STUDIES OF THE PHOSPHORYLATION OF THE HERPES SIMPLEX VIRUS TYPE 1 ENCODED DEOXYRIBONUCLEASE & RIBONUCLEOTIDE REDUCTASE

A thesis submitted to the University of Glasgow
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by

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'Sweet dreams are made of these
Who am I to disagree
I travel the world and the seven seas
Everybody's looking for something'

Sweet Dreams
Annie Lennox

I am the dark-avised, the widower, the inconsolable,
The Prince of Aquitaine before his ruined tower:
My only star is dead; and now my jewel-studded lute
Will only bear the blackened sun of Melancholia .....

My forehead is red yet with the kiss of the queen
I have dreamed in the grotto where the siren swims
And twice I have crossed the Acheron, triumphant .....

El Desdichado
Gerad de Nerval

'In my distress, well I wanted someone to blame me
In my devastation.... I wanted so to change
In my way.... disaster was the only thing that I
could depend on..
But it's alright,... I'm doing the best that I can'

S. Nicks

'She is my sun and ever she eclipses me, burning me with her fire. Yet before I go, for one time at least I shall outshine her ; supernova into oblivion, to end in darkness as it was begun.

Yet I cannot not love her for her spirit shines bright beyond belief for all to see. Yet ever it flames in my mind, slowly consuming me. She knows this not and I would never say. For she is my sun and no one wishes to live their life in perpetual darkness.'

D.G.H.

'Oh wad some power the giftie gie us
To see oursels as ithers see us!
It wad frae mony a blunder free us,
And foolish notion:
What airs in dress and gait wad lea'e us,
And even devotion!

To A Louse
Robert Burns

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ABBREVIATIONS

ADP	adenosine 5' diphosphate
AMP	adenosine 5' monophosphate
cAMP	3'5' cyclic adenosine monophosphate
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
AZT	3' azido deoxythymidine
BHK	Baby hamster kidney
bp	base pairs
BSA	bovine serum albumin
ccc	covalently closed circles
dCTP	deoxycytidine triphosphate
D(Da)	dalton
1D	one dimensional
2D	two dimensional
DAG	diacylglycerol
DBP	DNA binding protein
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DR	direct repeat
ds	double stranded
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol bis (β amino ethyl ether) N,N,N',N' tetra acetic acid
FPLC	fast protein liquid chromatography
GMEM	Minimal essential medium Glasgow modification
cGMP	3'5' cyclic guanosine monophosphate
GTP	guanosine triphosphate
HIV	human immunodeficiency virus
HCMV	human cytomegalovirus
HHV6	human herpes virus 6
HRP	horseradish peroxidase
HSV-1	herpes simplex virus type 1

HSV-2	herpes simplex virus type 2
IE	immediate early
l	linear
LAT	latency associated transcript
NAD	nicotinamide adenine dinucleotide
NP40	Nonidet 40
oc	open circles
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
pi	post infection
PI 3 kinase	phosphatidyl inositol 3 kinase
PMSF	phenyl methyl sulphonyl fluoride
PP	protein phosphatase
PRV	pseudorabies virus
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
RR	ribonucleotide reductase
RR1	ribonucleotide reductase : large subunit
RR2	ribonucleotide reductase : small subunit
RSB	reticulocyte standard buffer
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ss	single stranded
SVP	snake venom phosphodiesterase
SV40	simian virus 40
TCA	trichloroacetic acid
TEMED	NNN'N' tetra methylene diamine
αTIF	α trans-inducing factor
TPCK	tosyl-L-phenylalanine chloromethyl ketone
dTTP	deoxythymidine triphosphate
UL	unique long
US	unique small
VSV	vesicular stomatitis virus

CHAPTER 1

SUMMARY

SUMMARY

The herpes virus, herpes simplex type 1 (HSV-1) contains around 70 genes, most of which are expressed during lytic infection of BHK cells in culture. Two of the enzymes encoded by herpes simplex type 1 were studied using enzymatic, electrophoretic and immunological techniques.

The deoxyribonuclease

The deoxyribonuclease or DNase is encoded by gene UL12 of herpes simplex type 1. The enzyme has both exo- and endonuclease activity and appears to be essential for virus growth in cell culture. The function of the DNase during lytic infection is as yet unknown.

Modification of the DNase

Two dimensional gel electrophoresis and subsequent immunoblotting of the purified DNase showed a complex pattern made up of many spots which indicated that the protein was modified. The immunoblot pattern remained complex throughout the infection, although the distribution of protein between the spots varied and seemed to indicate a reduction in modification at late times in the infection. The effect of various phosphatases on the DNase indicated that at least part of the modification was due to phosphorylation of the enzyme. There may also be an element of ADP ribosylation but this remains unconfirmed.

Phosphorylation, *in vitro*, of the DNase by various kinases showed that cAMP dependent protein kinase, protein kinase C, casein kinase II and the US3 protein kinase of HSV-1 could utilise it as a substrate. Labelling with [^{32}P] in cell culture showed the DNase to be phosphorylated during HSV-1 infection.

Tryptic peptide analysis of *in vitro* phosphorylated DNase by the above kinases when compared to the tryptic peptide

analysis of [^{32}P] labelled DNase purified from infected cells failed to resolve which kinases were responsible for the phosphorylation of the DNase in cell culture.

DNase endonuclease activity

The DNase has an endonuclease function as well as exonuclease activity. This was studied in an assay where only the endonuclease function of the DNase is active. A plasmid containing the sequences required for the cleavage and packaging of concatenated HSV-1 DNA was used as a substrate for the enzyme. Under the conditions employed in the assays, purified DNase had no endonuclease activity. DNase treated with protein phosphatases 1 or 2A also had no endonuclease activity. High speed supernatants from both infected and uninfected cells had endonuclease activities. When they were used to cleave the substrate described above in the presence of purified DNase the pattern of digestion was altered from that in the absence of the purified enzyme. This suggested that the DNase was either affording protection to the plasmid or it was being activated by a component(s) of the supernatant. Samples collected from the various stages of the purification of the DNase all exhibited endonuclease activity against the plasmid except for the final purified enzyme. This may mean that a factor(s) required by the DNase for endonuclease activity was lost at the DNA Cellulose chromatography step of purification.

The Antiviral xanthates

The antiviral activity of xanthates has been reported against a wide range of both DNA and RNA viruses including herpes simplex. This activity was reported to have been mediated through the inhibition of protein kinases. This was investigated using partially purified protein kinases, cellular and viral, from HSV-1 infected BHK cells in culture and three xanthate compounds D609,D611exo and endo (isomers) synthesised by Glaxo Group Research chemists. The xanthates inhibited all of the kinases tested at mM concentrations, an order of magnitude higher than the concentrations required for antiviral activity in cell culture. The best inhibitor

was D609 with D611exo and D611endo similar in activity. Using the HSV-1 encoded US3 kinase the inhibition was shown to be competitive with respect to ATP.
Infection of HSV-1 in cell culture in the presence of the xanthate D609 with subsequent two dimensional electrophoresis and immunoblotting of the purified DNase showed that the presence of the xanthate significantly reduced the phosphorylation of the DNase. Since the xanthates have recently been reported as blocking the activation of protein kinase C at the phospholipase C level this indicates that the DNase is phosphorylated in cell culture by protein kinase C or a kinase activated by protein kinase C.

The Ribonucleotide reductase

The viral ribonucleotide reductase is composed of two subunits encoded by genes UL39 and UL40 of HSV-1. The enzyme has the overall structure $\alpha_2\beta_2$, with a small subunit (RR2) of 38kD and a large subunit (RR1) of 136kD. The enzyme catalyses the reduction of ribonucleotides to deoxyribonucleotides. The large subunit also contains an N terminal protein kinase domain, the function of which is as yet unknown.

Modification of ribonucleotide reductase large subunit

Although the intact large subunit was present on immunoblots of one dimensional gels, using the two dimensional electrophoresis system only a 60kD C terminal fragment of the large subunit could be obtained. The fragment was present at all time points and allowed comparisons to be made. The 60kD fragment at 3 - 9 hr. post infection was modified, this modification was reduced after 9 hrs. and further reduced up to 18 hrs. post infection. Literature reports showing that the large subunit is phosphorylated in cell culture allow this result to be interpreted as a decrease in the phosphorylation of the large subunit in the course of the infection. No information is available concerning the kinase responsible for this, however, the large subunit has been shown to undergo autophosphorylation.

CHAPTER 2

INTRODUCTION

INTRODUCTION

1. Herpes Viruses

1.1 Herpes Viruses (see The Herpes Viruses Vol. 1 - 4 ed Roizman B. 1982 - 1985)

Herpes viruses are a large family of viruses found to be widespread in vertebrates including fish, birds and mammals. The herpes viruses as a group are characterised by: 1. The structure of their virions which consist of an icosahedral capsid containing the viral DNA, surrounded by an amorphous layer known as the tegument, which in turn is surrounded by a lipid bilayer containing virally coded glycoproteins (Figure 2.1). 2. The basic infectious procedure, by which viral genes are transcribed by cellular RNA polymerase II and viral DNA synthesis and capsid assembly take place in the nucleus of the infected cell. The capsids then acquire the tegument and the lipid bilayer by budding through the inner nuclear membrane (Section 2.1). 3. The genomes of the herpes viruses are large double stranded DNA molecules, 120 - 230 kbp, and highly complex (See Section 1.2). 4. They have the ability to establish a latent infection in certain cell types in their hosts (Section 1.3).

The herpes viruses are subdivided into three subfamilies depending on their biological behaviour. The three sub-families are the alpha, beta and gamma herpes viruses and some examples of each are shown in Table 1.1. The alpha herpes viruses tend to replicate quickly in permissive cells, killing the host cell. They are also able to establish a latent infection in neurons near the site of infection. The beta herpes viruses relicate slowly in permissive cells, causing cell enlargement and eventual cell death. They are able latently to infect various tissues. The gamma herpes viruses tend to form chronic infections preferentially in lymphocytes which they also latently infect. They have also been linked to cellular transformation. There are six known human herpes viruses, five of these are shown in Table 1.1.

Table 1.1 The Human Herpes Viruses

Subfamily	Basis of Classification	Virus	Size (kbp)	Disease
Alpha	Neurotropic	Herpes Simplex Type 1 (HSV-1)	152	Cold sores, fever blisters, eye & brain infections
		Herpes Simplex Type 2 (HSV-2)	150	Genital ulcers
		Varicella zoster (VZV)	124	Chickenpox in children & adults, Shingles in adults
Beta	Cytomegalotropic	Human Cytomegalovirus (HCMV)	230	Mononucleosis, eye, kidney, brain & congenital infections
Gamma	Lymphotropic	Epstein-Barr (EBV)	172	Infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma

The sixth virus is human herpes virus-6 (HHV6) which can proliferate in T or B lymphocytes and has been implicated in the transient childhood illness exanthem subitum.

1.2 Herpes virus Genomes (McGeoch 1989)

The genomes of the six human herpes viruses have been isolated and their genomes either sequenced completely (HSV-1, VZV, EBV & HCMV (not published) or partially sequenced (HHV-6 & HSV-2). The HSV-1 genome of 152 kbp consists of two unique regions, the unique long (U_L) and the unique small (U_S) which are bordered by internal and terminal repeats (Figure 1.2). The U_L and U_S regions can invert with respect to each other and so produce the four possible genomic orientations shown in Figure 1.2. The so called a sequence is responsible for the genomic inversions (Mocarski & Roizman 1982, Chou & Roizman 1985). The a sequence also occurs as terminal redundancies and contains the signals necessary for the cleavage and packaging of unit genomic lengths (Mocarski & Roizman 1982, Stow et al 1983, Varmuza & Smiley 1985, Diess et al 1986, Diess 1986).

The HSV-1 genome contains at least 72 genes; 56 in U_L, 12 in U_S and one in each copy of the repeat units, which produce 70 distinct proteins, but it is possible that some genes remain undiscovered in the genome. When compared to HSV-2 the two are very similar, in overall genomic structure, they are colinear, and in the sequence homology between their respective genes and gene products. The proteins produced by the HSV-1 will be discussed in Section 2.2.

The genome of VZV is similar to that of HSV-1 (Figure 1.2) and has a similar gene organization in the U_L region. VZV has 70 genes which encode 67 proteins, most of which have counterparts in HSV-1, but some, notably the VZV U_L genes 1, 2, 13, 32 & 57, have no counterparts in HSV-1. The VZV genome also lacks equivalents to the HSV-1 U_S genes US2, 4, 5, 6, 11 & 12 and the U_L genes UL45 & 56.

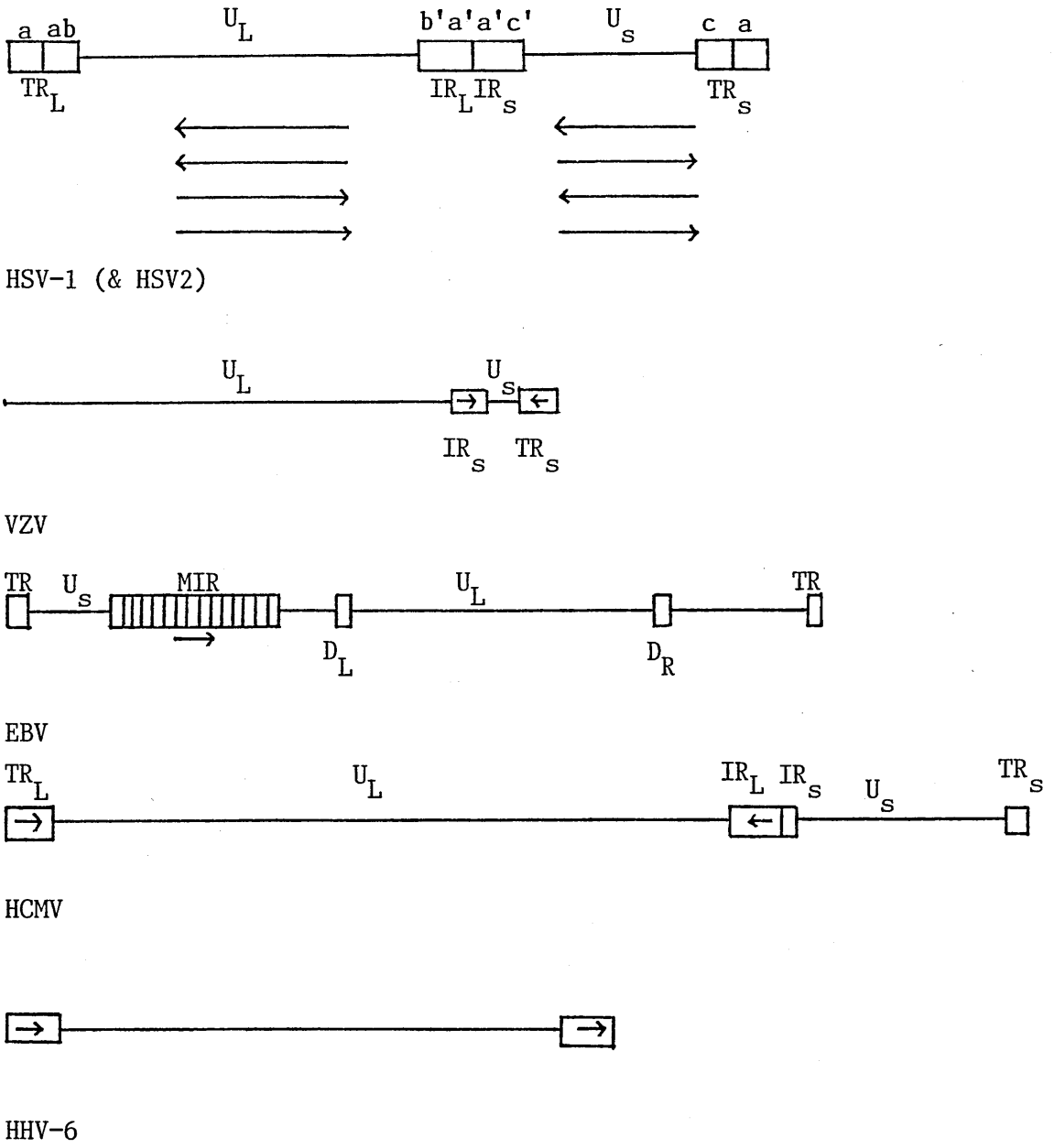


Figure 1.2 The overall structure of the human herpes viruses (not drawn to scale)

U_L = Unique long region
U_s = Unique short region
TR_L = Terminal repeat of U_L
IR_L = Internal repeat of U_L
TR_s = Terminal repeat of U_s
IR_s = Internal repeat of U_s

MIR = Major internal repeat
D_L & D_R = 1000bp regions whose sequences are almost identical

The arrows below the HSV genome indicate the possible genome orientations

EBV has little overall similarity to the HSV-1 genome (Fig. 1.2), though genes common to EBV, VZV & HSV-1 have been found. The genome organization differs in overall structure and local gene arrangements. EBV contains no equivalents to the genes in the U_S regions of VZV and HSV-1. 29 genes in EBV have been found to have equivalents in VZV and HSV-1 by amino acid homology of the predicted gene product. 14 genes have tentatively been identified by their genomic locus.

The HCMV genome has an overall similarilty to the HSV-1 genome but is much larger (Fig. 1.2). The full sequence of the HMCV genome has not yet been published but from the data available it has been shown that it contains a DNA polymerase gene with homology to VZV, EBV & HSV-1. An equivalent of the HSV-1 glycoprotein B has also been located. The U_S region has no similarities with any other herpes virus genes and contains around 38 open reading frames which make up five multigene families. The HCMV genome also contains the a sequence terminal redundancies which have been shown to direct cleavage/packaging in defective HSV-1 genomes (Spaete & Mocarski 1985).

The genome of HHV-6 has not yet been fully sequenced but several homologies to other herpes virus genes have been reported. Though the herpes virus genomes vary in size, composition and genetic layout, similarities also occur. The presence of certain genes in all herpes viruses suggests that these were present in the common ancestor of the viruses. The other genes acquired by the herpes viruses, presumably from their hosts genome , may represent adaptations to particular host cells and particular conditions.

1.3 Latency (Roizman & Sears 1987)

Latency is the ability of a virus to be present in cells without undergoing a productive infectious cycle. Only limited viral functions occur in this state and the process is poorly understood.

In the case of HSV-1, the virus can latently infect neurons near the site of the initial productive infection. The latent virus under certain conditions can be reactivated causing a productive infection at or near the site of the initial infection e.g. cold sores produced by HSV-1 heal with time but occur again at any time.

The viral DNA has been shown to occur in two forms in the nuclei of latently infected cells. It is either found integrated into the host cell genome or as circular DNA molecules. EBV has also been shown to occur in these two forms in its latently infected host cells. Viral DNA has also been shown to be amplified in latently infected neurons, but the mechanism involved is not viral in origin (i.e. not the viral DNA polymerase) and is not understood.

Animal models have been used to study HSV-1 latency, as no cell culture system is available for this, but each system has its own drawbacks as outlined in Roizman & Sears (1987).

It has been reported that in neurons latently infected with HSV-1 certain RNA transcripts exist (Wagner et al 1988, Spivack & Fraser 1988) which are known as latency associated transcripts or LAT's. One of the LAT's has been shown to be complementary (antisense) to the ICP 0 (α0) gene (Gordon et al 1988, Spivack & Fraser 1988). The function of these LAT's remains unclear but they have been shown to decrease prior to reactivation of the infectious cycle.

Reactivation of latent virus to a productive infectious cycle has been shown to be induced by various conditions such as stress. Reactivation of HSV-1 latently infected neurons *in vitro* has been induced by the deprivation of nerve growth factor (Wilcox & Johnson Jr. 1987, 1988) and

by the addition of 5'Azacytidine to HSV-2 latently infected neurons *in vitro* (Stephanopoulos et al 1988). The mechanism for the nerve growth factor deprivation reactivation is not known but 5'Azacytidine reduces deoxy-cytidine methylation in DNA and has been reported to activate quiescent virus genes which suggests a role for DNA methylation in HSV latency and reactivation.

2. Herpes Simplex Virus Type 1 Infection

2.1 The Viral Infectious Cycle

The infectious particle, known as the virion, (Fig. 2.1) is composed of the viral DNA enclosed in a protein capsid. This nucleocapsid (capsid plus viral DNA) is surrounded by an amorphous, protein containing matrix known as the tegument which in turn is enclosed by a lipid bilayer, which is derived from the inner nuclear membrane of infected cells, into which are embedded viral glycoproteins. The infectious cycle (Figure 2.2) starts with the binding of the virion to a receptor, as yet unknown, on the surface of the permissive cell. This binding may initially involve cellular heparan sulphate (Lycke et al 1991), it also involves the viral glycoproteins on the virion surface and can be blocked by various compounds such as heparin and polylysine (Langeland et al 1988). The viral glycoproteins are also involved in the internalisation of the nucleocapsid which involves the fusion of the cell membrane and the virion bilayer. Internalisation and subsequent transport of the nucleocapsid and associated tegument proteins in someway involves the cytoskeleton (Rosenthal et al 1985).

The nucleocapsid is transported to the nucleus where the viral DNA is released and circularises (Mocarski & Roizman 1982).

A component (or components) of the virion, released on nucleocapsid entry mediates the shutdown of cellular protein synthesis and the degradation of cellular mRNA (Fenwick & Clark 1982, Kwong et al 1988).

Viral gene transcription and translation are regulated by a sequential cascade mechanism (Honess & Roizman 1974) and the viral genes can be divided into three main types: immediate-early or αgenes, early or βgenes and late or γ genes. Transcription utilises the cellular RNA polymerase II enzyme, αgene transcription is initiated by a protein associated with the capsid, the α transinducing factor (αTIF), UL48) (Kristie & Roizman 1988, Ace et al 1988).

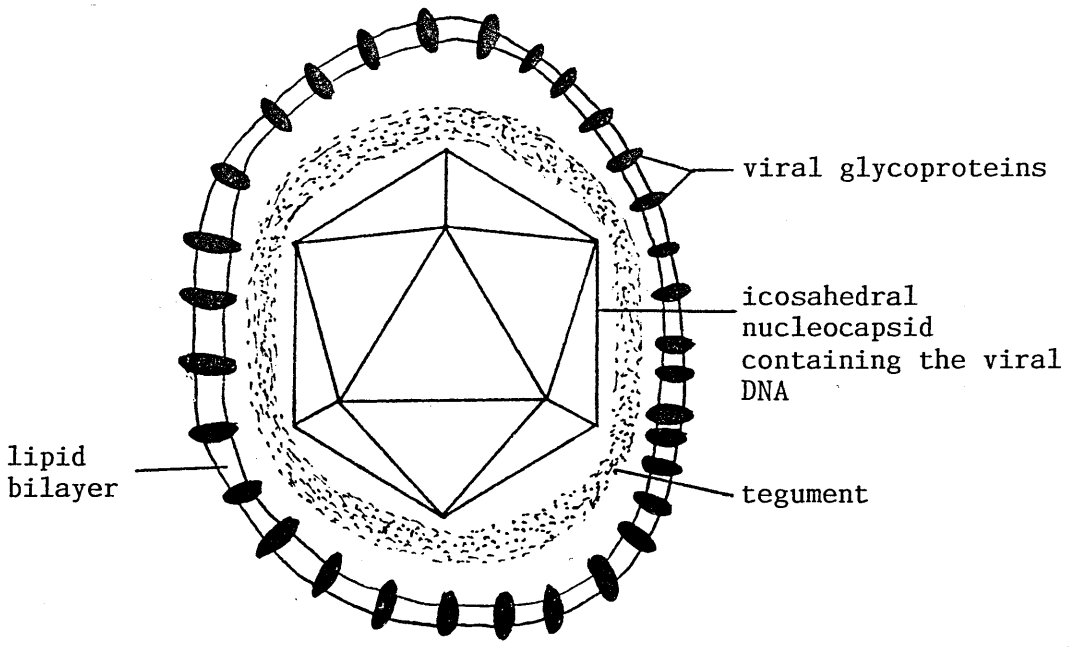


Figure 2.1 The structure of the HSV-1 virion

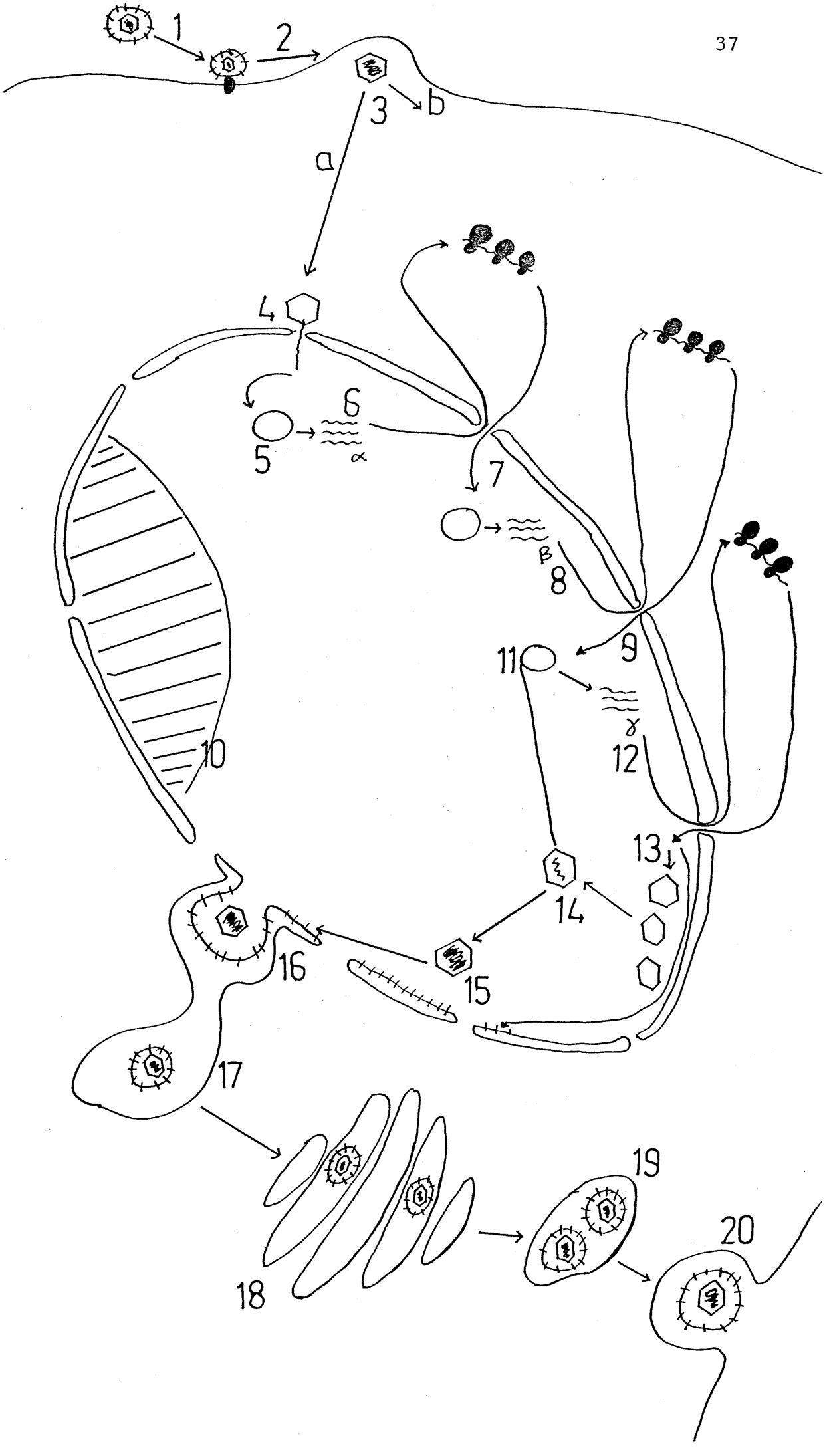
The α gene products are involved in the regulation of subsequent transcription from the viral genome. The α genes are also themselves regulated by some of their subsequent gene products (Mackem & Roizman 1982, De Luca and Schaffer 1988, Hubenthal-Voss et al 1988). The β genes are regulated by the products of the α genes, the products of the β genes include those involved in viral DNA synthesis and replication such as the viral DNA polymerase, thymidine kinase and ribonucleotide reductase. The γ genes require DNA synthesis in addition to the trans-acting factors for their expression (Mavromara-Nazos & Roizman 1987). The γ genes encode structural proteins involved in the production of the protein capsid, the tegument and the glycoproteins.

The viral DNA circularises in the nucleus (Challberg & Kelly 1989) and is replicated, probably by a rolling circle mechanism, the details of which are not well understood. Replication results in the production of large head-to-tail viral concatemers. This concatenated viral DNA is cleaved to unit genomic lengths, with the cleavage linked to the packaging of the genome into the capsid. The signals responsible for the cleavage and subsequent packaging of viral DNA are contained within the terminally redundant a sequence (Vlazny et al 1982, Moscarski & Roizman 1982, Stow et al 1983, Varmuza & Smiley 1985, Diess et al 1986, Diess 1986).

The capsid proteins assemble in the nucleus, along with the tegument proteins and the viral glycoproteins which are inserted into the inner nuclear membrane (Stephens & Compans 1988). The viral DNA is packaged into the assembled capsid and its associated proteins and the whole structure buds through the inner nuclear membrane into the perinuclear space. The perinuclear space is contiguous with the endoplasmic reticulum through which the immature virion passes. In the Golgi apparatus the virion glycoproteins are processed and the mature virions pass to the cell surface via. exocytotic vesicles. These vesicles fuse with the cell membrane releasing the virions into the extracellular space.

Figure 2.2 Schematic representation of the HSV-1 productive life cycle.

The virion binds to a receptor on the cell surface (1) and introduces the nucleocapsid into the cell via. a membrane fusion event (2). The nucleocapsid is transported to the nucleus (3a) and a tegument protein mediates the shut-off of host protein synthesis (3b). The viral DNA is ejected into the nucleus (4) and circularises (5). The α mRNA's are transcribed, translocated to the cytoplasm and translated (6). The α proteins mediate transcription of the β genes (7). The β mRNA's are transcribed, translocated to the cytoplasm and translated (8). β proteins are involved in DNA metabolism prior to viral DNA replication. Cellular chromatin breaks down and is marginated to the sides of the nucleus (10). Viral replication occurs via rolling circle methods (11). γ genes are transcribed, translocated to the cytoplasm and translated (12). The capsid proteins are transported to the nucleus where they are assembled (13). Concaten ated viral DNA is cleaved and packaged into capsids (14). Nucleocapsids bind additional proteins (15). Capsids bud through the inner nuclear membrane acquiring a lipid bilayer containing viral glycoproteins (16). Immature virions are translocated via the peri-nuclear space to the endoplasmic reticulum (17). The virion glycoproteins are matured in the Golgi apparatus (18) and the mature virions travel via exocytotic vesicles (19) to the cell surface where the vesicles fuse with the cell membrane releasing the virus (20).


1
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γ
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2.2 The HSV-1 Virally Coded Proteins

2.2.1 Viral Proteins

The 70 proteins encoded by the HSV-1 genome (McGeoch 1989, McGeoch et al 1988) can be loosely divided into the three classes into which their genes are grouped, namely α, β & γ. The α proteins appear mainly to be involved in the subsequent regulation of the viral genes, the β proteins are mainly enzymes some of which are required for DNA metabolism and replication and the γ proteins are involved in the formation of the virion. The proteins involved in control and regulation of the virus life cycle consist of the immediate-early proteins and UL48 which is synthesised as a γ protein, and is a component of the virion. UL48 or the α-transinducing factor (Ace et al 1988, Kristie & Roizman 1988), αTIF, initiates the transcription of the α genes (IE110, UL54, IE175, US1, US12) by binding to specific sequences in the regulatory domains of these genes. Three of these gene products IE110, IE175 & UL54 (or α0, α4 & α27 respectively) are known to be transcriptional regulatory proteins. The α gene products are known to repress the expression of their own genes (De Luca & Schaffer 1988).

The early proteins are predominantly enzymes or have functions in viral DNA replication. The enzymes found to date are mainly those involved in DNA metabolism (thymidine kinase, deoxyuridine triphosphatase, ribonucleotide reductase (Section 4)), DNA replication (DNA polymerase, helicase-primase ssDNA binding protein, ori binding protein) or DNA repair (Uracil DNA glycosylase). In vitro studies have shown that of the herpes proteins involved in DNA metabolism, 7 are necessary and sufficient for origin dependent replication (Challberg & Kelly 1989, Wu et al 1988, McGeoch et al 1988, Crute & Lehman 1991).

These proteins are the viral DNA polymerase (UL30), the helicase-primase (UL5/UL8/UL52), the single stranded DNA binding protein (UL29), the origin binding protein (UL9) and the processivity enhancing factor (UL42).

The deoxyribonuclease (UL12) may be involved in the cleavage/packaging of concatenated DNA and will be discussed further in Section 3.2.

The other group of enzymes so far found in HSV-1 are the protein kinases, US3 and the predicted kinase of UL13. These will be discussed in Section 5.3.2.

The γ proteins include those required for the production of the virion, the capsid proteins, the tegument proteins and the glycoproteins. The virion consists of ~30 proteins, some of which have a purely structural role, e.g. the formation of the capsid (major capsid protein UL19). Other proteins, including UL48, the αTIF, or UL41 which is responsible for the virally mediated host shut-off mechanism (Kwong et al 1988), are required in the early stages of the virus life cycle for a productive infection to be established. Proteins associated with the nucleo capsid may also have a role in the packaging of viral DNA into newly formed capsids (UL26, UL33, UL6).

The seven viral glycoproteins found so far are synthesised as γ proteins and are inserted into the inner nuclear membrane. The glycoproteins are required by the virion in order to form an infectious particle. gB is required for the entry of the nucleocapsid via a membrane fusion event (Stephens & Compans 1988, Cai et al 1988), gD is also involved in virus penetration into cells (Petrovski et al 1988, Ligas & Johnson 1988), gH is required for the virion to be infectious (Desai et al 1988) and gE and gI form a complex which acts as an IgG Fc receptor (Johnson et al 1988) which may reduce immune cytolysis of virally infected cells.

Many of the HSV-1 virally coded proteins have no assigned function, but with advancing techniques and increasing sequence information in the various data banks, this is not likely to remain the case for long.

Table 2.3 The HSV-1 Encoded Proteins *

Gene	Function (where known)	Class	Requirement for growth in cell culture
IE110	IE transcription regulator	α	n
UL1			
UL2	Uracil DNA glycosylase	β	n
UL3			n
UL4			n
UL5	helicase/primase component	β	e
UL6	Virion protein : DNA packaging		e
UL7			
UL8	helicase/primase component	β	e
UL9	ori binding protein	β	e
UL10			n
UL11			
UL12	Deoxyribonuclease	β	e
UL13	predicted protein kinase		
UL14			
UL15			
UL16			n
UL17			
UL18			
UL19	Major capsid protein	γ	e
UL20			
UL21			
UL22	glycoprotein H	γ	e
UL23	thymidine kinase	β	n
UL24			n
UL25	Virion protein	γ	e
UL26	DNA packaging		e
UL27	Glycoprotein B	γ	e
UL28	Structural protein	γ	e
UL29	ssDNA bindng protein	β	e
UL30	DNA polymerase	β	e

Gene	Function (where known)	Class	Requirement for growth in cell culture
UL31			
UL32	Structural protein	γ	e
UL33	Structural : DNA packaging	γ	e
UL34	Virion protein		
UL35			
UL36	very large tegument protein	γ	e
UL37			
UL38	Capsid assembly		e
UL39	Ribonucleotide reductase large subunit	β	n
UL40	" " small "	β	n
UL41	host shut off protein	γ	
UL42	processivity enhancing factor	β	e
UL43			
UL44	Glycoprotein C	γ	n
UL45			
UL46			n
UL47			n
UL48	Major tegument protein αTIF	γ	
UL49			
UL50	Deoxyuridine triphosphatase	β	n
UL51			n
UL52	primase/helicase component	β	e
UL53			
UL54	IE transcriptional regulator	α	e
UL55			n
UL56			n
IE175	IE transcriptional regulator	α	e
US1	IE protein	α	n
US2			n
US3	Protein kinase	β	n
US4	Glycoprotein G	γ	n
US5			n
US6	Glycoprotein D	γ	e

Gene	Function (where known)	Class	Requirement for growth in cell culture
US7	Glycoprotein I	γ	n
US8	Glycoprotein E	γ	n
US9	Virion protein	γ	n
US10	Virion protein	γ	n
US11			n
US12	IE protein	α	n

n = non essential to virus growth in cell culture

e = essential to virus growth in cell culture

* After McGeo ch et al 1988, McGeo ch 1989 & Baines & Roizman 1991

2.2.2 Viral Proteins: Cell Culture Requirements

Much of the work that has been reported on productive infection by HSV-1 has been done in cell culture systems. Many groups have noticed that certain viral genes and their products are not required for HSV-1 growth in cell culture. Mutational analysis has shown these to include the ribonucleotide reductase, (Goldstein & Weller 1988), thymidine kinase (Kit & Dubbs 1963), the US3 protein kinase (Purves et al 1987a), glycoprotein C (Heine et al 1974), and all but one of the genes in the Us region of the genome, glycoprotein D being an essential protein. Some of these results can be explained by the fact that in the cell culture system host DNA metabolising enzymes such as thymidine kinase, ribonucleotide reductase and dUTPase are present which can compensate for the loss of the viral enzyme. In the animal host the cells infected may not be dividing and therefore there is a requirement for viral enzymes which can carry out the necessary metabolism. Another possible function for some of the proteins reported as unnecessary in cell culture is in the production of, maintenance of or reactivation from the latent form of the virus (Roizman & Sears 1989).

Studies using mutants with deletions in the genes that are unnecessary for viral growth in cell culture have shown that certain mutants have much lower virulence in animal hosts than the wild type virus. This has been found to be the case for the ribonucleotide reductase (Cameron et al1988), the thymidine kinase (Tenser 1991) and the US3 protein kinase (Meignier et al 1988). The lower virulence (the ability of the virus to infect cells and set up productive infection and produce the symptoms of viral infection) of these mutants implies that the protein functions are required by the virus for a productive infection to be set up in the animal host.

3. Deoxyribonucleases

3.1 Deoxyribonucleases In General

Deoxyribonucleases or DNases are the enzymes which cleave deoxyribonucleic acid in prokaryotes and eukaryotes. The basic division between DNases is whether they act in an endo or exonuclease mode. Endonucleases can cleave DNA at internal sites in the molecule (e.g. circular DNA), exonucleases require free termini for their action either 5' or 3' termini and therefore intact circular DNA is resistant to their action. DNases also differ in the types of DNA they cleave, some utilise only double stranded (ds) DNA, others single stranded (ss) DNA and some can utilise both, but to varying degrees. Some DNases cleave DNA at specific sites whilst others cleave it at random. Endonucleases can either nick dsDNA (produce a single stranded break) or cleave both strands. Exonucleases can work in either a 3'$\longrightarrow$5' or 5'$\longrightarrow$3' direction and in a processive (degrades one DNA molecule completely before starting a new one) or dispersive manner (i.e. degrades one DNA molecule partially before dissociating and starting on a new one).

The possible functions of DNases and some examples are shown in Table 3.1.

Many of the enzymes shown in Table 3.1 are discrete entities which have deoxyribonuclease activity (e.g. Type II restriction enzymes) but many are also directly associated with other enzyme functions (e.g. E.coli B Type 1 restriction enzyme) in a multi subunit enzyme or are domains in a multi functional protein (e.g. Exo III, Exo V in E.coli, DNA polymerases I, III in E.coli, phage T4 UV DNA incising enzyme), some examples of these are given in Table 3.2.

Regulation of DNases varies from source to source and depends on the type of enzyme studied. In the case of the bacterial restriction enzymes which recognise specific sequences in the DNA and cleave at these sites (type II restriction enzymes). Regulation is achieved by the fact that if the

References

1. Moore S. 1981
2. Lehman I. R. 1981 a
3. Lehman I. R. 1981 b
4. McHenry & Kornberg 1981
5. Weissbach A. 1981
6. Endlich & Linn 1981
7. Wells et al 1981
8. Friedberg et al 1981
9. Weiss 1981
10. Bohr & Wasserman 1989

Table 3,1 The function, substrate specificity & cleavage pattern of various deoxyribonucleases

Function	Ref.	Nuclease	Type	Substrate	Direction and type of cleavage	Requirement
Salvage & Scavenger	1	Pancreatic DNase I	Endo	dsDNA	3'⟶5' non specific sequence dependent	Ca^{2+}
	2	ss endonuclease for N.crassa	Endo	ssDNA ssRNA	non specific	Zn^{2+} Co^{2+} Mg^{2+}
	2	Nuclease S1 for Aspergillus oryzae	Endo	ssDNA ssRNA	non specific	Zn^{2+} Co^{2+}
DNA replication	3	E coli DNA polymerase I	EXO	ssDNA	3'⟶5' non specific	
			EXO	dsDNA	5'⟶3' non specific	
	4	E coli DNA polymerase III	EXO	ssDNA	3'⟶5'	
			EXO	ssDNA	5'⟶3'	
	5	HSV DNA polymerase	EXO	N.D.	3'⟶5'	

Function	Ref.	Nuclease	Type	Substrate	Direction and type of cleavage	Requirement
Genetic recombination	6	Type I restriction enzymes	Endo	dsDNA	non-specific cleavage 1-5kb from recognition sequence	SAM * ATP Mg^{2+}
	7	Type II restriction enzymes	Endo	dsDNA	cleavage at specific recognition sites	Mg^{2+}
Conjugation & transformation						
DNA repair	8	phage T4 UV DNA incising enzyme	Endo	ds or ss DNA containing pyrimidine dimers	cleavage of phospho-diester bond 3' to to AP^{o} site	
	9	Exo III *E coli*	Endo	ds DNA containing AP sites	cleavage of phospho-diester bond 5' to AP site	Mg^{2+}
	8, 10	UVR ABC *E coli*	Endo	DNA modifications that disrupt duplex structure	cleavage of phospho-diester bond 5' to damage	ATP

Function	Ref.	Nuclease	Type	Substrate	Direction and type of cleavage	Requirement
Protection against foreign DNA	6	Type I restriction enzymes *E coli* B & K enzymes	Endo	dsDNA	non-specific cleavage 1-5kb from non-methylated recognition sites	SAM Mg^{2+} ATP
	7	Type II restriction enzymes EcoRI Hind III Pst I etc.	Endo	dsDNA	specific ds cleavage at non-methylated recognition sites	Mg^{2+}

* SAM = S adenosyl methionine

o AP = apurinic or apyrimidine site
a site where the base has been removed either chemically or enzymatically

Table 3,2 Examples of DNases or DNase containing Enzymes

Enzyme	Structure	Source	Nuclease Function	Other Enzymic Function
EcoR I	α_2	E.coli	endonuclease at specific sites	
ECOB	$\alpha_2 \beta_4 \gamma_2$	E.coli	endonuclease at site distant from recognition site (α subunit)	Methylase [β, γ] ATPase [α]
EXO III	monomeric	E.coli	i) 3'→5' ds exonuclease endonuclease ii) cleavage of phospho-diester bond 5' to AP[o] site	iii) RNase H iv) DNA 3' phosphatase
ECO EXO V	$\alpha\beta\gamma$	E.coli	i) ATP dependent ds exonuclease 5'→3' & 3'→5' ii) ssDNA exonuclease ATP dependent iii) ssDNA endonuclease ATP dependent	iv) DNA dependent ATPase v) ATP dependent dsDNA unwinding
DNA polymerase I	monomeric	E.coli	i) 3'→5' Exonuclease ssDNA ii) 5'→3' Exonuclease dsDNA	DNA polymerase
phage T4 UV DNA incising enzyme	monomeric	bacteriophage T4	endonuclease i) cleavage of phospho-diester bond 3' to AP[o] site	DNA glycosylase

[o] AP = apurinic or apyrimidinic site

recognition sequence is methylated then the restriction enzyme cannot recognise it and cleave at that site. All the bacterial sites are methylated and so only foreign DNA which is not methylated is recognised and degraded by the enzymes.

The DNase's which are involved in the repair of damaged DNA are also regulated by their specificity, since they will only act on DNA which has been damaged, for example, DNA that contains thymidine dimers (For reviews on DNA repair see Sancar & Sancar 1988, and Bohr & Wasserman 1988).

The single stranded endonuclease produced by Neurospora crassa is first synthesised as an inactive proenzyme (88kDa), in new mycelia. As the mycelia age the proenzyme is converted to a 61kDa protein which has endonuclease activity towards ssDNA and exonuclease activity towards dsDNA and is thought to be involved in recombination and DNA repair in vivo. During exponential growth the enzyme is cleaved to a 55kDa form which is exported to the culture medium and has only endonuclease activity towards ssDNA and ss RNA.

In eukaryotes pancreatic DNase I is inhibited by globular actin which has been found in pancreatic juice. It is proposed that actin inhibits the enzyme after secretion until it reaches the duodenum where bile from the liver neutralises the inhibition of the DNase allowing it to degrade DNA.

Much of the work done with DNases has been in genetic engineering and molecular biology using the DNases as tools rather than studying the enzymes themselves. The type II restriction enzymes are widely used in cloning, DNase I or S1 nuclease is used in "DNA finger printing".

Recent advances in DNase work have included the publication of the crystal structure of DNase I and as a result of this a possible mechanism of action has been put forward (Suck & Oefner 1986). It has also been shown that the diptheria toxin A subunit contains an intrinsic endonuclease activity which rapidly degrades host DNA (Chang et al 1989).

3.2 The Deoxyribonuclease of Herpesvirus

A virally induced deoxyribonuclease activity in cells infected with HSV-1 was first reported by Keir & Gold in 1963. The activity was found to be maximal at 8 - 12 hr. post infection. The activity was shown by Morrison & Keir (1968) to have different properties to the host enzyme and was not present in infected cells treated early in infection with puromycin or actinomycin D.

The enzyme responsible for this activity has been purified from infected cells by various groups (Hoffman & Cheng 1978 & 1979, Strobel-Fidler & Francke 1980, Banks et al 1983). Discrepancies between the molecular weight of the purified enzyme, 49.4kDa by sedimentation, 90kDa by SDS PAGE, have been resolved and the enzyme has a Mr on SDS PAGE of around 85kDa. The molecular weight calculated from the amino acid sequence of the protein is 67.5kDa.

The DNase has exonuclease activity towards double stranded and single stranded DNA. It appears to digest in a dispersive manner in either the 5'$\longrightarrow$3' or 3'$\longrightarrow$5' direction with the end product being 5' monophosphate deoxyribonucleotides. The exonuclease function has an absolute requirement for Mg^{2+} ions (5mM optimum) and other divalent cations cannot substitute. In the presence of Mg^{2+} other divalent cations such as Ca^{2+}, Zn^{2+} and Mn^{2+} become inhibitory. The pH optimum of the exonuclease is high between 9 - 10 pH units (hence the name alkaline DNase) and Banks et al (1983) reported no inhibitory pH could be found. Strobel-Fidler & Francke (1980) showed the high pH optimum was a feature of the enzyme itself and not the substrate. The enzyme was found to be inhibited by KCl concentrations above 0.2M, sulphyryl blocking reagents such as p-hydroxymercuribenzoate (IC_{50} 1.9mM) and the polyamines spermine (IC_{50} 0.6mM) and spermidine (IC_{50} 1.4mM). The HSV-1 and HSV-2 exonuclease activities failed to activate calf thymus DNA for the viral DNA polymerase which implied that both strands were degraded to the same degree (Hoffman 1981).

The DNase purified from infected cells also has an endonuclease function (Hoffman & Cheng 1979, Hoffman 1981, Strobel-Fidler & Francke 1980). The endonuclease activity requires Mg^{2+} or Mn^{2+} ions at 2 mM for optimal activity. In the presence of Mn^{2+} ions at concentrations greater than 1mM only endonuclease activity remains. In the presence of Mg^{2+} ions, Ca^{2+} and Zn^{2+} are inhibitory. The endonuclease function can act on closed circular double stranded DNA, preferring super coiled DNA, nicking it to form the open circular form. The enzyme then cleaves at the nick to produce linear duplex DNA. In the presence of the exonuclease function (i.e. with Mg^{2+} ions present) the linear DNA is degraded to small oligonucleotides and mononucleotides. The endonuclease appears to preferentially recognise ssDNA regions in dsDNA circles. The cleavage appears not to be base specific. In the presence of Mn^{2+} ions the HSV-1 and HSV-2 nucleases cleave opposite nicks and gaps in dsDNA. No distinct fragments were produced in DNA which contains no nicks. The endonuclease activity is also inhibited by the polyamines, and spermine inhibits at a 10 fold lower concentration than for the exonuclease function.

Using antibodies raised against the enzyme it has been shown to be present in the nucleus of infected cells (Banks L. et al 1983, Banks L. et al 1985) by 2 hr. post infection. At this time it appears diffusely in the nucleus but as the infection proceeds it becomes localised in globular structures. These workers also reported a possible change in antigenic structure of the enzyme 5 - 6 hr. pi. as shown by the loss of reactivity of the DNase with one of their monoclonal antibodies after 5 - 6 hr. pi.

The DNase has been shown to be phosphorylated in cell culture (Banks et al 1985). The enzyme has also been synthesised, by the micro-injection of mRNA and DNA into, Xenopus oocytes (Preston & Cordingley 1982). The protein synthesised was around 85kDa and they showed the presence of a new exonuclease activity with similar characteristics to those reported for the viral DNase. It follows that if phosphorylation is required for an active exonuclease function, it must be carried out in the oocyte by cellular

Studies using HSV mutants with temperature sensitive lesions provided evidence that the DNase was virally coded (Francke et al 1978, Moss et al 1979, Francke & Garret 1982, Moss 1986). The DNase has been mapped to the HSV genome (Preston & Cordingley 1982) and Costa et al (1983) identified a 2.3 kb mRNA which encoded an 82 kDa protein that reacted with a monoclonal antibody against the HSV-2 DNase. The temperature sensitive mutants showed initially that the mutation in the DNase gene was not lethal to the virus. Francke & Garret (1982) showed that mutants with a single lesion in the DNase gene had a reduced rate of viral DNA synthesis at the non-permissive temperature, and the level of the viral DNA polymerase activity was also reduced. Moss (1986) studied this mutant in greater detail and showed that the DNase was required for viral DNA synthesis and virus growth.

The sequence of the genes of the HSV-1 and HSV-2 enzymes are known (Draper et al 1986, McGeo ch et al 1986) and from this the predicted amino acid sequences have been determined. The HSV-1 enzyme has 626 amino acids and the HSV-2 620 amino acids. Sequence analysis showed significant differences in the amino acid sequences in the N terminal domain, compared to the C terminal domain which had a high amino acid homology. Computer predictions indicated a similar 3D structure. McGeo ch et al (1986) showed the HSV-1 enzyme to be related to a sequence in the γ herpes virus EBV (BGLF5) and a similar enzyme has been reported in cells infected with EBV (Clough 1980). It was noted that the EBV enzyme lacked 110 amino acids present in the N terminal of the HSV-1 enzyme. The possible production of a truncated HSV-1 protein was also suggested.

The function of the DNase in herpesvirus infection is unknown. The most obvious role is the degradation of cellular DNA to provide deoxyribonucleotides for viral DNA synthesis. Francke (1977) reported no evidence that cellular DNA was degraded during HSV infection and that virally 'stimulated' DNase activity appeared to be directed against viral DNA. This was reported from HSV infection in cell culture where cells are dividing, but cells infected

by HSV-1 in an animal host maybe terminally differentiated and therefore not dividing. In these conditions the virus may be required to degrade cellular DNA for its replication.

When a monoclonal antibody against the DNase was used as an affinity column on infected cell extracts, the DNase was found to bind large quantities of the major DNA binding protein (DBP) (Vaughan et al 1984). Studies using virus with a temperature sensitive lesion in the major DBP gene showed that the DNase was destabilised, as well as the viral DNA polymerase, in infected cells (Littler et al 1983). This may implicate the major DNA binding protein in the function of the DNase.

The DNase has been shown not to be required for viral DNA synthesis in in vitro replication assays, which requires minimally 7 viral proteins: UL9, UL30, UL42, UL29, UL5, UL8 and UL52 and a viral origin of replication (Wu et al 1988, McGeoch et al 1988). It may still be required for the maturation of concatenated viral DNA into genomic lengths with subsequent encapsidation.

Chou & Roizman (1989) showed that they could purify the DNase from HSV-1 infected cells by its ability to bind to a DNA probe derived from the 'a' sequence of the genome. The a sequence (see Section 1.2) occurs at the termini of the genome (direct repeats) and in the junction between the UL & US regions (inverted repeats). The a region has been shown not only to be responsible for inversions of the UL & USregions of the genome (Mocarski & Roizman 1982, Chou & Roizman 1985) but also contains the signals required for the cleavage to and packaging of genomic DNA lengths into capsids (Vlazny et al 1982, Moscarski & Roizman 1982, Stow et al 1983, Varmuza & Smiley 1985, Diess et al 1986, Diess 1986). The a sequence (Fig. 3.2.1) contains two signals Pac 1 and Pac 2 which direct the cleavage of concatenated viral DNA at a site in DR1. The cleavage in DR1 does not appear to be sequence specific but rather occurs at a certain distance from the recognition sites (Fig. 3.2.1).

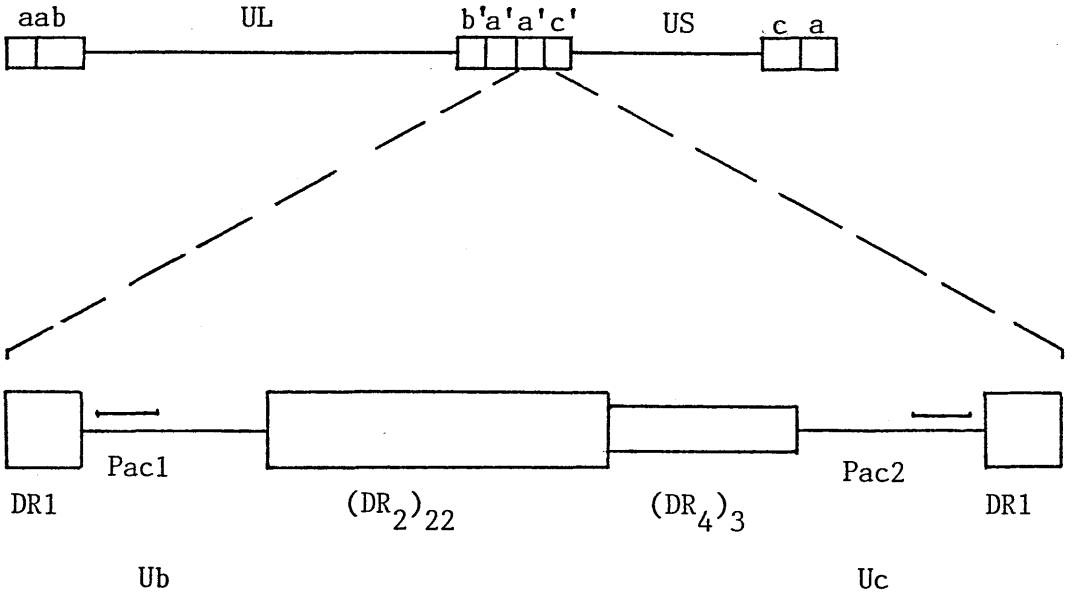
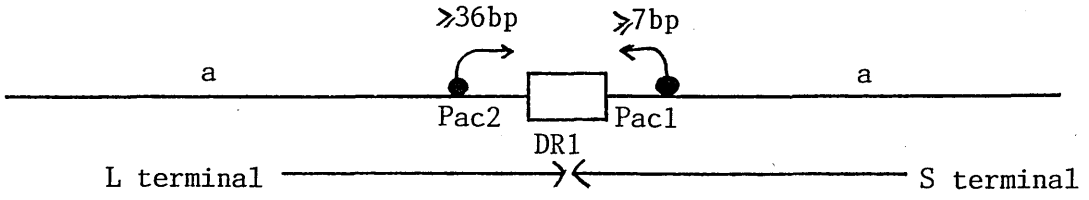


Figure 3.2.1 a) Structure of the a region of HSV-1



Pac2 CGCCGCG | GC rich 32bp | TTTTTT | GC rich 34bp | cleavage site

L terminal

Pac1 cleavage site | GC rich 32bp | CCCCC GGGGGG TGTGTTTC GGGGGGGG

S terminal

Figure 3.2.1 b) Cleavage of concatenated DNA by signals in Pac1 & Pac2. and the structure of the two signals

The finding that the DNase binds though non-specifically, to DNA probes from this region including the sites for Pac 1 and Pac 2 may indicate a function for the enzyme in the cleavage of viral concatemers into unit genomic lengths perhaps as part of a multienzyme complex.

Section 4 Ribonucleotide Reductase

4.1 Cellular Ribonucleotide Reductase

During cell division in the S phase when DNA synthesis is underway the cell requires a regulated supply of deoxyribonucleoside triphosphates. This is achieved, in prokaryotes and eukaryotes, by the enzyme ribonucleotide reductase (ribonucleoside diphosphate reductase E.C. 1.17.4.1). The enzyme reduces ribonucleotides (in most cases the diphosphate form although in some cases the triphosphate form can be utilised) by means of a protein radical and a metal centre. Cysteine residues are involved in hydrogen transfer and the final hydrogen donor is cellular thioredoxin. The enzyme is under stringent allosteric control which involves feedback inhibition by dATP and dTTP (Thelander & Reichard 1979, Reichard & Ehrenberg 1983). The enzymes examined to date fall into three classes depending on their subunit composition and cofactor requirements (Stubbe 1990). The largest group consists of those enzymes which have a subunit composition $\alpha_2\beta_2$. Their cofactors (on each of the small α subunits) are a binuclear iron centre (Nordlund et al 1990), and a protein radical which in this case happens to be a tyrosine radical (Larsson & Sjöberg 1986, Sjöberg et al 1985). The subunits exist as homodimers. The large subunit, β, (B1 in Ecoli, M1 in mouse) contains the substrate binding sites, the sites for allosteric control and the cysteine residues involved in hydrogen transfer.

The small subunit, α, (B2 in Ecoli, M2 in mouse) contains the cofactors described above which are essential for catalysis. The catalytic site is formed by the binding of the homodimers.

The second class of reductase has a single α subunit and requires adenosylcobalamin as a cofactor. It is thought that a protein radical may also be involved in the reaction.

The final class of reductase has been the least studied, it has a subunit composition $\alpha\beta_2$ and may require a binuclear manganese centre and a protein radical for catalysis (Stubbe 1990).

The first class of enzyme has been found in both eukaryotes and prokaryotes (Caras et al 1985 , Carbon et al 1984, Elledge & Davis 1987, Standart et al 1985), the other two classes have, so far, only been found in prokaryotes (Thelander & Reichard 1979, Stubbe 1990).

4.2 Herpesvirus Ribonucleotide Reductases

During infection of tissue culture cells with herpesviruses in the presence of hydroxyurea or excess thymidine where the cellular ribonucleotide reductase was rendered inactive, it was found that viral replication could still occur (Cohen 1972 a, Cohen et al 1974, Cohen et al 1977, Henry et al 1978, Cohen 1972b, Roller & Cohen 1976). The enzyme responsible for this activity was subsequently shown, in the case of HSV-1, to be a virally coded ribonucleotide reductase (Dutia 1983). The viral enzyme consisted of two subunits, a large subunit known as RR1 (140K Daltons) and a small subunit known as RR2 (40K Daltons) (Preston et al 1984, Cohen E et al 1985, Frame et al 1985, Bacchetti et al 1986, Swain & Galloway 1986). The subunits form an $\alpha_2\beta_2$ complex, as described earlier, and the same cofactors are involved (a tyrosine radical and a binuclear iron centre) but the enzyme is not under allosteric control (De Leon et al 1977).

Other herpesviruses have been shown to encode ribonucleotide reductases and sequence comparisons of the reductase genes in EBV and VZV have shown them to have regions of homology, particularly in the C terminal domain of RR1 and the whole of RR2, with the HSV-1 and HSV-2 genes (Nikas et al 1986, Gibson et al 1984). The HSV-1 and HSV-2 RR1 genes have a high degree of homology with the

other herpes RR1 genes except for the N- terminal domains which in both cases appear to have extensions when compared to other reductase large subunits (Nikas et al 1986). The N- terminal domains of HSV-1 and 2 large subunits have been found to have sequence homology with protein kinases (See Section 5.1.4) and recently have been shown to possess intrinsic protein kinase activities (Chung T.D. et al 1989, Paradis H. et al 1990). The large subunit of HSV-2 has been implicated in the transforming potential of the virus (Huszar & Bacchetti 1983, McLauchlan & Clements 1983, Galloway & McDougall 1981) and a protein kinase activity could possibly explain that role.

Analysis of mutants has shown that the ribonucleotide reductase of HSV-1 is not required for virus growth in cell culture (Goldstein & Weller 1988a), but temperature sensitive mutants in RR1 or RR2 have greatly reduced virulence in mice, (10^6 fold reduction) (Cameron et al 1988).

Further studies using a deletion mutant (deletion in RR1 gene) has shown that the ability of the mutant to grow in cell culture was dependent on the state of the infected cells. The mutant was severely compromised in non dividing cells or in cells at 39.5^{o}C (Goldstein & Weller 1988b) and it was suggested that the cellular ribonucleotide reductase could compensate the deletion in dividing cells. Turk et al (1989) showed that, using a similar deletion mutant to Goldstein & Weller (1988b), the mutant virus could produce cutaneous lesions in guinea pigs and that these lesions resulted from the *in vivo* replication of the mutant virus. They concluded that further work was required before it could be certain whether the ribonucleotide reductase was essential for the *in vivo* replication of HSV-1.

In vitro the ribonucleotide reductase is inhibited by the decapeptide YAGAVVNDL which corresponds to the C terminal end of the small subunit (Gaudrea P. et al 1987).

The peptide causes the dissociation of the holoenzyme into the homodimers α_2 & β_2 with a consequent loss of activity.

The N terminal domain of the HSV-1 large subunit is highly susceptible to proteolysis in infections in cell culture which results in the production of a number of lower molecular weight species (Lankinen et al 1989). Some of the partially degraded subunits are still enzymatically active and were located in the nuclei of infected cells.

The only known translational modification of the HSV-1 enzyme is the phosphorylation of both subunits (Preston et al 1984).

Section 5 Protein Phosphorylation : dephosphorylation

The reversible covalent attachment of phosphate to proteins (phosphorylation), and its removal (dephosphorylation), has been found to be a widespread control mechanism in both prokaryotes and eukaryotes. Although phosphorylation has been the most widely studied, recent reports have shown that dephosphorylation may have an equally important role in cellular control mechanisms.

Phosphorylation has now been found to be involved in virtually all aspects of biochemistry including the control of enzymes in central metabolism (Cohen 1985, Krebs 1985, Hardie et al 1989), protein synthesis (Rhoads 1988), transcription in prokaryotes (Magasanik 1988) and eukaryotes (Corden 1990), photosynthesis (Gal et al 1987, Wollman & Lemaire 1988, Coughlan 1988), cell division (Dumont et al 1989, Featherstone 1989, Gould & Nurse 1989, Draetta et al 1988, Moria et al 1989, Dunphy & Newport 1989, Draetta 1990), intracellular reponses to extra cellular stimuli (Cohen 1985), regulation of signal transduction (Katada et al 1985, Zick et al1986, Sagi-Eisenberg et al 1989), regulation of T-lymophycte function (Bolen & Veillette 1989, Tonks & Charbonneau 1989), release of secretory granuoles from adrenal chromaffin cells (Knight et al 1988, Burgoyne et al 1988), phermone responses in the yeast Saccharomyces cerevisae (Fields 1990) and possible regulation of membrane bound guanyl cyclase activity (Hanks et al 1988, Chinkers et al 1989) (Table 5.0).

Dephosphorylation of protein phosphates reverses the action of protein phosphorylation and so the cell can control, via the enzymes responsible for the two activities, to a high degree the above functions. Dephosphorylation of protein phosphates has been implicated as means of signal transduction (Tonks & Charbonneau 1989, Alexander 1990) and has been shown to be necessary in cell division (Gould & Nurse 1989, Moria et al 1989, Dunphy et al 1989).

Table 5.0 Examples of Protein Phosphorylation

Cellular Function	Phosphorylation	Result	Ref.
Metabolism	Acetyl CoA Carboxylase by AMP protein kinase	Inhibition of carboxylase & fatty acid synthesis	Hardie D. et al 1989
	Phosphorylase kinase by cAMP dependent protein kinase	Activation of the kinase & glycogenolysis	Cohen 1985
	Tyrosine hydroxylase by cAMP dependent protein kinase	Activation of the hydroxylase & catecholamine synthesis	Cohen 1985
Protein Synthesis	eIF2α by haem regulated protein kinase	Inhibition of protein synthesis initiation	Edelman et al 1987
Cell division	cdc2, p34 by kinase kinase	Inhibition of entry into mitotic stage of cell division	Moria A. et al 1989 Dunphy & Newport 1989
Signal transduction	Giα, transducin Gα by protein kinase C	Regulation of signal transduction	Katada T. et al 1985 Zick Y. et al 1986

5.1 Protein Kinases

The enzymes responsible for the covalent attachment of phosphate to proteins are known collectively as protein kinases (Hunter 1987). They catalyse the transfer of the γ phosphate group of ATP (and in some cases GTP) to the hydroxy amino acids : either serine/threonine or tyrosine. The kinases are subdivided into protein serine/threonine kinases (Edelman et al 1987) and protein tyrosine kinases (Hunter & Cooper 1985). Another group of kinases exist which transfer the γ phosphate of ATP to lysine or histidine residues on the protein substrate (Chen et al 1974, Smith et al 1974).

In some cases the transfer of phosphate does not require a protein kinase directly and involves the transfer of the phosphate group directly between proteins (Magasanik 1988, Wilcox et al 1980).

The kinases also differ in their substrate specificity, some kinases have only one known protein substrate (e.g. rhodopsin kinase Kuhn 1978) and others have a broad range of substrates (e.g. protein kinase C Nishizuka Y 1986). The kinases also have a variety of regulatory effectors. A short list of protein kinases exemplifying these features in shown in Table 5.1.

5.1.1 Regulation of kinase activity

Protein kinases are highly regulated since they control much of cellular activity, and this regulation can be divided into four types. The first type of regulation occurs in those kinases which are domains of transmembrane receptors. These domains are protein tyrosine kinase activities and are found on the cytosolic face of the membrane, (Figure 5.1.1). The binding of the ligand to the receptor causes activation of the tyrosine kinase activity and the phosphorylation of its substrates (e.g. epidermal growth factor receptor kinase, Cohen S et al 1982, Cooper J.A. et al 1982). Removal of the ligand terminates the kinase activity, the number of receptors can also be controlled by endocytosis or at the level of transcription or translation.

References:

1. Beebe & Corbin 1986
2. Nishizuka 1986
3. Edelman et al 1987
4. Stull et al 1986
5. Hardie et al 1989
6. London et al 1986
7. Nurse & Gould 1989
8. Hathaway & Traugh 1982
9. Kuhn 1978
10. Cohen S. et al 1982
11. Cooper J.A. et al 1982
12. Bolen & Veillette 1989

Table 5.1 Protein kinases, their substrates and regulatory effectors

Protein Serine/threonine kinase

Kinase	Effector	Substrate(s)	Reference
cAMP dep Protein kinase	cAMP	Phosphorylase kinase Acetyl CoA carboxylase myosin light chain kinase	1
cGMP dep protein kinase	cGMP		1
Protein kinase C	Ca^{2+} DIACYL-glycerol	EGF receptor IL-2 receptor myelin basic protein	2
Ca^{2+}: Calmodulin dep protein kinase			
I	Ca^{2+}:calmodulin	synapsin I protein III	3
II	"	synapsin I tryptophan hydroxylase MAP - 2	4
III	"		3
Myosin light chain kinase	Ca^{2+}:Calmodulin	Myosin light chain	4
AMP Protein kinase	AMP phosphorylation by a kinase kinase	HMG CoA reductase acetyl CoA carboxylase hormone sensitive lipase	5
Haem regulated Protein kinase	haem	eIF 2 α	6
dsRNA dependent protein kinase	dsRNA	eIF 2 α	6
cdc 2	phosphorylation by a kinase kinase		7
Casein kinase I	unknown	amino acyl tRNA synthase glycogen synthase	8
Casein kinase II	unknown inhibited by heparin	glycogen synthase acetyl CoA carboxylase	8

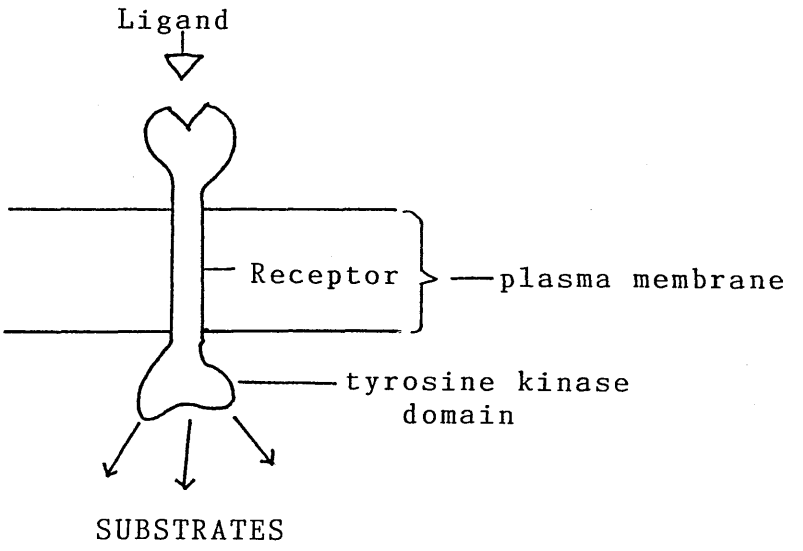
Kinase	Effector	Substrate(s)	Reference
S6 kinase	unknown	ribosomal protein S6	3
Rhodopsin kinase	unknown	rhodopsin	9
Protein tyrosine kinases			
EGF receptor kinase	EGF	p42	10,11
$p56^{lck}$	CD4 or CD8 receptor	T cell receptor ζ subunit	12

Figure 5.1.1 Regulation of protein kinases

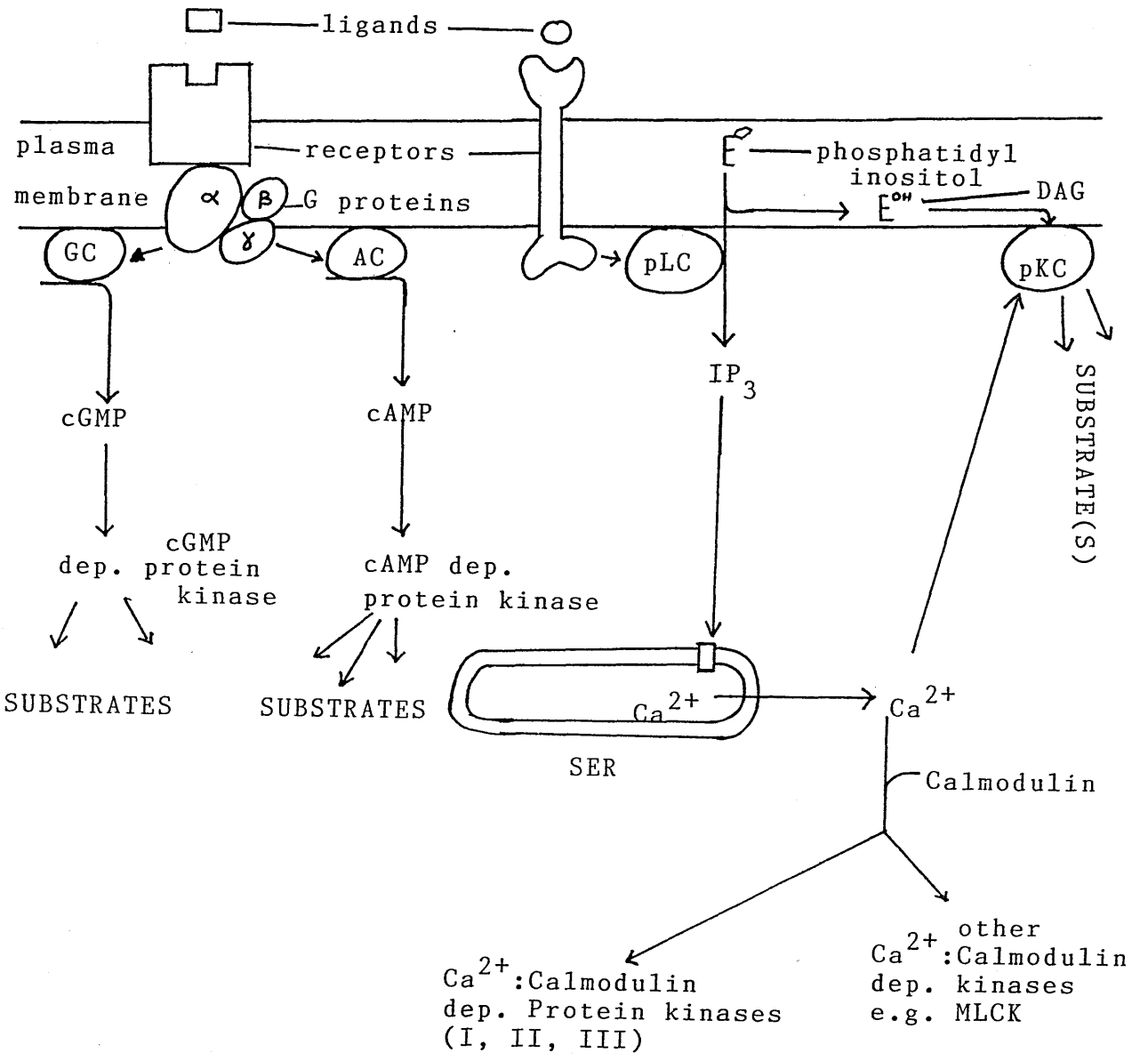
i) receptor-ligand regulated
ii) 2nd messenger regulated

GC Guanyl cyclase
AC Adenylate cyclase
pLC Phospholipase C
pKC Protein kinase C
IP_3 Inositol trisphosphate
SER Smooth Endoplasmic Reticulum
DAG Diacyclglycerol

MLCK Myosin light chain kinase


Ligand
Receptor
plasma membrane
tyrosine kinase domain
SUBSTRATES

(1)


ligands
plasma membrane
receptors
G proteins
GC
AC
pLC
phosphatidyl inositol
DAG
pKC
cGMP
cAMP
IP3
SUBSTRATE(S)
cGMP dep. protein kinase
cAMP dep. protein kinase
SUBSTRATES
SUBSTRATES
Ca2+
SER
Ca2+
Calmodulin
Ca2+:Calmodulin dep. Protein kinases (I, II, III)
other Ca2+:Calmodulin dep. kinases e.g. MLCK

(11)

The second type of regulation involves the 2nd messengers generated by signal transduction (Figure 5.1.1 (11)) namely cAMP, cGMP, diacylglycerol (DAG) and inositol triphosphate (IP_3). The IP_3 released from the plasma membrane brings about the release of Ca^{2+} from intracellular stores in the smooth endoplasmic reticulum (Spat A. et al 1986, Dawson A. 1985), cAMP activates the cAMP dependent protein kinase (Beebe & Corbin 1986), cGMP activates the cGMP dependent protein kinase (Beebe & Corbin 1986), DAG & Ca^{2+} activate protein kinase C (Nishizuka 1986) and Ca^{2+} bound to calmodulin activates a number of kinases including the Ca^{2+}:calmodulin dependent protein kinases I (Edelman et al 1987), II (Stull J. et al 1986, Schulman & Lou 1989), III (Edelman et al 1987) and the myosin light chain kinase (Stull J. et al 1986, Sellers & Aldstein 1986).

The third type of regulation is by intracellularly generated activators such as haem which regulates the haem regulated protein kinase (London I. et al 1986), double stranded RNA which regulates the ds RNA dependent protein kinase (London I. et al 1986), AMP and phosphorylation by a 'kinase kinase' which regulates the AMP dependent protein kinase (Hardie D. et al 1989) and the regulation of the Schizosaccharomyces pombe cdc2 protein kinase by phosphorylation (Gould & Nurse 1989).

The last group of kinases have, at this present time, no known regulators and they include casein kinases I and II (Hathaway & Traugh 1982), and the hormone-stimulated ribosomal S6 kinase (Edelman et al 1987).

5.1.2 Site specificity of protein kinases

Many protein kinase substrates have been analysed to see which sites are phosphorylated and if there are any similarities between sites on different substrates. Along with studies using artificial peptides these have been used to produce consensus phosphorylation sequences for those kinases with broad substrate specificities. Table 5.2 shows some of these consensus sequences, but it must be said that these are not hard and fast sequences which can be used to identify possible phosphorylation

Table 5.2 Protein kinase phosphorylation site motifs*

	Protein kinase	Phosphorylation Site
BROAD SUBSTRATE RANGE	cAMP dep protein kinase	RRX<u>S</u>X
	cGMP dep protein kinase	X<u>S</u>RX
	protein kinase C	XRXX<u>S</u>XRX
	Ca^{2+}:Calmodulin dep protein kinase II	XRXX<u>S</u>X
	Casein kinase I	XS(P)XX<u>S</u>X or XEXX<u>S</u>X
	Casein kinase II	X<u>S</u>XXEX
SINGLE SUBSTRATE	Myosin light chain kinase	KRRXXRXX<u>S</u>X
	Haem regulated kinase on eIF 2 α	LSEL<u>S</u>RR
	dsRNA dependent protein on eIF 2α	SEL<u>S</u>RR
	rhodopsin kinase	DEA<u>STT</u>VKTET<u>S</u>QVA
TYROSINE KINASES	EGF receptor kinase	TAENAE<u>Y</u>LRVAP
	$p56^{LCK}$	RLIEDNE<u>Y</u>TAREGAK

* For original references see Kemp & Pearson 1990

sites on a particular protein as the tertiary structure of the protein probably plays some role in determining the sites phosphorylated by a particular kinases.

From the table it seems that many of the kinases require multiple arginine residues around the site of phosphorylation. These are serine/ threonine protein kinases. The casein kinases, however, appear to require acidic residues as do the protein tyrosine kinases.

5.1.3 Autophosphorylation of protein kinases

Many of the known protein kinases undergo an autophosphorylation event via mainly an intramolecular catalytic reaction, but in some cases intermolecular catalysis may be involved. Both protein serine/threonine and protein tyrosine kinases can undergo this phenomenon. For example, the serine/threonine Ca^{2+}:calmodulin dependent multifunctional protein kinase II (Schulman & Lou 1989) undergoes autophosphorylation at two distinct sites. This is thought to be involved in the activation and deactivation of the enzyme. The EGF receptor tryosine kinase also undergoes autophosphorylation at several residues on the binding of EGF (Hunter & Cooper 1985). This event is an intermolecular one brought about by receptor oligomerization (Schlessinger J. 1988).

The function of the autophosphorylation may be to retain activity in the tyrosine kinase after the removal of EGF. The function of autophosphorylation in many kinases remains unclear, but it has been suggested that autophosphorylation takes place at a pseudo-substrate site in the kinase which when phosphorylated is displaced and so activates the kinase. It must be noted that in some cases autophosphorylation occurs at many sites and it is not clear what the significance of this is.

5.1.4 The Primary Sequence of Protein Kinases

The amino acid sequence data for over 30 protein kinases is now available and using computer analysis, sequence comparisons have shown a region of approximately 240 amino acids where the homology between protein kinases is the greatest. This region corresponds to the catalytic domains of the protein kinases and contains residues essential for catalysis (Figure 5.2). The domain contains residues conserved in the majority of kinases, both serine/threonine and tyrosine kinases, but the function of many of these remains unclear (Kemp & Pearson 1990).

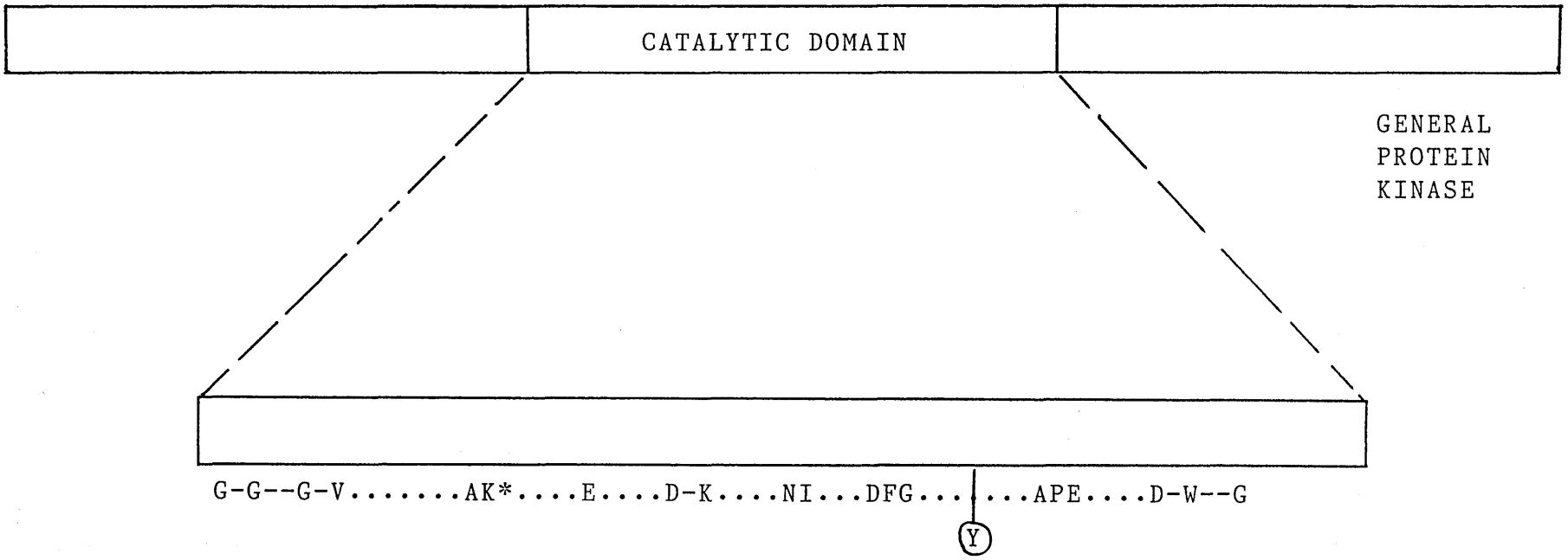


Figure 5.2 The general structure of protein kinase's catalytic domain showing residues conserved in most kinases - serine/threonine and tyrosine kinases. (Y) site of autophosphorylation in many tyrosine kinases

A: Alanine, D: Aspartic Acid, E: Glutamic Acid, F: Phenylalanine,

G: Glycine, I: Isoleucine, K: Lysine, L: Leucine, N: Asparagine,

R: Arginine, S: Serine, V: Valine, W: Trytophan, Y: Tyrosine

The sequence motif G-G--G-V represents the nucleotide binding sequence, and has been found in many proteins that bind nucleotides. Also involved in ATP binding, and shown to be essential for kinase activity, is the Lysine residue (K) marked with an asterisk in Figure 5.2 (Zoller & Taylor 1979). The APE motif is thought to be located near or at the active side of the kinase.

The presence of these conserved motifs in the catalytic domain allows the sequences of putative protein kinases to be analysed to see if they contain any of these motifs as in the case of the UL13 gene sequence in HSV-1 (Chee et al 1989).

5.2 Protein Phosphatases

5.2.1 Protein Serine/Threonine Phosphatases (Cohen P. 1989)

In animal cells so far 4 types of protein serine/threonine phosphatase have been identified. The 4 types of phosphatase are classified depending on their preference to dephosphorylate either the α or β subunits of phosphorylase kinase *in vitro* and their requirement for divalent cations (Table 5.2.1).

Sequence data for the genes of phosphatases has shown that protein phosphatase type 1 (PP1), protein phosphatase type 2A (PP2A) and protein phosphatase type 2B (PP2B) belong to the same gene family whilst protein phosphatase type 2C (PP2C) appears to be distinct.

The 4 protein serine/threonine phosphatases have a broad range of overlapping substrates *in vitro* but *in vivo*, this range may be narrowed by their subcellular location and specific regulation of the enzymes (Table 5.2.2).

Protein phosphatase type 1 catalytic subunit in striated skeletal muscle is found associated with glycogen, the sarcoplasmic reticulum and myofibrils. These associations are mediated through two subunits, the G subunit which binds to glycogen and the M subunit which binds to actinomyosin. Thus binding to either of these subunits (PP1-G, PP1-M) directs the phosphatase to a specific subcellular location with a limited number of substrates. The catalytic subunit PP-1C is also found bound to the inhibitor 2 protein (PP1I) in the cytosol, this can be activated by phosphorylation of inhibitor 2 by glycogen synthase kinase 3. The physiological significance of this is unclear. The enzyme in skeletal muscle is regulated by the second messengers cAMP and Ca^{2+}. Inhibitor 1 is activated by phosphorylation by cAMP dependent protein kinase and the G subunit is also phosphorylated by this enzyme which causes release of the catalytic subunit into the cytosol and inhibition by activated inhibitor 1.

Table 5.2.1 Protein Serine/Threonine Phosphatases

	PP1	PP2A	PP2B	PP2C
Subunit of Phosphorylase kinase preferred	β	α	α	α
Inhibition by inhibitor 1 & inhibitor 2	Yes	No	No	No
Divalent ion requirement	-	-	Ca^{2+}	Mg^{2+}
Inhibition by okadaic acid	I_{50} 20nm	I_{50} 0.2nm	I_{50} 5 M	No
Subunit composition	PP1-G PP1-C G subunit PP1-M PP1-C M subunit PP1-I PP1-C I2	AB'C (PP2AO) ABC (PP2A1) AC (PP2A2) AB''C	AB	Monomeric C

o Hydroxymethylglutaryl CoA reductase

Δ Dopamine & cyclic AMP-regulated phosphoprotein - an isoform of inhibitor 1 found in the brain

* The phosphatases given are not definitive, their function has not been proved directly but by knowledge of their *in vivo* cellular location and studies of their activities *in vitro*

Table 5.2.2 Protein Serine/Threonine Phosphatases : Substrates

Substrate	Phosphatase in vitro	Phosphatase in vivo *
Phosphorylase kinase		
∝ subunit	PP2A, PP2B, PP2C	PP2A
β subunit	PP1	PP-1G
Glycogen Phosphorylase a	PP1, PP2A	PP1G
Glycogen synthase	PP1, PP2A, PP2C	PP1G
Myosin light chain	PP1, PP2A, PP2C	PP1M
Ribosomal protein S6	PP1, PP2A	PP-1
eIF2 ∝	PP1, PP2A	PP-1
HMG CoA reductase °	PP1, PP2A, PP2C	PP-1
6 phospho fructo2-kinase / fructose 2, 6 bisphosphatase	PP1, PP2A, PP2C	PP2A
Pyruvate kinase		
6 phospho fructo1-kinase		
Fructose-1, 6-bisphosphatase		
Acetyl CoA carobxylase		
ATP citrate lyase		
Phenylalanine hydroxylase		
Inhibitor 1	PP2B	PP2B
DARPP ▲		
RII of cAMP dep PK		
Calmodulin dep cAMP phosphodiesterase		

Dephosphorylation of inhibitor 1 is by PP2B which is regulated by Ca^{2+}. Hepatic PP1-G is sensitive to inhibition by phosphorylase a and Ca^{2+} which prevents the activation of glycogen synthase. It is also regulated by insulin.

Protein phosphatase type 2A has been purified in four major forms (Table 5.2.1). The catalytic subunit C is found associated with subunits A & B (PP2AO), subunits A & B' (PP2A1), subunit A (PP2A2) or with subunits A & B''. The A subunit directly interacts with PP2A C and the B, B', B'' subunits interact with A. The binding of the A & B (or B', B'') subunits suppresses the activity of the catalytic subunit towards certain substrates producing the enzyme in a low activity form. In vitro polyamines (spermine) and basic proteins (protamine) stimulate the activity of all forms of the enzyme, but the physiological significance of this is not understood.

Protein phosphatase type 2B purified from brain consists of a heterodimer AB. The A subunit contains the catalytic site and the calmodulin binding site. The B subunit contains the Ca^{2+} binding site(s). The B subunit may also be myristylated in some cases. The enzyme is regulated by Ca^{2+} levels on which it is completely dependent. Calmodulin appears to neutralize the effects of an inhibitory 4kD domain on the A subunit.

Protein phosphatase type 2C has been purified as a monomeric protein. In rabbit skeletal muscle and liver two isozymes have been found. The mechanism of its regulation is unknown.

5.2.2 Protein Tyrosine Phosphatases

The protein tyrosine phosphatases found to date fall into two classes, those which are cytosolic and those which are domains of integral membrane proteins (Tonks & Charbonneau 1989). Sequence data from the cytosolic protein tyrosine phosphatases (PTP) allowed the identification of other possible phosphatases. Little is known about the physiological substrates or the regulation of either class of PTPase.

The CD45 integral membrane proteins found on cells of haematopoietic origin contain cytosolic domains which have homology with PTPases. The protein has intrinsic phosphatase action *in vitro* but no known physiological substrates. The CD45 protein has structural homology with receptor proteins and may represent a new class of receptor which on binding of their specific ligand initiate novel signal transduction pathways by protein tyrosine dephosphorylation. Possible *in vivo* substrates of the CD45 phosphatase include fodrin (A & B subunits), the ζ subunit of the CD3 receptor and $p56^{lck}$ protein tyrosine kinase (Tonks & Charbonneau 1989).

5.3 Protein Phosphorylation & HSV Infection

(for review Leader & Katan 1988).

5.3.1 Viral Infection & Protein Phosphorylation

In HSV-1 infection of BHK cells in culture certain viral and cellular proteins undergo phosphorylation. The viral proteins which are phosphorylated include the immediate early proteins IE 1, 2,3 & 4 (or α0, α27, α4, and α22) (Ackermann et al 1984), the DNase (Banks et al 1985), and the large subunit of ribonucleotide reductase (Preston et al 1984).

The functional and regulatory significance of these phosphorylations is still not understood and it may well be that some of the phosphorylations observed are not relevant to viral infection.

Many of the virion proteins of HSV-1 are phosphorylated and this appears to be mediated by cellular kinases such as casein kinase II (Stevely et al 1985) but again the functional significance of these is not understood.

5.3.2 HSV Viral Kinases

HSV-1 has been shown to encode a protein kinase activity which is the product of the US3 gene (Purves et al 1987a, Frame et al 1987) and has equivalent genes in HSV-2, VZV (McGeoch & Davison 1986) and PRV (Zhang et al 1990). The protein has been purified to homogeneity (Purves et al 1987b) and shows different characteristics from cellular kinases (Purves et al 1986a). The enzyme is a serine\threonine kinase, it is not affected by the physiological effectors of the most common kinases, i.e. cAMP, DAG, Ca^{2+}, cGMP and it is active at high salt concentrations (Katan et al 1985). The enzyme prefers basic artifical substrates (protamine) and appears to have a different site specifity from known kinases (Purves et al 1986b) [RRXXSZ Z not Glu/Pro]. As yet no physiological substrate for the enzyme is known, and no cellular equivalent has been found that may give a clue to its function.

The US3 protein kinase is not required for virus growth in cell culture conditions (Purves et al 1987a), but mutant virus with a deletion in the US3 gene had a greatly reduced virulence in mice (Meignier et al 1988) which may indicate a role for the kinase in the infection of host cells.

Another protein kinase has been postulated in the HSV-1 genome by its homology to other protein kinases (Smith & Smith 1989, Chee et al 1989). The UL13 gene of HSV-1 and its equivalents in VZV, EBV, HCMV and HHV6 has the characteristic motifs of protein kinases (see Section 5.1.4), but as yet the protein products of these genes have not been shown to have kinase activity.

The N terminal extensions of the large subunits of ribonucleotide reductase (see Section 4.2) of HSV-2 and HSV-1 have been shown to have sequence homology with protein kinases and they have both been shown to possess intrinsic protein serine/threonine kinase activities (Chung et al 1989, Paradis et al 1990). Both of the kinase activities undergo autophosphorylation and the HSV-1 RR1 has been shown to phosphorylate the small subunit of ribonucleotide reductase.

The HSV-2 RR1 subunit is myristylated (Chung et al 1990) and the enzyme has been found in cytosolic, cytoskeletal and plasma membrane fractions of infected cells. The kinase activity of RR1 was found to be stimulated by polylysine *in vitro*.

The function of these protein kinase domains in RR1 of HSV-1 and HSV-2 is not known but the HSV-2 enzyme may have a role in the transforming potential of the virus. Seuqences containing a large subunit of HSV-2 are known to cause transformation in hamster cells (Hayashi et al 1985, Iwasaka et al 1985).

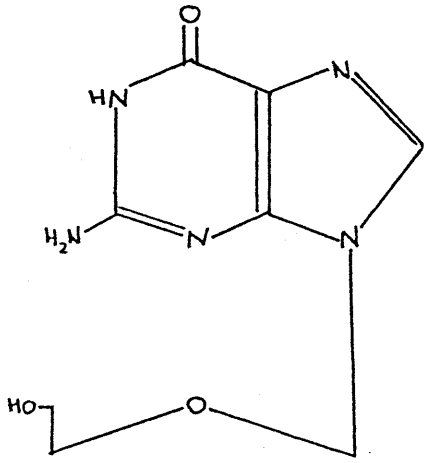
Section 6 Antiviral Agents

6.1 Antiviral Agents in General

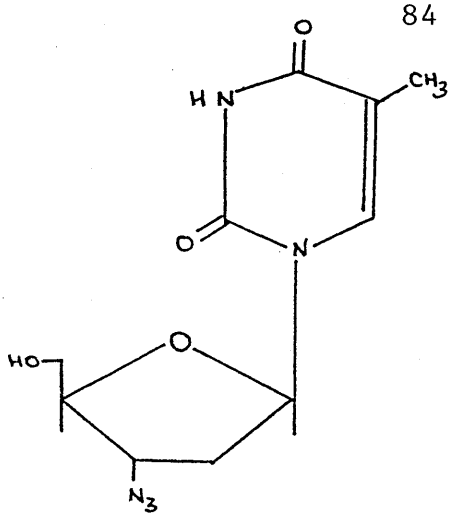
In recent years the search for compounds with antiviral activity has greatly increased, especially in the case of viruses such as human immunodeficiency virus-1 (HIV-1) and HSV-1 where vaccines are proving difficult to produce (De Clercq 1989, Dimmock, Griffiths & Madeley (Ed) Control of Virus Diseases Part II 1990 Cambridge University Press). The rationale behind many of the known antiviral compounds has been that they are nucleoside analogues (Table 6.1) which interfere with viral DNA or RNA synthesis in either a specific or non-specific manner. More recent attempts to identify novel antiviral drugs have concentrated on inhibitors of specific viral functions (Cameron 1990). The commercially available anti-herpes drug, acyclovir [9-(2-hydroxyethoxy-methyl) guanine] (Figure 6.1) can only be phosphorylated by the virally coded thymidine kinase and so will not affect uninfected cells. The mono-phosphorylated form of acyclovir is an even more potent inhibitor of viral DNA synthesis (Elion et al 1977). The triphosphorylated derivative of the compound is produced by cellular enzymes. The compound can then be incorporated into viral DNA causing termination of the nascent viral DNA chain. The triphos-phorylated derivative is also an inhibitor of the viral DNA polymerase.

AZT (3' azido-3' deoxythymidine) (Fig. 6.1) the commercially available anti-HIV drug does not have the same degree of specific inhibition. AZT is phosphorylated by cellular enzymes and the triphosphorylated form is a highly potent inhibitor of the HIV reverse transcriptase.

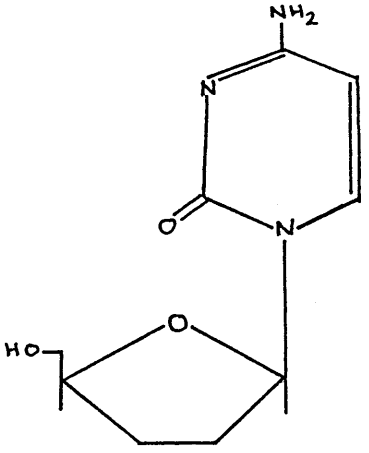
The utilisation of this form of AZT by the reverse transcriptase leads to premature chain termination of nascent viral DNA chains. The fact that AZT can be phosphorylated by cellular enzymes leads to the accumulation of its intermediary phosphorylated derivatives (e.g. 5' monophosphate form) in dividing non infected cells.



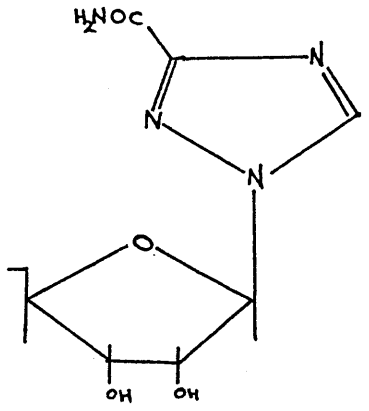
Acyclovir



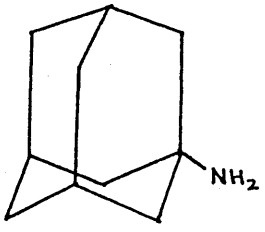
Azidothymidine



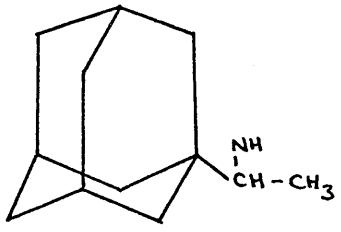
Dideoxycytidine



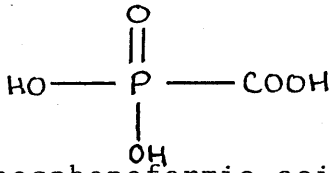
Ribavirin



Amantadine



Rimantadine



Phosphonoformic acid

Figure 6.1 Structures of known antiviral agents

Table 6.1 Antiviral compounds: their target viruses and sites of action

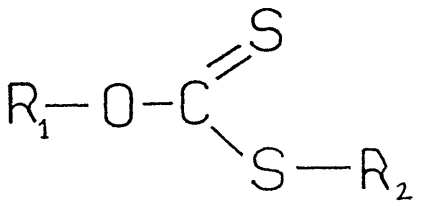
Antiviral compound	Virus inhibited	Site of action
Acyclovir	HSV-1, HSV-2	Viral DNA polymerase
Azidothymidine (AZT)	HIV-1	Viral reverse transcriptase
Dideoxycytidine (ddc)	HIV-1	Viral reverse transcriptase
Ribavirin	Influenza A & B respiratory syncytical virus	viral RNA polymerase
Phosphonoformate	Cytomegalovirus	Viral DNA polymerase
Amantadine Rimantadine	Influenza A	Viral penetration and uncoating
Interferon	Many viruses, including rhinovirus & Influenza A & B	Virus release

The AZT triphosphorylated form is a poor inhibitor of cellular DNA polymerase and so will cause little chain termination, but AZT's accumulated derivatives inhibit essential enzymes required in DNA metabolism. These intermediate derivatives may account for the cytotoxic effects of the compound found in many patients (De Clercq 1989).

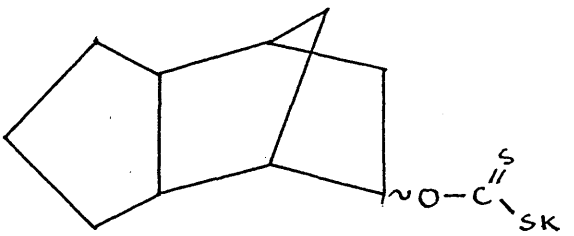
6.2 Xanthates

Xanthates, a novel group of compounds with the general structure shown in Figure 6.2 have been shown to have antiviral activity against a wide range of DNA and RNA viruses including HSV-1, Vaccinia, Coxsackie B, SV40 (Sauer G. et al 1984) and HIV-1 (Mellert et al 1988). The compounds were shown to have low cytotoxicity at the levels used for their antiviral activity. In HSV-1 infected cells the xanthate D609 (Figure 6.2) inhibited viral DNA synthesis but was not incorporated into viral or host DNA. Drug resistant mutants to the xanthate were not isolated (Sauer G. et al 1984). The compound D609 healed HSV-1 and HSV-2 induced skin lesions on guinea pigs when applied in an acidic ointment. Viral DNA synthesis and virus production were inhibited in the skin (Amtmann E. et al 1985a) by the xanthate. The xanthate D609 was able to interrupt the viral life cycle of SV40 in infected cells at any point. Viral replication and transcription was inhibited (Sauer G. et al 1984) by the compound. In bovine papilloma virus transformed cells treatment with D609 resulted in reversion of the transformed cells to their normal phenotype with a limited *in vitro* lifetime and contact inhibition (Sauer G. et al 1984, Amtmann E. et al 1985b).

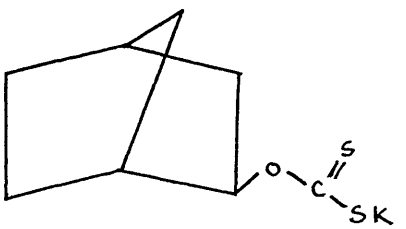
Problems with the acidic pH required for antiviral activity (pH 6.8) were solved when it was found that co-administration of a monocarboxylic acid C_{10}-C_{14} in chain length had a synergistic antiviral effect at physiological pH (pH7.4) (Amtmann E. et al 1987). This effect was found to be caused by the fact that the short chain fatty acids



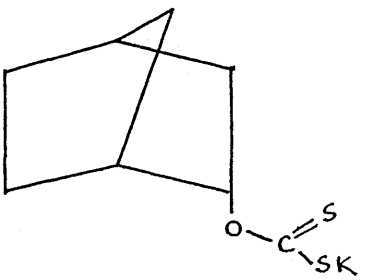
General structure of the xanthates



D609 (endo/exo)



D611 exo



D611 endo

Figure 6.2 Structure of the xanthate antiviral agents

(C_{10}-C_{14}) occupied the same binding site on BSA as the xanthate (Music L. et al 1989).

Detailed studies on vesicular stomatis virus (VSV) infection indicated that the xanthate D609 inhibited viral replication and late gene transcription. It was also shown to have significantly reduced the phosphorylation of the virally coded NS protein which is required for viral transcription (Müller-Decker K. et al 1987). Further studies on the signal transduction system of uninfected cells suggested that the xanthate acted at the level of phospholipase C to block the activation of protein kinase C through provision of diacylglycerol (DAG) (See Figure 5.1) one of its cellular activators (Müller-Decker K. et al 1988).

CHAPTER 3

MATERIALS & METHODS

1. Materials

1.1 Chemicals & Reagents

All chemicals used were of Analar grade and obtained from appropriate sources as stated below.

1.1.1 Enzymes

3'5' cyclic AMP dependent protein kinase, snake venom phosphodiesterase I, tpck-treated trypsin and alkaline phosphatase were obtained from the Sigma Chemical Co., Poole, Dorset, U.K..

Acid phosphatase was obtained from Böehringer Mannheim (U.K.), East Sussex, U.K..

Protein phosphatases 1 & 2A were a gift from Dr.D.G. Hardie, Protein Phosphorylation Group, Dept. of Biochemistry, University of Dundee, Dundee, U.K..

The pseudorabies virus protein kinase was a gift from Dr. D.P.Leader, Dept. of Biochemistry, University of Glasgow, Glasgow, U.K..

1.1.2 Enzyme Assays

Casein, protamine sulphate, histone IIIs; histone IIAS, calmodulin, cyclic AMP, ATP, myosin, phosphatidyl serine, ethidium bromide, agarose and double stranded calf thymus DNA were obtained from the Sigma Chemical Co., Poole, Dorset, U.K..

DE81 paper was obtained from Whatman Ltd., Maidstone, Kent, U.K..

1.1.3 Polyacrylamide gel Electrophoresis & Immunoblotting

SDS molecular weight markers, Coomassie brilliant blue, 1 - chloro - 4 - napthol, tween 20, protein A peroxidase conjugate, BSA (fraction V) and NNN'N' tetra methylene diamine (TEMED) were obtained from the Sigma Chemical Co., Poole, Dorset, U.K..

Ampholines were obtained from LKB, Sweden.

Nitrocellulose membrane was obtained from Schleicher & Schuell, Anderman & Co., Ltd., Surrey, U.K..

PVDF membrane was obtained from Millipore, Watford, Hertfordshire, U.K..

The monoclonal antibody Q1 against the HSV DNase was a gift from Dr. K. Powell, Molecular Sciences Dept., Wellcome Research Laboratories, Beckenham, Kent, U.K..

Anti-mouse IgG (sheep) horse-radish peroxidase conjugate was obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, U.K..

The antibodies against the ribonucleotide reductase large subunit were a gift from Dr. H. Marsden, and Dr. H. Lankinen, Institute of Virology, Glasgow, U.K..

1.1.4 Chromatography

DE52 Cellulose, phosphocellulose P11, and cellulose C11 were obtained from Whatman Ltd., Maidstone, Kent, U.K..

Single stranded DNA cellulose, Sephadex G50, phenyl Sepharose CL4B, and the Mono Q FPLC column were obtained from Pharmacia Ltd., Milton Keynes, U.K..

1.1.5 Tissue Culture

Minimum essential medium, Glasgow modification (GMEM), 200mM glutamine, 7.5% sodium bicarbonate, trypsin, penicillin: streptomycin and new born calf serum were obtained from Gibco-BRL, Paisley, U.K..

Essential amino acids, non-essential amino acids and vitamins were obtained from Flow Laboratories, Irvine,U.K..

Versene (0.14M NaCl, 2.7mM KCl, 10mM$NaHPO_4$, 2.4mM KH_2PO_4, 2mM EDTA, 0.015% phenol red) was prepared and autoclaved by the department's tissue culture staff.

$25cm^2$, $75cm^2$ & $150cm^2$ sterile tissue culture flasks were obtained from Corning Glassworks, New York, U.S.A..

1.1.6 Autoradiography

X-ray film was obtained from the Fuji Photo Film Co., Ltd., Tokyo , Japan.

1.1.7 Scintillation Fluid

Ecoscint A was obtained from National Diagnostics, Aylesbury, U.K..

1.1.8 General

Nonidet P40, undecanoic acid, phenyl methyl sulphonyl fluoride (PMSF), ethylene glycol bis (β amino ethyl ether) N,N,N',N' - tetra acetic acid (EGTA) and tosyl-L-phenyl-alanine chloromethyl ketone (TPCK) were obtained from the Sigma Chemical Co., Poole, Dorset, U.K..

Poly (dI.dC).Poly (dI.dC) was obtained from Pharmacia Ltd., Milton Keynes, U.K..

dATP, dTTP and dGTP were obtained from Böehringer Mannheim, East Sussex, U.K..

Bradford reagent was obtained from Biorad Ltd., Richmond, California, U.S.A.

Aquacide II was obtained from Calbiochem-Behring Corporation, U.S.A..

The restriction enzymes and react buffers were obtained from Bethesda Research Laboratories, Cambridge, U.K..

The plasmid G35a was a gift from Dr. N. Stow, Institute of Virology, Glasgow, U.K..

The xanthates were synthesised by chemists at Glaxo Group Research, Greenford, Middlesex, U.K., by established methods (Moller & Ottel 1967).

The nick translation kit was obtained from Amersham International, Amersham, U.K..

1.2 Radiochemicals

Deoxy (5-^{3}H) cytidine 5' triphosphate, ammonium salt, adenosine 5' (γ^{32P}) triphosphate, triethyl ammoni um salt and orthophosphate [^{32P}] in dilute HCl were obtained from Amersham International plc, Amersham, U.K..

1.3 Cells & Viruses

BHK 21|C13 baby hamster kidney fibroblasts originally produced by MacPherson & Stoker (1962) were maintained in continuous cell culture.

HSV-1 strain F was obtained from the Institute of Virology, Church Street, Glasgow (Ejecito & Kieff 1968).

2 Solutions

2.1 Cell Culture Medium

Normal medium:	Distilled water	365ml
Minimum essential medium Glasgow modification	GMEM	50ml
	New born calf serum	50ml
	7.5% sodium bicarbonate	15ml
	200mM glutamine	10ml
	Penicillin/Streptomycin	10ml

Phosphate Reduced medium:

Sterile water	244.5ml	Phenol Red 0.17mg/ml	50ml
Penicillin/Streptomycin	10ml	Salts	
New born calf serum	50ml	2.65mg/ml $CaCl_2.6H_2O$	
7.5% sodium bicarbonate	15ml	4 mg/ml KCl	
200mM gluatime	10ml	2mg/ml $MgSO_4.7H_2O$	
essential amino acids	10ml	64mg/ml NaCl	50ml
non essential amino acids	5ml		
vitamins	5ml	D Glucose 45 mg/ml	50ml
		Ferric chloride 0.1mg/ml	0.5ml

2.2 Viral Infections

Phosphate Buffered Saline PBS

A: 10g NaCl
0.25g KCl
1.44g NaH_2PO_4
0.25g KH_2PO_4 pH to 7.4 with HCl
H_2O to 1l

B: 0.25g $CaCl_2$/l C: 0.25g $MgCl_2$/l

Solutions A:B:C mixed in the ratio 8:1:1 before use.

Reticulocyte standard buffer	RSB 10mM TrisHCl pH7.5 10mM KCl 1.5mM magnesium acetate
Medium K	250mM Tris-HCl pH7.5 1.25M KCl 50mM magnesium acetate 50mM 2-mercaptoethanol
PMSF	500mM in DMSO
EGTA	250mM in dilute alkali

2.3 Column buffers

DE Buffer	20mM Tris-HCl pH 7.5 1mM EDTA 1mM EGTA 1mM PMSF 10mM 2-mercaptoethanol 10% glycerol
DC Buffer	20mM Tris-HCl pH 7.5 50mM KCl 1mM EDTA 1mM EGTA 1mM PMSF 0.5mM dithiothreitol 20% glycerol 0.1% NP40
Phenyl Sepharose Buffer	20mM Tris-HCl pH 7.5 1mM EDTA 1mM EGTA 1mM PMSF 10mM 2-mercaptoethanol

2.4 DNA work buffers

STE Buffer	10mM Tris-HCl pH 8.0 1mM EDTA 100mM NaCl
TE Buffer	10mM Tris-HCl pH 8.0 1mM EDTA

2.5 Assay buffers

Exonuclease assay buffer	500mM Tris-HCl pH 9.0 20mM $MgCl_2$ 100mM 2-mercaptoethanol
Endonuclease assay buffer	300mM Tris-HCl pH 7.0 24mM 2-mercaptoethanol
General kinase assay mix	20mM Tris-HCl pH 7.4 50mM KCl 10mM 2-mercaptoethanol 10mM $MgCl_2$ 0.1mM ATP 0.2μCi [^{32}P] ATP substrate

2.6 Lowry protein estimation

A: 4% Na_2CO_3, 0.8% 0.2M NaOH

B: 1% potassium sodium tartrate

C: 0.5% $CuSO_4 \cdot 5H_2O$

Solutions D & E were prepared just before use

D: 50:1:1 of A:B:C

E: Folin-Ciocalteau reagent diluted 1:1 with water

2.7 SDS PAGE Solutions

Electrophoresis buffer	28.8g Glycine 6g Tris 2g SDS H_2O to 2l
Sample buffer	1.25ml 1M Tris-HCl pH 7.0 5 ml 20% SDS 2.5ml 2-mercaptoethanol 2.5ml 1% Bromophenol blue 10ml glycerol
Coomassie stain	0.1% Coomassie brilliant blue R250 50% methanol 10% glacial acetic acid
Destain	10% methanol 10% glacial acetic acid

Silver staining

stain { Solution A: 0.8g $AgNO_3$ in 4 ml H_2O
Solution B: 21ml 0.36% NaOH, 1.4ml 14.8M NH_4OH

Solution A was added dropwise to B with mixing, and then diluted to 100ml with deionised water.

Developer:
0.12ml 1M Citric Acid
0.25ml 38% formaldehyde
to 500ml with deionised water

2.8 2 Dimension gels

Sample buffer

Urea 0.57g
10% NP40 200 µl
ampholines pH 3.5-10 50 µl
2-mercaptoethanol 50 µl
1% Bromophenol blue 1µ 1
H_2O 300 µ 1

Overlay solution

Urea 1.2g
ampholines 40 µ 1
H_2O 3.9ml

Gel solutions

Urea 4.76g

45% Acrylamide, 0.6% bisacrylamide 0.84ml
10% NP40 1.6ml
40% ampholines pH3.5-10 0.4ml
H_2O 3.23ml
to polymerize
TEMED 6 µ 1
10% ammonium persulphate 30 µ 1

Interdimensional soaking buffer

0.5M Tris-HCl pH6.8	10ml
10% SDS	30ml
10mM dithiothreitol	3ml
glycerol	10ml
H_2O to	100ml

2.9 Western Blotting

Transfer buffer

25mM Tris
192mM Glycine
0.02% SDS
20% methanol

Ponceau S stain

0.1% Ponceau S
3% TCA
in H_2O

2.9.1 DNase

Blocking buffer 20mM Tris-HCl pH 7.2
0.15M NaCl
0.5% Tween 20

prepared as a (X10) stock without Tween. The Tween 20 was added just prior to use.

1st Antibody solution: Blocking buffer containing 5% heat inactivated horse serum and the Q1 monoclonal at a dilution of 1:1000.

2nd Antibody solution: Blocking buffer without tween containing 3% BSA and the sheep antimouse Ig G HRP conjugate at a dilution of 1:100.

Developer:

Solution 1: 20mg 1-chloro-4-napthol in 6ml methanol (-20°C)

Solution 2: 30ml of blocking buffer without tween + 20µl 30% H_2O_2.

Solutions 1 & 2 were mixed together just prior to use.

2.9.2 Ribonucleotide Reductase

TBS 20mM Tris-HCl pH 7.5
500mM NaCl

TTBS 20mM Tris-HCl pH 7.5
500mM NaCl
0.05% Tween 20

Blocking buffer: TBS containing 3% Gelatin

1st Antibody Solution: TTBS containing 1% Gelatin
0.01% sodium azide and antibody 20208 at a dilution of 1:50.

Conjugate solution: TTBS containing 1% Gelatin and the protein A-HRP conjugate at a dilution of 1:1000.

Developer

Solution 1: 60mg 1-chloro-4-napthol in 20ml methanol (-20^{0}C)

Solution 2: 100ml TBS + 60µl 30% H_2O_2

Solutions 1 & 2 were mixed just prior to use.

2.10 Invitro/In vivo labelled peptides

Transfer buffer
- 25mM Tris
- 10mM Glycine
- 0.5mM Dithiothreitol

2.11 Agarose gel Electrophoresis

Sample buffer: Glycerol : 0.025% Bromophenol blue [1:1]

Electrophoresis buffer (x10)

TBE
- 108g Tris
- 55g Boric Acid
- 93g edta

1l with H_2O

Ethidium bromide was included in the final buffer at a concentration of 0.5 µg/ml.

2. Methods

1 Cell Culture

1.1 Subculture of BHK cells

Trypsin: versene (See Materials)

In the ratio 1:4 was warmed to 37°C. The growth medium (Materials 2.1) was removed from an 80oz. glass winchester (roller bottle) of confluent BHK cells and 10ml of the trypsin:versene mixture added. This was swirled around in the bottle for 3 - 5 mins. and then discarded. 15 - 20 ml fresh trypsin:versene was added and gently swirled around the bottle and the cells observed under an inverted microscope every few minutes. When the cells had started to round up the trypsin:versene was poured off and the bottle left until the majority of the cells had detached from the glass. 20 - 30 ml of culture medium was added and the bottle shaken vigorously to detach all of the cells. The cell suspension was counted using a haemocytometer and new roller bottles seeded with 10×10^6 cells in 130ml culture medium and $120cm^3 CO_2$.

1.2 Propagation of cells from frozen stock

A vial of frozen cells was removed from the liquid nitrogen vat and immediately placed in a water bath at 37°C. When defrosted the cell suspension was transferred to $25cm^2$ tissue culture flasks containing 10ml culture medium at a seeding level of 0.5×10^6 cells per flask. When the cells were confluent, they were subcultured into $150cm^2$ flasks until sufficient numbers were obtained to seed 80 oz. roller bottles.

2 Virus

2.1 Virus stocks for infection

Confluent roller bottles of BHK cells were taken and the culture medium removed. To each was added 50 ml. fresh culture medium containing 0.01pfu/cell HSV-1(F). The roller bottles were left rotating at 37°C for 48 hours and then shaken vigorously to remove the infected cells. The virus suspension was sonicated for 20 mins. in a sonicating water bath to release cell associated virus particles, and then stored at -80°C.

2.2 HSV-1(F) infection of BHK cells

20 - 30 roller bottles of confluent BHK cells were used. After the culture medium had been removed, 20 ml. of the virus suspension described in Methods 2.1 was added to give infection at 20pfu/cell and the bottles returned to 37°C for 1 hour. 60 ml. of culture medium was then added to each bottle and they were returned to 37°C and left for the required post infection time.

The cells were harvested on ice into PBS (Materials 2.2) containing 1mM EGTA and 1 mM PMSF to inhibit proteolysis. The cells were pelleted by centrifugation at 2000 rpm for 5 mins. in a Beckman TJ6 centrifuge and the pellet resuspended in an equal volume of RSB (Materials 2.2) containing 1mM EGTA and 1 mM PMSF. The cell suspension was left on ice for 5 mins. then homogenised by 20 strokes in a teflon-glass homogeniser. The homogenate was subjected to centrifugation at 30,000xg for 30 mins. at 4°C, after the addition of Medium K (Materials 2.2) [0.11 x volume of RSB added]. The supernatant was either shock frozen in liquid nitrogen and stored at -80°C or was used immediately for the purification [Methods 6] of the enzyme required.

3 DNA Cellulose Preparation

(after Alberts & Herrick) 1971

3.1 Pretreatment of Cellulose

10g. of cellulose C11 was washed 3 times in boiling ethanol using a distillation flask and heater. The cellulose was then washed at the pump with 100ml each of 0.1M HCl, 1 mM EDTA and 10mM HCl. It was then washed with distilled water until the filtrate had a neutral pH and lyophilised to dryness.

3.2 Preparation of denatured DNA

3 x 10 ml. aliquots of a solution of 2 mg/ml calf thymus DNA, 10 mM K_2HPO_4 and 1 mM EDTA were heated in test tubes at 100°C for 15 mins. The tubes were then placed in ice cold water and the solutions made to 20mM Tris-HCl pH 7.4.

3.3 DNA Cellulose production

The solution prepared in Section 3.2 was placed in a clean dry beaker and to it was added the dry cellulose (see Section 3.1) with occasional mixing with a glass rod. The mixture was then spread out in an evaporating dish, covered with gauze and left at room temperature until dry (2 - 3 days). The dried mixture was ground to a powder with a glass rod and lyophilised overnight. The lyophilised powder was suspended in 20 volumes of 10mM Tris HCl pH 7.4, 1mM EDTA and left at 4°C for 24 hours. The slurry was then washed twice with 10 mM Tris HCl pH 7.4, 1 mM EDTA and stored at -20°C in 10 mM Tris HCl pH 7.4, 1mM EDTA and 0.15 MKCl.

4 Preparation of [^{3}H] labelled DNA

4.1 Synthesis of [^{3}H] DNA

25µl of dCTP[^{3}H] (≡ 25µCi) was placed in a 1.5ml Eppendorf tube and lyophilised to dryness. To the tube was added:

5 µl calf thymus ds DNA (2mg/ml)
10 µl Nick translation buffer } Amersham Nick
10 µl Enzyme solution } Translation Kit
10 µl Nick translation buffer } (Materials
2 µl 10 µl Enzyme solution } solutions)
73 µl H_2O

Solution mixed and incubated at 15°C for 2 hours.

4.2 Purifcation of [^{3}H] DNA

A 1ml disposable syringe was plugged with a small amount of glass wool and sterilised by running 100% ethanol through it and allowing it to dry. A 0.9 ml. bed volume of Sephadex G50 in STE pH8 (Materials 2.4) was prepared in the syringe by centrifugation at 1600 rpm for 4 mins. in a Beckman TJ6 centrifuge. The column was washed with several 100 µl aliquots of STE pH8.

The collecting system was then set up as shown in Figure 4.2.1 below, the reaction mixture was loaded onto the column and it was subjected to centrifugation as above. The run through was taken and diluted to 1ml. with STE pH8. It was stored in aliquots at -20°C. 10µl was added to 5ml. Ecoscint and placed in a scintillation counter to check incorporation.

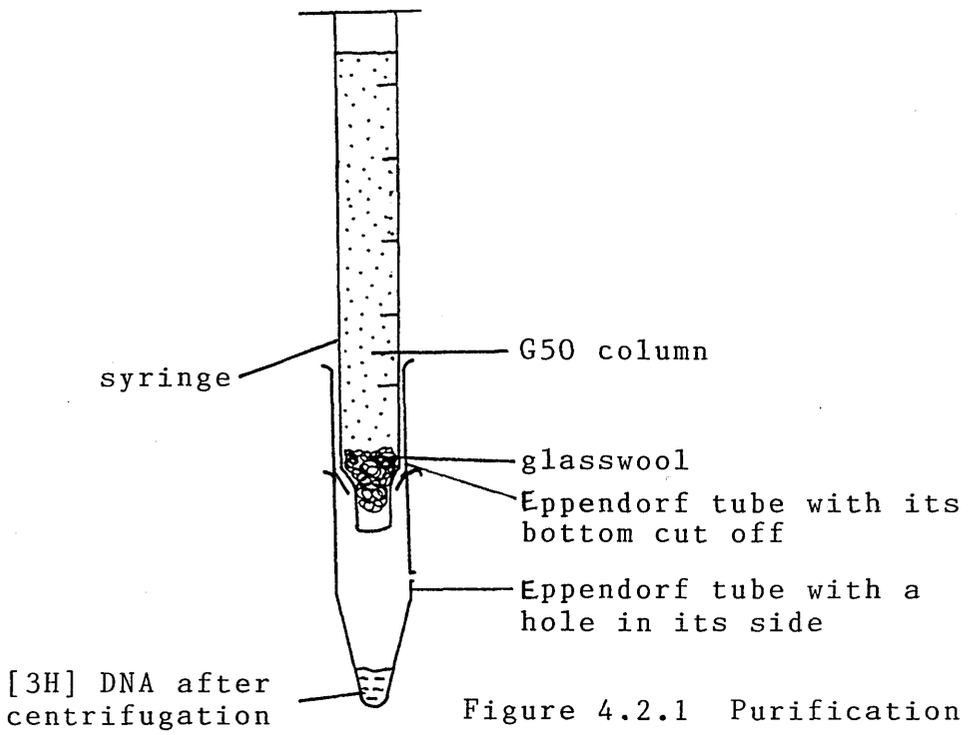


Figure 4.2.1 Purification system for nick translated [3H] DNA

5. Enzyme assays

5.1 DNase assay: exonuclease

(after Purifoy et al 1976)

Assay:
- 20µl Assaybuffer (Materials 2.5)
- 10µl [^{3}H] DNA (Methods 4)
- 10µl sample
- 160µl Water

After mixing, the assays were incubated at 37°C for 30 mins. 10µl of a 25mg/ml BSA solution and 1.3ml. of a 5% TCA solution were then added and the assays kept on ice for 30 mins. They were then subjected to centrifugation for 5 mins. in a microcentrifuge. To 1 ml. of each supernatant 5 ml. of ecoscint was added and the radio activity of the samples measured in a scintillation counter.

5.2 Kinase assays

The general kinase assay mix (Materials 2.5) was used for the assay of the US3 protein kinase, protein kinase C, casein kinase II and the cAMP dependent protein kinase. The substrates used were at a concentration of 800µg/ml. and were protamine sulphate for US3 protein kinase and protein kinase C, casein for casein kinase II and histone IIAS for cAMP dependent protein kinase. In the cAMP dependent protein kinase assay, cAMP was included at a concentration of 10µM. The final volume of all the above assays was 0.25ml..

A specific assay for protein kinase C was also used and consisted of: 5mM Hepes pH7.5, 12.5mM $MgCl_2$, 8mM2 mercaptoethanol, 0.5mM EGTA, 0.3mM EDTA, 50µM ATP, 0.2 mg/ml phosphatidyl serine, 0.2µCiɣ [^{32}P]ATP, 0.17mg/ml BSA, 1.2mg/ml histone III-s in a final volume of 0.12ml.

The assay for calcium: calmodulin dependent protein kinase (after Fukunaga K. et al 1982) was 25mMTris-HCl pH 7.5, 1mM dithiothreitol, 0.1mM$CaCl_2$, 10mM $MgCl_2$, 0.1mM ATP, 0.2µCiɣ[^{32}P]ATP,100 units calmodulin, 800µg/ml myosin

in a final volume of 0.2ml..

The assays were initiated by the addition of the enzyme: 100µl in the case of the 0.25ml and 0.2 ml. assays and 40µl in the case of 0.12ml. assays. They were incubated either for 30 mins. at 37°C (column assays) or 15 mins. at 35°C (xanthate studies).

After incubation 100µl aliquots of column assays were spotted onto 2 cm. x 2 cm. squares of DE81 filter paper whilst 3 x 50µl aliquots of xanthate assays were spotted onto a DEAE block filter. In both cases the filters were then washed twice for 30 mins. in 20% TCA, then twice for 15 mins. in 10% TCA. The DE81 filters were then rinsed in ethanol, whilst the DEAE block filter was rinsed in methylated spirits and both allowed to dry. The Cherenkov activity of the DE81 filters was then measured in a scintillation counter whilst the radioactivity of the DEAE block filter was measured using a LKB β plate scintillation counter.

6. Enzyme Purification

6.1 DNase Purification (after Banks et al 1983)

An extract prepared from 30 roller bottles as described in Methods 2.2 was loaded onto a 10 x 1.5cm column of DE52 cellulose. The column was then washed with 2 column volumes of DE Buffer (Materials 2.3) and eluted with a linear gradient of 0-0.5M KCl in DE Buffer (500ml gradient for a 30-40 roller bottle prep.). Every 2nd fraction was assayed for DNase activity (Methods 5.1) and the active fractions were pooled and dialysed against DE Buffer overnight.

The dialysed sample was then loaded onto a 7 x 1.5cm. phosphocellulose column and the column washed with 2 column volumes of DE Buffer. Elution was carried out with a linear gradient of 0.1M-0.4M KCl in DE Buffer (Volume 150-400ml.). Every 2nd fraction collected was assayed for enzyme activity.

The active fractions were pooled, and the BSA concentration adjusted to 500 µg/ml.. The preparation was dialysed overnight against DC Buffer (Materials 2.3).

The dialysed sample was loaded onto an 8 x 1 cm. DNA cellulose column which was composed of a mixture of commercial DNA Cellulose (Pharmacia) and denatured DNA Cellulose prepared as detailed in Methods 3. The column was washed with 2 column volumes of DC buffer and eluted using a 0.05M-1M KCl gradient (Volume: 100-200ml.). Every 2nd fraction was assayed for DNase activity and the active fractions pooled and dialysed against DC buffer. The sample was then reverse dialysed against Aquacide to reduce the volume, and then stored in 1 ml. aliquots at -80°C.

6.2 Kinase Purification

6.2.1 Protein Kinase C (after Kikkawa V. et al 1986)

An extract prepared from 20 - 30 roller bottles as described in Methods 2.3 was loaded onto a 10 x1.5cm. DE52 cellulose column. The column was washed with 2 column volumes of DE Buffer and then eluted with a 500 ml. gradient of 0-0.5M KCl. Every 2nd fraction was assayed using protamine sulphate as a substrate (Methods 5.2), the active fractions were pooled and dialysed against Phenyl Sepharose buffer (Materials 2.3) containing 1M $(NH_4)_2SO_4$ overnight.

The dialysed sample was loaded onto an 8 x 0.75 cm. Phenyl Sepharose column, which was then washed with 2 column volumes of Phenyl Sepharose buffer containing $1M(NH_4)_2SO_4$. Elution was carried out with a 150 ml. gradient ($1M(NH_4)_2SO_4$-$0M(NH_4)_2SO_4$) followed by 50 ml. of Phenyl Sepharose buffer. Every 2nd fraction was assayed for protamine kinase activity, the active fractions were then pooled and dialysed against DE Buffer. The sample was then concentrated in Aquacide, made to 50% glycerol and stored at -80°C.

6.2.2 US3 Protein kinase

This purification was essentially the same as that for protein kinase C except that this enzyme eluted at 0.2-0.22MKCl on DE52 Cellulose. The kinase from 3-4 DE52 cellulose columns was pooled and concentrated in Aquacide before being dialysed against Phenyl Sepharose buffer containing $1M(NH_4)_2SO_4$ and subsequent hydrophobic interaction chromatography.

6.2.3 Casein kinase II (after McGarvey 1981)

2 rat livers were chopped and homogenised in 10ml. RSB containing 1mM PMSF and 1mM EGTA (Materials 2.2). The homogenate was centrifuged at 30000xg for 30 mins. at 4°C and the supernatant loaded onto a 10 x 1.5cm. DE52 cellulose column. The column was then washed with 3-5 column volumes of DE Buffer and eluted with a 500ml. gradient of 0-0.4M KCl. Every 2nd fraction was assayed for casein kinase activity. Active fractions were pooled and dialysed overnight against DE Buffer containing 0.25 M KCl.

The dialysed sample was loaded onto a 7 x 1.5cm. phosphocellulose column and the column washed with 2 column volumes of DE Buffer containing 0.25M KCl. Elution was carried out with a 250ml 0.25M-1M KCl in DE Buffer gradient. Every 2nd fraction was assayed for casein kinase activity. Active fractions from0.5-0.75M KCl were pooled and dialysed against DE Buffer. The sample was concentrated in Aquacide, made to 50% glycerol and stored at -80°C.

7. Protein Estimation

7.1 Bradford Method

The test solutions were diluted to 800 µl with distilled water and 200µl Bradford reagent added and mixed immediately. Samples were left for at least 5 mins. and then their absorbance at 595nm measured. A standard curve was prepared using BSA at specific concentrations and from this the test sample protein concentrations were estimated.

7.2 Lowry Method

The test samples were made to 1.5ml. with distilled water and 1.5ml. of solution D (Materials 2.6)added, mixed and left for 10 mins. 0.3ml. of solution E (Materials 2.6) was added with immediate mixing.

Samples were left for 10 mins. and then their absorbance at 750nm was measured. A standard curve was constructed using dilutions of a BSA solution and from this unknown protein concentrations were estimated.

8. Protein Concentration

8.1 Acetone precipitation

To the sample in a test tube 5 volumes of acetone at room temperature was added. The tube was sealed with Nescofilm and placed at $-20^{o}C$ overnight. The precipitate was collected by repeated centrifugation ina 1.5ml. Eppendorf tube in a microcentrifuge (5 mins. per spin). The pellet was air dried.

8.2 TCA precipitation

Samples were made to 1 ml. with distilled water and 0.1ml. 0.15% sodium deoxycholate added. The sample was allowed to stand for 10 mins. at room temperature. 0.1ml. of 72% TCA was then added and after mixing the precipitate was collected by centrifugation in a microcentrifuge for 5 mins. The pellet was washed twice with distilled water.

9. SDS polyacrylamide gel electrophoresis

The gels were prepared using BRL apparatus with a thickness of either 1.5mm or 3 mm. The final concentration of acrylamide in the main gel was 10%.

Samples were prepared by the addition of sample buffer (Materials 2.7) either to protein precipitates (Methods 8) or directly to the sample and then heated to 100°C for 2 - 5 mins..

Gels:	MAIN GEL (ml)	STACKER GEL (ml)
30% Acrylamide : 0.8% bisacrylamide	16.6	2.5
1.5M Tris HCl pH8.8	12.5	-
0.5M Tris HCl pH6.8	-	3.8
H_2O	20.3	8.8
20% SDS	0.25	75µl
TEMED	18µl	5µl
10% Ammonium persulphate	0.5	0.3
	50	15

The gels were set up in a BRL vertical electrophoresis system in the electrophoresis buffer (Materials 2.7) and run under the following conditions:-

Gel thickness (mm)	1st Hr.	Next 4-5 hrs.	Overnight
1.5	25mA	40mA	10mA
3	40mA	60mA	20mA

10. Protein gel staining

10.1 Coomassie staining

SDS gels were subjected to electrophoresis as described in Methods 9. After removal from the electro phoresis apparatus each gel was placed in the staining solution (Materials 2.7) for 1 - 2 hrs. at room temperature. The gel was then removed and placed in the destaining solution (Materials 2.7) at 37°C. The gel was destained with several changes of solution until the individual bands could clearly be seen.

10.2 Silver staining (after Wray W. et al 1981)

The gel was removed from the electrophoresis apparatus and placed in a 50% methanol solution using double deionised water. The gel was allowed to soak, with several changes of 50% methanol, for at least 24 hours. The silver stain was prepared (Materials 2.7) and the gel soaked in this for 10-15 mins.. The gel was then rinsed in double deionised water (2 changes) for 1 hr.. The gel was then placed in the developer (Materials 2.7) until the first black bands appeared then removed and placed in water while the bands continued to develop.

11 2 Dimensional gel electrophoresis (after O'Farrell 1975)

11.1 1st Dimension: Isoelectric focusing gels

The gel tubes were sealed with Nescofilm and 10 cm. x 1.5 mm. gels were poured with a gel solution (Materials 2.8) which had been incubated at 37°C to dissolve the urea. The gels were overlaid with water and left to polymerize overnight. Samples were prepared by the addition of 30µl of sample buffer (Materials 2.8) to a protein precipitate, (Methods 8), and then it was heated to 100°C for 5 mins.. The samples were loaded onto the 1st Dimension gels then overlaid with an overlay solution (Materials 2.8) and run under the following conditions:-

Top tank	0.4% Ethanol amine -ve terminal
Bottom tank	0.2% Phosphoric acid +ve terminal

The gels were run at 200V for the first 30 mins., 300V for a further 3 hrs., 400V overnight and at 500V for an hour the next day. The gels were then removed from their tubes using a glass syringe and a long needle. They were then placed in 5 ml. interdimensional soaking buffer (Materials 2.8) for 1 hr. at room temperature.

11.2 2nd Dimension: SDS gels

A 1.5mm., 10% acrylamide gel was prepared as descibed in Methods 9. The 1st Dimension gel was loaded onto the stacker gel and sealed using a 1% agarose solution containing bromophenol blue. The gel was then subjected to electro phoresis under the conditions stated in Methods 9 until the dye front had run off the end of the gel. The gel was then stained with Coomassie blue (Methods 10.1) or used for Western blotting (Methods 12).

12 Western or Immunoblotting

12.1 Transfer conditions

The transfer apparatus was set up in a plastic box containing transfer buffer (Materials 2.9)in the following order: the plastic holder, rough padding, 2 sheets of 3 mm. filter paper and a nitrocellulose sheet. The 2nd Dimension gel or SDS gel was laid on top of the nitro-cellulose and any air bubbles removed. A further 2 sheets of filter paper were then placed on the gel and then a second rough pad followed by the second half of the plastic holder. The whole arrangement was then placed in the transfer tank and transfer buffer added to cover it. Transfer was either at 40V for 3 hrs. or 50mA overnight.

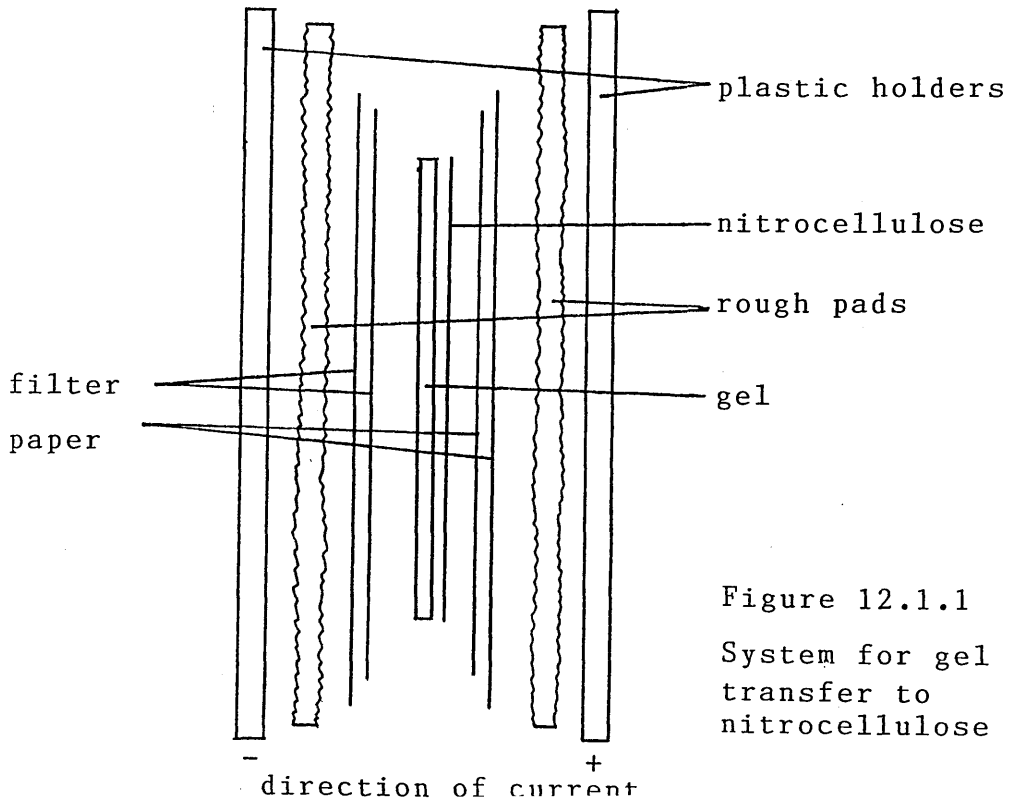


Figure 12.1.1
System for gel transfer to nitrocellulose

12.2 Ponceau S staining of nitrocellulose

The nitrocellulose sheet was removed from transfer and cut to match the shape of the gel. The sheet was then placed in the staining solution (Materials 2.9) for 30 mins. then rinsed in water. The molecular weight markers and proteins were stained red. The markers were marked with pencil and the sheet rinsed in water until the red staining had disappeared.

12.3 Immuno blotting for the DNase

The destained nitrocellulose sheet was incubated in blocking buffer (Materials 2.9) for 1 hr. at room temperature then incubated with the 1st Antibody solution (Materials 2.9) for at least 2 hrs. at room temperature. The sheet was then washed with 5 changes of blocking buffer for 5 mins. each, rinsed in water for 5 mins. and then incubated with the 2nd Antibody solution (Materials 2.9) for at least 3 hrs. at room temperature. The sheet was then washed with 5 changes of blocking buffer (5 mins. each) and rinsed in water. The developer was prepared as described in Materials 2.9 and added to the sheet at room temperature. The sheet was left until development had taken place, then it was rinsed in water, placed between filter papers and stored in aluminium foil.

12.4 Immuno blotting for the Ribonucleotide reductase (large subunit)

The destained sheet was incubated for 1 hr. in 2 changes of blocking buffer (Materials 2.9) at 37^{o}C and then washed in 2 changes of TTBS (Materials 2.9) for 5 mins. each. The sheet was then incubated in the 1st Antibody solution (Materials 2.9) overnight at room temperature, then washed for 15 mins. each in 3 changes of TTBS and incubated in the conjugate solution (Materials 2.9) for 1 - 2 hrs. at room temperature. The nitrocellulose sheet was washed a further 3 times in TTBS (15 mins.) before being rinsed in TBS (Materials 2.9) (2 x 15 mins.).

The developer was prepared as described in Materials 2.9 and 20 - 30 ml. added to the sheet, which was left at room temperature until development was complete. The sheet was then rinsed in water, stored in filter paper and wrapped in aluminium foil.

13 Modification of the DNase

13.1 Alkaline phosphatase

Buffer	500mM Glycine pH10
	40mM $MgCl_2$
	5mM $ZnCl_2$
Reaction	500µl Buffer
	500µl DNase sol^n (Methods 6.1)
	100 units Alkaline phosphatase

The reagents were mixed and incubated at 37°C for 1 - 2 hrs. The assay was then split into 2 x 500µl aliquots. Each 500µl was made to 1 ml. and TCA precipitated (Methods 8.2). The pellets were dissolved in 20µl 1st Dimension sample buffer and run in the 2 Dimension gel system (Methods 11) in a pH gradient 3.5 - 10. One gel was coomassie blue stained (Methods 10.1) and the other was transferred to nitrocellulose and immuno blotted for the DNase (Methods 12.3).

13.2 Acid phosphatase

Buffer	250mM MES pH6.5
	5mM EGTA
	5mM $MgSO_4$
Reaction	500µl Buffer
	450µl DNase (Methods 6.1)
	50µl Acid phosphatase

The reagents were mixed and incubated at 37°C for 2 hrs. and then treated in the same way as those in Methods 13.1.

13.3 Snake venom phosphodiesterase (after Yoshihara K. et al 1978)

2 x 1 ml. aliquots of a DNase solution (Methods 6.1) were acetone precipitated (Methods 8.1) overnight. The pellets were washed twice with ethanol : 150mM Tris HCl pH7.4 (2:1, v:v), and then dissolved in 200µl of 0.1M NaOH at 25°C for 30 mins.. The samples were neutralized by the addition of 20µl 1MHCl and to each sample was added 20µl 1MTris HCl pH8.0, 4µl 1M $MgCl_2$ and 10µl 10mM 5'AMP. To one tube was added 20µg snake venom phosphodiesterase and to the other 20µl H_2O. The samples were incubated at 37°C for 1 hr. then a further 30µg of the enzyme was added and the samples incubated for a further 2 hrs., after which they were acetone precipitated overnight. The samples were then run on the 2 Dimension gel system (Methods 11) in a pH range 3.5 - 10 and Immuno blotted for the DNase (Methods 12).

13.4 Protein phosphatase 1

Phosphatase Buffer	200mM Tris HCl pH7.0 10mM Dithiothreitol
Reaction	500µl DNase (Methods 6.1) 50µl buffer 50µl Protein phosphatase 1

The reagents were mixed and incubated at 30°C for 1 hr., The sample was then acetone precipitated (Methods 8.1) overnight and run on the 2 Dimension gel system (Methods 11) in a pH range 3.5 - 10, then Immuno blotted for the DNase (Methods 12).

14 In vitro phosphorylation

These reactions were based on the kinase assays given in Methods 5.2. The kinases available were those purified in Methods 6.2 (protein kinase C, US3 protein kinase, casein kinase II), cAMP dependent protein kinase and the PRV protein kinase.

14.1 1 Dimension in vitro phosphorylation

The assays used were those given in Methods 5.2 except that the final volume for the US3 protein kinase, PRV protein kinase, cAMP dep protein kinase and casein kinase II assays was 50μl. The final volume of the protein kinase C assay was 40μl. DNase was used as a substrate, as well as the artificial substrates given in Methods 5.2, and the γ [^{32}P]ATP concentration was 0.5 μ Ci per assay. The assays were incubated at 37°C for 30 mins., 30μl of SDS sample buffer (Materials 2.7) was added and they were heated to 100°C for 5 mins.. The samples were then run on 3 mm. 10% acrylamide gels (Methods 9), coomassie blue stained (Methods 10.1), dried down and subjected to autoradiography at -80°C.

14.2 2 Dimension in vitro phosphorylation

The assays used were those given in Methods 5.2 except that the final volume of each assay was 1 ml.. The kinases used were cAMP dep protein kinase, protein kinase C and US3 protein kinase. 2 assays for each kinase were set up, one had an ATP concentration in the assay of 2 mM and no [^{32}P] labelled ATP was added to those assays. The other set had an ATP concentration in the assay of 50μM and 3μCi of γ [^{32}P] ATP was added to the assays. All the assays were incubated at 37°C for 1 hr., TCA precipitated (Methods 8.2) and run on the 2 Dimension gel system (Methods 11) pH range 3.5 - 10. The labelled gels were then coomassie blue stained (Methods 10.1), dried down and subjected to autoradiography at -80°C. The unlabelled gels were transferred to nitrocellulose and immuno blotted for the DNase (Methods 12).

15 In vivo labelling with [^{32}P] orthophosphate

In vivo labelling was carried out in plastic 150cm^2 tissue culture flasks, 3-5 per labelling experiment. BHK cells were grown to confluence and 2 hrs. prior to infection the culture medium was removed and phosphate reduced medium (Materials 2.1) introduced. After infection (Methods 2.2) for 1 hr., the virus preparation was removed and 20 ml. phosphate reduced medium containing 1-2MCi [^{32}P] orthophosphate added. After the specified infection time the cells were harvested as described in Methods 2.2 and the DNase purified as described in Methods 6.1. The purified DNase was then acetone precipitated (Methods 8.1), run on the 2 Dimension gel system pH 3.5-10 (Methods 11), transferred to nitrocellulose and Immuno blotted for the DNase (Methods 12). The nitrocellulose sheet was then subjected to autoradiography at -80^{o}C.

16 In vitro [^{32}P] labelled peptides

The kinase assays used were based on those given in Methods 5.2, and the kinases used were cAMP dep protein kinase, protein kinase C, casein kinase II and the US3 protein kinase.

In each case 4 x 500µl assays were set up for each kinase using the DNase as a substrate and 25µCi of [^{32}P] ATP per assay. Heparin at a concentration of 5µg/ml. was included in the US3 protein kinase assays.

The assays were incubated at 37^{o}C for 1 hr., then pooled and acetone precipitated (Methods 8.1) overnight. The samples were then subjected to electrophoresis on a 3mm. 10% acrylamide gel (Methods 9). The gel was then transferred to PVDF membrane in a similar manner to nitrocellulose transfer (Methods 12.1) except that a different transfer buffer was used (Materials 2.10), and transfer was overnight at 75mA. The membrane was then stained with coomassie blue and destained in 50% methanol

to reveal the molecular weight markers.

The membrane was then dried, wrapped in cling film and subjected to autoradiography at room temperature for 5 - 6 hrs.. The band containing the [^{32}P] labelled DNase was cut out, rehydrated in 500µl 20mMTris HCl pH7.5 to which was added 100µg tpck-treated trypsin. This was allowed to digest overnight at room temperature and the membrane removed. The supernatant was chromatographed on a Pharmacia Mono Q column using a 0-1MKCl gradient in 20mM Tris HCl pH 7.5 on the Pharmacia FPLC system. The 1ml. fractions were collected and their Cherenkov activity measured by a scintillation counter.

17 <u>In vivo [^{32}P] labelled peptides</u>

5 plastic roller bottles of BHK cells were grown to confluence and 2 hrs. prior to infection phosphate reduced medium (Materials 2.1) was placed in the roller bottles. After infection for 1 hr. the virus was removed and replaced by 20-30ml. phosphate reduced medium containing 2mCi [^{32}P] orthophosphate. The infection was left to proceed for 12 - 15 hrs. and then the cells were harvested as described in Methods 2.2 except that NaF was added to 20mM. The DNase was purified as described in Methods 6.1 and then acetone precipitated (Methods 8.1) overnight. The sample was then subjected to electrophoresis on a 3 mm. 10% acrylamide gel (Methods 9) and transferred overnight to a PVDF membrane at 75mA. The DNase band was cut out and incubated overnight at room temperature in 500µl 20mM Tris HCl pH 7.5 containing 100µg tpck-treated trypsin. The supernatant was then chromatographed on the Mono Q using a gradient 0-1M KCl in 20mM Tris HCl pH7.5 on the Pharmacia FPLC system. The 1 ml. samples collected were taken and their Cherenkov activity measured by a scintillation counter.

18 DNase endonuclease studies

18.1 Ethanol precipitation

To the DNA sample 0.1 volumes of 3M sodium acetate pH6 and 2.5 volumes of cold ethanol (-20°C) were added and the mixture was kept on dry ice for 10 mins. The DNA was collected by centrifugation for 15 mins. in a microcentrifuge, washed with ethanol and lyophilised for 5 mins.. The pellet was resuspended in TE Buffer (Materials 2.4).

18.2 Agarose gel electrophoresis

A 1% agarose solution was made up in TBE (Materials 2.11) and dissolved by heating in a microwave. Ethidium bromide was added to a concentration of 0.5µg/ml.. The agarose solution was allowed to cool to hand heat before the gel was poured and allowed to solidify.

Samples were prepared by the addition of 10µl of sample buffer (Materials 2.11) to the endonuclease assay. 20µl of this mixture was then run on a gel. The gels were run in a horizontal slab electrophoresis system in TBS buffer containing 0.5µg/ml Ethidium bromide (Materials 2.11) at 60-100v. for 1 - 2 hours.

18.3 Restriction Enzyme digestions

Enzymes: Hind III, EcoR I & Pst I

Reaction:

3µl React Buffer x 10 (BRL)

React 3 — EcoR I

React 2 — Hind III, Pst I

1µl Enzyme (≡ 10 units)

10µl G35a DNA solution (≡10µg) or ethanol precipitated sample

16µl H_2O

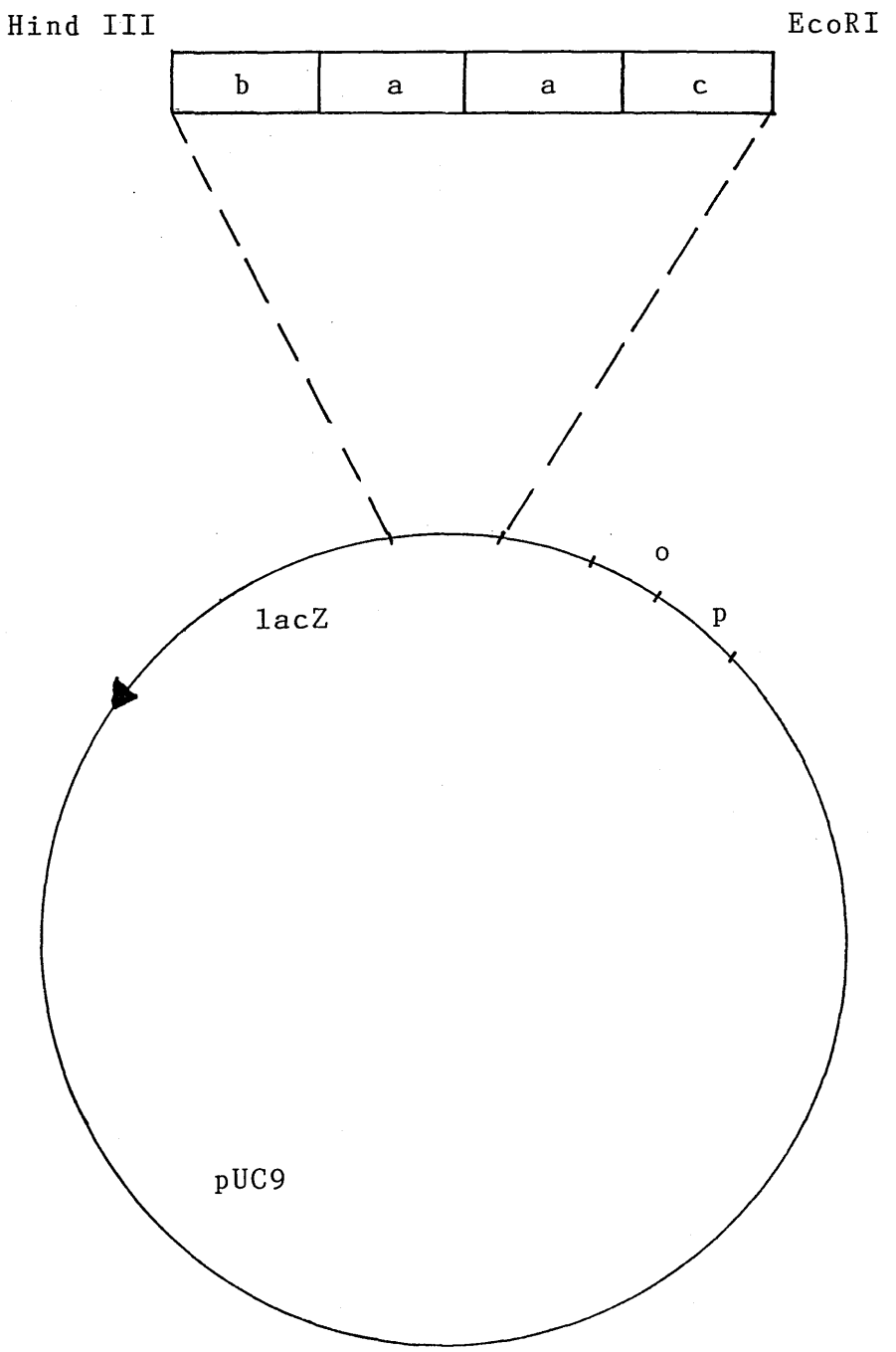


Figure 18.1 Plasmid G35a

Nuc. Acid Res. 11 8205 Stow et al (1983)

The digestions were carried out for 1½ hrs. at 37°C and the samples were then subjected to agarose gel electrophoresis (Methods 18.2).

18.4 Endonuclease assays

The standard assay used was 50mM Tris HCl pH7.0, 4mM 2 mercaptoethanol (Materials 2.5), 5mM $MnCl_2$ and 10µg G35a DNA (Figure 18.1)in a final volume of 30µl. The reaction was initiated by the addition of the enzyme and the incubation, at 37°C, varied depending on the experiment. In some experiments poly (dI.dC).poly (dI.dC) was present to prevent non specific protein:DNA binding and in some experiments a series of buffers at different pH's were used.

19 Xanthate studies

19.1 Kinase partial purification

20 roller bottles of BHK cells were infected and harvested as described in Methods 2.2. The cell extract was then loaded onto a 6.0 x 2.5 cm. DE52 cellulose column, and washed with 2 column volumes of DE Buffer (Materials 2.3). Elution was carried out with a 300 ml. gradient 0-0.5M KCl in DE Buffer, and 5 ml. fractions were collected. Fractions were stored at -20°C.

Protein kinase C and the US3 protein kinase were located by protamine kinase assays (Methods 5.2) of the column fractions. The protein kinase C fractions were then reassayed in a specific protein kinase C assay. The positions of the other kinases were estimated from their known KCl elution concentrations from DE52 cellulose. Specific assays (Methods 2.3) were then carried out to determine their positions. In all cases the most active fractions were used in the inhibitor studies.

19.2 Inhibitor studies

The xanthate stock solutions used were 1mM, 10mM, 50mM and 1M in water and were stored at -20°C. In the assays the xanthates were added after the substrate, the labelled ATP was then added and these were incubated at 35°C for 5 - 10 mins. before the addition of the kinase. The reactions were then carried out as described in Methods 5.2.

CHAPTER 4

R E S U L T S

Section 1 Purification of the DNase

The purification of the DNase was carried out, after the method of Banks et al 1983. A broad peak of exonuclease activity was found on DE52 cellulose chromatography of an extract of infected cells. This eluted at 0.1 - 0.25M KCl (Figure 1.1). The exonuclease activity in this peak resolved into two peaks on subsequent Phosphocellulose chromatography. The DNase eluted at 0.1 - 0.15M KCl and the virally coded DNA polymerase coeluted with the second peak at 0.225 - 0.275M KCl (Figure 1.2).

The DNA polymerase is known to have an exonuclease activity (Weissbach A. et al 1973). The DNase was finally purified as a single peak of activity on DNA Cellulose chromatography from which it eluted at 0.1 - 0.3M KCl (Figure 1.3).

The purity of the final concentrated DNase varied from preparation to preparation. In some cases essentially a single protein of homogeneous enzyme was found on Coomassie blue stained polyacrylamide gels (Figure 1.4). On other occasions a small number of additional proteins was seen, one of which was normally BSA.

Quantitative and qualitative analysis of a typical purification are shown in Table 1.1 and Figure 1.4. The final purification by a factor of approximately 400 over the starting material compares favourably with published data (Banks et al 1983). The Coomassie blue stained polyacrylamide gel showed that both the DE52 Cellulose and phosphocellulose DNase pools contained a highly complex mixture of proteins (Figure 1.4) which, in the particular preparation illustrated were resolved into one band which migrated at a molecular weight 85000 after chromatography on DNA Cellulose.

This is in agreement with other published data on the molecular weight of the enzyme. Coomassie blue staining of a two dimensional polyacrylamide gel (Methods 11) on which a TCA precipitated (Methods 8.2) sample of purified DNase had been run (Figure 1.5) also showed a single stained region at 85000 Daltons.

Figure 1.1 DE52 Cellulose chromatography (Methods 6.1) of an 18 hr. HSV-1(F) infected BHK cell extract (Methods 2.2). Elution was carried out using a 250 ml. gradient of 0-500 mMKCl in DE Buffer (Materials 2.3).

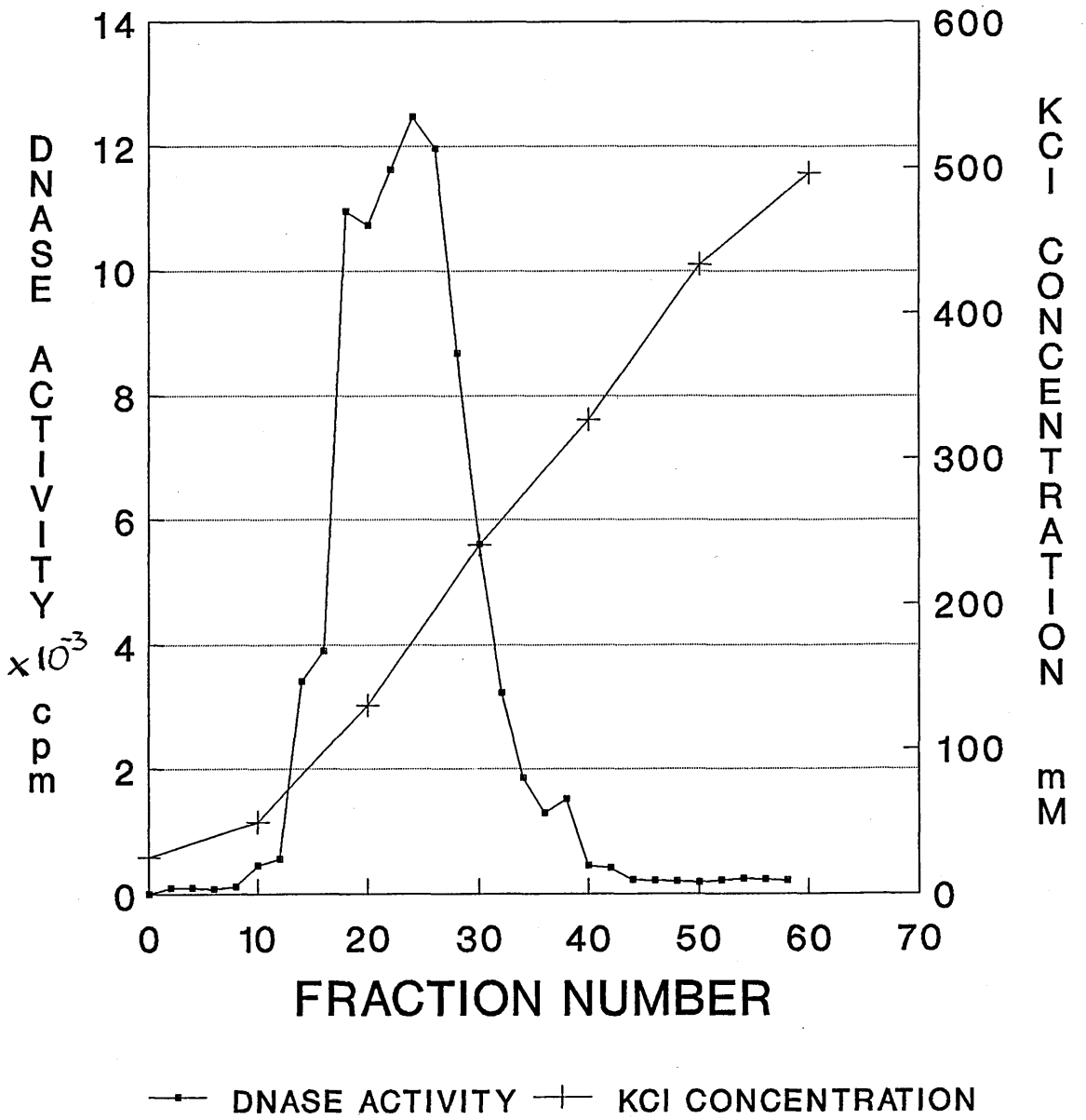

DNASE ACTIVITY x10^-3 cpm
KCl CONCENTRATION mM
FRACTION NUMBER
0
2
4
6
8
10
12
14
0
100
200
300
400
500
600
0
10
20
30
40
50
60
70
DNASE ACTIVITY
KCl CONCENTRATION

Figure 1.2 Phosphocellulose chromatography (Methods 6.1) of fractions 16-31, from the DE52 cellulose chromatography shown in Fig. 1.1, which had been dialysed against DE Buffer (Materials 2.3). Elution was carried out using a 150 ml. gradient of 0.1-0.4MKCl in DE Buffer.

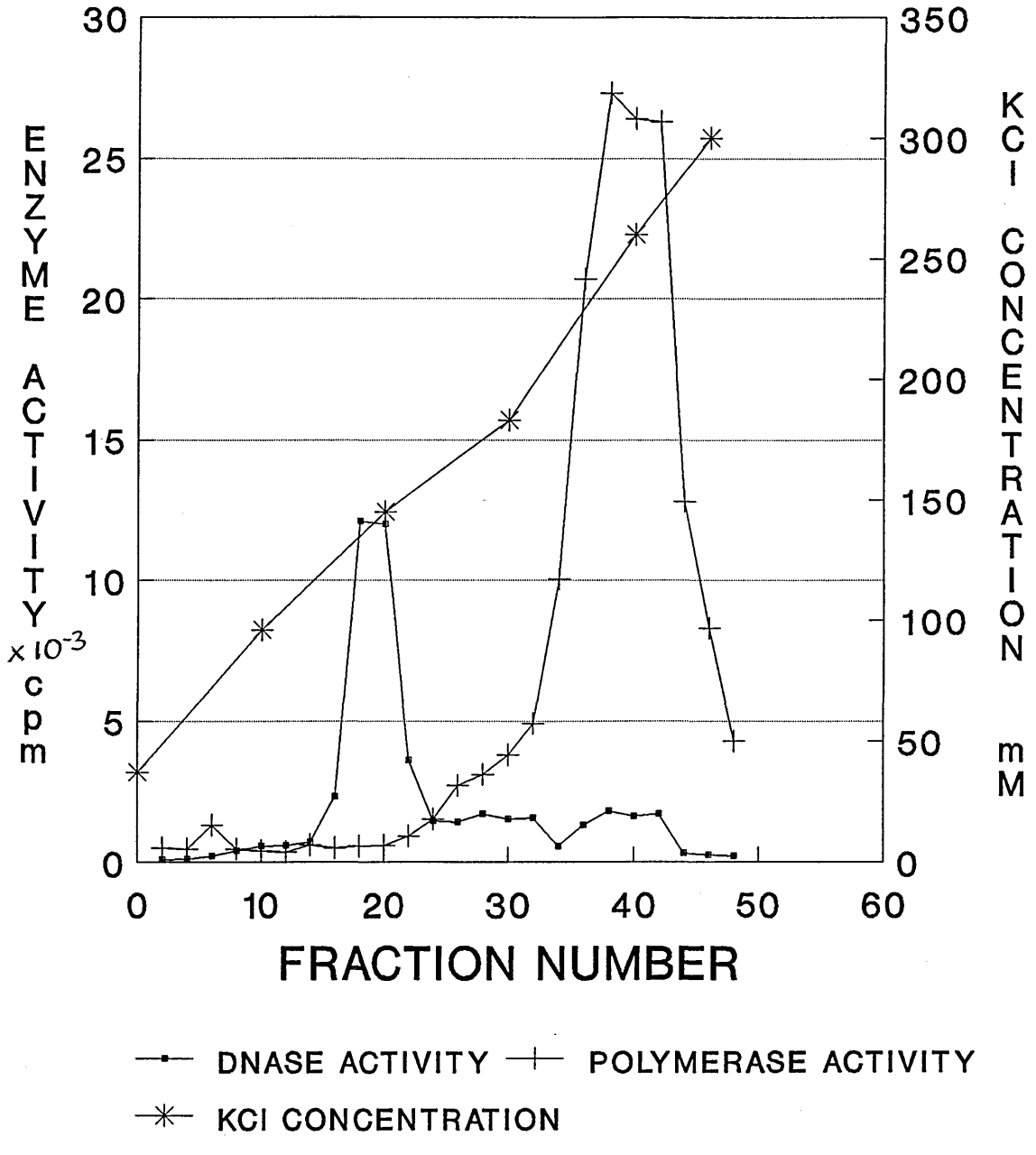

ENZYME ACTIVITY x 10-3 cpm
KCl CONCENTRATION mM
FRACTION NUMBER
0
5
10
15
20
25
30
50
100
150
200
250
300
350
0
10
20
30
40
50
60
DNASE ACTIVITY
POLYMERASE ACTIVITY
KCl CONCENTRATION

Figure 1.3 DNA Cellulose chromotography (Methods 6.1) of fractions 16-22, from the Phosphocellulose chromatography shown in Fig. 1.2 which had been dialysed against DC Buffer (Materials 2.3). Elution was carried out using a 100 ml. gradient of 0-1MKCl in DC Buffer.

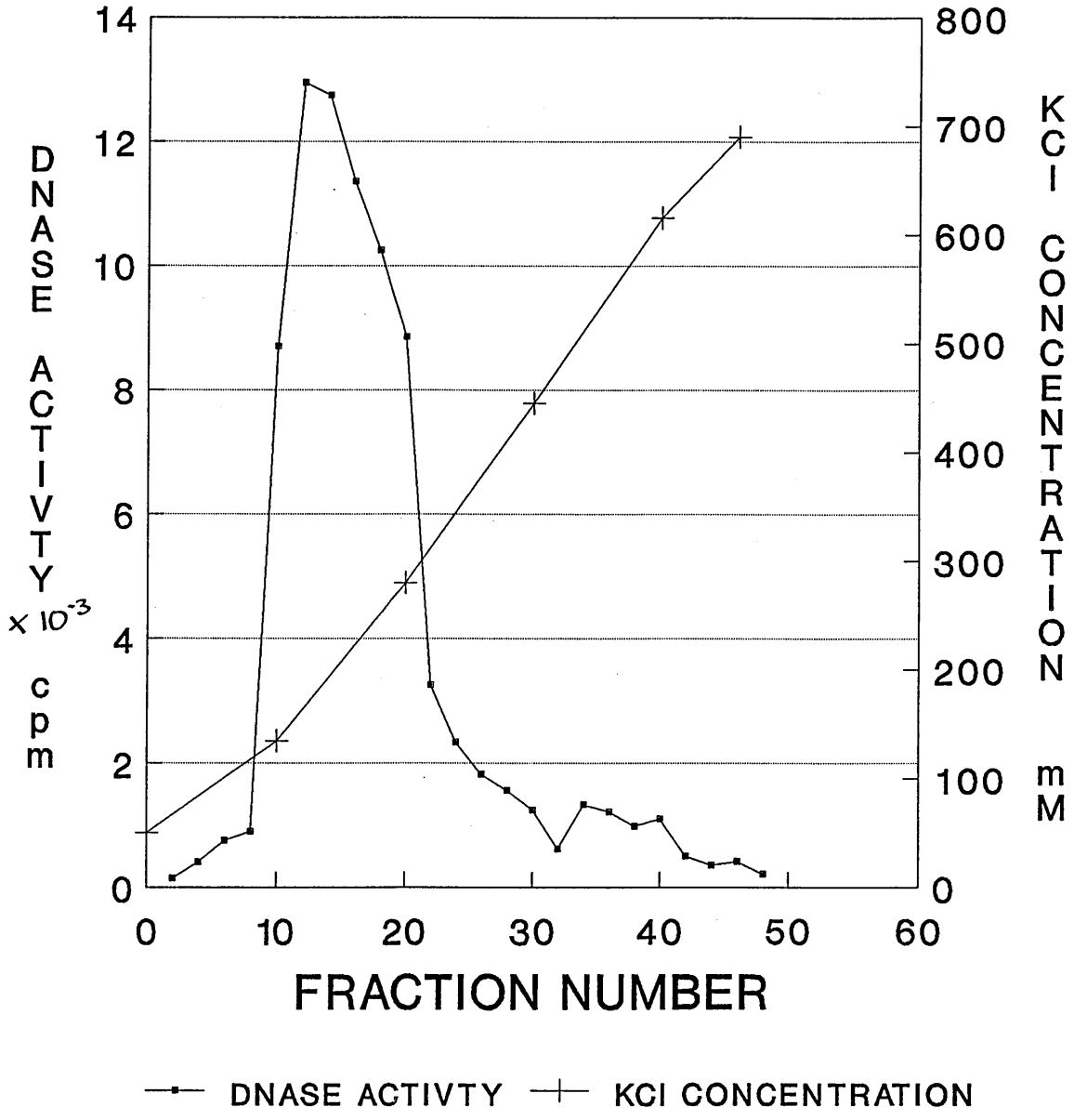

DNASE ACTIVTY x 10⁻³ cpm
KCl CONCENTRATION mM
FRACTION NUMBER
0
2
4
6
8
10
12
14
100
200
300
400
500
600
700
800
0
10
20
30
40
50
60
DNASE ACTIVTY
KCl CONCENTRATION

Table 1.1 Purification data for a typical DNase preparation

Purification Step	Volume (ml)	Total Protein (mg)	Specific Activity *	Purification
Cell Homogenate	14	115	19.6×10^4	1
10K spin supernatant	9.5	61	25.2×10^4	1.3
DE52 Cellulose pool	83	20.1	1.7×10^6	9.0
Phosphocellulose pool	50	4.5	6.2×10^6	31.6
Concentrated DNA Cellulose pool	12.5	1.0	75×10^6	382.7

* Specific Activity = cts released from a standard calf thymus DNA substrate per min. per mg protein at $37^{\circ}C$.

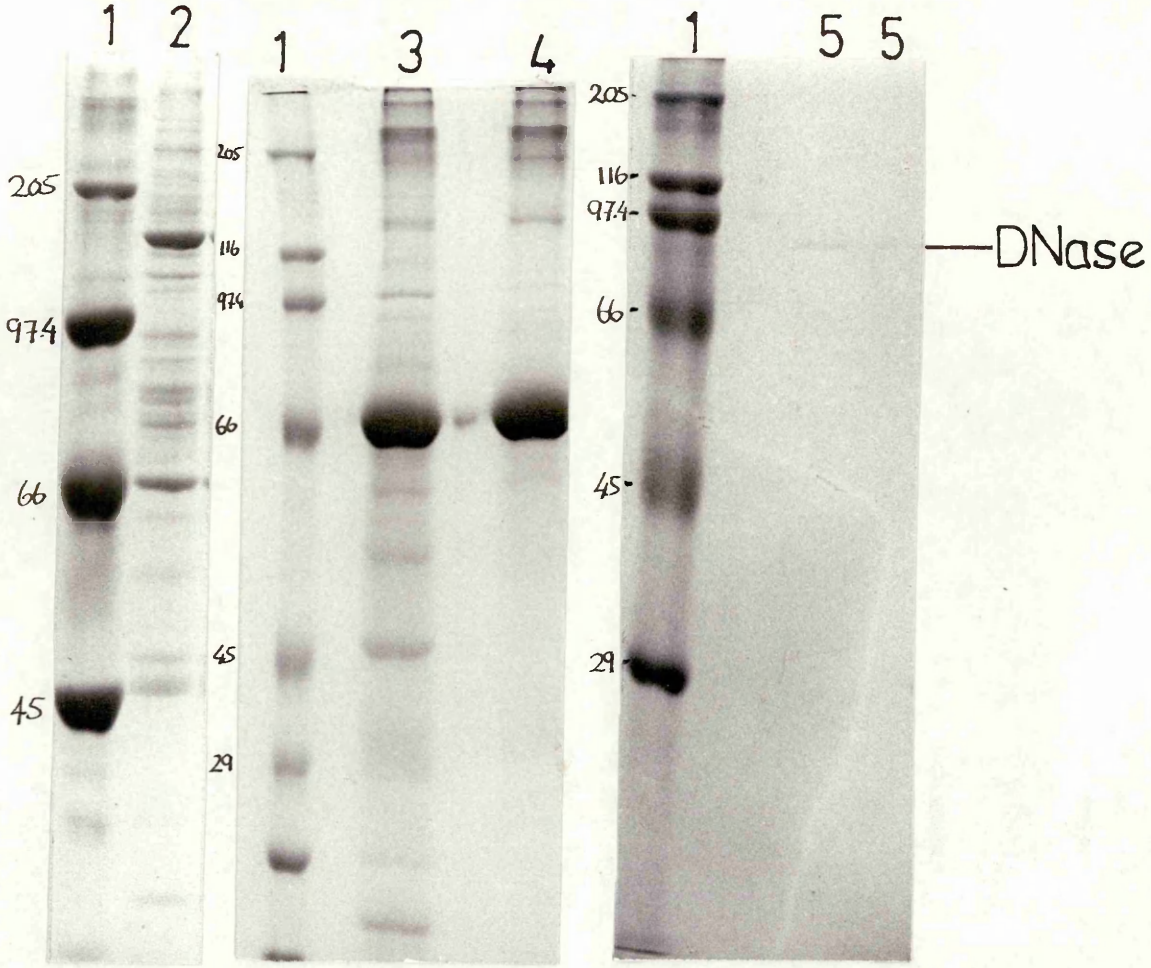


Figure 1.4 SDS polyacrylamide gel analysis of the purification of DNase. Samples collected during the purification of DNase from BHK cells infected for 18 hrs. with HSV-1(F) (Methods 2.2, 6.1) were subjected to electrophoresis (Methods 9) on SDS polyacrylamide gels containing 10% acrylamide. The gels were stained with Coomassie blue (Methods 10.1).

Key Lane 1 Markers Myosin 205kDa, β Galactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg Albumin 45kDa, Carbonic anhydrase 29kDa.

Lane 2 10K spin supernatant from 18 hr. infected BHK cells (Methods 2.2).

Lane 3 DNase activity pool from DE52 Cellulose chromatography (Methods 6.1).

Lane 4 DNase activity pool from Phosphocellulose chromatography (Methods 6.1).

Lane 5 Purified DNase from DNA cellulose chromatography (Methods 6.1).

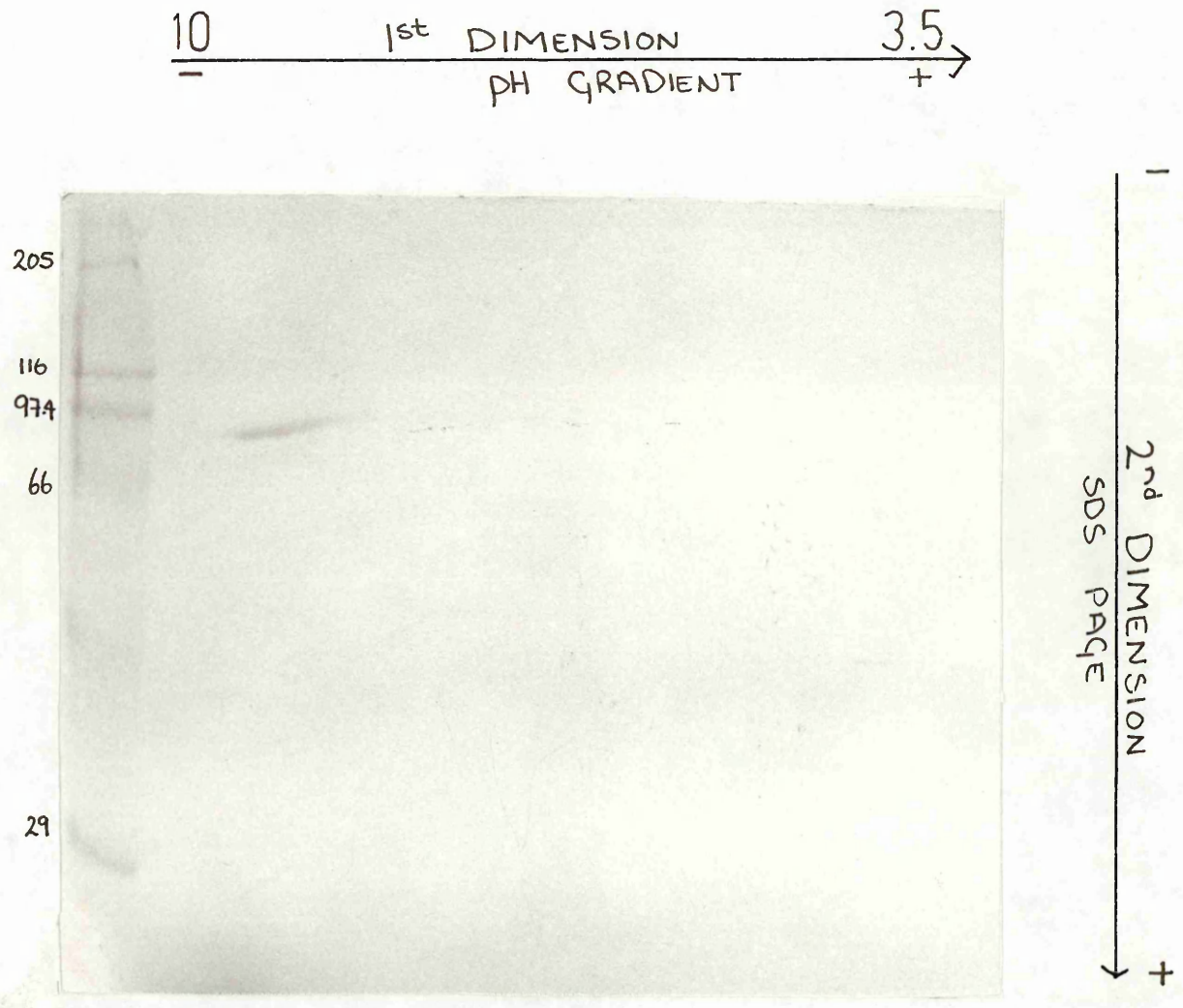


Figure 1.5 Two dimensional polyacrylamide gel electrophoresis (Methods 11) of a TCA precipitated (Methods 8.2) purified DNase sample. In the first dimension the sample was subjected to electrophoresis in a pH gradient of 3.5-10 (Methods 11.1). In the second dimension an SDS PAGE system was used, the gel contained 10% acrylamide (Methods 11.2). The gel was stained with Coomassie blue (Methods 10.2). A marker lane was run at the side of the second dimension.

Key Marker Lane Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg Albumin 45kDa, Carbonic anydrase 29kDa.

Section 2 Analysis of the DNase by two dimensional polyacrylamide gel electrophoresis

2.1 Time course analysis

DNase was prepared from cells harvested at various times after infection (Methods 2.2 & 6.1). The purified enzyme was subjected to two dimensional PAGE (Methods 11). The protein was transferred to nitrocellulose and probed using an antibody specific for the viral DNase (Methods 12). Typical results are shown in Figure 2.1.1. At all times apart from 3 hrs. the protein had a highly complex profile made up of many spots. These complex profiles indicated that the DNase was highly modified, from an early stage and throughout the infectious cycle. The enzyme is known to be phosphorylated in cell culture (Banks L. et al 1985).

The enzyme from 6 hrs. onwards appeared to be highly modified with a large dark spot at lower pI values and in some cases smaller spots at higher pI values. At 18 hrs. the orientation of the large spot at lower pI values was inverted with most of the protein being at the more basic end of the spot.

A quantitative analysis of these profiles was carried out using Laser densitometry (LKB Ultroscan XL) of the photographs of the blots. The data are reported in Table 2.2 and confirm the qualitative evaluation given above.

2.2 The effect of Alkaline phosphatase on the DNase

Alkaline phosphatase treatment (Methods 13.1) of DNase purified from 18 hr. infected cells (Methods 2.2 & 6.1) had an effect on its 2D gel profile after immunoblotting. The more basic of the smaller spots had increased in area (Figure 2.2.1). This indicated a possible reduction in the modification of the DNase by the removal of phosphate.

2.3 The effect of Acid phosphatase on the DNase

Acid phosphatase treatment (Methods 13.2) of DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) had no significant effect on its 2D gel profile after immunoblotting (Figure 2.3.1).

2.4 The effect of Snake venom phospho-diesterase on the DNase

Snake venon phosphodiesterase treatment (Methods 13.3) of DNase purified from cells infected for 12 & 18 hrs. (Methods 2.2, 6.1) resulted in similar profiles on 2D gels after immunoblotting (Figure 2.4.1). These both showed a small spot at lower pI values and a much larger spot at slightly higher pI values. These profiles were both very different from those of the original 12 & 18 hr. samples. This indicated that a significant reduction in their modification had occurred. Snake venon phospho-diesterase is an exonuclease which degrades poly (ADP-ribose) covalently attached to proteins (Yamada M. & Surgimura 1973).

2.5 The effect of Protein phosphatase 1 on the DNase

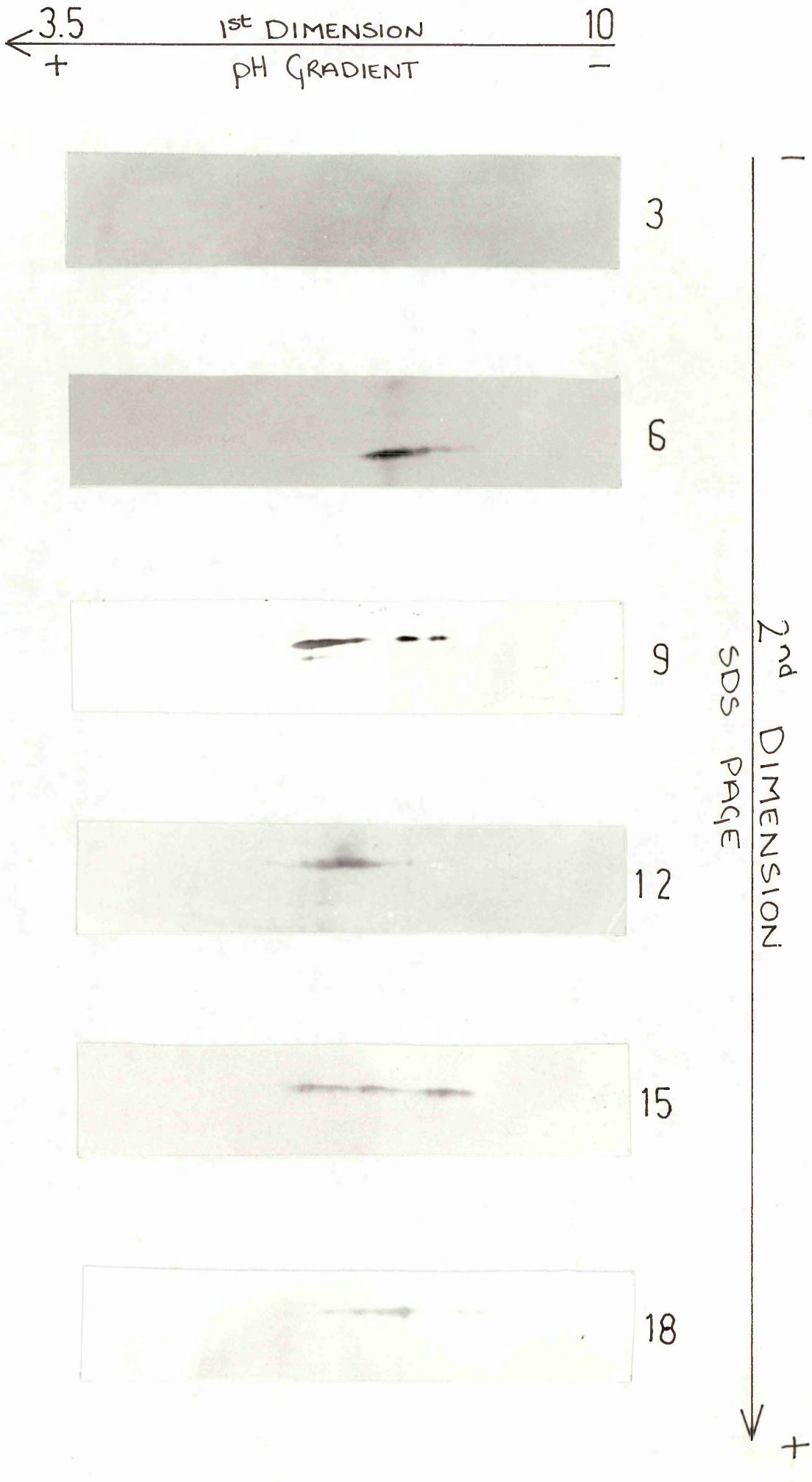
Protein phosphatase 1 treatment (Methods 13.4) of DNase purified from cells infected for 15 hrs. (Methods 2.2, 6.1), resulted in a significant change in its 2D gel profile after immunoblotting (Figure 2.5.1). The large spot that was present at more acidic pI values was altered in size and the orientation within the spot. This profile also indicated a reduction in the modification of the DNase due to the loss of phosphate.

Quantitative analysis of these modified profiles by Laser densitometry (LKB Ultroscan XL) of the photographs of the blots (Table 2.1) confirmed the qualitative evaluation given above. (Figure 2.6)

These results indicate that one form of modification of the DNase is phosphorylation. In addition ADP ribose or poly (ADP ribose) may be covalently attached to the enzyme.

Figure 2.1.1 Immunoblots of two dimensional polyacrylamide gels of DNase purified from cells infected for 3, 6, 9, 12, 15 & 18 hrs. (Methods 2.2, 6.1). In the first dimension the acetone precipitated (Methods 8.1) samples were subjected to electrophoresis in a 3.5 - 10 pH gradient. The second dimension was an SDS polyacrylamide gel containing 10% acrylamide (Methods 11). The protein on the gel was transferred to nitrocellulose by electrophoresis (Methods 12.1) and probed with an antibody against the DNase (Methods 12.3). A marker lane was run at the side of the second dimension and was visualised by Ponceau S staining (Methods 12.2) of the nitrocellulose membrane before it was blotted.

Marker lane: Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4 kDa, BSA 66kDa, Egg Albumin 45kDa, Carbonic anhydrase 29kDa.


3.5
1st DIMENSION
10
+
pH GRADIENT
−
3
6
9
12
15
18
−
2nd DIMENSION
SDS PAGE
+

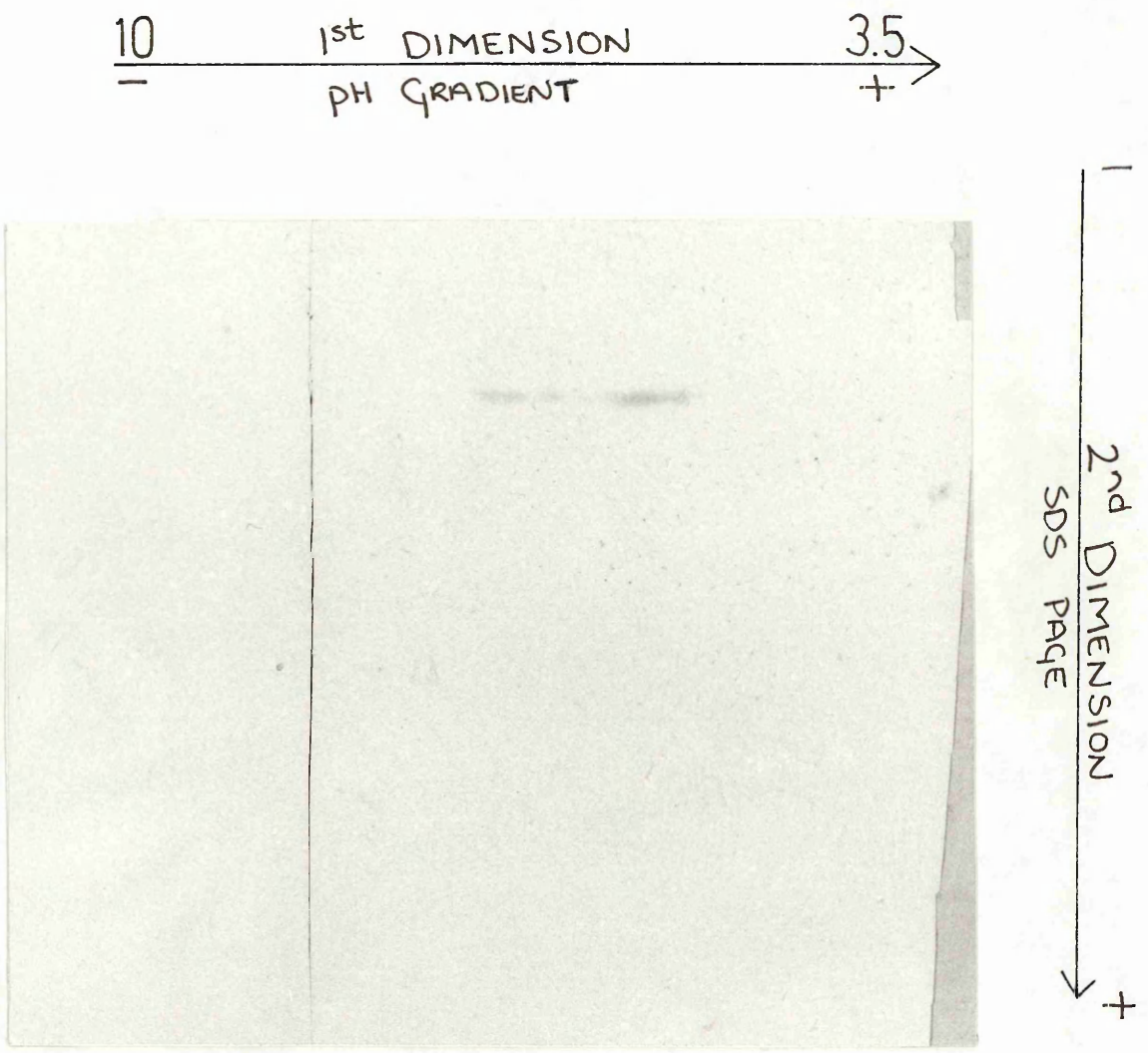


Figure 2.2.1 Immunoblot of a two dimensional polyacrylamide gel of DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) and treated with alkaline phosphatase for 2 hrs. (Methods 13.1). In the first dimension the TCA precipitated sample (Methods 8.2) was subjected to electrophoresis in a pH gradient of 3.5 - 10. The second dimension was an SDS polyacrylamide gel containing 10% acrylamide (Methods 11). The protein on the gel was transferred to nitrocellulose by electrophoresis (Methods 12.1) and probed with an antibody against the DNase (Methods 12.3). A marker lane was run at the side of the second dimension and was visualised by Ponceau S staining of the membrane (Methods 12.2). Marker lane Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA66kDa, Egg albumin 45kDa, Carbonic anhydrase 29kDa.

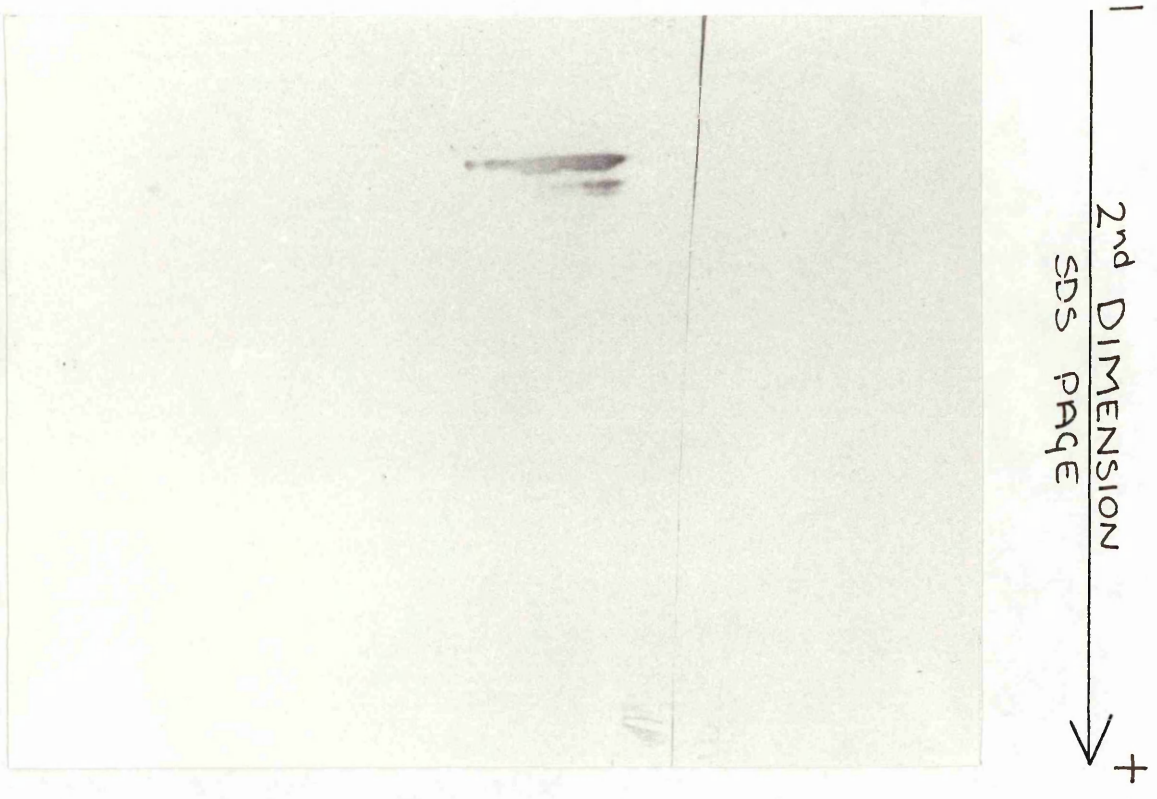


Figure 2.3.1 Immunoblot of a two dimensional polyacrylamide gel of DNase purified from 18 hr. infected cells (Methods 2.2, 6.1) and treated with Acid phosphatase for 2 hrs. (Methods 13.2). The TCA precipitated sample (Methods 8.2) was subjected to electrophoresis in the first dimension in a pH gradient of 3.5-10. The second dimension was an SDS polyacrylamide gel containing 10% acrylamide (Methods 11). The protein on the gel was transferred to nitrocellulose by electrophoresis (Methods 12.1) and probed with an antibody against the DNase (Methods 12.3). A marker lane was run at the side of the second dimension and was visualised by Ponceau S staining (Methods 12.2). Marker lane Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg Albumin 45kDa, Carbonic anhydrase 29kDa.

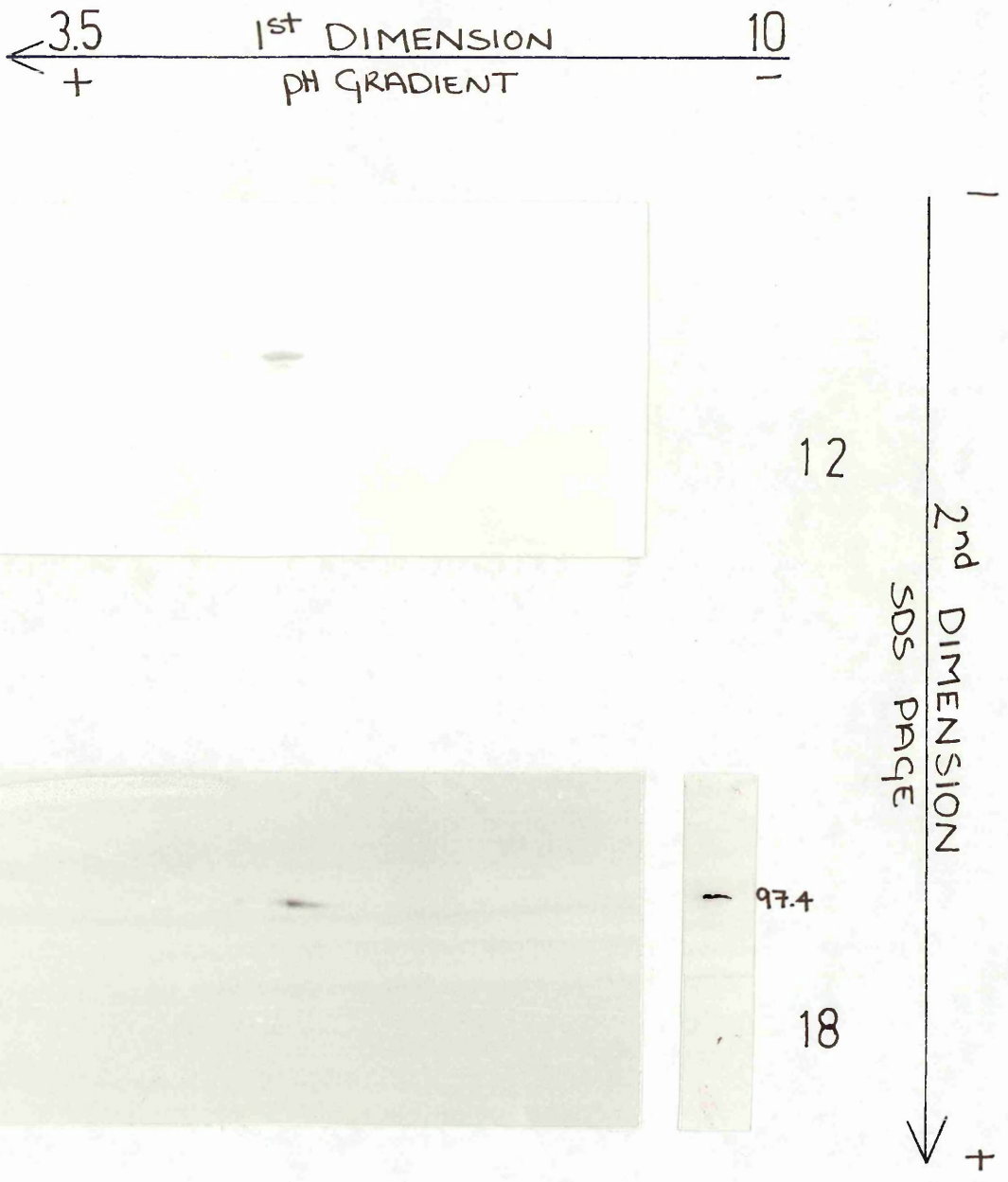


Figure 2.4.1 Immunoblots of two dimensional polyacrylamide gels of DNase purified from cells infected for either 12 or 18 hrs. (Methods 2.2., 6.1) and treated with snake venom phosphodiesterase for 3 hrs. (Methods 13.3). The acetone precipitated samples (Methods 8.1) were subjected to electrophoresis, in the first dimension in a pH gradient of 3.5-10 and in the second dimension on an SDS polyacrylamide gel containing 10% acrylamide (Methods 11). The protein in the gels was then transferred to nitrocellulose by electrophoresis (Methods 12.1) and probed with an antibody against the DNase (Methods 12.3). A marker lane was run at the side of the second dimension and was visualised by Ponceau S staining (Methods 12.2). Marker lane Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg Albumin 45kDa, Carbonic anhydrase 29kDa.

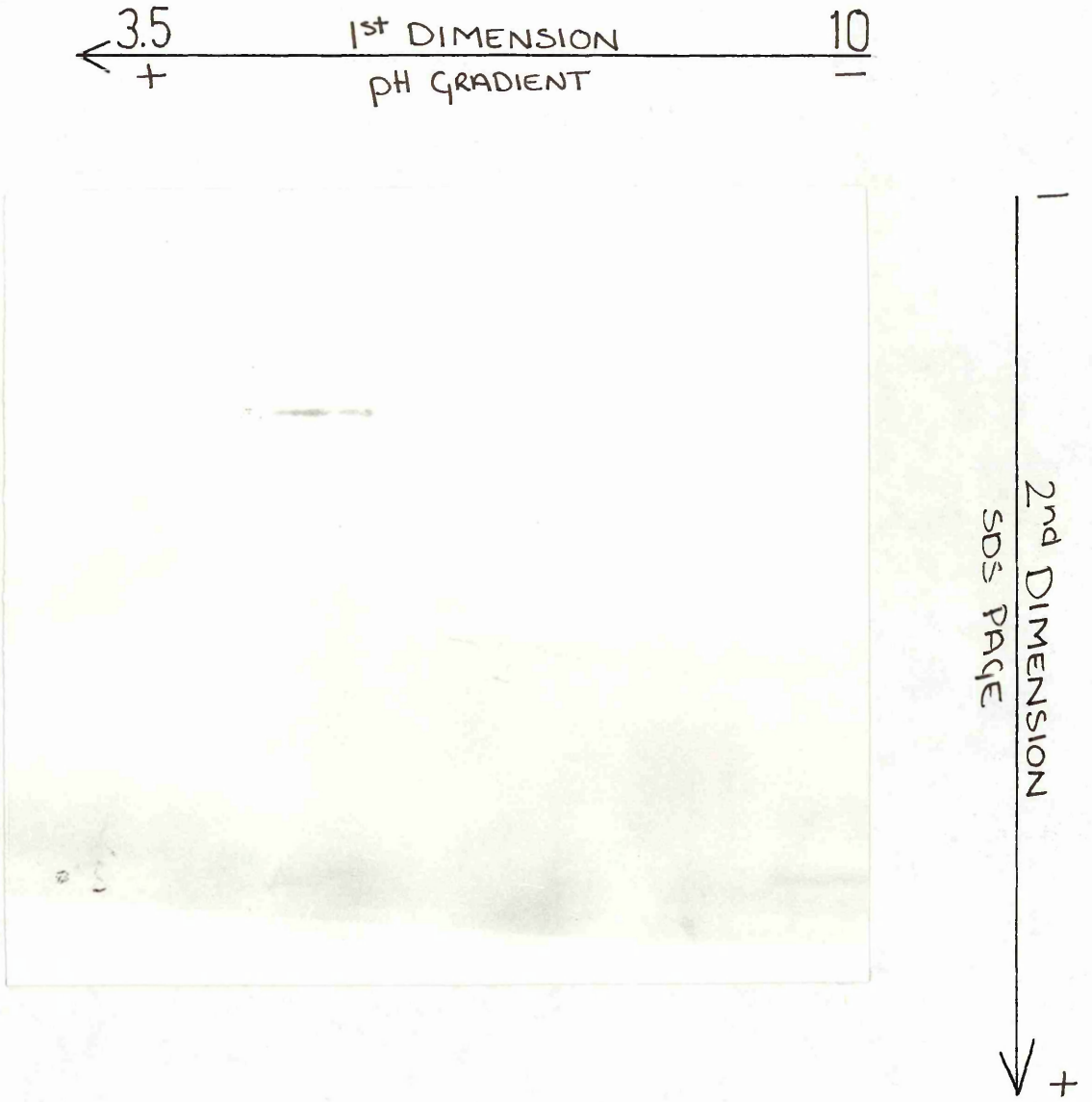


Figure 2.5.1 Immunoblot of a two dimensional polyacrylamide gel of DNase purified from cells infected for 15 hrs. (Methods 2.2, 6.1) and treated with Protein phosphatase 1 for 1 hr. (Methods 13.4). The acetone precipitated sample (Methods 8.1) was subjected to electrophoresis, in the first dimension in a pH gradient of 3.5 - 10 and in the second dimension on an SDS polyacrylamide gel containing 10% acrylamide (Methods 11). The protein on the gel was transferred to nitrocellulose (Methods 12.1) and probed with an antibody against the DNase (Methods 12.3). A marker lane was run at the side of the second dimension and visualised by Ponceau S staining (Methods 12.2). Marker lane Myosin 205kDa, β Galactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg Albumin 45kDa, Carbonic anhydrase 29kDa.

Table 2.1 Quantitative analysis of the DNase time course profiles and modified DNase profiles by laser densitometry

Profile	% area of each region in the large spot at more acidic pI values (3.5 $^{+}\leftarrow$ $^{-}$10) 1	2	3
6 hr.	10%	75%	15%
9 hr.	35%	43%	22%
12 hr.	16%	75%	9%
15 hr.	33%	38%	29%
18 hr.	10%	33%	57%
Acid phosphatase treated 18 hr. DNase	20%	48%	32%
SVP treated 12 hr. DNase		11%	89%
SVP treated 18 hr. DNase		4%	96%
PP1 treated 15 hr. DNase	12%	17%	71%

Figure 2.6

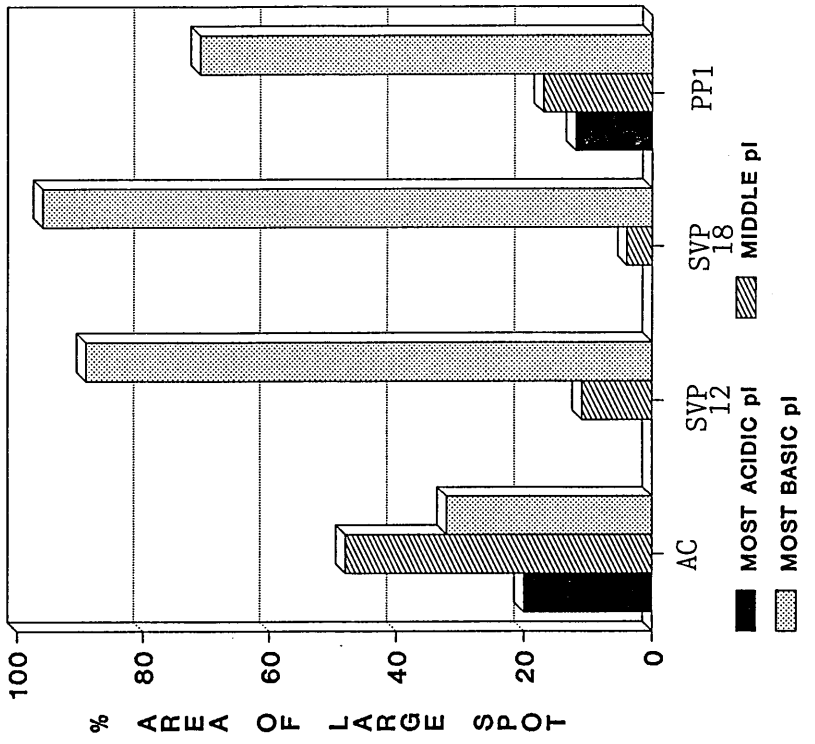
Quantitative analysis of the DNase time course profiles and modified DNase profiles by laser densitometry

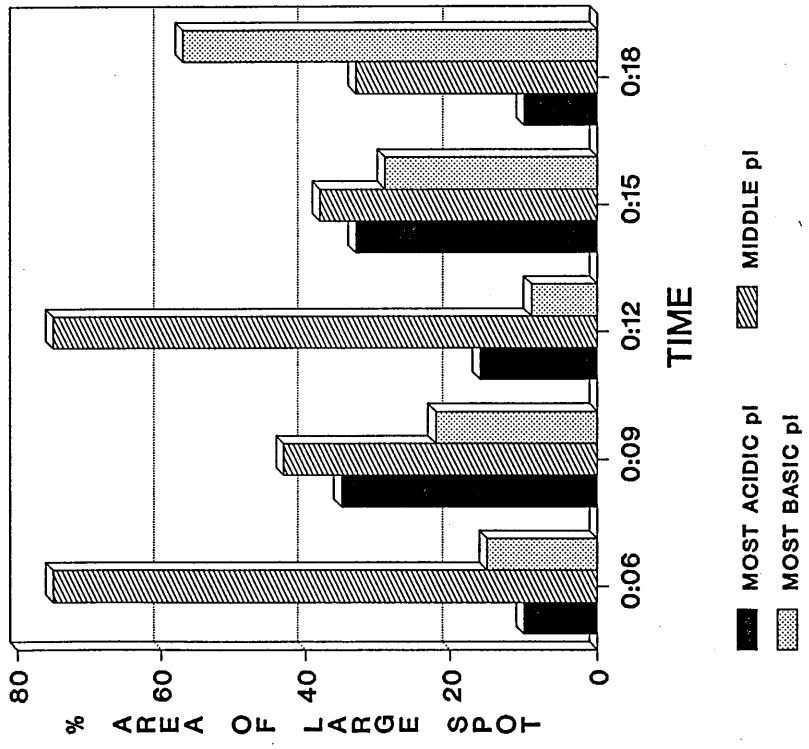
AC: Acid phosphatase treated 18 hr. DNase

SVP12: Snake Venom Phosphodiesterase treated 12 hr. DNase

SVP18: Snake Venom Phosphodiesterase treated 18 hr. DNase

PP1: Protein Phosphatase 1 treated 15 hr. DNase


% AREA OF LARGE SPOT
100
80
60
40
20
0
AC
SVP 12
SVP 18
PP1
MOST ACIDIC pI
MOST BASIC pI
MIDDLE pI


% AREA OF LARGE SPOT
80
60
40
20
0
0:06
0:09
0:12
0:15
0:18
TIME
MOST ACIDIC pI
MOST BASIC pI
MIDDLE pI

3 Phosphorylation : in vitro & in vivo

3.1 Phosphorylation : in vitro

The purified DNase was incubated *in vitro* with protein kinases in the presence of $\gamma[^{32}P]$ ATP. The sample was then subjected to electrophoresis on one dimensional polyacrylamide gels. The autoradiographs of these gels (Figures 3.1.1, 3.1.2, & 3.1.3) showed that of the kinases used only one could not phosphorylate the protein. Casein kinase II, cAMP dependent protein kinase (Figure 3.1.1) and protein kinase C (Figure 3.1.3) were all able to phosphorylate the DNase under the conditions employed. The PRV virus kinase (Figure 3.1.2) was unable to utilise the DNase as a substrate and the HSV-1 US3 protein kinase (Figure 3.1.3) may have been able to phosphorylate the DNase but because of the impure nature of this preparation of the kinase the result was not conclusive. However,the autoradiographs from the two dimensional gel analysis of the *in vitro* phosphorylations (Figures 3.1.4, 3.1.5, 3.1.6) showed that the HSV-1 US3 protein kinase was able to phosphorylate the DNase (Figure 3.1.6).

The autoradiographs also showed that the incorporated $[^{32}P]$ was present almost exclusively in the large spot at lower pI values (see Results Section 2). This may confirm that the spot at lower pI values contains the phosphorylated enzyme.

The Immuno blots of the two dimensional gels of the *in vitro* phosphorylations (Figure 3.1.7) showed a similar pattern to the autoradiographs, but in the case of protein kinase C and cAMP dependent protein kinase (Figure 3.1.7) the spots at higher pI values were present. Since they were not present in the autoradiographs it again confirms that the spot at lower pI values contains the phosphorylated DNase.

3.2 Phosphorylation : in vivo with [^{32}P] inorganic phosphate

DNase was purified from infected cells labelled with [^{32}P] inorganic phosphate for 1 - 6 hr. post infection and subjected to two dimensional gel analysis and immunoblotting. The autoradiograph (Figure 3.2.1a) showed the label to be distributed in the large spot at lower pI values. This confirmed that this was the region which contained the modified DNase.

During the purification of the labelled enzyme unlabelled DNase was added to stabilize it and so the immunoblot (Figure 3.2.1b) provided evidence that it was the DNase alone that was responsible for the pattern seen on the autoradiograph.

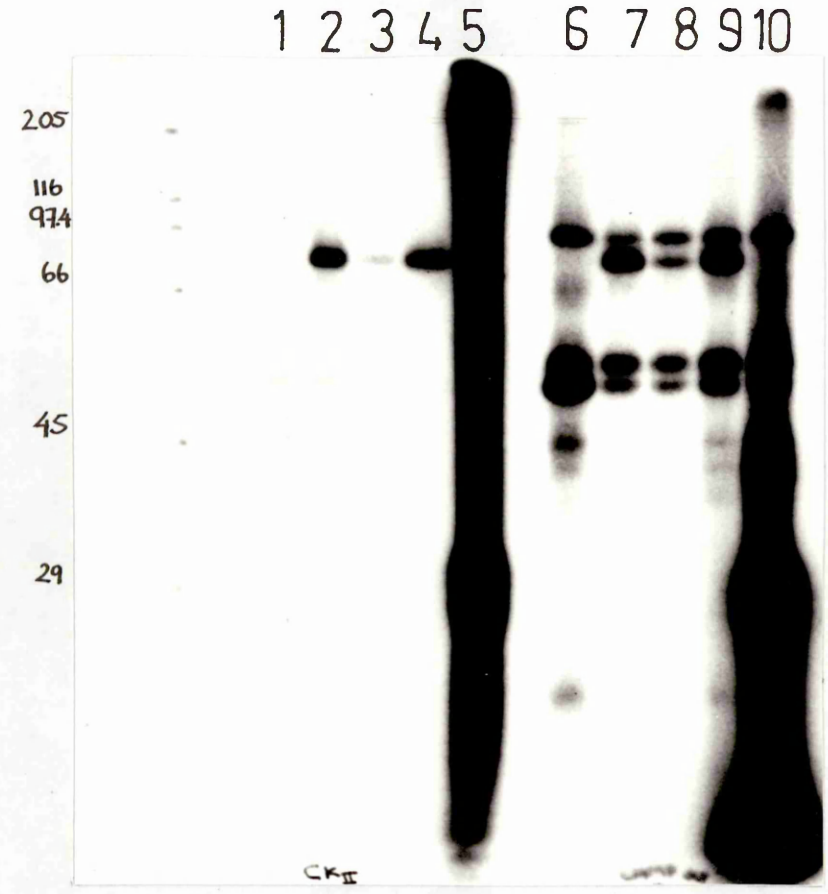


Figure 3.1.1 In vitro phosphorylation of the DNase by casein kinase II and cAMP dependent protein kinase. DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) was incubated with casein kinase II (Methods 6.2.3) or cAMP dependent protein kinase (Materials) in the presence of $\gamma[^{32}P]$ATP (Methods 14.1). The samples were then subjected to electrophoresis on an SDS polyacrylamide gel, 3 mm. thick containing 10% acrylamide (Methods 9). The gel was stained with Coomassie blue (Methods 10.1), dried and subjected to autoradiography at -80°C.

Key: Marker lane Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg albumin 45kDa, Carbonic anhydrase 29kDa.

Lane 1 = CKII + $\gamma[^{32}P]$ATP

Lane 2 = CKII + DNase + $\gamma[^{32}P]$ATP

Lane 3 = CKII + DNase + heparin + $\gamma[^{32}P]$ATP

Lane 4 = CKII + DNase + cAMPdep.PKinhibitor + $\gamma[^{32}P]$ATP

Lane 5 = CKII + Casein + $\gamma[^{32}P]$ATP

Lane 6 = cAMPdep.PK + $\gamma[^{32}P]$ATP

Lane 7 = cAMPdep.PK + DNase + $\gamma[^{32}P]$ATP

Lane 8 = cAMPdep.PK + DNase + heparin + $\gamma[^{32}P]$ATP

Lane 9 = cAMPdep.PK + DNase + cAMPdep.PKinhibitor + $\gamma[^{32}P]$ATP

Lane10 = cAMPdep.PK + histone IIAS + $\gamma[^{32}P]$ATP

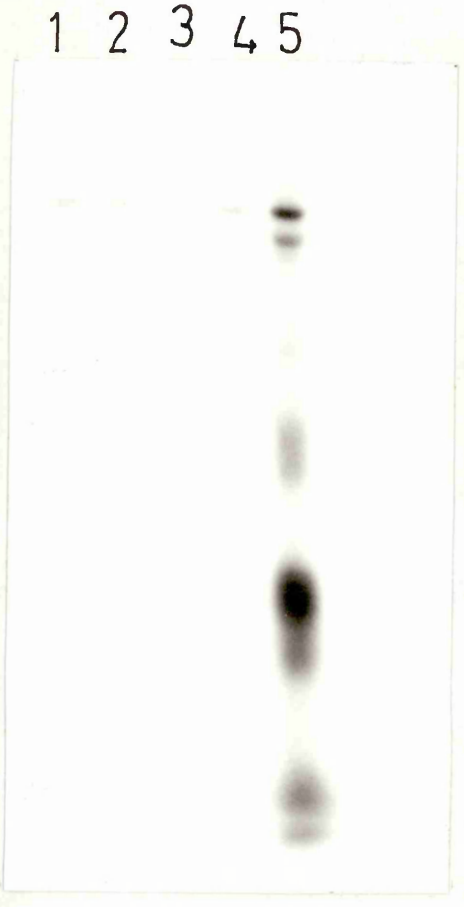


Figure 3.1.2 In vitro phosphorylation of the DNase by the PRV viral kinase. D Nase purified from cells infected for 18 hrs. (Methods 2.2., 6.1) was incubated with PRV viral kinase (Materials) in the presence of γ [^{32}P] ATP (Methods 14.1). The samples were then subjected to electrophoresis on an SDS polyacrylamide gel, 3 mm thick containing 10% acrylamide (Methods 9). This was stained with Coomassie blue (Methods 10.1), dried and subjected to autoradiography at -80^{o}C.

Key Lane 1 = Kinase + γ [^{32}P] ATP
Lane 2 = Kinase + DNase + γ [^{32}P] ATP
Lane 3 = Kinase + heparin + DNase + γ [^{32}P] ATP
Lane 4 = Kinase + cAMPdepPKinhibitor + DNase + γ [^{32}P] ATP
Lane 5 - Kinase + histone IIAS + γ [^{32}P] ATP

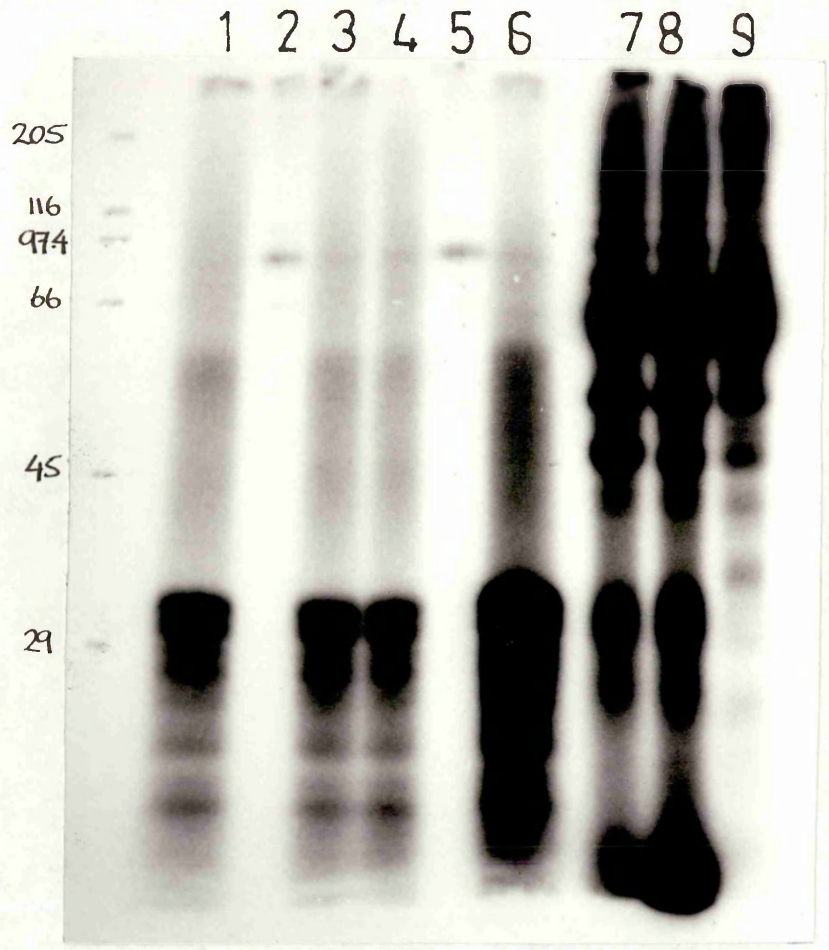


Figure 3.1.3 In vitro phosphorylation of the DNase by protein kinase C and the US3 protein kinase. DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) was incubated with protein kinase C (Methods 6.2.1) or the US3 protein kinase (Methods 6.2.2) in the presence of γ [^{32}P] ATP (Methods 14.1). The samples were then subjected to electrophoresis on an SDS polyacrylamide gel, 3 mm thick containing 10% acrylamide (Methods 9). The gel was stained with Coomassie blue (Methods 10.1), dried and subjected to autoradiography at -80°C.

Key: Marker lane Myosin 205kDa, β Galactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg albumin 45kDa, Carbonic anhydrase 29kDa.

Lane 1 = pKC + histone IIIs + γ [^{32}P] ATP
Lane 2 = pKC + γ [^{32}P] ATP
Lane 3 = pKC + histone IIIs + γ [^{32}P] ATP - Ca^{2+}
Lane 4 = pKC + histone IIIs + γ [^{32}P] ATP - phosphatidylserine
Lane 5 = pKC + DNase + γ [^{32}P] ATP
Lane 6 = pKC + histone IIIs + γ [^{32}P] ATP
Lane 7 = US3pK + histone IIAS + γ [^{32}P] ATP
Lane 8 = US3pK + histone IIAS + heparin + γ [^{32}P] ATP
Lane 9 = US3pK + DNase + γ [^{32}P] ATP

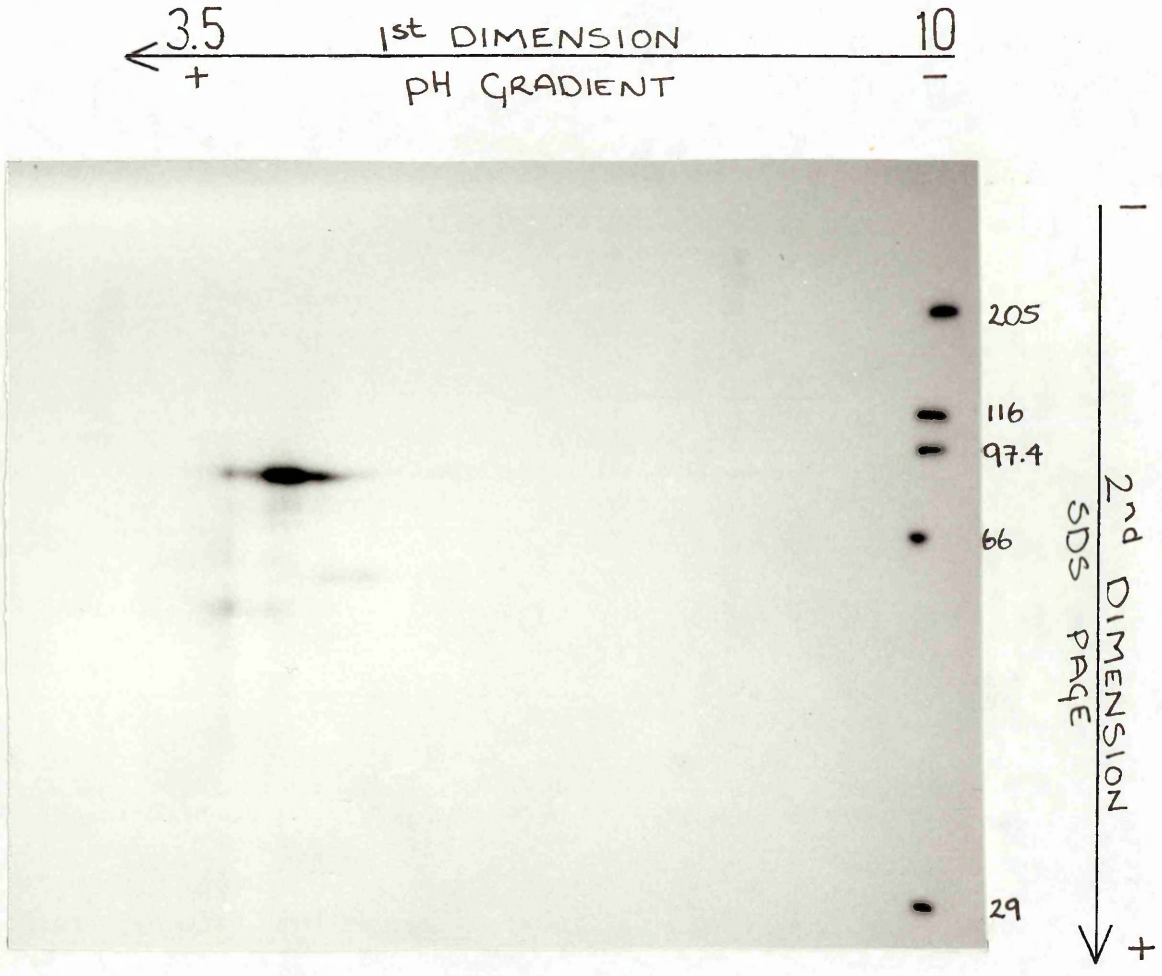


Figure 3.1.4 In vitro phosphorylation of DNase by protein kinase C. DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) was incubated with protein kinase C (Methods 6.2.1) in the presence of γ [^{32}p]ATP (Methods 14.2). The sample was then TCA precipitated (Methods 8.2) and subjected to two dimensional polyacrylamide gel electrophoresis (Methods 11). In the first dimension a pH range of 3.5 - 10 was employed and in the second dimension the SDS polyacrylamide gel contained 10% acrylamide. The gel was stained with Coomassie blue (Methods 10.1), dried and subjected to autoradiography at -80°C.

Marker lane Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg albumin 45kDa, Carbonic anhydrase 29kDa.

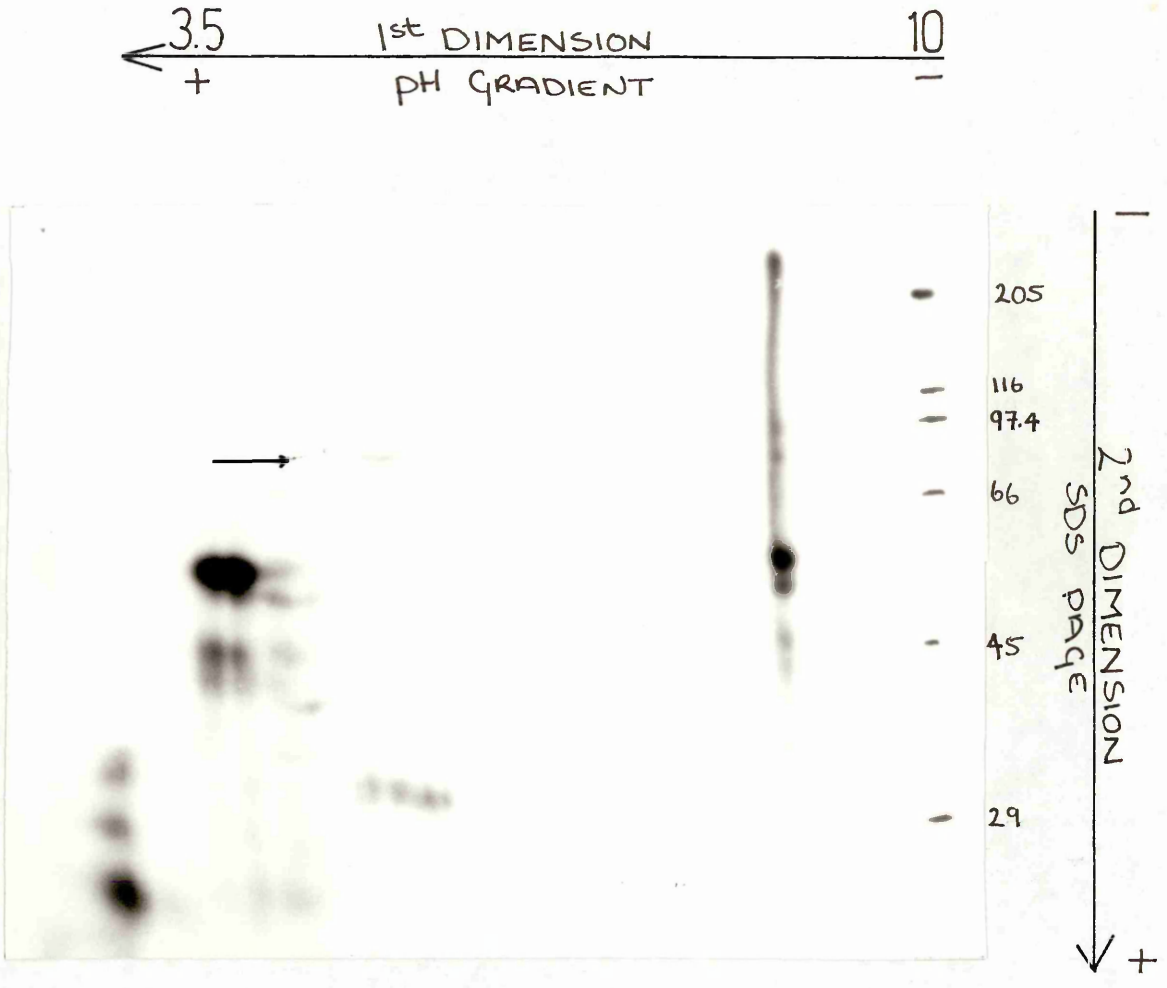


Figure 3.1.5 In vitro phosphorylation of the DNase by cAMP dependent protein kinase. DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) was incubated with cAMP dep protein kinase (Materials) in the presence of γ [^{32}P] ATP (Methods 14.2). The sample was then TCA precipitated (Methods 8.2) and subjected to two dimensional polyacrylamide gel electrophoresis (Methods 11). In the first dimension a pH gradient of 3.5 - 10 was employed and in the second dimension the SDS polyacrylamide gel contained 10% acrylamide. The gel was stained with Coomassie blue (Methods 10.1), dried and subjected to autoradiography at -80°C.

Marker lane Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg albumin 45kDa, Carbonic anhydrase 29kDa

⟶ position of the DNase.

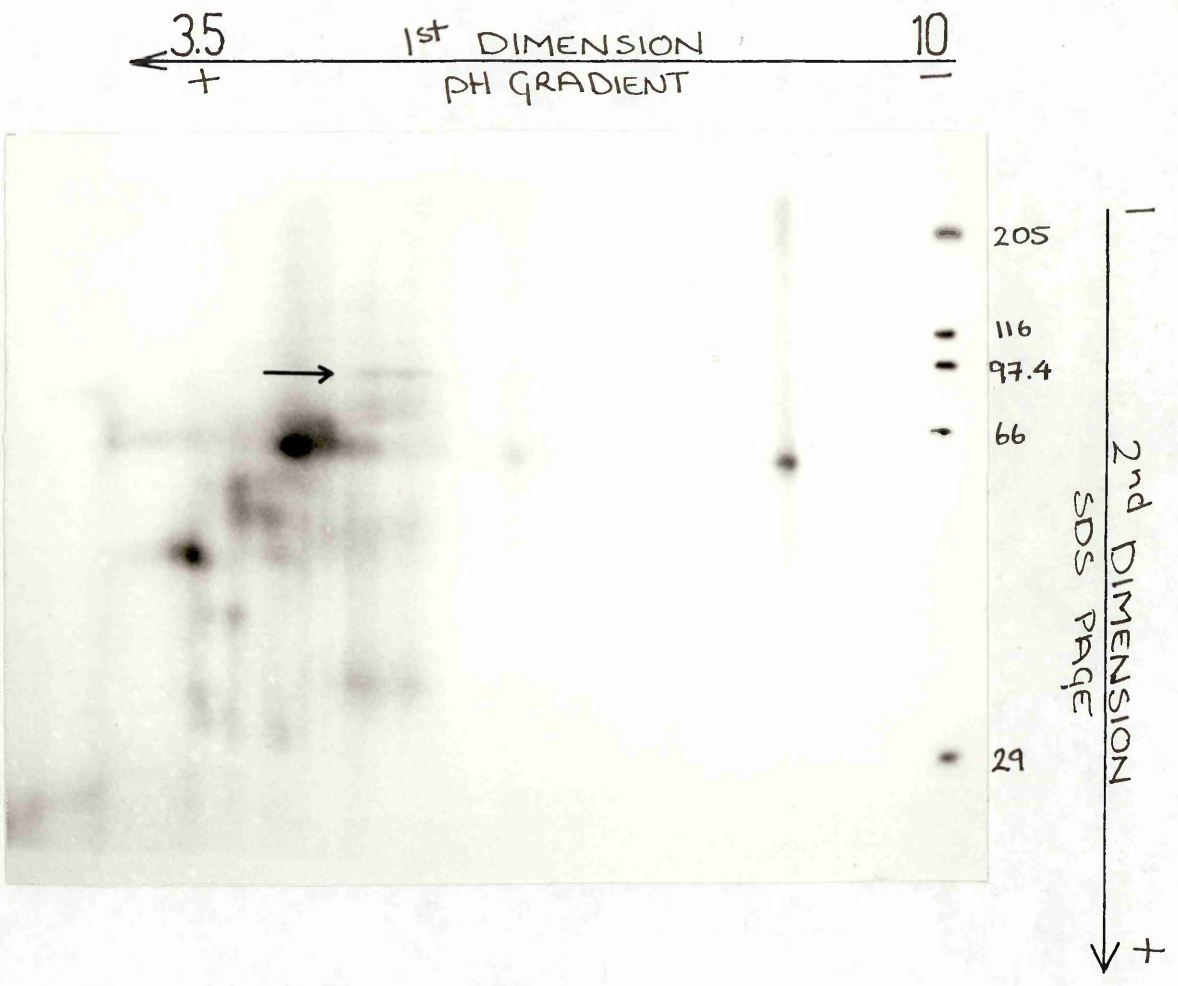


Figure 3.1.6 In vitro phosphorylation of the DNase by the HSV-1, US3 protein kinase. DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) was incubated with the US3 protein kinase (Methods 6.2.2) in the presence of $\gamma[^{32}p]$ATP (Methods 14.2). The sample was then TCA precipitated (Methods 8.2) and subjected to two dimensional polyacrylamide gel electrophoresis (Methods 11). In the first dimension a pH gradient of 3.5 - 10 was employed and in the second dimension the SDS polyacrylamide gel contained 10% acrylamide. The gel was stained in Coomassie blue (Methods 10.1), dried and subjected to autoradiography at -80°C.

Marker lane: Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg albumin 45 kDa. Carbonic anhydrase 29kDa.

⟶ position of the DNase.

Figure 3.1.7 *In vitro* phosphorylation of the DNase by protein kinase C, cAMP dep. protein kinase and the US3 protein kinase. DNase purified from cells infected for 18 hrs. (Methods 2.2.,6.1) was incubated with protein kinase C (Methods 6.2.1), cAMP dep. protein kinase (Materials) or the US3 protein kinase (Methods 6.2.2) in the presence of 2mM ATP (Methods 14.2). The samples were then TCA precipitated (Methods 8.2) and subjected to two dimensional gel electrophoresis (Methods 11). In the first dimension a pH gradient of 3.5 - 10 was employed and in the second dimension the SDS polyacrylamide gel contained 10% acrylamide. The protein was then transferred to nitrocellulose by electrophoresis (Methods 12.1) and probed with an antibody against the DNase (Methods 12.3). A marker lane was run at the side of the 2nd dimension and was visualized by Ponceau S staining (Methods 12.2).

Marker lane: Myosin 205kd, β Galactosidase 116kd, Phosphorylase b 97.4kd, BSA 66kd, Egg Albumin 45kd, Carbonic anhydrase 29kd.

a = DNase phosphorylated by protein kinase C
b = DNase phosphorylated by cAMP dep. protein kinase
c = DNase phosphorylated by US3 protein kinase
o = DNase

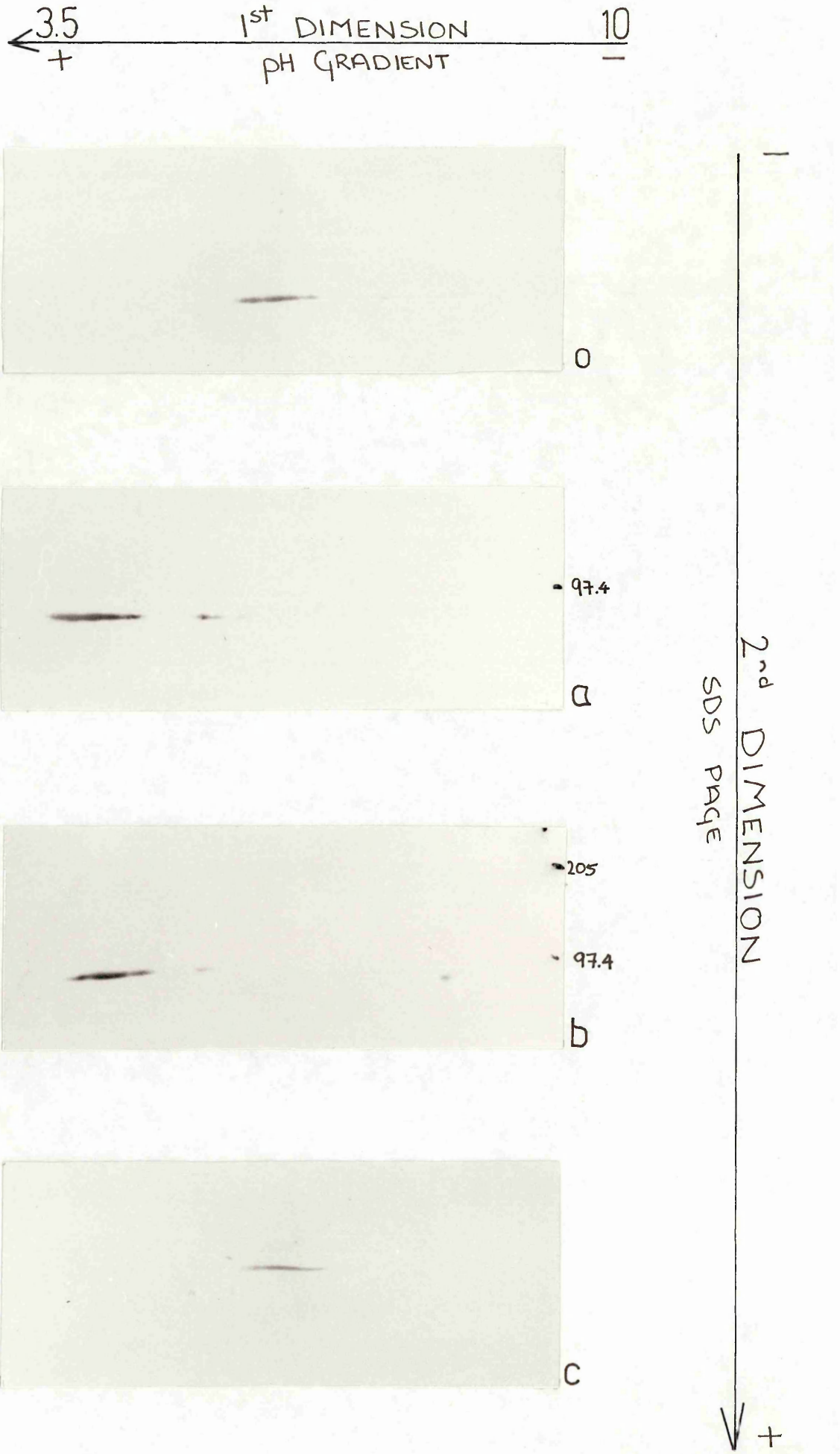

3.5
+
1st DIMENSION
pH GRADIENT
10
−
0
97.4
a
205
97.4
b
c
−
2nd DIMENSION
SDS PAGE
+

Figure 3.2.1 *In vivo* labelling of the DNase with [^{32}P] inorganic phosphate. DNase was purified from cells infected for 6 hrs. (Methods 2.2, 6.1) and which had been labelled with [^{32}P] Pi for 1 - 6 hrs. post infection (Methods 15). The purified DNase was acetone precipitated (Methods 8.1) and subjected to two dimensional polyacrylamide gel electrophoresis (Methods 11). In the first dimension a pH gradient of 3.5 - 9 was employed and in the second dimension the SDS polyacrylamide gel contained 10% acrylamide. The protein on the gel was transferred to nitrocellulose by electrophoresis (Methods 12.1) and probed with an antibody against the DNase (Methods 12.3). The blot was then dried and subjected to autoradiography at -80^{o}C.

Marker lane: Myosin 205kDa, β Galactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg albumin 45kDa, Carbonic anhydrase 29kDa.

a = autoradiography
b = immunoblot

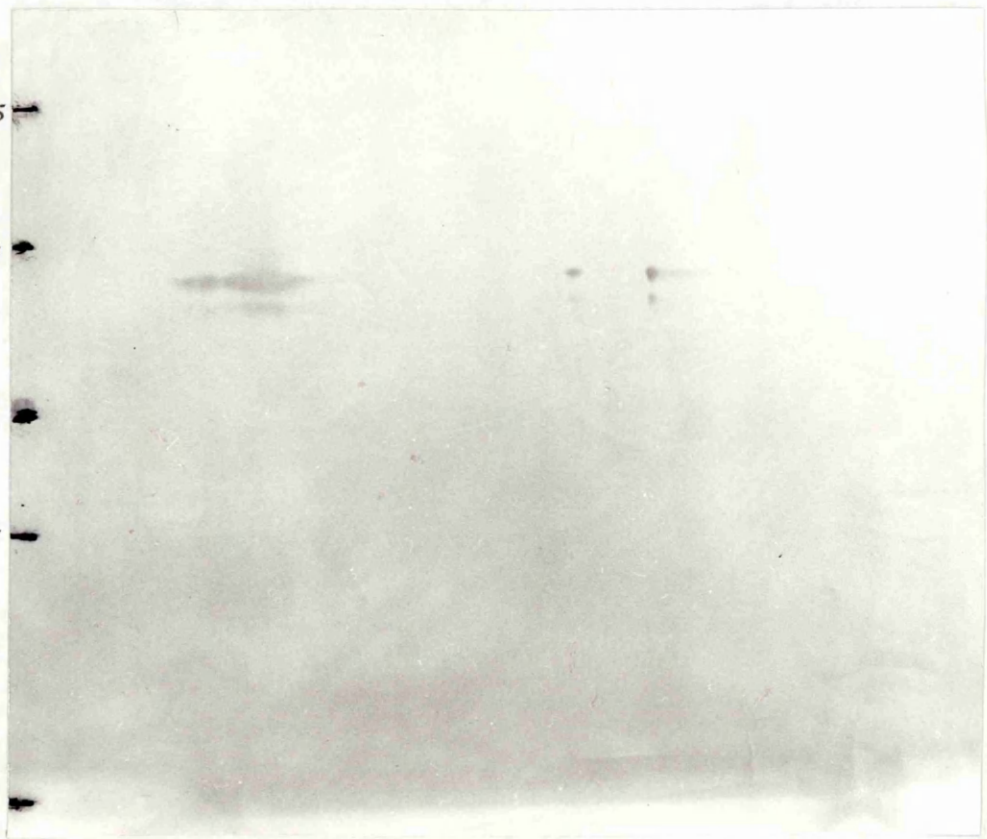
9 — 1st DIMENSION pH GRADIENT — 3.5 +

a

2nd DIMENSION SDS PAGE

−

+



b

4. Phosphopeptide analysis of DNase phosphorylated *in vitro* & *in vivo*

DNase phosphorylated *in vitro* (Methods 16) or *in vivo* (Methods 17) was subjected to one dimensional polyacrylamide gel electrophoresis (Methods 9) and transferred to a PVDF membrane (Methods 16). After autoradiography the DNase region was cut from the membrane and the protein digested with tpck-treated trypsin. The digest was then subjected to chromatography on a Pharmacia FPLC system (Methods 16). The resultant phosphopeptide FPLC profiles are shown in Figures 4.1 & 4.2.

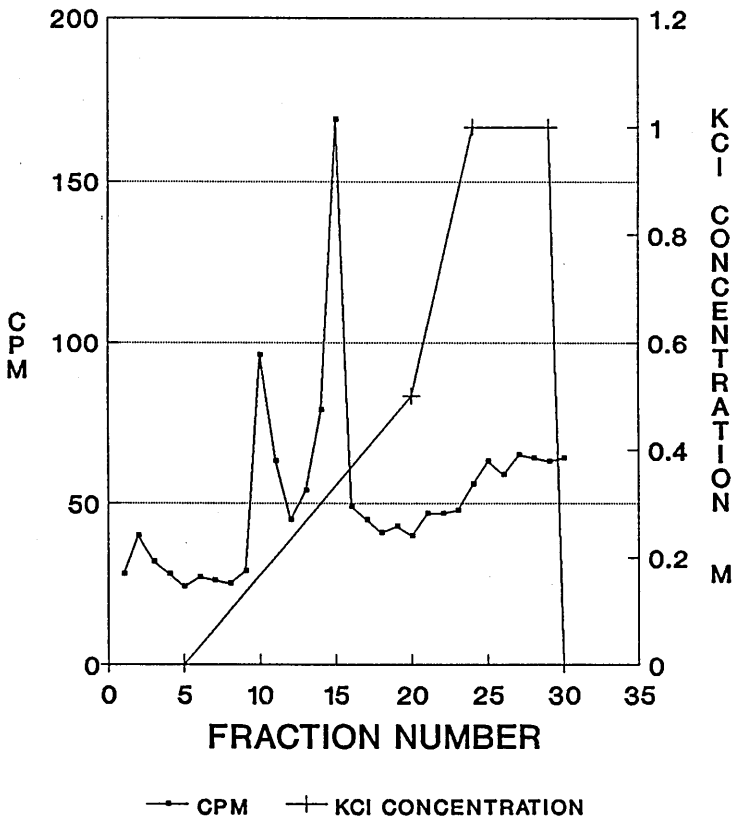
The cAMP protein kinase produced one labelled peak which eluted at fraction 25, protein kinase C produced two labelled peaks which eluted at fractions 11 & 16, and casein kinase II and the US3 protein kinase both produced two labelled peaks which co-eluted at fractions 10 & 15.

The FPLC profile of the cell culture [^{32}p] labelled DNase (Figure 4.2) showed no recognisable peaks and when compared to the *in vitro* profiles (Figure 4.3) none of the peaks present in those profiles could be aligned with it. The problem was that not enough [^{32}p] was incorporated into the DNase during the labelling experiment.

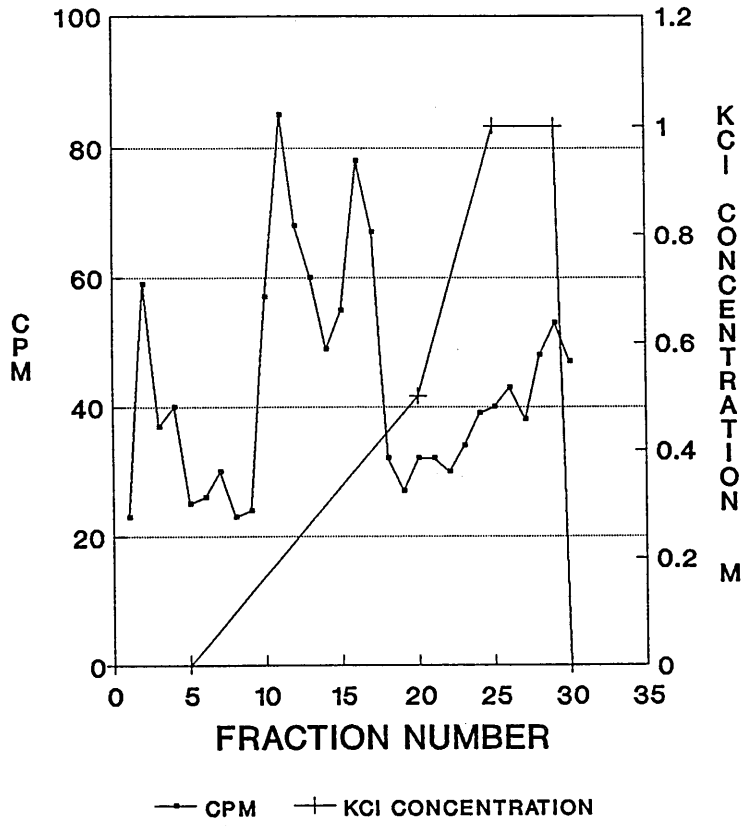
Figure 4.1 F.P.L.C. analysis of the tryptic digests of *in vitro* phosphorylated DNase. DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) was incubated with cAMP dep. protein kinase (Materials), protein kinase C (Methods 6.2.1), casein kinase II (Methods 6.2.3) or the US3 protein kinase (Methods 6.2.2) in the presence of $\gamma[^{32}P]$ATP (Methods 16). The samples were then acetone precipitated (Methods 8.1) and subjected to electrophoresis on an SDS polyacrylamide gel, 3 mm. thick containing 10% acrylamide (Methods 9). The proteins were then transferred to a PVDF membrane (Methods 16) by electrophoresis. The membrane was subjected to autoradiography and the DNase band cut out and digested with tpck-treated trypsin (Methods 16). The digest was then subjected to chromatography on a Mono Q column on a Pharmacia FPLC system using a 0-1M KCl gradient in 20mMTris-HClpH7.5 (Methods 16).

Cherenkov activity of the fractions was then measured in a scintillation counter.

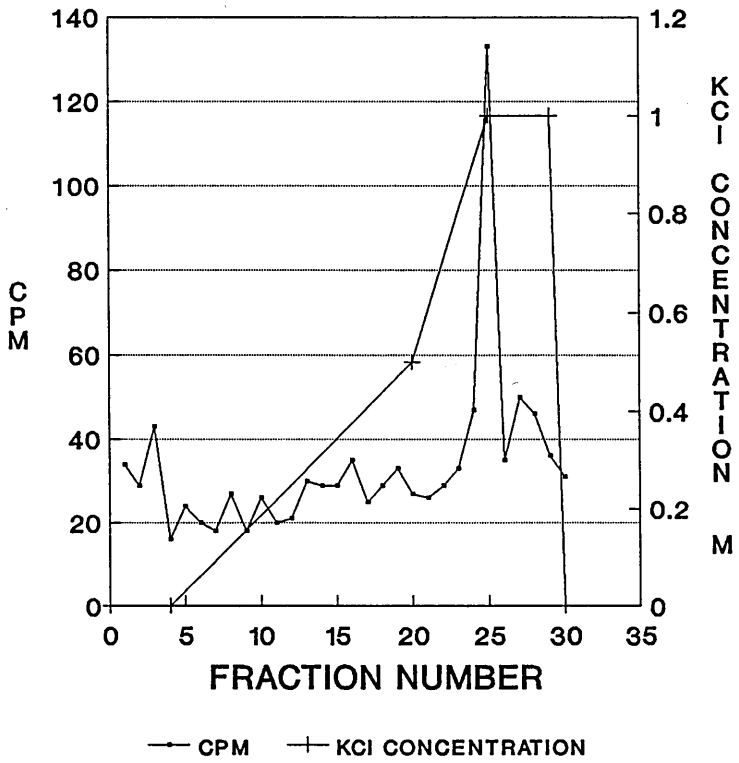
CASEIN KINASE 2



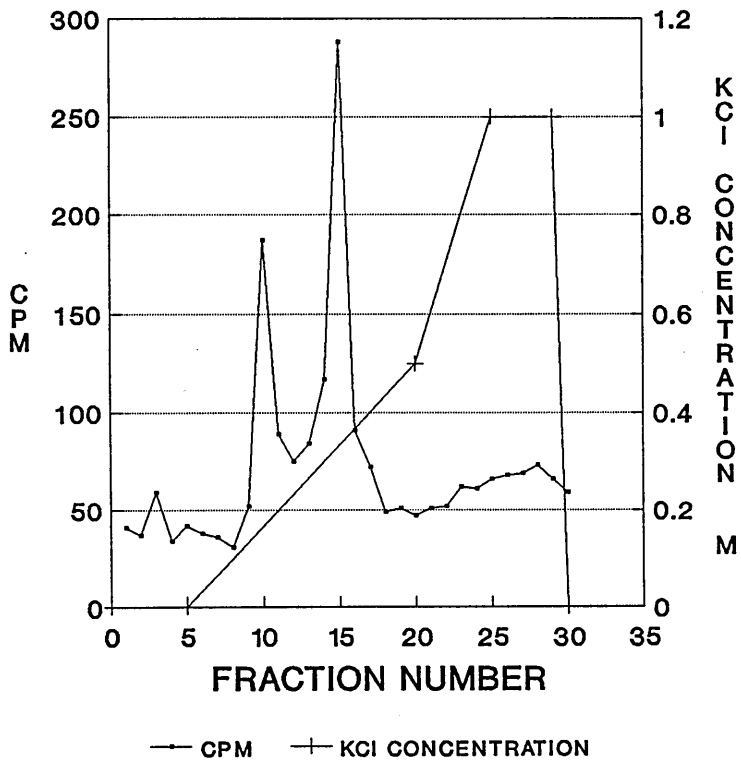
PROTEIN KINASE C



cAMP dep. PROTEIN KINASE



HSV-1 US3 PROTEIN KINASE



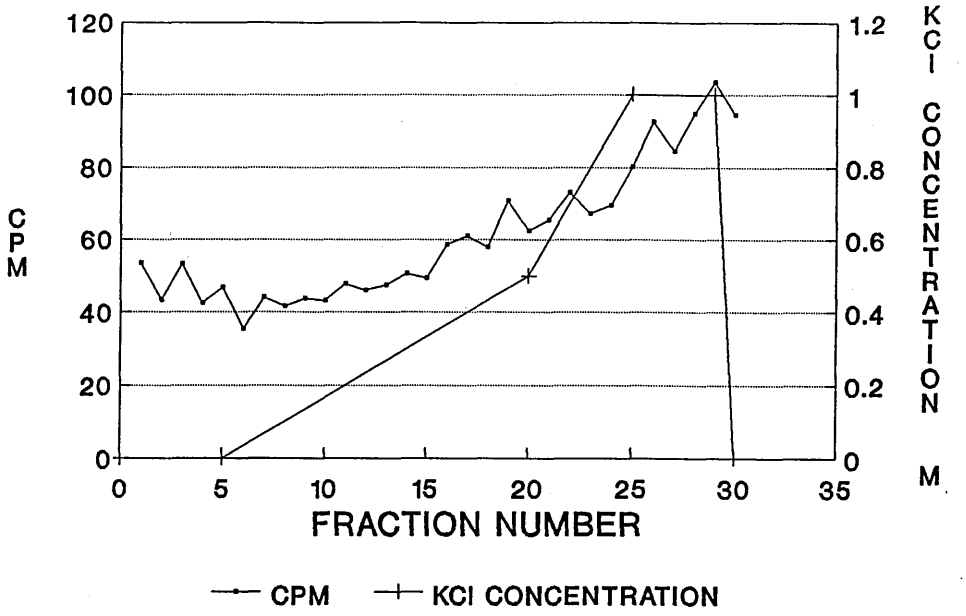
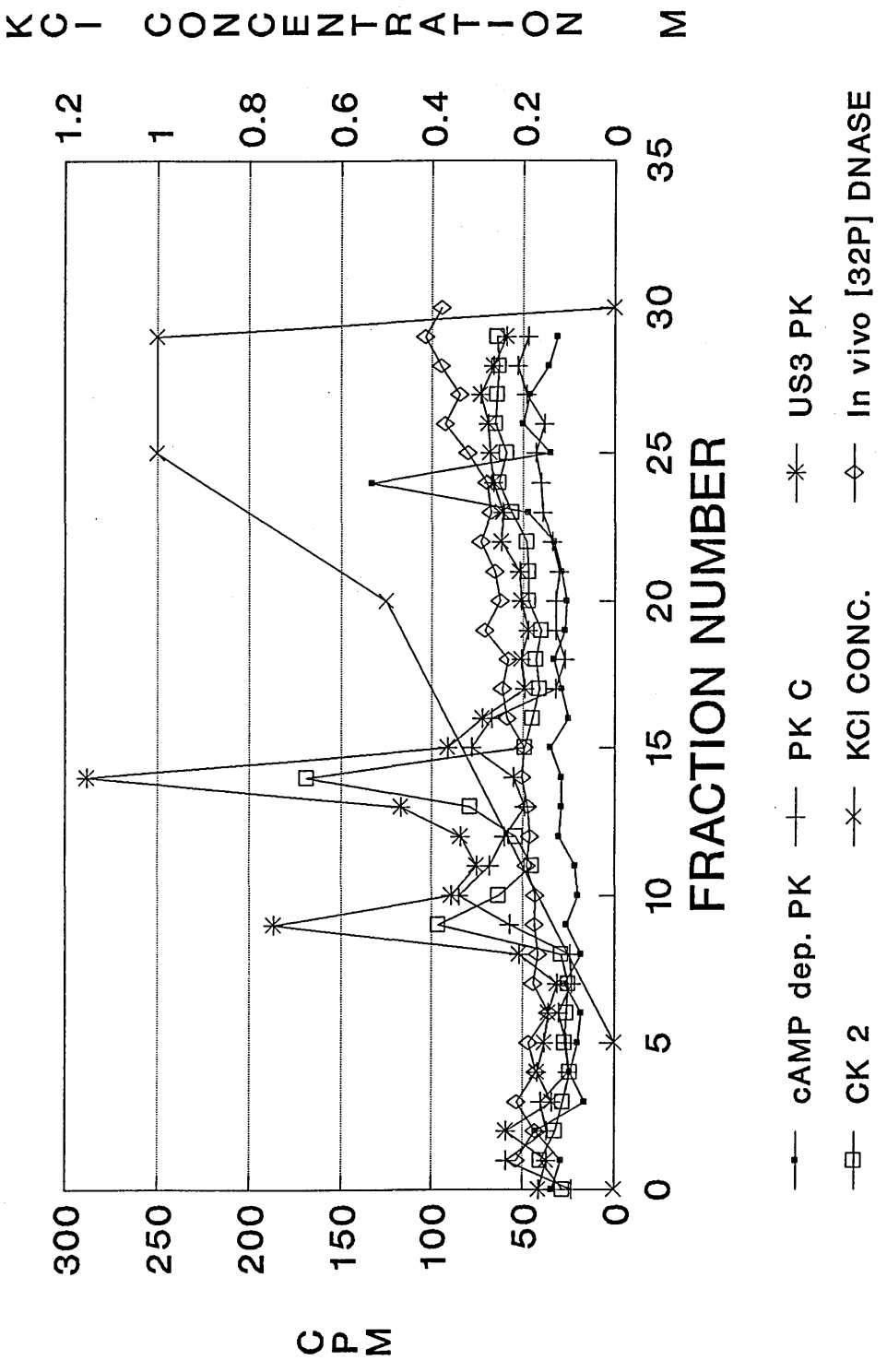


Figure 4.2 F.P.L.C. analysis of the tryptic digest of cell culture [^{32}P] labelled DNase. DNase was purified from cells infected for 12 - 15 hrs. (Methods 2.2, 6.1) and which had been labelled with 2 - 3mCi [^{32}P] for 11 - 14 hrs. (Methods 17). The purified DNase was then acetone precipitated (Methods 8.1) and subjected to electrophoresis on a 10% acrylamide SDS gel. The protein was then transferred to PVDF membrane by electrophoresis (Methods 17). The membrane was then stained with Coomassie blue (Methods 10.1), destained in 50% methanol and the DNase band cut out and digested overnight with tpck-treated trypsin (Methods 17). The digest was then subjected to chromatography on a Mono Q column on a Pharmacia FPLC system using a 0-1MKCl gradient in 20mMTris-HCl pH 7.5 (Methods 17). The radioactivity of each sample was measured in the presence of scintillant in a scintillation counter.

Figure 4.3 Comparison of the FPLC phosphopeptide profiles produced from *in vitro* phosphorylated DNase & (Figure 4.1) cell culture [^{32}P] labelled DNase (Figure 4.2).

cAMPdep.PK	cAMPdependent protein kinase
PKC	Protein kinase C
US3 PK	US3 protein kinase
CK2	Casein kinase 2
KCl Conc.	KCl concentration
In vivo [^{32}P] DNase	DNase purified from [^{32}P] labelled infected cells


KCl CONCENTRATION M
1.2
1
0.8
0.6
0.4
0.2
0
CPM
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
FRACTION NUMBER
cAMP dep. PK
PK C
US3 PK
CK 2
KCl CONC.
In vivo [32P] DNASE

5. Studies of the endonuclease activity of the DNase

The DNase enzyme has an endonuclease activity (Hoffman & Cheng 1979, Hoffman P. 1981) as well as the exonuclease activity which was used to monitor its purification (Methods 5.1, 6.1). A plasmid G35a (Methods Figure 18.1) containing a restriction fragment from the HSV-1 genome was used as a substrate. The inserted fragment contains the cleavage site for the production of unit genomic lengths from concatenated viral DNA (see Introduction 1.2).

The plasmid was used to see if the endonuclease activity of the DNase could act in a site specific manner under conditions where only this activity was active.

5.1 The effect of pH on endonuclease activity

The assays were set up as described in Methods 18.4 except that the activity of the enzyme was tested in a series of buffers at pH's 5.5, 6.0, 6.5, 7.0, 8.0 & 9.0. The samples used were DNase purified from 18 hr. infected cell lysates (Methods 2.2,,6.1), the 10K spin supernatant from 18 hr. infected cell lysates (Methods 2.2) and a mixture of both. The samples were incubated at 37^{o}C for 5 mins. and then subjected to electrophoresis on agarose gels (Methods 18.2).

The purified DNase alone had no effect on the plasmid (Figure 5.1.1) at any pH. The infected supernatant digested the plasmid at all pH's, it degraded the covalently closed circular form to mainly the open circular form with some linear forms produced at pH's 5.5 to 7.0. At pH's 8.0 and 9.0 it degraded the plasmid equally to the open circular form and linear form. The mixture of the purified DNase and infected supernatant also degraded the plasmid, it degraded the closed circular from to the open circular and linear forms at all pH's.

5.2 The effect of time on the endonuclease activity

The assays were set up as described in Methods 18.4.

The Effect of time I

The samples used were DNase purified from 18 hr. infected cells (Methods 2.2, 6.1), the 10K spin supernatant from 18 hr. infected cells (Methods 2.2) and a mixture of both. Assays were incubated for 5, 30 or 60 mins. at 37°C and then subjected to electrophoresis on agarose gels (Methods 18.2). The purified DNase had no effect on the plasmid under the conditions employed (Figure 5.2.1), the infected supernatant had completely digested the plasmid by 5 mins. of incubation. The purified DNase and infected supernatant mixture had digested the plasmid to the open circular and linear forms by 5 mins. and had completely digested the plasmid by 30 mins..

The Effect of time II

In this experiment the sample used was DNase purified from 18 hr. infected cells (Methods 2.2, 6.1). The assays were incubated for 1, 5, 10, 20, 30 or 60 mins. at 37°C and the DNA was ethanol precipitated (Methods 18.1). The DNA was then digested with the restriction enzyme Hind III (Methods 18.3) and the samples subjected to electrophoresis on agarose gels (Methods 18.2). At each time point employed only the linearized plasmid form was found (Figure 5.2.2) which indicated that the DNase had not digested the plasmid in any way.

5.3 The endonuclease activity of partially purified DNase

The assays were set us as described in Methods 18.4. The samples used were those collected during the purification of the DNase from 18 hr. infected cell lysates (Methods 2.2, 6.1) and used to determine the purification data (Results Table 1.1). The samples were the infected cell homogenate, 10K spin infected supernatant, the DNase pool from DE52 Cellulose chromatography, the DNase pool from Phosphocellulose chromatography and the Aquacide concentrated DNase pool from DNA Cellulose chromatography. The assays were incubated

for 5 mins. at 37°C and then subjected to electrophoresis on agarose gels (Methods 18.2). The infected cell homogenate completely digested the plasmid (Figure 5.3.1) as did the 10K spin supernatant except for a faint band of linearized plasmid. The DNase pool from DE52 Cellulose chromatography completely digested the plasmid to the linear form whilst the DNase pool from phosphocellulose chromatography digested the plasmid to the open circular form with only limited linear production. The purified DNase (the concentrated DNase pool from DNA Cellulose chromatography) had no effect on the plasmid under the conditions employed.

5.4 The effect of uninfected cell supernatant on the endonuclease activity

The assays were set up as described in Methods 18.4 except that poly (dI.dC).poly (dI.dC) was included in the assays in order to prevent non-specific binding to the plasmid. The samples used were DNase purified from 18 hr. infected cell lysates (Methods 6.1), the 10K spin supernatant from uninfected cells (Methods 2.2 except uninfected cells used) and a mixture of the above. The assays were incubated at 37°C for 5 mins. and then subjected to electrophoresis on agarose gels (Methods 18.2). The purified DNase had no effect on the plasmid (Figure 5.4.1), the uninfected supernatant had completely digested the plasmid by 5 mins.. The mixture digested the plasmid to the open circular and linear forms.

5.5 Double digestion of the plasmid to determine site specificity

The duplicate assays were set up as described in Methods 18.4 except that poly (dI.dC).poly (dI.dC) was added to prevent non-specific binding. The samples used were a mixture of DNase purified from 18 hr. infected cell lysates (Methods 6.1) and uninfected 10K spin supernatant (Methods 2.2 except uninfected cells used) or infected 10K spin supernatant from 18 hr. cell lysates (Methods 2.2). The assays were incubated for

2 mins. at 37°C then the DNA was ethanol precipitated (Methods 18.1). The DNA was then digested with the restriction enzymes Hind III or Pst I (Methods 18.3) and the samples subjected to electrophoresis on agarose gels (Methods 18.2). The only distinct bands produced by the digestions were those of linearized plasmid (Figure 5.5.1). These would result from the action of the restriction enzymes on covalently closed or circular forms of the plasmid.

5.6 The effect of dephosphorylation of the DNase on its endonuclease activity

The assays were set up as described in Methods 18.4. The samples used were DNase purified from 18 hr. infected cell lysates (Methods 6.1) which had been treated by the protein phosphatases 1 (PP1) or 2A (PP2A) (Methods 13.4) and the protein phosphatases themselves.

The assays were incubated at 37°C for 2 mins. and then subjected to electrophoresis on agarose gels (Methods 18.2).

The purified DNase which had been treated with the phosphatases digested the plasmid (Figure 5.6.1). The DNase treated with PP1 digested the plasmid to the open circular and linear forms whilst that treated with PP2A digested the plasmid almost completely to the linear form.

The samples treated with phosphatase alone gave similar digestion patters (Figure 5.6.2). Phosphatase 1 and PP1 treated DNase digested the plasmid to the open circular and linear forms and the pattern obtained was identical. PP2A treated DNase digested the plasmid to the open circular and linear forms with the linear form dominating. The phosphatase 2A completely digested the plasmid.

When the digestions were carried out in the presence or absence of 20mM pyrophosphate, a phosphatase inhibitor, it was observed that where the pyrophosphate was present no digestion of the plasmid occurred (Figure 5.6.3). In the absence of the pyrophosphate, the phosphatases and

the phosphatase treated DNase samples all digested the plasmid. In the case of the PP1 samples mainly the open circular form resulted with some linears and for the PP2A samples mainly linear forms resulted.

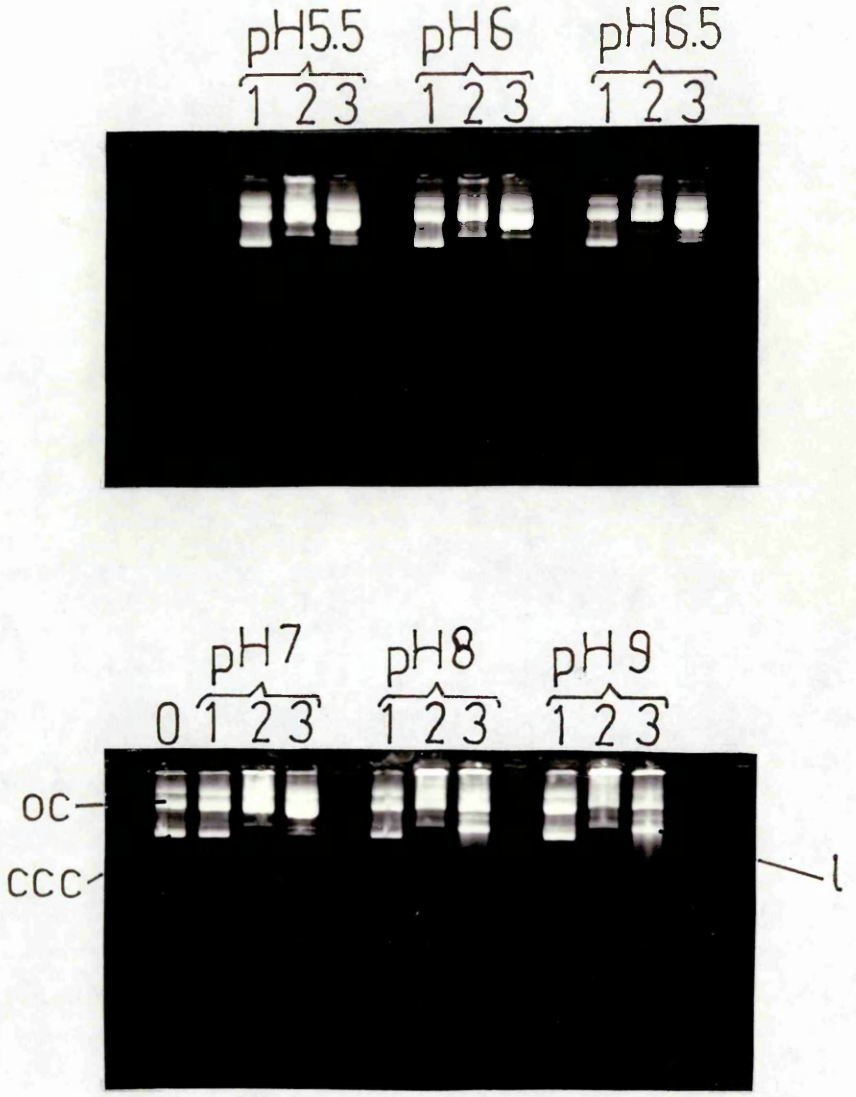


Figure 5.1.1 The effect of pH on the ability of DNase to digest plasmid G35a. The endonuclease activity of DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) was tested over a range of pH 5.5, 6.0, 6.5, 7.0, 8.0 & 9.0, the 10K spin infected supernatant from cells infected for 18 hrs. (Methods 2.2) and a mixture of both were also tested. The conditions chosen allowed only the DNase's endonuclease function to be active. The samples were incubated at 37°C for 5 mins. and then subjected to electrophoresis on a 1% agarose gel at 100V for 2 hrs.

Key 0 = original plasmid
1 = purified DNase
2 = infected supernatant
3 = mixture of 1 & 2
CCC = covalently closed circular plasmid
OC = open circular plasmid
L = linear plasmid

Figure 5.2.1 The effect of time on the endonuclease activity of DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1),the 10K spin supernatant from cells infected for 18 hrs. (Methods 2.2) and a mixture of both. The conditions chosen allowed only the DNase's endonuclease function to be active. The samples were incubated at 37°C for 5, 30 or 60 mins. and then subjected to electrophoresis on a 1% agarose gel for 2 hrs. at 80V.

Key 0 = original plasmid
E = EcORI digest of the plasmid
1 = 5 mins. digestion
2 = 30 mins. digestion
3 = 60 mins. digestion
I.S.= Infected Supernatant

Figure 5.2.2 The effect of time on the endonuclease activity of DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1). The conditions chosen allowed only the DNase's endonuclease function to be active. The samples were incubated for 1, 5, 10, 20, 30 or 60 mins. at 37°C, then ethanol precipitated (Methods 18.1) and digested for $1\frac{1}{2}$ hrs. at 37°C with Hind III. The samples were then subjected to electrophoresis on a 1% agarose gel at 100V. for 2 hrs..

Key 0 = original plasmid
1,5,10,20,30,60 = time of DNase digestion
λ = λ markers

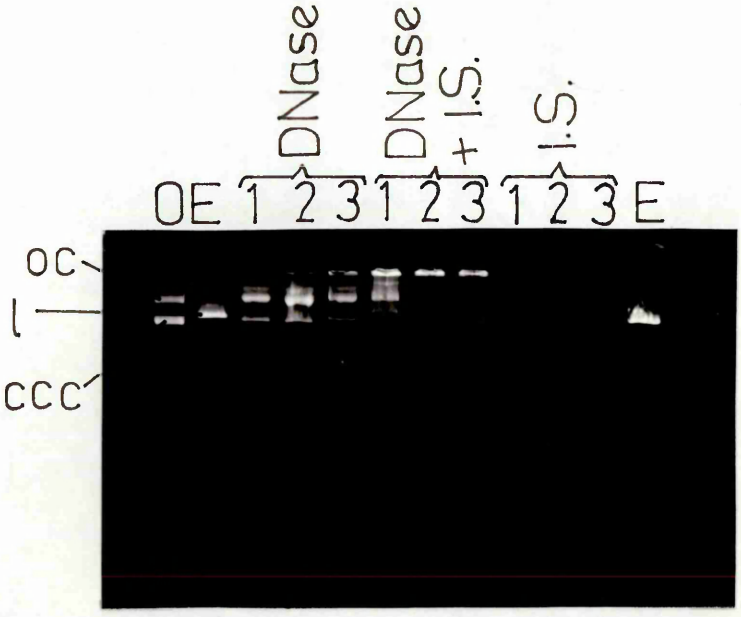


Figure 5.2.1

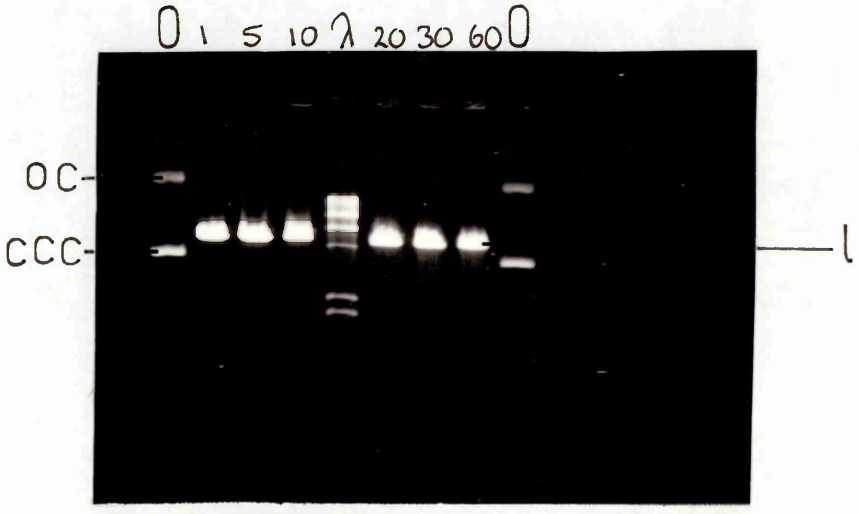


Figure 5.2.2

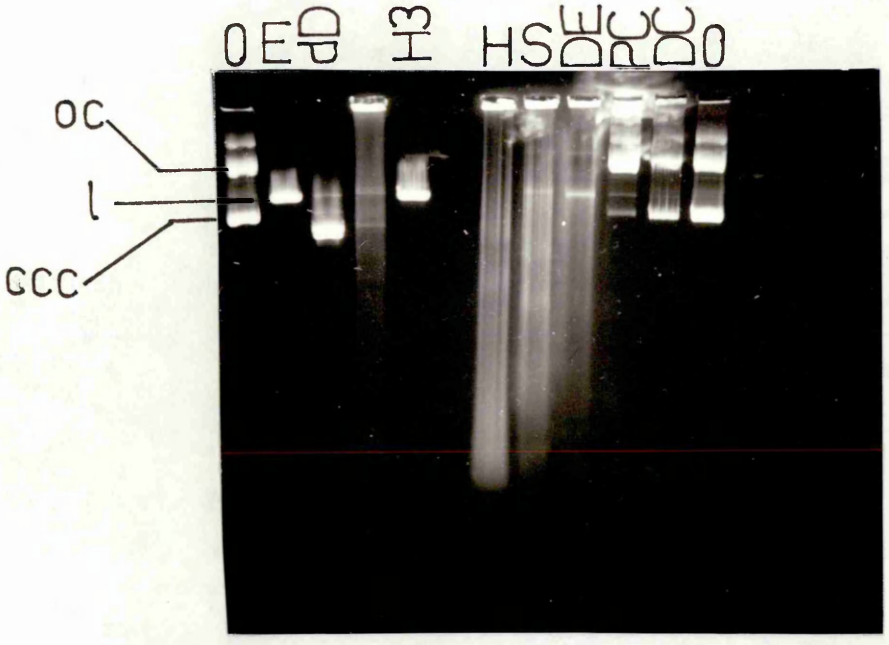


Figure 5.3.1 The endonuclease activity of partially purified DNase fractions obtained during the purification of DNase from cells infected for 18 hrs. (Methods 2.2, 6.1). The conditions chosen allowed only the DNase's endonuclease function to be active. The samples were incubated at 37°C for 5 mins. and then subjected to electrophoresis on a 1% agarose gel for 2 hrs. at 100V..

Key
O = original plasmid
E = EcOR1 digest of the plasmid
dD = EcOR1 | Hind III double digest of the plasmid
H3 = Hind III digest of the plasmid
H = Cell homogenate
S = 10K spin supernatant
DE = DE52 Cellulose DNase pool
PC = Phosphocellulose DNase pool
DC = DNA Cellulose DNase pool = purified DNase

OC = open circular plasmid
L = linear plasmid
CCC = covalently closed plasmid

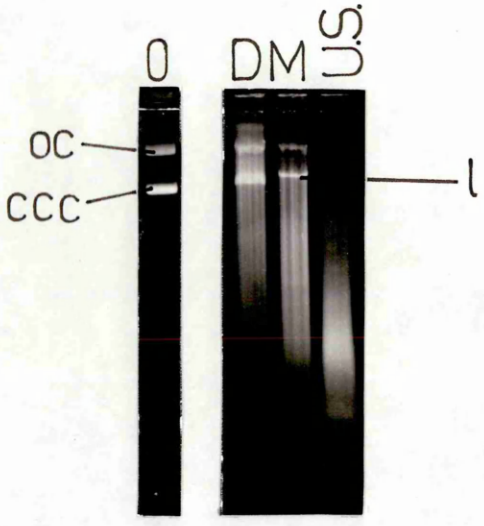


Figure 5.4.1 The effect of uninfected supernatant on the endonuclease activity. DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1), 10K spin supernatant from uninfected cells (Methods 2.2) and a mixture of both were assayed in conditions where only the DNase endonuclease function was active. The samples were incubated at 37°C for 5 mins. and then subjected to electrophoresis on a 1% agarose gel at 80V. for 2 hrs.

Key O = original plasmid
D = purified DNase
US = uninfected supernatant
M = mixture of D & US

OC = open circular plasmid
L = linear plasmid
CCC = covalently closed circular plasmid

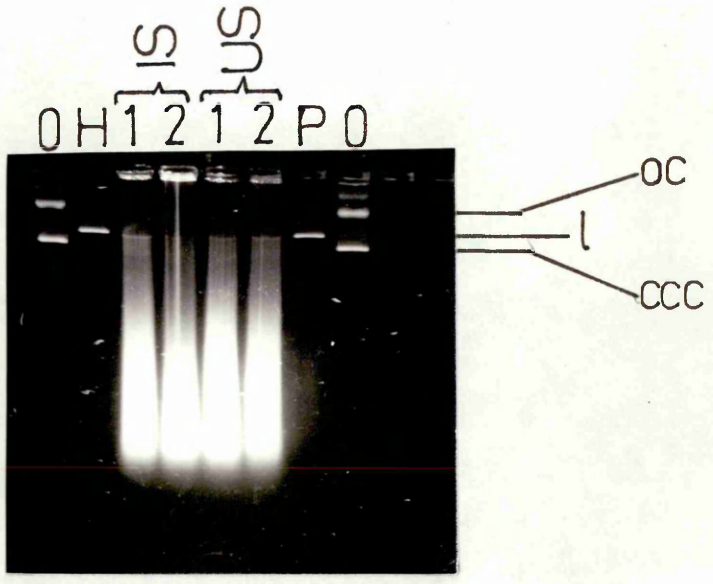


Figure 5.5.1 Double digestions of the plasmid. DNase purified from 18 hr. infected cells (Methods 2.2, 6.1) together with either 10K spin supernatant purified from 18 hr. infected cells (Methods 2.2) or 10K spin supernatant purified from uninfected cells (Methods 2.2 uninfected cells) was incubated with the plasmid for 2 mins. at 37°C. The conditions chosen allowed only the DNase's exonuclease function to be active. The DNA was then ethanol precipitated (Methods 18.1) and digested with either Hind III or PstI for 1½ hrs. at 37°C. The samples were then subjected to electrophoresis on a 1% agarose gel at 100V. for 2 hrs..

Key
- O = original plasmid
- H = Hind III digestion of the plasmid
- P = Pst1 digestion of the plasmid
- IS = DNase + infected supernatant
- US = DNase + uninfected supernatant
- 1 = 2nd digestion with Hind III
- 2 = 2nd digestion with Pst1

- OC = open circular plasmid
- L = linear plasmid
- CCC = covalently closed circular plasmid

Figure 5.6.1 The effect of dephosphorylation on endonuclease activity. DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) was treated with either protein phosphatase 1 or 2A (Methods 13.4) and then its endonuclease activity was assayed. The conditions chosen allowed only the DNase's endonuclease activity to be active. The samples were incubated for 2 mins. at 37°C and then subjected to electrophoresis on a 1% agarose gel for 2 hrs. at 100V..

Key
O = original plasmid
H = Hind III digestion of the plasmid
P1 = PP1 treated DNase
P2 = PP2A treated DNase
D = Untreated DNase
oc = Open circular plasmid
1 = linear plasmid
ccc = covalently closed circular plasmid

Figure 5.6.2 The effect of protein phosphatases on endonuclease activity. DNase purified from cells infected for 18hrs. (Methods 2.2, 6.1) and treated with either protein phosphatase 1 or 2A (Methods 13.4) was assayed for endonuclease activity as were the protein phosphatases. The conditions chosen allowed only the DNase's endonuclease function to be active. The assays were incubated at 37°C for 2 mins. and then subjected to electrophoresis on a 1% agarose gel at 80V. for 2 hrs.

Key
O = original plasmid
1D = PP1 treated DNase
2D = PP2A treated DNase
1P = PP1
2P = PP2A

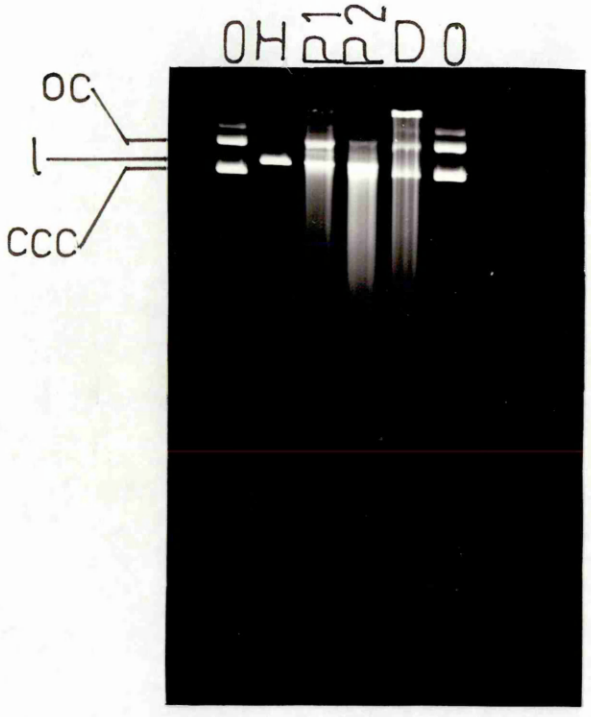


Figure 5.6.1.

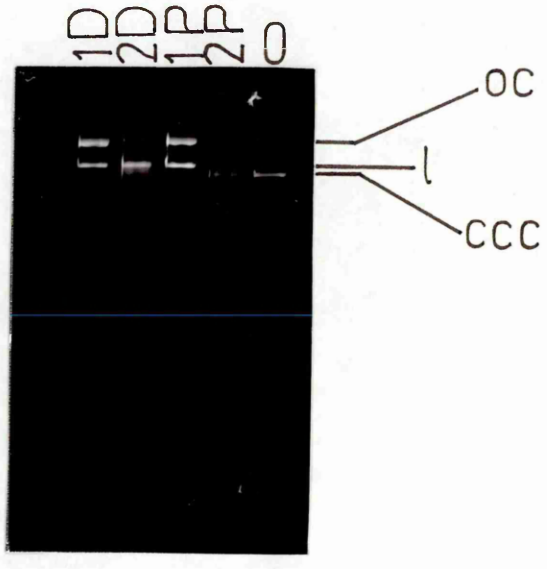


Figure 5.6.2.

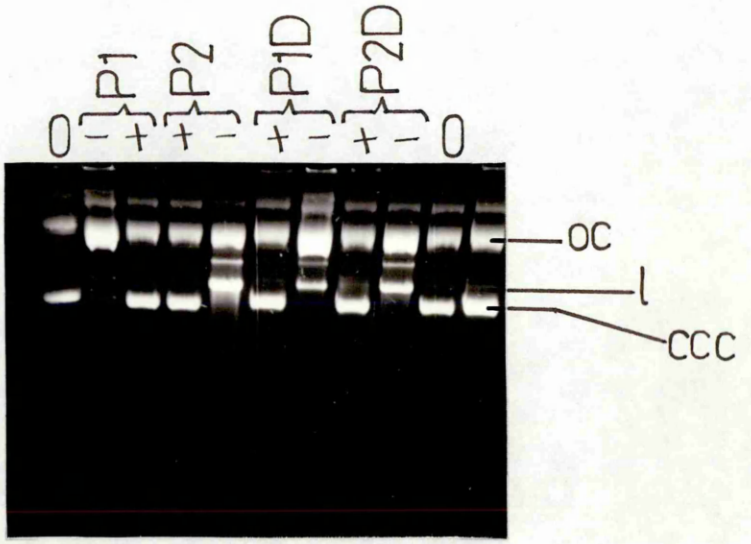


Figure 5.6.3 The effect of phosphatase inhibitors on the endonuclease activity. DNase purified from cells infected for 18 hrs. (Methods 2.2, 6.1) and treated with protein phosphatase 1 or 2A (Methods 13.4) and the phosphatases themselves were assayed for endonuclease activity in the presence or absence of 20mM pyrophosphate, a phosphatase inhibitor. The conditions chosen allowed only the DNase's endonuclease function to be active. The samples were incubated at 37°C for 2 mins. and then subjected to electrophoresis on a 1% agarose gel at 90V. for 2 hrs..

Key
0 = original plasmid
+ = 20mM pyrophosphate present
- = no inhibitor present
P1 = PP1
P2 = PP2A
P1D = PP1 treated DNase
P2D = PP2A treated DNase

6. Xanthate studies

The virally coded US3 protein kinase and other cellular protein kinases were partially purified to examine what effect the xanthates D609, D611 endo and D611exo, known antiviral agents (Sauer G. et al 1984, Amtmann E. et al 1987, Müller-Decker K. et al 1987), would have on their *in vitro* activities.

6.1 Kinase purification

The protamine kinase profile from the DE52 cellulose column (Methods 19.1) shown in Figure 6.1.1 gave the position of the US3 protein kinase which eluted at 200mMKCl. Further specific kinase assays (Methods 5.2) determined the positions of the cellular kinases. The fractions used for the inhibitor studies were: US3 protein kinase fraction 37, protein kinase C fraction 16, cAMP dep. protein kinase fraction 11, Ca^{2+}/Calmodulin dep. protein kinase fraction 36, casein kinase I fraction 16 and casein kinase II fraction 38.

6.2 Inhibitor studies

Xanthate concentrations in the μM range had no significant effect on the activity of the US3 protein kinase (Figure 6.2.1) but an increase into the mM concentration range resulted in up to 90% inhibition of the kinase (Figure 6.2.2). The xanthate D609 was found to have the greatest inhibitory activity which was reflected in its IC_{50} value of 5mM (Table 6.2.1), compared to values of 15mM and 18mM for the xanthate isomers D611 exo and D611 endo respectively.

This was found to be repeated throughout the inhibitor studies (Figures 6.2.3 - 6.2.7; Table 6.2.1). The xanthate D609 had the greatest inhibitory activity with IC_{50} values of 4 - 5mM. The isomers D611 exo and D611 endo had a lesser inhibitory activity with IC_{50} values at least double that of the corresponding D609 value.

The only exception to this was in the case of cAMP dep. protein kinase where the IC_{50} values for all three xanthate compounds were in the range 4 - 7mM.

Lineweaver - Burk analysis of the inhibition of US3 protein kinase by the xanthates relative to the ATP concentration was carried out. In the case of D609 (Figure 6.2.8), Vmax remained unchanged at each xanthate concentration showing that the inhibition was competitive in nature. A Dixon plot of $\frac{1}{v}$ against [I] for the D609 (Figure 6.2.9) compound gave a value for its Ki of 850μM. Ki is the dissociation constant for the enzyme: inhibitor complex and can be used as a measure of the relative activity of inhibitors.

In the case of the two D611 isomers, D611 exo (Figure 6.2.10) and D611 endo (Figure 6.2.12) a similar analysis suggests that they too function as competitive inhibitors. Dixon plots of the two isomers (Figures 6.2.11 & 6.2.13) gave Ki values of 2.5mM for D611 exo & 2mM for D611 endo. These values agree with the IC_{50} values (Table 6.2.1) which showed the D609 compound to be the best inhibitor of kinase activity.

6.3 The effect of the xanthate D609 on the DNase in cell culture

A 15 hr. HSV-1(F) infection (Methods 2.2) was carried out in the presence of 20μg/ml xanthate D609 and 10μg/ml undecanoic acid. The resultant 10K spin supernatant (Methods 2.2) was acetone precipitated (Methods 8.1) and subjected to electrophoresis on a two dimensional polyacrylamide gel system (Methods 11). The first dimension used a pH gradient of 3.5 - 9 and the second dimension an SDS polyacrylamide gel containing 10% acrylamide. The gel was transferred to nitrocellulose and probed with an antibody against the DNase (Methods 12).

When compared to the control, the blot of the DNase prepared in the presence of the xanthate (Figure 6.3.1) showed a shift in the distribution of the spots. More protein appeared to be concentrated in the spots at more

basic pI values and less protein was present in the spots at the more acidic pI values. This suggested that the xanthate had prevented the modification of the DNase.

Quantitative analysis of the two profiles by laser densitometry (LKB Ultroscan XL) confirmed the qualitative analysis above (Table 6.3.1).

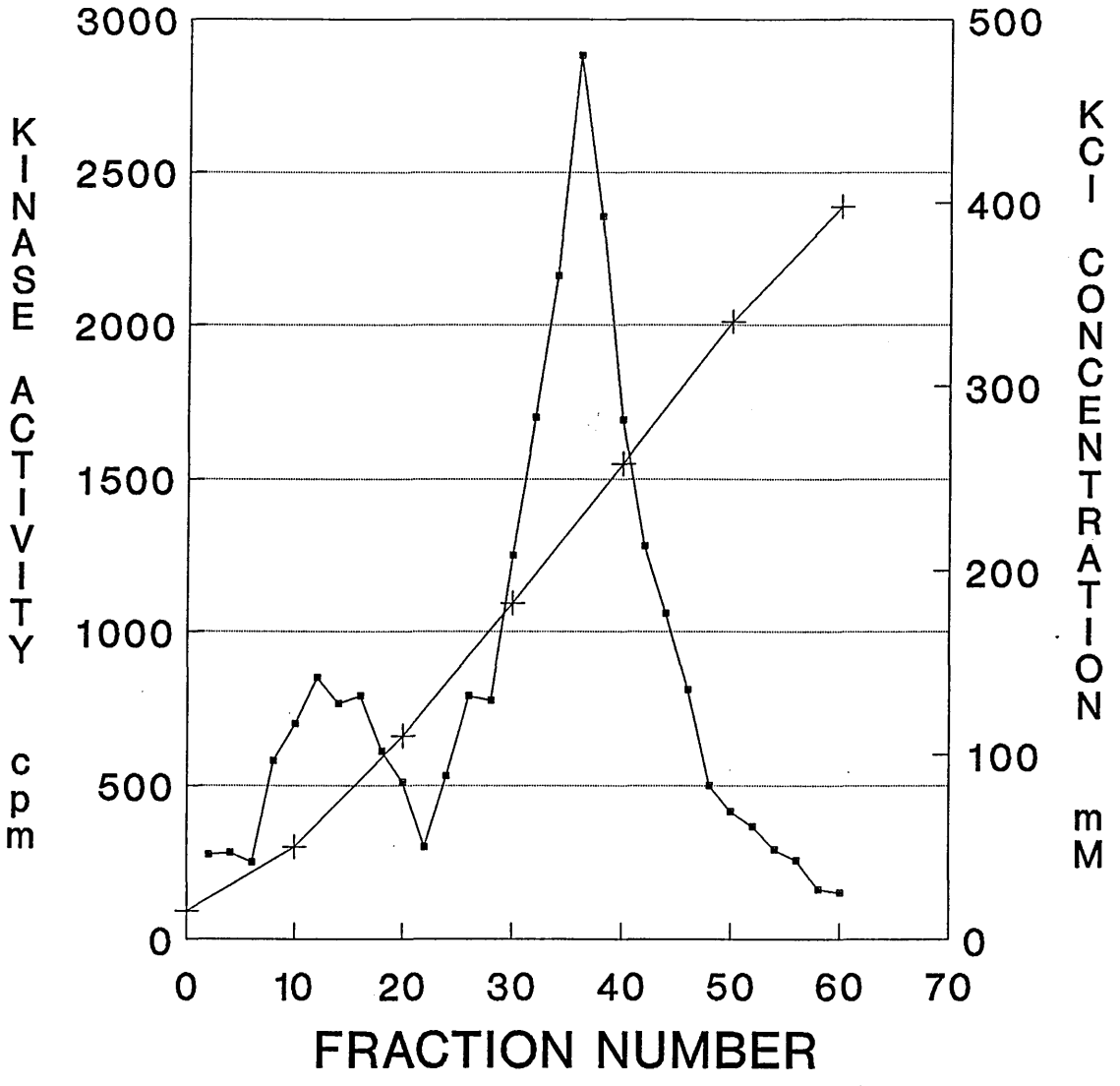


Figure 6.1.1 Protamine kinase activity (Methods 5.2) of an HSV-1(F) infected cell lysate prepared from cells infected for 18 hrs. (Methods 19.1) and subjected to chromatography on DE52 Cellulose (Methods 19.1). Elution was carried out with a linear gradient of 0 - 0.5M KCl in DE Buffer (Materials 2.3).

Key ■ - ■ Kinase activity + KCl concentration

Figure 6.2.1 The effect of the xanthates D609, D611exo and D611endo at μM concentrations on the activity of the US3 protein kinase (fraction 37 Figure 6.1.1) using protamine as a substrate (Methods 5.2, 19.2).

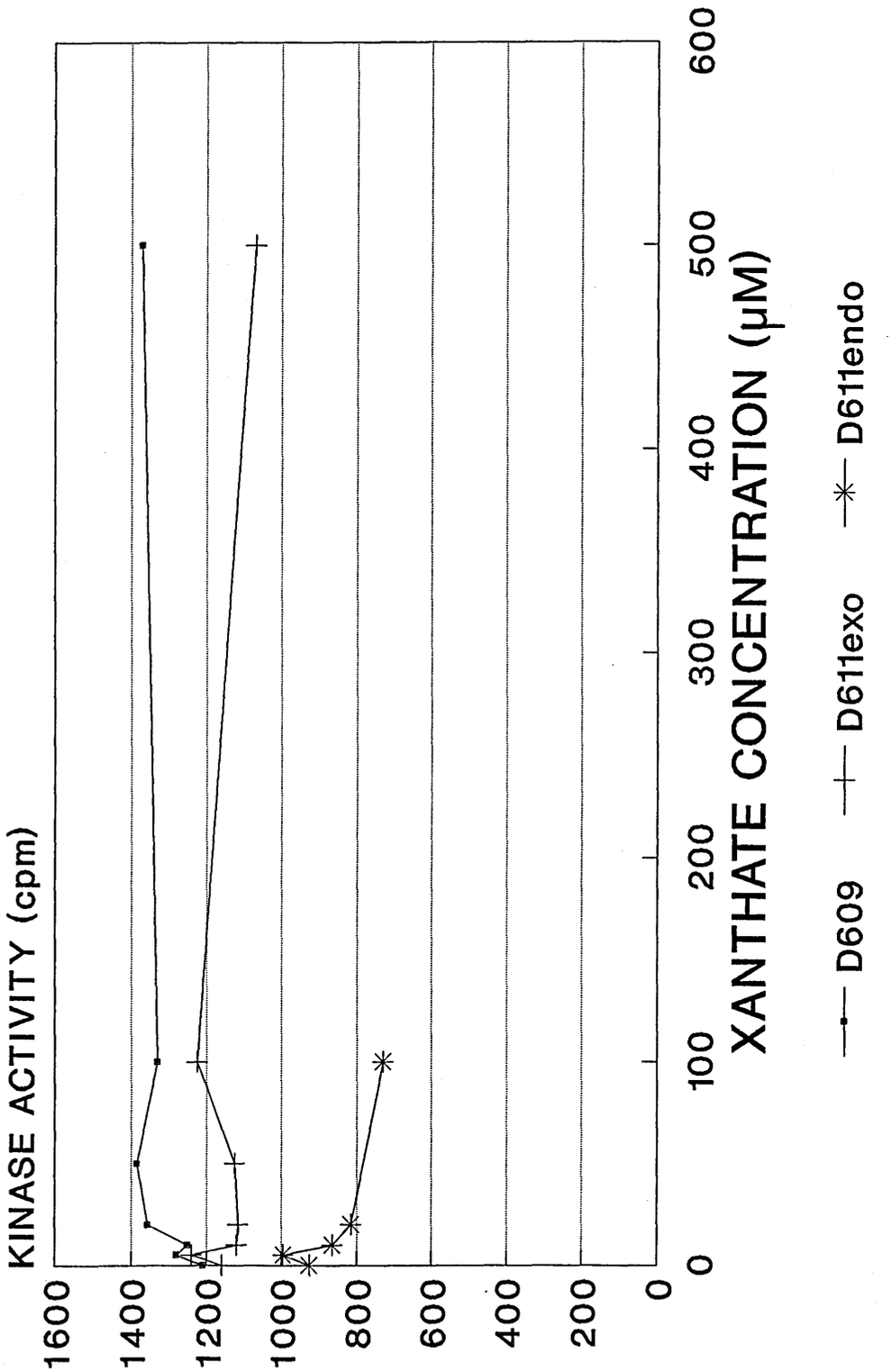
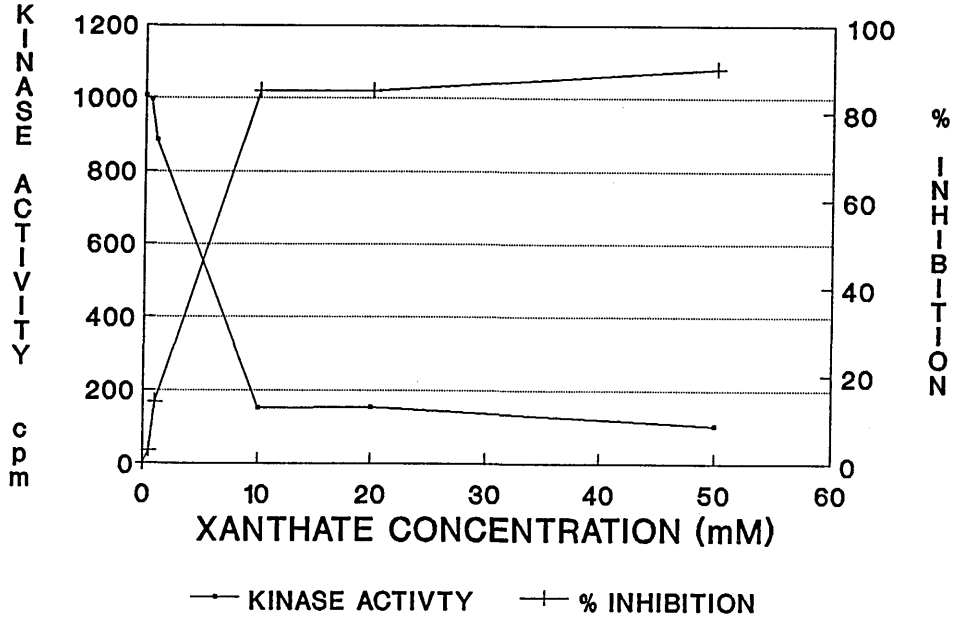

KINASE ACTIVITY (cpm)
1600
1400
1200
1000
800
600
400
200
0
0
100
200
300
400
500
600
XANTHATE CONCENTRATION (μM)
D609
D611exo
D611endo

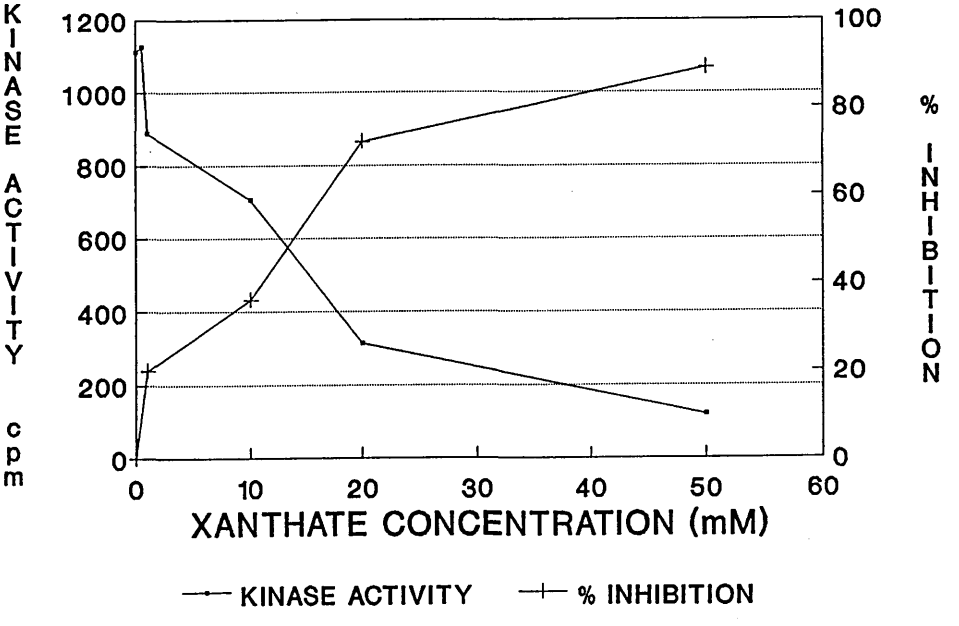
Figure 6.2.2 The effect of the xanthates D609, D611 exo & D611 endo at mM concentrations on the activity of the US3 protein kinase (fraction 37 Figure 6.1.1) using protamine as a substrate (Methods 5.2, 19.2).

Key A = D609 B = D611 exo C = D611 endo

A


KINASE ACTIVITY cpm
1200
1000
800
600
400
200
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVTY
% INHIBITION

B


KINASE ACTIVITY cpm
1200
1000
800
600
400
200
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

C

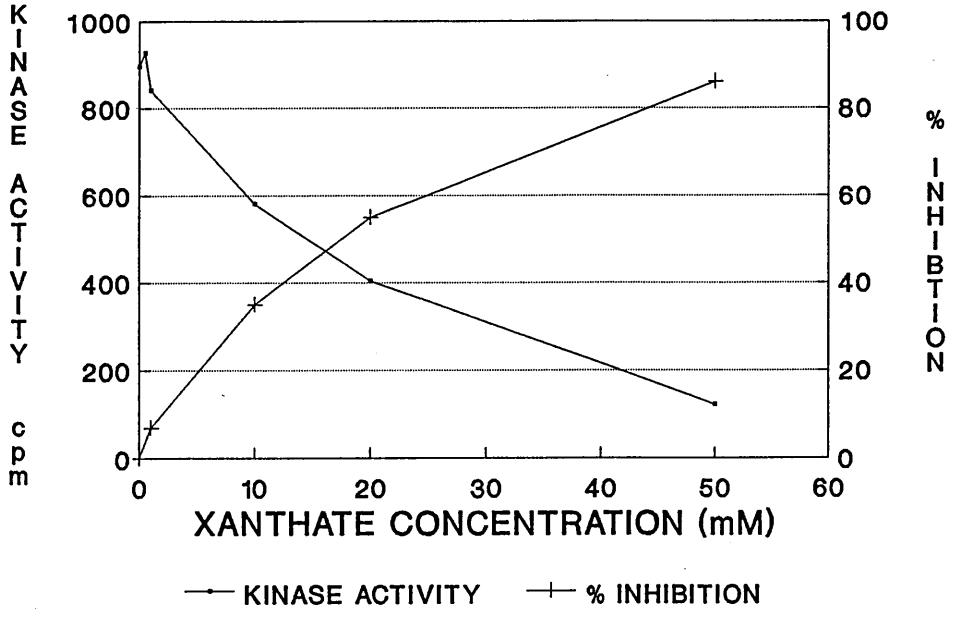
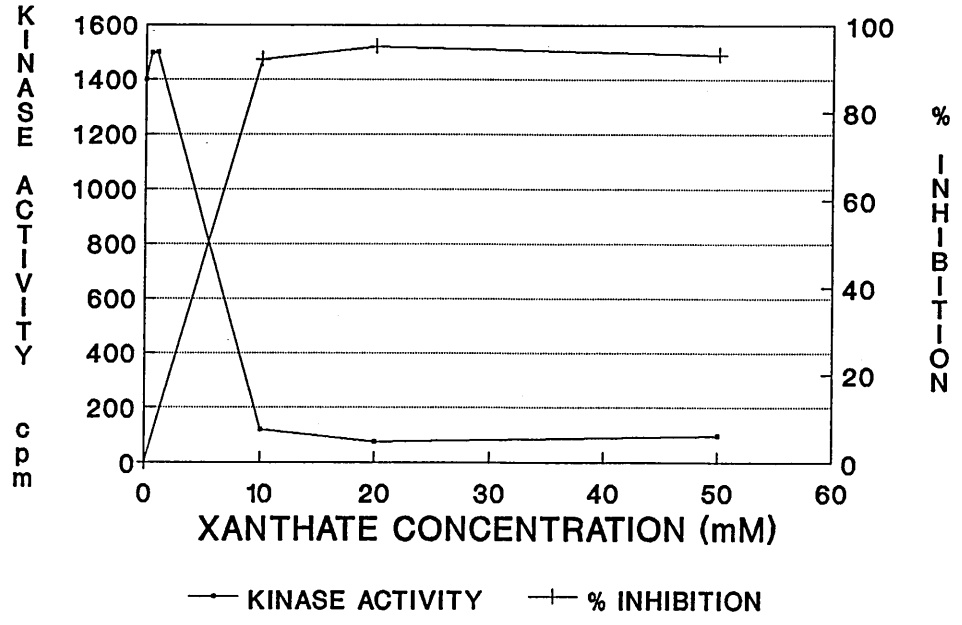

KINASE ACTIVITY cpm
1000
800
600
400
200
0
100
80
60
40
20
0
% INHIBTION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

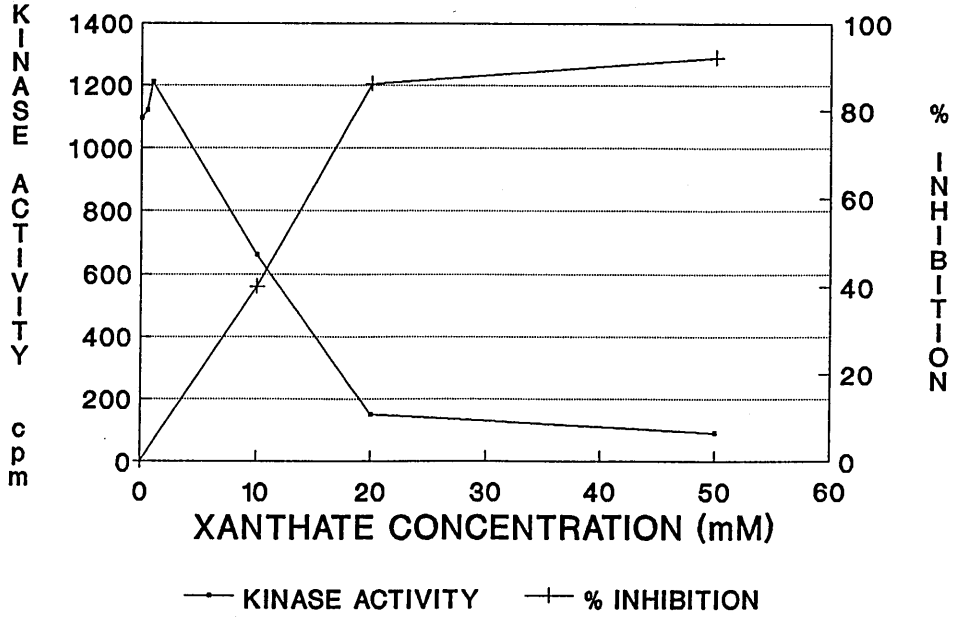
Figure 6.2.3 The effect of the xanthates D609, D611 exo and D611 endo at mM concentrations on the activity of protein kinase C (fraction 16 Figure 6.1.1) using histone IIIs as a substrate (Methods 5.2, 19.2).

Key A = D609 B = D611 exo C = D611 endo

A


KINASE ACTIVITY cpm
1600
1400
1200
1000
800
600
400
200
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

B


KINASE ACTIVITY cpm
1400
1200
1000
800
600
400
200
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

C

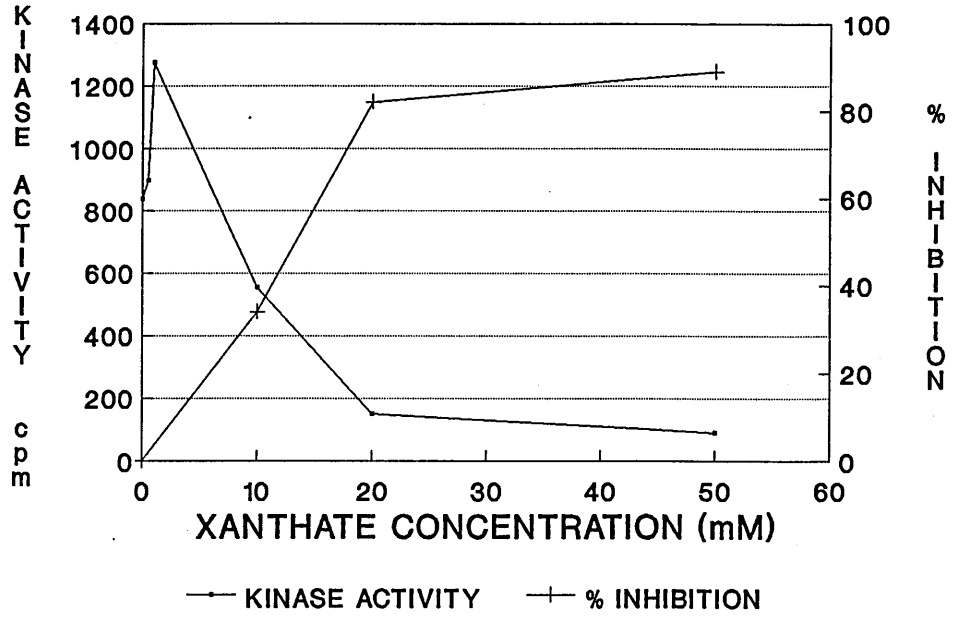
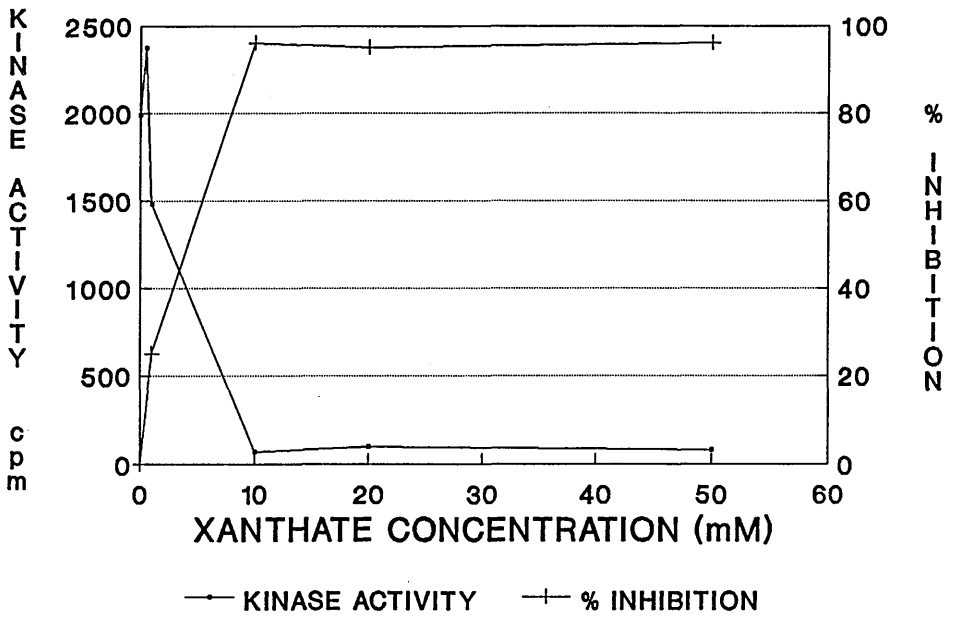

KINASE ACTIVITY cpm
1400
1200
1000
800
600
400
200
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

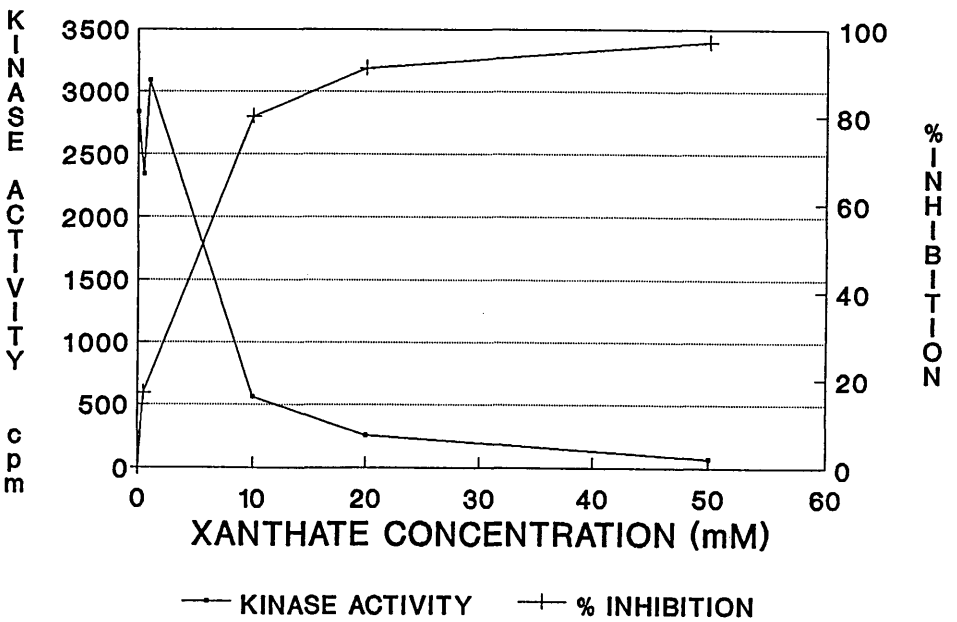
Figure 6.2.4 The effect of the xanthates D609, D611 exo and D611 endo at mM concentrations on the activity of cAMP dependent protein kinase (fraction 11, Figure 6.1.1) using histone IIAS as a substrate (Methods 5.2, 19.2).

Key A = D609, B = D611 exo, C = D611 endo

A


KINASE ACTIVITY cpm
2500
2000
1500
1000
500
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

B


KINASE ACTIVITY cpm
3500
3000
2500
2000
1500
1000
500
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

C

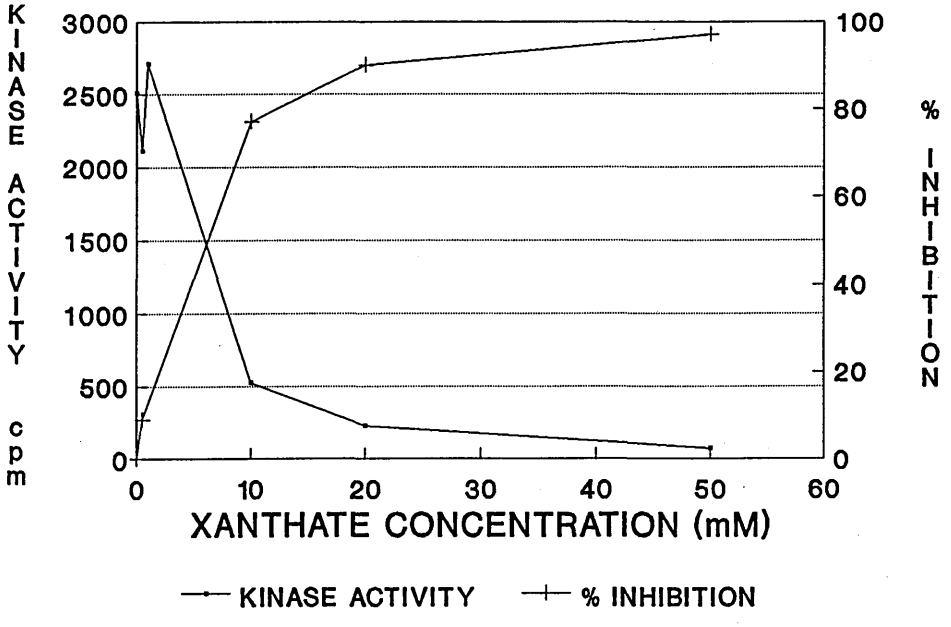
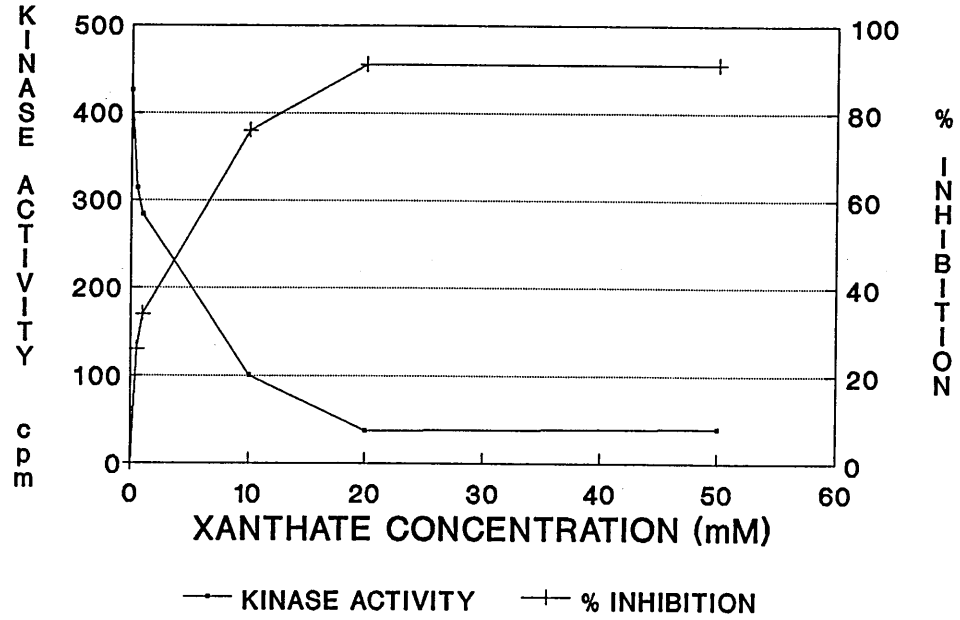

KINASE ACTIVITY cpm
3000
2500
2000
1500
1000
500
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

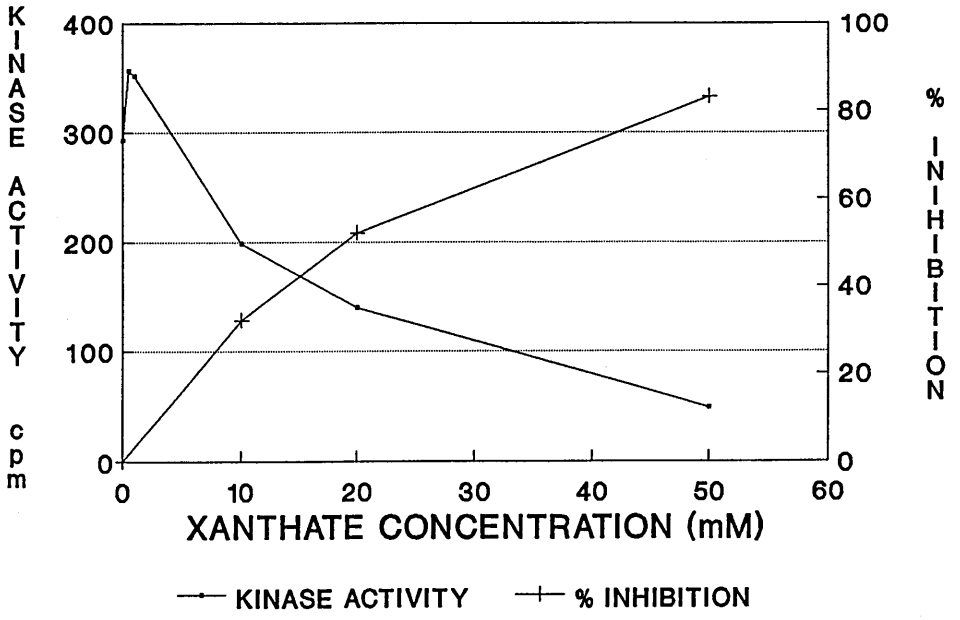
Figure 6.2.5 The effect of the xanthates D609, D611 exo and D611 endo at mM concentrations on the activity of Ca^{2+}: calmodulin dependent protein kinase (Fraction 36, Figure 6.1.1) using myosin as a substrate (Methods 5.2, 19.2).

Key A = D609 B = D611 exo C = D611 endo

A


KINASE ACTIVITY cpm
500
400
300
200
100
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

B


KINASE ACTIVITY cpm
400
300
200
100
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

C

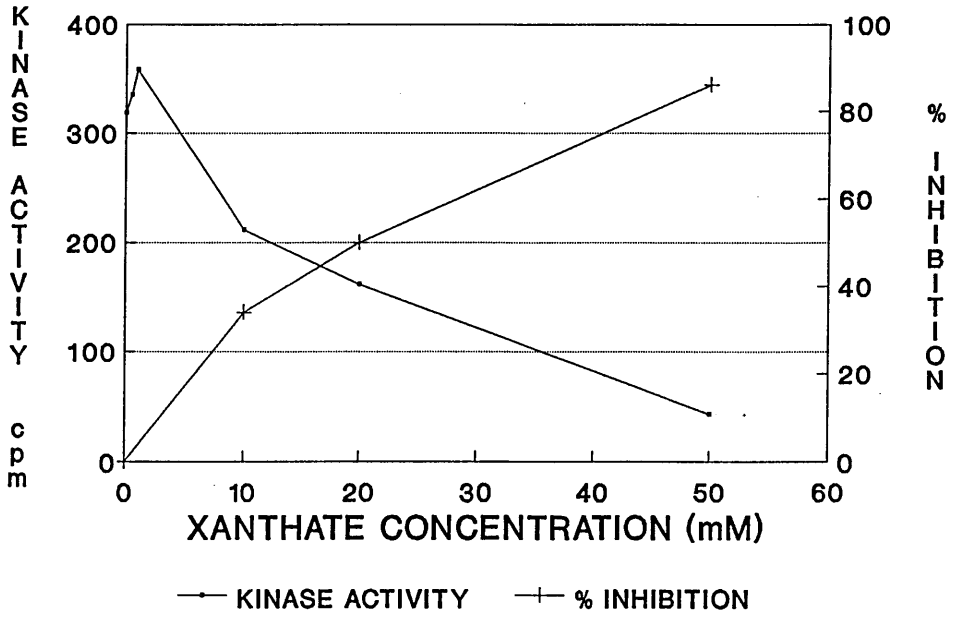
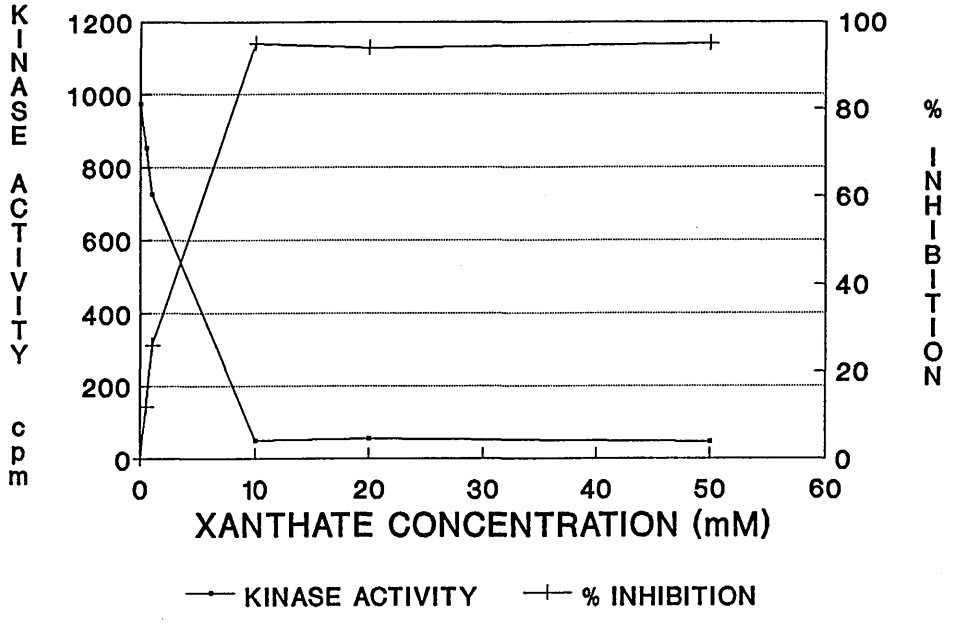

KINASE ACTIVITY cpm
400
300
200
100
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

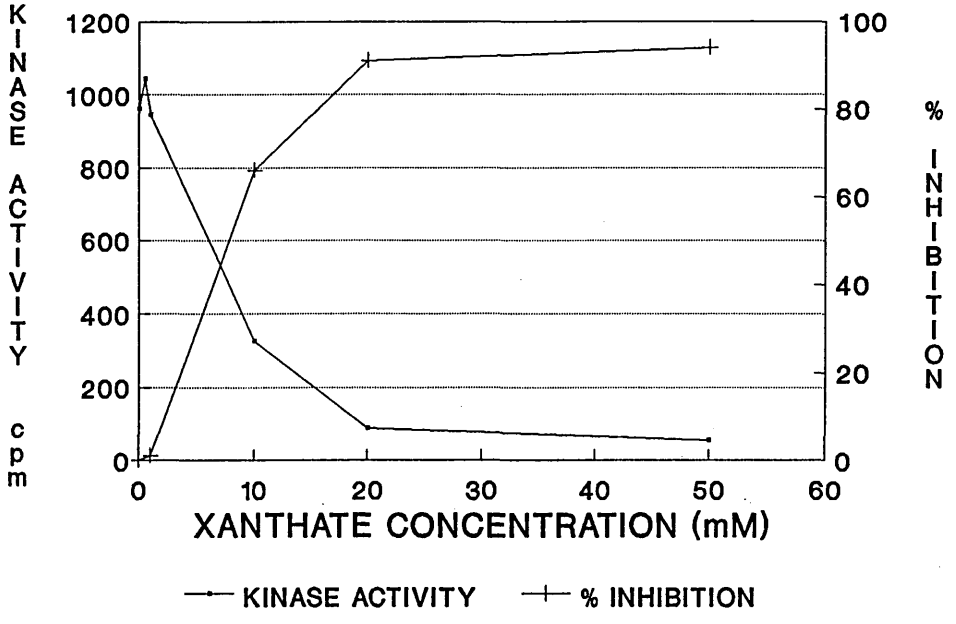
Figure 6.2.6 The effect of the xanthates D609, D611 exo and D611 endo at mM concentrations on the activity of casein kinase I (fraction 16, Figure 6.1.1) using casein as a substrate (Methods 5.2, 19.2).

Key A = D609 B = D611exo C = D611 endo

A


KINASE ACTIVITY cpm
1200
1000
800
600
400
200
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

B


KINASE ACTIVITY cpm
1200
1000
800
600
400
200
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

C

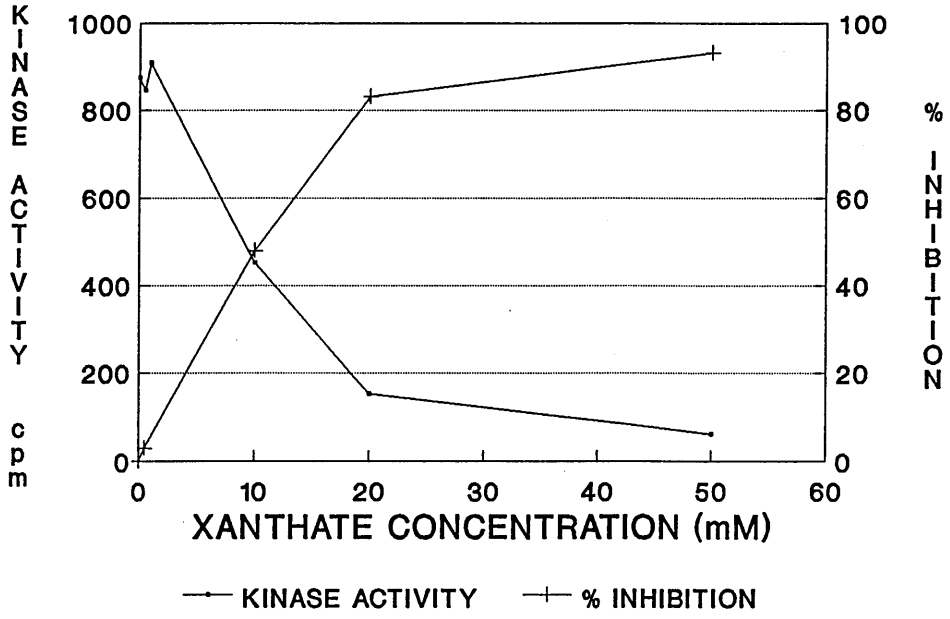
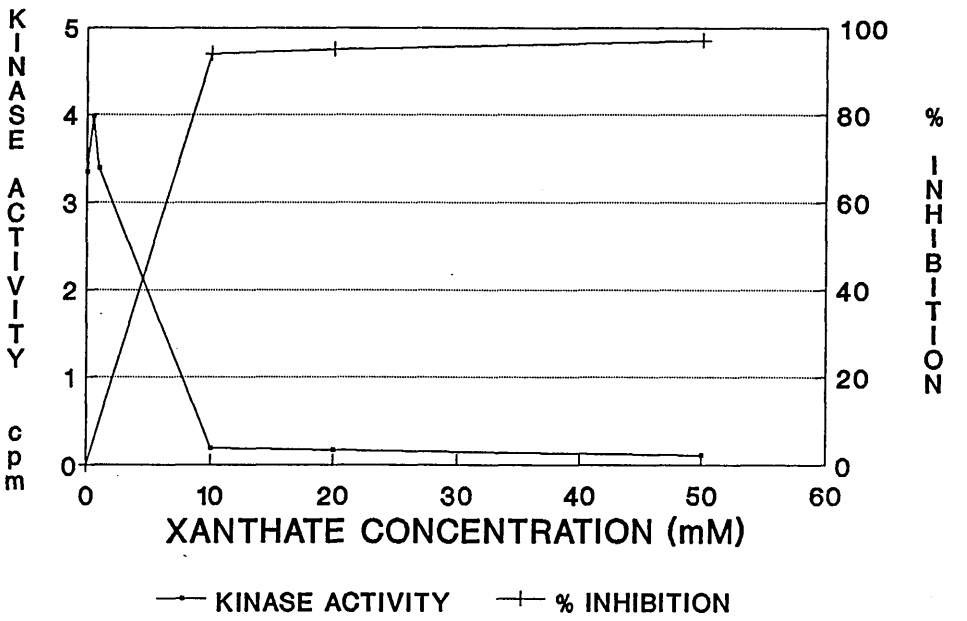

KINASE ACTIVITY cpm
1000
800
600
400
200
0
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

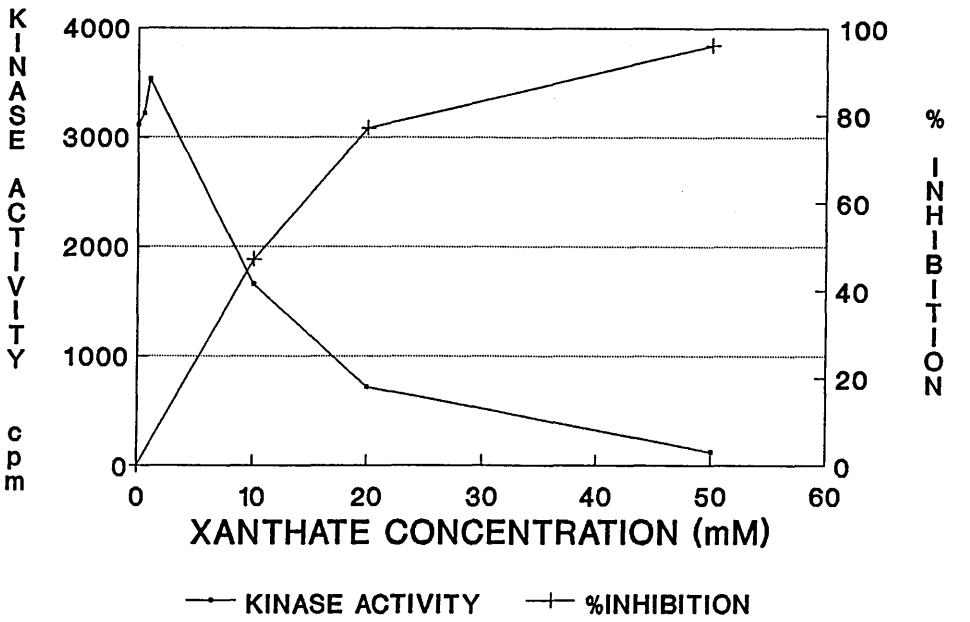
Figure 6.2.7 The effect of xanthates D609, D611 exo and D611 endo at mM concentrations on the activity of casein kinase II (fraction 38, Figure 6.1.1) using casein as a substrate (Methods 5.2, 19.2).

Key A = D609 B = D611 exo C = D611 endo

A


KINASE ACTIVITY cpm
% INHIBITION
0
1
2
3
4
5
0
20
40
60
80
100
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

B


KINASE ACTIVITY cpm
% INHIBITION
0
1000
2000
3000
4000
0
20
40
60
80
100
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
%INHIBITION

C

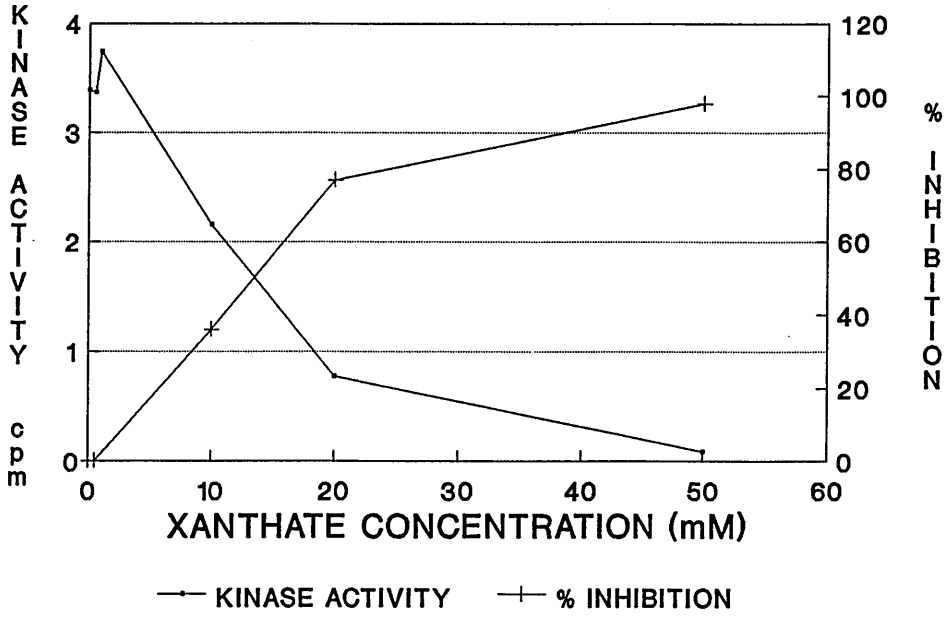

KINASE ACTIVITY cpm
4
3
2
1
0
120
100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
XANTHATE CONCENTRATION (mM)
KINASE ACTIVITY
% INHIBITION

Table 6.2.1 The inhibitory effect of the xanthates D609, D611exo & D611endo on the HSV-1 viral kinase & cellular kinases

KINASE	IC_{50} * (mM)		
	D609	D611exo	D611endo
US3 protein kinase	5	15	18
Protein kinase C	5	12	12
cAMP dep protein kinase	4	5	7
Ca^{2+}:Calmodulin dep protein kinase	4	18	20
CKI	4	8	10
CKII	5	12	13

* IC_{50} is the concentration of xanthate required to inhibit kinase activity by 50%

Figure 6.2.8 Lineweaver-Burk analysis of the effect of the xanthate D609 on the US3 protein kinase (fraction 37, Figure 6.1.1) activity relative to the ATP concentration (Methods 5.2, 19.2).

Key

□ 0mM D609
▽ 1mM D609
◇ 5mM D609
△ 10mM D609

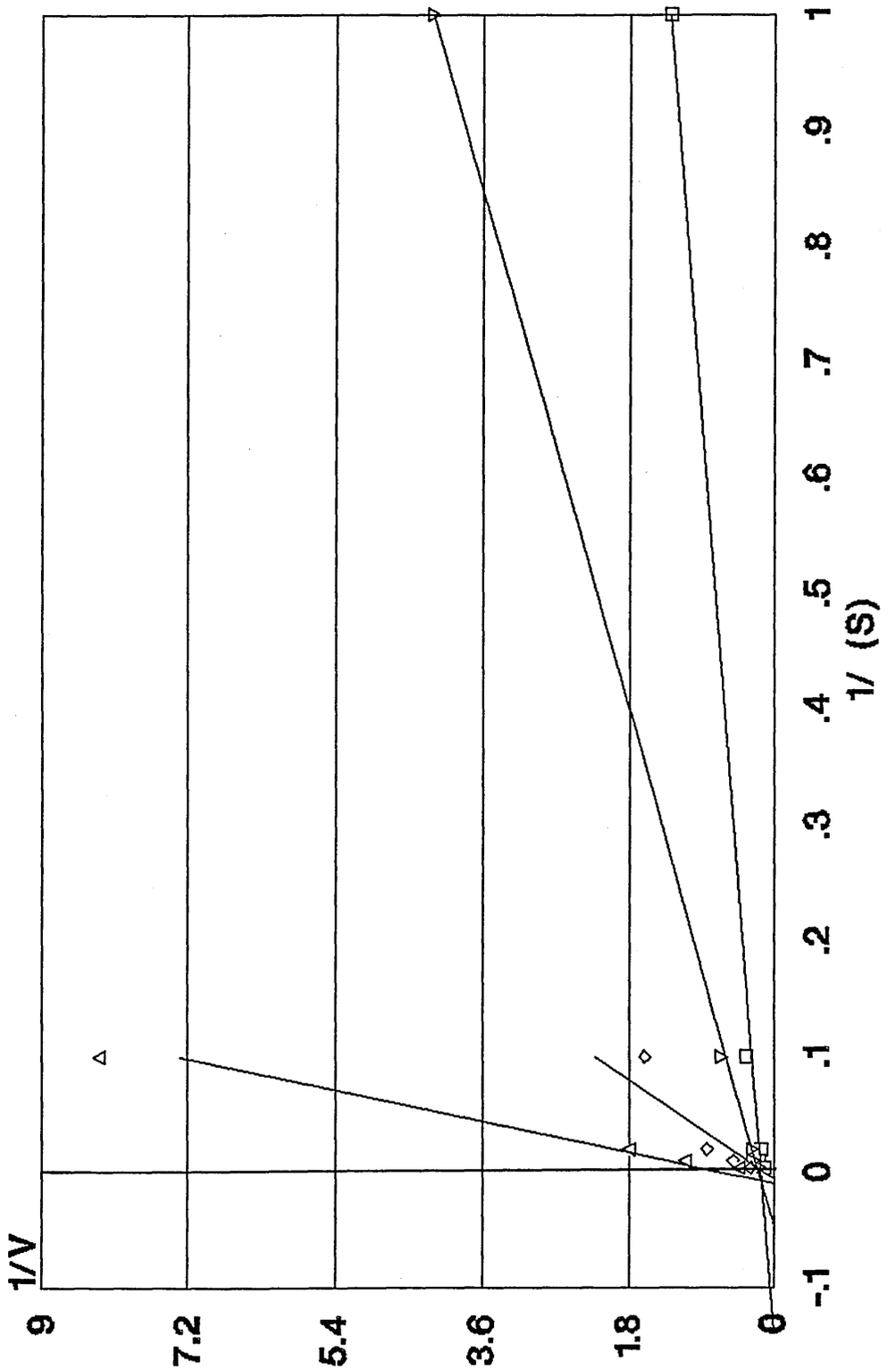

1/V
9
7.2
5.4
3.6
1.8
0
-.1
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
1/ (S)

Figure 6.2.9 Dixon analysis of the effect of the xanthate D609 on the US3 protein kinase (fraction 37, Figure 6.1.1) activity relative to the ATP concentration (Methods 5.2, 19.2).

Key

□ 1μM ATP

▽ 10μM ATP

× 50μM ATP

△ 100μM ATP

◇ 250μM ATP

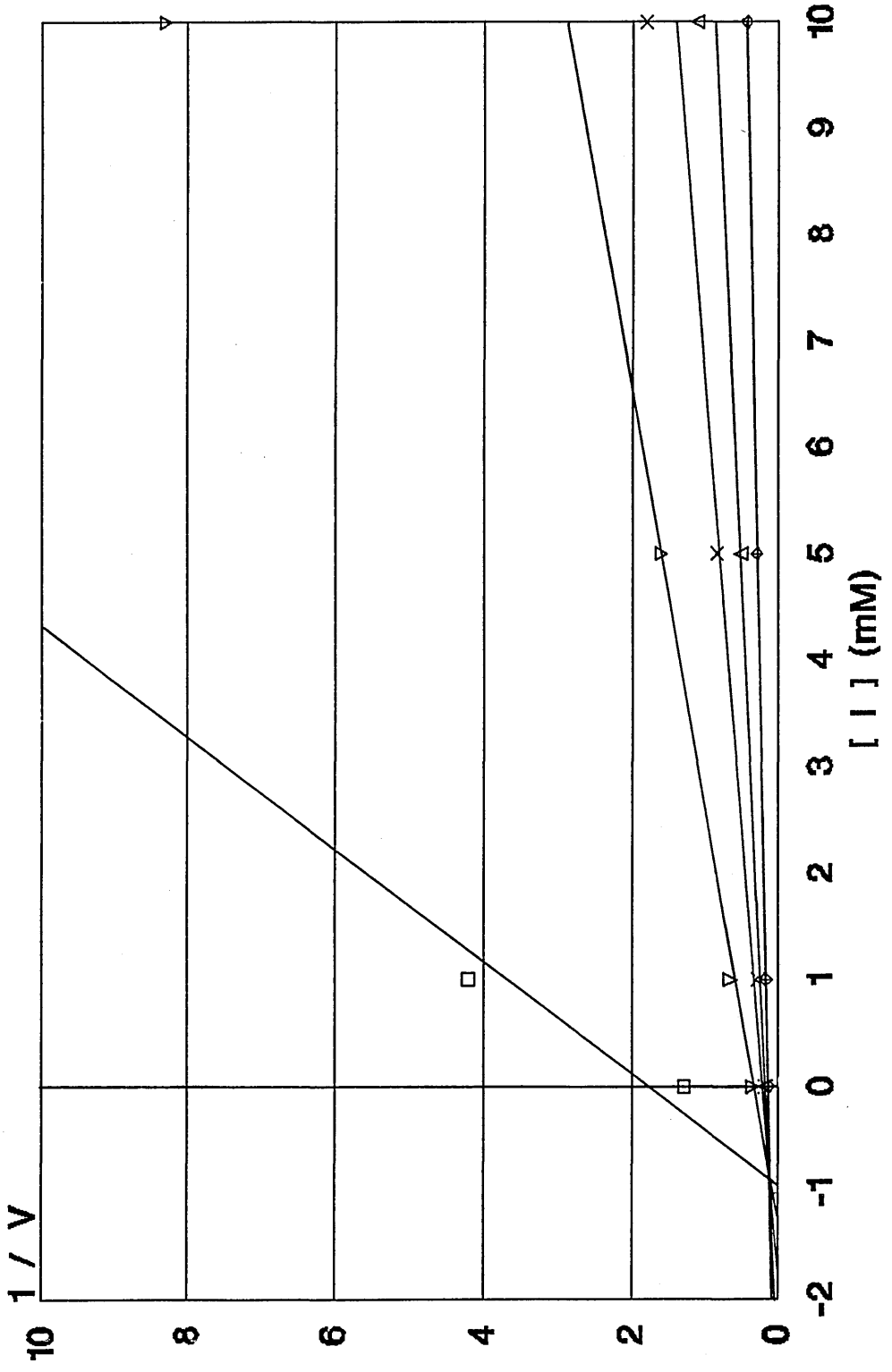

1 / V
10
8
6
4
2
0
-2
-1
0
1
2
3
4
5
6
7
8
9
10
[I] (mM)

Figure 6.2.10 Lineweaver-Burk analysis of the effect of the xanthate D611 exo on the US3 protein kinase activity (fraction 37, Figure 6.1.1) relative to the ATP concentration (Methods 5.2, 19.2).

Key

□ 0mM D611 exo
▽ 1mM D611 exo
◇ 5mM D611 exo
△ 10mM D611 exo

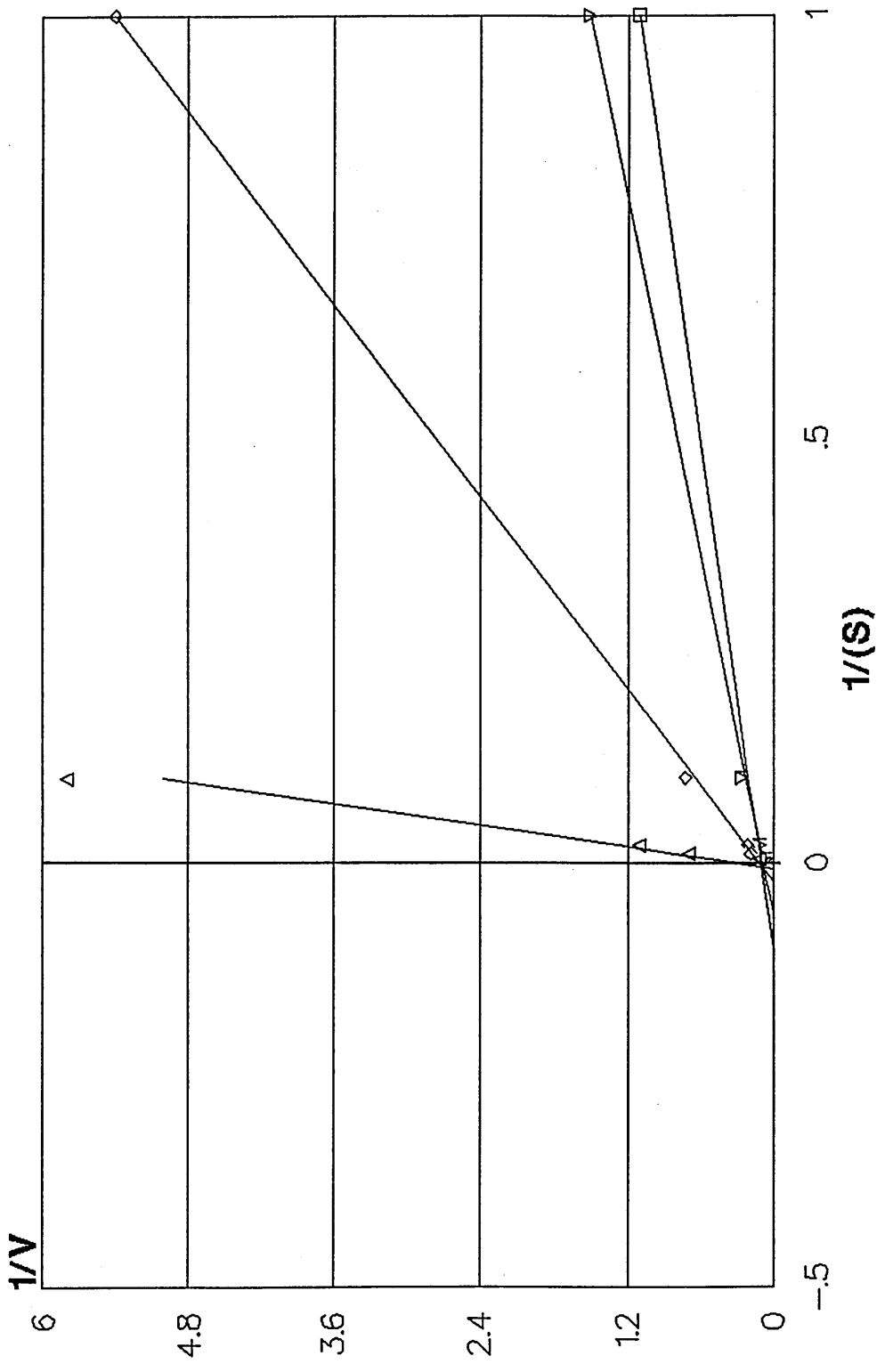

1/V
6
4.8
3.6
2.4
1.2
0
−.5
0
.5
1
1/(S)

Figure 6.2.11 Dixon analysis of the effect of the xanthate D611 exo on the US3 protein kinase (fraction 37, Figure 6.1.1) activity relative to the ATP concentration (Methods 5.2, 19.2).

Key

□ 1μM ATP

▽ 10μM ATP

✕ 50μM ATP

△ 100μM ATP

◇ 250μM ATP

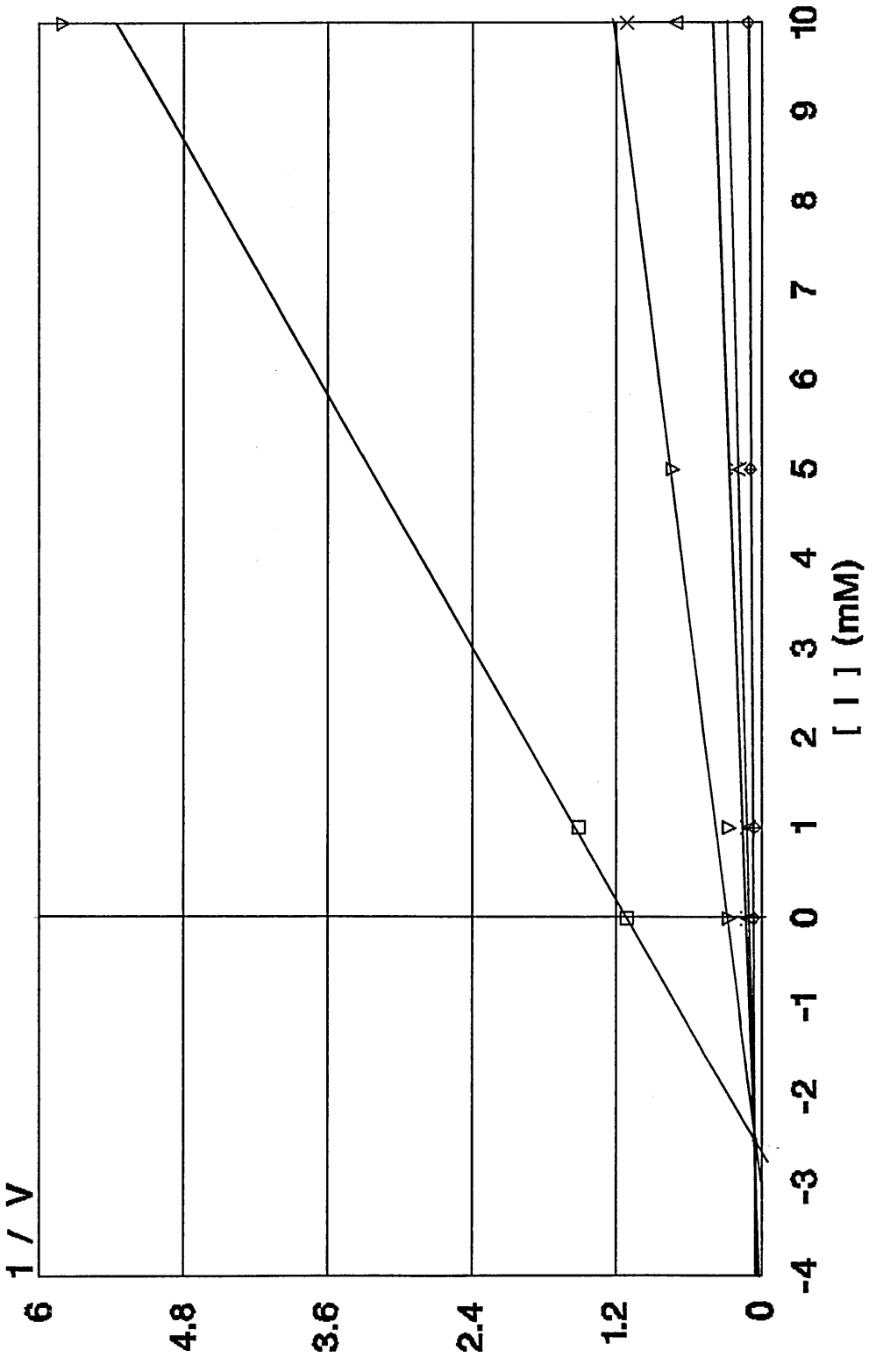

1 / V
6
4.8
3.6
2.4
1.2
0
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
9
10
[I] (mM)

Figure 6.2.12 Lineweaver-Burk analysis of the effect of the xanthate D611 endo on the US3 protein kinase (fraction 37, Figure 6.1.1) activity relative to the ATP concentration (Methods 5.2, 19.2).

Key

□ 0mM D611 endo
▽ 1mM D611 endo
◇ 5mM D611 endo
△ 10mM D611 endo

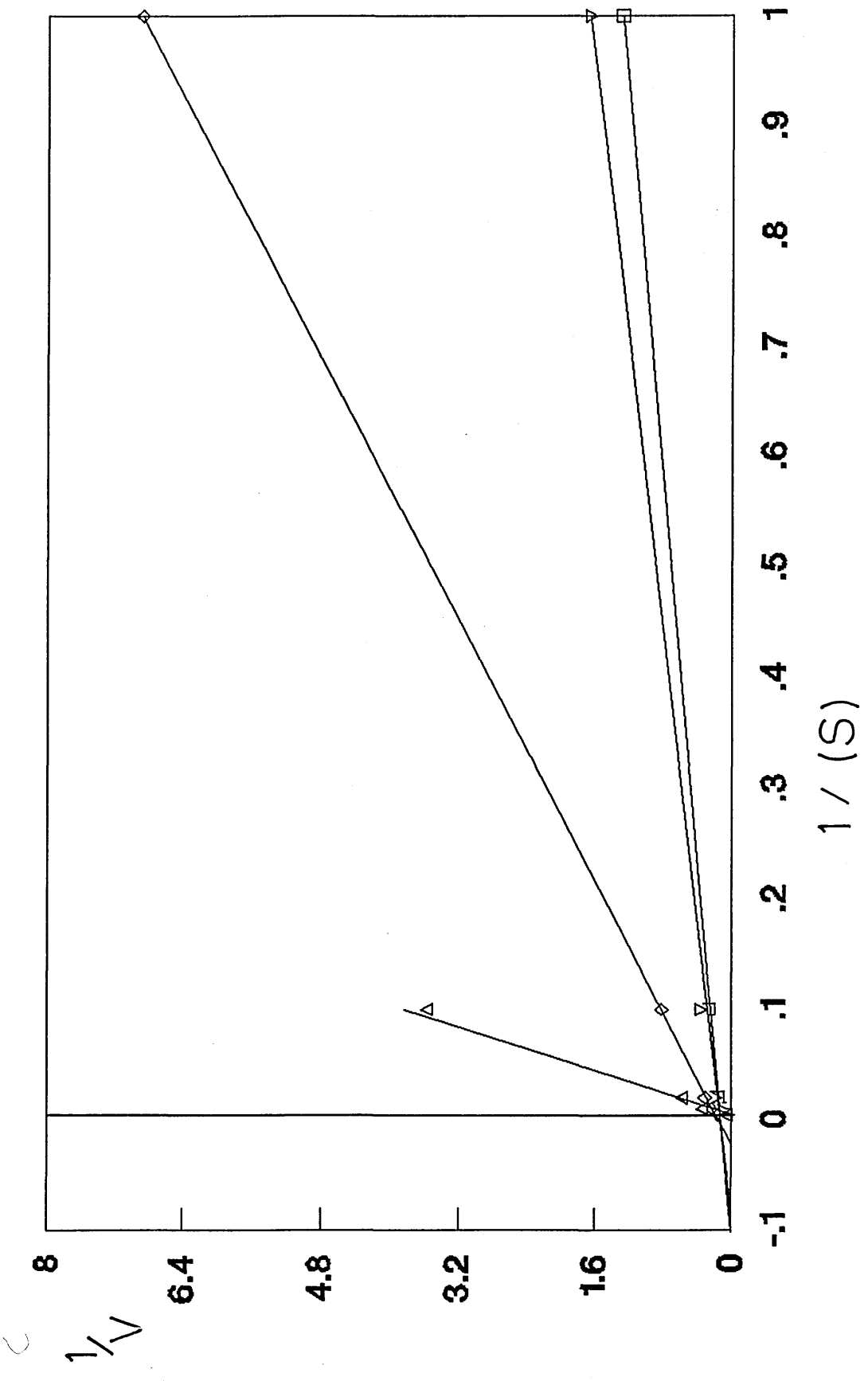

8
6.4
4.8
3.2
1.6
0
1/V
-.1
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
1 / (S)

Figure 6.2.13 Dixon analysis of the effect of the xanthate D611 endo on the US3 protein kinase (fraction 37, Figure 6.1.1) activity relative to the ATP concentration (Methods 5.2, 19.2).

Key

□ 1μM ATP

▽ 10μM ATP

× 50μM ATP

△ 100μM ATP

◇ 250μM ATP

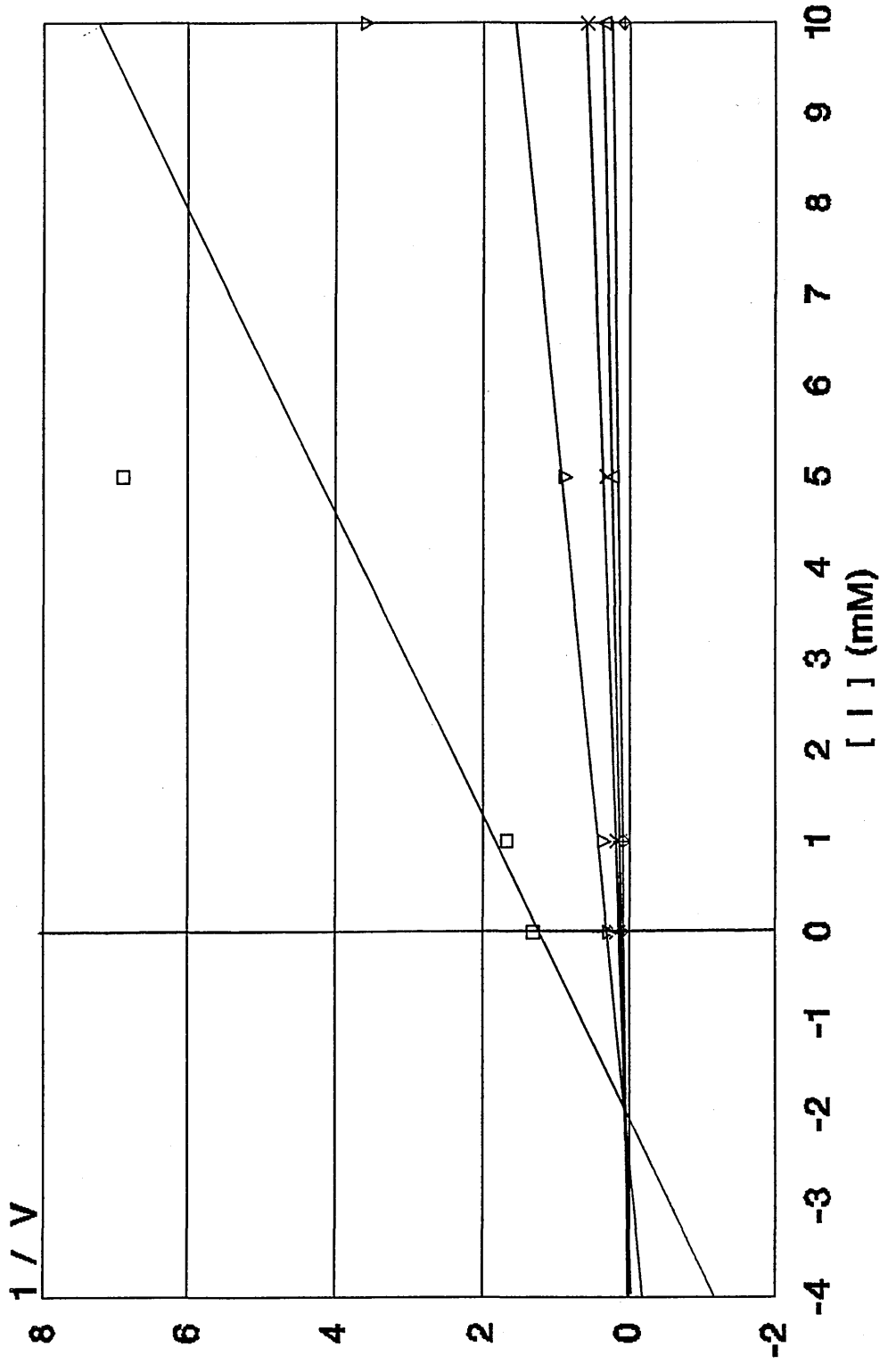

1 / V
8
6
4
2
0
-2
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
9
10
[I] (mM)

Figure 6.3.1 The effects of the xanthate D609 in cell culture.

A 15 hr. HSV-1(F) infection (Methods 2.2) was carried out in the present of 20μg/ml. D609 and 10μg/ml. undecanoic acid. The resultant 10K spin supernatant was acetone precipitated (Methods 8.1) and subjected to electrophoresis on a two dimensional polyacrylamide gel system (Methods 11).

The first dimension used a pH gradient of 3.5 - 9 and the second dimension an SDS polyacrylamide gel containing 10% acrylamide. The proteins were transferred to nitrocellulose by electrophoresis (Methods 12.1) and probed with an antibody against the DNase (Methods 12.3). A marker lane was run at the side of the second dimension and was visualised by Ponceau S staining (Methods 12.2).

Marker lane: Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg albumin 45kDa, Carbonic anhydrase 29kDa.

a = control
b = + xanthate

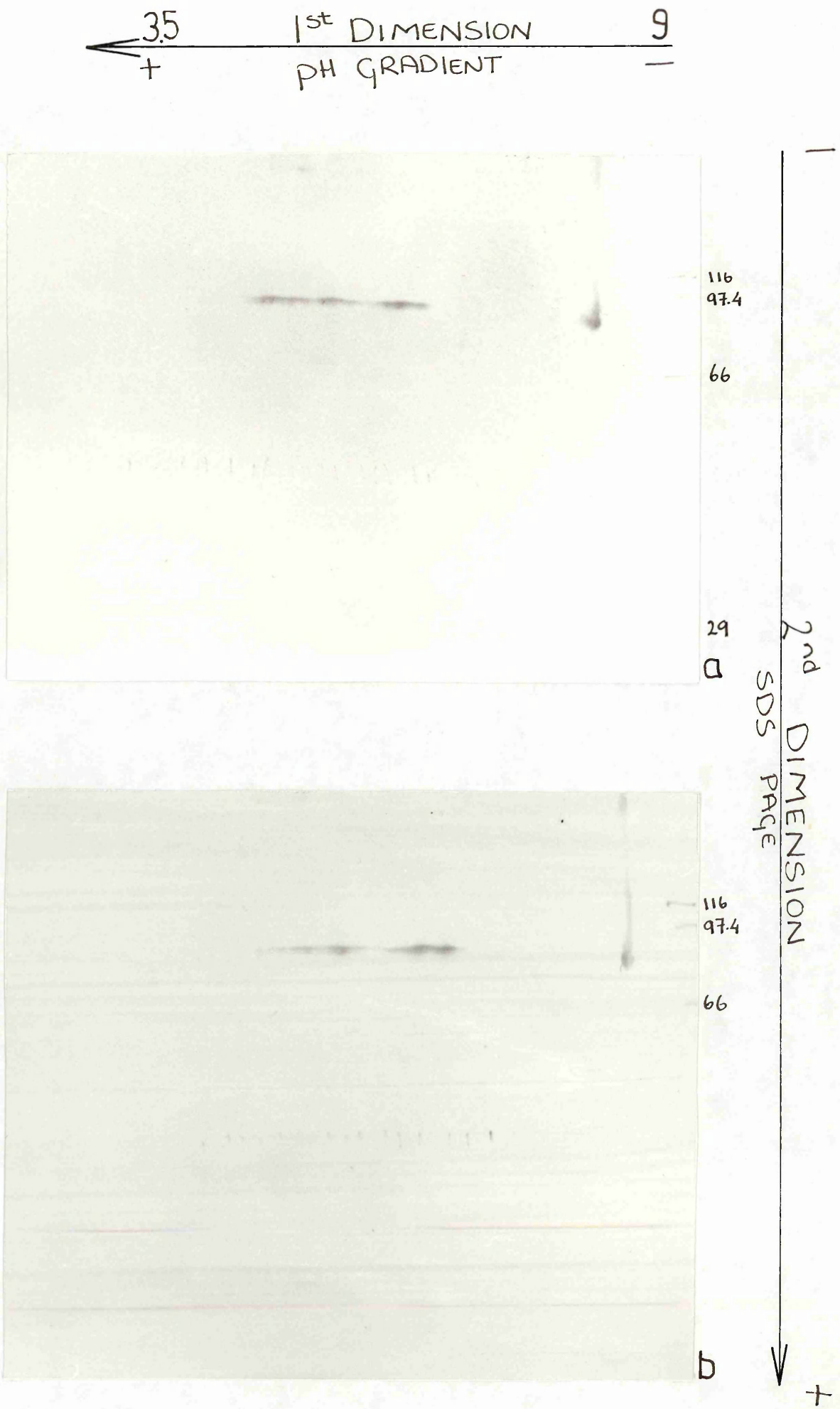
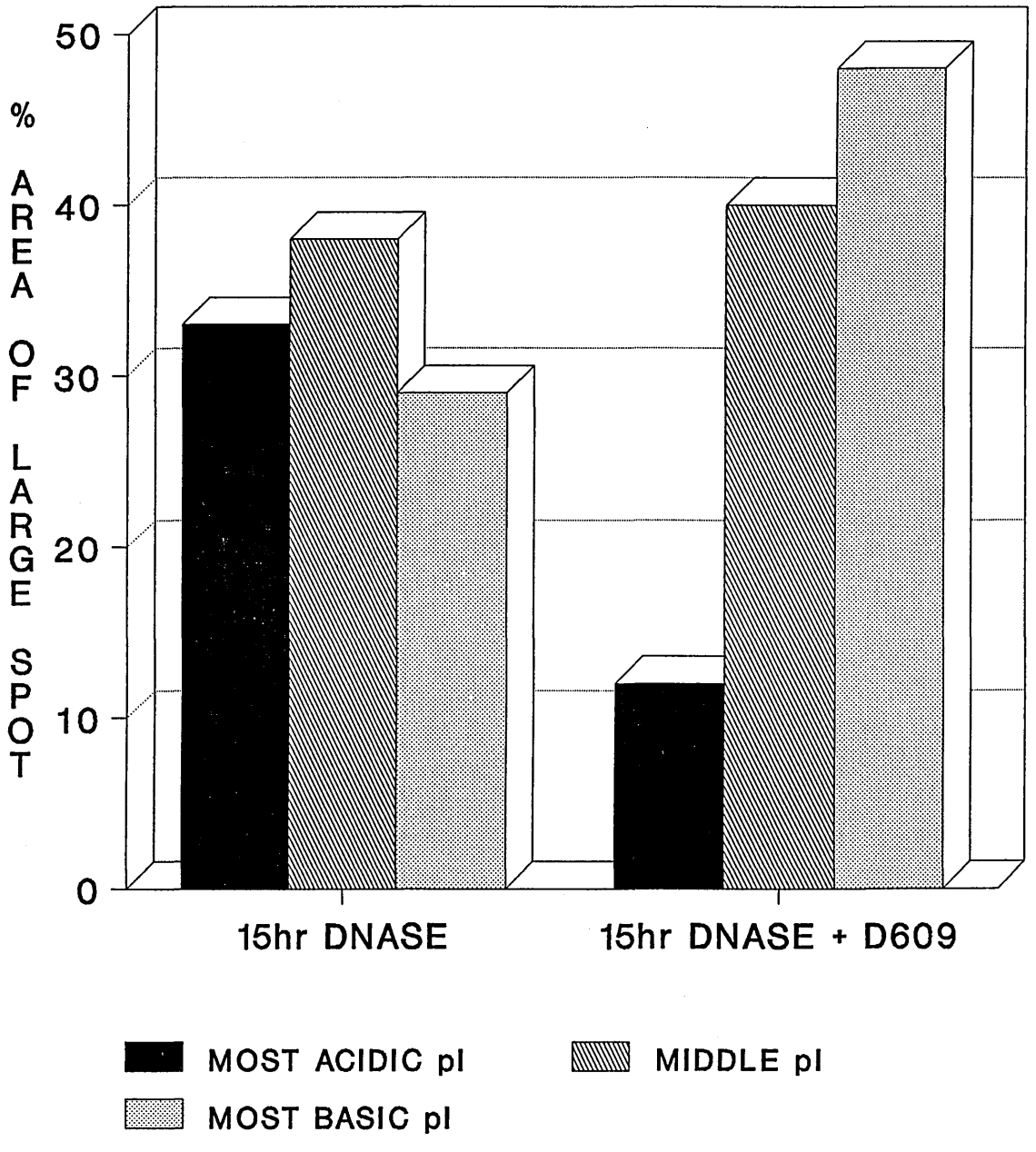

3.5
1st DIMENSION
9
+
PH GRADIENT
−
116
97.4
66
29
a
116
97.4
66
b
2nd DIMENSION
SDS PAGE
−
+

Table 6.3.1 Quantitative analysis of DNase purified from 15 hr. infected BHK cells in the presence or absence of xanthate D609 by laser densitometry

Profile	% area of each region in the large spot at more acidic pI values (3.5^{+} ← $^{-}$10)		
	1	2	3
15 hr. DNase	33%	38%	29%
15 hr. DNase + Xanthate	12%	40%	48%

Figure 6.3.2

Quantitative analysis of DNase purified from 15 hr. infected BHK cells in the presence or absence of xanthate D609 by laser densitometry


50
40
30
20
10
0
% AREA OF LARGE SPOT
15hr DNASE
15hr DNASE + D609
MOST ACIDIC pI
MIDDLE pI
MOST BASIC pI

7. Ribonucleotide reductase : 2 dimensional gel analysis of the large subunit

A two dimensional polyacrylamide gel analysis (Methods 11) of ribonucleotide reductase was carried out at various times after infection (Methods 2.2) using acetone precipitates (Methods 8.1) of the infected cell supernatants.

Immunoblotting of these gels (Method 12) resulted in the profiles shown in Figure 7.1.

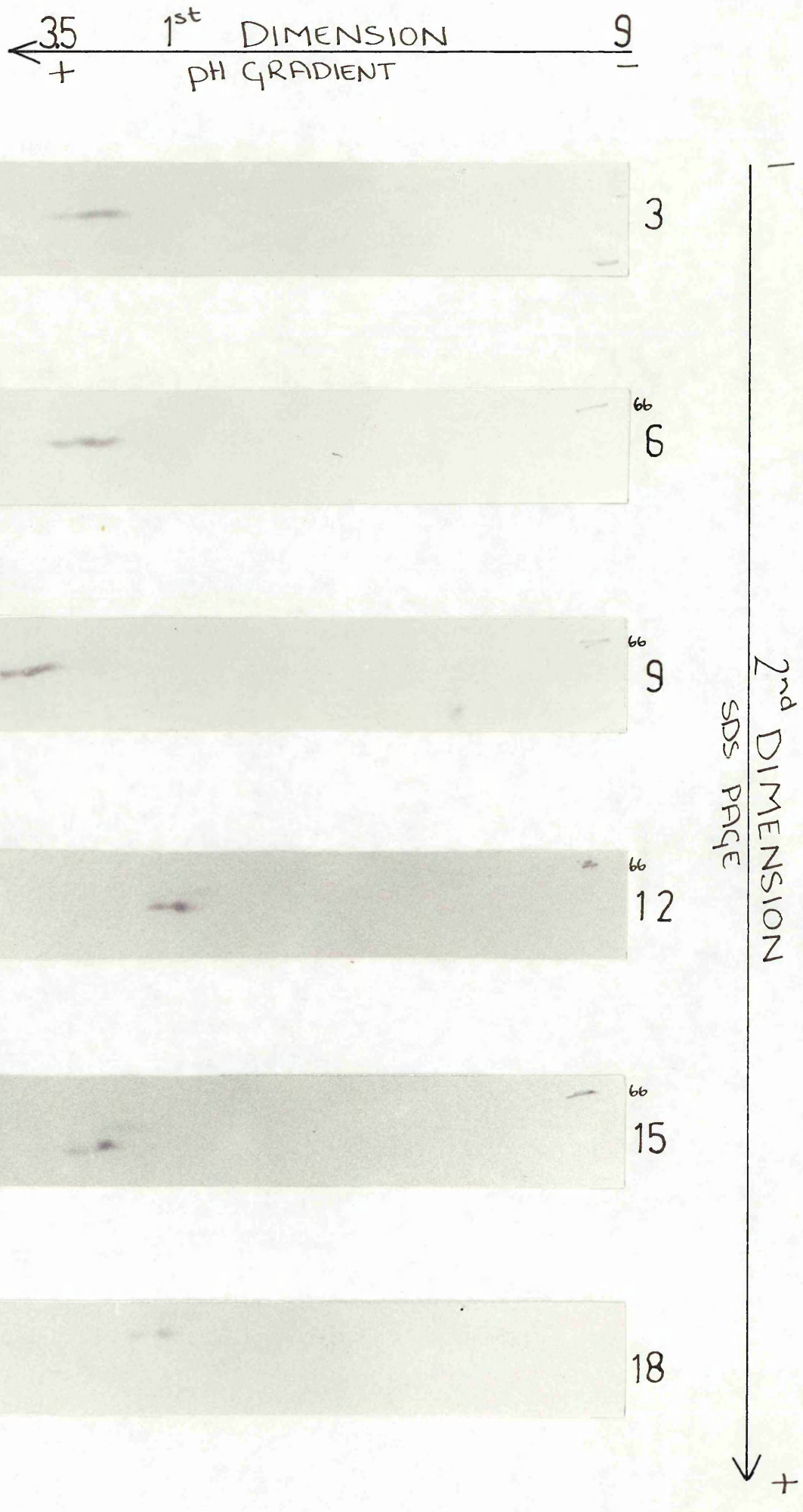
The profiles for the 3, 6 & 9 hr. large subunit contained an elongated spot which appeared to be made up of at least three identifiable sub-regions. The 12, 15 & 18 hr. large subunit profiles had a similar pattern present in each. The profile was made up of two spots, the spot at more basic pI values being the larger and darker of the two. The spot at more acidic pI values was more pronounced in the 12 hr. profile and in the 15 & 18 hr. profiles became much fainter. The molecular weight of the reductase large subunit on polyacrylamide gels is 140kDa, the molecular weight of the species observed in these experiments was 60kDa. Intact large subunit was seen on blots of one dimensional polyacrylamide gels (Figure 7.2) but the intact subunit could not be observed on blots of two dimensional gels under any of the conditions employed in this study.

The 60kDa species was found in all the time course blots and so allowed comparisons to be made between them. The nature of the modification of the intact subunit remains unclear, but it is known to be phosphorylated in cell culture (Preston V. et al 1984).

Quantitative analysis of the profiles by laser densitometry (LKB Ultroscan XL) confirmed the qualitative analysis given above (Table 7.1). The analysis showed a reduction in modification between 9 hr. & 12 hr..

Figure 7.1.1 Immunoblots of two dimension polyacrylamide gels of the large subunit of ribonucleotide reductase at 3, 6, 9, 12, 15 & 18 hrs. after infection. Acetone precipitates (Methods 8.1) of infected cell supernatants (Methods 2.2) were subjected to electrophoresis. In the first dimension a pH gradient of 3.5 - 9 was employed and in the second dimension the SDS polyacrylamide gel contained 10% acrylamide (Methods 11). The protein in the gels was transferred to nitrocellulose by electrophoresis (Methods 12.1) and probed with an antibody against the large subunit (Methods 12.4). A marker lane was run at the side of second dimension and visualised by Ponceau S Staining (Methods 12.2).

Marker lane: Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg albumin 45kDa, Carbonic anhydrase 29kDa.


3.5
1st DIMENSION
9
+
pH GRADIENT
−
3
66
6
66
9
66
12
66
15
18
−
2nd DIMENSION
SDS PAGE
+

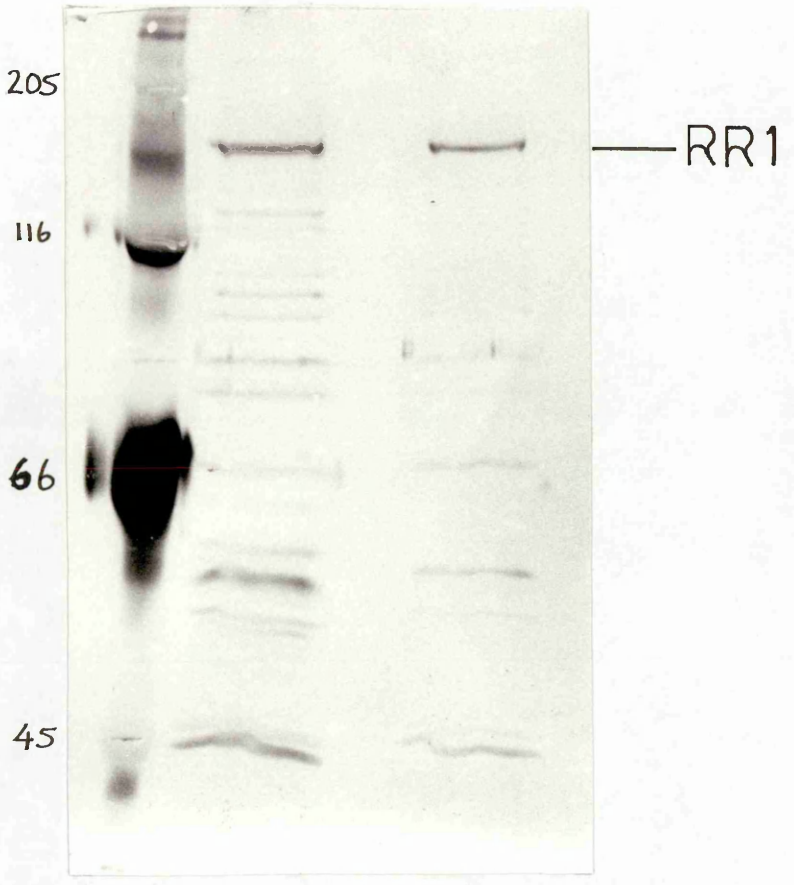


Figure 7.2 The Immunoblot of a one dimensional polyacrylamide gel of the large subunit of ribonucleotide reductase. The 10K spin supernatant from cells infected with HSV-1 for 6 hrs. (Methods 2.2) was subjected to electrophoresis on an SDS polyacrylamide gel containing 10% acrylamide (Methods 9). The gel was transferred to Nitrocellulose and probed with an antibody against the reductase large subunit (Methods 12). A marker lane was run and was visualized by Ponceau S staining (Methods 12.2).

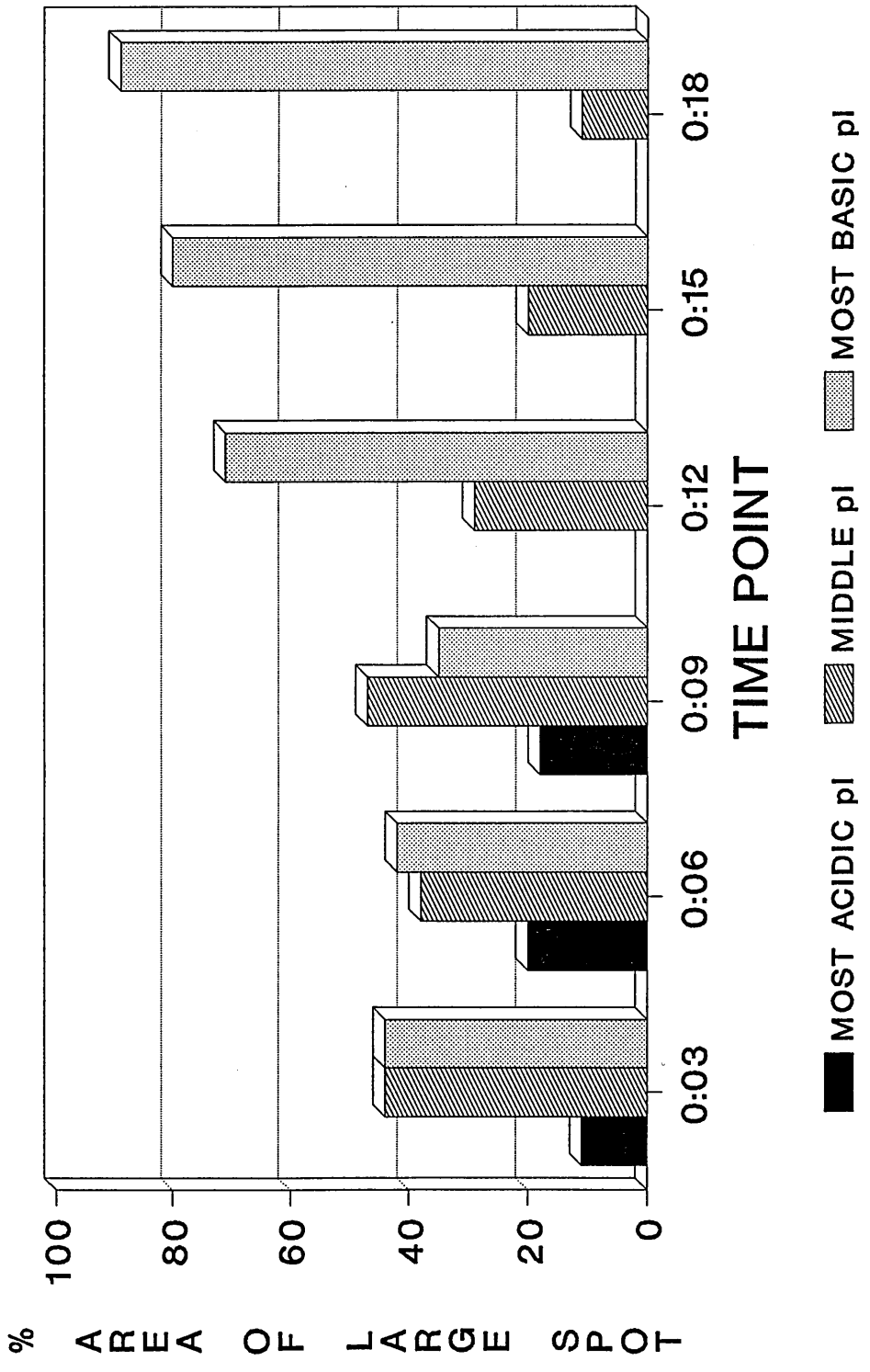
Marker lane Myosin 205kDa, βGalactosidase 116kDa, Phosphorylase b 97.4kDa, BSA 66kDa, Egg Albumin 45kDa, Carbonic anhydrase 29kDa.

Table 7.1 Quantitative analysis of ribonucleotide reductase large subunit time course profiles by laser densitometry

	% area of each region of the spot (3.5 $^{+}$ ← $^{-}$ 9.0)		
Profile	1	2	3
3 hr.	11%	44.4%	44.4%
6 hr.	20%	38%	42%
9 hr.	18%	47%	35%
12 hr.		29%	71%
15 hr.		20%	80%
18 hr.		11%	89%

Figure 7.3

Quantitative analysis of ribonucleotide reductase large subunit time course profiles by laser densitometry


% AREA OF LARGE SPOT
100
80
60
40
20
0
0:03
0:06
0:09
0:12
0:15
0:18
TIME POINT
MOST ACIDIC pI
MIDDLE pI
MOST BASIC pI

CHAPTER 5

DISCUSSION

1. The Viral Deoxyribonuclease

1.1 The two dimensional profile of the DNase

The viral DNase purified from HSV-1 infected BHK cells was shown, by two dimensional polyacrylamide gel analysis and immunoblotting to be highly modified (Results Section 2). The antibody used was (Q1) a gift from Dr. K. Powell and is a monoclonal which reacts against a site-common epitope on HSV-2 and HSV-1 DNase. The Q1 monoclonal has been shown to be specific for the DNase by Banks et al (1983 & 1985) and binding of the monoclonal inhibits the exonuclease activity of the enzyme.

Enzyme activity was present at 3 hr. post infection but no signal was obtained after two dimensional gel analysis and immunoblotting. Banks et al (1985) using immunofluoresence showed that the DNase in HSV-1 infected cells was just starting to be synthesised at 3 hr. p.i.. It is probable that there was not enough DNase protein after purification, to produce a signal with the antibody after immunoblotting. At 6 hr. p.i. and throughout the rest of the infection the majority of the DNase appeared highly modified. The highly modified DNase profiles contained discrete spots which varied in their respective proportions (Results Figure 2.1.1 and Table 2.1) during the infection as judged by laser densitometry. The variation in the relative proportions of the large highly modified region of the DNase suggested the return to less modified forms of the enzyme at late times in infection. This may reflect a less stringent requirement for the degree of modification of the enzyme by the virus at these times or the fact that at these late times in the infection the ATP reserves of the host cell may be depleted and so the modification cannot be maintained. The reason for the presence of the two regions of less modified DNase (Results Figure 2.1.1) at some times (9, 15 & 18 hr. p.i.) and not others is unclear.

As stated above towards late times in infection the enzyme may not be required by the virus and so the presence of these less modified DNase species occurs because the enzyme is less stringently controlled. At earlier times the less modified species of the enzyme may be required by the virus for a particular function. Modification then alters the enzyme structure and function and allows it to play another role in the viral infection.

1.2 Phosphorylation of the DNase

The modification of the enzyme has been shown to be due in part to phosphorylation (Results Section 3 & 4). Banks et al (1985) showed using the Q1 monoclonal that DNase purified from [^{32}P] labelled cells infected with HSV-2 was a phosphoprotein. In this study we have taken this further to investigate which kinases are responsible for the phosphorylation of the enzyme.

Labelling of HSV-1 infected BHK cells in culture with [^{32}P] inorganic phosphate has shown the DNase to incorporate the isotope by 6 hr. p.i. (Results Figure 3.2.1). In in vitro studies the purified DNase can be utilised as a substrate by the cellular kinases: protein kinase C, cAMP dependent protein kinase and casein kinase II and by the HSV-1 encoded US3 protein kinase (Results Section 3). Purified DNase was also a substrate for protein phosphatase 1 as judged by the altered 2 dimensional gel profile obtained after incubation of the DNase with this enzyme, indicating the removal of phosphate from the nuclease.

Tryptic peptide analysis of in vitro phosphorylated DNase resulted in the production of two unique profiles, for cAMP dependent protein kinase and protein kinase C, and two profiles which had identical peaks, the US3 protein kinase and casein kinase 2, (Results Figure 4.1).

This finding maybe the result of an incomplete digestion of the PVDF bound DNase by tpck-treated trypsin. This would release large peptides which may contain phosphorylation sites for both the US3 protein kinase and casein kinase 2, and result in the identical pattern observed. On the other hand the result may also be due

to similar or identical phosphorylation sites on the DNase for the two kinases, which on trypsin digestion would result in identical phosphopeptide profiles.

The cell culture [^{32}P] labelled DNase phosphopeptide profile containedno defined peaks, which is a direct result of not having enough [^{32}P] incorporated into the DNase. This is due to the fact that the labelling process is inefficient and only a small proportion of the label added to the medium ends up in the cells. Subsequently very little label is incorporated into the protein of interest. This could be overcome to some extent by increasing the amount of label used in the experiment, but this introduces problems in handling the labelled material, disposing of contaminated waste materials and the health and safety regulations.

Comparisons between <u>in vitro</u> and cell culture phosphopeptides failed to resolve which kinases are responsible for the phosphorylation of the DNase in cell culture, (Results Fig. 4.1, 4.2 & 4.3).

However, the xanthate studies (See Discussion Section 3) suggested that protein kinase C (or a kinase activated by protein kinase C) was responsible, in part, for the phosphorylation of the DNase in cell culture.

The effect of phosphorylation on the function of the DNase is not known, as the function of the enzyme in the viral infection still remains unclear. Preston & Cordingley (1982) expressed the DNase in <u>Xenopus</u> oocytes using viral DNA or mRNA and showed after expression the presence of a new exonuclease activity. They did not check to see if the newly synthesised protein contained phosphate. However if phosphorylation is required by the DNase for exonuclease activity kinases homologous to those operative in natural infection must be present in the <u>Xenopus</u> oocyte and cellular in nature.

Phosphorylation may not be required for the exonuclease function but for the endonuclease function of the enzyme though no cellular or viral deoxyribonuclease has been reported to be regulated by phosphorylation. This may be a result of the lack of research into the regulation and study of deoxyribonucleases in general. A possible role for phosphorylation in the regulation of the enzyme is in the mediation of its interactions with other viral or cellular proteins. The DNase is known to interact with the viral major DNA binding protein (Vaughan et al 1984) which stabilises the enzyme in infected cells. Phosphorylation of the DNase may be a requirement for this interaction, or others. It maybe these proteins which in turn modify the activity of the DNase.

Examples where phosphorylation affects the interaction of two proteins include the p85 subunit of the phosphatidyl inositol 3 kinase (PI 3 kinase), which phosphorylates the inositol ring of phosphatidyl inositol at position 3. p85 mediates the binding of the PI3 kinase to the ligand activated tyrosine autophosphorylated PDGF receptor kinase domain, binding does not occur when the receptor is not activated and the kinase domain is not auto-tyrosine-phosphorylated (Escobedo et al 1991, Ostu et al 1991).

Another example is in the product of the retinoblastoma gene pBR, which plays a crucial role in the regulation of normal cell growth. Loss or alteration of this gene can lead to cell transformation. pBR is phosphorylated in a cell-cycle dependent manner by the cdc2 kinase (Cooper & Whyte 1989, Taya et al 1989), with phosphate first detected on entry to the Sphase and lost on emergence from the Mphase. Hyperphosphorylation of the pBR protein is thought to be a regulatory event which leads to the inactivation of its growth repressing functions. The hyperphosphorylated pBR is easily extracted from nuclei but the un- or underphosphorylated pBR species are tightly associated with the nuclear structure (Mittnacht & Weinberg 1991). The underphosphorylated species of pBR

has been found associated with the E2F transcription factor and an inhibitor of E2F, E2F-I (Chellappan et al 1991, Bagchi et al 1991).

The transcription factor AP-1, inducible by phorbol-esters, consists a heterodimer of the products of the cellular oncogenes fos and jun. AP1 is modulated by an inhibitory protein IP1, which specifically blocks the DNA binding of AP1. Phosphorylation of IP-1 prevents or disrupts its association with AP-1 thus allowing it to bind to TPA responsive elements in the DNA (Auwerx & Sassone-Corsi 1991).

Phosphorylation may be sequential with phosphorylation at one site allowing the phosphorylation of another site and the kinase involved using the initial phosphate group as a site determinant such as glycogen synthase kinase 3 (Kemp & Pearson 1990) which requires a phospho serine group four amino acids C terminal of its target serine.

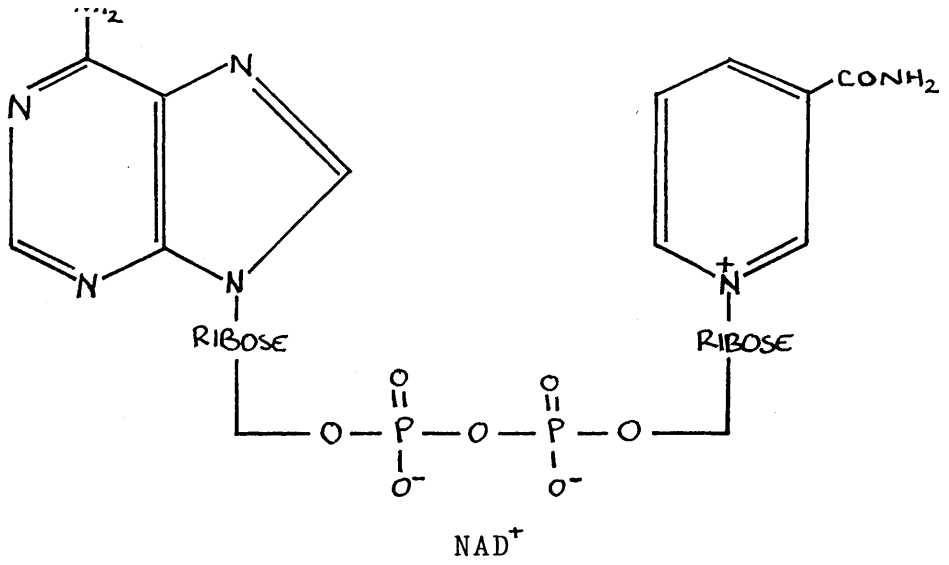
Other HSV-1 viral proteins that are phosphorylated and have known functions include the immediate-early protein $\alpha 4$ (Ackermann et al 1989) and the ribonucleotide reductase (Preston et al 1984). It is still unclear what role phosphorylation plays in the function or control of these proteins. In the field of virology, in general, phosphorylation of viral proteins and the role of this modification in their function or control is not well understood, (for a review see Leader & Katan 1988). One of the best studied examples is the NS protein of the enveloped RNA virus, vesicular stomatitis virus (VSV). The NS protein is required by the virus for secondary transcription. The protein is found in two forms, one of which is highly phosphorylated and one of which has a low state of phosphorylation. In in vitro transcription assays only the highly phosphorylated NS protein enhanced viral transcription (Kingsford & Emerson 1980) and this activity could be abolished by dephosphorylation (Hsu et al 1982). The phosphorylation of the NS protein maybe due in part to the cellular protein kinase C (Müller Decker et al 1987).

The studies carried out in this Thesis used BHK cells in culture as the host for HSV-1 infection but it must be remembered that these cells are fibroblastic in origin and have been adapted to grow in cell culture. It is probable that protein kinases are present in BHK cells which are not present in the cells infected in the animal host. The cells infected in the animal host are differentiated and have stopped dividing. The protein kinases present in dividing BHK cells may phosphorylate the DNase in a fortuitous manner which has no effect on its function. The virally coded US3 protein kinase, which may phosphorylate the DNase in cell culture, is not required for virus growth in cell culture (Purves et al 1987a) and so presumably has a cellular equivalent which can substitute for its role in viral infection. This cellular homologue may affect the phosphorylation of the DNase. The US3 protein kinase is similar to the ribonucleotide reductase and thymidine kinase which are also not required for growth in cell culture (See Introduction Section 2.2) but may well be required in the animal host for infection in cells which are terminally differentiated and so do not contain the enzymes required for DNA metabolism.

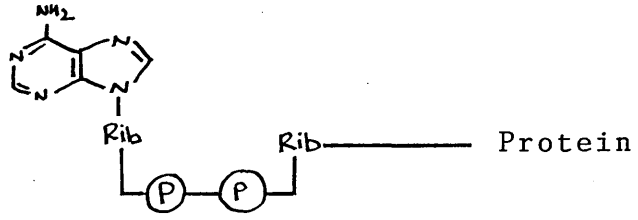
1.3 ADP ribosylation of the DNase

The other type of modification which may be present on the DNase is mono- or poly ADP ribosylation. Treatment of the purified DNase with snake venom phosphodiesterase, an exonuclease which cleaves -XpYpZ to -XpY+pZ, resulted in the production of two regions, on the 2D gel profile which we were unable to relate to those seen in the time course immunoblots. The fact that the 2D gel profile was altered suggested that the DNase had contained ADP ribose or poly (ADP ribose).

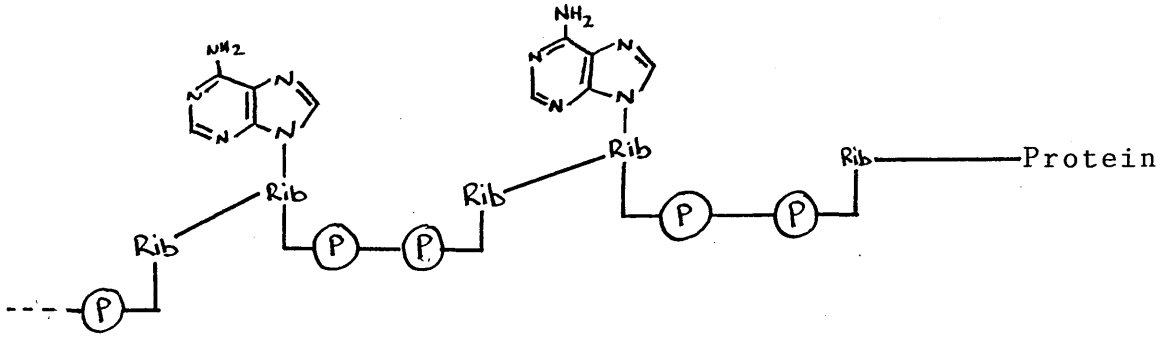
ADP ribosylation (for review see Ueda & Hayaishi 1985) is a type of covalent modification which involves the transfer of ADP ribose from NAD^+ to an amino acid on a receptor protein (Fig. 1.1). There are two types of this modification, mono ADP ribosylation (e.g. the bacterial toxins cholera and diptheria) and poly ADP ribosylation (e.g. eukaroytic chromatin bound poly (ADP ribose) polymerase). Poly ADP ribosylation has been implicated in NAD^+ metabolism,cell differentiation and RNA metabolism. It has also been shown to be involved in the repair of DNA strand breaks, and in cell death when DNA damage has become extensive (Gaal & Pearson 1985, Berger 1985, Gaal et al 1987, Denhardt & Kowalski 1988). In the context of viral infections the best known example of ADP ribosylation is during the infection of E.coli by bacteriophage T4. When phage T4 infects E.coli it utilises the host RNA polymerase to transcribe its own genes. In order to enable the E.coli RNA polymerase to carry this out the enzyme is specifically modified by ADP ribosylation (Cohen 1983). In the case of HSV-1, the immediate-early protein $\alpha 4$ has been ADP ribosylated *in vitro* (Preston & Natarianni 1983) but the protein has not been shown to be modified *in vivo*



NAD^+



mono ADP ribosylation



poly (ADP ribosyl)ation

	Mono (ADP)	Poly (ADP)
Amino Acid acceptors	Arginine	Glutamic acid
	Asparagine	COOH terminus
	Lysine	(lysine)

Figure 1.1 ADP ribosylation

The function of the modification of the DNase, if confirmed, is unclear but it may be involved in the interaction of the protein with viral DNA or with viral (or cellular) proteins. It must be noted that ADP ribosylation activity is higher in dividing cells and the modification maybe the consequence of the cell culture system used rather than a necessary modification.

1.4 Comments

The role of covalent modification in the function and/or control of the HSV-1 DNase is unclear, though this may also be due to the fact that the function of the enzyme in viral infection is still unknown, and so we lack a specific assay for its function. The use of an expression system which could produce completely unmodified DNase would be a useful tool in the further study of this enzyme. This would allow the effect of the covalent modifications to be judged separately or in conjunction, on firstly its known *in vitro* functions and then in time on its *in vivo* functions.

2. The endonuclease activity of the HSV-1 deoxyribonuclease

The virally coded deoxyribonuclease maybe essential for virus growth and production in cell culture systems (Moss 1986) but not viral DNA synthesis (Weller et al 1990). The enzyme is highly modified and its function in the viral infection which makes it essential is unclear. The exonucleolytic function of the enzyme seems an unlikely candidate for this essential function. Its endonucleolytic function, seems a more likely candidate, although it is not sequence specific. It may be involved in the cleavage of concatenated viral DNA which also does not appear to be sequence specific but occurs at a certain distance from each of the recognition sites (Varmuza & Smiley 1985) (cf EcoK, EcoB type 1 restriction enzymes from E.coli which cleave dsDNA at a site 1-5kb from their recognition sequences).

Chou & Roizman (1989) showed that the DNase could bind to sequences in the 'a' region containing Pac1 and Pac2, the cleavage and packaging signals (See Introduction 1.2 and 3.2). The DNase also bound to other sequences but the binding appeared to be less strong. The cleavage of concatenated viral DNA occurs in the DR1 sequence between two adjacent 'a' sequences (Varmuza & Smiley 1985). The plasmid used in our studies (Methods Fig. 18.1) contained two adjacent 'a' sequences, derived from the junction between U_L and U_S, in the vector pUc9.

Under the conditions used the purified DNase itself had no endonuclease activity against the plasmid as judged by agarose gel electrophoresis. This result disagrees with all the published data (Hoffman & Cheng 1979, Hoffman 1981, Strobel-Fidler & Francke 1980 & Banks et al 1983). These groups obtained endonuclease activity from their purified preparations. The purification procedures in all cases differed as did the purity of each group's final preparations, Hoffman & Cheng (1978) did not show a gel of their purified enzyme, Strobel-Fidler & Francke's (1980) preparation contained at least three other proteins, Banks et al's (1983) final preparation contained one protein but used a different assay to determine endonuclease activity.

The enzyme preparations used in this study were active exonucleolytically and the conditions used for the endonuclease assay were those of Hoffman (1981). The reason for the lack of endonuclease activity remains unclear.

The addition of infected supernatant to DNase in the assay produced a digestion pattern on agarose gels which was distinct from that of the infected supernatant alone. A similar result was obtained using uninfected supernatant, which suggested the DNase had in some way protected the plasmid by either binding to it itself or binding the protein(s) in the supernatants responsible for the digestion. Digestions carried out with the DNase in the presence of infected or uninfected supernatants showed no evidence of specific cleavage of the plasmid. These experiments were difficult to resolve because of the presence of other exonucleolytic activities at 5mM Mn^{2+}.

The fractions obtained during the purification of the DNase all had endonucleolytic activity up to the DNA cellulose purified DNase which did not. This suggested that either the endonuclease activity seen in the above assays was a distinct protein which did not purify with the DNase on DNA cellulose chromatography or that the DNase required a protein cofactor(s) for the cleavage of the plasmid (no evidence for specific cleavage) and this protein was lost on the DNA cellulose chromatography step.

Treatment of the DNase by either protein phosphatase 1 or protein phosphatase 2A had no effect on its endonuclease activity. It must be noted that treatment of the DNase with protein phosphatase 1 (Results Figure 2.5.1) did not result in a complete reduction in the modification of the enzyme. It is impossible to say if the remaining modification was due solely to the possible poly ADP ribose or to it and residual phosphate moieties. As stated in Section 1 the phosphorylation of the DNase may not affect its activity directly but rather its interactions with other proteins which indirectly modulate its activity. The surprising result was the digestion of the plasmid by the protein phosphatases themselves. Since the

digestion of the plasmid was inhibited by 20mM pyrophosphate, a protein phosphatase inhibitor, we can attribute this property to the phosphatases rather than to an unknown protein in their preparations.

The role of this endo/exo nuclease activity, if confirmed, in the protein phosphatases in vivo functions is unclear.

The assay used in these experiments permitted the study of endonuclease activity of simple systems such as the purified DNase itself but problems occurred when trying to study its possible in vivo role of cleaving concatenated viral DNA. Encapsidation of and cleavage to unit lengths of viral DNA are linked together and require specific proteins some of which may not have been identified. This leads to problems in the assay system: 1) which proteins are required for cleavage and can these be purified and added back to the assay, 2) can an extract from virus infected cells substitute, which may contain all the proteins required, but also some which may interfere with the assay.

3. Xanthate studies

The studies of Müller-Decker et al (1987) on the effect of the antiviral xanthate D609 in cell culture indicated a role as possible protein kinase inhibitors (Introduction Section 6). This possibility was investigated using partially purified protein kinases from HSV-1 infected BHK cells. The results from the inhibitor studies (Results Section 5) showed that the xanthates, D609, D611 exo and D611 endo (Introduction Figure 6.2), could inhibit all the protein kinases used in the study in a non-specific manner. The xanthate D609 had the greatest inhibitory activity with an average IC_{50} value of 4.5 ($\pm$0.5)mM, the two isomers D611 exo & D611 endo had values more than double this at 11.7($\pm$4.3)mM and 13.3($\pm$4.4)mM respectively. The xanthates acted as competitive inhibitors with respect to ATP. However, the concentrations at which they inhibited the protein kinases did not correlate with the concentrations

at which they act as antiviral agents in cell culture which were in the μM range. The difference makes it highly unlikely that they act as protein kinases inhibitors in cell culture at μM concentrations.

Müller-Decker et al (1988) indicated a possible role for the xanthate D609 as an inhibitor of phospholipase C and so as an indirect inhibitor of protein kinase C. Protein kinase C is activated by diacylglycerol (& Ca^{2+} ions) which is produced by the action of phospholiphase C on phosphatidyl inositol in the cell membrane. It has been shown recently that the activiation of phospholipase C γ (one of the four isozymes of the enzyme) is achieved by the phosphorylation of specific tyrosine residues by the platlet derived growth factor (PDGF) receptor kinase (cf EGF receptor kinase Introduction Section 5.1.1) (Kim et al 1991). It has not yet been determined whether the xanthate inhibits the activation of the phospholipase C or inhibits the enzyme directly.

The cell culture experiment using the xanthate D609 showed that at 15 hr. p.i. the two dimensional gel profile of the DNase after immunoblotting was altered. The change in profile was confirmed by laser densitometry (Results Figure 6.3.1 & Table 6.3.1). This provides evidence that protein kinase C or a protein kinase activated by protein kinase C is responsible for some of the phosphorylation of the DNase. If however, phosphorylation is sequential and certain protein kinases require a protein kinase C phosphorylated residue on the enzyme as a site determinant, as discussed in Section 1.2, then this would also lead to the reduced modification seen in Figure 6.3.1.

The antiviral activity of the xanthates against a wide range of viruses (Sauer et al 1984) suggests a role for protein kinase C in the infectious cycle of these viruses, e.g. phosphorylation of the VSV NS protein (Müller-Decker et al 1987). Other groups have also reported evidence which implies a role for protein kinase C in viral infections, these include HIV (Kinter et al 1990), Influenza Type A virus (Kurokawa et al 1990) and Polyomavirus (Asano et al 1990).

The inhibition of the activation of protein kinase C by xanthates provides an antiviral agent with a widespread range (Sauer et al 1984), as long as protein kinase C is essential for the virus life cycle then the xanthates will have an antiviral effect. Problems, however, arise in the nature of the interaction between the xanthate and its inhibition of phospholipase C. If the interaction is specific to virally infected cells then the xanthates constitute a new group of antiviral agents with great potential. If the inhibition, however, is non-specific and occurs in other cells, as has been shown (Müller-Decker et al 1988), then problems will arise in cells which require to activate protein kinase C via. phospholipase C in response to certain growth factors such as PDGF. So far no studies on the toxic effects on whole animals, if any, have been reported.

4. Ribonucleotide reductase

The virally coded ribonucleotide reductase, which produces deoxyribonucleotides from ribonucleotides, is not required for virus growth in cell culture (See Introduction 2.2) but is probably required for the virus' infectious cycle in its host animal cells. The ribonucleotide reductase function is located in the C terminal domain of the large subunit and the whole of the small subunit. The N terminal domain of the large subunit contains a protein kinase activity which autophosphorylates and phosphorylates exogeneous substrates. The N terminal domain of the HSV-1 reductase large subunit is highly susceptible to proteolysis in vivo and many related species of lesser molecular weight than the large subunit are found. Some of these species are enzymatically active in the ribonucleotide reductase function. The HSV-2 large subunit protein kinase domain has been implicated in the ability of the virus to transform certain cells. It is possible that degradation of the HSV-1 large subunit protein kinase domain prevents transformation in cells which contain the necessary proteases. The function of the N terminal protein kinase domains and their substrates, (although the small subunit of the enzyme has been suggested as a possibility) remains unclear, but

the enzyme is known to be phosphorylated in cell culture.

The results of the two dimensional gel electrophoresis and immunoblotting with an antibody raised against the C terminus of the large subunit of HSV-1 ribonucleotide reductase (Results Section 7) are difficult to analyse. They contain only a 60kDa C terminal fragment of the 140kDa protein. They clearly showed that the C terminal fragment was modified at early times, in the infection, and that this modification was maintained up to 9 hr. p.i.. The immunoblots showed the presence of three species of the C terminal fragment. Between 9 and 12 hr. p.i. the modification was reduced to two species, the species at more acidic pI values decreased from 12 to 18 hr. p.i. where it was barely visible. These results provided an inconclusive view of the large subunits modification because they lack the N terminal domain and its modification, if any. We can speculate that the decrease in modification, probably phosphorylation though this requires confirmation, between 9 and 12 hr. p.i. may indicate that the reductase function is no longer required by the virus and so its modification does not need to be maintained.

This assumes that the modification is required for reductase activity which has not yet been demonstrated. Another possibility is that the decrease in modification between 9 and 12 hr. p.i. may indicate a switch from the reductase function to the protein kinase function of the N terminal domain. The third possibility is that modification somehow affects the interaction of the two subunits and a decrease in modification may cause dissociation of the enzyme.

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