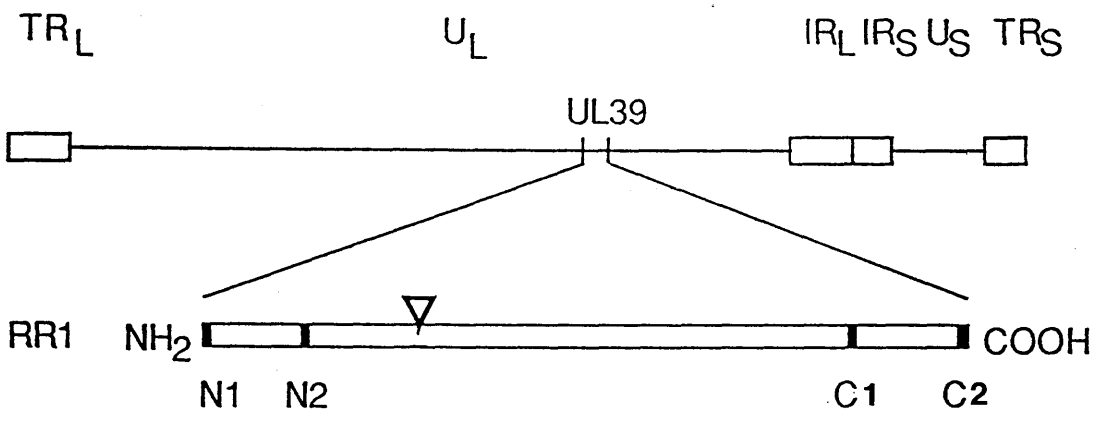
peptide 1 precipitated only RR2 from HSV-1 infected cells. Inclusion of 0.5% SDS in the precipitation buffer, however, resulted in the coprecipitation of RR1 with RR2. Figure 25 shows the result of immunoprecipitations with antisera against peptides 1,2,3 and 4 in the presence of 0.5% SDS. Antisera raised against peptide 3 coprecipitated RR1 with RR2 in the same way as the antiserum against peptide 1. Antisera against peptide 4 also coprecipitated RR1 with RR2 but relatively more RR1 was precipitated by antiserum 4 than by antisera 1 or 3. In each case, RR1 appeared as a doublet and the upper band was preferentially coprecipitated in the presence of SDS. The double bands may represent two different forms of the RR1 polypeptide. The antiserum against peptide 2 precipitated a polypeptide which migrated just below RR1 and failed to precipitate more than background levels of RR1. Both antisera 2 and 4 precipitated very weak bands coresponding to RR2.

To determine whether the faster migrating band precipitated by antiserum 2 was a result of proteolytic activity present in the serum, IgG was purified from the antiserum against peptide 2 and immune precipitations were carried out with HSV-1 infected cell extracts. Figure 26 shows that IgG purified from antiserum 2 precipitated RR1 and the faster migrating band was no longer detectable.

IgG was purified from each of the antisera raised against the carboxy-terminal region of RR2 and tested for their ability to inhibit HSV-1-induced ribonucleotide reductase activity. However, none were found to neutralise enzyme activity.

Figure 27. A schematic presentation of the linearised RR1 polypeptide with the location of peptides against which the antibodies were raised.

The HSV-1 genome is shown with the unique long (U_L) and unique short (U_S), the terminal repeats (T_{RL} and T_{RS}), internal repeats (I_{RL} and I_{RS}) and the locus of gene UL39 coding region indicated. The position and sequence of the peptides used to produce amino-terminal (N1 and N2) and carboxy-terminal (C1 and C2) sera are shown. The tyrosine (Y) residues in parentheses are not part of the RR1 sequence but were added to facilitate coupling of the peptide to the carrier protein. The amino acid residue numbers of each peptide in this 1137 amino acid protein are also indicated. The triangle (∇) depicts the boundary of the unique amino-terminal domain. Antiserum C1 was raised by Dr H. Lankinen.



Antiserum designation	Peptide	Amino acid numbers
N1	MASRPAASSPVE(Y)	1-12
N2	TQTADVPTEAL(Y)	137-147
C1	RNSQFVALMPTA	959-970
C2	(Y)FGGDDNIVCMS	1114-1134

V.2 Production of anti-oligopeptide sera against HSV-1 RR1.

Figure 27 shows peptide sequences of RR1 against which antisera were raised. The HSV-1-induced RR1 polypeptide is sequentially degraded by proteolytic activity into three breakdown products M_r 100K, 90K and 80K of which at least the 90K species is enzymatically active (Ingemarson and Lankinen, 1987). From comparisons with equivalent RR1 polypeptides, there is an additional amino-terminal region of some 320 amino acids present in the HSV-1-induced RR1 which, except for HSV-2, is absent from all other RR1 polypeptides so far examined (Nikas et al. 1986). These results suggest that part of the 136K polypeptide may have functions not related to ribonucleotide reduction. Oligopeptide-induced sera were raised against sequences representing the unique amino domain and the carboxy domain of the HSV-1-induced RR1.

V.3 Reactivity of anti-oligopeptide sera with HSV-1-infected cells.

V.3(a)Immunoprecipitations.

The first RR1 peptides to be injected into rabbits were (Y)FGGDDNIVCMS and MASRPAASSPVE(Y). Two rabbits were injected with each peptide. Rabbits numbered 18379 and 18380 were immunised against (Y)FGGDDNIVCMS and rabbits numbered 18381 and 18382 were immunised with MASRPAASSPVE(Y). As shown in Figure 28, neither sera from the rabbits immunised with MASRPAASSPVE(Y) precipitated RR1 from HSV-1 infected cell extracts. This was probably due to the fact that this

Figure 28. Reactivities of antisera N1 and C2 in immunopreciptations.

Immunoprecipitations of polypeptides from [^{35}S]methionine-labelled HSV-1-infected cell extracts were carried out with antisera raised against peptide (Y)FGGDDNIVCMS (C2) and the first batch of peptide MASRPAASSPVE(Y) (N1) received from CRB. 18379 and 18380 represent sera from rabbits immunised against peptide (Y)FGGDDNIVCMS and 18381 and 18382 represent sera from rabbits immunised against peptide MASRPAASSPVE(Y). Monoclonal antibody 1100 which reacts specifically with the HSV-1-induced RR1 and antiserum 1149 which was raised against the carboxy-terminal seven amino acids of RR2 (peptide 1, Figure 24) were included as controls. Polypeptides labelled with [^{35}S] were eluted from Protein A-Sepharose-antigen-antibody complexes and separated on a 5% to 12.5% polyacrylamide gel.
MI mock infected cell extract; EXT HSV-1 infected cell extract; NRS normal rabbit serum; MA1100 monoclonal antibody 1100; CA control ascites.

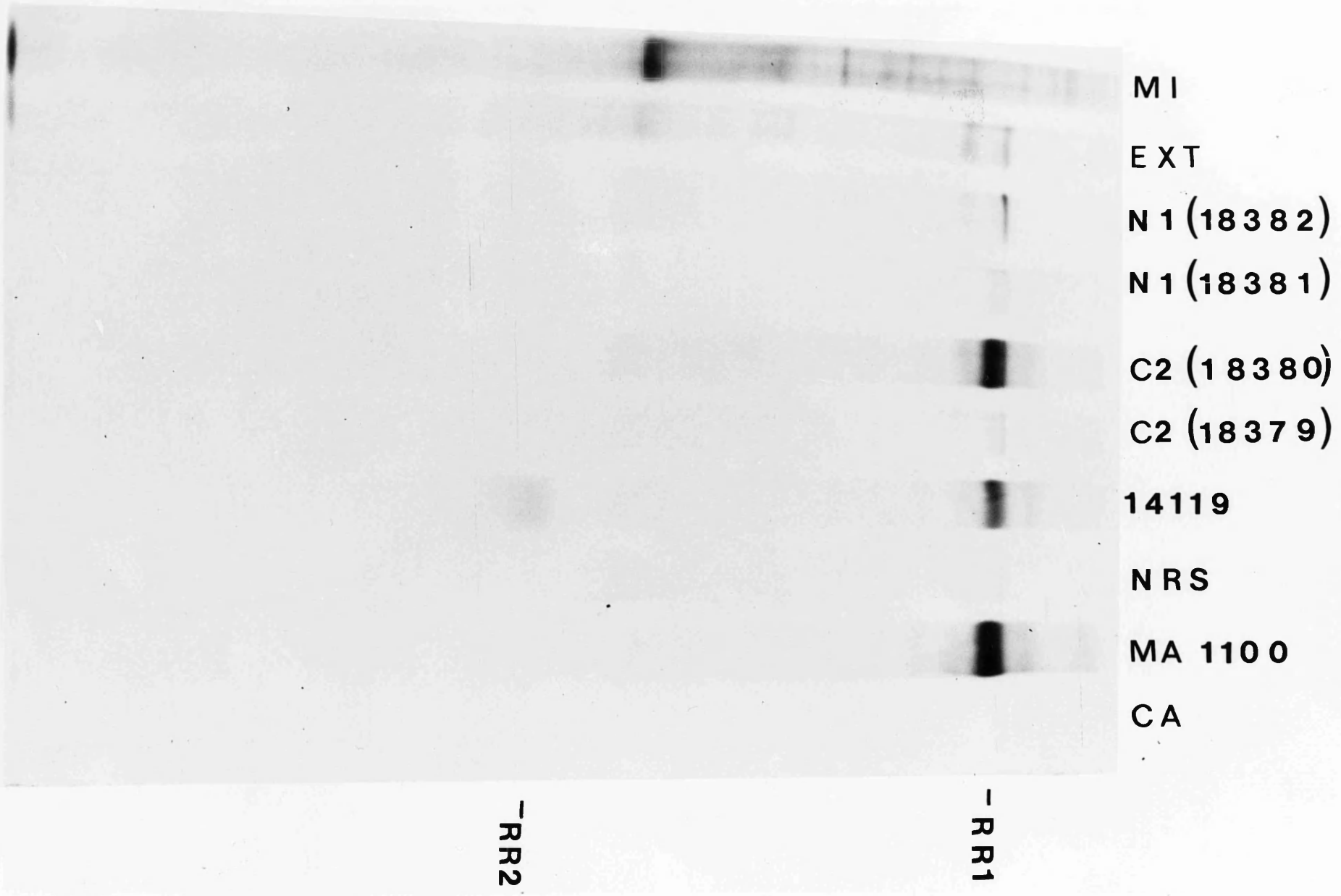
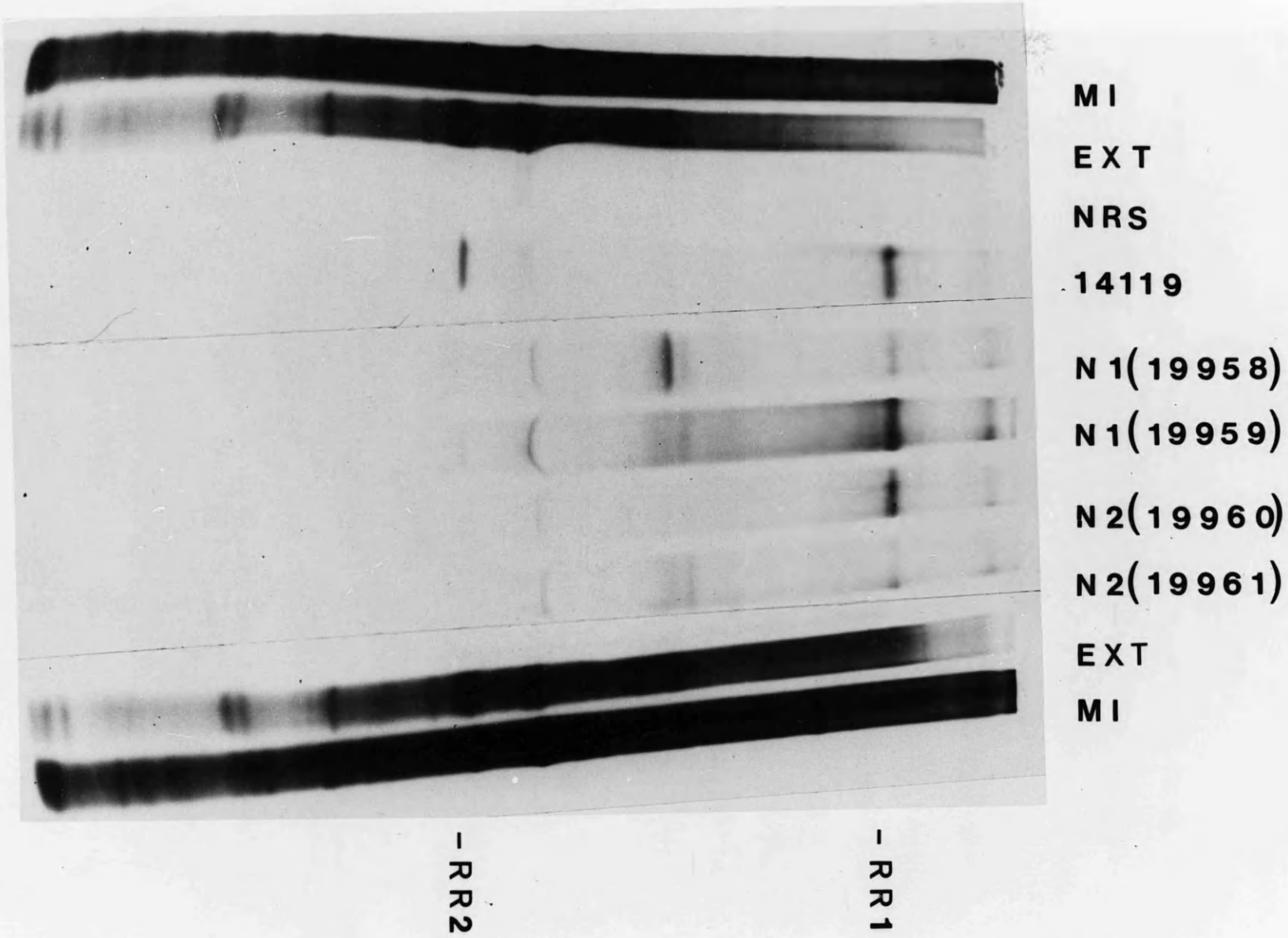

MI
EXT
N1 (18382)
N1 (18381)
C2 (18380)
C2 (18379)
14119
NRS
MA 1100
CA
RR2
RR1

Figure 29. Reactivities of antisera N1 and N2 in immunoprecipitations.

Polypeptides from HSV-1-infected cells were immunoprecipitated with antisera raised against peptides MASRPAASSPVE(Y) (N1) and TQTADVPTEAL(Y) (N2). Peptide MASRPAASSPVE(Y) was readily coupled to the BSA carrier molecule unlike the previous batch (Figure 28). 1995♂ and 1995♀ represent sera from rabbits immunised against MASRPAASSPVE(Y) and 1996○ and 1996◐ represent sera from rabbits immunised against TQTADVPTEAL(Y). Polypeptides labelled with [^{35}S] were separated on a 5% to 12.5% polyacrylamide gel.


MI
EXT
NRS
14119
N1(19958)
N1(19959)
N2(19960)
N2(19961)
EXT
MI
-RR2
-RR1

peptide was not readily soluble in the borate buffer used for coupling to the carrier protein and so the immunising dose of peptide may have been lower than expected. The serum from one of the rabbits (18380) immunised with the (Y)FGGDDNIVCMS precipitated a strong band corresponding to RR1 from HSV-1.

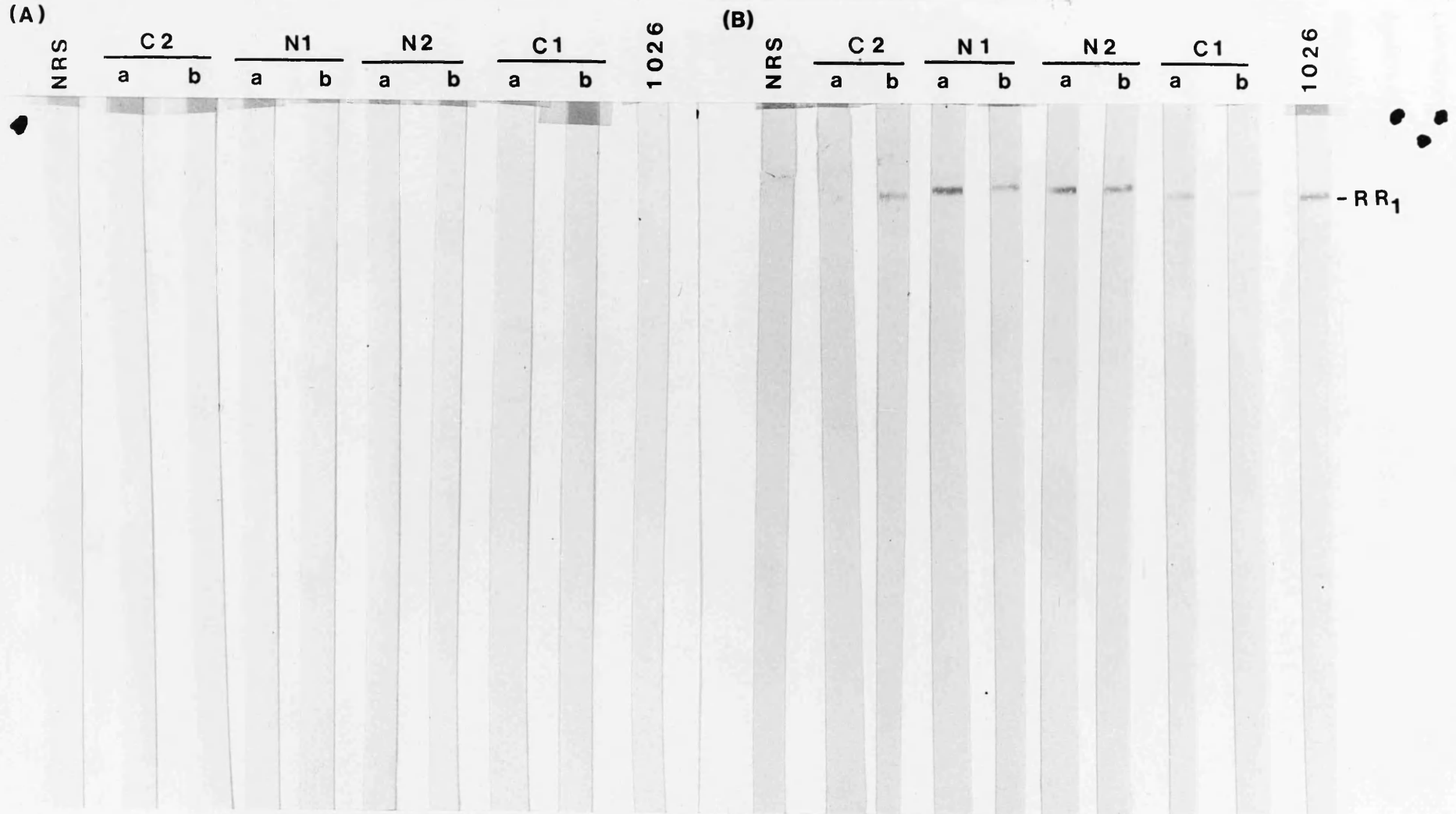
A new batch of MASRPAASSPVE(Y) was purchased which had a different appearance from the previous batch (a white fluffy powder as opposed to a fine grey powder) and was readily soluble in borate buffer. Two rabbits were injected with MASRPAASSPVE(Y) or TQTADVPTEAL(Y) Figure 29 shows the result of immunoprecipitations with the antisera obtained. Each of the four antisera precipitated levels of RR1 above background, however, the best results were obtained with antiserum 19959 (N1) and 19960 (N2). Antiserum 19958 precipitated a polypeptide M_r 60K which was not identified. None of the antisera raised against RR1 peptide sequences precipitated RR2.

V.3(b)Immunoblotting.

Figure 30 shows the result of immunoblotting with each of the antisera raised against RR1 peptides. Mock- or HSV-1-infected cell extracts were run on SDS polyacrylamide gels and the polypeptides were transferred to nitrocellulose. Each of the antisera was diluted either 1 part in 4 or 1 part in 20 and incubated with infected or mock-infected cell polypeptides bound to nitrocellulose. Protein A-peroxidase was added as a second antibody and proteins which had reacted with the antisera were visualised

Figure 30. Immunoblots of HSV-1-infected cell extracts with antisera raised against peptide sequences representing the HSV-1-induced RR1.

Cells were either mock-infected (A) or infected with HSV-1 (B) and [^{35}S]methionine-labelled extracts were prepared as described in the Methods section IX.1. Extracts were electrophoresed on a 5% to 12.5% SDS polyacrylamide gel and polypeptides were transferred to nitrocellulose filters. The filters were incubated with normal rabbit serum (NRS) at a 1 in 4 dilution or with antisera against each of the peptides C1, C2, N1, or N2 at either a 1 in 4 dilution (a) or a 1 in 20 dilution (b). Monoclonal antibody 1026 (provided by Dr. A Cross) was included as a control as it is known to react specifically with the HSV-1-induced RR1 in immunobots. Polypeptides which reacted with the antisera were visualised by staining with horseradish peroxidase conjugated Protein A.


(A)
NRS
C 2
a
b
N1
a
b
N2
a
b
C1
a
b
1026
(B)
NRS
C 2
a
b
N1
a
b
N2
a
b
C1
a
b
1026
- R R1

by staining with horseradish peroxidase development solution (Methods section IX). Each of the antisera reacted specifically with a high M_r band at both dilutions except C2 which only reacted with the band at a 1 in 20 dilution. This band was only present in infected cell extracts and from the [^{35}S]methionine-labelled polypeptide profile of the nitrocellulose strips was identified as RR1.

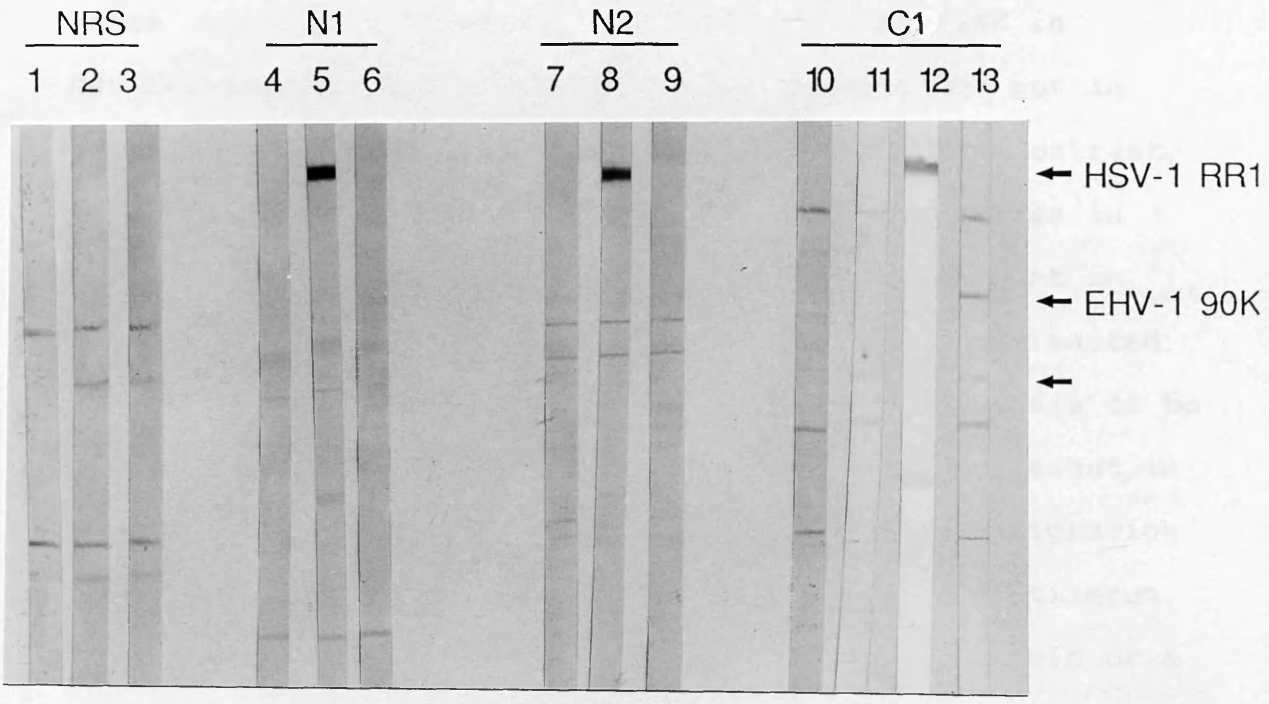
V.4 Reactivity of anti-oligopeptide sera with EHV-1-infected cells.

Dutia et al. (1986) showed that RR2$_\lambda$ (M_r 33K) was specifically immunoprecipitated from extracts of cells infected with EHV-1 by anti-peptide 1 serum but this antiserum failed to coprecipitate an EHV-1-induced RR1 polypeptide. Similarly, antisera against the HSV-1-induced RR1, described here, failed to react with any EHV-1-induced proteins in immunoprecipitations. Monoclonal antibody 7602, which reacts with both HSV-1- and HSV-2-induced RR1 in immunoprecipitations did not react with an EHV-1-induced polypeptide although IgG purified from this antibody could reduce the activity of the enzyme. It may be that the EHV-1-induced RR1 contains few methionine residues such that it is not detectable by [^{35}S]methionine-labelling or alternatively that the epitope recognised by monoclonal antibody 7602, which is conformational rather than sequential since the antibody does not react with blotted proteins, is not sufficiently conserved in the EHV-1 ribonucleotide reductase. It was therefore decided to try to identify the EHV-1-induced RR1 by immunoblotting with anti-oligopeptide sera raised against HSV-1-induced RR1.

Figure 31. Identification of the EHV-1-induced RR1 by immunoblotting with antisera raised against peptides representing the HSV-1-induced RR1.

Cells were either mock-infected (1,4,7,10,11) or infected with HSV-1 (2,5,8,12) or EHV-1 strain Kentucky A (3,6,9,13) and immunoblotting was carried out as described in Methods section IX. Extracts were electrophoresed on a 5% to 12.5% polyacrylamide gel and polypeptides were transferred to nitrocellulose membranes. Nitrocellulose filters were incubated with either normal rabbit serum (NRS), N1, N2 or C1 antisera. Antisera were at a 1 in 10 dilution (tracks 1 - 10) or a 1 in 20 dilution (tracks 11, 12 and 13). Polypeptides which reacted with the antisera were visualised by staining with horseradish peroxidase conjugated Protein A.

(Telford, Lankinen and Marsden, manuscript submitted for publication).


NRS
N1
N2
C1
1 2 3
4 5 6
7 8 9
10 11 12 13
HSV-1 RR1
EHV-1 90K

90mm plates of RK13 cells were either mock-infected or infected with 10pfu HSV-1 or EHV-1 (strain Kentucky A) and labelled overnight with [35S]methionine. The cells were then harvested, infected cell polypeptides were transferred to nitrocellulose and reacted with antisera N1, N2 and C1 which were raised against the HSV-1-induced RR1. The result is shown in Figure 31. As found previously, all three sera react with a polypeptide of apparent M_r 136K in HSV-1-infected cells (tracks 5, 8 and 12) but not in mock-infected cells (tracks 4, 7, 10 and 11). In contrast, neither N1 nor N2 were reactive with polypeptides in EHV-1-infected cells (tracks 6 and 9) not present in uninfected cells (tracks 4 and 7) while serum C1 reacted with two proteins (track 13). One of them appears to be a viral protein of apparent M_r 90K as it is not present in mock-infected cells (tracks 10 and 11). A faster migrating protein (arrowed) was also detected with the C1 antiserum. It could be a proteolytic fragment of the 90K protein or a cellular protein as there is a weakly reactive protein of this mobility in mock-infected cells. The control non-immune rabbit sera (NRS) reacted with some proteins in mock-infected and infected cells (tracks 1, 2 and 3) but these bands did not comigrate with the 90K protein.

Taken together these results suggest that EHV-1 encodes a smaller RR1 polypeptide than that of HSV-1 or HSV-2 which does not contain the amino-terminal domain that is so far unique to HSV types 1 and 2.

DISCUSSION

I Stability of the nonapeptide.

One of the aims of this project was to determine whether the inhibitory nonapeptide YAGAVVNDL was modified in cellular extracts and, if so, to identify the modified species produced. Results show that in the presence of HSV-infected cell extracts the nonapeptide was modified by hydrolysis of the peptide bond between the amino-terminal tyrosine and the neighbouring alanine to generate the octapeptide AGAVVNDL and free tyrosine. The octapeptide has previously been shown to have a markedly decreased inhibitory potency (IC_{50} = 283uM) compared with the nonapeptide (IC_{50} = 36uM) (Gaudreau *et al*. 1987).

This proteolysis of the nonapeptide could be prevented to some extent by the use of protease inhibitors PMSF and TPCK while pepstatin A, leupeptin and alpha 2 macroglobulin gave no protection to the peptide bond. The best protection was conferred by bacitracin, a cyclic polypeptide. However, the nonapeptide was still progressively modified in the presence of 1mM bacitracin. Other peptide bonds present in the nonapeptide sequence may also be susceptible to hydrolysis by cellular proteases but this would seem unlikely as no other modification products apart from tyrosine or the octapeptide were ever detected.

It is believed that proteins contain sets of amino acids that act to determine protein half-lives *in vivo*. Using an approach which makes it possible to expose *in vivo* different amino acids at the amino termini of otherwise identical test proteins, it has been shown that the amino-terminal residue

of the target protein was either stabilising or destabilising (Bachmair et al. 1986). In the yeast Saccharomyces cerevisiae the stabilising amino-terminal residues (Met, Gly, Val, Pro, Cys, Ser, Ala, Thr) confer a long half-life (> 20 hours) on the test protein β-galactosidase whereas the destabilising amino-terminal residues (Glu, Gln, Asp, Asn, Ile, Leu, Phe, Trp, Tyr, His, Lys, Arg) confer half-lives ranging from less than 2 minutes to approximately 30 minutes. In particular the test protein bearing tyrosine as an amino-terminal residue had a half-life of approximately 10 minutes whereas alanine conferred a half-life of more than 20 hours. This may therefore explain the instability of the nonapeptide YAGAVVNDL and the relative stability of the octapeptide AGAVVNDL which bears a stabilising alanine residue at its amino-terminus.

These results suggest that a means of protecting the peptide bond between the amino-terminal tyrosine and the neighbouring alanine of YAGAVVNDL would be necessary to produce an antiviral drug based on the nonapeptide. This may be possible by producing non-peptide analogues of the inhibitory nonapeptide.

II Inhibition of the EHV-1-induced ribonucleotide reductase.

II.1 Inhibition of EHV-1-induced ribonucleotide reductase by YAGAVVNDL.

Serological evidence for the conservation of the carboxy-terminal region of the HSV-1-induced RR2 in HSV-2, PRV, VZV and EHV-1 made it likely that the nonapeptide

YAGAVVNDL would inhibit a broad range of herpesviruses (Dutia et al. 1986). It has already been shown that a polyclonal antiserum directed against the carboxy-terminus of RR2 neutralised the PRV-induced reductase as well as the HSV enzyme and that the nonapeptide itself could specifically inhibit the PRV-induced ribonucleotide reductase activity (Cohen et al. 1987). The proposal that the nonapeptide might be inhibitory over a wide range of herpesviruses (Dutia et al. 1986) was further tested on the novel ribonucleotide reductase activity induced in cells infected with different strains of EHV-1.

In two separate experiments, ribonucleotide reductase partially purified from cells infected with EHV-1 strain Vol939 or Kentucky A was assayed in the presence of increasing concentrations of YAGAVVNDL. The results show the peptide inhibits the ribonucleotide reductase activity induced by both strains of EHV-1 (Figure 21 and Figure 22). Parallel experiments with the HSV-1-induced enzyme and the EHV-1 strain Kentucky A-induced enzyme showed that the concentration of peptide required to inhibit 50% of the ribonucleotide reductase activity was 28uM. This is approximately five times less than that required, in a separate experiment, to inhibit 50% of the ribonucleotide reductase activity induced by strain Vol939 (110uM). However, in that experiment with the Vol939 enzyme, the IC_{50} for the HSV-induced enzyme was 160uM. The difference in inhibitory potency of the nonapeptide in each of these experiments results from the fact that the strain Kentucky A-induced enzyme was assayed in the presence of 1mM bacitracin which protected the nonapeptide from modification

to the less inhibitory octapeptide AGAVVNDL. Strain Vol939-induced ribonucleotide reductase was assayed in the absence of bacitracin and therefore YAGAVVNDL was probably modified by proteolytic cleavage to produce the less inhibitory octapeptide thereby increasing the value of the IC_{50}. Both the EHV-1 strain Kentucky A-and the HSV-1-induced ribonucleotide reductase activities were unaffected by the presence of a control peptide (TSRLSDPNSSAY) in the assay mixture.

These data and the finding that the PRV-induced enzyme is inhibited by the nonapeptide support the proposition that an antiviral drug based on the nonapeptide might have activity against a broad range of herpesviruses.

II.2 Inhibition of EHV-1-induced ribonucleotide reductase activity by monoclonal antibody 7602.

Monoclonal antibody 7602 reacts specifically with the RR1 polypeptide in immune precipitations with HSV-1- and HSV-2-infected cell extracts (Dr A. Cross, unpublished data). This result suggests that monoclonal antibody 7602 recognises an antigenic domain on the HSV-1-induced polypeptide which is conserved on the HSV-2-induced polypeptide. IgG purified from monoclonal antibody 7602 can neutralise ribonucleotide reductase activity induced in both HSV-1- and HSV-2-infected cells (Dr A. Darling, unpublished data) and therefore the epitope which is conserved between these two viruses would appear to be important for enzyme activity. To determine whether the antigenic domain which this antibody recognises is conserved in another herpesvirus, the EHV-1-induced ribonucleotide reductase

activity was assayed in the presence of IgG purified from monoclonal antibody 7602 at either a 1 in 10 or a 1 in 100 dilution. In a parallel experiment with HSV-1-induced reductase, the enzyme activity was reduced to approximately 10% of the control enzyme activity at both concentrations of IgG. The EHV-1-induced ribonucleotide reductase activity was reduced to 50% of the control enzyme activity by IgG at a 1 in 10 dilution but enzyme activity was unaffected by IgG at a 1 in 100 dilution.

This result suggests that monoclonal antibody 7602 recognises an antigenic domain on the HSV-1-induced RR1 which is conserved in both the HSV-2 and the EHV-1 RR1 polypeptides. The fact that the virus-induced enzyme activity is neutralised in HSV-1- and HSV-2-infected cell extracts and at least reduced in EHV-1-infected cell extracts could suggest that the monoclonal antibody may bind to a site on RR1 to prevent the association of RR1 with RR2. Alternatively, the antibody may bind to a nearby site sterically hindering the subunit association, cause a conformational change in RR1 to block the association with RR2 or bind to the active site of the enzyme. Monoclonal antibody 7602 does not react with virus specific RR1 on immunoblots suggesting that the epitope is conformational rather than sequential. The observation that the antibody was considerably less inhibitory to the EHV-1-induced ribonucleotide reductase activity than to that induced by HSV-1 and HSV-2 is not yet understood but could be a consequence of only a partial amino acid sequence homology between shared epitopes or, less likely, of the epitope being less accessible.

III Regions of the carboxy-terminal domain of RR2 which are involved in subunit interaction.

Cohen et al. (1986) have shown that an antiserum raised against the inhibitory nonapeptide, YAGAVVNDL, had specific neutralising activity against the HSV ribonucleotide reductase. To determine whether other regions of the RR2 carboxy-terminus were involved at the site of interaction with RR1, antisera were raised against synthetic peptides 2, 3 and 4 (Figure 24). However, none of the antisera, including that raised against the inhibitory nonapeptide, were found to neutralise enzyme activity. It is likely that the antibody titre of the antiserum against YAGAVVNDL was too low to be inhibitory to the enzyme. As none of the antisera inhibited the ribonucleotide reductase activity, nothing could be concluded about other sequences which may be involved in interactions between RR1 and RR2.

Each of the antisera raised against the RR2 subunit coprecipitated RR1 in immune precipitations. However, each antiserum differed in the relative amounts of RR1 and RR2 which were precipitated. The reason for this is not clear.

IV Immunoblotting with antisera against peptides representing different regions of the RR1 polypeptide.

IV.1 Identification of a region of the RR1 polypeptide which is not required for enzyme activity.

Previous studies have revealed that truncated forms of the HSV RR1 polypeptide exist which can form a functional enzyme (Ingemarson and Lankinen 1987). To determine whether the amino-terminal domain, which is so far unique to the HSV-1 and HSV-2 RR1, is essential for ribonucleotide

reductase activity, antisera were raised against peptides corresponding to different regions of the HSV RR1. These antisera have been used in immunoblotting experiments with extracts of HSV-1-infected cells containing the full length RR1 as well as major truncated species of M_r 93K and 81K (Lankinen, Telford, MacDonald and Marsden, manuscript in press). The results of these experiments show that antisera N1 and N2 react with RR1 but fail to react with the truncated forms of the polypeptide whereas antisera C1 and C2 reacted with proteolytic fragments of M_r 93K and 81K in addition to the RR1 polypeptide. These data show that the enzymatically active proteolytic fragments of RR1 contain the carboxy-terminus but lack at least part of the amino-terminus.

Nikas et al. (1986) predicted that the amino-terminal domain of RR1 consisted of two distinct similar sized regions divided by a short stretch of prolines the first of which (amino acids 1-149) was predicted to form a domain of β-sheets with a well buried core of hydrophobic residues. The observation that antiserum N2, which does not react with the functional 93K species, is directed to a sequence just at the end of this region demonstrates that it is not necessary for enzyme activity. A third antiserum (N3) directed to an epitope within amino acids 297-311 which represents the junction between the amino and carboxy domains reacted with the 93K polypeptide (Lankinen et al. manuscript submitted for publication). The cleavage site for the 93K species must lie between amino acids recognised by antisera N2 (140) and N3 (308) and therefore its amino-terminus extends minimally to amino acid 309.

Antiserum N3 does not react with the 81K species and it is not known whether this polypeptide is enzymatically active.

Paradis et al. (1988) showed that the inhibitory nonapeptide YAGAVVNDL bound not only to intact RR1 but also to proteins with apparent M_rs 95K and 85K. These appear to be the two stable degradation products of RR1 equivalent to the 93K and 81K fragments. It can therefore be concluded that the nonapeptide interacts with the conserved carboxy 826 amino acids of RR1.

IV.2 Structural features of the EHV-1-induced ribonucleotide reductase.

In an attempt to identify the EHV-1-induced RR1 polypeptide, EHV-1-infected cell polypeptides were immune precipitated with each of the antisera raised against the HSV-1-induced RR1 but no viral specific polypeptides reacted with any of these antisera. However, in an immunoblotting experiment with EHV-1-infected cell extracts, antiserum C1 raised against the carboxy-domain of the HSV-1 RR1 reacted specifically with a viral protein of approximate M_r 90K. Antisera raised against peptides corresponding to the unique amino-terminal domain failed to react with any viral protein. It may be possible that the EHV-1 RR1 contains the amino-terminal domain and that peptide sequences MASRPAASSPVE(Y) and TQTADVPTEAL(Y) are not conserved between HSV-1 and EHV-1. However, this would appear unlikely as the EHV-1-induced polypeptide which reacts with antiserum C1 has a molecular weight similar to equivalent RR1 polypeptides which do not contain the amino terminal domain. Therefore these results suggest that the EHV-1 RR1 does not contain

the amino-terminal domain that is so far unique to HSV-1 and HSV-2.

The role of the unique terminus of the HSV RR1 subunit is not understood. However, the finding that a 93K truncated product which lacks at least the amino-terminal 140 amino acids of RR1 is enzymatically active (Lankinen et al. manuscript submitted for publication) suggests that at least part of the amino-terminal domain of the HSV amino-terminal domain is not required for ribonucleotide reduction. Efforts are at present directed toward the purification of enzyme containing native and truncated forms of the RR1 subunit to determine the role of the amino-terminal domain in ribonucleotide reduction. It is possible that the amino domain plays some other role unrelated to the reduction of ribonucleotides in the life cycle of the virus; perhaps in some aspects of regulation or in latency.

V Future Aims

Ribonucleotide reductase proteins induced by the human herpesviruses, HHV-6 and HCMV, have not yet been identified and therefore studies with antisera raised against peptides representing th carboxy-terminus of RR2 may be extended by attempting to identify the RR1 and RR2 polypeptides induced by these viruses.

Early attempts to investigate the proposed interaction between HSV-1-induced RR1 and RR2 using an ^{125}I-labelled cross-linking failed as the ^{125}I label was transferred not only to RR1 but also to other major proteins in the extract in amounts proportional to their relative abundance.

Failure to demonstrate specific transfer may have been caused by an alteration of the conformation of the peptide hindering the specific interaction with RR1. It may now be possible to perform these experiments using the tritium-labelled nonapeptide to show directly by UV cross-linking the tritiated nonapeptide to proteins in infected cell extracts that the nonapeptide binds specifically to RR1 and from there to identify the amino acid sequence of RR1 involved in the interaction with RR2.

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IgG against peptide	% enzyme activity
2	87
3a	95
3b	96
4a	86
4b	95

IgG from antisera against peptides YAGAVVNDL (3a, 3b) HTNFFECRSTSY (2) and RSTSYAGAVV (4a and 4b) were tested in ribonucleotide reductase assays for their ability to neutralise enzyme activity. The enzyme activity in a control assay with no IgG was taken as 100%.