A PROTEIN KINASE INDUCED BY PSEUDORABIES VIRUS IN INFECTED CELLS

Matilda Katan

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Department of Biochemistry,

University of Glasgow.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Abbreviations

The abbreviations recommended by the Biochemical Journal in its Instructions to Authors [Biochem. J. (1981) 193, 4-27] have been used throughout the thesis with the following additions:

BHK cells	Baby Hamster Kidney Fibroblasts
PRV	Pseudorabies virus
Bis-tris	bis (2-hydroxyethyl)imino-tris-(hydroxymethyl) methane
Mops	morpholino propane sulphonic acid
Hepes	[4-(2-hydroxyethyl)-1-piperazine -ethane-sulphonic acid]
SDS	Sodium dodecyl sulphate
TEMED	NNN'N'- tetramethylethylene-diamine
HPLC	High Performance Liquid Chromatography
FPLC	Fast Protein Liquid Chromatography
pfu	plaque forming unit
1 A_{260} unit	the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm, when measured in a cell with a 1 cm light path
1 unit of protein kinase activity	amount of enzyme that catalyses the incorporation of 1nmol phosphate per min into protein substrate under standard assay conditions
ViPK	virus-induced protein kinase

Summary

This thesis describes studies of the phosphorylation of proteins during the infection of baby hamster kidney fibroblasts (BHK cells) with the swine herpes virus, pseudorabies virus, with particular emphasis on the protein kinase activites in infected cells.

Protein kinases present in the cytosol of BHK cells infected with pseudorabies virus were compared with those present in the cytosol of uninfected cells. The cytosol was first subjected to chromatography on DEAE-cellulose and fractions analysed for protein kinase activities. In the fractions from uninfected cells the activities were found to correspond to the following previously characterized protein kinases: i) cyclic AMP-dependent protein kinase type I; ii) cyclic AMP-dependent protein kinase type II; iii) protein kinase C; iv) casein kinase I; v) casein kinase II; vi) a less clearly defined protamine kinase which is probably the proteolytic fragment of protein kinase C. In BHK cells infected with pseudorabies virus the protein kinases listed above were present in amounts comparable to those in uninfected cells. However, a new protein kinase activity was detected in infected cells, appearing about 4 h after infection and increasing during the following 6 h, at least. This protein kinase designated virus-induced protein kinase (ViPK), was the subject of subsequent studies.

The new protein kinase was further purified either by Blue A dye-ligand chromatography or by high-performance size-exclusion and ion- exchange chromatography. The latter procedure resulted in 100-fold purification of the enzyme. The partially-purified preparations were used to characterize the new protein kinase.

It had an apparent molecular weight of 68,000 on the basis of size-exclusion chromatography, and had a sedimentation coefficient of 4.3S. It catalysed the phosphorylation of serine residues of basic proteins in vitro, with protamine a better substrate than mixed histones; and used ATP (apparent Km=60 μM), but not GTP, as phosphate donor. Molecules that can serve as effectors for other protein kinases (cyclic AMP, cyclic GMP, Ca^{2+} + calmodulin, Ca^{2+} + phospholipid, double-stranded RNA, and heparin) did not significantly alter the activity of this enzyme. A striking characteristic of the protein kinase was a high KCl concentration optimum with the persistence of activity up to 800 mM KCl, at least. These characteristics distinguish it from other protein kinases found in BHK cells or reported to be present in other eukaryotic cells.

Further studies attempted to relate the new protein kinase activity to proteins which become phosphorylated during viral infection. The results obtained show that the major virion phosphoproteins were not phosphorylated by the new protein kinase in vitro, even though that activity was also present in the virion. An enzyme activity, present in BHK cells and a similar activity from virus particles, both with characteristics consistent with those of casein kinase II, phosphorylated these proteins in vitro. Thus, it is possible that these viral proteins synthesized de novo in infected cells can be recognized as substrates by cellular casein kinase II. Analysis of viral non-structural proteins showed that one of the proteins tested, viral DNase, served as a substrate in vitro for a partially-purified preparation of the new protein kinase. Studies of the phosphorylation of ribosomal protein S6 in vitro by protein kinase activities from infected cells showed that the new protein kinase and also some other cellular protein kinases (cyclic AMP-dependent protein kinases and

protein kinase C) can catalyse this reaction. Specific phosphorylation of S6 by the new protein kinase was only observed in certain conditions, e.g. 600 mM KCl or 7-10 mM spermine. Phosphopeptide maps of S6 following phosphorylation *in vivo* and *in vitro* by the new kinase were similar. However, the enzyme catalysed the incorporation of only two phosphate groups per molecule of S6 while the stoichiometry of phosphorylation *in vivo* was up to five phosphate groups per S6 molecule. This finding does not support the possibility that the new protein kinase is responsible for the complete phosphorylation of ribosomal protein S6 observed *in vivo*. One of the cellular kinases, protein kinase C appears to be a better candidate, on the basis of the results of studies of stoichiometry and phosphopeptides.

INTRODUCTION

1. PHOSPHORYLATION OF PROTEINS

It is now well recognized that the phosphorylation and dephosphorylation of proteins is an important regulatory process applicable to many different cellular functions. The best documented example is the neural and hormonal control of glycogen metabolism (Cohen, 1982), and other examples include the regulation of fatty acid synthesis by hormones (Denton and Brownsey, 1983), the control of muscle contraction (Kamm and Stull, 1985), the regulation of protein synthesis (Hunt, 1980), etc. The enzymes which catalyse phosphorylation and dephosphorylation of proteins have been extensively studied.

1.1. PROTEIN KINASES

Protein kinases (EC 2.7.1.37, ATP: protein phosphotransferases) represent a multiplicity of enzymes which catalyse the phosphorylation step in various phosphorylation - dephosphorylation systems. They are regulated by different specific agents, possess either relatively broad or limited substrate specificities, use either ATP or both ATP and GTP as a phosphate donor and transfer the phosphoryl group of this to one or more of serine, threonine or tyrosine residues in characteristic recognition sequences.

One system of classification of protein kinases (Krebs, 1983) is based on the amino acid residue which serves as the phosphate acceptor. Two classes, serine-threonine and tyrosine protein kinases, can further be subdivided according to the regulatory agent(s) of protein kinase activity (Table 1).

The individual enzymes listed in Table 1 have been described in detail in many recent reviews [e.g., Malencik and Fischer (1982), Kamm and Stull (1985), Beavo and Mumby (1982), Hathaway and Traugh (1982), Sefton and Hunter (1984), Nishizuka (1983), etc]. In the following sections they will be discussed with respect to the general characteristics of protein kinase molecules and the characteristics of phosphorylation reaction they catalyse.

(1) Structural Properties

The structural properties of some protein kinases purified to apparent homogeneity are described in Table 2. It is clear that protein kinases have a wide range of molecular weights and a variety of different subunit structures. However, as protein kinases play a transducing role in nature, acting as mediators between effector molecules and specific protein substrates, it has been possible to identify within the structure of most protein kinases a regulatory domain or subunit (to which the effector binds) and a catalytic domain or subunit (Flockhart and Corbin, 1982).

The catalytic domain or subunit is involved in transferring the terminal phosphate of ATP to specific serine, threonine or tyrosine residues. Several groups of investigators have speculated that some of the kinases might share an underlying structural basis for this common function. In support of this view it has been found that there is homology between the primary structures of the catalytic subunit of cyclic AMP-dependent protein kinase and i) cyclic GMP-dependent protein kinase (Takio et al., 1984), ii) the γ subunit of phosphorylase b kinase (Reimann et al., 1984), iii) the epidermal growth factor (Downward et al., 1984) and insulin (Ullrich et al., 1985)

receptors, and iv) certain members of a family of transforming retroviral tyrosine protein kinases, typified by $pp60^{src}$ (reviewed by Sefton and Hunter, 1984). There is currently only limited chemical evidence to identify the residues in the homologous regions that comprise the active sites of protein kinases. The affinity label, [(fluorosulphonyl)-benzoyl] adenosine, has identified lysyl residues that appear to be in the vicinity of the binding sites of the γ-phosphate of ATP in the catalytic subunit of cyclic AMP-dependent protein kinase (Zoller et al., 1981), the catalytic domain of cyclic GMP-dependent protein kinase (Hashimoto et al., 1982) and $pp60^{src}$ (Kamps et al., 1984). In other protein kinases there is a lysine residue at a similar position to those which have been found labelled in the protein kinases mentioned above (Kamps et al., 1984). In all these proteins, a cluster of glycines with the sequence Gly-X-Gly-X-X-Gly lies 16-28 residues to the amino-terminal side of the lysine implicated in binding ATP.

In contrast to the catalytic domain, the structure of the domain or subunit involved in binding the effector is highly specific and depends on the nature of the particular effector molecule. Thus, protein kinases regulated by cyclic nucleotides (type I and type II cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase) have similar nucleotide binding sites to the unrelated cyclic AMP-binding protein (CAP) of E. coli (Titani et al., 1984; Takio et al., 1984 and 1984a; Weber et al., 1982). On the basis of the homology between the sequences, and from the known X-ray crystal structure of CAP, a possible conformation was proposed for the cyclic nucleotide binding sites of the protein kinases (Hoppe, 1985).

Protein kinases which require Ca^{2+} and calmodulin for their activity share this property with other unrelated enzymes (e.g. adenylate cyclase and cyclic-AMP phosphodiesterase). A peptide fragment has been obtained from skeletal muscle myosin light chain kinase that appears to represent the calmodulin-binding domain of this enzyme (Blumenthal et al., 1985). However, the sequence of the peptide shows no significant homology to any other known sequences of peptides that bind calmodulin.

(2) Regulation

The following types of regulatory effectors have been identified for the protein kinases known at present: i) extracellular signals (EGF, PDGF and insulin), ii)molecules termed "second messengers", produced as a result of the action of extracellular signals (cyclic AMP, cyclic GMP, Ca^{2+} and diacylglycerol) and iii) other specific regulatory molecules (double-stranded RNA, haemin, polyamines and acetyl CoA, NADH, pyruvate and ADP) (Table 1). The tyrosine protein kinases of EGF, PDGF and insulin receptors are stimulated following binding of the respective agonist. In the class of serine-threonine protein kinases, those regulated by calcium are, in addition to the cyclic nucleotide-dependent enzymes, especially prominent. The effects of calcium are mediated by a protein, calmodulin, to which calcium ions bind tightly. In the case of phosphorylase kinase calmodulin is, in fact, one of the subunits (δ) of the enzyme. Another serine-threonine protein kinase, protein kinase C, requires the presence of Ca^{2+} and phospholipids for its activity. It has been demonstrated that diacylglycerol, one of the earliest products of the breakdown of inositol phospholipids in response to extracellular signals, greatly increases the affinity of protein kinase C for Ca^{2+}, thereby activating it.

Another subset of protein kinases consists of those enzymes for which no regulatory agents or effectors are known to exist (e.g. casein kinase I and some tyrosine protein kinases). This does not exclude the possibility that these enzymes may be subject to regulation by mechanisms other than the direct interaction of the protein kinase with an effector molecule. For example, they could be regulated by covalent modification, the enzymes serving as substrates for other protein kinases.

The mechanism by which the binding of the ligand is though to activate the protein kinase by inducing a conformational change is best documented for cyclic AMP-dependent protein kinases (Hoppe,1985). The regulatory (R) and catalytic (C) subunits of this enzyme have an exceptionally high affinity for each other in the absence of cyclic AMP, but this affinity is decreased 10^4-fold by binding of the nucleotide. In the holoenzyme (R_2C_2) one part of the R molecule directly impedes the active site of the catalytic subunit, resulting in the inhibition of its catalytic activity towards added protein substrates. It has been proposed that cyclic AMP binds to the R subunits of the holoenzyme (rather than to free R), forming an intermediate ternary complex of the nucleotide and holoenzyme (cyclic AMP R_2C_2). The next step of interaction between the cyclic AMP-binding sites and cyclic AMP leads to a conformational change in the R subunits, accompanied by their dissociation from the C subunits [R_2(cyclic AMP)$_4$ + 2C].

(3) Determination of Physiological Substrates

The properties of many enzymes can be altered by covalent modification, and this in turn can effect changes in cellular metabolism. Historically, the evidence that the modulation of the function of a protein led to a significant change in metabolism often pre-dated the discovery that the protein in question was a phosphoprotein and the isolation of the regulatory

protein kinase. Before it can be regarded as proven that the phosphorylation of an enzyme by a particular protein kinase leads to a physiological change in its activity, a set of established criteria has to be satisfied. The criteria (Krebs and Beavo, 1979) include:

i) Demonstration in vitro that the enzyme can be phosphorylated stoichiometrically at a significant rate in a reaction catalysed by the protein kinase.

ii) Demonstration that functional properties of the enzyme undergo meaningful changes that correlate with the degree of phosphorylation.

iii) Demonstration that the enzyme can be phosphorylated and dephosphorylated in vivo.

iv) Correlation of cellular concentrations of protein kinase effectors and the extent of phosphorylation of the enzyme.

On the basis of these criteria several enzymes have been accepted as physiological substrates for cyclic AMP-dependent protein kinase (e.g. phosphorylase kinase, glycogen synthase and pyruvate kinase). Some other protein kinases have more limited substrate specificity: examples include glycogen phosphorylase kinase, myosin light chain kinase, Ca^{2+}/calmodulin glycogen synthase kinase and pyruvate dehydrogenase kinase.

It is not easy to identify the substrate for a protein kinase when there is nothing to suggest that its activation is related to a change in the activity of a particular protein involved in regulation of a cellular function. Among examples of such protein kinases are some relatively recently discovered enzymes, protein kinase C and tyrosine kinases. The strategy used to try to identify their physiological substrates can be illustrated by one specific example: pp $60^{v\text{-}src}$ tyrosine protein kinase and its possible substrate - the cytoskeleton protein, vinculin (Cooper and Hunter, 1983). The question first examined was,

under what conditions, and to what extent is the possible substrate phosphorylated on tyrosine *in vivo*. The results showed that vinculin contained some phosphotyrosine in normal cells, but the extent of phosphorylation was increased 20-fold in cells transformedby Rous sarcoma virus containing $pp60^{v-src}$. In further experiments it was demonstrated that purified vinculin can be phosphorylated *in vitro* by a purified fragment of $pp60^{v-src}$. Another question addressed the function of the possible substrate in cell physiology. It has been found that 20% of vinculin is concentrated in adhesion plaques. In the adhesion plaques, the surface of the membrane is bonded to the extracellular matrix and the inner surface provides an anchorage point for stress fibres composed of actin microfilaments. This location of vinculin suggested that it was important for maintaining the cell in a particular shape. The shape of a cell transformed by Rous sarcoma virus is very different from that of a normal cell, and both the total cellular microfilament system and the adhesion plaques are reorganized. On the basis of these data, an explanation of how phosphorylation of vinculin contributes towards changes in cell physiology was suggested. The greatly increased phosphorylation of tyrosine in vinculin, possibly as a result of activity of $pp60^{v-src}$, might reduce the tenacity of vinculin as a linker and lead to the release of the actin filaments. Some recent findings (Rohrschneider and Rosok, 1983) suggest,however, that vinculin phosphorylation alone may not be enough to cause such disruption.

(4) Recognition Sequences in Protein Substrates

Analysis of physiological substrates for protein kinases and analysis of other proteins and polypeptides which are phosphorylated only *in vitro*, has allowed identification of the characteristics required for recognition of the substrates by individual protein kinases. For many protein kinases

(e.g. cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase and casein kinases, Table 3) it has become apparent that the selection of serine and threonine residues in substrates is, at least in part, dependent on the primary sequence near the target amino acid. As shown in Table 3, acidic or basic residues with a particular location relative to the target residue are important determinants of the recognition. Nevertheless, the presence of the appropriate features of primary structure is not always sufficient to ensure recognition. For example, cyclic AMP-dependent protein kinase does not recognize some serines in the native lysozyme molecule that it can phosphorylate if the lysozyme is denatured (Bylund and Krebs, 1975).

(5) Amino Acids Phosphorylated

Phosphorylation of proteins at serine, threonine and tyrosine residues is now well established. In the proteins of normal cells most of the phosphorylated residues are phosphoserine, while phosphotyrosine accounts for only about 0.05% of the total acid-stable phosphate in protein. There is evidence (Smith et al., 1974) that some other amino acid residues, histidine and lysine, can be phosphorylated. However, work on the protein kinases responsible for phosphorylation of such residues has been limited.

(6) Phosphate Donors and Divalent Metal Ion Requirements

All protein kinases that have been characterized can use ATP as a phosphate donor. The Km values for ATP (3-400μM) are well below the concentration of the nucleotide (2-6mM) thought to exist in most cells. The only other significant phosphate donor known is GTP, which can be utilised by casein kinase II equally as well as ATP. It is not known if GTP is a physiologically significant phosphate donor.

As for other phosphoryl transferase enzymes, divalent metal ions are necessary for the reaction. Mg^{2+} is normally the preferred cation, but Mn^{2+} can often substitute for this.

Table 1. Classification of Protein Kinases (Krebs,1983)

CLASS 1: TYROSINE PROTEIN KINASES

REGULATORY AGENT	PROTEIN KINASE
EGF	EGF - receptor
PDGF	PDGF - receptor
insulin	insulin - receptor
unknown	$pp60^{v\text{-}src}/pp60^{c\text{-}src}$ and other viral and homologous cellular kinases (Table 6)

CLASS 2: SERINE-THREONINE PROTEIN KINASES

REGULATORY AGENT	PROTEIN KINASE
cyclic AMP	type I, type II (heart) and type II (brain) cyclic AMP-dependent
cyclic GMP	cyclic GMP-dependent
Ca^{2+} (calmodulin)	phosphorylase kinase myosin light-chain kinase liver glycogen synthase kinase
diacylglycerol (Ca^{2+}, phospholipid)	protein kinase C
double-stranded RNA	double-stranded RNA-dependent
haemin (inhibition)	eIF-2 kinase
acetyl-CoA, NADH, pyruvate, ADP	pyruvate dehydrogenase kinase
polyamines	polyamine-dependent
unknown	casein kinase I and II glycogen synthase kinase 3 and 4

Table 2. Structural Properties of Some Purified Protein Kinases

PROTEIN KINASE	MONOMER MOLECULAR WEIGHTS	SUBUNIT STRUCTURE	AUTOPHOSPHORYLATED SUBUNITS
cyclic AMP-dependent type I	49,000(R) 39-42,000(C)	R_2C_2	C
cyclic AMP-dependent type II	54-56,000(R) 39-42,000(C)	R_2C_2	C,R
cyclic GMP-dependent	74-81,000(E)	E_2	E
phosphorylase kinase	118-145,000(α) 108-128,000(β) 41-42,000 (γ) 17,000 (δ)	$(\alpha\beta\gamma\delta)_4$	α,β
myosin light-chain kinase	77-125,000	monomer	+
protein kinase C	77-87,000	monomer	+
eIF-2 kinase	80-95,000		±
casein kinase I	37-42,000	monomer	+
casein kinase II	42-44,000 (α) 38-40,000 (α') 24-26,000 (β)	$\alpha\alpha'\beta_2$	β
EGF-receptor	170-180,000	monomer	+
$pp60^{v\text{-}src}/pp60^{c\text{-}src}$	60,000	monomer	+
insulin-receptor	120-130,000 (α) 90,000 (β)	$\alpha_2\beta_2$	β

References: Flockhart and Corbin (1982), Nishizuka (1983) and Ullrich et al. (1985)

Table 3. Amino Acid Sequences at the Phosphorylation Sites of Protein Substrates (Data from Engström et al., 1984)

PROTEIN KINASE	SUBSTRATE	SEQUENCE
cyclic AMP--dependent	phosphorylase kinase (α-subunit)	phe-arg-arg-leu-ser(P)-ile
	glycogen synthase (site 1a)	gln-trp-pro-arg-arg-ala-ser(P)-cys
	phenylalanine hydroxylase	ser-arg-lys-leu-ser(P)-asp
	troponin-I	val-arg-arg-ser(P)-asp
cyclic GMP--dependent	R subunit of cyclic AMP-dependent protein kinase type I	arg-gly-ala-ile-ser(P)-ala-glu
	H2B	lys-arg-ser(P)-arg-lys-glu-ser(P)-tyr
	HMG 14	lys-arg-lys-val-ser(P)-ser-ala
casein kinase II	casein (β)	glu-gln-gln-gln-thr(P)-glu-asp-glu
	glycogen synthase (site 5)	ser-pro-his-gln-ser(P)-glu-asp-glu
	troponin-T	Ac-ser(P)-asp-glu-glu-val
casein kinase I	casein (αs_2)	glu-glu-asn-ser(P)-lys-lys-thr
	casein (β)	ser(P)-ser(P)-glu-glu-ser(P)-ile-thr

1.2 PROTEIN PHOSPHATASES

Protein phosphatases which catalyse the dephosphorylation of proteins containing phosphoserine and phosphothreonine residues (reviewed by Ingebritsen and Cohen, 1983) and protein phosphatases which act on phosphotyrosyl proteins (reviewed by Foulkes,1983) have been described.

Studies of protein phosphatases acting on proteins phosphorylated at serine and threonine residues suggested that four enzymes, termed protein phosphatases 1, 2A, 2B and 2C, account for virtually all protein phosphatase activity in cellular extracts. The phosphoproteins that are substrates for such phosphatases include enzymes involved in regulating many different metabolic processes: glycogen metabolism, glycolysis/gluconeogenesis, fatty acid synthesis,cholesterol synthesis and protein synthesis. In addition, these four protein phosphotases appear to explain most of the protein phosphatase activities that have been reported.

The four protein phosphatases (Table 4) have different structural and catalytic properties. A study of the substrate specificities of the enzymes demonstrated that protein phosphatases 1, 2A and 2C each have broad but distinct substrate specificities. In contrast, protein phosphatase 2B has significant activity towards only three substrates, namely inhibitor-1, the α subunit of phosphorylase kinase and the P light chain of myosin.

Like protein kinases, protein phosphatases are important targets for cellular regulation. Two mechanisms for regulating protein phosphatase 1 have been identified. One involves regulation by a protein called inhibitor-1. The inhibitor-1 itself can be regulated by phosphorylation: the phospho form of the protein is a potent inhibitor of protein phosphatase 1, and the dephospho form is inactive. It is likely that phosphorylation of the inhibitor is catalysed by cyclic AMP-dependent protein kinase. The dephosphorylation could be a result of the activity of Ca^{2+}-dependent protein phosphatase 2B. It is therefore possible that the activity of protein

phosphatase 1 is regulated by cyclic AMP and Ca^{2+} via phosphorylation and dephosphorylation of inhibitor-1. The second mechanism for regulating protein phosphatase 1 is related to the phosphatase molecules present in the complex with a protein called inhibitor-2. The activation of protein phosphatase 1 is triggered by the phosphorylation of inhibitor-2 by glycogen synthase kinase 3.

Protein phosphatase 2B is a Ca^{2+}-dependent enzyme (activation constant, $A_{0.5}$ = 0.5 to 1.0 μM) the activity of which is stimulated tenfold by calmodulin ($A_{0.5}$ = 6.0 nM). Protein phosphatase 2C is absolutely dependent on Mg^{2+} ($A_{0.5}$ = 1.0 mM) for activity with all known substrates, but protein phosphatase 2A is active in the absence of divalent cations. It has recently been reported (Tung et al., 1985) that the activity of protein phosphatase 1 (oligomeric form) and the activities of four forms of protein phosphatase 2A can be stimulated by polyamines in vitro. Optimal effects were obtained with 1-2 mM spermine.

Table 4. Dephosphorylation of Proteins with Phosphoserine and Phosphothreonine Residues: Protein Phosphatases

(Ingebritsen and Cohen, 1983)

CLASSIFICATION		PROTEIN PHOSPHATASE	STRUCTURE		SUBSTRATE SPECIFICITY	REGULATORS
GROUP	BASIS FOR CLASSIFICATION		MOLECULE WITH CATALYTIC ACTIVITY (approx. mol. weight)	OTHER SUBUNITS OR ASSOCIATED PROTEINS		
1	-specificity for β subunit of phosphorylase kinase -inhibition by inhibitors 1 and 2	PrP 1	35,000	+	Broad	inhibitor 1 inhibitor 2 glycogen synthase kinase 3
		PrP 2A	38,000	+	Broad	unknown (polyamines ?)
2	-specificity for α subunit of phosphorylase kinase	PrP 2B	61,000	+	Narrow	Ca^{2+} and calmodulin
	-not affected by inhibitors 1 and 2	PrP 2C	43,000	-	Broad	Mg^{2+}

2. PROTEIN PHOSPHORYLATION IN INFECTION OF CELLS WITH ANIMAL VIRUSES

2.1 EVIDENCE TO SUPPORT THE ROLE OF PROTEIN PHOSPHORYLATION DURING VIRAL INFECTION

Most information concerning the role of protein phosphorylation in the interaction of animal viruses with host cells has emerged from studies of viral transforming genes and proteins. Transformation of cells by some viral oncogenes is due to the ability of the oncogenes to affect particular steps of normal cellular growth (Heldin and Westermark, 1984). Thus, some of the viral oncogenes are related to polypeptide growth factors or to growth factor-receptors with tyrosine kinase activity. Some other viral oncogenes are related to cellular serine-threonine protein kinases, but their role in transformation (or the role of the homologous cellular kinases in growth) is unknown.

Different mechanisms from those operative in transformation operate during productive infection of cells by animal viruses. Productive infection of cells involves several separable events: i) adsorption and uncoating of the virus particle; ii) inhibition of cellular functions; iii) transcription of the viral genome into mRNA; iv) replication of the viral genome; v) morphogenesis of the virion and release of the virus from the cell. The characteristics of these events are dependent on the specific properties of the virus (Luria et al., 1978). Although it is still uncertain whether protein phosphorylation plays a role in any of these various processes, the following findings suggest that this may, in fact,be the case:

(1) Protein kinase activity is present in purified virions of many completely unrelated viruses.

(2) Some virally-coded proteins have associated protein kinase activity.

(3) Some viral structural and functional proteins are phosphoproteins.

(4) The phosphorylation of cellular proteins can be altered as a result of viral infection

These different points will be discussed in turn in the following sections.

(1) Protein Kinase Activities Present in Virus Particles

Protein kinase activity has been found associated with the virions of many different viruses. These include adenoviruses (Akusjärvi et al., 1978; Blair et al., 1978), herpesviruses (Randall et al., 1972; Flügel and Darai, 1982; Lemaster and Roizman, 1980; Rubenstein et al., 1972), iridoviruses (Silberstein and August, 1976; Monnier and Devauchelle, 1980), poxviruses (Kleiman and Moss, 1975), retroviruses (e.g. Sen and Todaro, 1979; Owada et al., 1981; Weis and Faras, 1983), togaviruses (Waite et al., 1974; Tan and Sokol, 1974), picornaviruses (Grubman, 1982), rhabdoviruses (Imblum and Wagner, 1974; Clinton et al., 1982), and mixoviruses (Lamb, 1975; Kamata and Watanabe, 1977). It is therefore clear that the presence of protein kinases in virions cannot be related to any particular group of viruses (RNA or DNA), to the presence or absence of a viral envelope acquired by budding at cellular membranes, or to the ability of a virus to cause infection rather than transformation of host cells, or vice versa.

The existence of protein kinases in viral particles allows many possibilities for the physiological significance of their presence. These possibilities range from the chance incorporation of cellular protein kinases into the virions, to the possibility that they represent important components of the virion i.e. viral regulatory proteins. It was therefore necessary to try to characterize the protein kinases in relation to their

molecular and enzymic properties as well as to their origin from cellular or viral genome.

Some properties of protein kinases isolated from various virus particles are shown in Table 5. The protein kinases isolated from FV_3 (Silberstein and August, 1976 and 1976a), MSV-MuLV (Blaas et al., 1979; Sen and Todaro,1979), AMV (Rosok and Watson, 1979; Houts et al., 1978; Tsiapalis, 1977) and RSV (Weis and Faras,1983) have molecular weights between 40,000 and 50,000, they are probably all monomeric in structure, preferentially phosphorylate acidic proteins at serine and threonine residues, and use ATP more efficiently than GTP as the phosphate donor. A similar protein kinase is present in animal cells and has been termed casein kinase I (Table 2). This enzyme has been isolated from the cytoplasm, and some variants of casein kinase I have also been isolated from the nucleus (NI) (Baydoun et al., 1981) and the plasma membrane (M) (Tao et al., 1980 and 1981). So far, there has been no direct experimental comparison between this cellular kinase and the similar group of kinases from virus particles.

The protein kinases isolated from Ad2 (Akusjärvi et al., 1978), Mo-Mu SV (Sen and Todaro, 1979; Sen, 1981) and RSV-PrC (Hizi et al., 1979) are distinct from other characterized viral and cellular protein kinases (Table 2 and 5) in their small size (11-14,000 Da). Mo-Mu SV kinase phosphorylates tyrosine in an autocatalytic reaction but is not associated with the viral transforming protein, in contrast to tyrosine kinases of other retroviruses (Table 6).

The protein kinases isolated from the virions of RSV (a retrovirus) and VSV (a rhabdovirus) have been shown to be identical to the tyrosine protein kinase, $pp60^{src}$ (the transforming protein of RSV and its cellular homologue) on the basis of specific immunoprecipitation, purification and

peptide analysis (Owada et al., 1981 ; Clinton et al., 1982).

The second important question regarding protein kinases associated with the virion, is whether they are coded by the viral or cellular genome. In the case of enveloped viruses, which obtain their coat by budding from the host cell, it is likely that the virions contain host cell membrane protein kinase(s). One enveloped virus, vesicular stomatitis virus (VSV), contains, among several protein kinases, the tyrosine kinase $pp60^{c-src}$ (Clinton et al., 1982). This protein kinase, as well as $pp60^{v-src}$ of Rous sarcoma virus, is present in the cellular membrane (Burr et al., 1981). To establish whether the tyrosine protein kinase is taken up from the membrane during the budding of VSV, virus was grown in normal cells, or in cells transformed by Rous sarcoma virus containing increased amounts of $pp60^{src}$ tyrosine protein kinase activity. VSV particles isolated from the transformed cells were found to contain more $pp60^{src}$ activity than particles from normal cells (Clinton et al., 1982). Uptake of cell-membrane components by budding viruses is not a random process but involves some specificity. In the case of VSV most cell-surface proteins as well as most enzymes assoicated with the membrane are excluded from purified virions (Lodish and Porter, 1980). Thus, the presence of membrane protein kinases in enveloped viruses must be a result of specific rather than nonspecific incorporation.

Analysis of the subviral compartments of enveloped viruses indicated that some protein kinases are located in the virus core (Monnier and Devauchelle, 1980; Kleiman and Moss, 1975; Blaas et al., 1979). Furthermore some non-enveloped viruses possess protein kinase activity (Akusjärvi et al., 1978; Blair and Russell, 1978; Grubman, 1982). Different approaches have been used to answer the question of whether these kinases (clearly not derived from cell membrane) are coded by the virus or host cell.

Table 5. Protein Kinases Isolated from Virus Particles

VIRUS (REFERENCE)	APPROX. MOL WEIGHT	PHOSPHATE DONOR	SUBSTRATE PREFERENCE in vitro	AMINO ACID PHOSPHORYLATED
Adenovirus type 2 Ad-2 (1)	14,000	ATP	phosvitin casein	N.D.
Frog virus 3 FV3 (2,3)	50-53,000	ATP>GTP	casein phosvitin	P-Ser P-Thr
Vaccinia virus (4, 5)	62,000	ATP	casein	P-Ser P-Thr
Murine sarcoma leukemia virus MSV-MuLV (6, 7)	40,000	ATP	casein phosvitin	P-Ser P-Thr
Moloney murine sarcoma virus Mo-MuSV (7, 8)	14-16,000	ATP>GTP	phosvitin casein	P-Ser
Rous sarcoma virus RSV (9)	120-130,000	ATP>GTP	phosvitin casein	N.D.
Rous sarcoma virus RSV (9)	43,000	ATP	phosvitin casein	N.D.
Rous sarcoma virus RSV (10)	11,000	ATP>GTP	casein phosvitin	P-Ser P-Thr
Avian sarcoma virus ASV (11)	60,000	ATP>GTP	casein	P-Tyr
Avian mieloblastosis virus AMV (12)	63,000	ATP	histone protamine	N.D.
Avian mieloblastosis virus AMV (12,13,14)	45-50,000	ATP	phosvitin casein	N.D.
Vesicular stomatitis virus VSV (15)	60,000	ATP>GTP	casein	P-Tyr

Key to references:

1) Akusjarvi et al. (1978)

2 and 3) Silberstein and August (1976 and 1976a)

4 and 5) Keliman and Moss (1975 and 1975a)

6) Blaas et al. (1979)

7) Sen and Todaro (1979)

8) Sen (1981)

9) Weis and Faras (1983)

10) Hizi et al. (1979)

11) Owada et al. (1981)

12) Rosok and Watson (1979)

13) Tsiapalis (1977)

14) Houts et al. (1978)

15) Clinton et al. (1982)

The most direct, genetic approach is at present only applicable to relatively simple viruses with extensively characterized genomes and gene products. The available evidence for Rous sarcoma virus clearly shows that kinases such as those of 43,000 Da (Weis and Faras,1983) and 120,000 Da (Weis and Faras, 1983) are not products of the viral genome. In contrast, the 15,000 Da protein kinase from Moloney murine sarcoma virus (Mo-Mu-SV) seems to be virally-coded. When the 15,000 Da protein kinase is isolated from the virions of a mutant of Mo-Mu-SV temperature sensitive for transformation, its ability to transfer phosphate is about four times more thermolabile than that of the wild-type enzyme (Sen et al., 1979). Another example when the genetic approach was used is the protein kinase associated with the virions of frog virus 3 (FV3) and also present in infected cells (Silberstein and August,1976 and 1976a). Temperature-sensitive mutants of this virus have been isolated. In normal cells and cells separately infected with two of these mutants no protein kinase can be detected using antibodies raised against the enzyme isolated from wild-type virus particles. Thus, expression of the viral genome is necessary for the synthesis of this protein kinase, although this still does not prove that the kinase is itself a virally-coded protein.

The evidence presented in this section leads to the conclusion that, although protein kinases in the virions may originate from both the viral or cellular genome, it is unlikely that their incorporation into the virions results from nonspecific interaction with viral components during the processes of assembly and budding.

(2) Protein Kinase Activities Associated with Virally-coded Proteins

Besides the protein kinases associated with components of the virions of various animal viruses, protein kinase activities have also been found in

association with certain virally-coded proteins present in infected and transformed cells. The main objective of the studies concerning such viral proteins was to establish whether these proteins possess intrinsic protein kinase activity, or whether the kinase activity is due to the association of the viral protein with a cellular kinase(s).

Among the first examples of protein kinase activity found in association with virally-coded proteins was a tyrosine protein kinase associated with $pp60^{v-src}$ (Collett and Erikson, 1978; Levinson et al., 1978). This finding stimulated a series of investigations to test whether there were protein kinase activities associated with the virally-coded proteins of other (especially tumour) viruses. The assay most commonly employed has been simple incubation of an immunoprecipitate containing the viral protein(s) with [γ -^{32}P]ATP, and analysis of the ^{32}P-labelled products. The specific presence of phosphorylated proteins in samples containing viral proteins was taken as evidence for an associated protein kinase activity. As a result it has been demonstrated that the transforming proteins of some of the highly oncogenic retroviruses (Table 6) and some early proteins of papovaviruses, adenoviruses and herpesviruses (Table 7) contain a protein kinase activity in their immunoprecipitates.

Highly oncogenic retroviruses represent a distinct group in the retrovirus family as regards the organisation of their genome and their ability to cause acute diseases with a short latent period (Cooper and Hunter,1983). A common feature of this group of retroviruses is that their genomes are chimeric. Both termini are derived from the genome of a non-defective weakly oncogenic retroviruses; However, the central part of the genome originates from cellular genes, presumably by a recombinant event. This part of the genome (the viral oncogene), which represent a transduced cellular gene sequence, is responsible for transformation. The evolutionary

Table 6. Retroviral Transforming Proteins with Protein Kinase Activity

RETROVIRUS	GENE NAME	PROTEIN KINASE	NORMAL CELLULAR HOMOLOGUE
		1. tyrosine	
Rous sarcoma virus (RSV)	src	$pp60^{v-src}$	$pp60^{c-src}$
Y73 avian sarcoma virus	yes	$P90^{gag-yes}$	?
Esh sarcoma virus (ESV)		$P80^{gag-yes}$	
Fujinami sarcoma virus (FSV)	fps	$P140^{gag-fps}$	$p98^{c-fps}$
PRC II sarcoma virus		$P105^{gag-fps}$	
UR-1 sarcoma virus		$P150^{gag-fps}$	
Snyder-Theilen feline sarcoma virus	fes	$P85^{gag-fes}$	$p92^{c-fes}$
Gardner-Arnstein feline sarcoma virus		$P95^{gag-fes}$	
UR-2 sarcoma virus	ros	$P68^{gag-ros}$	?
Abelson murine leukemia virus (AMuLV)	abl	$P120^{gag-abl}$	$p150^{c-abl}$
Garder-Rasheed feline sarcoma virus	fgr	$P72^{gag-fgr}$	?
Avian erythroblastosis virus	erbB	$gp65^{erbB}$	intracellular part of EGF receptor
		2. serine-threonine	
Moloney murine sarcoma virus	mos	$p37^{mos}$ $P85^{gag-mos}$	?
Murine sarcoma virus 3611 (3611-MSV)	raf	$P75^{gag-raf}$	?
Avian carcinoma Mill Hill No.2 (MH2)	mil	$P100^{gag-mil}$	?

References: Cooper and Hunter (1983), Bishop (1983), Heldin and Westermark (1984), Gilmore et al. (1985), Kris et al. (1985), Maxwell and Arlinghaus (1985), Kloetzer et al. (1984) and Moelling et al. (1984)

Table 7. Viral Proteins with Associated Protein Kinase Activity

VIRUS	PROTEIN WITH ASSOCIATED KINASE ACTIVITY	REFERENCE
Papovaviruses		
SV 40	large T (85,000 Da)	1 - 3
Polyoma	middle T (50,000 Da)	4 - 6
Adenoviruses		
Ad 5	58,000 Da	7
Herpesviruses		
Epstein-Barr	48,000 Da	8
Human cyto-megalovirus	68,000 Da	9

Key to references:

1) Griffin et al. (1979)
2) Bradly et al. (1982)
3) Tjian and Robbins (1979)
4) Eckhart et al. (1979)
5) Schaffhausen and Benjamin (1979)
6) Smith et al. (1979)
7) Yee and Branton (1983)
8) Kamata et al. (1981)
9) Michelson et al. (1984 and 1985)

progenitors of viral oncogenes are present in normal cells (proto-oncogenes). In cells transformed by retroviruses the oncogenic protein is made in large amounts because the oncogene is expressed like an active viral gene despite not having a function in viral replication. In papovaviruses, adenoviruses and herpesviruses, the proteins with associated protein kinase activity have not so far been related to any cellular gene and some of them have functions during lytic infection as well as cell transformation.

Because of the possibility that the immunoprecipitates of the proteins discussed above and listed in Table 6 and 7, contain an associated or contaminating cellular protein kinase, it is important to demonstrate that the detected protein kinase activity is an intrinsic property of a specific viral protein. Several lines of experimental evidence support the identification of the retroviral transforming proteins as protein kinases. It has been shown that many transforming proteins of conditional mutants, temperature-sensitive for transformation, have a more thermolabile protein kinase activity than their respective wild type (Hunter and Sefton, 1982). Furthermore, highly purified preparations of $pp60^{src}$ display tyrosine kinase activity and the v-src and, v-abl gene products expressed in bacteria retain their kinase activity (Cooper and Hunter, 1983). Molecular-cloning techniques have made it possible to determine the nucleotide sequence of almost every viral oncogene, and, by applying the genetic code, to predict the amino acid sequence of its product. A comparison of these sequences has not only revealed homology between the transforming proteins themselves but also homology between the transforming proteins and the catalytic subunit of cyclic AMP-dependent protein kinase (Privalsky et al., 1984; Kamps et al., 1984).

The relationship between proteins encoded by other animal viruses (Table 7) and the protein kinase activities detected in their immuno-precipitates is less clear. The large transforming (T) antigen of simian

virus 40 (SV 40) becomes phosphorylated at multiple sites in both infected and transformed cells. A protein kinase activity present in immunoprecipitates can phosphorylate large T antigen *in vitro* at serine and threonine residues. Some of these residues were also phosphorylated *in vivo* (Van Roy *et al.*, 1984). Several investigators (Griffin *et al.*, 1979; Bradly *et al.*, 1982; Tjian and Robbins, 1979) have reported that large T antigen, highly purified by biochemical means, displays protein kinase activity. However, others either were unable to detect this kinase activity of purified large T antigen, or managed to separate large T antigen from an associated protein kinase activity (Giacherio and Hager,1979; Tjian *et al.*, 1980), suggesting that T antigen is not itself a protein kinase. Another protein, middle T antigen of polyoma virus, is associated with a protein kinase activity in immunoprecipitates, with the major phosphate-acceptor species being the antigen itself (Eckhart *et al.*, 1979; Schaffhausen and Benjamin,1979; Smith *et al.*, 1979). *In vitro*, the protein kinase activity transfers phosphate from [γ -^{32}P]ATP to tyrosine (Eckhart *et al.*,1979). Currently, there is no evidence to demonstrate that the protein kinase activity is an intrinsic property of middle T antigen. The antigen synthesized in a translation system *in vitro*, or expressed in *E. coli* (Schaffhausen *et al.*, 1982), does not possess detectable protein kinase activity. Recently, it has been demonstrated that middle T antigen is associated with pp60^{c-src}. Since pp60^{c-src} possesses intrinsic tyrosine protein kinase activity, it would appear that the kinase associated with middle T antigen is, at least in part, a property of pp60^{c-src} (Courtneidge and Smith,1983; Bolen *et al.*, 1984). Other viral proteins listed in Table 7 have been studied less extensively. A serine-threonine protein kinase activity copurified with the early protein (58,000 Da) of adenovirus type 5 through several stages of purification (Yee and Branton,1983). Similar results were obtained for the nuclear

antigen specified by Epstein-Barr virus (Kamata et al., 1981). In the case of a serine-threonine protein kinase found associated with a virally-coded protein of 68,000 Da in cells infected with human cytomegatovirus, the only purification was by immunoprecipitation (Michelson et al., 1984 and 1985).

The data presented in this section do not provide convincing evidence that viral genomes other than those of the highly oncogenic retroviruses encode protein kinases. However the tyrosine protein kinases of these viral oncogenes cannot be regarded as normal viral proteins as the genes which code for them originate from host cells. Nevertheless, it has been suggested that specific association of a virally-coded protein and cellular protein kinase may have a physiological significance. Thus, specific association of polyoma virus middle T antigen with $pp60^{c-src}$ increases the specific activity of $pp60^{c-src}$ tyrosine protein kinase in both infected and transformed cells (Bolen et al., 1984; Courtneidge, 1985).

(3) Phosphorylation of Viral Proteins

Many structural and regulatory proteins of animal viruses undergo phosphorylation. In virtually every animal virus, some of the protein components of the virion can be phosphorylated by endogenous kinase activity (e.g. Blaas et al., 1979; Imblum and Wagner,1974; Lamb,1975; Akusjärvi et al., 1978; Lemaster and Roizman,1980). In addition, some viral structural and regulatory proteins are phosphorylated in the host cell during the infection cycle (e.g. Jeng et al., 1977; Russell and Blair, 1977; Wilcox et al., 1980; Hizi and Joklik,1977; Clinton et al., 1979; Moyer and Summers,1974; Lamb and Choppin,1977). The question to be considered here is, regardless of the origin of the kinase responsible, whether the phosphorylation of these viral proteins has any physiological function.

The phosphoproteins present in virus particles are frequently associated with the viral nucleic acid, and there is evidence that the degree of their phosphorylation determines the extent of their binding to the viral nucleic acid. This binding in turn may be the regulatory mechanism for replication or transcription of the viral genome. A role in assembly or uncoating of the virus particle has also been suggested. There are several examples which illustrate some of these possibilities.

The low molecular weight proteins from avian and mammalian type C retroviruses are examples of phosphorylated structural proteins. These are found in close association with the RNA genome in the virion core, and bind specifically to their homologous RNA *in vitro* (Sen and Todoro, 1977). The specific RNA-binding protein of many different type C retroviruses is also the major phosphoprotein of the virion (Sen and Todaro, 1977). This phosphoprotein of avian retroviruses is termed pp12. When the phosphate associated with serine residues of pp12 was removed, the apparent binding constant for viral RNA decreased 100-fold (Leis and Jentoft,1983). The high affinity binding of pp12 to viral RNA was restored by phosphorylation of the protein at the same serine residues *in vitro* (Leis *et al.*,1984). On the basis of these data a biological model has been proposed in which phosphorylation of the protein is involved in the packing of the viral RNA, and dephosphorylation is involved in its uncoating (Leis *et al.*, 1984). The results obtained from analysis of an adenovirus also suggest a regulatory role of protein phosphorylation in the assembly of the virion (Weber and Khittoo, 1983). One of the core proteins (designed pV) was studied. This was detected both as a free protein in infected cells and also as a part of the "young" and "mature" virions. The free pV was present in both a phosphorylated and an unphosphorylated form, while "young" virions contained only

the phosphorylated form of the protein. Subsequent maturation of the virion resulted in the dephosphorylation of this core protein.

One phosphoprotein of the virions of vesicular stomatitis virus is protein NS, which, in association with another protein (L), constitutes the RNA-polymerase complex. This protein has a regulatory function during viral RNA synthesis and viral morphogenesis. The NS protein exists as multiply phosphorylated species, both in the cell and the virus particle (Clinton et al., 1978 and 1979). The different phosphorylated species of NS bind to different extents to the ribonucleoprotein cores during virus assembly (Clinton et al., 1978). The individual species, isolated and purified, were tested for their ability to reconstitute a transcription system in vitro, involving viral cores from which the NS protein had been removed. Only the most highly phosphorylated NS molecules restored full transcriptional activity (Kingsford and Emerson, 1980). This result was confirmed by the finding that the specific dephosphorylation of NS inhibited transcription in the viral core in vitro (Hsu et al., 1982). A similar example of the role of protein phosphorylation in the regulation of transcription is provided by influenza virus. The major phosphoprotein of this virus is the NP protein which constitutes the capsid around highly packed viral RNA. Influenza virus contains protein kinase activity as well as viral transcriptase in, or in association with, the nucleocapsid structure (Sugiyama et al., 1976). The phosphorylation of the NP protein by the endogenous protein kinase in vitro stimulated viral transcription in isolated nucleocapsids (Kamata and Watanabe, 1977).

In another study, performed with vesicular stomatitis virus, an uncoating function for phosphorylation of the virion components has been suggested. Enveloped viruses generally enter host cells by viropexis (a variant of phagocytosis) or by fusion with the cellular plasma membrane.

The predominant mode of entry of vesicular stomatitis virus is viropexis. This virus, in contrast to viruses uncoating by membrane fusion and subsequent release of the genome, requires an additional step of uncoating prior to activation of virus replication. It seems that the function of the matrix protein (M) is to mediate the interactions between the envelope and internal core structure (Lenard and Compans, 1974). This protein, unlike the proteins described previously, is not associated with the viral genome. It has been shown that activation of the kinase activity present in the virions resulted in phosphorylation of protein M and some other proteins in the nucleocapsid (Witt et al., 1981). The electron microscopic analysis showed that following this phosphorylation of vesicular stomatitis virus protein in vitro, the virion envelope was disrupted (Witt et al., 1981). A similar electron microscopic study showed that activation of the kinase in the purified nucleocapsid structure of granulosis virus resulted in release of DNA from the nucleocapsid (Wilson and Consigli,1985). The main substrate for the kinase activity was the basic DNA-binding protein VP-12 (Wilson and Consigli,1985a). The phosphorylation of VP-12 reduced its binding capacity for viral DNA (Wilson and Consigli,1985).

Besides the possible roles for phosphorylation of viral proteins described above, the fact that some virally-coded enzymes become phosphorylated in infected cells (Hizi and Joklik, 1977; Lee et al., 1975; Schiff and Grandgenett, 1980; Banks et al., 1985) suggested a role of phosphorylation in the regulation of their enzyme activity. However, the only example where clear correlation between the extent of phosphorylation and enzyme activity has been found, is that of the RNA-dependent DNA polymerase of avian tumour viruses. The enzyme is composed of two subunits, one of them (β) a phosphoprotein (Hizi and Joklik,1977). The enzyme of Rous sarcoma virus (RSV) was phosphorylated in vitro with kinase purified from RSV particles (11,000 Da), with protein kinase from another retrovirus-

AMV (40,000 Da) or with the catalytic subunit of beef heart cyclic AMP-dependent protein kinase. The correlation between the phosphorylation state of RNA-dependent DNA polymerase and its enzymic activity was shown only when the 11,000 Da protein kinase of RSV was used (Hizi, 1982). This finding suggests a specific role of the RSV virion kinase in the regulation of reverse transcription.

(4) Phosphorylation of Cellular Proteins During Infection with Animal Viruses: Ribosomal Protein S6

Discussion of phosphorylation in cells infected with animal viruses, has so far concentrated on viral proteins. However in some cases there are also changes in the phosphorylation of certain cellular proteins, and one of these, ribosomal protein S6, has been found phosphorylated following infection with several different animal viruses. It has been demonstrated that infection with vaccinia virus (Kaerlein and Horak, 1978), herpes simplex virus type 1 (Kennedy et al., 1981) and pseudorabies virus (Kennedy et al., 1981), increases the extent of phosphorylation of S6. Two other reports that infection with adenovirus type 5 (Blair and Horak, 1977) and mengovirus (Rosnitschek et al., 1978) could produce the same effect, are less well documented. However, this effect is not specific for cells infected with the animal viruses mentioned above, since the extent of phosphorylation of S6 can be altered by a wide variety of physiological and pathological stimuli (Leader, 1980). Some of the physiological stimuli (e.g. cyclic AMP) lead to incorporation of 1-2 moles of phosphate per S6 molecule. Other stimuli (e.g. some secretagogues) can cause the conversion of unphosphorylated S6 to derivatives containing 3-4 moles of phosphate per S6 molecule. The most dramatic change, however, occurs in response to growth stimuli. Viral transformation, like growth stimuli, leads to the maximum phosphorylation of S6 to state in

which the protein contains five phosphoryl groups per molecule. The precise extent of phosphorylation of S6 in cells infected with animal viruses has not always been determined, but at least in some cases, the highest phosphorylated derivatives of S6 have been detected (Kennedy et al., 1981).

The phosphorylation of S6 in cells infected with animal viruses has been studied less extensively than in other biological systems. Nevertheless, it is possible to make certain generalisations about the characteristics of this phosphorylation and also to point out important problems which are not yet resolved in any of the biological systems studied.

Identification of some of the sites in S6 which are phosphorylated in vivo has come from analysis of the tryptic phosphopeptides generated from S6 following various stimuli, and from determination of the primary structure of the protein. Analysis of phosphopeptide maps of S6 has suggested that there are no subclasses of sites uniquely associated with the action of a single growth-promoting agent (Trevillyan et al., 1985; Martin-Perez et al., 1984; Blenis et al., 1984). Furthermore, the sites phosphorylated in response to cyclic AMP are identical to some of the sites affected by growth stimuli (Wettenhall and Morgan, 1984; Martin-Perez et al., 1984). Analysis of the primary structure of the region of S6 which is phosphorylated suggests that the phosphorylated sites may be clustered within a 15- residue segment at the COOH - terminus of the molecule: -Ser-Ser-Leu-Arg-Ala-Ser-Thr-Ser-Lys-Ser-Glu-Glu-Ser-Gln-Lys-COOH (Wettenhall and Morgan,1984). The first three serine residues are phosphorylated in vivo, but the identity of the other phosphoserines is uncertain. The clustering of several phosphorylation sites appears to be a general occurrence in phosphoproteins (Picton et al., 1982).

The function of the phosphorylation of S6 has not been established, but in some circumstances there is a suggestive correlation between the extent of phosphorylation of S6 and the rate of protein synthesis. Nevertheless this correlation between phosphorylation and protein synthesis is not always stringent: some stimuli for increased phosphorylation, such as cyclic AMP, have no obvious influence on protein biosynthesis, and most attempts to detect effects of the phosphorylation on the functional activity of 40S subunits in more physiological cell-free systems have been unsuccessful (Leader, 1980). It has been suggested that a role of S6 phosphorylation, at least in some cases, may be related to recruitment of specific mRNAs. In 3T3 cells, ribosomes containing maximally phosphorylated S6 molecules are incorporated preferentially into new polysomes after stimulation of growth by serum (Thomas et al., 1982; Duncan and McConkey, 1982). An increase in S6 phosphorylation correlates with increased protein synthesis in Xenopus oocytes induced to mature with progesterone or insulin, and the increased protein synthesis has been reported to occur largely as a result of mRNA recruitment (Nielsen et al., 1982; Richter et al., 1982; Stith and Maller,1984). Infection of cells with animal viruses results in formation of new polysomes containing viral mRNA (Luria et al., 1978). As mentioned above, in the infected cells where the extent of phosphorylation of S6 was analysed, it was found to be increased compared with the extent of phosphorylation in uninfected cells.

In general, little is known about the mechanisms that control the overall state of phosphorylation of S6. It has been suggested that the growth factors and certain viral transforming proteins have a common initial signal, related to the increase in tyrosine kinase activity, which probably leads to phosphorylation of several cellular proteins (Cooper and Hunter,1983) including S6 (Blenis et al., 1984; Spivack et al.,1984). It has been recently found that vaccinia virus encodes a polypeptide (expressed

during the early stages of infection) which is related to transforming growth factor type 1 (TGF 1) and epidermal growth factor (EGF) (Blomquist et al., 1984; Brown et al.,1985; Reisner, 1985). This finding opens the possibility that the increase in phosphorylation of S6 during the infection with vaccinia virus occurs through the action of this growth factor-like polypeptide. As the vaccinia virus polypeptide is as structurally similar to EGF as it is to TGF, it is believed that, like TGF, it might be able to bind to and activate the EGF-receptor (Brown et al., 1985).

An understanding of the control of the phosphorylation of S6 by various stimuli requires that the enzymes responsible for the phosphorylation/dephosphorylation of this protein be identified. The conditions for a putative S6 kinase(s) to fulfill are at present limited to evidence that the enzyme activity is increased in stimulated cells and that it phosphorylates the same sites in vitro as are phosphorylated under conditions in which it is active in vivo. For the control of phosphorylation by cyclic AMP the conditions described above have been satisfied by the cyclic AMP-dependent protein kinase (Wettenhall et al., 1982). However, as already mentioned, serine residues other than the one or two that serve as substrates for the cyclic AMP-dependent protein kinase are phosphorylated in vivo, suggesting the existence of other S6 kinase(s). In cells infected with animal viruses the kinases and phosphatases involved in S6 phosphorylation have not yet been studied. As a result of analysis in other biological systems, several S6 kinases of uncertain identity have been found to respond to treatment of cells with insulin and growth factors: i) One protein kinase activity from 3T3 cells stimulated by insulin was fractionated on DEAE-cellulose and identified as the activated form of an enzyme termed protease-activated kinase II (Perišić and Traugh, 1983). It has been shown that this enzyme, extensively purified from rabbit reticulocytes, is able to convert unphosphorylated ribosomal S6 to

triphosphorylated forms (Perišić and Traugh, 1983a). The phosphopeptides generated from S6 following phosphorylation by this kinase were found in cells stimulated with insulin (Perišić and Traugh, 1983) and growth factors (Perišić and Traugh, 1985). Many of the characteristics of protease-activated kinase II resemble those of protein kinase C. ii) More recently, a comparison of extracts from quiscent 3T3 cells and cells stimulated with serum has revealed a highly specific S6 kinase activity (Novak-Hofer and Thomas, 1984). This activity, which is 25-fold greater in extracts from cells stimulated with serum, phosphorylates most of the sites on S6 found to be phosphorylated in vivo, as concluded from comparison of phosphopeptide maps. To obtain maximum enzymic activity, phosphatase inhibitors were required in all extraction buffers. This suggests that this particular protein kinase may be modified by phosphorylation, which may be involved in regulating its activity. iii) Analysis of S6 kinase activity in extracts of Xenopus leavis eggs showed another specific S6 kinase with 5- to 10-fold greater activity than in extracts of oocytes (Erikson and Maller, 1985). The phosphopeptides of S6 phosphorylated by this kinase in vitro and the phosphopeptides observed in maximally phosphorylated derivatives of S6 in vivo, were identical.

Clearly, more data are needed to clarify the possible relationship between the S6 kinases described above and to establish how these enzyme(s) are regulated by various stimuli.

2.2 PROTEIN PHOSPHORYLATION IN INFECTION OF CELLS WITH HERPESVIRUSES

This thesis is concerned with the phosphorylation of proteins in cells infected with herpesviruses, and it is therefore necessary to present briefly the general characteristics of these viruses and previous results relevant to protein phosphorylation in the infected cells.

Herpesviruses are among the largest and most complex DNA viruses. Members of the herpes family have linear double stranded genomes ranging in molecular weight from approximately $80x10^6$ to $150x10^6$. This DNA, and the proteins surrounding it, constitute a structural unit termed the viral core. Other structural units are the icosadeltahedral capsid (composed of 162 prismatic capsomers), the tegument (a layer of fibrous material asymmetrically surrounding the capsid), and the envelope which encloses the capsid-tegument structure. The capsid is assembled in the nucleus, with the viral DNA being packed into preformed capsids, and the envelope is acquired from the nuclear membrane in a budding process. The viral envelope appears to be assembled from cellular phospholipids and virus-specific glycoproteins (Roizman, 1982).

Herpes simplex virus type 1 (HSV-1) has long served as the prototype of the herpesvirus family. Viruses closely related to HSV-1 are herpes simplex virus type 2 (HSV-2), pseudorabies virus (PRV), equid herpes virus 1 (EHV-1) and varicella-zoster virus (VZV). Infection of cells with HSV-1 has been studied more extensively than has infection with others, but it seems that the major characteristics, at least, are common to all members of this group (Roizman, 1982).

Productive infection of cells with HSV-1 is accompanied by shut-off of host DNA, RNA and protein synthesis, and the appearance of virus-specific macromolecules. On the basis of the differential transcription patterns of the virus genome at various stages if infection, viral mRNA and proteins

may be subdivided into at least three classes: immediate-early (α), early (β) and late (γ) (Spear and Roizman, 1980). It seems that proteins synthesized earlier during infection (α and some β) have a regulatory role in the viral replicative cycle. However, the functions of only a few such proteins have been identified to date (Wagner,1985). One of the proteins (molecular weight approx. 160,000) has a role in the transition from the first to the second phase of transcription of viral mRNA. Other proteins have been identified as part of the machinery for DNA replication (DNA-polymerase, thymidine kinase, alkaline exonuclease, DNA-binding proteins, ribonucleotide reductase and deoxypyrimidine triphosphatase). Structural proteins are synthesized later during infection.

A role of protein phosphorylation of cells by herpesviruses has been indicated by several lines of experimental evidence. This evidence, like that already described for animal viruses in general (previous section), includes i) the presence of protein kinases in purified virions; ii) the association of protein kinase activity with virally-coded proteins; iii) the phosphorylation of viral and cellular proteins in infected cells.

The virus particles of several members of the herpes family, have, associated with them, a protein kinase activity which can phosphorylate some of the proteins present in the virion (Randall et al., 1972; Tan,1975; Lemaster and Roizman,1980; Flügel and Darai, 1982). The protein kinase activity from HSV-1 particles, located in the capsid-tegument structure, phosphorylated several viral proteins (Lemaster and Roizman, 1980). The major phosphorylated species were polypeptides of molecular weights of 90,000, 55,000 and 35,000. The presence of protein kinase activity in PRV particles has also been demonstrated (Tan, 1975). When the virus was grown in cells labelled with ^{32}Pi, two of the virion proteins (115,000 and 120,000 Da) were phosphorylated (Stevely, 1975).

Protein kinase activity was found associated with one protein coded

by Epstein-Barr virus and one protein of human cytomegalovirus [Introduction, 2.1 (2), Table 7]. As discussed previously, identification of these viral proteins as molecules with intrinsic protein kinase activity has not been established. The function of these proteins in viral infection is not known.

When the phosphorylation of viral proteins was analysed in cells infected with HSV-1, as many as 11 phosphoproteins were detected (Wilcox et al., 1980; Marsden et al., 1978). The major phosphopeptides have molecular weights of about 145,000, 64,000 and 68,000. The protein of molecular weight 145,000 was identified as viral ribonucleotide reductase. Among other proteins phosphorylated to a lesser extent, two were idenitifed as the regulatory protein of early transcription (160,000 Da) and viral DNase (82,000 Da).

As already mentioned [Introduction, 2.1. (4)] one protein of the host cell which is more phosphorylated in cells infected with HSV-1 and PRV, has been identified as ribosomal protein S6 (Kennedy et al., 1981). In addition, in cells infected with HSV-1, another host protein associated with polysomes (48,000 Da) becomes phosphorylated early after infection (Fenwick and Walker, 1979).

The number of proteins which become phosphorylated in infected cells raises the question of the identity of the protein kinase(s) responsible for the phosphorylation. In an attempt to answer this question, changes in protein kinase activities following infection with HSV-1 were analysed (Blue and Stobbes, 1981), and the induction of a new casein kinase activity dependent on the expression of the viral genome, was observed. However, the relation of this enzyme to known cellular casein kinases or to virally-coded proteins has not been examined.

MATERIALS

1. BIOLOGICAL

1.1 CELLS

BHK 21/C13 cells are an established line of Hamster kidney fibroblasts (MacPherson and Stocker, 1962).

1.2 VIRUS

Pseudorabies virus (PRV), a member of the Herpesvirus group, was originally obtained from Kaplan and Vatter (1959) and subsequently passaged in culture.

1.3 RATS

The rats, derived from the Wistar strain, were supplied by the departmental Animal House. The weight of the animals was between 100-150 g.

2. CHEMICAL

2.1 GENERAL

All laboratory chemicals were analytical reagent (ANALAR) grade from British Drug House Ltd., England, except those listed below.

2.2 RADIOCHEMICALS

[^{14}C] amino acid mixture and [^{32}P] orthophosphate were purchased from/

from Amersham International Ltd., England.

[γ -^{32}P] ATP and [γ -^{32}P] GTP were either from Amersham International or synthesised according to Maxam and Gilbert (1980).

2.3 ENZYMES, OTHER PROTEINS AND AMINO ACIDS

The catalytic subunit of cyclic AMP-dependent protein kinase from rabbit muscle : a gift from P.J. Parker (ICRF - London), prepared according to Beavo et al.(1974).

Protein kinase C, homogenous preparation isolated from bovine brain according to Parker et al.(1984) : a gift from P.J. Parker (ICRF - London).

Haemin-regulated inhibitor (HRI) : a gift from Professor H.O. Voorma (University of Utrecht), prepared according to Amesz et al. (1979).

Casein kinase II from pig liver : a gift from O.G. Issinger (University of Saarland).

Trypsin (for Method 10.1) - Sigma Chemical Co., U.S.A.

Trypsin (for Method 7) (L-1-tosylamido-2-phenylethyl-chloromethyl-ketone-treated) : Worthington Biochemical Corporation, U.S.A.).

Viral DNA polymerase (prepared according to Powell and Purifoy, 1977), major DNA-binding protein (prepared according to Powell et al., 1981) and DNase (prepared according to Banks et al., 1985) : gifts from K.L. Powell (University of Leeds).

Calmodulin : Boehringer Mannheim, West Germany.

Heparin : Glaxo Pharmaceuticals Ltd., England.

Mixed histones type 11-A, lysine-rich histones type III-S, histone type V-S, VI-S, VII-S and VIII-S, protamine sulphate, casein and phosvitin/

phosvitin : Sigma Chemical Co., U.S.A.

Soybean trypsin inhibitor : Sigma Chemical Co., U.S.A.

Eukaryotic initiation factor eIF-2 from rabbit reticulocytes: a gift from Professor H.O. Voorma (University of Utrecht), prepared according to Voorma et al.(1979).

Heat-stable inhibitor protein of cyclic AMP-dependent protein kinase, isolated from rabbit muscle : a gift from H.G. Nimmo (University of Glasgow), prepared according to Walsh et al. (1971).

Low molecular weight protein standards : Pharmacia, Sweden.

o-phosphoserine, o-phosphothreonine and o-phosphotyrosine : Sigma Chemical Co., U.S.A.

2.4 NUCLEIC ACIDS AND NUCLEOTIDES

polyI-polyC : Pharmacia, Sweden.

ATP and GTP : P-L Biochemicals Inc., U.S.A.

cyclic AMP and cyclic GMP : Boehringer Mannheim, West Germany.

2.5 OTHER COMPOUNDS

Phosphatidylserine : Sigma Chemical Co., U.S.A.

Spermine : Sigma Chemical Co., U.S.A.

Puromycin : Boehringer Mannheim, West Germany.

2.6 CHROMATOGRAPHIC MATERIAL

DE-52 : Whatman Biochemicals Ltd., England.

DEAE-Sephacel : Pharmacia, Sweden

Blue A : Amicon Co., U.S.A.

Phosphocellulose/

Phosphocellulose P-11 : Whatman Biochemicals Ltd., England.

High-performance hydroxylapatite (HPHT) column (7·8 mm x 100 mm) : Bio-Rad Laboratories, U.S.A.

TSK-GEL G3000SW column (7·5 mm x 600 mm) : Toyo Soda Manufacturing Co., Japan.

Mono Q^{TM} HR 5/5 column (5 mm x 50 mm) : Pharmacia, Sweden.

μBondapak C_{18} column (3·9 mm x 30 cm) : Waters Associates, U.S.A.

Vydac 5 TP C18 column (4·6 mm x 7·5 cm) : HPLC Technology, Ltd., England.

Aquacide (type) II ; Calbiochem Behring Corporation, U.S.A.

Polyethyleneimine (PEI) impregnated cellulose : Macherey-Nagel and Co., West Germany.

2.7 REAGENTS FOR ELECTROPHORESIS

Urea [for Method, 6.1 (3)] : Aristar, British Drug House Ltd., England.

Ampholines pH5·7 and pH3·5-10 : LKB, Sweden.

Acrylamide, N-methylene-bis-acrylamide and ammonium persulphate : Bio-Rad Laboratories, U.S.A.

Agarose (type VI), Coomassie Brilliant Blue R-250 and Coomassie Brilliant Blue G-250 : Sigma Chemicals Co., U.S.A.

NNN'N' tetraethylmethylethylenediamine (TEMED) and Bromophenol Blue : Eastman Kodak Co., U.S.A.

Pyronine Y : G.T. Gurr Ltd., England.

2.8/

2.8 REAGENTS FOR TISSUE CULTURE

Eagle's minimum essential medium MEM, Glasgow modification (described by Eagle, 1959, and incorporates the Glasgow modification of MacPherson and Stoker, 1962), MEM vitamins, MEM amino acids, MEM amino acids minus methionine and bovine calf serum : Gibco Bio-cult, Scotland.

Trypsin and PPLO agar : Difco Laboratories, U.S.A.

Tryptose phosphate broth, blood agar, brain and heart infusion broth, and Sabouraud's medium : Oxoid Ltd., England.

Penicillin and streptomycin : Glaxo Pharmaceuticals Ltd., England.

Phenol Red and Giemsa Stain : BDH Chemicals, England.

L-Glutamine : Sigma Chemical Co., U.S.A.

2.9 PHOTOGRAPHIC MATERIALS AND REAGENTS

DX10 photographic developer, FX40 photographic fixer, X-ray (no screen) film (NS-2T) and X-ray (screen) film (X-omat XAR5) : Eastman Kodak Co., U.S.A.

Cronex intensifying screens and cassettes : du Pont de Nemours & Co. Inc., U.S.A.

2.10 SCINTILLATION REAGENTS

2,5-Diphenyloxazole (PPO), p-bis-[2-(5-phenyloxazole)]-benzene (POPOP) and Protosol (gel solubiliser) : New England Nuclear, U.S.A.

3. COMPOSITION OF STANDARD BUFFER SOLUTIONS

3.1/

3.1 PHOSPHATE BUFFERED SALINE (PBS)

This buffer was prepared according to Dulbecco and Vogt (1954) and contained 0·17 M NaCl, 3·4 mM KCl, 10 mM $NaHPO_4$, 2·4 mM NaH_2PO_4, 0·49 mM $MgCl_2$ and 0·68 mM $CaCl_2$ at a pH of 7·4.

3.2 SCINTILLATION FLUID

(1) For Method 10.1:

66% (v/v) toluene, 33% (v/v) 2-methoxyethanol, 0·4% (w/v) 2,5-diphenyloxazole, 0·01% (w/v) p-bis-[2-(5-phenyloxazolyl)]-benzene.

(2) For Method 6.2 (5)

0·4% (w/v) 2,5-diphenyloxazole and 0·02% (w/v) p-bis-[2-(5-phenyloxazolyl)]-benzene in toluene.

METHODS

1. METHODS FOR CELL CULTURE

1.1 GROWTH OF BHK CELLS

BHK 21/C13 cells were grown as monolayers in rotating 2·5 l roller bottles in 180 ml of Eagle's medium per bottle. This medium contained BSS/B, Eagle's minimum essential medium (Glasgow modification), 2·8 g/l tryptose phosphate broth, 10^5 units/l penicillin, 100 mg/l streptomycin, 100 ml/l bovine calf serum and 40 ml/l 5·6% (w/v) $NaHCO_3$. Cultures were seeded at 2-3 x 10^7 cells per bottle and "gassed" with 100 ml 5% (v/v) CO_2 in oxygen to maintain buffering capacity. The bottles were rotated at 37°C for 3 days, by which time the cells had just reached confluence.

1.2 INFECTION OF BHK CELLS WITH PSEUDORABIES VIRUS

BHK cells grown to confluence for 3 days at 37°C were infected with 20 pfu/cell of PRV in 25 ml of the original medium and the virus allowed to adsorb to the cells for 1 h at 37°C. After this adsorption period the medium was removed and replaced with 50 ml of the original medium and the infection allowed to continue at 37°C until harvesting.

1.3 GROWTH OF PSEUDORABIES VIRUS

PRV stocks were produced by infecting confluent BHK cells at a multiplicity of 1 pfu/300 cells, and, after the initial adsorption period, allowing the infection to continue for a further 3 days. After this time all the cells had been infected and death of the cells

and/

and release of virus had occurred. The bottles were shaken gently to detach remaining cells from the glass surface, and the cell debris removed by centrifugation at 500 g for 10 min. The virus was sedimented by centrifugation at 20,000 g for 90 min in an MSE-18 centrifuge. The sedimented virus was resuspended, plaque assayed, and stored as aliquots at -70°C.

1.4 PLAQUE ASSAY OF PSEUDORABIES VIRUS

Plaque assay was carried out to titre virus stocks and to follow release of virus particles into the medium during infection of BHK cells.

Serial logarithmic dilutions of PRV were prepared in Eagle's medium (lacking serum). 50 mm petri dishes were seeded with 3×10^6 BHK cells, 4 ml Eagle's medium added and the cells allowed to grow in a CO_2 incubator at 37°C. Four plates were used per dilution. After the cells had grown for 24 h the medium was removed and the cell monolayer infected with 0·2 ml of the various virus dilutions or 0·2 ml of Eagle's medium was added as control. After adsorption of the virus to the cells for 1 h, 5 ml of Eagle's medium was added and the incubation continued for a further 2 h. After this time 230 µg heparin was added to each dish to prevent the virus spreading, and the cultures again returned to the incubator for a further 28 h. After this time the medium was removed from the cells and the infected monolayer fixed with 4% formal saline for 10-20 min. After fixing, the monolayer was stained with Giemsa stain for 10-20 min and then washed with distilled water. The plaques were viewed with a plate microscope and counted. Plates containing between 100-200 plaques were chosen and the average

number/

number taken over the 4 dishes was used in calculation of the virus concentration.

1.5 LABELLING OF BHK CELLS

In those experiments in which the cells were labelled with ^{32}Pi, the medium was replaced by 30 ml of one in which the phosphate had been decreased to 1/10 of its normal concentration and from which tryptose phosphate broth had been excluded. After 30 minutes 1-3 mCi [^{32}P] orthophosphate was added per roller bottle, and incubation was continued for a further 3 h before the cells were harvested.

When cells were labelled with [^{14}C] amino acid mixture, the replacement medium contained 1/10 of the normal concentration of amino acids. After 30 min of incubation, 10 μCi of [^{14}C] amino acids was added per roller bottle and cells incubated for another 30 min and harvested.

2. PREPARATION OF SOLUBLE EXTRACT FROM PSEUDORABIES VIRIONS

2.1 ISOLATION OF VIRIONS

Pellets of infected cells (obtained as described in Methods, 1.3) were suspended in 2 vol 1 mM phosphate buffer (pH7·4) and disrupted with four strokes of a Dounce homogenizer. The cytoplasm was then separated from the nuclei by centrifugation at 500 g for 10 min. Samples (2 ml) of the supernatant were layered on 15-40% (w/v) sucrose density gradients (36 ml) made up in 1 mM phosphate buffer. The gradients were centrifuged for 1 h at 53,000 g (20,000 rpm) in the Beckman/

Beckman SW27 rotor. After centrifugation, the diffuse light-scattering band of virions was aspirated with a needle and syringe. This virus suspension was diluted tenfold with 10 mM Tris-HCl (pH7·2) and centrifuged at 53,000 g (20,000 rpm) in the SW27 rotor to sediment the particles.

2.2 EXTRACTION OF PROTEINS FROM VIRUS PARTICLES

Virus particles were suspended in 10 mM Tris-HCl (pH7·5). Aliquots (0·2 ml) containing 2 to 4 mg of protein were adjusted to 10% (v/v) Nonidet P-40 and 0·6 M NaCl. The suspension was allowed to stand at room temperature for 30 min after which it was diluted tenfold either with 10 mM Tris-HCl (pH7·5) or with the same buffer containing 0·6 M NaCl. This diluted material was centrifuged at 10,000 g for 2 h. The supernatant was dialysed overnight against the appropriate equilibration buffer in preparation for either DEAE-cellulose or phosphocellulose column chromatography.

3. PREPARATION OF CELLULAR FRACTIONS

3.1 POST-RIBOSOMAL SUPERNATANT FROM BHK CELLS

BHK cells (either uninfected or infected with PRV) were harvested by scraping into PBS - Phosphate Buffered Saline. At this stage and in all other procedures the temperature was 0-4°C. The cells were sedimented by low-speed centrifugation (500 g), washed in PBS and suspended in buffer containing 10 mM KCl, 1·5 mM magnesium acetate and 10 mM Tris-HCl (pH7·5). The cells were then broken open with a teflon-glass homogenizer (20 strokes) and the ionic composition of the homogenate/

homogenate was adjusted to 125 mM KCl, 5 mM magnesium acetate, 5 mM 2-mercaptoethanol, 25 mM Tris-HCl (pH7·5). The homogenate was sedimented by centrifugation for 30 min at 30,000 g. The post-mitochondrial supernatant was sedimented by ultracentrifugation at 165,000 g (50,000 rpm) for 2·5 h in the Ti50 rotor of a Beckman ultracentrifuge. The post-ribosomal supernatant (containing approximately 5 mg of protein per ml) was dialysed overnight against DEAE-equilibration buffer [20 mM Tris-HCl (pH7·5), 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol] and either subjected to immediate anion-exchange chromatography or shock-frozen in liquid nitrogen and stored at -70°C.

3.2 RIBOSOMES FROM BHK CELLS

For preparation of ribosomes from BHK cells a modified version of the methods described by Ascione and Arlinghaus (1970) and Thomas et al. (1977) was used.

Cell pellets were resuspended in ice-cold Swell Buffer containing 20 mM Tris-HCl (pH7·5), 50 mM KCl, 5 mM $MgCl_2$ and 5 mM $CaCl_2$, and kept on ice for 10 min. An equal volume of Lysis Buffer [0·5 M sucrose, 0·55 M KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM EDTA, 40 mM Tris-HCl (pH7·5), 1% Triton X-100] was then added and cells left on ice for another 5 min. The cells were homogenized with 6 strokes of a teflon-glass homogenizer. The homogenate was sedimented by centrifugation at 500 g for 10 min. Sodium deoxycholate was then added to the supernatant to give a final concentration of 0·5% (w/v). The supernatant was layered over a step gradient of 8 ml of buffer A [0·7 M sucrose in 20 mM Tris-HCl (pH7·6), 100 mM KCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol] on top of 8 ml of buffer B [1·6 M sucrose in 20 mM Tris-HCl (pH7·6), 500 mM KCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol]. The gradients were centrifuged at 96.000/

96,000 g (27,000 rpm) for 16 h at 4°C in the SW27 rotor. Any light material was washed off the pellet and, if required, ribosomes were stored at -70°C.

3.3 RIBOSOMES FROM RAT LIVER

Six to ten rats were killed by cervical dislocation, their livers were then removed and transferred into buffer containing 50 mM Tris-HCl (pH7·6), 25 mM KCl, 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. The final volume of the buffer was adjusted to 3,5 x the liver volume. At this stage and in all subsequent procedures the temperature was 0-4°C. The livers were homogenized with 12 strokes of a teflon-glass homogenizer. Unbroken cells and nuclei were sedimented by centrifugation at 500 g for 10 min and the supernatant futher sedimented by centrifugation at 30,000 g for 30 min. The post-mitochondrial supernatant was then adjusted to 5% (w/v) sodium deoxycholate and layered over 10 ml of 1 M sucrose in 50 mM Tris-HCl (pH7·6), 500 mM KCl, 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. It was then subjected to centrifugation for 5 h at 96,000 g (27,000 rpm) in a SW27 (or SW28) rotor and the pellet was washed and, if required, stored at -70°C.

3.4 RIBOSOMAL SUBUNITS

Ribosomal subunits were prepared by dissociation at high ionic strength using a modification of the method of Leader and Wool (1972).

Ribosomes were suspended at 4°C in 10 mM Tris-HCl (pH7·5), 80 mM KCl and 5 mM $MgCl_2$. The concentration of KCl was adjusted to 500 mM and the supernatant clarified by centrifugation at 100 g for 5 min. To promote separation of the subunits the clarified suspension was incubated/

incubated at 37°C for 15 min with 0·1 mM puromycin and 20 mM 2-mercaptoethanol. Aliquots containing approx. $80A_{260}$ units of ribosomes were then layered directly on to 37 ml of linear 10-30% (w/v) sucrose gradient containing 10 mM Tris-HCl (pH7·6), 300 mM KCl, 5 mM $MgCl_2$ and 20 mM 2-mercaptoethanol. After centrifugation at 96,000 g for 4 h at 28°C in a Beckman SW27 (or SW28) rotor, the gradients were pumped through a Gilford flow-cell (Gilford model 240) and the absorbance at 260 nm was monitored. The subunit fractions were collected and the total 40S and 60S subunits obtained from 6-12 gradients were separately pooled and sedimented at 177,000 g for 16 h at 4°C in a Beckman Ti60 rotor. The pellets were resuspended in a buffer containing 20 mM Tris-HCl (pH7·6), 100 mM KCl, 4 mM magnesium acetate, 7 mM 2-mercaptoethanol and 250 mM sucrose, so that the final concentration of 40S or 60S subunits was $100A_{260}$ units per ml. Aliquots (100 µl) were stored at -70°C.

3.5 EXTRACTION OF PROTEINS FROM RIBOSOMES AND RIBOSOMAL SUBUNITS

Ribosomal proteins were extracted by a modification of the methods described by Sherton and Wool (1972) and Barritault et al. (1976).

For extraction of proteins, ribosomes or ribosomal subunits were suspended in 10 mM Tris-HCl (pH7·6) so that the final concentration was 50-100 A_{260} units per ml. Magnesium acetate was added to give a final concentration of 200 mM. To this was added 2 volumes of glacial acetic acid and the mixture stirred at 0°C for 1 h. Ribosomal RNA was removed by centrifugation at 10,000 g for 10 min (in a Eppendorf microcentrifuge). The supernatant (containing the protein) was removed and the yield of protein increased by re-extraction of the pellet with the 10 mM Tris-HCl (pH7·6)/

(pH7·6)/100 mM magnesium acetate/67% (v/v) acetic acid mixture. The supernatants were pooled, diluted with an equal volume of water, and the protein precipitated with 5 volumes of cold (-20°C) acetone. Proteins were allowed to precipitate overnight at -20°C (or -70°C). The precipitate was then collected by centrifugation at 30,000 g for 30 min.

4. SEPARATION OF PROTEINS BY CHROMATOGRAPHY

4.1 DE-52 AND DEAE-SEPHACEL ANION-EXCHANGE COLUMN CHROMATOGRAPHY

For analytical studies of protein kinases present in the post-ribosomal supernatant of BHK cells, chromatography was on DEAE-Sephacel packed in a column of 2·5 cm x 1 cm (height x diameter) and equilibrated with DEAE-equilibration buffer. Post-ribosomal supernatant containing 4-15 mg protein was applied, the column washed with 25 ml of DEAE-equilibration buffer, and then eluted with 84 ml of a linear gradient of 0-0·4 M KCl in the same buffer at a flow rate of 12 ml per h, 1 ml fractions being collected. Samples (50 µl) of the fractions were assayed for protein kinase activity. The conductivity of the fractions was measured to allow calculation of the salt concentration.

Protein kinases present in the extracts from pseudorabies virions were analysed in the same manner, approx. 4 mg of protein being applied to the column.

For the preparation of the viral-induced protein kinase, chromatography was on pre-equilibrated DEAE-cellulose, DE-52, packed in a 16 x 6 cm column, to which was applied 100-150 mg protein, the yield from/

from approx. 10^9 cells, 6-9 h after infection with PRV. The column was washed with 150 ml buffer and eluted with 500 ml of a linear gradient of 0-0·4 M KCl in the same buffer at a flow rate of 70 ml per h, 6 ml fractions being collected. The peak fractions of the ViPK (approx. 40 ml) were concentrated to 4-5 ml with Aquacide and, if not used immediately, stored at -70°C, under which conditions the activity was stable for several months at least.

4.2 PHOSPHOCELLULOSE COLUMN CHROMATOGRAPHY

Protein kinases in soluble extracts of pseudorabies virions were analysed on a phosphocellulose column under conditions described by Hathaway et al. (1979).

The phosphocellulose column (3·2 x 1 cm) was equilibrated with buffer containing 50 mM Tris-HCl (pH7·5), 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol and 250 mM NaCl. Protein (4 mg), extracted from virions, was applied to the column in 1·5 ml of the equilibration buffer. The unbound material was removed by 12 ml of the same buffer. This was followed by washing with 92 ml of a linear gradient of 0·25-1·25 M NaCl in the equilibration buffer. The column was eluted at a flow rate of 15 ml per h and fractions of 1·3 ml were collected. Aliquots (40 µl) of the fractions were assayed for protein kinase activity. The conductivity of the fractions was measured to allow calculation of the salt concentration.

4.3 CHROMATOGRAPHY ON IMMOBILIZED BLUE-A DYE-LIGAND

A sample of ViPK from the DE-52 column, concentrated by Aquacide, was dialyzed overnight against DEAE-equilibration buffer with 10 mM $MgCl_2$/

$MgCl_2$. A 1-2 ml aliquot (2 mg protein) was loaded on to a 3·5 x 1 cm column of Blue-A, pre-equilibrated with the above buffer. The sample was allowed 30 min to bind to the matrix at zero flow-rate, then washed with 15 ml of starting buffer and eluted with 20 ml of a linear gradient of 0·3-1·5 M KCl in the same buffer at a flow rate of 6 ml per h. Fractions containing the peak of ViPK activity were combined and stored at -70°C.

4.4 SIZE-EXCLUSION CHROMATOGRAPHY ON SEPHADEX G-150

Samples of ViPK obtained after DE-52 chromatography were concentrated by Aquacide and dialysed overnight against buffer containing 20 mM Tris-HCl (pH7·5), 1 mM EDTA, 10 mM 2-mercaptoethanol, 3% (v/v) glycerol and 500 mM KCl. The same buffer was used for equilibration of the column and subsequent elution. A sample containing 0·5 mg of protein in 0·6 ml was loaded on to a pre-equilibrated column (40 x 1 cm) of Sephadex G-150. The elution was performed at a flow rate of 0·5 ml per min and fractions of 0·75 ml were collected. The void volume of the column was determined using Dextran Blue (1 mg). Bovine serum albumin (2 mg), ovalbumin (2 mg) and cytochrome c (2 mg) were used as protein size standards.

4.5 HIGH-PERFORMANCE HYDROXYLAPATITE CHROMATOGRAPHY

Fractions containing ViPK activity, obtained after DE-52 chromatography of BHK cytosol, were concentrated by Aquacide and dialysed against equilibration buffer containing 10 mM sodium phosphate (pH8·0) and 0·03 mM $CaCl_2$. The sample (0·3 mg of protein in 0·5 ml) was applied to a pre-equilibrated column for high-performance hydroxy-

lapatite/

lapatite chromatography. The column was eluted with 60 ml of a linear gradient of sodium phosphate buffer (pH8·0), containing $CaCl_2$. The gradient was from 10 mM sodium phosphate with 0·03 mM $CaCl_2$ to 300 mM sodium phosphate with 0·001 mM $CaCl_2$. The flow rate was 0·5 ml per min and fractions of 1 ml were collected.

4.6 HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY

Concentrated aliquots (1 ml) of ViPK from DE-52 chromatography were applied to a TSK G3000SW column, pre-equilibrated with a buffer containing 20 mM Bis-Tris (pH7·0), 1 mM EDTA, 10 mM 2-mercaptoethanol and 500 mM KCl. The sample was eluted with the same buffer at a flow rate of 0·5 ml per min and 1 ml fractions were collected.

4.7 HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY

The fractions containing the ViPK from 4-5 size-exclusion columns (i.e. the total material originating from a single DE-52 column) were combined and dialysed against DEAE-equilibration buffer from which glycerol had been excluded. After dialysis the sample (approximately 10 ml) was loaded on to a Mono Q column, washed with 10 ml of 0·25 M NaCl in the above buffer, and eluted with 30 ml of a linear gradient of 0·25-0·8 M NaCl at a flow rate of 1 ml per min, 0·5 ml fractions being collected.

4.8 HIGH-PERFORMANCE REVERSED-PHASE CHROMATOGRAPHY

The method for rapid chromatography of histones by high-performance reversed-phase chromatography, described by Gurley et al.(1983), was used to separate histones H1 and H2B from a lysine-rich histone fraction (Type/

(Type V-S) obtained commercially from Sigma.

The histone, 400 μg in 140 μl 0·1% (v/v) trifluoroacetic acid, was applied to a μBondapak C_{18} column. Protein was eluted with a linear gradient of 20-60% (v/v) acetonitrile in 0·1% (v/v) trifluoroacetic acid over 2 h at a flow rate of 1 ml per min. The histones eluting from the column were detected by uv absorbtion at 206 nm and fractions containing histones H1 and H2B were lyophilized and stored at -70°C.

5. SUCROSE DENSITY GRADIENT ANALYSIS

5.1 DETERMINATION OF SEDIMENTATION COEFFICIENT

The sedimentation coefficient of the ViPK was determined on 10-30% (w/v) linear gradients of sucrose in a buffer containing 20 mM Tris-HCl (pH7·5), 1 mM EDTA, 10 mM 2-mercaptoethanol and either 1 M or 0·1 M KCl, as indicated in Fig. 2.23. Concentrated ViPK prepared by DE-52 chromatography was dialysed against DEAE-equilibration buffer minus glycerol and an aliquot (0·2 ml) layered over the 4·6 ml gradient and subjected to centrifugation at 4°C for 18 h at 345,000 g (58,000 rpm) in the SW60 rotor of a Beckman ultracentrifuge. Aldolase (0·2 mg), bovine serum albumin (0·2 mg) and myoglobin (0·4 mg) were subjected to concurrent centrifugation on separate gradients. The gradients were pumped through the flow cell of a Gilford spectrophotometer and the absorbance at 280 nm monitored. Fractions of 0·22 ml were collected and aliquots (40 μl) assayed for protein kinase activity.

6./

6. SEPARATION OF PROTEINS BY GEL ELECTROPHORESIS

6.1 SEPARATION METHODS

(1) One-Dimensional Gel Electrophoresis in the Presence of Sodium Dodecyl Sulphate:

SDS slab gels were prepared and samples subjected to electrophoresis on them by the method of Laemmli (1970).

The dimension of the gel plates was 19 x 16 cm, to fit the BRL-V-16-2 slab apparatus, and the thickness of gels was 1·5 mm or 0·75 mm.

The lower separation gels contained 8·5, 12·5 or 15% (w/v) acrylamide. The ratio of acrylamide : methylene-bis-acrylamide was 30 : 0·8. When optimal separation of proteins of about 30kDa was required, gels containing 15% (w/v) acrylamide, 0·09% (w/v) methylene-bis-acrylamide were used (i.e. the ratio of acrylamide : methylene-bis-acrylamide changed to 30 : 0·18). The concentration of the other components present in the separation gel was as follows: 0·375 M Tris-HCl (pH8·8), 0·1% (w/v) SDS, 0·03% (v/v) TEMED and 0·1% (w/v) ammonium persulphate. The upper stacking gels contained 5% (w/v) acrylamide, 0·13% (w/v) methylene-bis-acrylamide, 0·12 M Tris-HCl (pH6·8), 0·1% (w/v) SDS, 0·03% (v/v) TEMED and 0·2% (w/v) ammonium persulphate.

Samples were mixed with 0·5 volumes of buffer containing 0·05 M Tris-HCl (pH6·8), 4·5% (w/v) SDS, 45% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0·002% (w/v) Bromophenol Blue. The maximum amount of protein in samples was 100 µg and the volume applied was never larger than 120 µl. The protein samples were completely reduced and denatured by heating at 100°C for 2 min.

The/

The electrophoresis buffer contained 0·05 M Tris, 0·192 M glycine and 0·1% (w/v) SDS at a pH of 8·5. The samples were subjected to electrophoresis towards the anode at 10 mA per gel for about 16 h, until the Bromophenol Blue dye marker was about 1 cm from the bottom of the gel. The gels were then processed as described in Methods, section 6.2.

Molecular weights of proteins in SDS gels were determined from a plot mobility versus log (molecular weight), constructed using standards supplied as a low-molecular weight kit from Pharmacia. This contained: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and alpha-lactalbumin (14,400).

(2) Two-Dimensional Kaltschmidt/Wittmann Gel Electrophoresis:

The original method of Kaltschmidt and Wittmann (1970), for separation of ribosomal proteins, was modified as described by Lastick and McConkey (1976). The nomenclature for ribosomal proteins was according to McConkey et al. (1979).

Isolated ribosomal proteins, obtained after precipitation with acetone, were dissolved in buffer containing 8 M urea, 20 mM Tris, 26 mM boric acid, 1 mM EDTA and 5% (v/v) 2-mercaptoethanol, at a pH of 8·2. Generally, 100-200 µg of protein was analysed in each gel.

The first dimension gels were 10 cm long and prepared in glass tubes of 3 mm inside diameter. The gel mixture contained 6 M urea, 4% (w/v) acrylamide, 0·13% (w/v) methylene-bis-acrylamide, 0·2 M Tris, 0·26 M boric acid, 0·01 M EDTA, 0·2% (v/v) TEMED and 0·05% (w/v) ammonium persulphate, at a pH of 8·7. When basic proteins were analysed, electrophoresis was towards the cathode at 3 mA per gel for 3 h. For analysis of acidic proteins electrophoresis was in the opposite/

opposite direction, for 1·5 h at 3 mA per gel. The electrophoresis buffer (pH8·6) contained 0·06 M Tris, 0·078 M boric acid and 3 mM EDTA. At the end of the electrophoresis, gels were removed from the tubes and frozen in hexane at -70°C until further processing. Just before annealing to the second-dimensional gel, they were equilibrated with 6 M urea, 0·35 M acetic acid and 5% (v/v) 2-mercaptoethanol at room temperature for less than 5 min.

The second-dimensional gel (pH4·0) contained 6 M urea, 15% (w/v) acrylamide, 0·47% (w/v) methylene-bis-acrylamide, 0·44 M acetic acid, 25 mM KOH, 0·5% (v/v) TEMED and 0·3% (w/v) ammonium persulphate. Once the first-dimensional gel had been equilibrated, it was annealed at -20°C in contact with the slab gel (7 x 6·5 x 0·3 cm) with a solution containing 6 M urea, 0·35 M acetic acid, 5% (v/v) 2-mercaptoethanol and 1% (w/v) agarose. Electrophoresis in the second dimension was towards the cathode at 8 mA per gel for 16 h in a buffer (pH4·0) containing 93 mM glycine and 13 mM acetic acid.

When resolution of the phosphorylated forms of the basic protein S6 was required, several modifications of the method described above were introduced. Electrophoresis in the first dimension was in gels of 10 cm length and 0·2 cm diameter for 16 h at 100 V, the electrophoresis buffer for the first dimension present in the upper reservoir contained one additional component, 0·04% (w/v) mercaptoethylamine, and the second dimension was on slab gels (19 x 16 x 0·15 cm) at 150 V for 16 h.

(3) Two-Dimensional O'Farrell Gel Electrophoresis:

The procedure for high resolution of basic as well as acidic proteins/

proteins was as described by O'Farrell et al. (1977). The method combines nonequilibrium pH gradient electrophoresis (resolution of proteins with pH3·5-10 Ampholines) - NEPHGE, and sodium dodecyl sulphate slab gel electrophoresis.

Protein (25 µg) obtained after acetone precipitation, was dissolved in 50 µl of buffer containing 9 M urea, 5% (v/v) 2-mercaptoethanol, 2% (v/v) Nonidet P-40 and 0·4% (v/v) Ampholines pH3·5-10 and 1·6% (v/v) Ampholines pH5-7.

The first dimension gels were poured to a height of 12 cm in glass tubes of 2 mm inside diameter. The gel mixture was composed of 9·2 M urea, 2% (v/v) Nonidet P-40, 4% (w/v) acrylamide, 0·1% (w/v) methylene-bis-acrylamide, 5% (v/v) Ampholines pH3·5-10, 0·02% (v/v) TEMED. The lower reservoir was filled with 0·02 M NaOH, and the tubes were placed in the electrophoresis chamber. The samples were loaded and overlayed with 20 µl of the sample buffer diluted with water (1 : 1), and the tubes were filled with 0·01 M phosphoric acid. The upper reservoir was filled with 0·01 M phosphoric acid. Electrophoresis was towards the cathode at 400 V for 4·5 h. At the end of electrophoresis, the NEPHGE gels were removed, equilibrated for 2 h in SDS sample buffer [10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2·3% (w/v) SDS, 0·1% (w/v) Bromophenol Blue and 0·0625 M Tris-HCl (pH6·8)] and frozen at -70°C until second dimension electrophoresis was performed.

The second dimension was on 10% (w/v) acrylamide/0·26% (w/v) methylene-bis-acrylamide SDS slab gels, as described in Methods, 6.1 (1). The first dimensional gel was placed directly over the surface of the SDS separation gel (no stacking gel was used) and electrophoresis/

electrophoresis carried out at 10 mA per gel for 16 h.

Measurements of the pH gradient in the first dimensional gels (NEPHGE) do not give absolute isoelectric points of proteins (unlike isoelectric focusing, where a stable pH gradient is formed). For that reason, proteins of known isoelectric points were used as standards. The mixture of standard proteins, applied on one gel, consisted of 8 µg 3-phosphoglycerate kinase (pI6·4), 10 µg carbonic anhydrase (pI5·85), 5 µg bovine serum albumin (pI4·8), 10 µg actin (pI4·65) and 8 µg soybean trypsin inhibitor (pI4·55).

6.2 PROCESSING OF GELS

(1) Staining of Gels with Coomassie Brilliant Blue:

Gels were stained for 2-3 h at room temperature in 0·1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid. Gels were destained for about 24 h at 37°C with several changes, using a solution containing 10% (v/v) acetic acid and 10% (v/v) methanol.

(2) Silver Staining:

When the Coomassie Brilliant Blue staining revealed that insufficient protein was present for adequate visualisation in this way, the same gels were re-stained using the Bio-Rad silver stain. This method is 10-50 fold more sensitive than staining with Coomassie Brilliant Blue R-250. The Bio-Rad silver stain derived from the method of Merril et al. (1981).

All solutions for silver staining were prepared in deionized water (conductivity less than 1 µmho) and were warmed to 25°C immediately/

immediately prior to use. Staining was performed in a glass vessel (placed in a water bath with a shaker platform) at 25°C.

Gels, 1·5 cm thick, were kept in 40% (v/v) methanol/10% (v/v) acetic acid for at least 60 min and treated with 10% (v/v) ethanol/ 5% (v/v) acetic acid for another 60 min. Gels were than incubated in the presence of Oxidizer (containing potassium dichromate and nitric acid) for 10 min, Silver Reagent (containing silver nitrate) for 30 min, and Developer (containing sodium carbonate and paraformaldehyde) for 3-10 min. Between these steps the gels were washed with deionized water. The reaction was stopped by incubation of the gels in 5% (v/v) acetic acid for 5 min. The gels were stored in deionized water.

(3) Autoradiography of Gels:

Destained gels, containing ^{32}P-labelled proteins, were incubated in destain solution with 3% (v/v) glycerol for 1 h and then dried on to Whatman 3 MM filter paper under suction from an electric vacuum pump. Autoradiography was at -70°C on Kodak X-omat XAR5 film, using an intensifying screen (Cronex).

For some purposes (Methods, section 7) autoradiography was on unprocessed gels. At the end of the run, one gel plate was replaced by plastic cling film and the gel subjected to autoradiography at 4°C for no more than 12 h.

(4) Fluorography of Gels:

Gels containing ^{14}C-labelled proteins were processed according to the method of Bonner and Laskey (1974).

Destained gels were soaked in three changes of dimethyl sulphoxide for at least 30 min each change, and then soaked for 3 h in 22% (w/v) diphenyl/

diphenyl-oxazole in dimethyl sulphoxide. The diphenyl-oxazole was precipitated into the gels by thorough washing under running water for at least 1 h. The gels were then dried and fluorographed on Kodak X-omat SAR5 film at -70°C.

(5) Quantitative Measurements of Radioactivity in Gels:

Proteins of interest, labelled with ^{32}P, were cut out of dried gels using the corresponding autoradiograph as a guide. The excised areas of the gel were reswollen in 7% (v/v) acetic acid, the paper removed, and protein solubilised in 1 ml of 90% (v/v) Protosol (NEN) at 37°C for 16 h. The radioactivity was determined in 9 ml of scintillant in a scintillation spectrometer.

When the amount of radioactivity in the protein bands was high enough, Cherenkov radiation was measured in the areas excised from dried gels with an efficiency of approx. 30%.

7. ANALYSIS OF TRYPTIC PHOSPHOPEPTIDES BY HIGH-PERFORMANCE REVERSED-PHASE CHROMATOGRAPHY

The tryptic mapping was done according to the method described by Mayes and Waterfield (1984).

Ribosomal proteins after labelling with ^{32}P either *in vitro* (Methods, 10.3) or *in vivo* (Fig. 3. 2) were separated by polyacrylamide gel electrophoresis and subjected to autoradiography at 4°C [Methods, 6.2 (3)]. The region of the gel containing phosphorylated ribosomal protein S6 was excised with a scalpel and washed in four changes of 0·5 ml acetone for 15 min each. After evaporation of acetone, the gel was equilibrated with 50 mM ammonium bicarbonate using three successive

0·5/

0·5 ml aliquots for 5 min each. To the last aliquot of ammonium bicarbonate was added 0·1 mg of trypsin (TPCK - Worthington) and the protein digested overnight at room temperature. The sample was then re-digested with a further 0·1 mg of trypsin for 4 h at 37°C. The supernatant was applied to a Vydak TPC18 reversed-phase HPLC column and peptides eluted with a 0-40% (v/v) linear gradient of acetonitrile in 0·1% (v/v) trifluoroacetic acid at a flow rate of 1 ml per min. The Cerenkov radiation of 0·4 ml fractions was measured in a liquid scintillation spectrometer.

8. SEPARATION OF ATP AND Pi BY PEI-CELLULOSE THIN LAYER CHROMATOGRAPHY

This method, described by Cashel et al. (1969), was used for determination of the percentage of ATP and inorganic phosphate (Pi) present in [γ -^{32}P] ATP preparations made according to Maxam and Gilbert (1980). The method was also used for measurements of ATPase activity (Fig. 2.8).

Samples, 1 μl aliquots, were applied to PEI-cellulose plates (10 x 5 cm) prerun in water (approx. 1·5 cm from the bottom), allowed to dry, and chromatography performed with 0·75 M potassium phosphate buffer (pH3·5). In 30 min the buffer travelled about three-quarters of the way up the plate and gave good separation of ATP from Pi (Pi migrated near the front, ATP had an R_F of approx. 0·3). The plates were dried and subjected to autoradiography, and the areas containing ATP and Pi cut out. The relative amounts of radioactive ATP and Pi were determined from measurements of Cerenkov radiation in the excised spots.

9./

9. ANALYSIS OF PHOSPHOAMINO ACIDS BY HIGH-VOLTAGE PAPER ELECTROPHORESIS

Protamine, phosphorylated *in vitro* by ViPK in the presence of [γ-^{32}P] ATP (Methods, 10.1), was analysed for amino acids containing covalently bound ^{32}P. At the end of incubation, the phosphorylation reaction was terminated by adding HCl to a final concentration of 2 M and the sample dialysed overnight against 2 M HCl at 4°C. Protamine (0·3 mg in 0·4 ml of 2 M HCl) was partially hydrolysed by heating at 105°C for 16 h in a sealed evacuated glass ampoule. The hydrolysate was evaporated to dryness in a vacuum desiccator and the residue resuspended in 100 µl of electrophoresis buffer [2·5% (v/v) formic acid and 7·8% (v/v) acetic acid (pH1·85)]. Aliquots of this sample (50 µl) were applied to Whatman 3 MM paper, separately and in a mixture with phosphoamino acid standards. Other samples applied on the chromatography paper were o-phosphoserine (20 µg), o-phosphothreonine (20 µg), o-phosphotyrosine (20 µg) and ^{32}Pi (5 µCi). The paper was wetted with buffer and subjected to electrophoresis towards the anode at 2,500 V for approx. 2 h (until the ^{32}Pi had migrated across the paper) in a high-voltage electrophoresis apparatus. After electrophoresis, the paper was allowed to dry and then cut up for analysis. The portion containing phosphoamino acid markers was stained with a ninhydrin/cadmium acetate solution (Dreyer and Bynum, 1967). The portion containing ^{32}P-labelled samples was subjected to autoradiography using Kodak X-omat XAR5 film and an intensifying screen at -70°C.

10. PHOSPHORYLATION REACTION IN VITRO

10.1/

10.1 MEASUREMENT OF PROTEIN KINASE ACTIVITY

Protein kinases were assayed by measuring the incorporation of ^{32}P from [γ-^{32}P] ATP into various protein substrates. The procedure followed in principle the method of Corbin and Reiman (1974).

The standard assay mixture for protein kinase activity contained in a total volume of 0·12 or 0·25 ml: 20 mM Tris-HCl (pH7·4), 50 mM KCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0·1 mM ATP containing 1-3 μCi [γ-^{32}P] ATP, and either protamine sulphate (0·8 mg per ml), mixed histones (0·8 mg per ml) or dephosphorylated casein (0·8 mg per ml) as a protein substrate. Assays for cyclic AMP-dependent protein kinase contained 10 μM cyclic AMP.

A different assay mixture for protein kinase C was employed and this contained in a total volume of 0·12 ml : 1·25 mg/ml lysine-rich histone (enriched for histone H1), 6·25 mM Hepes (pH7·5), 12·5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0·5 mM EGTA, 0·25 mM EDTA, 0·125 mg/ml bovine serum albumin, 0·05 mM ATP containing 1-3 μCi [γ-^{32}P] ATP, 1 mM $CaCl_2$, and 0·5 mg/ml phosphatidyl serine. Phosphatidyl serine was prepared by the method of Parker et al. (1984). Phosphatidyl serine [10 mg/ml in chloroform (95%)/methanol (5%)] was concentrated by drying 70 μl under a stream of nitrogen followed by redissolving the residue in 30 μl of 10 mg/ml phosphatidyl serine (in chloroform/methanol). This solution was dispersed in 470 μl 20 mM Hepes (pH7·5) using a Kontex Sonicator.

When kinase C was activated by limited proteolysis, the reaction mixture for measuring enzyme activity contained the same components present in the assay for native kinase C but $CaCl_2$ and phospholipid

were/

were excluded. Proteolytic activation of kinase C was in a reaction mixture containing : 10 mM Tris-HCl (pH8·0), 5 mM 2-mercaptoethanol, 2 mg/ml bovine serum albumin, 2-8 units of kinase C and 4 μg/ml of trypsin (Sigma). Incubation was for 3 min at 30°C and the reaction was terminated by the addition of 16 μg (10 x molar excess) of soybean trypsin inhibitor.

Aliquots of samples in which protein kinase activity was measured were added to the reaction mixture (40 μl to a 120 μl reaction mixture or 50 μl to a 250 μl reaction mixture) and incubation carried out at 30°C. After 30 min, or 15 min for assay of kinase C, the reaction was terminated by removing 100 μl from assay tubes and applying this on to Whatman 3 MM paper discs. The discs were then washed in two changes of 20% (w/v) trichloroacetic acid and four changes of 10% (w/v) trichloroacetic acid for 15 min each, and rinsed in 100% ethanol before being dried and their radioactivity measured by liquid scintillation spectrometry.

10.2 MEASUREMENT OF DEPHOSPHORYLATION OF PROTAMINE

For some experiments (Results, section 2.2) it was important that the preparations of ViPK were free of protein phosphatase activity, at least under the conditions employed to assay the kinase activity.

The preparations of ViPK were incubated under the conditions used to assay enzyme activity (Methods, section 10.1) but with the following changes: (1) protamine was replaced with ^{32}P-phosphoprotamine prepared by incubation with ViPK, phosphoprotamine being precipitated from the reaction mixture with 5 volumes of acetone and protein collected by centrifugation/

centrifugation; (2) [γ-^{32}P] ATP was omitted.

The radioactivity of protamine (measured as in Methods, section 10.1) incubated with the preparation of ViPK was compared with the value obtained from a similar reaction mixture but without added ViPK.

10.3 PHOSPHORYLATION OF RIBOSOMAL SUBUNITS IN VITRO

Ribosomal subunits were prepared as described in Methods, section 3.4. The assay conditions for phosphorylation or ribosomal subunits were as described in Methods, section 10.1, but with some modifications.

The standard reaction mixture contained 20 mM Tris-HCl (pH7·4), 100 mM (or higher) KCl, 4 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 0·1 mM ATP.

The reaction mixture for native kinase C contained 6·25 mM Hepes (pH7·5), 150 mM KCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0·5 mM EGTA, 0·25 mM EDTA, 0·125 mg per ml bovine serum albumin, 0·15 mM $CaCl_2$, 0·5 mg per ml phosphatidyl serine, and 0.05 mM ATP.

Assay conditions for proteolytically activated kinase C were as for the native enzyme but $CaCl_2$ and phosphatidyl serine were excluded. Proteolytic activation of kinase C was as described in Methods, section 10.1.

For phosphorylation of 40S ribosomal subunits by the catalytic subunit of cyclic AMP-dependent protein kinase (Materials, 2.3), the reaction mixture was according to Wettenhall and Cohen (1982) and contained 15 mM Tris-HCl (pH7·2), 15 mM Mops (pH7·2), 25 mM KCl, 5 mM $MgCl_2$, 0·1 mM EGTA, 1 mM dithiothreitol and 0·1 mM ATP.

Samples for subsequent analysis by one-dimensional sodium dodecyl sulphate/

sulphate gel electrophoresis contained 3 μCi [γ-^{32}P] ATP in a reaction volume of 40-120 μl; those for analysis by two-dimensional gel electrophoresis contained 30 μCi [γ-^{32}P] ATP in a reaction volume of 800 μl.

The quantities of enzyme and ribosomal subunits, and the time of incubation are indicated in the figure legends of individual experiments.

For separation of phosphorylated ribosomal proteins by one-dimensional gel electrophoresis in the presence of SDS, the reaction was terminated by addition of an aliquot of the sample buffer [Methods, section 6.1 (1)]. When two-dimensional gel electrophoresis, 20 mM Tris-HCl (pH7·5) and 0·1 mM ATP were added to the mixture, and the ribosomes sedimented in a SW60 Beckman rotor for 3 h at 260,000 g (50,000 rpm). They were then processed as described in Methods, sections 3.5 and 6.1 (2).

10.4 PHOSPHORYLATION OF VIRAL PROTEINS IN VITRO

Protein was extracted from pseudorabies virions as described in Methods, section 2.2. Viral non-structural proteins, the major DNA-binding protein, DNA-polymerase and DNase had been partially purified from cells infected with herpes simplex virus type 1 (Materials, section 2.3).

When proteins in a soluble extract from pseudorabies virions were phosphorylated by exogenous kinase, kinases present in the extract were inactivated by preincubation at 60°C for 15 min. Quantities of viral proteins and enzymes in the assay mixture are indicated in the figure/

figure legends of individual experiments. Phosphorylation of viral (structural or non-structural) proteins was under standard conditions (Methods, section 10.1) in a reaction volume of 120 µl. At the end of the incubation (30 min at 30°C), the reaction was terminated by applying 100 µl aliquots on to Whatman 3 MM paper discs or by addition of sample buffer for SDS gel electrophoresis.

10.5 PHOSPHORYLATION OF eIF-2 IN VITRO

Preparations of eukaryotic initiation factor eIF-2, haemin-regulated inhibitor (HRI) and the casein kinase II are described in Materials, section 2.3.

The reaction mixture for phosphorylation of eIF-2 by HRI contained, in a final volume of 25 µl, 20 mM Hepes-KOH (pH7·6), 10 mM 2-mercapto-ethanol, 0·1 mM spermine, 120 mM potassium acetate, 2 mM magnesium acetate, 0·025 mM ATP containing 2 µCi of [γ-^{32}P] ATP, 1 µg of HRI and 0·8 µg of eIF-2. When eIF-2 was phosphorylated by casein kinase II the concentration of $MgCl_2$ was 10 mM. ViPK was assayed for phosphorylation of eIF-2 under conditions described for HRI and also under conditions for casein kinase II.

Incubation was at 37°C for 15 min and the reaction was terminated by addition of sample buffer for SDS gel electrophoresis.

10.6 PHOSPHORYLATION OF DIFFERENT HISTONE FRACTIONS IN VITRO

Various commercial (Sigma) histone fractions, 0·8 mg per ml, were phosphorylated under standard conditions (Methods, section 10.1) in a reaction volume of 120 µl. At the end of the incubation (30 min

at/

at 30°C), a 40 µl aliquot of reaction mixture was mixed with sample buffer for SDS gel electrophoresis and analysed as described in Methods, section 6.1 (1).

11. DETERMINATION OF THE CONCENTRATION OF PROTEIN

The concentration of protein was measured by the dye binding assay of Bradford (1976) modified by Spector (1978).

Coomassie Brilliant Blue G-250 solution (1 ml), at a concentration of 0·01% (w/v) in 0·95% (v/v) ethanol and 8·5% (w/v) phosphoric acid, was mixed with an aliquot of the protein solution (containing up to 10 µg of protein) and the absorbance at 595 nm measured in a spectrophotometer. A standard curve was constructed using known amounts of bovine serum albumin.

RESULTS

1. PROTEIN KINASES IN BHK CELLS INFECTED WITH PSEUDORABIES VIRUS

1.1 CHARACTERIZATION OF PROTEIN KINASES

In order to identify protein kinases that might be involved in the phosphorylation of proteins during infection of BHK cells with PRV an attempt was made to examine as many as possible of the different protein kinase activities present in infected cells. To analyse protein kinase activities, the cytosol fraction, where most of the new phosphorylation was observed, was chosen. The cytosol fraction was first fractionated by DEAE-cellulose chromatography and the column fractions were then assayed for phosphorylation of several commonly used substrates, including histone, protamine, casein and phosvitin. In addition, the fractions collected were tested for protein kinase activity under the various conditions known to be required for individual protein kinases that have been characterized in other cell types.

With casein as a substrate, two peaks of protein kinase activity were partially resolved (Fig. 1.1 A). These two peaks have chromatographic properties similar to those previously described for casein kinase I and casein kinase II (Hathaway and Traugh, 1982). Both peaks also phosphorylated phosvitin, another (acidic) substrate for casein kinase I and II. The identification of the second activity as casein kinase II was supported by its ability to use GTP as well as ATP as a phosphoryl donor, and the inhibition of its activity by 0·5 µg/ml heparin (not shown).

With the basic proteins, mixed histone or protamine, as substrate

several protein kinase activities were detected (Fig. 1.1 B). For their identification further characterization was required.

Two of the peaks, eluting at 30 mM and 130-160 mM KCl, phosphorylated both histone and, to a lesser extent, protamine. Both kinase activities were stimulated by 10 μM cyclic AMP and inhibited by the rabbit muscle heat-stable inhibitor of the catalytic subunit of the cyclic AMP-dependent protein kinase (Fig. 1.1 C). These properties are consistent with the identification of the two peaks as the type I and type II holoenzymes of the cyclic AMP-dependent protein kinase (Nimmo and Cohen, 1977).

Other peaks in the column fractions corresponded to protein kinase activities that phosphorylated only protamine or protamine in preference to histone. The first such peak of protamine kinase activity eluted at 50-80 mM KCl (Fig.1.1 B). A protamine kinase with similar chromatographic properties to this activity is protein kinase C (Inoue et al., 1977), a Ca^{2+} and phospholipid-dependent enzyme that is thought to be activated in vivo by diacylglycerol, which lowers the Ka of the enzyme for Ca^{2+} (Nishizuka, 1983). The column fractions were therefore assayed for protein kinase C activity using a lysine-rich histone fraction enriched in histone H1 (the best histone substrate for protein kinase C and, unlike protamine, dependent on Ca^{2+} and phospholipid for phosphorylation) in the presence or absence of phosphatidyl serine and Ca^{2+}, at a concentration of the latter (1 mM) which activates protein kinase C in the absence of diacylglycerol. It can be seen from Fig. 1.1 D that the first peak of protamine kinase activity also exhibited Ca^{2+} and phospholipid-dependent protein kinase activity with the lysine-rich histone fraction as substrate, and it was demonstrated by polyacrylamide gel electrophoresis that the

incorporation was into histone H1 (results not shown), thus supporting the identification of the kinase as protein kinase C.

The second peak of protamine kinase activity eluted at 150 mM KCl and partially copurified with cyclic AMP-dependent protein kinase type II (Fig. 1.1 B). Better separation of these two activities was achieved on DEAE-Sephacel columns (Fig. 1.2). Many workers who have studied the protein kinase activities of the supernatant fraction have observed elution of cyclic AMP-dependent protamine or histone kinase activity at a similar or slightly higher salt concentration than was required to elute cyclic AMP-dependent protein kinase type II. One such protein kinase activity was further purified and characterized in bovine brain (Takai et al., 1977). It has been shown that this protein kinase (kinase M) was generated from kinase C by Ca^{2+}-dependent proteolysis. Kinase M is not dependent on Ca^{2+} and phospholipid for activity and has a molecular weight of about 60,000. The apparent molecular weight of the activity from BHK cells is approx. 50,000 on Sephacryl S-100 (Watson, 1985), consistent with the possibility that it represents a proteolytic fragment of protein kinase C. Often the yield of this second protamine kinase activity did, in fact, appear to be inversely related to that of the first (protein kinase C); however, a complicating factor here was the selective loss of protein kinase C activity when too dilute a sample was applied to the column (see below).

The other clear peak of protamine kinase activity (Fig. 1.1 B, C and D) eluted at 200-240 mM KCl, also phosphorylated mixed histone, although to a lesser extent. This protein kinase did not immediately appear to correspond to any of the well-characterized protein kinases of animal cells.

Fig. 1.1 DE-52 Anion-exchange Chromatography of Protein Kinases from BHK Cells Infected with PRV

BHK cells were infected with PRV, harvested 6 h later, post-ribosomal supernatant prepared and subjected to column chromatography on DE-52, as described in Methods, sections 3.1 and 4.1. Column fractions (50 µl aliquots) were assayed for protein kinase activity with various substrates:

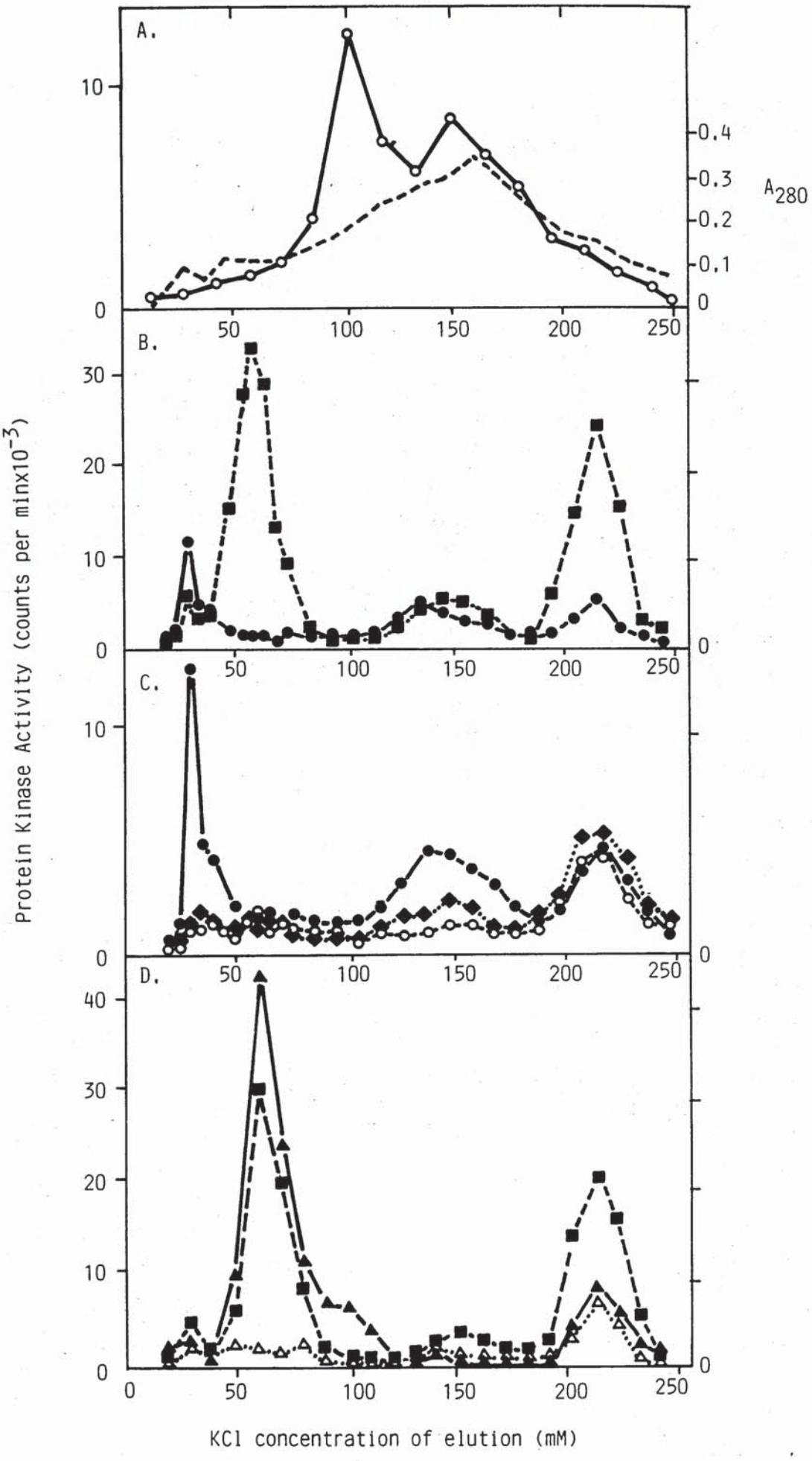
(A) Casein as substrate (—○—), A_{280} (– – –);

(B) Protamine as substrate (– –■– –), mixed histone as substrate in the presence of 10 µM cyclic AMP (—●—);

(C) Mixed histone as substrate alone (– –○– –), or in the presence of cyclic AMP (—●—), or cyclic AMP and heat-stable inhibitor (··· ◆ ···);

(D) Protamine as substrate (– –■– –), lysine-rich histone as substrate, alone (···· Δ ····), or in the presence of 1 mM $CaCl_2$ and 500 µg/ml phosphatidyl serine (—▲—).

The different frames represent different experiments, performed in an identical manner. The position of the (alternate) column fractions assayed has therefore been plotted in terms of KCl concentration (estimated from conductivity measurements) to allow better comparison.


A.
B.
C.
D.
Protein Kinase Activity (counts per minx10^{-3})
A_{280}
KCl concentration of elution (mM)

1.2 EFFECT OF VIRAL INFECTION ON PROTEIN KINASES IN THE CYTOSOL OF BHK CELLS: INDUCTION OF A NEW KINASE ACTIVITY

The protein kinase activities described in Fig. 1.1 were compared in samples from uninfected cells and cells infected with PRV for different lengths of time. The results are presented in Fig. 1.2, 1.3 and 1.4.

One clear difference was that the protamine and histone kinase activity eluting from DE-52 at 200-240 mM KCl, was only found in infected cells (Fig. 1.2 A and B). This activity was therefore designated viral-induced protein kinase (ViPK).

The profiles of elution from DEAE-Sephacel of other protein kinase activities, including the two isoforms of cyclic AMP-dependent protein kinase (Fig. 1.2 A and B), a presumed proteolytic fragment of kinase C (Fig. 1.2 A and B), and casein kinase I and II (Fig. 1.3) did not change significantly during viral infection. However, in the experiment from which these data are taken the total activity of protein kinase C was very low, and, because it was important (see Discussion) to monitor the effect of infection on all the protamine kinases, the 0 and 6 h time points were repeated on a larger scale so that more protein (15 mg rather than 4·5 mg) could be applied to the same size of column, hence avoiding the loss of protein kinase C referred to above. It can be seen from Fig. 1.4 that there was no decrease in the total assayable protamine kinase identified as protein kinase C on the induction by viral infection of quantitatively comparable ViPK protamine kinase activity.

The quantitative comparison of protein kinases during viral infection was based on the enzyme activity eluted from DEAE-Sephacel measured <u>in vitro</u> in the presence of an excess of the respective

regulatory molecule (if one was required). The values so obtained do not necessarily represent a quantitation of active enzyme in the intact cell, as this depends on the intracellular concentration of regulatory molecules or on other conditions. Different methods [such as that described by Litvin *et al.*(1984) for cyclic AMP-dependent protein kinases] are required to determine the enzyme activity in the intact cell. The data presented here more likely reflect, although they cannot be taken as a measure of, the amount of the enzyme present in the cytosol of BHK cells. (The determination of the amount of an enzyme requires the quantitation of enzyme molecules.)

Fig. 1.2 DEAE-Sephacel Column Chromatography of Protein Kinases from BHK Cells Infected with PRV for Different Lengths of Time

I Phosphorylation of Histone and Protamine

(A) BHK cells were infected with PRV virus and harvested after 0, 2, 4, 6, 8 and 10 h. Post-ribosomal supernatant containing 4·5 mg protein from each condition was subjected to chromatography on DEAE-Sephacel, as described in Methods, sections 3.1 and 4.1. Protein kinase activity in the column fractions was determined using as substrate: protamine (—■—), or mixed histones in the presence of 10 μM cyclic AMP (– –O– –). The KCl gradient (← – –) was estimated from conductivity measurements.

(B) The central fractions of histone and protamine kinase activities from A, were separately pooled and reassayed.

(—·-△-·—) cyclic AMP-dependent protein kinase type I

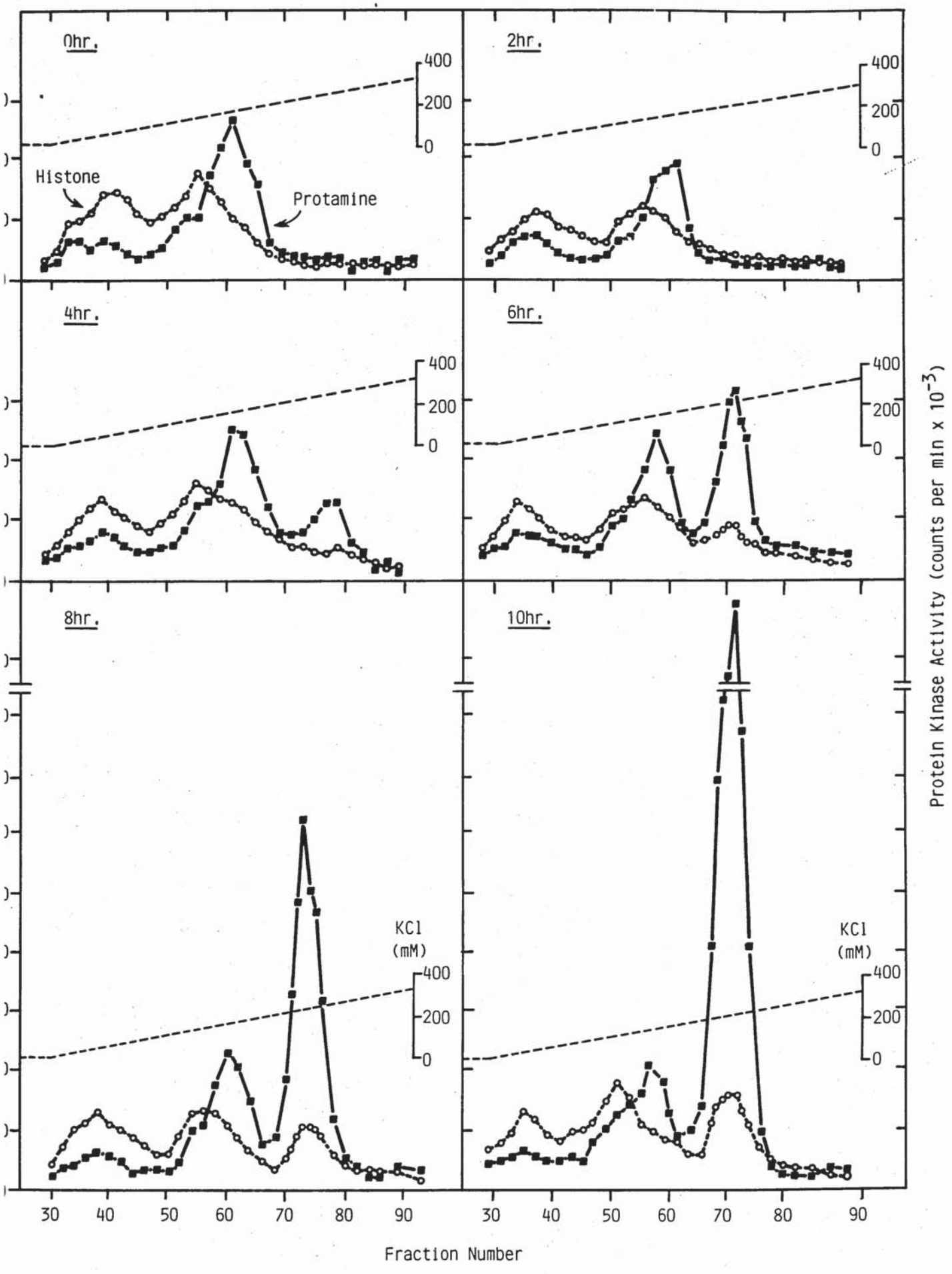
(··· ▲ ···) cyclic AMP-dependent protein kinase type II

(– –O– –) protamine kinase (eluted from DEAE-cellulose column at approx. 150 mM KCl)

(– –■– –) ViPK assayed for phosphorylation of histone

(——●——) ViPK assayed for phosphorylation of protamine.

This figure is presented on page 82 .


A
0hr.
2hr.
4hr.
6hr.
8hr.
10hr.
Histone
Protamine
KCl
(mM)
400
200
0
30
40
50
60
70
80
90
Fraction Number
Protein Kinase Activity (counts per min x 10^{-3})

B

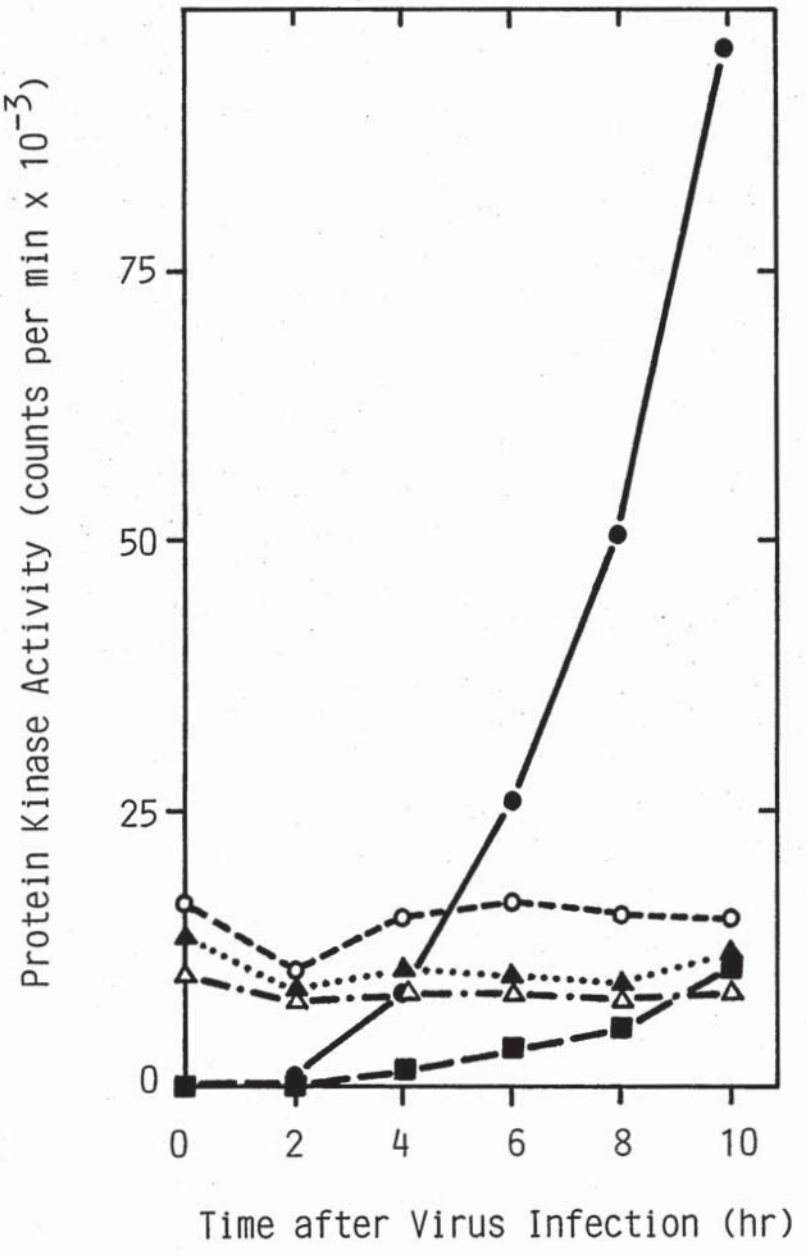

Protein Kinase Activity (counts per min x 10^{-3})
75
50
25
0
0
2
4
6
8
10
Time after Virus Infection (hr)

Fig. 1.3 DEAE-Sephacel Column Chromatography of Protein Kinases from BHK Cells Infected with PRV for Different Lengths of Time

II Phosphorylation of Casein

The column fractions from the experiment described in the legend to Fig. 1.2 A were assayed for protein kinase activity with casein as substrate.

(A) DEAE-Sephacel chromatography of the casein kinase activities of the post-ribosomal fraction from uninfected cells.

(B) The total activity in the hatched areas was estimated for each time point of infection and taken as a measure of casein kinase type I (—○—) and casein kinase type II (—●—).

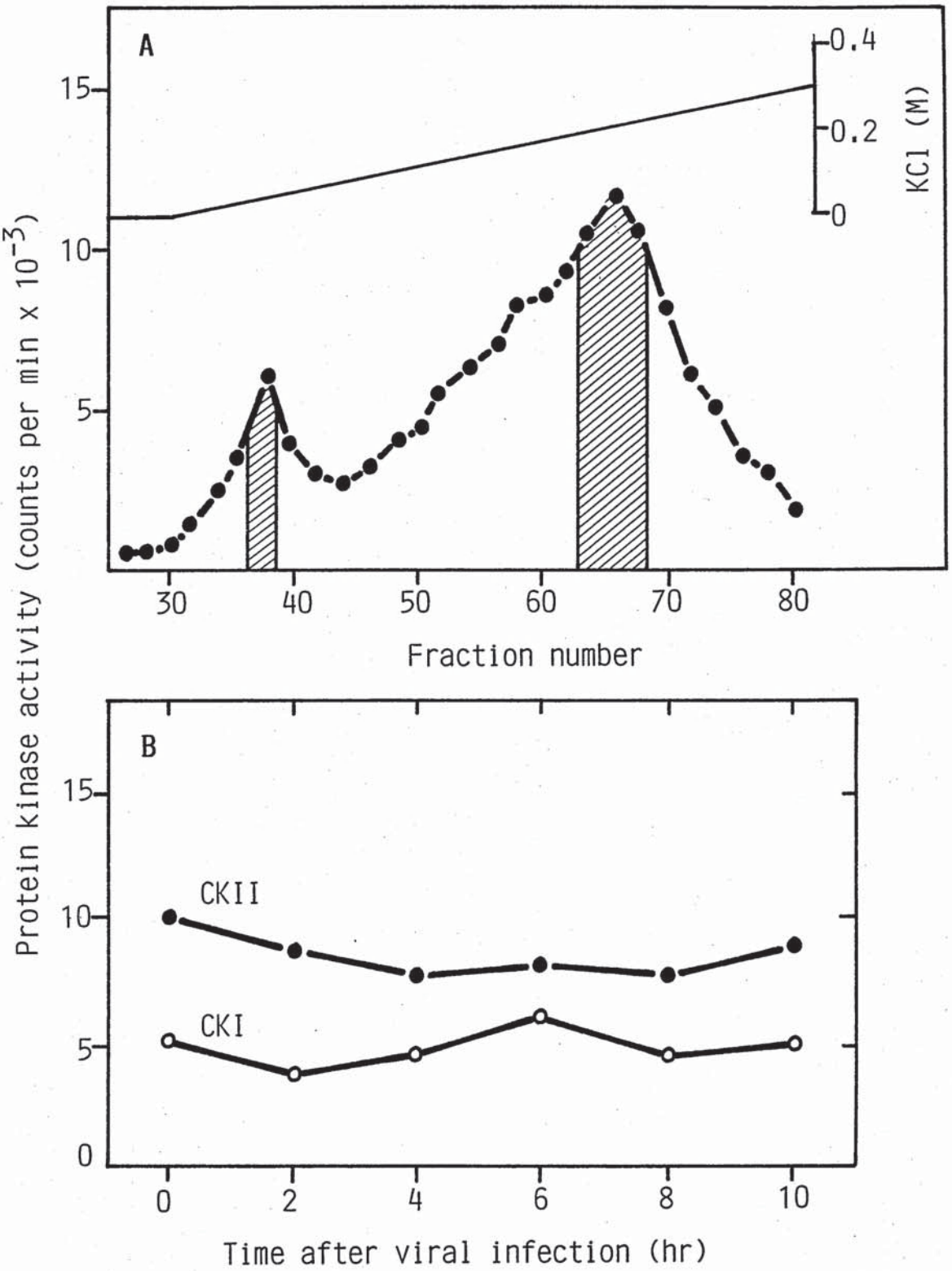
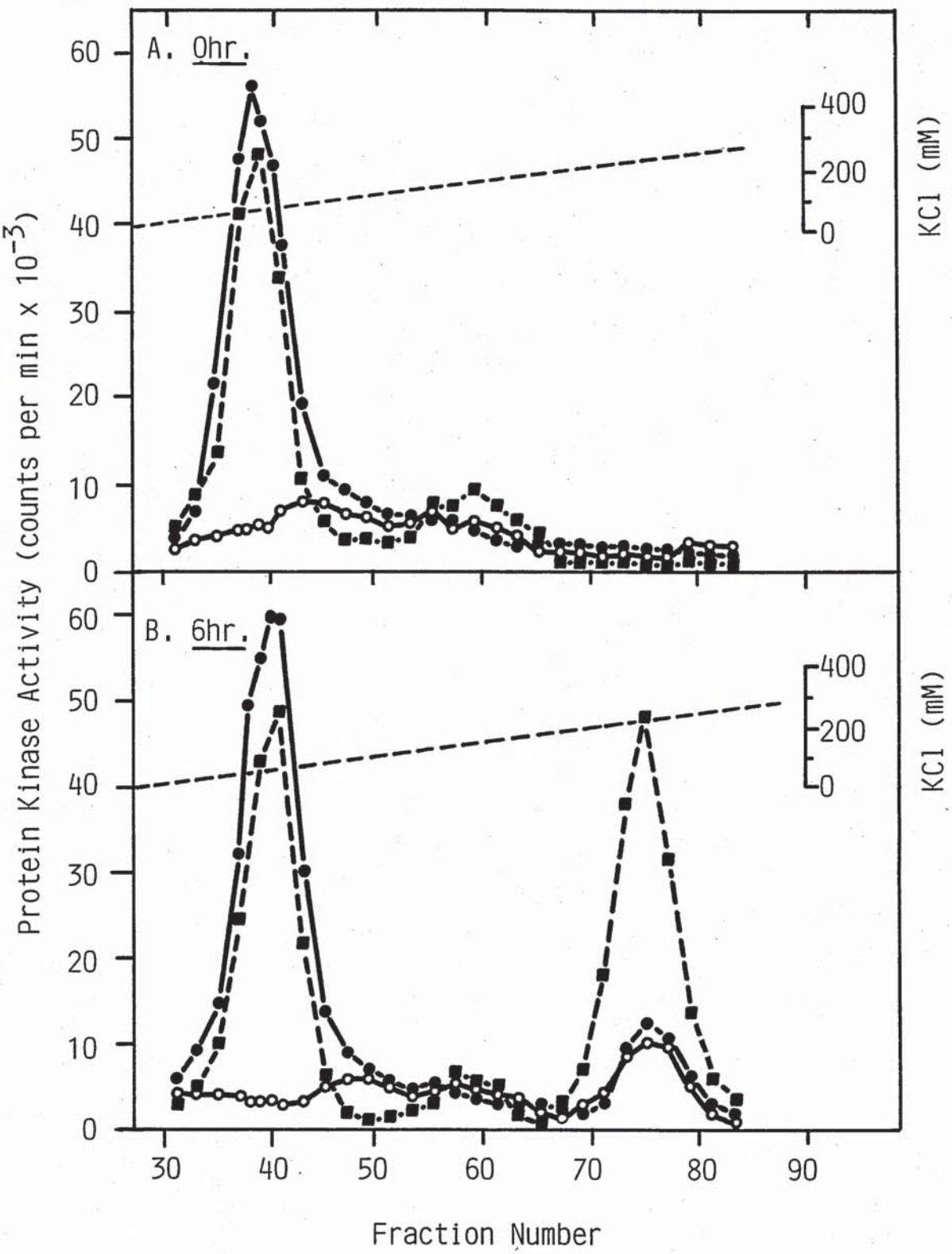

A
B
15
10
5
0
0.4
0.2
0
KCl (M)
30
40
50
60
70
80
Fraction number
CKII
CKI
0
2
4
6
8
10
Time after viral infection (hr)
Protein kinase activity (counts per min x 10^{-3})

Fig. 1.4 DEAE-Sephacel Column Chromatography of Protein Kinase C Before and After Infection of BHK Cells with PRV

BHK cells were infected with pseudorabies virus and harvested after (A) 0 h or (B) 6 h. Post-ribosomal supernatant containing 15 mg protein from each condition was subjected to chromatography on DEAE-Sephacel as described in Methods, section 4.1. Protein kinase activity in column fractions was determined using as substrate: protamine (--■--), lysine-rich histone, alone (—○—) or in the presence of 1 mM $CaCl_2$ and 500 µg/ml phosphatidyl serine (—●—).


A. 0hr.
B. 6hr.
Protein Kinase Activity (counts per min x 10^{-3})
KCl (mM)
400
200
0
60
50
40
30
20
10
0
30
40
50
60
70
80
90
Fraction Number

1.3 APPEARANCE OF ViPK DURING THE VIRAL REPLICATIVE CYCLE

It can be seen from Fig. 1.2 that ViPK was first detectable approx. 4 h after infection, and continued to increase throughout the remaining 6 h period studied. The time-scale of events which characterize the stages of virus infection is rather variable and depends on the conditions of infection which are not always easy to control. For that reason the time-course of appearance of ViPK was compared directly (i.e. in the same experiment) with other processes specific for cells infected with herpes viruses. The following were studied: (i) appearance of ViPK; (ii) release of progeny virus from infected cells; (iii) protein synthesis and (iv) induction of viral DNA-polymerase activity.

The amount of virus released from the cells at different times after infection is shown in Fig. 1.5. The progeny virus first appeared in the medium after approx. 4 h (the "lag period" of the viral replicative cycle). This was followed by rapid release of virus during the next 6 h studied (the period of "exponential growth"). Under similar conditions of infection in separate work (Kennedy, 1982), the accumulation of viral particles started to reach a plateau at about 12 h after infection.

PRV, in common with some other herpes viruses, inhibits synthesis of most of the host proteins during the period in which synthesis of viral functional and structural proteins takes place. As shown in Fig. 1.6, a decline in the synthesis of some polypeptides present in uninfected cells could first be detected at approx. 6 h after infection (e.g. polypeptides of 15-20,000 Da). Appearance of new

proteins in infected cells could clearly be seen 8 and 10 h after infection (e.g. polypeptides of 85-120,000 Da, a polypeptide of 40,000 and polypeptides of 30-35,000 Da). Some of these polypeptides can be identified as viral structural proteins which are known to appear in cells at later stages of infection ("late" or "γ" proteins).

Viral DNA-polymerase is a well-characterized enzyme coded by the virus genome and belongs to the group of "early" or "β" proteins (synthesized after the "immediate early" or "α" proteins). DNA-polymerase molecules appear in infected cells after a lag period, and their synthesis continues for the next several hours (Ben-Porat and Kaplan, 1985). The time-course of induction of DNA-polymerase activity observed in this experiment is shown in Fig. 1.5.

Taking all these results into consideration, one can conclude that the appearance of ViPK in infected cells occurs after the "lag period" of the viral replicative cycle and is roughly comparable with the kinetics of induction of one viral early (β) protein - DNA polymerase.

Fig. 1.5 Time-course of Appearance of ViPK: Correlation with Induction of Viral DNA Polymerase and Release of Virus Particles

BHK cells were infected with PRV as described in the legend to Fig. 1.2.

ViPK (—●—) was assayed in the DEAE-Sephacel column fractions, shown in Fig. 1.2. Fractions containing the enzyme from different time points after virus infection were separately pooled and re-assayed for phosphorylation of protamine.

DNA-polymerase activity (--○--) was measured in cytosol fractions, prepared as described in Methods, section 3.1, according to the method of Powell and Purifoy (1977).

The release of virus particles into the medium (····■····) was measured as follows: Aliquots of medium from BHK cell cultures, infected for various times with PRV, were removed before harvesting. The amount of viable virus in the medium from each time point was determined by plaque assay (Methods, section 1.5).

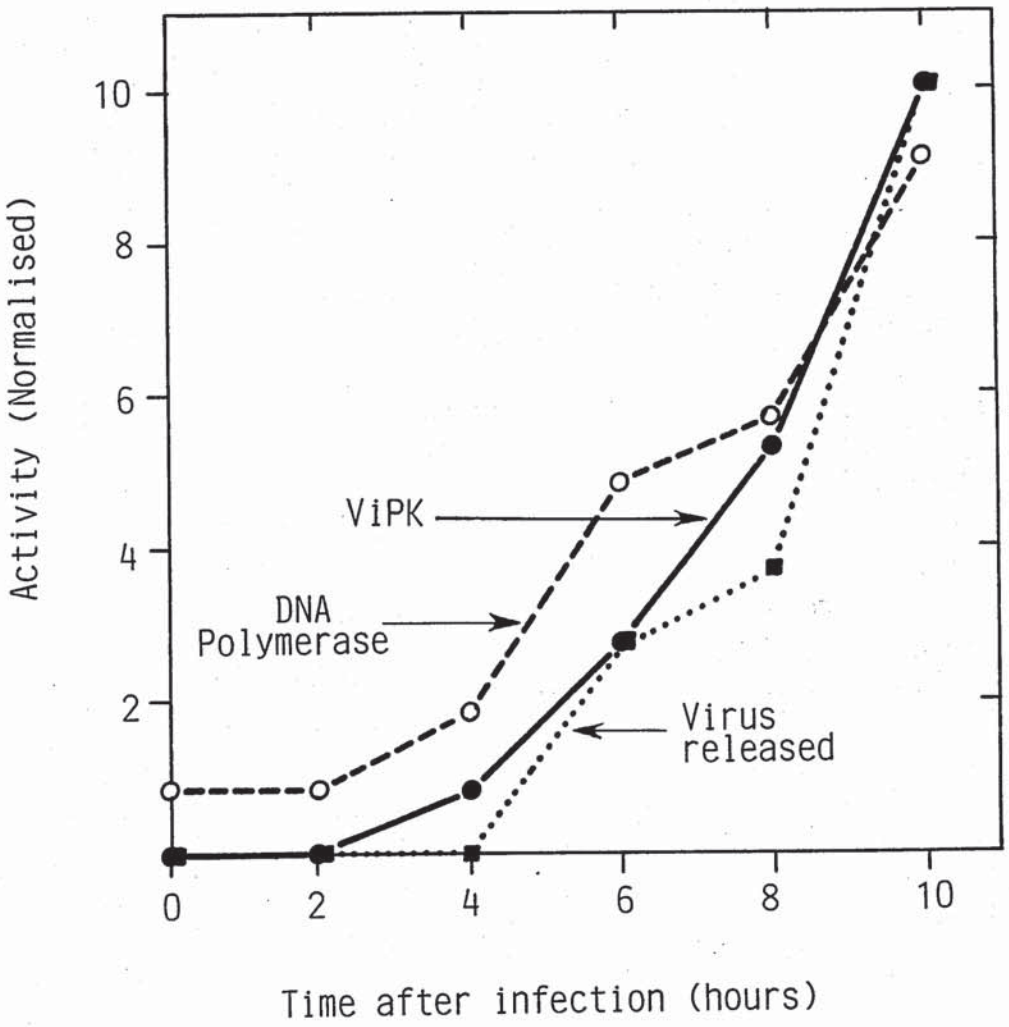
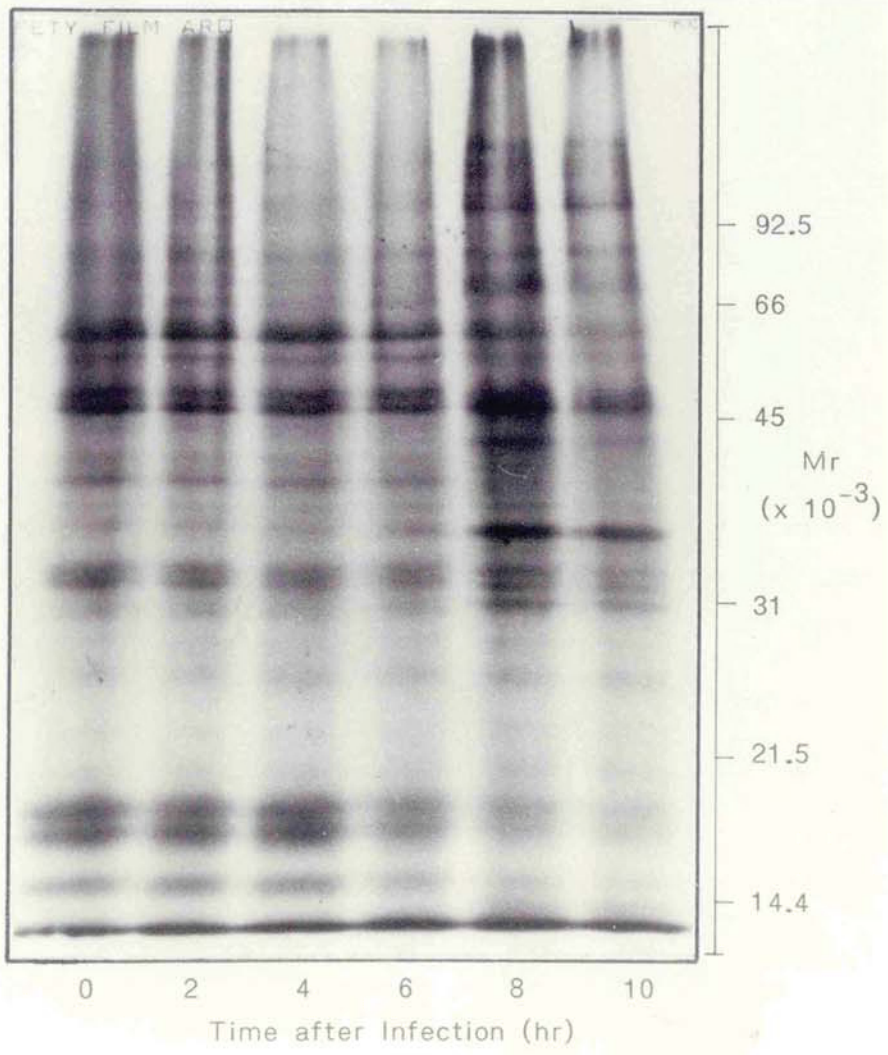

Activity (Normalised)
10
8
6
4
2
0
2
4
6
8
10
ViPK
DNA
Polymerase
Virus
released
Time after infection (hours)

Fig. 1.6 Proteins Synthesised at Different Times After Infection of BHK Cells with PRV

BHK cells were infected with PRV for various lengths of time and labelled with [^{14}C] amino acids as described in Methods, section 1.5. One culture of BHK cells (approx. 4 x 10^7 cells) was used for each time point of infection. The cells were harvested into 10 ml of PBS and 0·5 ml of the cell suspension was mixed with 2 volumes of 0·025 M Na_2CO_3/0·05 M NaOH solution. To this was added an equal volume of cold 10% (w/v) tricholoacetic acid. Precipitated protein was collected by centrifugation (30,000 g for 30 min) and washed with 5 volumes of acetone. The precipitated protein (approx. 100 µg) was then subjected to electrophoresis in the presence of SDS on a 15% (w/v) acrylamide/0·09% (w/v) methylene-bis-acrilamide gels [Methods, section 6.1 (1)].

The figure shows a fluorograph of the dried gel, processed as described in Methods, section 6.2 (4).


92.5
66
45
Mr
(x 10^{-3})
31
21.5
14.4
0
2
4
6
8
10
Time after Infection (hr)

2. PARTIAL PURIFICATION AND CHARACTERIZATION OF ViPK

2.1 PARTIAL PURIFICATION OF ViPK

For a typical purification of ViPK, about 10^9 BHK cells in monolayer culture were infected with 20 pfu of PRV per cell. Cells were harvested at 6-9 h after infection. (It has been reported by Weissbach *et al.*, 1973, that harvesting at later times resulted in a loss of total cell protein, due to cell death occurring at that stage of infection.)

The post-ribosomal supernatant was prepared as described in Methods, section 3.1. In this fraction ViPK was present, together with other protein kinases (some of them described in Results, section 1.1). For assaying ViPK activity in the presence of the latter, conditions in which the concentration of KCl was 800 mM were used. The activity of ViPK at 800 mM KCl was comparable with its activity at 100 mM KCl, while the activities of other protein kinases present in the preparation were suppressed (see Results, section 2.2). This allowed estimation of the total ViPK activity in the post-ribosomal fraction.

The post-ribosomal fraction was applied to a DE-52 column and fractions eluting at 200-240 mM KCl were pooled and concentrated (Fig. 2.1 A). The use of DE-52 anion-exchange chromatography as a first step was important not only because of the 9-fold purification achieved, but also because it allowed rapid separation of ViPK from most of the other protein kinase activities present. Nevertheless, DE-52 preparations of ViPK contained some casein kinase II activity (see Fig. 1.1). To study the characteristics of the ViPK it was necessary to remove this contaminating activity and further purify

the enzyme. This was achieved using the chromatographic steps described in Fig. 2.1 B.

Initially, for separation of ViPK from casein kinase II and its further purification, dye-ligand chromatography on Blue A was used. Casein kinase II was eluted from that resin with higher concentrations of KCl than were required for elution of ViPK (Fig. 2.2). Although ViPK was separated from many contaminating proteins by this method (Fig. 2.3), only 20-40% of the activity applied to the column was recovered.

An improved partial purification of the ViPK was achieved by subjecting the material from the DE-52 step to further chromatography (Fig. 2.4) on a high-performance size-exclusion column (TSK G3000SW), followed by chromatography (Fig. 2.5) on a high-performance anion-exchange resin (Mono Q). Results for the purification of ViPK using these chromatographic steps are summarized in Table 2.1. The enzyme was purified approx. 100-fold, with a recovery of about 20% of the original activity. The most purified preparation of ViPK had a specific activity of about 1000 units/mg of protein. Protein present in preparations of ViPK after the chromatographic steps described in Table 2.1, was analysed by gel electrophoresis in the presence of SDS (Fig. 2.6). The sample obtained after chromatography on the Mono Q column contained several protein bands in the range of molecular weights between 65,000 and 90,000, with the most prominent polypeptide being of approx. 90,000 Da. However, it is unlikely that this polypeptide is related to ViPK activity. Thus, when DE-52 preparations of ViPK were further purified using high-performance hydroxylapatite chromatography, the polypeptide of molecular weight 90,000 was not present in detectable amounts (Fig. 2.7). Also, the

molecular properties of the native enzyme (Results, section 2.3) do not support the possibility that ViPK activity is associated with a protein of 90,000 molecular weight.

The enzyme preparations of ViPK after the purification steps described in Table 2.1, or after dye-ligand chromatography (Fig. 2.3), were free of other protein kinases detected in BHK cells (Results, section 1.1). Furthermore, other enzyme activities which could interfere with the phosphorylation reaction, ATP-ases (Fig. 2.8) and phosphoprotein phosphatases (Methods, section 10.2) were not present in the preparations when assayed under standard conditions in vitro.

For studies of the characteristics of the phosphorylation reaction catalysed by ViPK, a dye-ligand preparation of the enzyme was used in many experiments. The more purified preparation obtained by high-performance size-exclusion and anion-exchange chromatography (Table 2.1) was used to confirm the main points emerging from these results.

In addition to the methods for partial purification of ViPK described above, several of other methods were tested in an attempt to purify the enzyme. However, these were found unsatisfactory for the following reasons:

(i) Chromatography of the DE-52 preparations on phosphocellulose separated ViPK from casein kinase II. Under conditions which allow binding of casein kinase II (Methods, section 4.2), ViPK was not bound to the resin. However, most of the other proteins applied to the column also eluted with the ViPK activity.

(ii) Affinity chromatography of ViPK on CNBr-activated Sepharose 4B with immobilized histone or protamine was tested as a possible purification step. ViPK was eluted with a concentration gradient

of KCl, but the degree of purification expected from an affinity chromatography method was not achieved.

(iii) Chromatofocusing of ViPK [using Polybuffer exchanger 94 and Polybuffer 74 (pH4·0), or Mono P column in the FPLC system under similar conditions] resulted in complete loss of the enzyme activity. It is possible that ViPK was inactivated at the pH of elution, or that inactivation was due to interaction with some component of Polybuffer.

(iv) Separation of proteins by non-denaturing polyacrylamide gels and assay of protein kinase activity in these gels according to Hirsch and Rosen (1974), was successfully applied to DE-52 preparations of ViPK. However, the resolution of proteins achieved using this method was not better than separation by chromatography on a Mono Q column, which also separates on the basis of the charge of the protein. The latter method allowed analysis of large (as well as small) amounts of protein and was much more convenient.

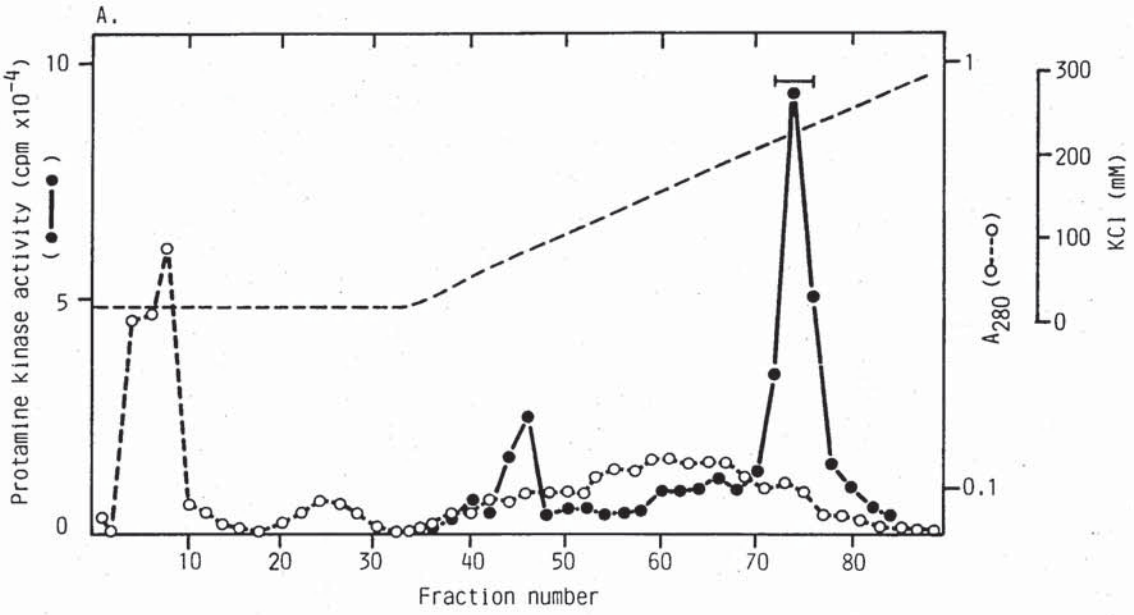
(v) A combination of high-performance size-exclusion chromatography performed at 0 mM KCl (where ViPK was eluted with an apparent molecular weight of approx. 200,000) with the same method performed at 500 mM KCl (where ViPK was eluted with an apparent molecular weight of 68,000) did not significantly improve the purification over that when only a single step using the buffer of high ionic strength was employed (Fig. 2.4).

(vi) The dye-ligand matrix, Red A, was able to bind ViPK under similar conditions to those described in Methods, section 4.3. However, the purification achieved was no better than the purification on Blue A, used for partial purification of the enzyme (Fig. 2.3).

Fig. 2.1 Scheme for the Partial Purification of ViPK

(A) The post-ribosomal supernatant from BHK cells (approx. 10^9 cells) was subjected to chromatography on a DE-52 column (Methods, section 4.1) and column fractions were assayed for phosphorylation of protamine (—●—). The KCl gradient (– – –) was determined by conductivity measurements and the absorbance of column fractions was measured at 280 nm (–○–).

(B) The fractions from the DE-52 column containing ViPK activity were pooled, concentrated by Aquacide II, and subjected to further purification by one of alternative schemes shown.


A.
Protamine kinase activity (cpm x10^{-4}) (●—●)
10
5
0
1
0.1
A_{280} (o--o)
300
200
100
0
KCl (mM)
10
20
30
40
50
60
70
80
Fraction number

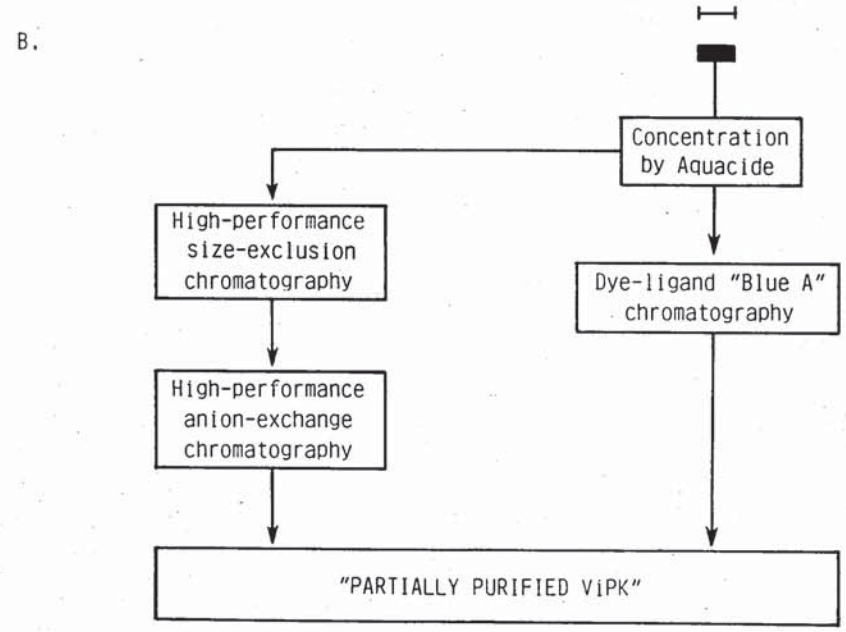

B.
Concentration by Aquacide
High-performance size-exclusion chromatography
Dye-ligand "Blue A" chromatography
High-performance anion-exchange chromatography
"PARTIALLY PURIFIED ViPK"

Fig. 2.2 Chromatography of ViPK and Casein Kinase II on a Blue A Dye-ligand Column

ViPK and casein kinase II, obtained by chromatography of post-ribosomal supernatant on a DE-52 column (Fig. 1.1 A and B) were concentrated by Aquacide and subjected to Blue A, dye-ligand chromatography. The chromatography was performed as described in Methods, section 4.3, but the linear elution gradient was replaced by a step-gradient employing 0·4 M, 0·8 M and 1·5 M KCl. The volume of elution buffer used in each step was 3 ml.

(A) Chromatography of casein kinase II on Blue A.

(—●—) Casein kinase activity.

(— — —) Absorbance at 280 nm.

(B) Chromatography of ViPK on Blue A.

(—●—) Histone kinase activity.

(— — —) Absorbance at 280 nm.

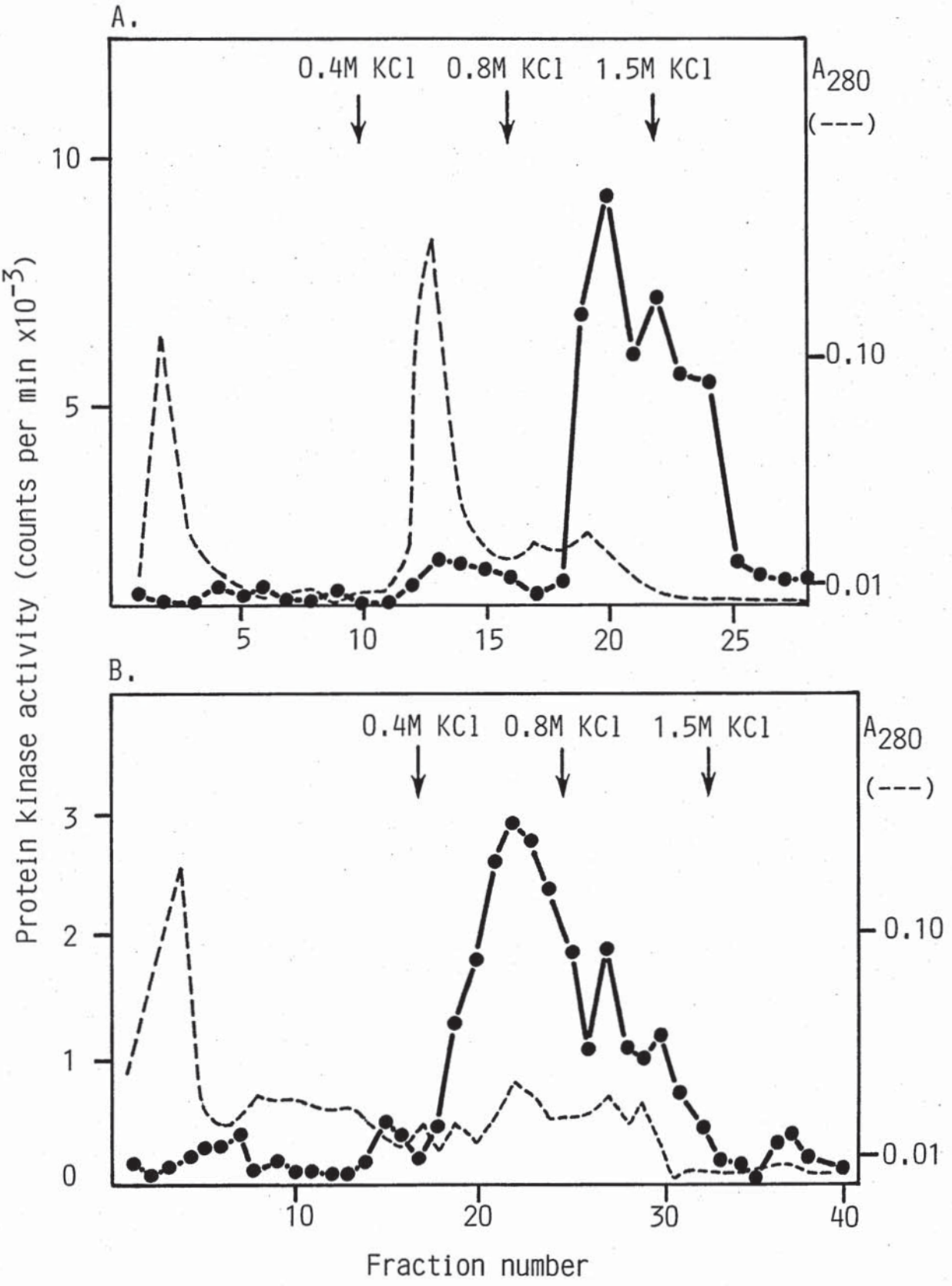
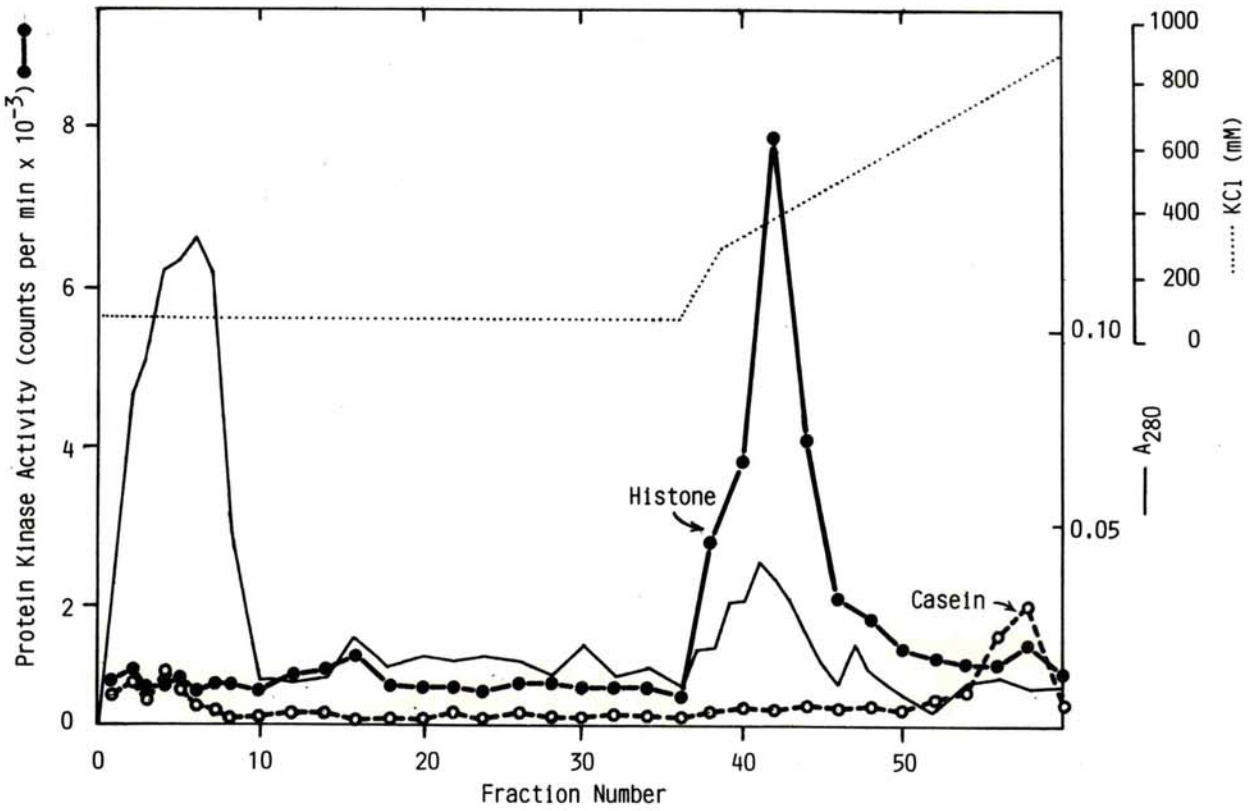

A.
0.4M KCl
0.8M KCl
1.5M KCl
A_{280}
(---)
10
5
0.10
0.01
5
10
15
20
25
B.
0.4M KCl
0.8M KCl
1.5M KCl
A_{280}
(---)
3
2
1
0
0.10
0.01
10
20
30
40
Protein kinase activity (counts per min x10^{-3})
Fraction number

Fig. 2.3 Dye-ligand, Blue A Chromatography of ViPK

(A) A preparation of ViPK purified by DE-52 chromatography was subjected to chromatography on a column of the dye-ligand, Blue A, as described in Methods, section 4.3. Aliquots (40 µl) of the fractions indicated were assayed for protein kinase activity using mixed histone (—●—) as substrate. The A_{280} (———) and KCl concentration (·····) of column fractions is also indicated.

(B) Protein present in the initial DE-52 preparation of ViPK (1), in Blue A column fractions 5-8 (2) and Blue A column fractions 39-43 with ViPK activity (3) was analysed by SDS gel electrophoresis on a gel containing 10% (w/v) acrylamide/0·27% (w/v) methylene-bis-acrylamide [Methods, section 6.1 (1)]. Samples contained approx. 5 µg of protein which was visualized by the silver stain method [Methods, section 6.2 (2)].

A


Protein Kinase Activity (counts per min x 10^{-3})
Fraction Number
Histone
Casein
A_{280}
KCl (mM)
0
2
4
6
8
0
10
20
30
40
50
0.05
0.10
0
200
400
600
800
1000

B

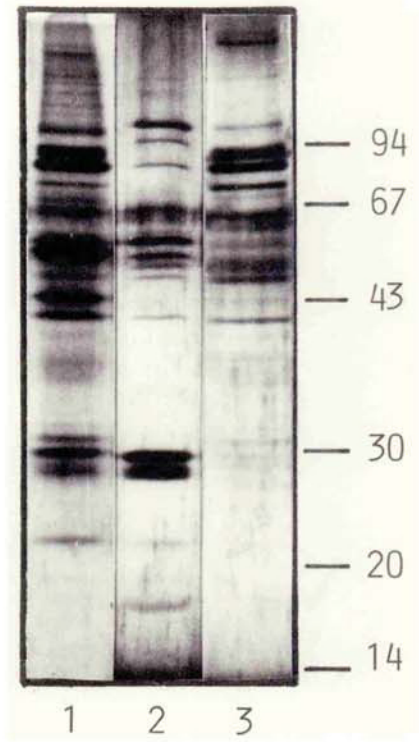

94
67
43
30
20
14
1
2
3

Fig. 2.4 High performance Size-exclusion Chromatography of ViPK

A preparation of ViPK purified by DE-52, anion-exchange chromatography was subjected to chromatography on a TSK G3000SW column as described in Methods, section 4.6. Aliquots (40 µl) of the column fractions were assayed for protein kinase activity (—●—) using protamine as substrate. The absorbance was monitored at 280 nm (———). The position of elution of protein standards is indicated.

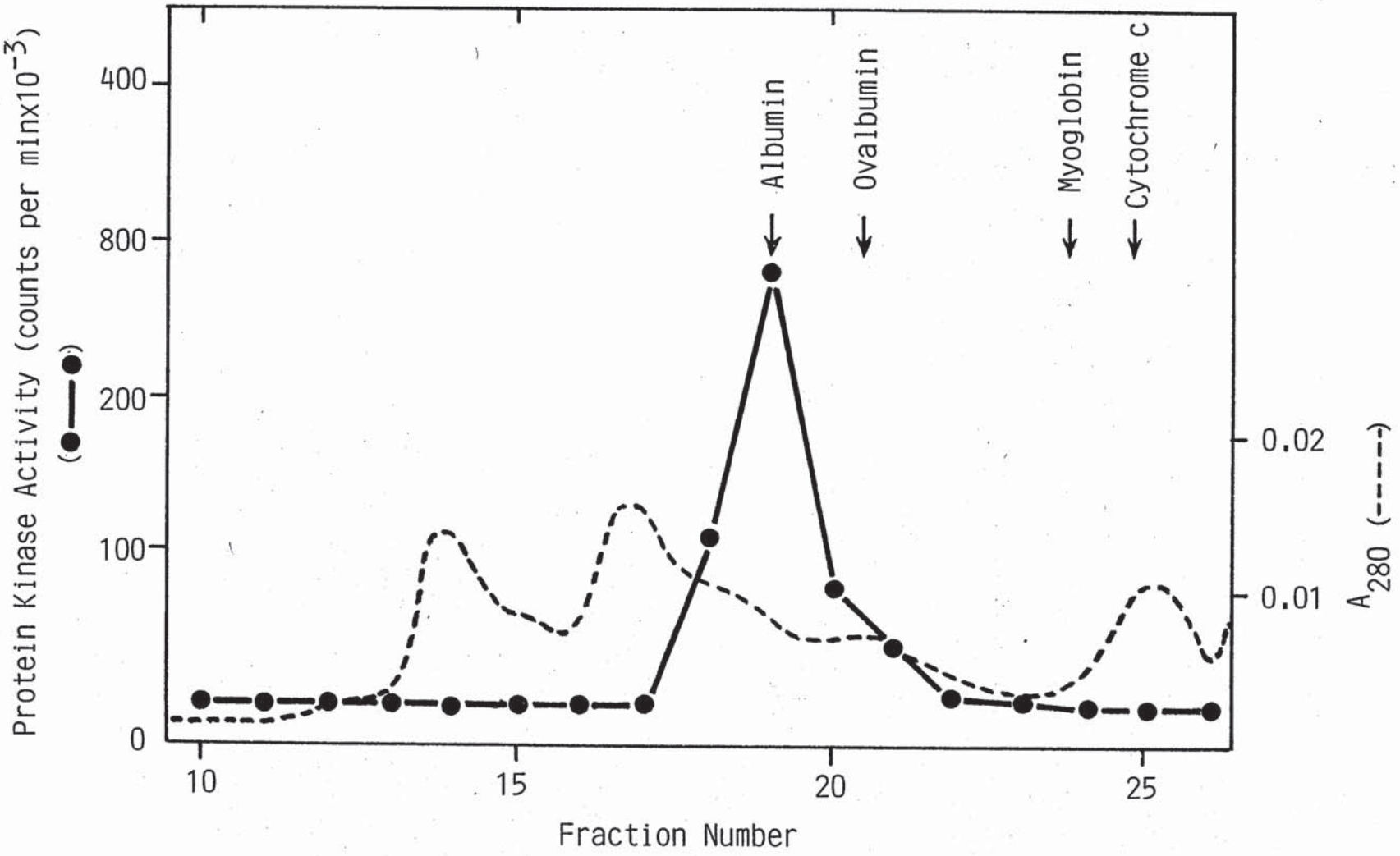

Protein Kinase Activity (counts per minx10^{-3})
(●—●)
400
800
200
100
0
10
15
20
25
Fraction Number
Albumin
Ovalbumin
Myoglobin
Cytochrome c
0.02
0.01
A_{280} (- - - -)

Fig. 2.5 High-performance Anion-exchange Chromatography of ViPK

A preparation of ViPK purified by DE-52 anion-exchange and high-performance size-exclusion chromatography was subjected to chromatography on a Mono Q column as described in Methods, section 4.7. Aliquots (40 µl) of the column fractions were assayed for protein kinase activity (—●—) using protamine as substrate. The A_{280} (———) and NaCl concentration (•••••) of column fractions is also indicated.

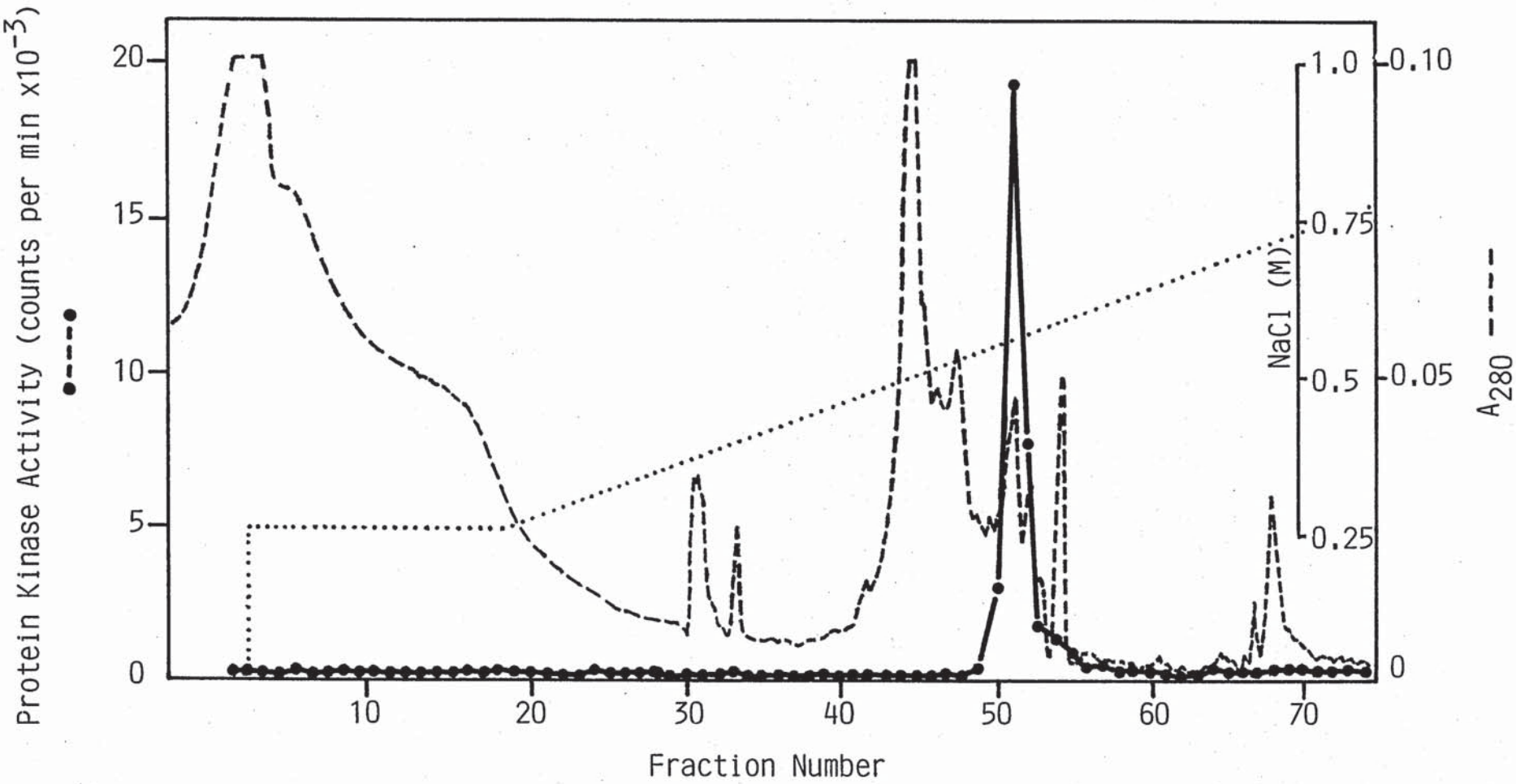

Protein Kinase Activity (counts per min x10^{-3})
20
15
10
5
0
10
20
30
40
50
60
70
Fraction Number
1.0
0.75
0.5
0.25
NaCl (M)
0.10
0.05
0
A_{280}

Table 2.1 Partial Purification of ViPK

BHK cells were infected for 6 h with PRV and ViPK purified from the post-ribosomal supernatant as shown in Fig. 2.1., 2.4 and 2.5. Protein kinase activity was assayed with protamine as substrate. Protein was assayed as described in Methods, section 1.1

Purification Step	Volume (ml)	Total protein (mg)	Total kinase activity (Counts/min x 10^{-6})	Purification (X)	Recovery (%)
Post-ribosomal supernatant	21	119	16·0*	1	100
DE-52	42	10·4	12·2	9	76
TSK-G3000SW	9	3·12	8·4	24·4	52
Mono Q	1	0·28	3·7	102	23

* Assayed at 800 mM KCl (see text).

Fig. 2.6 Partial Purification of ViPK

Protein (25 μg) present in samples from each step of purification of ViPK, described in Table 1, was analysed on a 10% (w/v) acrylamide/ 0·27% (w/v) methylene-bis-acrylamide gel in the presence of SDS. After electrophoresis [Methods, section 6.1 (1)], the gel was stained with Coomassie Brilliant Blue [Methods, section 6.2 (1)].

The samples applied were from:

(1) The post-ribosomal supernatant

(2) DE-52, anion-excahnge chromatography

(3) High-performance size-exclusion chromatography on a TSK G3000SW column

(4) High-performance anion-exchange chromatography on a Mono Q column.

The position of molecular weight standards is also indicated.

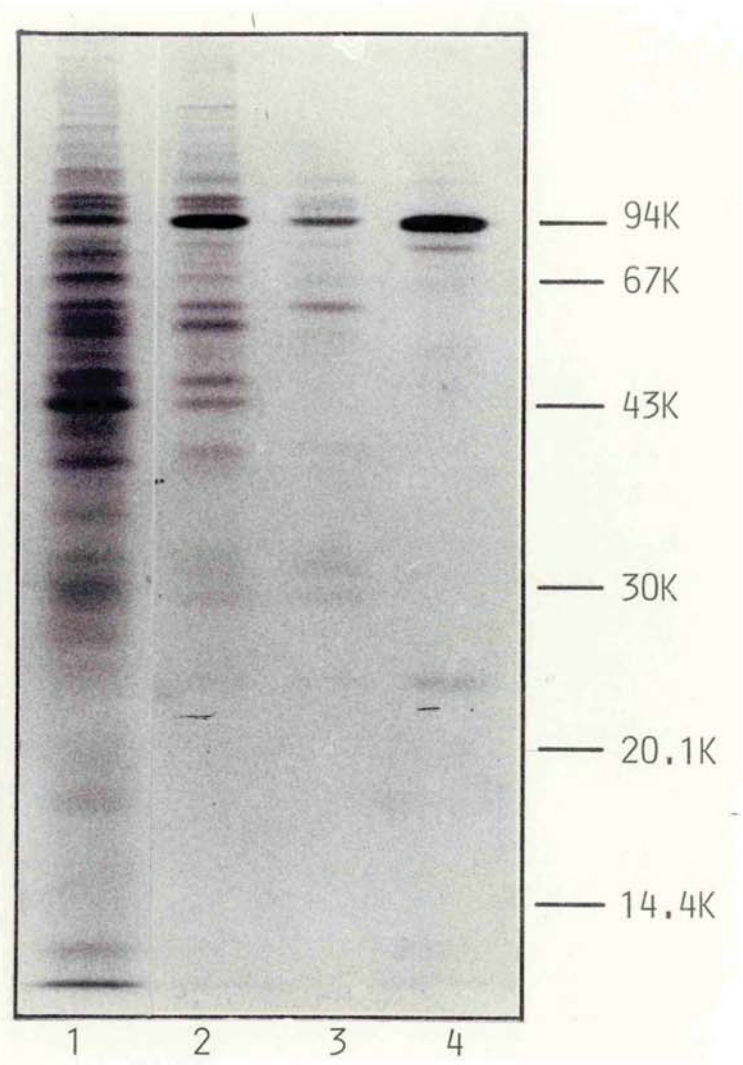

94K
67K
43K
30K
20.1K
14.4K
1
2
3
4

Fig. 2.7 High-performance Hydroxylapatite Chromatography of ViPK

A preparation of ViPK purified by DE-52 anion-exchange chromatography was subjected to chromatography on a hydroxylapatite column, as described in Methods, section 4.5. Aliquots (40 µl) of the column fractions were assayed for phosphorylation of protamine (—●—). The A_{280} (———) and concentration gradient of sodium phosphate buffer (– – –) are also indicated.

Protein present in the fractions containing ViPK after high-performance hydroxylapatite chromatography (1) and in the sample of ViPK activity applied to the column (2), was analysed by gel electrophoresis in the presence of SDS as shown in the insert.

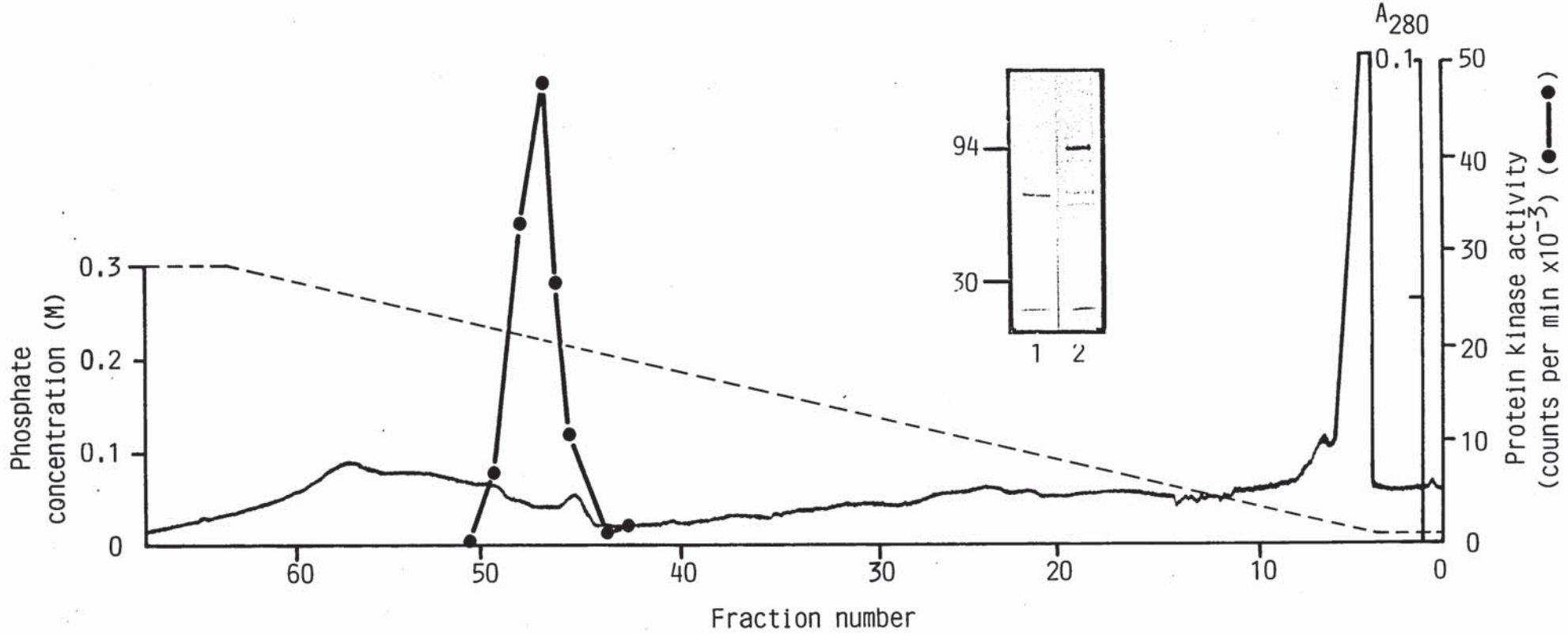
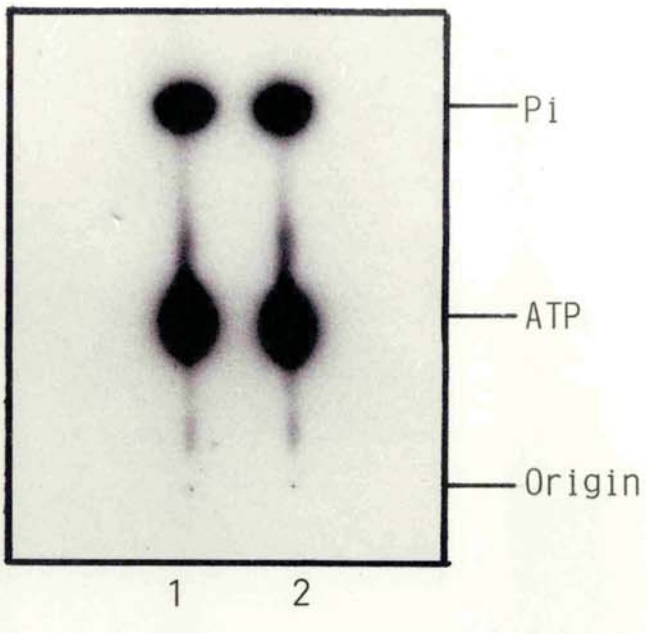

A280
0.1
94
30
1 2
Phosphate concentration (M)
0.3
0.2
0.1
0
60
50
40
30
20
10
0
Fraction number
Protein kinase activity (counts per min x10-3) (●—●)
50
40
30
20
10
0

Fig. 2.8 Determination of ATPase Activity in Standard Assay for ViPK

ViPK, partially purified as shown in Table 2.1, was assayed for phosphorylation of protamine in standard reaction mixture (Methods, section 10.1). An aliquot (1 µl) from that reaction mixture (1) and from a similar reaction mixture but without added ViPK (2), was taken at the end of incubation and subjected to PEI-cellulose thin-layer chromatography (Methods, section 8).

The figure shows an autoradiography of the dried plate. The amount of [γ-^{32}P] ATP present in samples 1 and 2 was determined from measurements of Cerenkov radiation in the excised spots. The values obtained were: 25,845 cpm in sample 1 and 27,413 cpm in sample 2.

A similar experiment, which gave essentially the same results, was performed with a ViPK preparation obtained after Blue A chromatography.


Pi
ATP
Origin
1
2

2.2 CATALYTIC PROPERTIES OF ViPK

(1) Conditions Affecting Catalytic Activity

Effect of ions - The effect of monovalent and divalent cations on the phosphorylation reaction catalysed by the ViPK was determined with mixed histone or protamine as a substrate. The characteristics of phosphorylation of these two substrates were generally similar. Fig. 2.9 A shows the effect of increasing concentrations of KCl on histone phosphorylation. The ViPK was active over a broad range of concentration of KCl, and its activity was actually stimulated between 300 and 500 mM KCl. In other experiments where concentrations higher than 600 mM KCl were tested (0·8 M and 1 M KCl), ViPK had as great an activity as at 100 mM KCl. The magnesium ion requirement of ViPK was examined in the presence of 110 or 500 mM KCl (Fig. 2.9B). At the lower concentration of monovalent cation (110 mM KCl) a very broad magnesium concentration optimum of 50–80 mM was observed. This is far in excess of that required for the formation of an ATP–magnesium complex with the 0·1 mM ATP in the assay mixture. At the higher concentration of monovalent cation (500 mM KCl) the optimum concentration of magnesium decreased to approx. 20 mM, suggesting that part of the effect of magnesium was through its contribution to the total ionic strength. ViPK also maintained its activity in the presence of relatively high concentrations of calcium (5 mM $CaCl_2$) or spermine (5 mM), as shown in Table 2.2

Effect of pH - The effect of pH on the ViPK activity (Fig. 2.10) was determined over a range of pH values from 4·5 to 10. In the

presence of 50 mM Tris-HCl buffer the activity of ViPK was inhibited compared with that in the presence of phosphate or glycine buffer at the same pH. The curve of the dependence of ViPK activity on pH, corrected for this inhibitory effect of Tris-buffer (Fig. 2.10, insert), showed a maximum between pH7·5 and 9.

Effect of sulphydryl-blocking reagents - The sulphydryl-blocking reagents, N-ethylmaleimide and p-hydroxymercuribenzoate which inhibit the activity of enzymes dependent on the structural requirement of sulphydryl groups, inhibited ViPK activity (Fig. 10.11).

Effect of temperature of incubation - The effect of temperature of incubation on ViPK activity was assayed with protamine and histone as a substrate. Maximal activity for histone phosphorylation was at approx. 30°C, while phosphorylation of protamine increased up to 40°C (Fig. 2.12).

Specificity for ATP as phosphate donor and determination of apparent Km - When [γ-^{32}P] GTP was substituted for [γ-^{32}P] ATP as phosphate donor for the phosphorylation at the same concentration of the nucleotide, no incorporation of ^{32}P was observed into protamine, indicating specificity of the enzyme for ATP.

To determine the apparent Km of ViPK for ATP, phosphorylation of protamine was examined as described in Fig. 2.13. A double-reciprocal plot of the initial velocity versus ATP concentration gave an apparent Km of 0·057 mM. This compares with values of 0·003-0·4 mM reported for other protein kinases (Roach, 1984).

Fig. 2.9 Effect of KCl and $MgCl_2$ on the Activity of ViPK

A preparation of ViPK purified by chromatography on DEAE-cellulose and Blue A was assayed for protein kinase activity using mixed histone as substrate as described in Methods, section 10.1, except that the concentration of KCl and $MgCl_2$ were varied as indicated.

(A) Effect of altering the concentration of the KCl at 10 mM $MgCl_2$.

(B) Effect of altering the concentration of $MgCl_2$ at 110 mM KCl (—●—) or 500 mM KCl (- -○- -).

In the same experiment cyclic AMP-dependent protein kinase type I was used as a control. This enzyme was most active between 5-10 mM $MgCl_2$ and 50% inhibition of its activity was achieved with 120 mM KCl, consistent with the results of others (Takai et al., 1977).

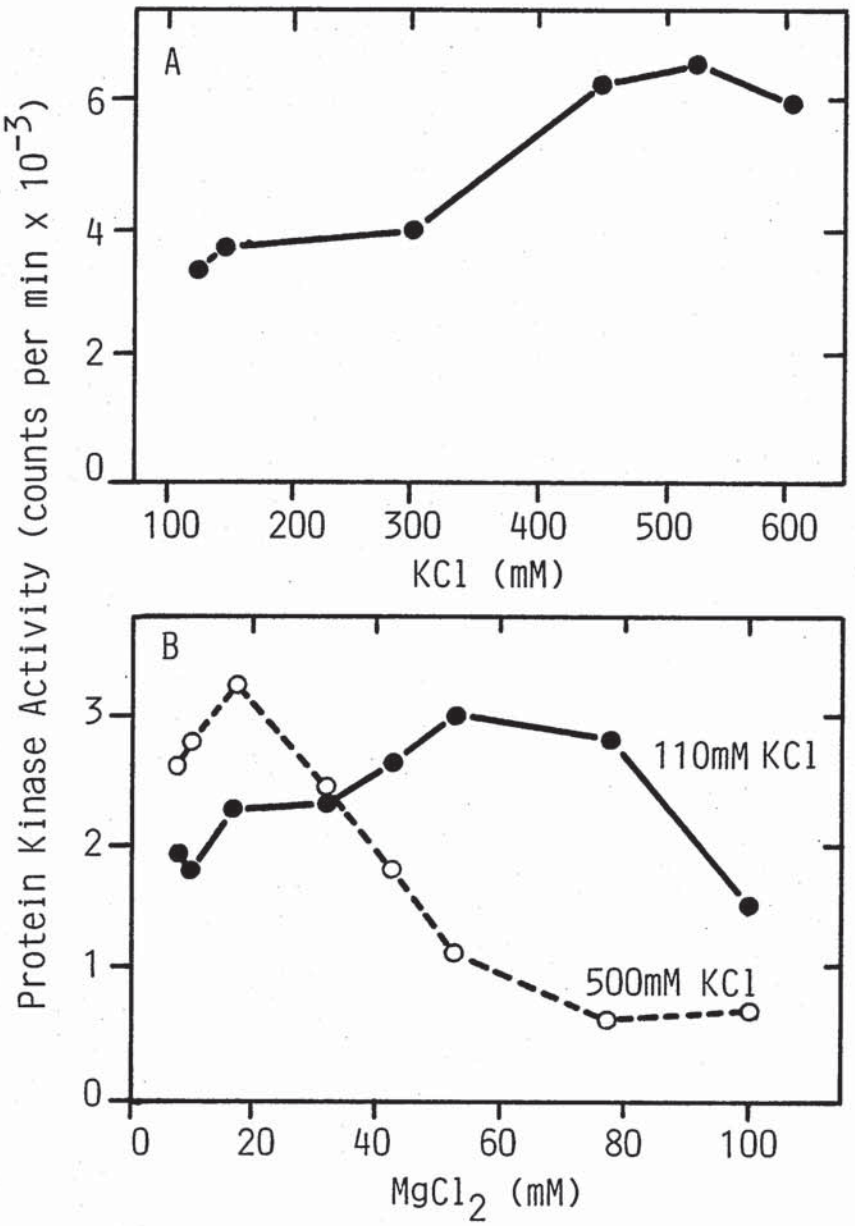

A
B
Protein Kinase Activity (counts per min x 10^{-3})
KCl (mM)
$MgCl_2$ (mM)
110mM KCl
500mM KCl
0
2
4
6
100
200
300
400
500
600
1
3
20
40
60
80

Fig. 2.10 Effect of pH on the Activity of ViPK

ViPK was partially purified by chromatography on DE-52 and Blue A. Protein kinase assay was carried out under standard conditions with mixed histones as a substrate (Methods, section 10.1) but the pH value of the reaction mixture varied between 4·5-10. The buffers used were: in the pH range 4·5-7, 50 mM sodium phosphate buffer (—●—); in the range 7-9, 50 mM Tris-HCl (- -▲- -); in the range 9-10, 50 mM Glycine-NaOH buffer (-·-■-·-).

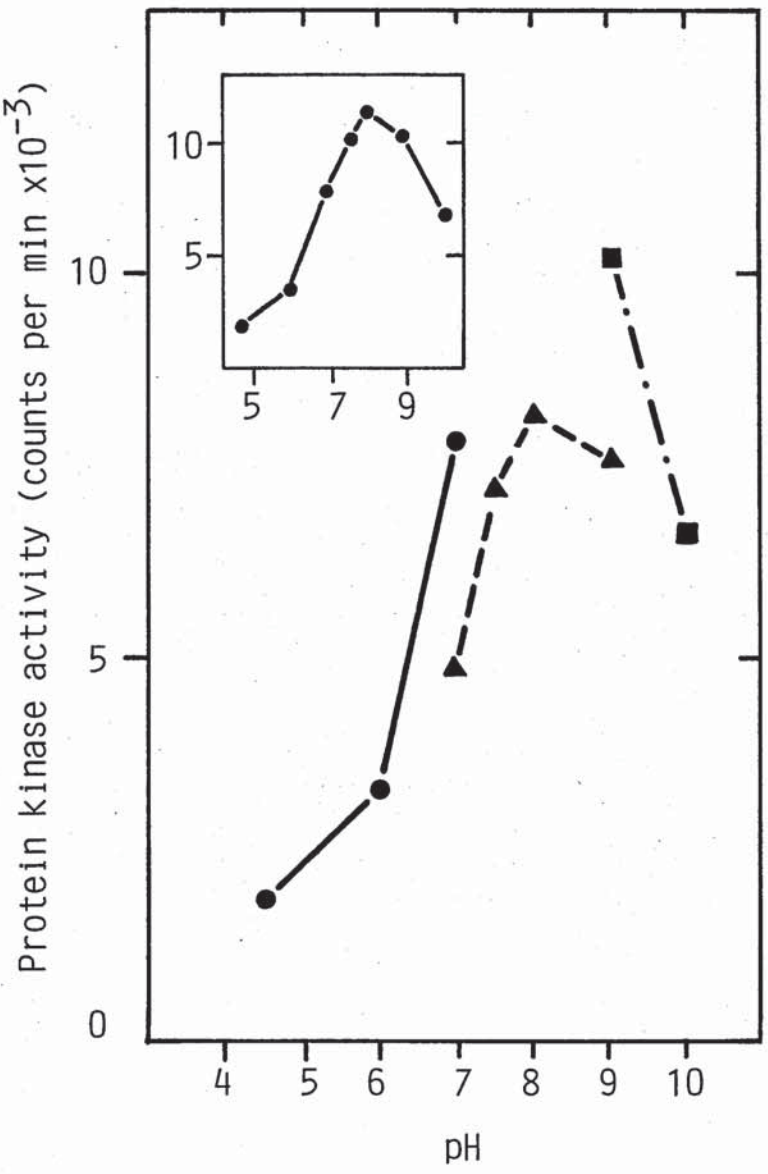

Protein kinase activity (counts per min x10^{-3})
10
5
0
4
5
6
7
8
9
10
pH
10
5
5
7
9

Fig. 2.11 Effect of Temperature of Incubation on the Activity of ViPK

ViPK was assayed as described in Methods, section 10.1, except that the temperature of incubation was varied as shown. Protamine (—●—) or histone (- -○- -) was used as substrate.

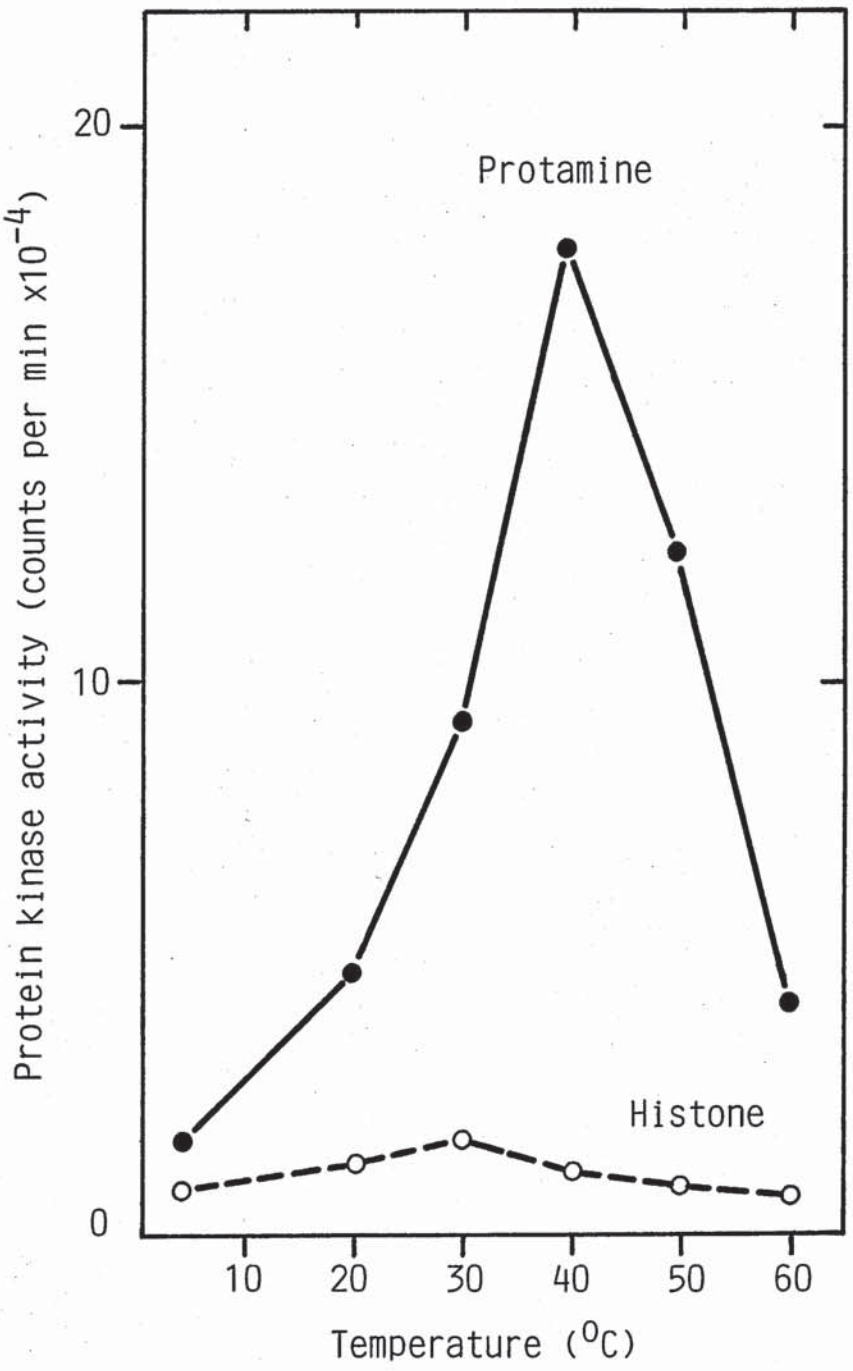

Protamine
Histone
20
10
0
10
20
30
40
50
60
Protein kinase activity (counts per min x10^{-4})
Temperature (^{o}C)

Fig. 2.12 Effect of Sulphydryl-blocking Reagents on the Activity of ViPK

A preparation of ViPK partially purified by DE-52 and Blue A chromatography, was assayed for histone kinase activity (Methods, section 10.1) in the presence of N-ethylmaleimide (—●—), or p-hydroxymercuribenzoate (—○—) at the concentrations shown.

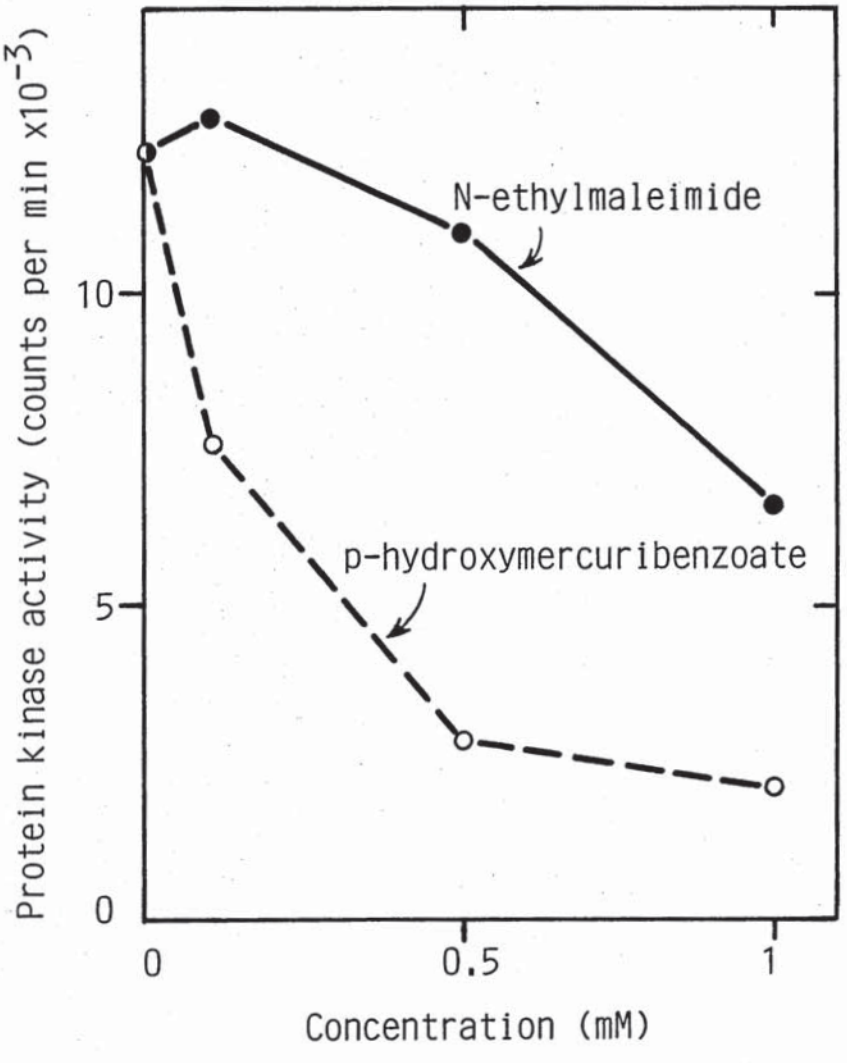
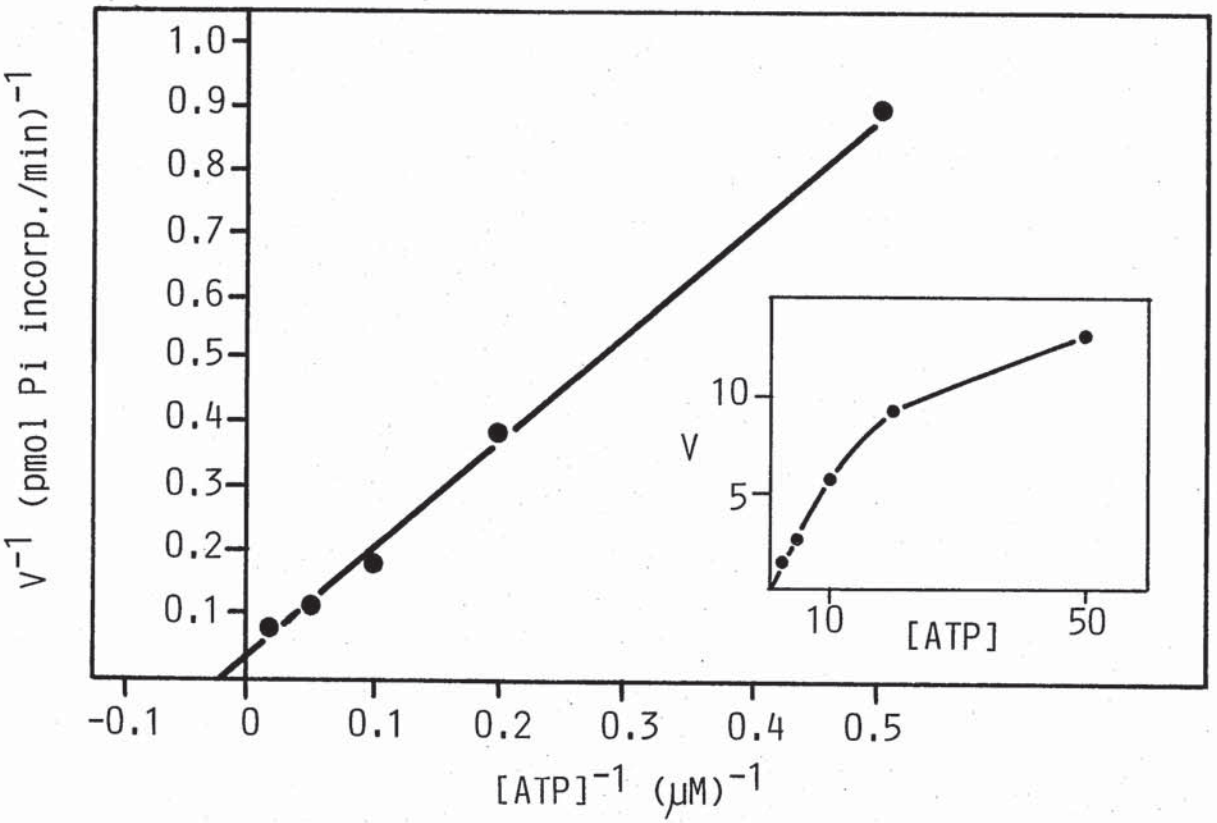

Protein kinase activity (counts per min x10^{-3})
10
5
0
N-ethylmaleimide
p-hydroxymercuribenzoate
0
0.5
1
Concentration (mM)

Fig. 2.13 The Apparent Km of ViPK for ATP

A preparation of ViPK purified by chromatography on Blue A, was assayed in standard reaction mixtures containing concentrations of ATP from 2 to 50 μM. The initial velocity was measured from the values obtained by terminating the reaction after 0, 5, 10 and 20 min at each of the ATP concentrations tested.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
V^{-1} (pmol Pi incorp./min)$^{-1}$
-0.1
0
0.1
0.2
0.3
0.4
0.5
[ATP]$^{-1}$ (μM)$^{-1}$
10
5
V
10
[ATP]
50

(2) Effect of Potential Regulatory Molecules on ViPK

The ability of the ViPK to phosphorylate protamine was examined in the presence of various molecules that have been found to affect the activity of other protein kinases. The results of these experiments together with the results of control experiments performed with other protein kinases are summarised in Table 2.2. The activity of ViPK was not affected by either cyclic AMP or the heat-stable protein inhibitor of the catalytic subunit of the cyclic AMP-dependent protein kinase. Cyclic GMP was also without effect on ViPK. (The stimulation of the cyclic AMP-dependent protein kinase by unphysiologically high concentration of 0·1 mM cyclic GMP was also observed by Reimann et al., 1971.) Heparin, at a concentration at which it specifically inhibits casein kinase II did not significantly inhibit the ViPK, although the ViPK did share with casein kinase II the property of being stimulated above its basal activity by spermine (Hathaway and Traugh, 1982). Concentrations of Ca^{2+} and phosphatidylserine that activate protein kinase C had no effect on the ability of the ViPK to phosphorylate protamine. It has already been shown (Fig. 1.1 D) that the ability of the ViPK to phosphorylate a lysine-rich histone fraction, which was enriched for histone H1, was not influenced by these molecules. (However, it is in fact histone H2B, and not histone H1, that ViPK phosphorylates in this histone fraction, Fig. 2.16.) Calmodulin and Ca^{2+} were also without effect on the ViPK, although in this case no calmodulin-dependent protein kinase was available to serve as a control. Double-stranded RNA also did not affect the ability of the ViPK to phosphorylate protamine.

Table 2.2 The Influence of Different Effectors and Inhibitors on the Activity of ViPK

The activity of ViPK, purified as shown in Table 2.1 or by chromatography on a Blue A column, was assayed using protamine as substrate. Enzymes used as controls were cyclic AMP-dependent protein kinase type I (cAMPdPKI), assayed with mixed histones as substrate, casein kinase II (CKII), assayed with casein as substrate, and protein kinase C (PKC) assayed with lysine-rich histone fraction as substrate. The results are expressed as a percentage of the activity in the absence of any addition.

Addition	Concentration or Amount	Protein Kinase			
		ViPK	cAMPdPKI	CKII	PKC
None	-	100	100	100	100
Cyclic AMP	$1{\cdot}6 \times 10^{-8}$M	101	201	-	-
	1×10^{-4}M	97	294	-	-
Cyclic GMP	$1{\cdot}6 \times 10^{-8}$M	97	87	-	-
	1×10^{-4}M	95	257	-	-
cAMPdPK inhibitor	1 unit	130	14	130	-
	10 units	149	2	121	-
$CaCl_2$	5×10^{-4}M	95	148	-	-
	5×10^{-3}M	99	46	-	-
	5×10^{-2}M	13	17	-	-
$CaCl_2$ + Calmodulin	5×10^{-3}M 60 µg/ml	107	-	-	-
$CaCl_2$ + Phosphatidyl Serine	1×10^{-3}M 0·5 mg/ml	125	-	-	1000
Spermine	5×10^{-4}M	187	-	-	-
	5×10^{-3}M	101	-	-	-
	5×10^{-2}M	5	-	-	-
Heparin	0·5 µg/ml	86	-	23	-
	5 µg/ml	96	-	19	-
polyI-polyC	0·1 µg/ml	111	-	-	-
	1 µg/ml	99	-	-	-
	10 µg/ml	89	-	-	-

3. Substrate Specificity of ViPK

Protamine and histone - The non-specific phosphate-acceptor proteins commonly used to assay protein kinases in vitro are histones, protamine, casein and phosvitin. The acidic proteins, casein and phosvitin, did not serve as a substrate for the ViPK in the range of concentrations 0·4-4 mg/ml, as shown in Fig. 2.14 B. Protamine was the best substrate for ViPK and mixed histones were phosphorylated to a lesser extent. The curve of activity of ViPK as a function of the concentration of protamine, and the corresponding curve for histone (Fig. 2.14 A) were similar, reaching maximal activity at a concentration of about 0·1 mg/ml for both substrates. The apparent Km value for protamine was determined by assaying kinase activity at concentrations of protamine from 0·005 to 0·05 mg/ml (Fig. 2.15). A double reciprocal plot of the initial velocity versus substrate concentration gave an apparent Km of 0·042 mg/ml. This compares with apparent Km values for histones of 0·14 and 0·4 mg/ml reported for the cyclic AMP-dependent and cyclic GMP-dependent protein kinases, respectively (Takai et al., 1975) and a Km of 0·2-0·3 mg/ml for lysine-rich histone reported for the protein kinase C (Ferrari et al., 1985).

Different protein kinases have specificities for different histones, and it was therefore of interest to examine the phosphorylation of histone subfractions by ViPK (Fig. 2.16). The results of this experiment showed that histones H3, H2A and H2B were good substrates, whereas histones H1 and H4 were very poor substrates (if at all) for ViPK. It should be pointed out that the subfractions used were in fact mixtures of different histones, and thus interactions between

individual histones or their interaction with ViPK cannot be excluded. For example, it has been reported that histone H1 was phosphorylated by protein kinase C only if histones H3 and H4 were not present in the assay. Histones H3 and H4 were poor substrates but potent inhibitors of the enzyme (Sahyoun et al., 1983). However, an additional experiment where purified histone H1 (Methods, section 4.8) was tested for phosphorylation by ViPK, confirmed that H1 was a poor substrate for the enzyme.

Ribosomal proteins - Certain basic proteins of ribosomal subunits also served as substrates for the ViPK. Phosphorylation of 40S ribosomal subunits was assayed under different ionic conditions (Fig. 2.17 and 2.18). At moderate ionic strength (110-350 mM KCl, 350 mM NaCl or 0·5 mM spermine) the major phosphorylated ribosomal protein had a molecular weight of 22,000, whereas at higher concentration of certain salts (660 mM KCl, 660 mM NaCl or 5 mM spermine) a protein of molecular weight 31,000 was a good substrate. Analysis of ribosomal proteins by two-dimensional gel electrophoresis (Fig. 2.19) showed that these two phosphoproteins correspond to ribosomal proteins S7 and S6, respectively. No other protein kinase with this substrate specificity for ribosomal proteins has been described.

Phosphorylation of eIF-2 - The α subunit of eukaryotic initiation factor eIF-2 can be phosphorylated in vivo and in vitro by a double-stranded RNA-dependent kinase found in both nucleated and non-nucleated cells, as well as by haemin-regulated kinase (HRI), found in reticulocytes (Ochoa, 1983). The other subunit, β , can be phosphorylated in vitro by several protein kinases including casein

kinase II, protease-activated kinase II (Tuazon et al., 1980) and kinase C (Schatzman et al., 1983). In the experiment shown in Fig. 2.20 eIF-2 was assayed for phosphorylation by ViPK, and HRI and casein kinase II were used as controls for phosphorylation of the α and β subunit, respectively. The ViPK was unable to phosphorylate either subunit of eIF-2.

Determination of the amino acid phosphorylated - Protein kinase activities can be classified in terms of the amino acids that they phosphorylate. Serine/threonine- and tyrosine-specific kinase activities are known (Krebs, 1983). Analysis of the phosphoamino acids in a protamine hydrolysate (Fig. 2.21) showed that only serine residues, and not threonine or tyrosine residues, were phosphorylated by ViPK.

Fig. 2.14 Activity of ViPK as a Function of Substrate Concentration

Partially-purified ViPK was assayed under standard conditions (Methods, section 10.1), but the concentrations of potential substrates were varied as indicated.

(A) (—○—) histone

(—●—) protamine

(B) (—●—) casein

(--○--) phosvitin

In the same experiment cyclic AMP-dependent protein kinase type I phosphorylated the histone fraction to a greater extent than protamine, consistent with the results of others (Takai et al., 1977).

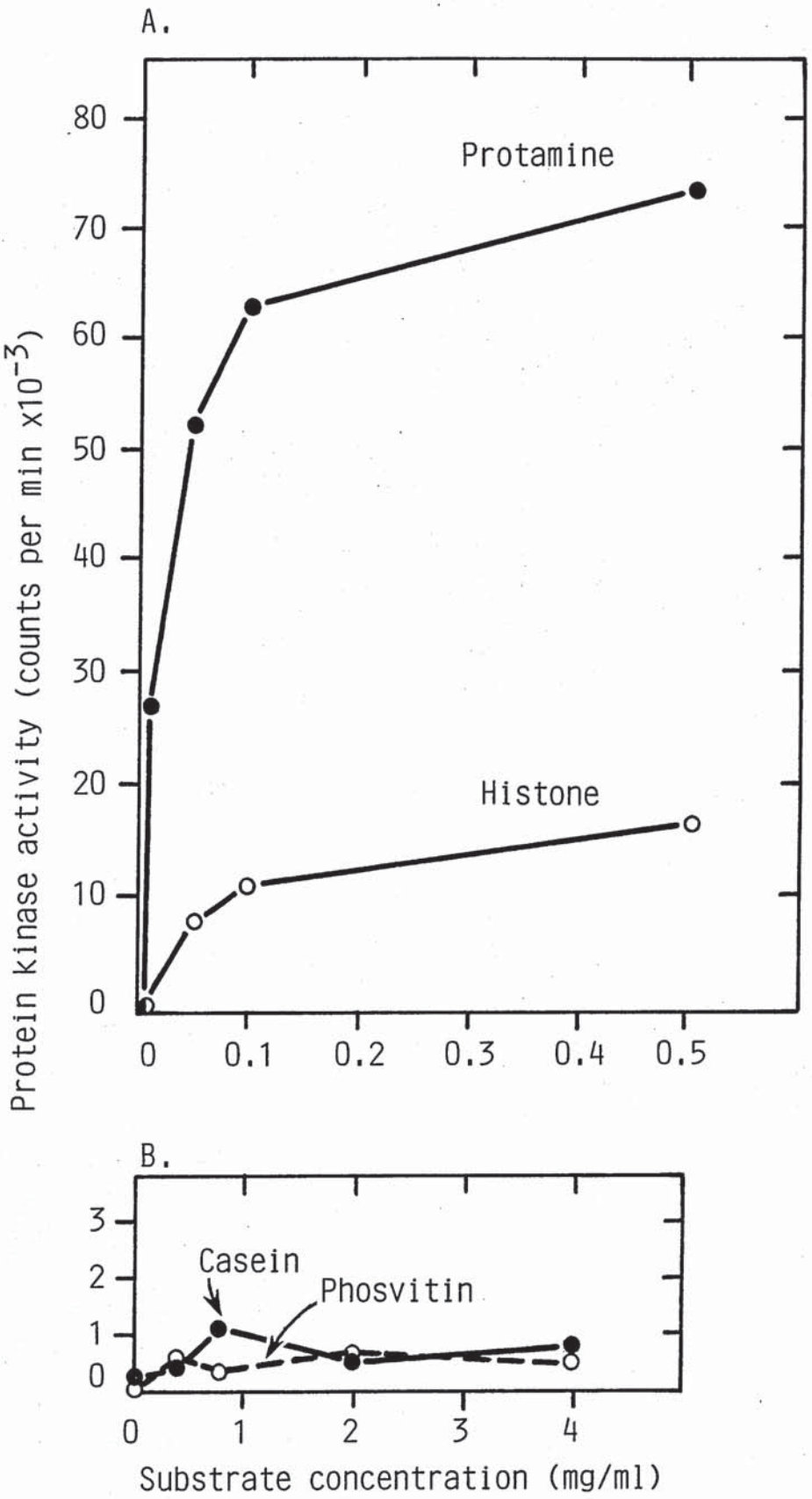

A.
Protamine
Histone
Protein kinase activity (counts per min x10^{-3})
80
70
60
50
40
30
20
10
0
0
0.1
0.2
0.3
0.4
0.5
B.
Casein
Phosvitin
3
2
1
0
0
1
2
3
4
Substrate concentration (mg/ml)

Fig. 2.15 The Apparent Km of ViPK for Protamine

A preparation of ViPK purified by chromatography on Blue A, was assayed in standard reaction mixtures containing concentrations of protamine from 0·0075 to 0·05 mg/ml. The initial velocity was measured from the values obtained by terminating the reaction after 0, 5, 10 and 20 min at each of the protamine concentrations tested.

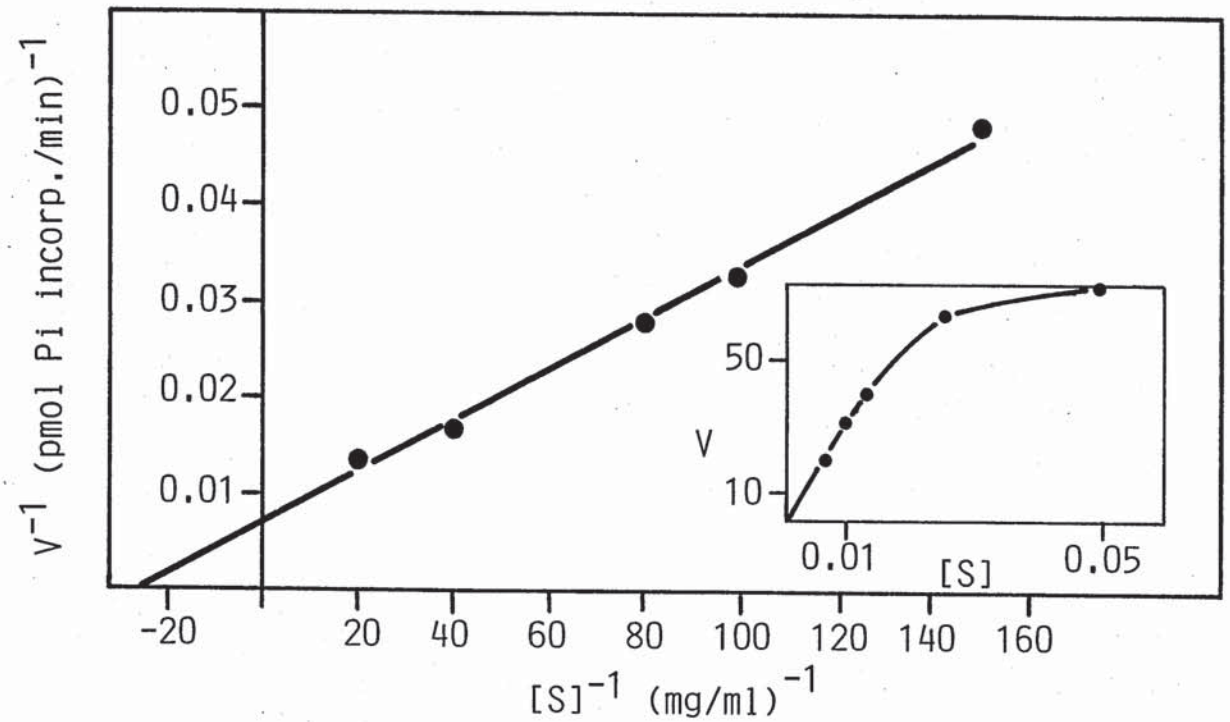

0.05
0.04
0.03
0.02
0.01
V^{-1} (pmol Pi incorp./min)$^{-1}$
50
V
10
0.01
[S]
0.05
-20
20
40
60
80
100
120
140
160
$[S]^{-1}$ (mg/ml)$^{-1}$

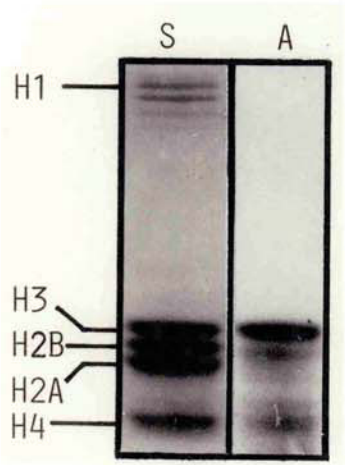
Fig. 2.16 Phosphorylation of Different Histones by ViPK

Partially-purified ViPK was assayed for protein kinase activity with various commercial (Sigma) histone fractions, and the products of the reaction (aliquots containing 32 μg of protein) subjected to electrophoresis in the presence of SDS on a gel containing 15% (w/v) acrylamide/0·4% (w/v) methylene-bis-acrylamide (Methods, section 10.6).

(A) Stained gel (S) and corresponding autoradiograph (A) with mixed histone type II-AS as substrate.

(B) Tracks 1-4 show the stained gels, and tracks 5-8 autoradiographs of these. The histone subfractions used (manufacturer's designations) were: (1, 5) Type V-S; (2, 6) Type VII-S; (3, 7) Type VI-S; (4, 8) Type VIII-S.

A


S
A
H1
H3
H2B
H2A
H4

B

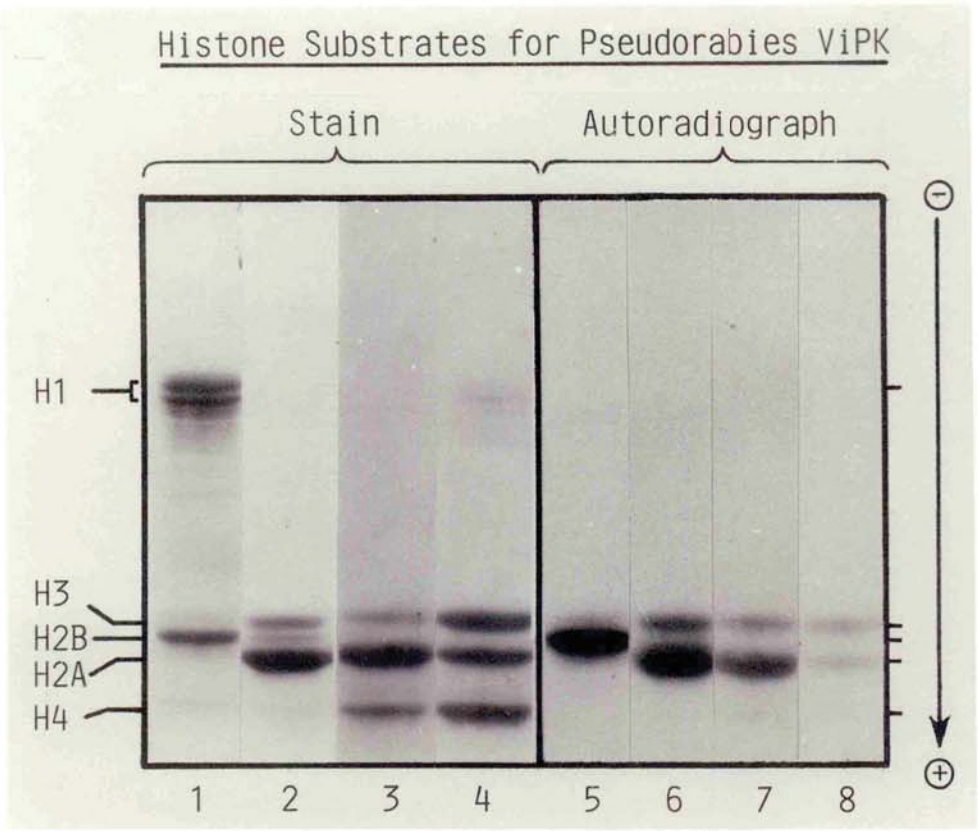

Histone Substrates for Pseudorabies ViPK
Stain
Autoradiograph
H1
H3
H2B
H2A
H4
1
2
3
4
5
6
7
8

Fig. 2.17 Phosphorylation of 40S Ribosomal Subunits by ViPK: Effect of KCl Concentration

ViPK (26 µl) partially purified by Blue A chromatography was used to phosphorylate rat liver 40S ribosomal subunits ($1A_{260}$ unit) under standard reaction conditions (Methods, section 10.3), but at the concentrations of KCl indicated in the figure. After incubation for 30 min at 30°C, the 40S subunits were subjected to SDS electrophoresis [15% (w/v) acrylamide/0·09% (w/v) methylene-bis-acrylamide] and the gel subjected to autoradiography.

The figure shows an autoradiograph of the dried gel. The apparent molecular weight and the ribosomal protein designation of each of these (based on two-dimensional analysis, Fig. 2.19) is indicated.

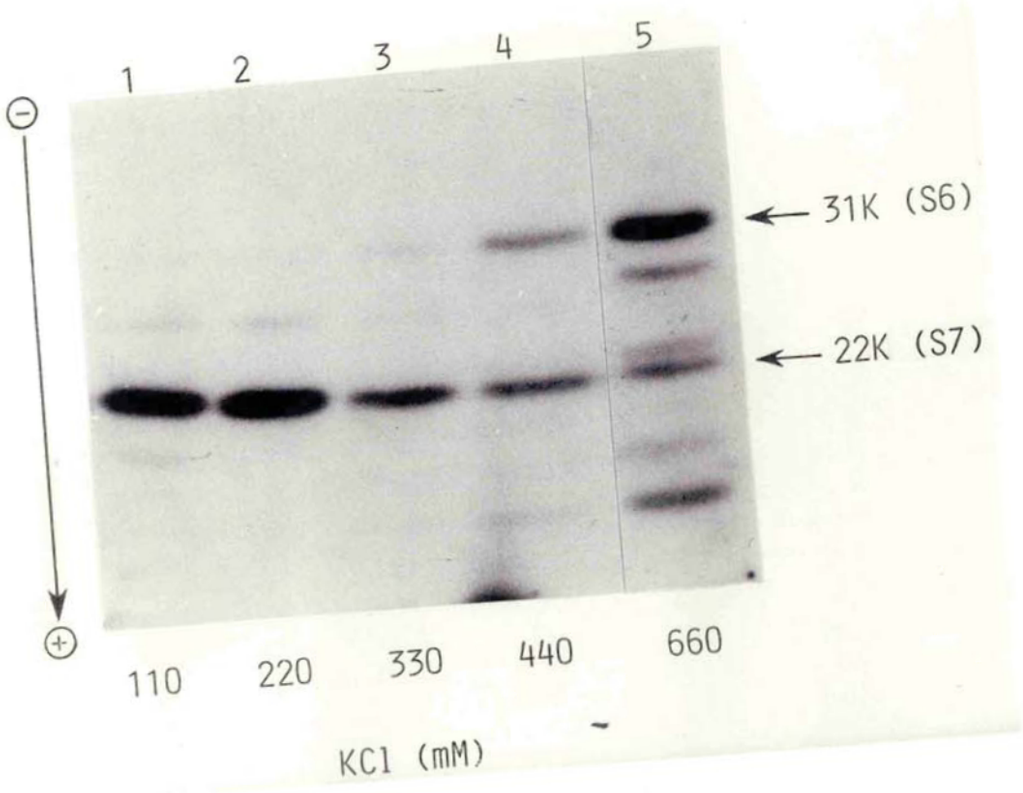

1
2
3
4
5
⊖
⊕
31K (S6)
22K (S7)
110
220
330
440
660
KCl (mM)

Fig. 2.18 Phosphorylation of 40S Ribosomal Subunits by ViPK: Effect of Various Cations

Conditions were generally as in Fig. 2.17, but the ionic composition of the reaction mixture was varied:

(1) 350 mM KCl (10 mM $MgCl_2$)

(2) 700 mM KCl (10 mM $MgCl_2$)

(3) 350 mM NaCl (10 mM $MgCl_2$)

(4) 700 mM NaCl (10 mM $MgCl_2$)

(5) 50 mM $MgCl_2$ (60 mM KCl)

(6) 100 mM $MgCl_2$ (60 mM KCl)

(7) 0·5 mM spermine (10 mM $MgCl_2$, 60 mM KCl)

(8) 5 mM spermine (10 mM $MgCl_2$, 60 mM KCl)

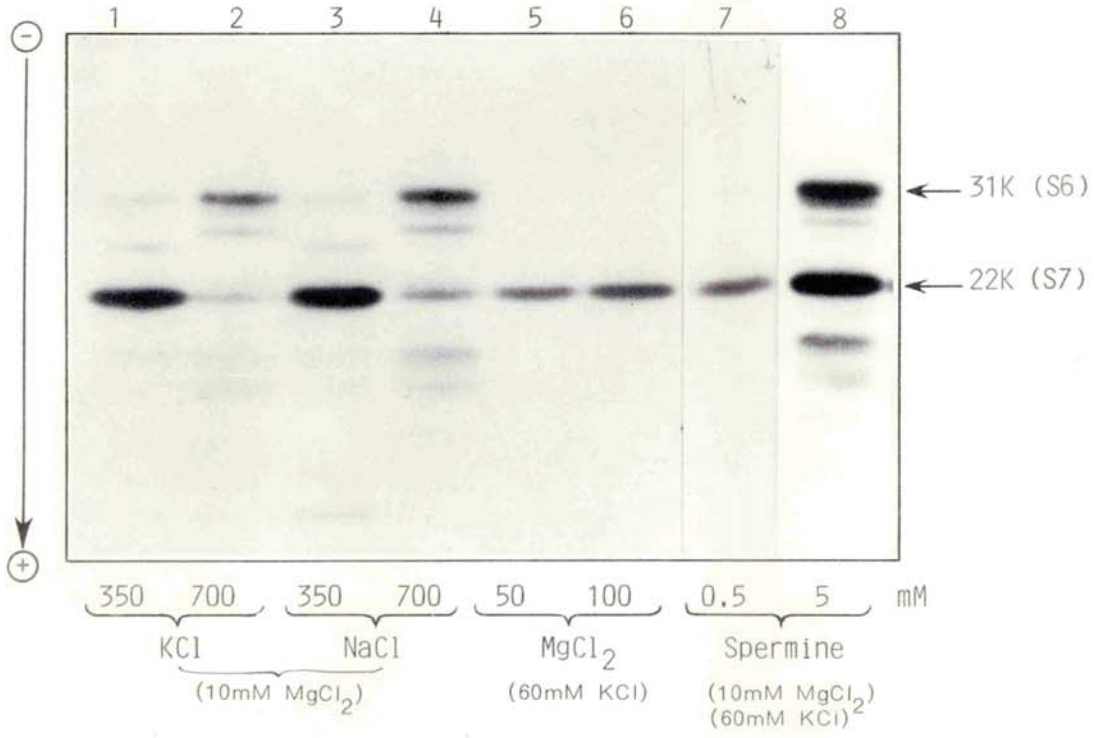

1
2
3
4
5
6
7
8
31K (S6)
22K (S7)
350
700
350
700
50
100
0.5
5
mM
KCl
NaCl
(10mM MgCl2)
MgCl2
(60mM KCl)
Spermine
(10mM MgCl2)
(60mM KCl)

Fig. 2.19 Two-Dimensional Gel Analysis of Ribosomal Proteins Phosphorylated by ViPK

A mixture of 40S and 60S ribosomal subunits ($15A_{260}$ units) was phosphorylated by ViPK (400 µl) partially purified on Blue A. The concentration of spermine in the reaction mixture was 0 mM (A, C) or 7 mM (B, D). Incubation was for 3·5 h at 30°C. The conditions of phosphorylation and preparation of the sample for analysis by two-dimensional gel electrophoresis, were as described in Methods, section 10.3.

(A, B) stained gel

(C, D) autoradiograph

The number 1 underneath protein S6 indicates the monophosphorylated derivative.

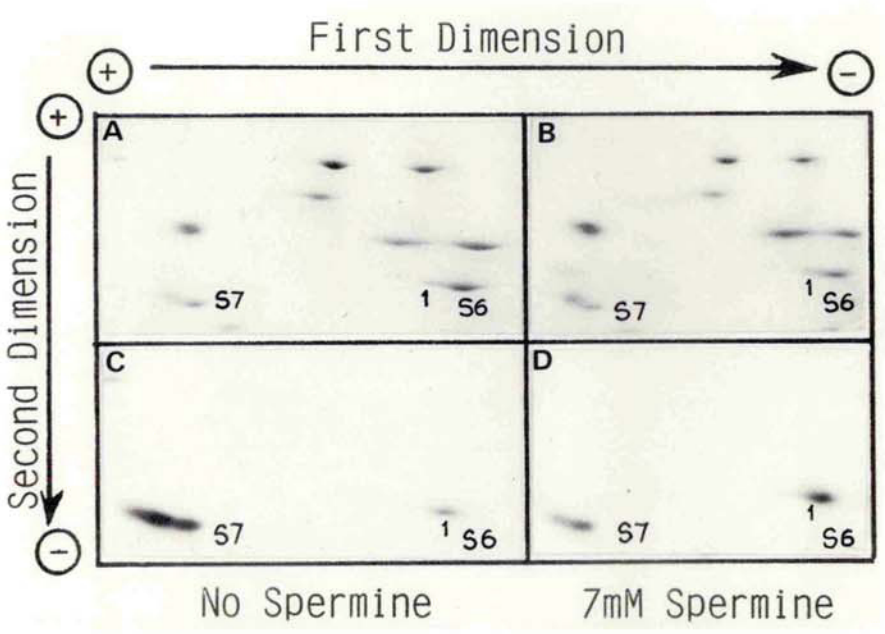

First Dimension
+
−
Second Dimension
+
−
A
B
C
D
S7
1
S6
No Spermine
7mM Spermine

Fig. 2.20 Phosphorylation of Initiation Factor eIF-2 by ViPK, Casein Kinase II and HRI

ViPK (partially purified by DE-52 anion-exchange chromatography), casein kinase II (Materials, section 2.3), and HRI (Materials, section 2.3) were used to phosphorylate eIF-2 (Materials, section 2.3) as described in Methods, section 10.5. The samples of eIF-2 were subjected to gel electrophoresis [15% (w/v) acrylamide/0·09% (w/v) methylene-bis-acrylamide] in the presence of SDS, and the phosphorylated proteins analysed by autoradiography.

Track 1: stained gel of eIF-2. Tracks 2-6: autoradiographs from reactions containing (2) HRI (1 μg); (3) HRI (1 μg) plus eIF-2 (0·8 μg); (4) ViPK (2 units); (5) ViPK (2 units) plus eIF-2 (0·8 μg); (6) casein kinase II (2 units) plus eIF-2 (0·8 μg).

The designation of the various subunits of eIF-2, are confirmed by tracks 3 and 6 for the α and β subunits. An unknown component, X, of the eIF-2 preparation, phosphorylated by ViPK and casein kinase II is also indicated.

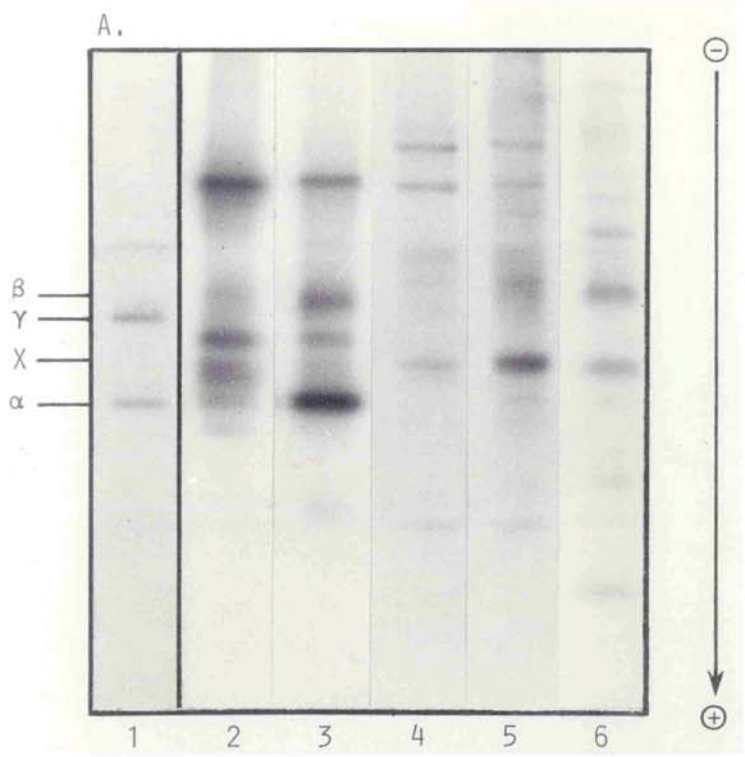
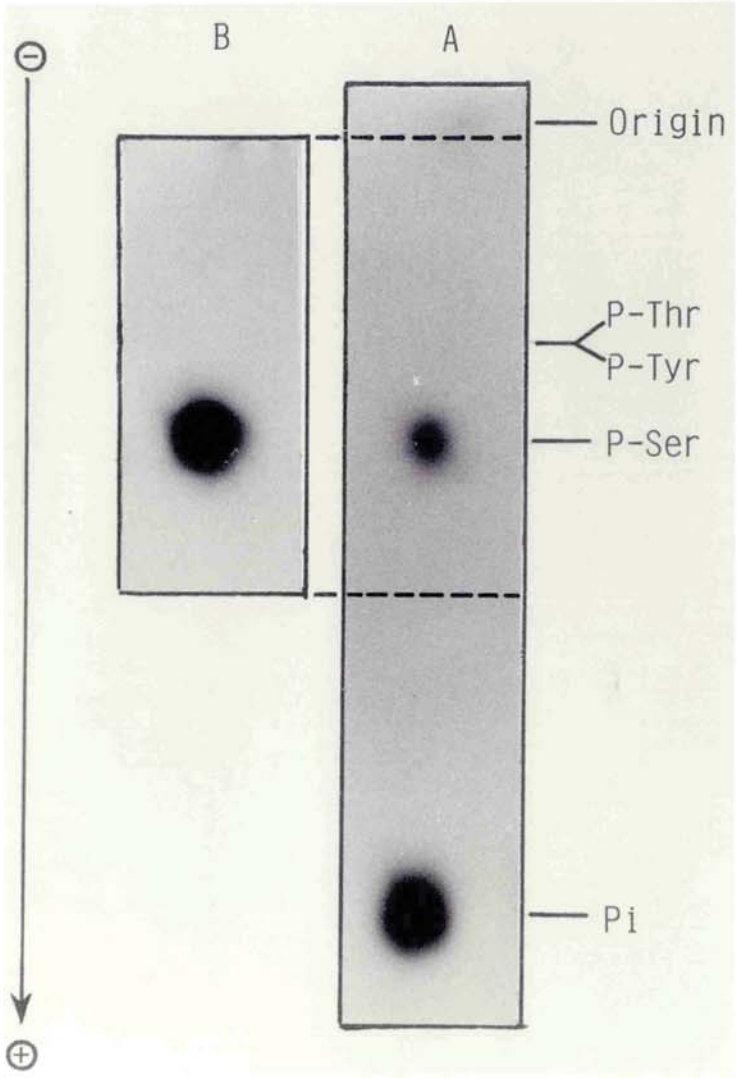

A.
β
γ
χ
α
⊖
⊕
1
2
3
4
5
6

Fig. 2.21 Phosphoamino Acid Analysis of Protamine Phosphorylated by ViPK

Protamine, phosphorylated *in vitro* by ViPK, was hydrolysed and subjected to high-voltage paper electrophoresis (Methods, section 9).

Autoradiographs of the paper electrophoretogram are shown: (A) exposure for 3 h; (B) exposure for 16 h.

The origin, the positions of the phosphoamino acid markers (P-Thr, phosphothreonine; P-Tyr, phosphotyrosine; P-Ser, phosphoserine) and the position of inorganic phosphate (Pi) are indicated.


B
A
⊖
⊕
Origin
P-Thr
P-Tyr
P-Ser
Pi

2.3 MOLECULAR PROPERTIES OF ViPK

(1) Apparent Molecular Weight

Chromatography on Sephadex G-150, using globular protein standards of known molecular weight, was used to estimate the molecular weight of native ViPK. From the experiment shown is Fig. 2.22 a value of 68,000 for the apparent molecular weight was obtained. A similar value was found when the estimation was performed by high-performance size-exclusion chromatography (see Fig. 2.4). Both these determinations were made at 500 mM KCl to prevent non-specific aggregation of proteins.

(2) Sedimentation Coefficient

Sedimentation studies of ViPK were performed in sucrose density gradients containing 0·1 M KCl or 1 M KCl (Fig. 2.23). As shown in Fig. 2.23 A, ViPK sedimented as a single peak with an S-value of approx. 4·3. When the salt concentation in the sucrose gradient was increased from 0·1 to 1 M the sedimentation profile of ViPK did not change significantly, showing the absence of non-specific aggregation.

Fig. 2.22 Size-Exclusion Chromatography of ViPK on Sephadex G-150

A preparation of ViPK purified by DE-52 anion-exchange chromatography was subjected to chromatography on Sephadex G-150 as described in Methods, section 4.4. Aliquots (40 µl) of the column fractions were assayed for protein kinase activity using protamine as substrate. The position of elution of Blue Dextran and protein standards is indicated.

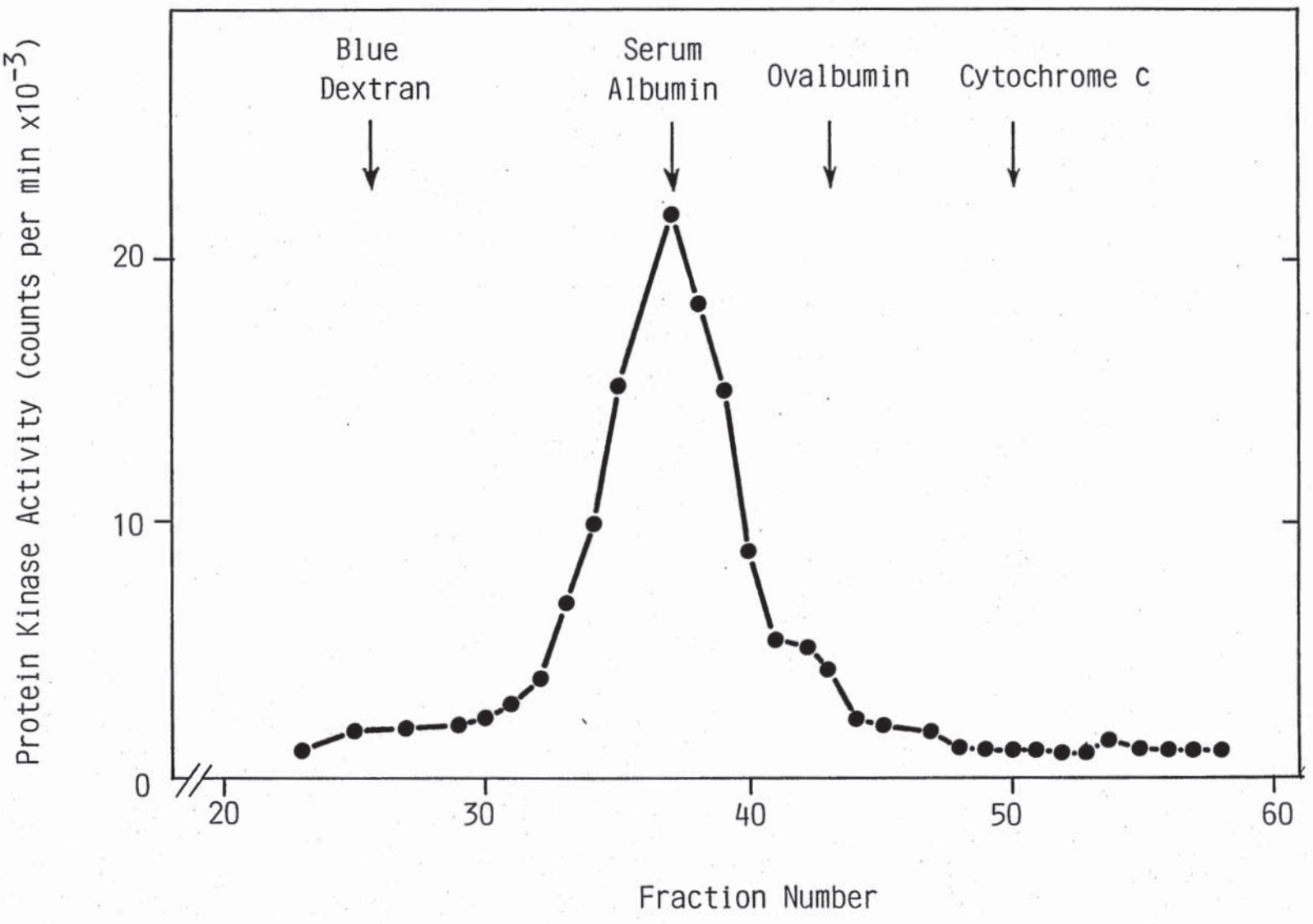
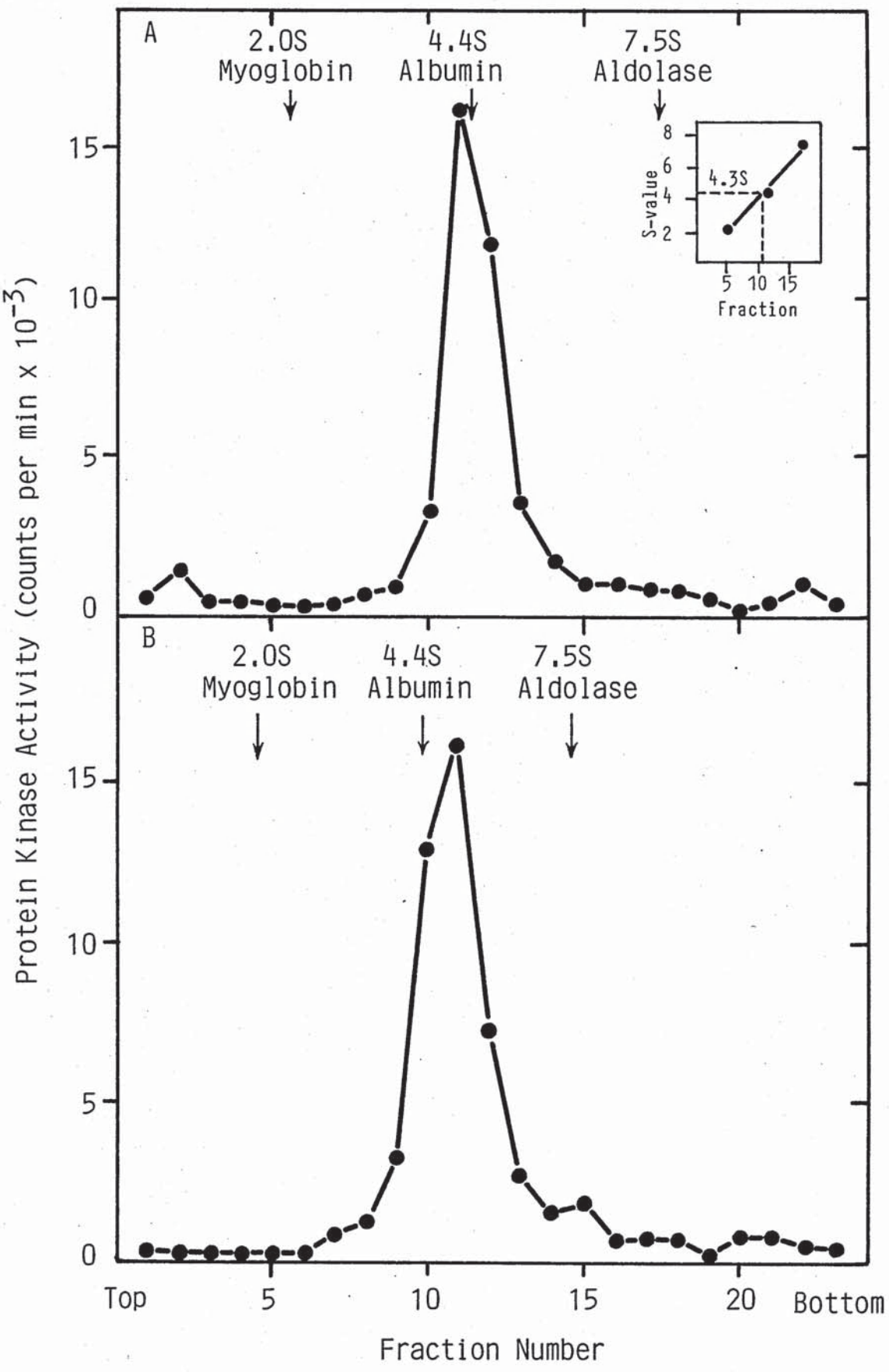

Blue
Dextran
Serum
Albumin
Ovalbumin
Cytochrome c
Protein Kinase Activity (counts per min x10^{-3})
20
10
0
20
30
40
50
60
Fraction Number

Fig. 2.23 Sucrose Density Gradient Sedimentation of ViPK

A preparation of ViPK purified by DE-52, anion-exchange chromatography was subjected to centrifugation on 10-30% linear sucrose gradients made in buffers containing (A) 0.1 M KCl, or (B) 1 M KCl, and the gradients fractionated, as described in Methods, section 5.1. Aliquots (40 μl) of the gradient fractions were assayed for protein kinase activity using mixed histones as substrate in a reaction mixture with a final concentration of 500 mM KCl in both cases. The position of sedimentation and published sedimentation coefficient of the protein standards is shown and presented graphically in the insert.


A
2.0S
Myoglobin
4.4S
Albumin
7.5S
Aldolase
S-value
4.3S
Fraction
B
2.0S
Myoglobin
4.4S
Albumin
7.5S
Aldolase
Protein Kinase Activity (counts per min x 10^{-3})
Top
Bottom
Fraction Number

3. ANALYSIS OF POTENTIAL PHYSIOLOGICAL SUBSTRATES FOR ViPK

3.1 PROTEINS PHOSPHORYLATED DURING VIRAL INFECTION

There are a number of proteins which become phosphorylated during infection of cells with herpes viruses and these include viral structural and non-structural proteins as well as proteins of the host cell. The presence of viral structural phosphoproteins has also been described in preparations of virus particles (Introduction, section 2.2). Fig. 3.1 illustrates the presence of some new phosphoproteins in the post-ribosomal fraction from BHK cells and Fig. 3.2 the increase in phosphorylation of ribosomal protein S6, after infection of the cells with PRV for 6 h.

As the appearance of a new protein kinase activity in the cytoplasm of infected BHK cells had been observed (ViPK, Fig. 1.2), an attempt was made to test whether the phosphorylation of certain proteins was a result of phosphorylation by this enzyme. Taking into account the importance, availability and appearance of specific phosphoproteins during the viral replicative cycle, the following were chosen for further studies:

(i) Two major structural (or late) phosphoproteins of the pseudorabies virion (molecular weights: 115,000 and 120,000).

(ii) Three non-structural β (early) proteins: DNase, DNA-polymerase and major DNA-binding protein.

(iii) One protein of the host cell, ribosomal protein S6.

These proteins were first tested to see whether they could serve as substrates for ViPK *in vitro*, and where this was the case the properties of the phosphoproteins from studies *in vitro* and *in vivo*

were compared. The possibility that other protein kinases could be involved in phosphorylation of spectific proteins *in vivo* was also tested.

Fig. 3.1 The Phosphorylation in vivo of Proteins in the Post-ribosomal Supernatant of Uninfected BHK Cells and Cells Infected with PRV

Uninfected BHK cells and cells infected with PRV were labelled with [^{32}P] orthophosphate for 3 h before harvesting, as described in Methods, section 1.5. Post-ribosomal supernatant was prepared (Methods, section 3.1) and protein precipitated with 5 volumes of acetone. Protein (25 μg) was subjected to two-dimensional O'Farrell gel electrophoresis [Methods, section 6.1 (3)].

The figure shows autoradiographs of the dried gels in which the protein was from:

(A) uninfected BHK cells

(B) BHK cells infected with PRV for 6 h.

Molecular weights of the standards used in the second dimension are indicated. For the first dimension the following standards were used: PGK - 3 phosphoglycerate kinase (pI 6·4), CA - carbonic anhydrase (pI 4·8), Actin - actin (pI 4·65) and STI - soybean trypsin inhibitor (pI 4·55).

The arrows indicate the phosphoproteins specific for post-ribosomal supernatant of infected cells.

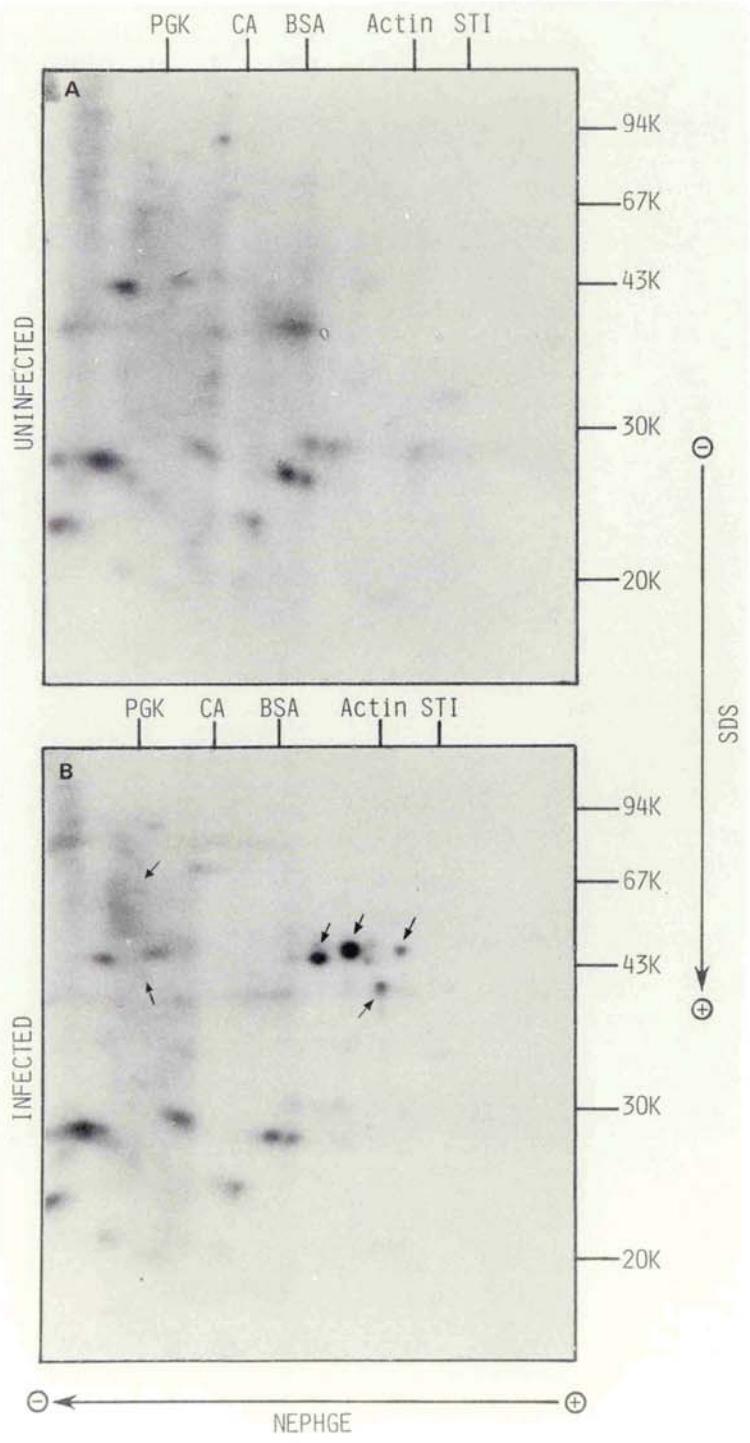

PGK
CA
BSA
Actin
STI
A
94K
67K
43K
30K
20K
UNINFECTED
SDS
B
INFECTED
NEPHGE

Fig. 3.2 The Phosphorylation in vivo of Ribosomal proteins of Uninfected BHK Cells and Cells Infected with PRV

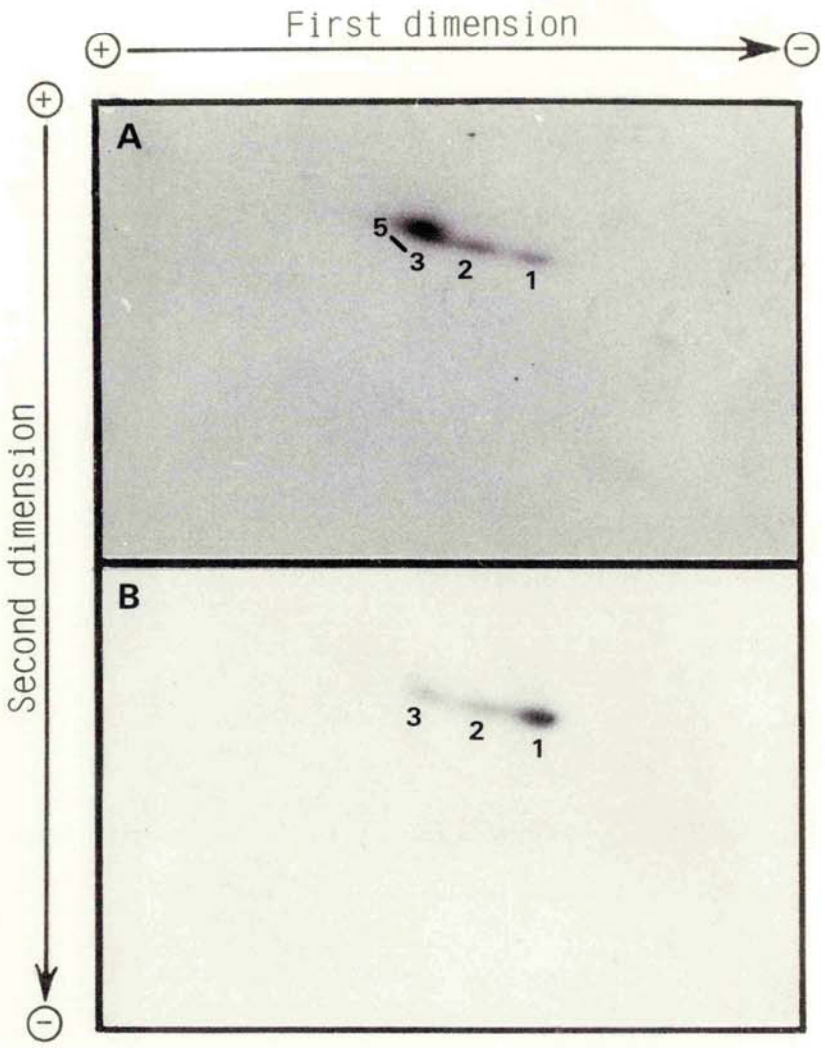
Uninfected BHK cells and cells infected with PRV, were labelled with [^{32}P] orthophosphate for 3 h before harvesting, as described in Methods, section 1.5. Ribosomes were prepared (Methods, section 3.2) and their proteins extracted (Methods, section 3.5). The protein labelled with ^{32}P (50 μg) was combined with 150 μg of the ribosomal proteins isolated from rapidly-growing cells (see Fig. 3.13) and subjected to two-dimensional gel electrophoresis [Methods, section 6.1 (2)].

The figure shows autoradiographs of the dried gels in which the protein was from:

(A) BHK cells infected with PRV for 6h.

(B) Uninfected BHK cells.

Different phosphorylated forms of ribosomal protein S6 are numbered (see Fig. 3.13).


First dimension
⊕
⊖
Second dimension
⊕
⊖
A
5
3
2
1
B
3
2
1

3.2 PHOSPHORYLATION OF VIRAL PROTEINS *IN VITRO*

(1) Structural Proteins

To study the phosphorylation of the two major structural phosphoproteins *in vitro*, proteins present in pseudorabies virus particles were solubilised in the presence of 10% NP-40 and 0·6 M KCl. The resulting protein extract contained endogenous kinase activity which phosphorylated the same two proteins (115,000 and 120,000 Da) which are labelled with ^{32}P *in vivo*. The endogenous phosphorylation was eliminated by heating the extract at 60°C for 15 min (Fig. 3.3). This heat-inactivated extract was used as a substrate for ViPK and preparations of other protein kinases.

Different fractions of protein kinases obtained from DE-52 chromatography of the post-ribosomal supernatant from infected BHK cells, were tested for phosphorylation of the heat-inactivated virion extract (Fig. 3.3). The fractions containing ViPK did not phosphorylate the viral proteins. The fractions in the range of 110-150 mM KCl strongly phosphorylated the 115,000 and 120,000 Da proteins. These fractions contain several protein kinase activities (Fig. 1.1), but when individual purified protein kinases were tested separately only casein kinase II used the two viral proteins as a substrate (not shown). These results therefore indicate that the candidates for the protein kinase responsible for phosphorylation *in vivo* include the endogenous activity of the virion and casein kinase II.

To analyse the protein kinase(s) present in pseudorabies virions, protein extracts were subjected to DEAE-cellulose or phosphocellulose chromatography and the column fractions assayed for phosphorylation of various substrates, including heat-inactivated virions (Fig. 3.4 and

3.5). It was clear from these experiments that the extract contains several kinase activities. The activity profiles were quite similar to those obtained for the protein kinases in the post-ribosomal supernatant of infected cells. Kinase activity similar to ViPK was also present (this eluted from DEAE-cellulose at 220 mM KCl and phosphorylated protamine). However, this activity was clearly separated by phosphocellulose column chromatography from the kinase which phosphorylated heat-inactivated virions (Fig. 3.5). The phosphorylation of the 115,000 and 120,000 Da proteins of the virion was by an activity which also phosphorylated casein, used GTP as well as ATP as phosphate donor and was strongly inhibited by heparin (Fig. 3.6). These properties are similar to those of host casein kinase II, as are the elution characteristics of the activity on DEAE-cellulose and phosphocellulose (Hathaway and Traugh, 1982). It is possible that the casein kinase II activity in the virion originates from the host cell. It could be incorporated into the virus particle during its assembly or during the budding process, as has been described for some other host proteins (Lodish and Porter, 1980).

(2) Non-structural Proteins

It has been demonstrated conclusively that herpes simplex virus (HSV) DNase is a phosphoprotein (Banks et al., 1985), and there is some indication that viral DNA-polymerase (Powell and Purifoy, 1977) and the major DNA-binding protein (Wilcox et al., 1980) are phosphorylated in infected cells. These three proteins (encoded by the HSV or PRV genome) are classified as β proteins (Honess and Roizman, 1974 and 1975) and appear in cells at the same stage of infection when ViPK activity has been detected. Direct comparison of the time-course

of induction of ViPK and DNA-polymerase activity is shown in Fig. 1.5.

Viral DNase, DNA-polymerase and the major DNA-binding protein were tested for phosphorylation by partially purified ViPK in vitro. As shown in Fig. 3.7, only viral DNase was phosphorylated. However, the studies with this viral enzyme were not extended further because insufficient purified protein was available.

Fig. 3.3 Phosphorylation of PRV Structural Proteins with Protein Kinase Activities from the Virion and from the Post-ribosomal Supernatant of Infected Cells

A protein extract of pseudorabies virions was prepared as described in Methods, section 2.2, and 2 µg of protein was assayed for phosphorylation by endogenous or added protein kinase activities under standard reaction conditions (Methods, section 10.4). The polypeptides of the virion were separated by electrophoresis in the presence of SDS on a 10% (w/v) acrylamide/ 0·26% (w/v) methylene-bis-acrylamide gel.

An autoradiograph of the dried gel, presented in the figure, shows endogenous phosphorylation in the extract of pseudorabies virions before (track 1) and after (track 2) incubation for 15 min at 60°C, and phosphorylation of the preincubated extract with DE-52 column fractions of the post-ribosomal supernatant from infected cells, combined within the range of 10-40 mM KCl (track 3), 110-150 mM KCl (track 4) and 200-240 mM KCl (track 5).


120
115
1
2
3
4
5

Fig. 3.4 DEAE-cellulose Chromatography of a Soluble Extract of Pseudorabies Virions

A protein extract of pseudorabies virions was subjected to DEAE-cellulose chromatography as described in Methods, section 4.1. Protein was eluted at increasing concentrations of KCl (←——). Aliquots (40 µl) of the column fractions were assayed, under standard reaction conditions (Methods, section 10.1), for the phosphorylation of various substrates:

(A) protamine (—●—) and histone (←—○—→);

(B) casein (—●—) and heat-inactivated pseudorabies virion extract (—■—).

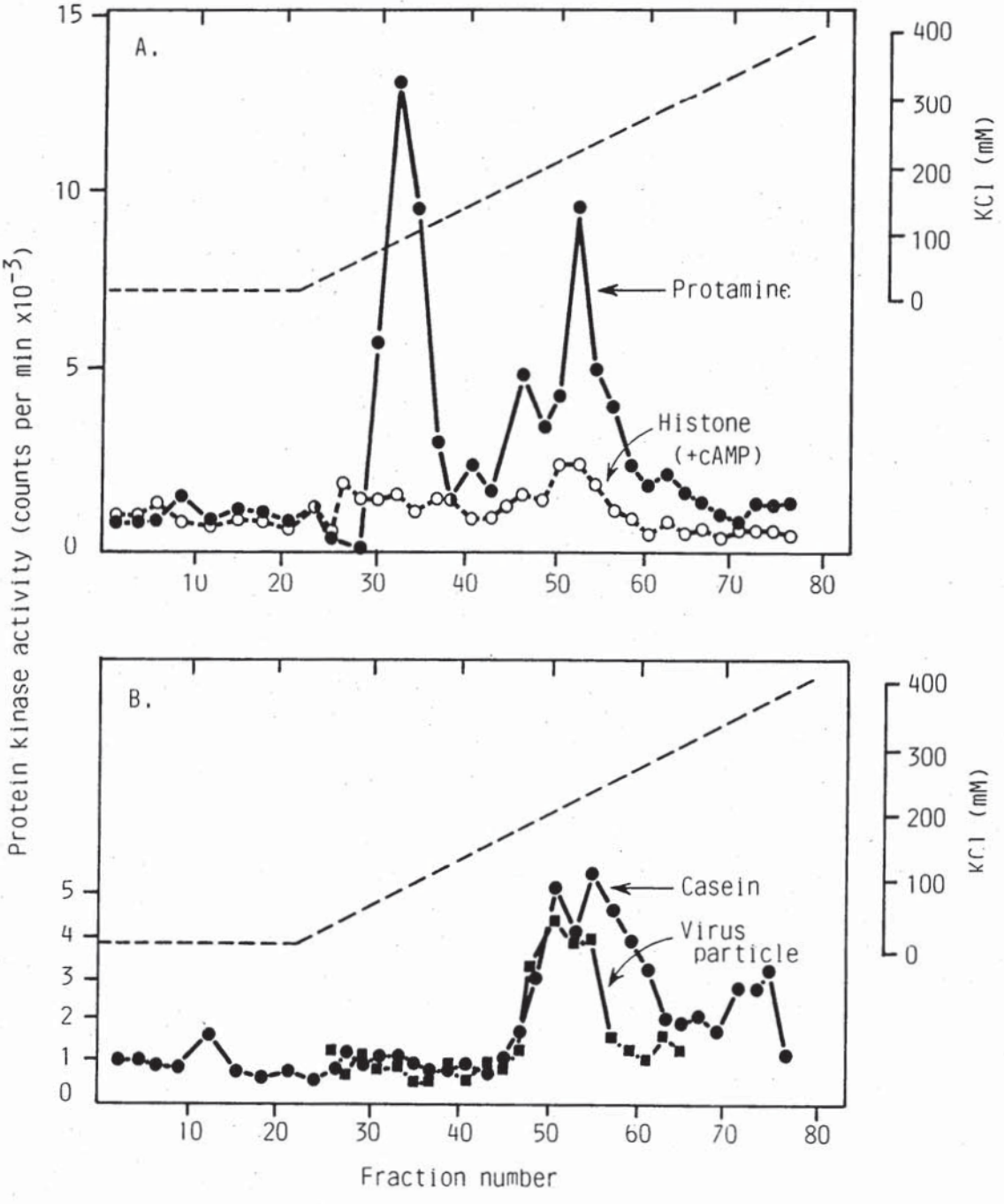

A.
B.
Protamine
Histone
(+cAMP)
Casein
Virus
particle
KCl (mM)
Protein kinase activity (counts per min x10^{-3})
Fraction number

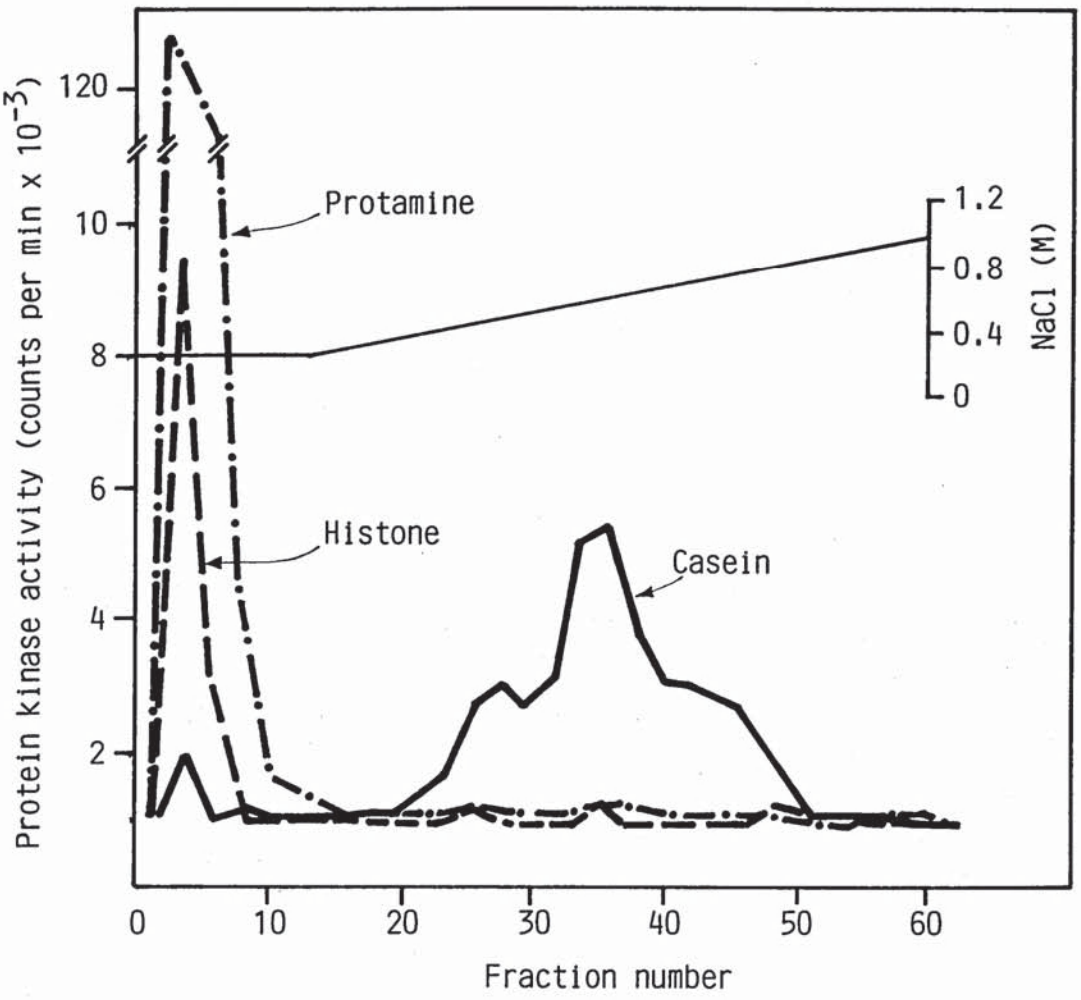
Fig. 3.5 Phosphocellulose Chromatography of a Soluble Extract of Pseudorabies Virions

A protein extract of pseudorabies virions was subjected to chromatography on a phosphocellulose column (Methods, section 4.2), and the concentration of NaCl in the column fractions was estimated from conductivity measurements (———). Aliquots (40 µl) of the column fractions were assayed for protein kinase activity with various substrates:

(A) Protamine as a substrate (—·—·→), mixed histone as a substrate in the presence of 10 µM cyclic AMP (— — —) and casein as a substrate (———).

(B) Heat-inactivated protein extract (1 µg) from pseudorabies virions as a substrate. Analysis of phosphorylation of 115,000 and 120,000 Da polypeptides was by SDS gel electrophoresis on a 10% (w/v) acrylamide/0·26% (w/v) methylene-bis-acrylamide gel, and subsequent autoradiography.

A


Protein kinase activity (counts per min x 10^{-3})
120
10
8
6
4
2
Protamine
Histone
Casein
1.2
0.8
0.4
0
NaCl (M)
0
10
20
30
40
50
60
Fraction number

B

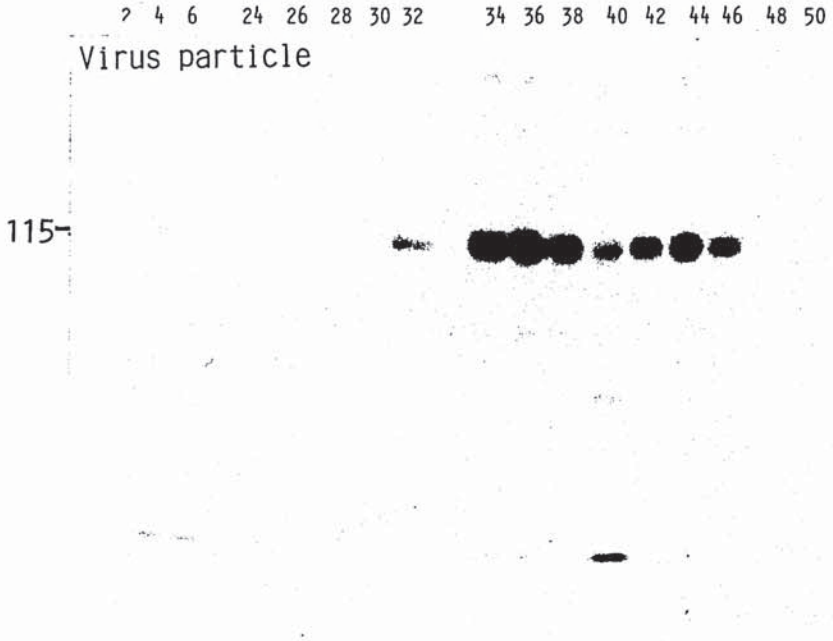

2 4 6 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Virus particle
115

Fig. 3.6 Characterization of Casein Kinase Activities in a Soluble Extract of Pseudorabies Virions after Fractionation by Phosphocellulose Column Chromatography

Fractions from the phosphocellulose column described in Fig. 3.5 were assayed for phosphorylation of casein using 0·1 mM [γ-^{32}P] ATP (—●—), phosphorylation of casein using 0·1 mM [γ-^{32}P] GTP (–·–○–·–), and phosphorylation of casein using [γ-^{32}P] ATP but in the presence of 1 µg/ml heparin (– – □ – –).

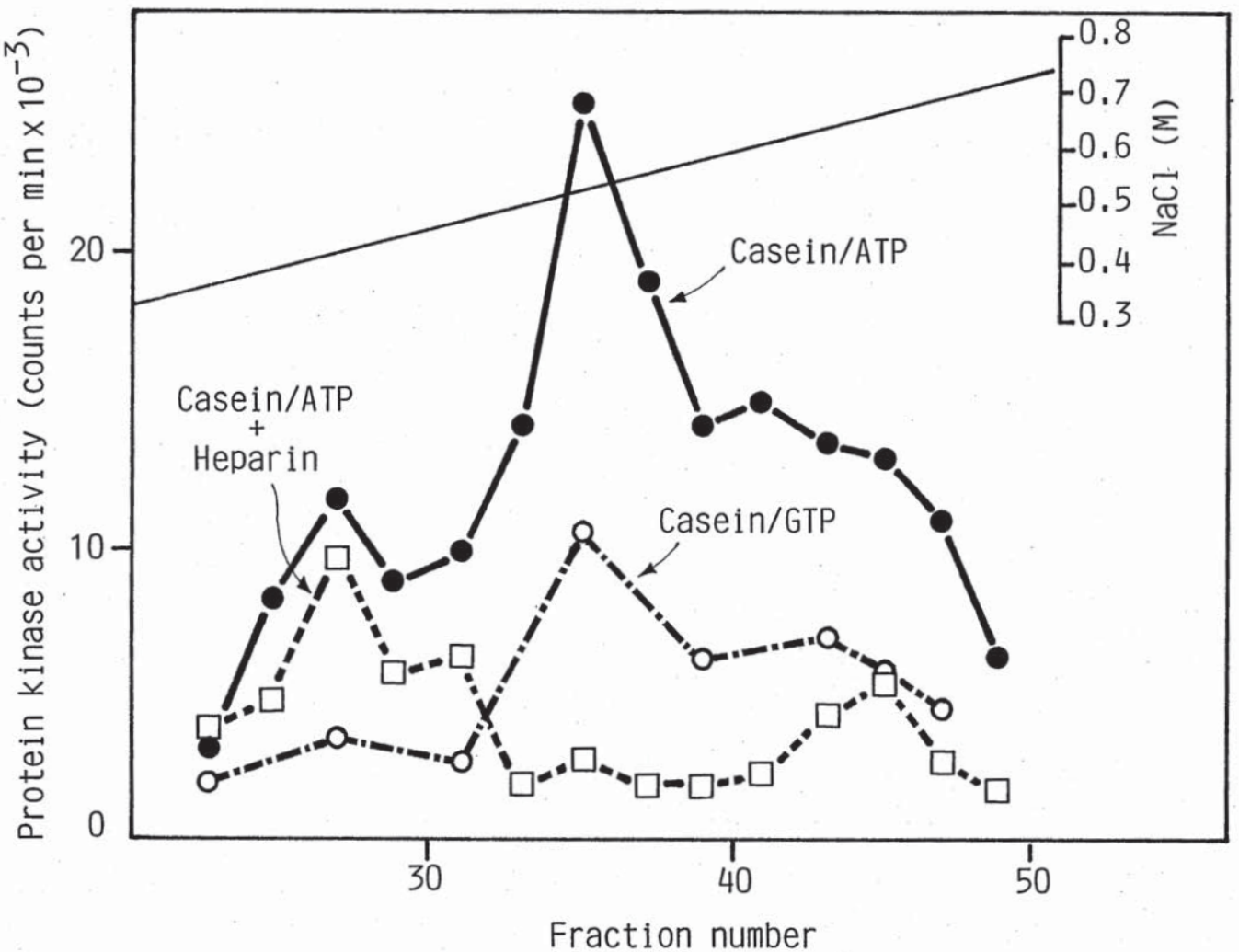
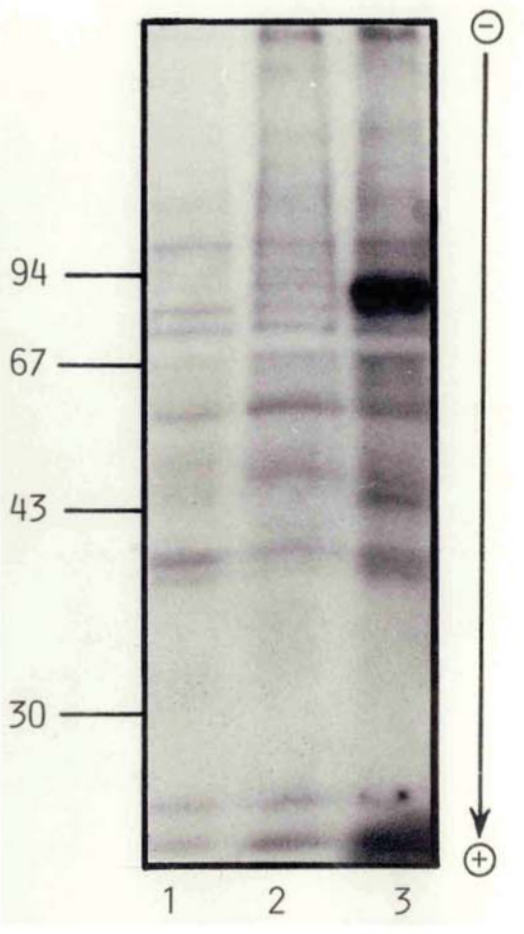

Protein kinase activity (counts per min x 10^{-3})
0
10
20
30
40
50
Fraction number
0.8
0.7
0.6
0.5
0.4
0.3
NaCl (M)
Casein/ATP
Casein/ATP
+
Heparin
Casein/GTP

Fig. 3.7 Phosphorylation of Non-structural Virally-coded Proteins by ViPK

ViPK (10 µl of 10 x concentrated DE-52 preparation) was assayed for phosphorylation of: (1) 1 µg of HSV major DNA-binding protein (140,000 Da); (2) 1 µg of HSV DNA-polymerase (150,000 Da); (3) 1 µg of HSV DNase (85,000 Da). The conditions for the phosphorylation reaction are described in Methods, section 10.4. The viral non-structural proteins were subjected to SDS gel electrophoresis on a 10% (w/v) acrylamide/0·26% (w/v) methylene-bis-acrylamide gel. The figure shows an autoradiograph of the dried gel.


94
67
43
30
1
2
3

3.3 PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6 *IN VITRO*

(1) Kinases in BHK Cells which Phosphorylate S6

Infection of BHK cells with PRV causes conversion of ribosomal protein S6 to a series of highly phosphorylated derivatives which contain up to 5 phosphoryl groups per molecule (Kennedy *et al.*, 1981; Fig. 3.2). In an attempt to test the involvement of ViPK or to identify another kinase(s) responsible for this phosphorylation, DE-52 fractions of post-ribosomal supernatant from infected cells were analysed for phosphorylation of S6 in 40S ribosomal subunits *in vitro*. This phosphorylation was assayed under ionic conditions which had been used by others to obtain optimal functional activity of 40S subunit *in vitro* (Voorma *et al.*, 1983). The results presented in Fig. 3.8 and 3.9 show that distinct peaks of S6 kinase activity could only be detected if the regulatory molecules of the previously identified protein kinases (Results, section 1.1) or, in the case of ViPK, high ionic strength, were present in the reaction assay. In their absence (Fig. 3.8) there was still some phosphorylation of S6 by fractions corresponding to the same protein kinase activities. Kinase activities which were able to phosphorylate S6 *in vitro* (Fig. 3.9) included the two isoforms of the cyclic AMP-dependent protein kinase, protein kinase C and ViPK.

Apart from S6, some other ribosomal proteins were phosphorylated *in vitro*. Two-dimensional gel analysis was used to identify these ribosomal phosphoproteins. DE-52 fractions which contained ViPK phosphorylated ribosomal protein S7 (Fig. 3.10 A and 2.19) and another protein which probably corresponds to S10 (Fig. 3.10 A). One basic protein of the 60S ribosomal subunit, L35, was extensively phosphorylated (Fig. 3.10 B), but like the phosphorylation of S7 and

S10, it most probably represents an artefact of the assay *in vitro*, since these proteins are not phosphorylated in uninfected or infected BHK cells (Fig. 3.2). Acidic proteins of 60S subunit, which are present as phosphoproteins in eukaryotic ribosomes (Leader, 1980), were also phosphorylated *in vitro* by fractions containing casein kinase activities (Fig. 3.10 C).

To further investigate the potential S6 kinases, the characteristics of S6 phosphorylation *in vitro* by ViPK, cyclic AMP-dependent protein kinase, and kinase C, were studied in more detail.

(2) Phosphorylation of S6 by ViPK, the Catalytic Subunit of Cyclic AMP-dependent Protein Kinase and Protein Kinase C

The enzymes used to phosphorylate S6 *in vitro* were: a homogenous preparation of the catalytic subunit of cyclic AMP-dependent protein kinase isolated from rabbit muscle, a homogenous preparation of kinase C from bovine brain, and partially purified ViPK from BHK cells infected with PRV.

Phosphorylation of S6 by the catalytic subunit of the cyclic AMP-dependent protein kinase *in vitro* has been studied in detail by others, and the conditions chosen for this study were those described by Wettenhall and Cohen (1982). Although phosphorylation of S6 by protein kinase C *in vitro* had been already reported, the conditions for maximal phosphorylation had not been investigated (Le Peuch *et al.*, 1983). Fig. 3.11 and 3.12 show the effect of $MgCl_2$, KCl, $CaCl_2$ and phosphatidyl serine on the phosphorylation of S6 by kinase C. Good phosphorylation of S6 was achieved in the presence of 150 mM KCl, 5 mM $MgCl_2$, 0·1 mM $CaCl_2$ and 0·5 mg/ml of phosphatidyl serine. The conditions for phosphorylation of S6 by ViPK were chosen according to

experiments already described [Results, 2.2(3)] and the reaction mixture contained 7-10 mM spermine, 100-150 mM KCl and 5 mM $MgCl_2$.

The phosphorylation of S6 *in vitro* was characterized and compared with the data from studies *in vivo* by analysis of (i) the stoichiometry of phosphorylation and (ii) the phosphopeptides generated from S6 by complete digestion with trypsin.

For the determination of the stoichiometry of phosphorylation two approaches were used. In both, the aim was to obtain maximum phosphorylation. However, the amounts of the enzymes and ribosomal subunits used were comparable with the amounts used by others (Martin-Perez *et al.*, 1984; Wettenhall and Morgan, 1984; Perišić and Traugh, 1983). One approach, described in Table 3.1, was based on measurements of the radioactivity incorporated into a known amount of protein S6 separated by SDS gel electrophoresis after phosphorylation *in vitro*. In the second approach, different phosphorylated derivatives of S6 were separated by two-dimensional gel electrophoresis (Fig. 3.13). Because of the amount of protein needed to visualize individual stained forms on two-dimensional gels it was not feasible to use the enzyme: substrate ratios used in the first approach. Instead, when it was necessary to approach saturation of S6 phosphorylation sites, the incubation time was prolonged. It was important in these determinations of stoichiometry to use ribosomes in which protein S6 was initially completely dephosphorylated (Fig. 3.14 A) and this was achieved by isolating ribosomes from BHK cells after 6 days of growth (Leader *et al.*, 1976).

The results of experiments using these two approaches showed that ViPK incorporated only 1-2 moles of phosphate per mol of S6 (Fig. 3.15 and Table 3.1).

The catalytic subunit of the cyclic AMP-dependent protein kinase incorporated 2-3 moles of phosphate per mole of S6 (Table 3.1) which is in agreement with data reported previously (Wettenhall and Morgan, 1984). Two-dimensional gel analysis (Fig. 3.14) showed that most of S6 was present as the diphosphorylated form.

Analysis of the phosphorylated forms of S6 after incubation in the presence of native kinase C showed the presence of multiple ^{32}P-labelled anodic derivatives which manifested themselves as a long "tail" on the autoradiograph, extending much further to the anode than the most highly phosphorylated form of S6 found in vivo (not shown). It is possible that the presence of phospholipid is somehow responsible for this artefact since it was eliminated by using protein kinase C activated by limited proteolysis. The use of this latter form of the enzyme, the activity of which is independent of Ca^{2+} and phospholipid, showed that the majority of stain and radioactivity was at the position of the triphosphorylated form of S6 (Fig. 3.16). The other approach, the results of which are presented in Table 3.1, gave a value of 4-5 moles of phosphate per mol of S6.

An assessment of the physiological significance of the phosphorylation in vitro, of a protein which can be phosphorylated at multiple sites, requires identification of the serine residues which are modified. This involves purification and sequencing of phosphopeptides generated from S6. Another although less precise method, which allows comparison of phosphorylated sites is analysis of the phosphopeptide patterns obtained after separation of peptides generated from S6. In the method used here, phosphopeptides were generated by complete digestion of S6 with trypsin and separated by reversed-phase high performance chromatography. As shown in Fig. 3.17 phosphopeptides

from S6 phosphorylated with ViPK *in vitro* were also present in the tryptic digest of S6 isolated from BHK cells infected with PRV. They included most, but not all, of the phosphopeptides found *in vivo*. The most prominent peptides phosphorylated by kinase C *in vitro* were unequivocally detected as phosphopeptides *in vivo*. The less prominent phosphopeptides were not detected in S6 phosphorylated *in vivo*. This could either be because less radioactivity was present in the sample of S6 phosphorylated *in vivo*, or could indicate that these phosphopeptides were artefacts of the phosphorylation by kinase C *in vitro*.

Fig. 3.8 Phosphorylation of 40S Ribosomal Proteins by DE-52 Column Fractions from BHK Cells Infected with PRV

Post-ribosomal supernatant from BHK cells infected with PRV was subjected to chromatography on a DE-52 column and assayed for protein kinase activities as described in Fig. 1.1 The column fractions (30 µl) were then assayed for phosphorylation of rat liver 40S ribosomal subunits ($1A_{260}$ unit) under standard conditions (Methods, section 10.3) in a reaction volume of 120 µl. Incubation was at 30°C for 30 min. The ribosomal proteins were separated by SDS gel electrophoresis [15% (w/v) acrylamide/0·09% (w/v) methylene-bis-acrylamide] and the dried gel subjected to autoradiography [Methods, sections 6.1 (1) and 6.2 (3)]. The autoradiograph was used to locate phosphorylated proteins, which were of 31,000 (—●—) and 22,000 (–-○-–) molecular weight, and these were cut out of the gel and their radioactivity quantitated as described in Methods, section 6.2 (5). Identification of phosphorylated proteins as S6 and S7, respectively, was based on two-dimensional gel analysis (see Fig. 3.10). The concentration of KCl in the column fractions, used to phosphorylate 40S ribosomal subunit, is also indicated.

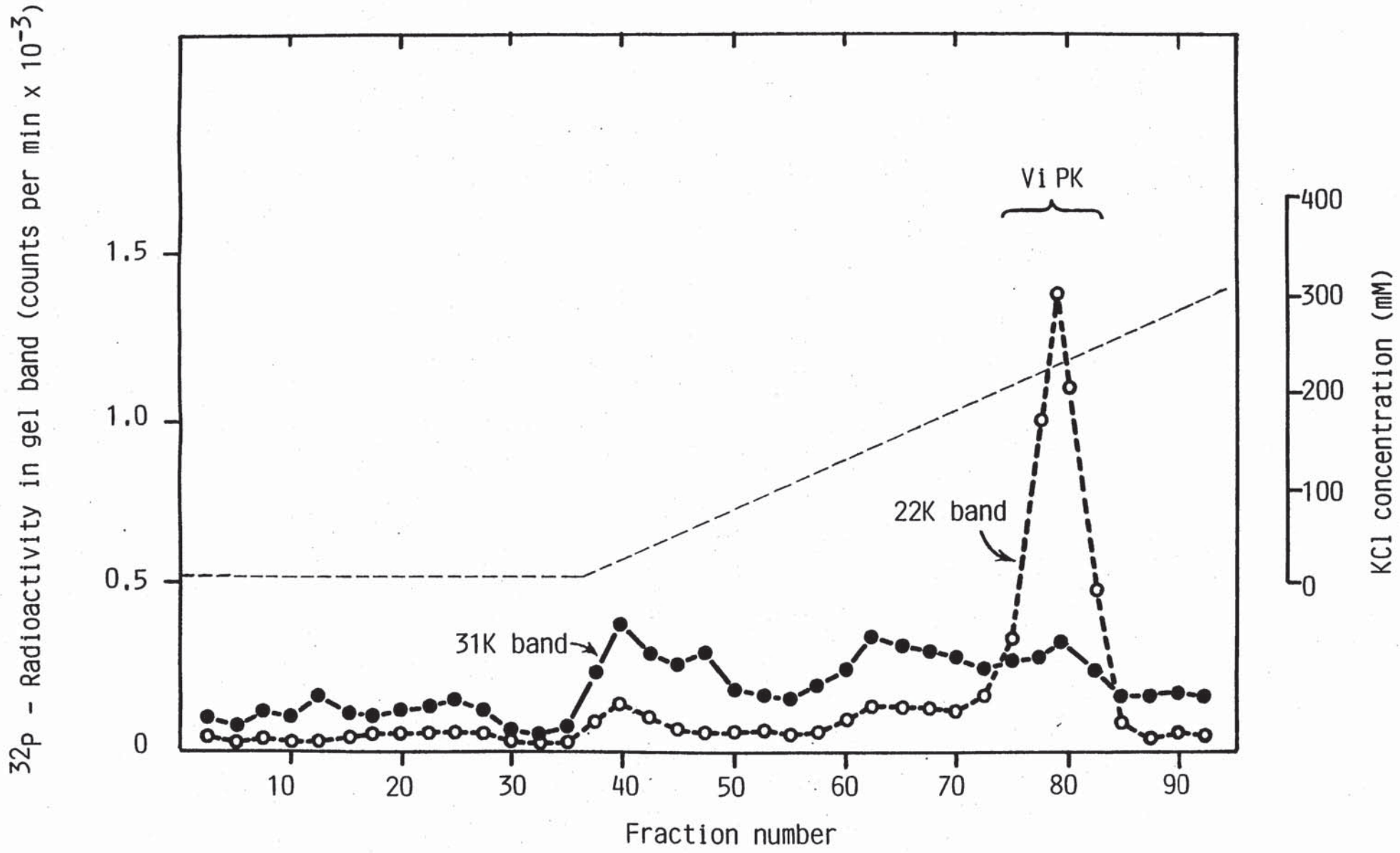

^{32}P - Radioactivity in gel band (counts per min x 10^{-3})
1.5
1.0
0.5
0
Vi PK
22K band
31K band
400
300
200
100
0
KCl concentration (mM)
10
20
30
40
50
60
70
80
90
Fraction number

Fig. 3.9 Ribosomal Protein S6 Kinase Activities in DE-52 Column Fractions from BHK Cells Infected with PRV

Post-ribosomal supernatant from BHK cells infected with PRV was subjected to DE-52 anion-exchange chromatography and column fractions analysed for protein kinase activities, as described in Fig. 1.1. The column fractions (30 µl) were then assayed for the phosphorylation of rat liver 40S ribosomal subunits ($1A_{260}$ unit) in a reaction volume of 120 µl. Incubation was at 30°C for 30 (A, B) or 15 min (C). Conditions of phosphorylation are described in Methods, section 10.3. The concentration of KCl and presence of regulatory molecules is indicated in the figure:

(A) 10 µM cyclic AMP, 100 mM KCl;

(B) 600 mM KCl;

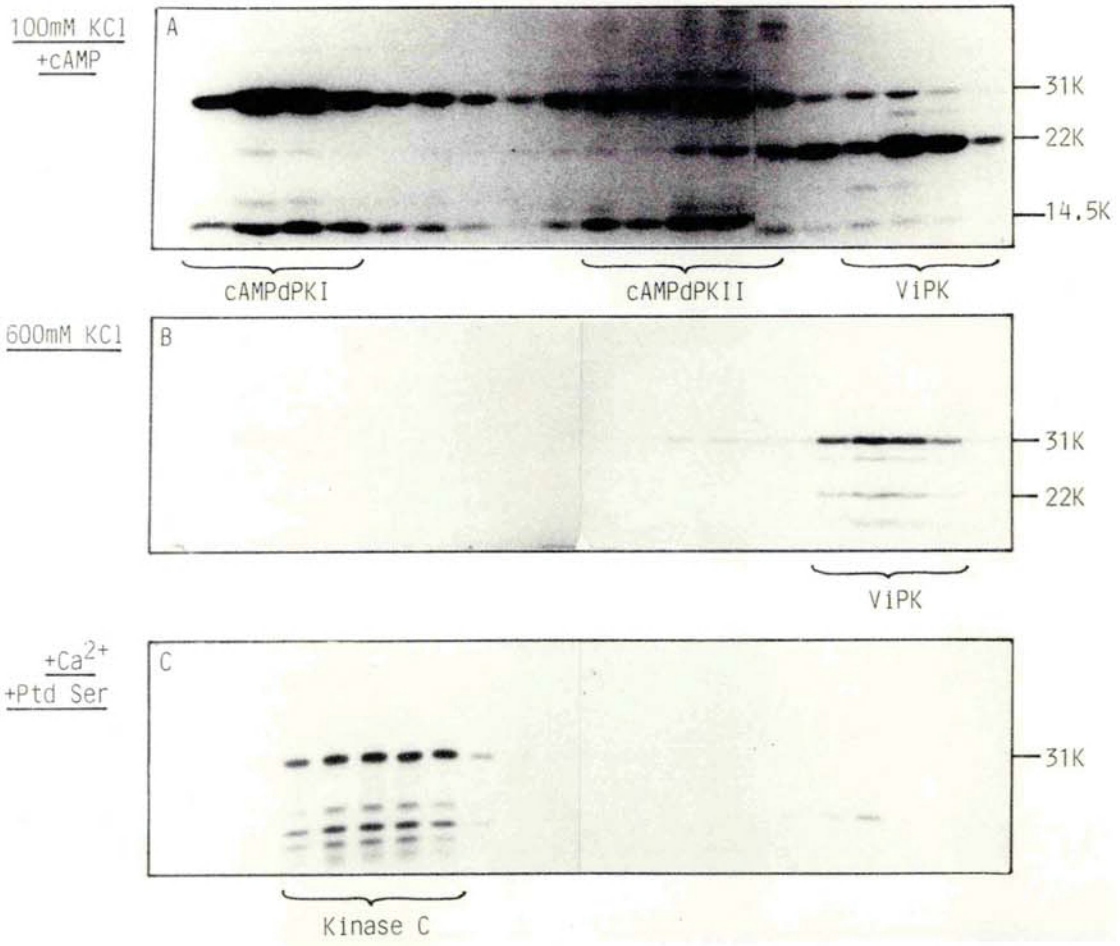
(C) 0·15 mM $CaCl_2$, 500 µg/ml phosphatidyl serine, 0 mM KCl.

Phosphorylation of ribosomal proteins was analysed as in Fig. 3.8, using SDS gel electrophoresis, autoradiography and quantitation of radioactivity in protein S6.

(a) Autoradiographs of the dried gels (opposite);

(b) ^{32}P-radioactivity in S6 (next page).

a


100mM KCl
+cAMP
A
31K
22K
14.5K
cAMPdPKI
cAMPdPKII
ViPK
600mM KCl
B
31K
22K
ViPK
+Ca2+
+Ptd Ser
C
31K
Kinase C

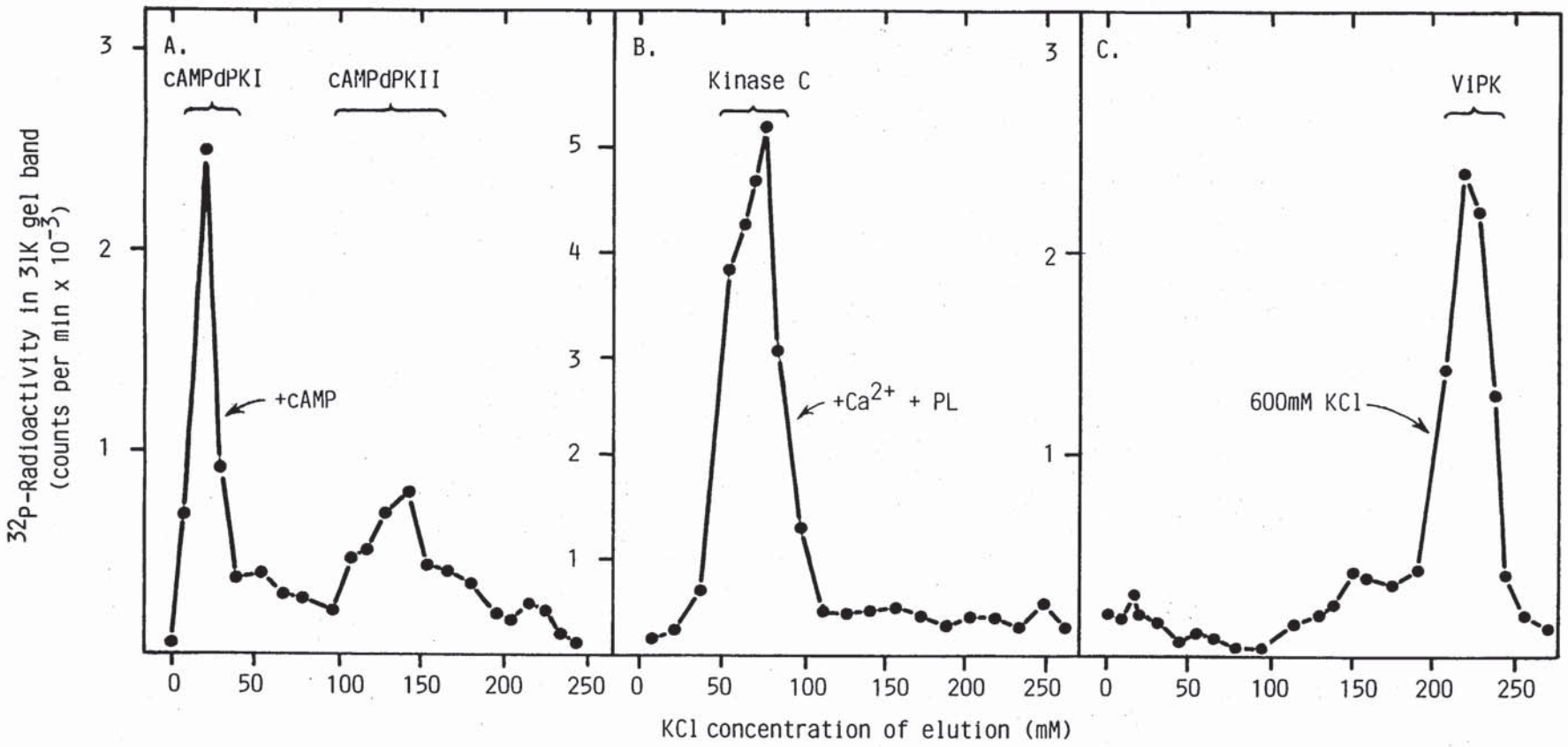

A.
cAMPdPKI
cAMPdPKII
+cAMP
B.
Kinase C
+Ca2+ + PL
C.
VIPK
600mM KCl
32P-Radioactivity in 31K gel band
(counts per min x 10-3)
KCl concentration of elution (mM)

Fig. 3.10 Two-dimensional Gel Analysis of Ribosomal Subunits Phosphorylated by DEAE-cellulose Fractions from BHK Cells Infected with PRV

DE-52 column fractions were combined within ranges of KCl concentrations of elution: 10-40 mM KCl (peak 1); 110-150 mM KCl (peak 2); 200-240 mM KCl (peak 3) and 120 µl of these used to phosphorylate rat liver ribosomal subunits in the standard reaction mixture in the presence of 10 µM cyclic AMP.

Ribosomal proteins were isolated and subjected to two-dimensional gel electrophoresis for:

(A) basic proteins from the 40S subunit ($5A_{260}$ units);

(B) basic proteins from the 60S subunit ($10A_{260}$ units);

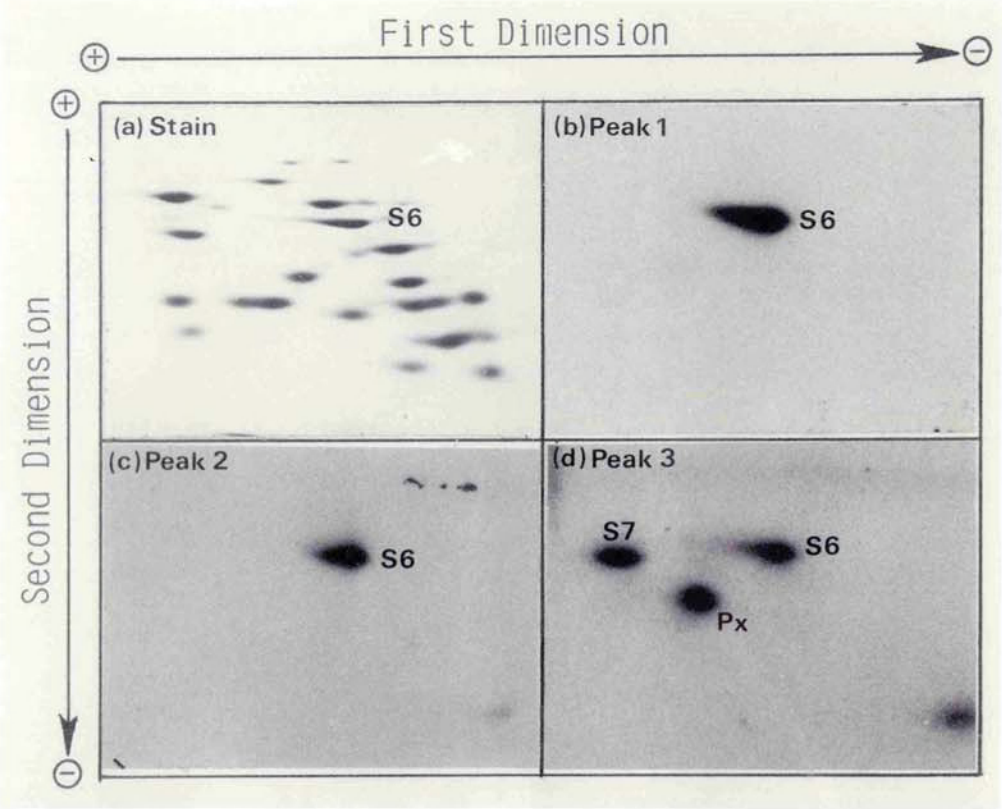
(C) acidic proteins from the 60S subunit ($15A_{260}$ units).

The details are in Methods, sections 10.3, 6.1 (2) and 6.2 (3).

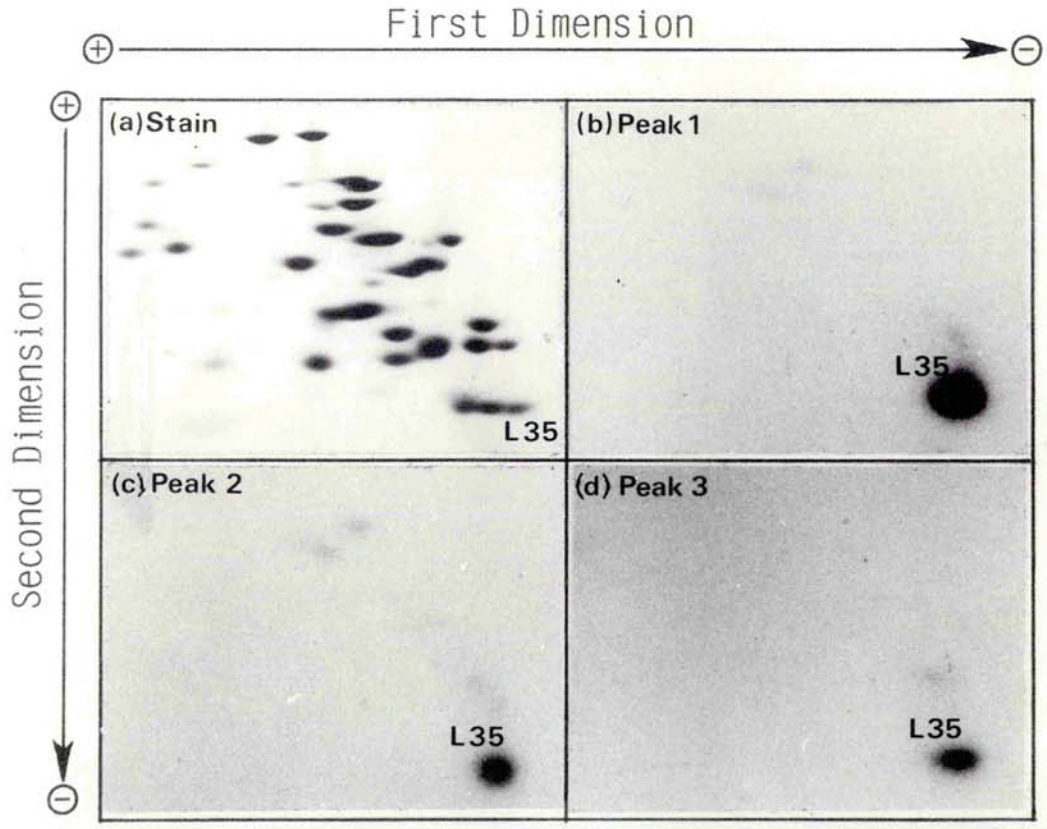
The figures show stained gels (a) and autoradiographs of ribosomal proteins phosphorylated by"peak 1" (b), "peak 2" (c) or "peak 3" (d).

The figures (A, B and C) are presented on the three following pages.

A


First Dimension
⊕
⊖
Second Dimension
(a) Stain
S6
(b) Peak 1
S6
(c) Peak 2
S6
(d) Peak 3
S7
S6
Px

B


First Dimension
⊕
⊖
Second Dimension
⊕
⊖
(a) Stain
L35
(b) Peak 1
L35
(c) Peak 2
L35
(d) Peak 3
L35

c

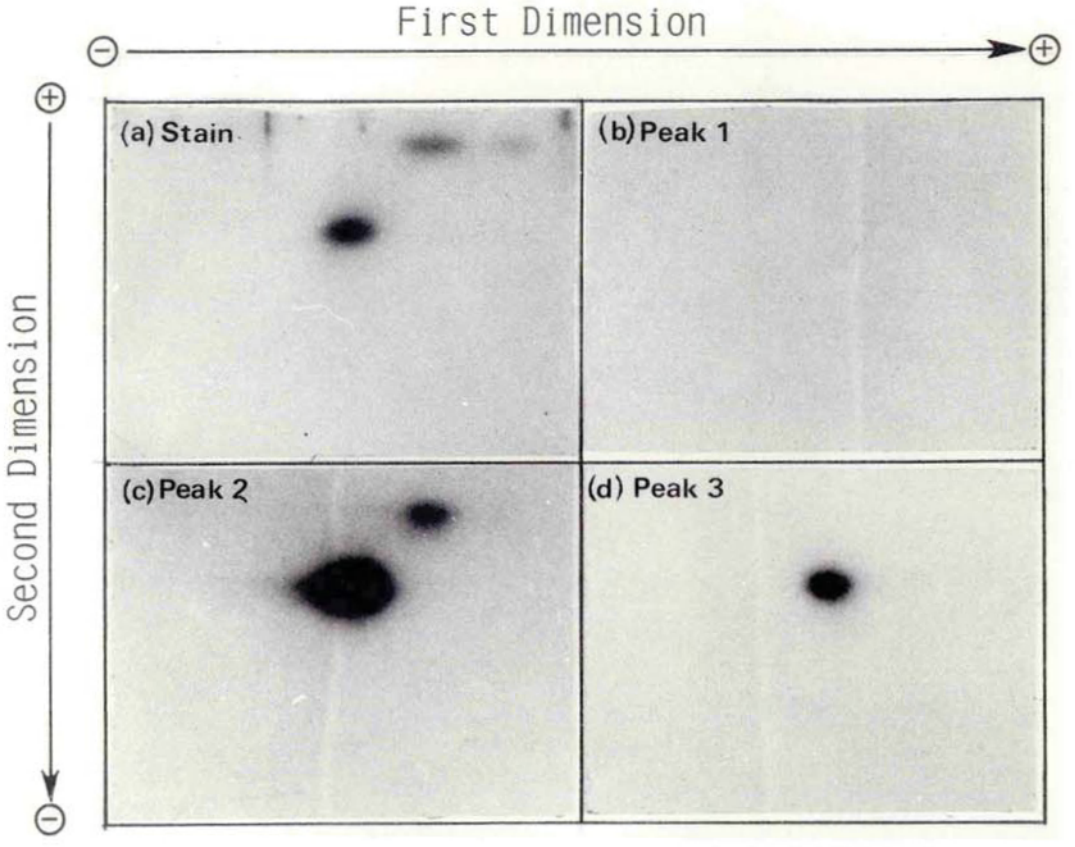

First Dimension
Second Dimension
(a) Stain
(b) Peak 1
(c) Peak 2
(d) Peak 3

Fig. 3.11 Ionic Dependency of the Phosphorylation of Ribosomal Protein S6 by Protein Kinase C

Rat liver 40S ribosomal subunits ($0{\cdot}5A_{260}$ units) were incubated for 5 min with homogenous native bovine brain protein kinase C (0·03 units) in 40 µl reaction as described in Methods, section 10.3, but at the ionic concentrations indicated, and subjected to gel electrophoresis in SDS.

The figure shows an autoradiograph of the dried gel. The designation of the position of migration of S6 was from the stained gel, based on previous analysis of this protein phosphorylated *in vivo* and *in vitro*.

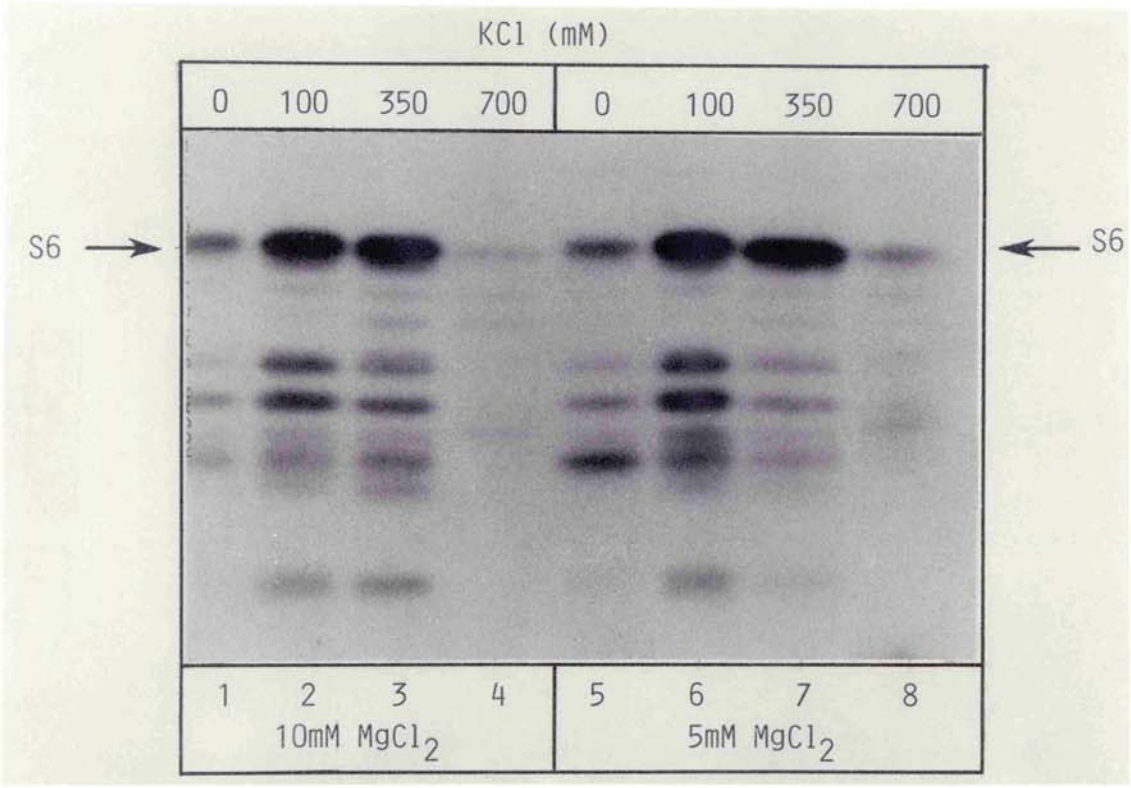

KCl (mM)
0
100
350
700
0
100
350
700
S6
S6
1
2
3
4
5
6
7
8
10mM MgCl2
5mM MgCl2

Fig. 3.12 Calcium Dependency of the Phosphorylation of Ribosomal Protein S6 by Protein Kinase C

Rat liver 40S ribosomal subunits ($0{\cdot}5A_{260}$ units) were incubated for 15 min with homogenous native bovine brain protein kinase C (0·03 units) in a 40 μl reaction as described in Methods, section 10.3, but with added $CaCl_2$ at the concentration shown, and phosphatidyl serine excluded in the one instance indicated. The 40S subunits were subjected to gel electrophoresis in the presence of SDS, and the gel dried and subjected to autoradiography. The autoradiograph was used to locate ribosomal protein S6, which was cut out of the gel and its Cerenkov radiation measured in a scintillation spectrometer.

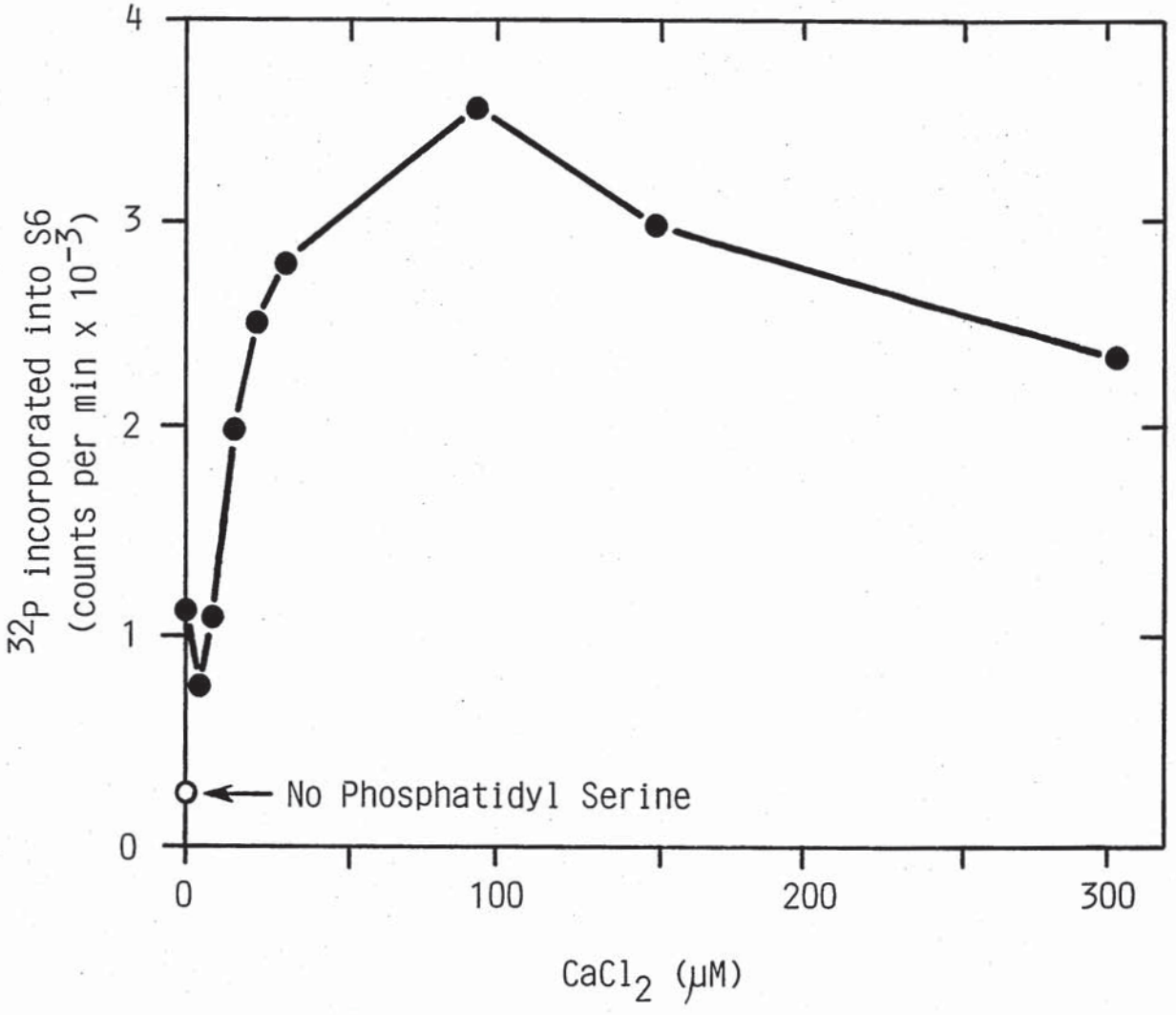

4
3
2
1
0
^{32}P incorporated into S6
(counts per min x 10^{-3})
No Phosphatidyl Serine
0
100
200
300
$CaCl_2$ (μM)

Table 3.1 Stoichiometry of Phosphorylation of Ribosomal Protein S6 in vitro

The table summarizes data from several experiments where 40S ribosomal subunits from rat liver were incubated with the protein kinases listed in the table in a 40 µl reaction mixtures under their respective optimal conditions described in Methods, section 10.3. The incubation time was 2 h.

The calculation of stoichiometry of the phosphorylation of ribosomal protein S6 was based on the radioactivity in the protein after excision from SDS gels, compared with the specific activity of the [γ-^{32}P] ATP and assuming that 1A_{260} unit of 40S ribosomal subunits is equivalent to 66·3 pmol.

TABLE 3.1

ENZYME	CONCENTRATION OF ENZYME (units/ml)	CONCENTRATION OF 40S (pmol/ml)	STOICHIOMETRY OF PHOSPHORYLATION (mol of phosphate per mol of S6)
KINASE C * activated by limited proteolysis or in the presence of Ca^{2+} and phospholipid	10-20	200	4-5
CATALYTIC SUBUNIT OF THE CYCLIC AMP-DEPENDENT PROTEIN KINASE *	10-20	200	2-3
ViPK+ in the presence of 7 mM spermine	10	200	2

* Purified to homogeneity (Materials, section 2.3)

+ Partially purified (Fig. 2.1)

Fig. 3.13 Two-dimensional Electrophoretic Resolution of Five Phosphorylated Forms of Ribosomal Protein S6

Ribosomes were prepared from rapidly growing cells (Methods, section 3.2), their proteins extracted (Methods, section 3.5) and 200 µg of the protein subjected to two-dimensional gel electrophoresis, modified to resolve five phosphorylated forms of ribosomal protein S6 [Methods, section 6.1 (2)].

In this and subsequent figures protein spots are designated at their immediate right hand side, and numbers 0, 1, 2 etc. underneath their different derivatives indicate the unphosphorylated and increasing phosphorylated states of these proteins respectively.

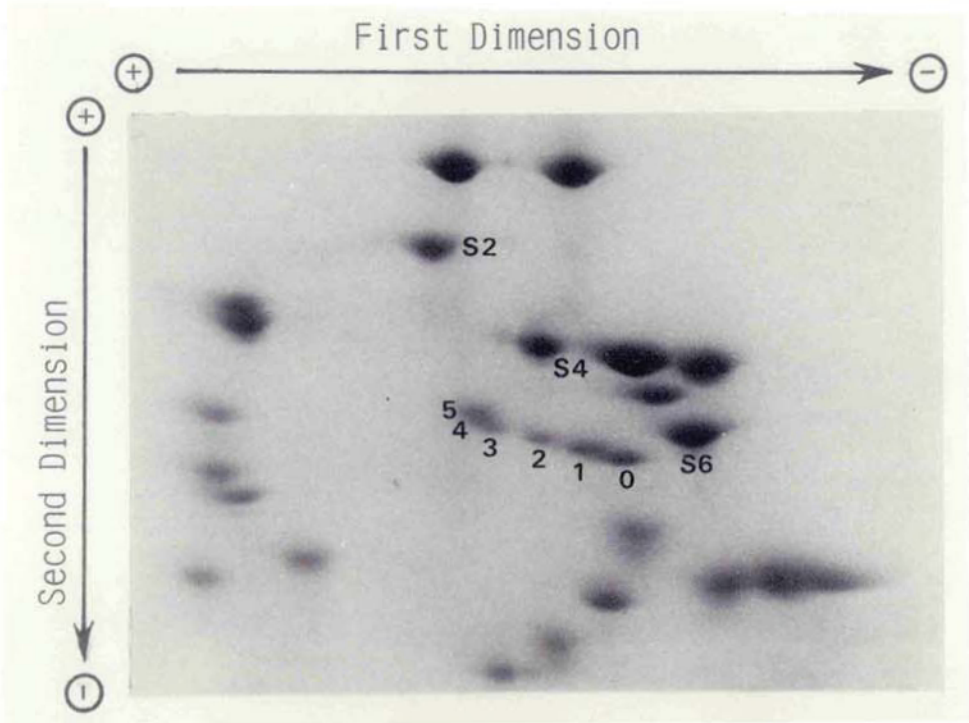

First Dimension
Second Dimension
S2
S4
5
4
3
2
1
0
S6

Fig. 3.14 Two-dimensional Gel Analysis of 40S Ribosomal Subunits Phosphorylated by the Catalytic Subunit of Cyclic AMP-dependent Protein Kinase

BHK cell 40S ribosomal subunits ($12A_{260}$ units) were phosphorylated with 5 units of the catalytic subunit of cyclic AMP-dependent protein kinase in 800 µl reaction mixture, as described in Methods, section 10.3. Incubation was for 4 h. The ribosomal protein was isolated and subjected to two-dimensional gel electrophoresis.

The figure shows a stained gel of unphosphorylated 40S ribosomal subunits (A), stained gel (B) and corresponding autoradiograph (C) of 40S subunits phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase.

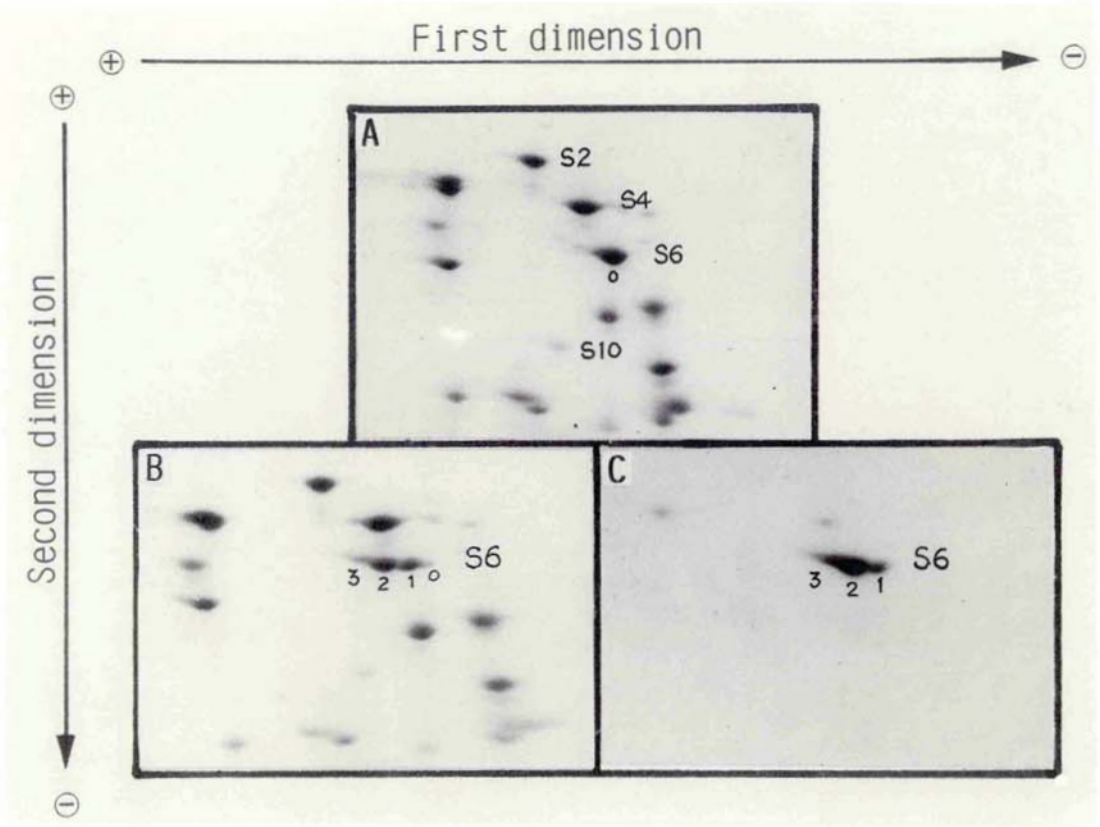

First dimension
⊕
⊖
Second dimension
⊕
⊖
A
S2
S4
S6
0
S10
B
S6
3 2 1 0
C
S6
3 2 1

Fig. 3.15 Two-dimensional Gel Analysis of 40S Ribosomal Subunits Phosphorylated by ViPK

Phosphorylation of 40S BHK ribosomal subunits ($12A_{260}$ units) with ViPK (800 units of 10 x concentrated DE-52 preparation) was in the 800 µl standard reaction mixture (Methods, section 10.3) in the presence of 10 mM spermine and 150 mM KCl. Incubation was for 3·5 h.

(A) Stained gel

(B) Autoradiograph

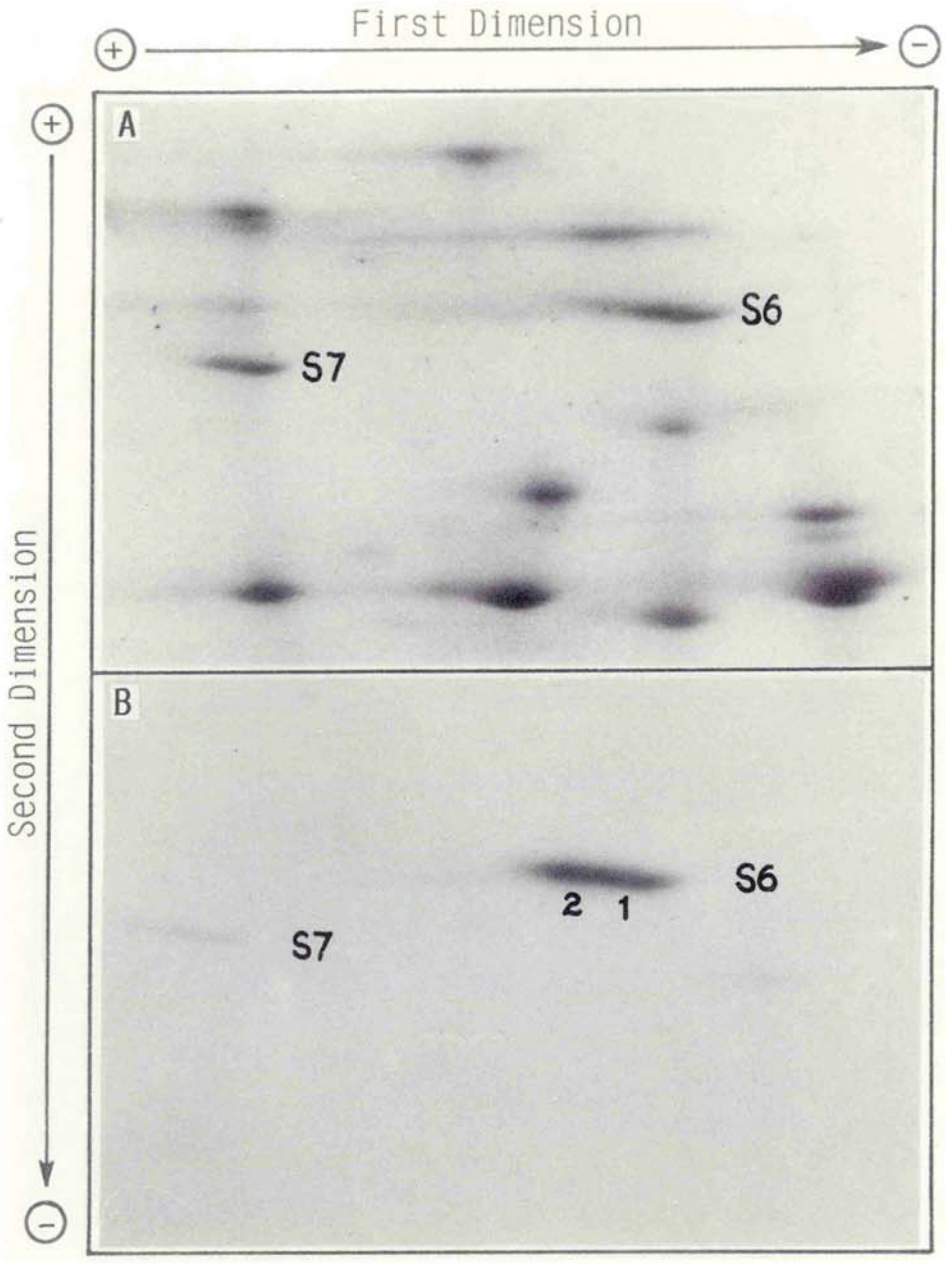

First Dimension
+
−
Second Dimension
+
−
A
S6
S7
B
S6
2
1
S7

Fig. 3.16 Two-dimensional Gel Analysis of 40S Ribosomal Subunits Phosphorylated by Protein Kinase C

BHK cell 40S ribosomal subunits ($12A_{260}$ units) were phosphorylated with proteolytically-activated kinase C in a 800 µl reaction mixture as described in Methods, section 10.3. Incubation was for 6 h, 2·4 units of the kinase being added at 0, 2 and 4 h.

(A) Stained gel

(B) Autoradiograph

The arrow indicates the same position in both frames. The position of migration of S6 relative to protein S4 in the first dimension shows that most of S6 was present as the triphosphorylated derivative.

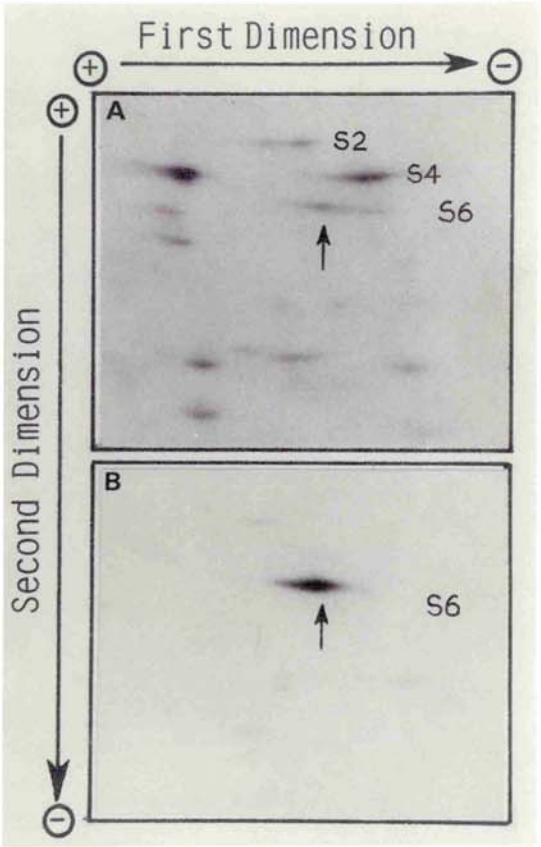
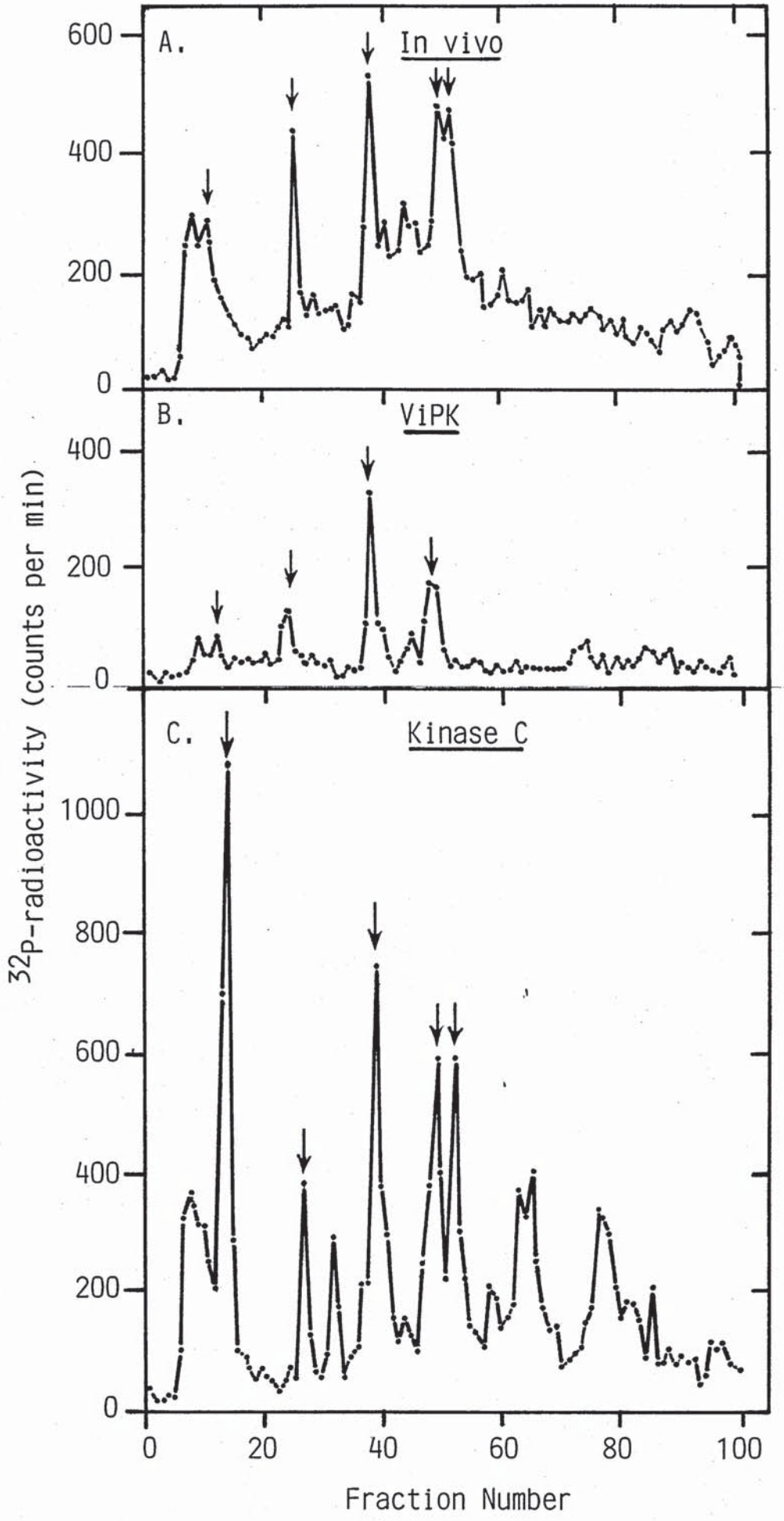

First Dimension
Second Dimension
A
S2
S4
S6
B
S6

Fig. 3.17 Analysis of Tryptic Phosphopeptides from Ribosomal Protein S6 Phosphorylated in vivo and in vitro

Tryptic phosphopeptides of ribosomal protein S6 were analysed by reversed-phase high-performance chromatography as described in Methods, section 9. Phosphorylation of S6 for this analysis was performed:

(A) in vivo, during infection with PRV. The aliquot of the same sample was analysed by two-dimensional gel electrophoresis shown in Fig. 3.2A.

(B) and (C) in vitro in the presence of protein kinase C (1 unit) and ViPK (1 unit), respectively. For the phosphorylation in vitro, BHK cell 40S ribosomal subunits ($0{\cdot}2A_{260}$ units) were used. Incubation was for 2 h in the reaction volume of 40 µl.


A.
In vivo
B.
ViPK
C.
Kinase C
600
400
200
0
400
200
0
1000
800
600
400
200
0
0
20
40
60
80
100
Fraction Number
32P-radioactivity (counts per min)

DISCUSSION

1. COMPARISON OF ViPK WITH OTHER PROTEIN KINASES

The results presented in section 1 demonstrate that a new protein kinase activity appears in the cytosol of cells infected with pseudorabies virus. The activity, termed virus-induced protein kinase (ViPK), was partially purified and its molecular and catalytic properties examined (Results, section 2). The fact that the enzyme was not purified to homogeneity leaves a possibility that some of the characteristics described (Results, section 2) may not be valid for the pure molecule. Nevertheless, these data provide an adequate basis on which to compare ViPK with other protein kinases that have been partially or extensively characterized from BHK cells (Results, Fig. 1.1) and other eukaryotic cells. They also allow comparison of ViPK with protein kinases reported to be induced in cells infected with other herpes viruses. To facilitate this comparison, those characteristics that are generally used to define a protein kinase (Introduction, section 1.1) are summarised for ViPK in Table 1D.

1.1. PROTEIN KINASES IN BHK CELLS

Comparison with protein kinases in the same cells was necessary to exclude the possibility that the new activity was, in fact, derived from one of these. The protein kinases in BHK cells were identified as cyclic AMP-dependent protein kinases (type I and type II), casein kinase I, casein kinase II and protein kinase C; and it was presumed that one of the protamine kinases corresponded to the proteolytic fragment of protein kinase C. The data presented

Table 1.D Summary of Some Characteristics of ViPK

Molecular size	- apparent molecular weight approx. 68,000 - sedimentation coefficient approx. 4.3S
Regulatory agent	- unknown (regulatory molecules of many other protein kinases excluded)
Substrate	
in vitro	- protamine histone H2A, H2B and H3 ribosomal proteins S7 and S6
physiological	- unknown
Phosphate donor	- ATP (Km = 57 μM)
Amino acid phosphorylated	- serine
Other properties	- broad magnesium concentration optimum - active at high concentrations of monovalent and polyvalent cations (e.g. 800 mM KCl)

here show clearly that none of the major pre-existing protein kinases from the cytosol are altered in infected cells at the time when ViPK is induced (Results, Fig. 1.2 - 1.4). In particular, although the ratio of total assayable enzyme in the two peaks of protamine kinase (protein kinase C and its presumed proteolytic derivative) did vary from experiment to experiment, this ratio was not altered by viral infection. The other properties of ViPK argue against the possibility that it might be derived from one of these protein kinases located either in the cytosol or in other cellular compartments. ViPK clearly differs from the cyclic AMP-dependent protein kinases and kinase C in its response to regulatory molecules (Results, Table 2.2). It is true that the dependence of cellular protein kinases on their regulatory molecules can be lost in certain circumstances. For example, it is known that proteolysis of certain kinases can generate new forms of these enzymes, which, in the case of the cyclic GMP-dependent protein kinase (Lincoln et al., 1978), protein kinase C (Inoue et al., 1977), phosphorylase kinase (Cohen, 1980) and myosin light chain kinase (Hathaway and Adelstein, 1979), no longer require the normal regulatory molecule for activity. Nevertheless, the experiments using inhibitors (in the case of cyclic AMP-dependent protein kinase) and different substrates (in the case of the casein kinases and protein kinase C, with its preference for histone H1) would seem to exclude the ViPK being derived from one of them.

1.2 OTHER PROTEIN KINASES FROM EUKARYOTIC CELLS

Among the well characterized protein kinases of eukaryotic cells which (as described for ViPK) phosphorylate serine and threonine

residues (Introduction, Table 1) are protein kinases regulated by Ca^{2+}/calmodulin, cyclic GMP and double-stranded RNA. Since they were not assayed in BHK cells, one cannot be quite so certain that they are not related to ViPK, despite the lack of stimulation by the latter by cyclic GMP, Ca^{2+} and calmodulin, or double-stranded RNA (Results, Table 2.2). However, the results with different substrates (Figs. 2.14, 2.16, 2.19 and 2.20) would argue against a relationship of ViPK to the cyclic GMP protein kinase [which has different ribosomal substrates (Issinger et al., 1980)], or the interferon-induced double-stranded RNA-dependent protein kinase (which phosphorylates the α-subunit of eIF-2). Two protein kinases of unknown mode of activation in vivo, but activated in vitro by limited proteolytic digestion with trypsin, were denoted as protease-activated kinase I (Tahara and Traugh, 1981; de la Houssaye et al., 1983) and protease-activated kinase II (Lubben and Traugh, 1983). The substrate specificities of these kinases, which have preferences for histone H4 and H1, respectively, differs from the substrate specificity of ViPK towards different histones (Results, Fig. 2.16). Furthermore, the preferences of ViPK for protamine, its activity at 600 mM KCl and above, and its molecular properties, make it most unlikely that it corresponds to any of the well-characterised cellular protein kinases.

It was also necessary to try to compare ViPK with some less well characterised cellular protein kinases which, like ViPK, use protamine as a good substrate. One protamine kinase activity from human promyelocyte leukemia cells (Durham et al., 1982) and one similar activity from trout testis (Jergil and Dixon, 1970) were eluted from DEAE-cellulose at a lower ionic strength (0·14 M NaCl or 0·15 M KCl at pH 7·4, respectively) than required to elute ViPK, and had

apparent molecular weights (about 110,000-150,000) greater than estimated for ViPK. The protamine kinase from trout testis phosphorylated protamine more efficiently in the presence of 1 M NaCl than in its absence, but unlike ViPK, did not phosphorylate histone under these conditions.

The presence of protamine kinase activities in some subcellular fractions other than the cytosol has also been reported. Analysis of protamine kinases in endoplasmic reticulum identified the enzymes as cyclic AMP-dependent protein kinases (type I and type II) and protein kinase C (Sommarin and Jergil, 1978 and 1983). In the nuclear fraction, apart from the cyclic AMP-dependent protein kinases (Jungmann et al., 1981) and protein kinase C (Kikkawa et al., 1982), some other protein kinases which can phosphorylate protamine have been reported. One protamine kinase purified from rat liver nuclei (Sikorska and Whitfield, 1982) also phosphorylated histone and, in addition, casein and synthetic basic polypeptides [poly (L-arginine) and poly (L-lysine)]. The latter two substrates were not phosphorylated by ViPK (Results, Fig. 2.14 and not shown, respectively). Although the activity from rat liver nuclei was eluted from DEAE-cellulose at a similar ionic strength to ViPK (0·25 M NaCl), it had a higher apparent molecular weight (105,000) and sedimentation coefficient (7·5) than ViPK. This enzyme had a broad optimum for Mg^{2+}, but its activity was completely inhibited by 0·5 M KCl. The other nuclear protamine kinase isolated from calf thymus (Kranias and Jungmann, 1978) had similar chromatographic properties on DEAE-cellulose to the one isolated from rat liver nuclei and was affected in a similar way by increasing concentrations of Mg^{2+} and KCl. However, it differed from this activity in its molecular size

(molecular weight of about 55,000). It would be interesting to compare directly a nuclear protein kinase with such characteristics (especially if present in BHK cells) with ViPK and also with the proteolytic fragment of protein kinase C, since it shares some characteristics with both of these two enzymes.

1.3 PROTEIN KINASES INDUCED DURING INFECTION OF CELLS WITH HERPES VIRUSES

The appearance of a new protein kinase has been resported in cells infected with another member of herpes virus family, herpes simplex virus type I (Blue and Stobbs,1981). The ViPK clearly differs from that activity as that protein kinase was specific for acidic rather than basic substrates. The size (sedimentation coefficient of about 2S) and time of induction in the infective cycle (maximum activity 4 h after infection), were also different from the pseudorabies ViPK. The appearance of the kinase activity in cells infected with herpes simplex virus was balanced by the disappearance of a pre-existing cellular protein kinase activity, and it is therefore possible that this activity is a proteolytic derivative of a pre-existing cellular protein kinase. However, no direct attempt to investigate the protein kinase reported by Blue and Stobbs was made in the work described here. The fact that in the present study and in a comparative study using both herpes simplex virus and pseudorabies virus (Leader et al., in preparation) no activity similar to the one described above was observed, could simply be due to the different chromatographic separations used (chromatography on carboxylmethyl cellulose) and the different stage of the infectious cycle examined.

It is also necessary to compare the protein kinase described here with an activity associated with a protein of molecular weight 68,000 (p68) precipitated by a monoclonal antibody from cells infected with another herpes virus, human cytomegalovirus (Michelson et al., 1984 and 1985). The latter activity is clearly different from the pseudorabies ViPK as it phosphorylates casein. The kinase activity associated with p68 has, apart from substrate specificity, some other characteristics in common with cellular casein kinase II. Thus, this activity used ATP and GTP as a phosphate donor equally well and was inhibited by heparin (although at a higher concentration than reported for purified preparations of casein kinase II) and quercetin. In view of the large amounts of casein kinase II present in cells, it is probably premature to conclude that the p68 protein specified by cytomegalovirus has an intrinsic protein kinase activity since the cellular kinase could be associated with this protein and present in its immunoprecipitate. Thus, the presence of a cellular kinase activity in an immune-complex of one virally-coded protein ($p37^{mos}$ of Moloney murine sarcoma virus) has been demonstrated (Maxwell and Arlinghaus, 1985). This cellular protein kinase activity was removed by preincubation of the cell extract with preimmune normal rabbit serum IgG and its properties were similar to those described for casein kinase II.

1.4 THE PROPERTIES OF ViPK IN RELATION TO THE POSSIBILITY THAT IT IS A VIRALLY-CODED ENZYME

For enzymes induced by herpes viruses, minor differences in properties, compared with those of similar activities of uninfected

cells, have usually been considered as a good indication that the induced enzyme is virally coded. For most such activities a genetic approach has subsequently proved their viral origin (Wagner, 1985). Unfortunately, the heterogeneity of protein kinases from normal cells does not allow application of the same logic to this class of enzymes. Nevertheless, it should be mentioned that, as for ViPK, the KCl optima for the DNA-polymerases coded by pseudorabies virus (Halliburton and Andrew, 1976) and herpes simplex virus (Powell and Purifoy, 1977), as well as for the uracil DNA glycolase of herpes simplex virus (Caradonna and Cheng, 1980), are higher than those of the corresponding cellular enzymes. For example, cellular DNA-polymerase has maximum activity at 0 mM KCl while DNA-polymerase coded by herpes simplex virus reaches maximum activity at 120 mM KCl. Furthermore, spermine [which is present in the virions of herpes viruses (Gibson and Roizman, 1971)] can, at a physiological concentration, stimulate the activity of the latter enzyme (Ostrander and Cheng, 1980). Nevertheless, it is difficult to say wether the stimulatory effect of spermine (or KCl) on the ViPK reflects the real properties of the enzyme, and hence a possible regulatory mechanism in vivo. Thus, a high concentration of these and other cations when ViPK was assayed in vitro [Results, section 2.2 (1)], could affect not only the conformation of the enzyme but also that of the artificial substrates. In addition it could have an influence on possible interactions between molecules in the preparation of partially-purified ViPK.

The question of the origin of ViPK has been addressed directly in separate work (Leader et al., in preparation). Theoretically, the appearance of ViPK in the cytosol of infected cells could be the

result of activation of a pre-existing protein, translocation of a protein kinase from another cellular compartment, or synthesis de novo. Studies of proteins synthesized de novo in cells infected with herpes viruses have shown that they originate not only from the viral genome (Wagner, 1985) but also, in some cases,from the cellular genome (e.g. heat shock proteins, La Thangue et al., 1984). To approach this question in relation to the origin of ViPK, agents which interfere with the viral replicative cycle and temperature-sensitive mutants of the virus were used. The results of these experiments showed that the appearance of ViPK required at least partial expression of the viral genome, but it is not yet clear whether the enzyme is a viral protein itself. Until this is demonstrated the other possibilities for the origin of ViPK cannot be ignored.

2. SUBSTRATES FOR ViPK

In vitro ViPK phosphorylated mainly basic protein substrates, e.g. protamine, histone and certain basic ribosomal proteins [Results, section 2.2 (3)]. Most of these proteins are good substrates for those protein kinases (e.g. cyclic AMP-dependent protein kinases, cyclic GMP-dependent protein kinase and protein kinase C) which phosphorylate serine and threonine residues in a sequence containing basic amino acids [Introduction, section 1.1 (4)]. Proteins such as the above, that are highly basic because of a role in interacting with nucleic acids, will have a relatively high probability of possessing such recognition sequences by chance, without this being of any physiological regulatory significance.

The identification of the physiological substrate for ViPK will be an important step towards clarifying its function in viral infection. In general, the identification of physiological substrates for protein kinases and the determination of the functional significance of the phosphorylation is no easy task. A recent illustration of this is the search for substrates for the tyrosine kinases described in the Introduction [section 1.1 (3)]. In spite of extensive information resulting from these studies and a large amount of work directly addressing the question, the physiological substrate(s) and significance of the phosphorylation at tyrosine residues are still incompletely understood. In the approach employed in the present work, some of the proteins which were found to be phosphorylated during viral infection were considered as candidate substrates, and their phosphorylation by ViPK *in vitro* was examined (Results, section 3).

The major virion phosphoproteins were obvious candidates. However, these were not phosphorylated by ViPK in vitro, even though an activity similar to ViPK was also present in the virion [Results, section 3.2 (1)]. One of the virally-coded enzymes, DNase, which is also phosphorylated in vivo (Banks et al., 1985) could be phosphorylated by preparations of ViPK (Results, Fig. 3.7). However, further studies of the phosphorylation are necessary to determine whether the viral DNase satisfies the accepted criteria for a physiologically relevant substrate (Krebs and Beavo, 1979). An important limitation in studies of phosphorylation of viral proteins is lack of understanding of the functional implication(s) of the phosphorylation in vivo [Introduction, section 2.1 (3)] so that the alteration(s) in the function of these specific proteins, due to the phosphorylation in vitro, cannot be easily tested. The viral enzymes are only synthesized in infected cells and their phosphorylation would not necessarily be expected to be involved in regulating their enzymic activity. One alternative possible function for the phosphorylation of viral non-structural proteins is suggested by the correlation (Wagner, 1985) between the phosphorylation of these and their transport from cytoplasm to nucleus.

Another phosphoprotein, ribosomal protein S6, has been extensively studied in various biological systems [Introduction, section 2.1 (4)]. This protein was phosphorylated by ViPK in vitro, but the enzyme catalysed the incorporation of only 2 moles of phosphate per S6 molecule (Results, Table 3.1 and Fig. 3.15), compared with the five moles of phosphate per S6 molecule observed in vivo (Kennedy et al., 1981; Results, Fig. 3.2). S6 was not the only ribosomal protein phosphorylated by ViPK (in contrast to the selective phosphorylation

of S6 *in vivo*) and specific phosphorylation of this ribosomal protein was only achieved in the presence of high concentrations of certain cations, e.g. KCl, NaCl, spermine (Results, Fig. 2.17, 2.18 and 2.19). As most of the studies *in vitro* were carried out under the conditions for the specific phosphorylation of S6 by ViPK, it was particularly important to check the physiological significance of the sites phosphorylated. Analysis of tryptic phosphopeptides generated from S6, indicated that the sites phosphorylated by ViPK *in vitro* are among those phosphorylated *in vivo* (Results, Fig. 3.17). Thus, the results regarding the stoichiometry of phosphorylation and analysis of tryptic phosphopeptides allow the possibility that ViPK could be involved in the phosphorylation of some, but not all, of the observed sites on S6 during viral infection.

Other criteria, apart from phosphorylation *in vitro*, must also be satisfied before a protein can be accepted as a substrate for ViPK *in vivo*. If ViPK were responsible for the phosphorylation of a specific protein, its phosphorylation should show a similar dependence on the expression of certain viral functions as the appearance of ViPK. However, experiments employing agents which interfere with the viral replicative cycle are not applicable to studies of virally-coded proteins since they can also interfere wuth the appearance of the proteins themselves. As ribosomal protein S6 is a host protein, however, it was suitable for this type of analysis. Thus, to test whether the observed phosphorylation of ribosomal protein S6 is a (direct or indirect) consequence of the induction of ViPK, the physiological requirements for the phosphorylation of S6 have been studied and compared with those for the appearance of ViPK. When BHK cells were exposed to PRV previously irradiated with uv light,

the phosphorylation of protein S6 was increased (Kennedy *et al.*, 1981) but ViPK was not induced (Leader *et al.*, in preparation). Thus, despite the results from comparison of S6 phosphorylation *in vivo* and *in vitro*, ViPK is not likely to be involved in the phosphorylation of this protein during viral infection.

The studies described above do not answer the question of what is the physiological substrate for ViPK. An extension of this type of approach to other proteins phosphorylated during viral infection is clearly required. In addition, analysis of specific proteins with an important function during viral infection may reveal some relevant phosphoproteins which escaped previous analysis (e.g. Fig. 3.1 in Results), which would only detect relatively abundant proteins. Should it transpire that the protein kinase is of viral origin, a genetic approach may be available to facilitate the task of determining the physiological substrate(s) and biological function of the enzyme.

3. INVOLVEMENT OF OTHER PROTEIN KINASES, IDENTIFIED AS CELLULAR ENZYMES, IN PHOSPHORYLATION DURING VIRAL INFECTION

Although the analysis of potential substrates for ViPK did not give a conclusive answer, the results obtained in those studies showed that some other protein kinases from BHK cells may be responsible for the phosphorylation of proteins during viral infection.

Viral proteins, which appear as new proteins in cells after infection, do not require any change in protein kinase activities to become phosphorylated if they can be recognized as suitable substrates by pre-existing enzymes. Analysis of the phosphorylation of the two major phosphoproteins of pseudorabies virions, illustrates this possibility. These proteins were phosphorylated *in vitro* only by casein kinase II isolated either from the cytosol of BHK cells or by a very similar activity from pseudorabies virions [Results, section 3.2 (1)]. The latter activity could be cellular casein kinase II incorporated into the virion during assembly of the nucleocapsid inside the nucleus of the host cell, or during the budding process when the virus acquires its envelope from the nuclear membrane (Ben-Porat and Kaplan, 1985). Studies of protein kinases present in the virions of other enveloped animal viruses showed that at least some of the activities are cellular enzymes (Clinton *et al.*, 1982; Harmon *et al.*, 1983). Furthermore, the involvement of cellular kinases in the phosphorylation of viral structural proteins has been demonstrated for one protein (M) of vesicular stomatitis virus (Clinton *et al.*, 1982) and one protein (pp12) of Rous sarcoma virus (Leis *et al.*, 1984).

It has been shown that the extent of phosphorylation of some

viral structural proteins determines the extent of their binding to viral nucleic acid (Leis and Jentoft, 1983; Wilson and Consigli, 1985; Kamata and Watanabe,1977). However, it is not clear whether the main phosphoproteins of pseudorabies virions interact with viral DNA like the proteins mentioned above (Stevely, 1975).

The analysis of the ability of protein kinases of the cytosol of infected cells to phorphorylate S6 [Results, section 3.3 (1)] showed that apart from ViPK (discussed in the previous section) at least two cellular enzymes, the cyclic AMP-dependent protein kinase and kinase C, can phosphorylate this protein _in vitro_. Detailed studies of the phosphorylation of S6 mediated by cyclic AMP showed that this kinase physiologically phosphorylates one or two serine residues in S6 (Wettenhall _et al_., 1982; Wettenhall and Morgan, 1984). As the phosphorylation of S6 during viral infection occurs at more than two sites, cyclic AMP-dependent protein kinase can only be responsible for part (at the most) of the effect observed _in vivo_. The possibility that part of the phosphorylation of S6 is catalysed by cyclic AMP-dependent protein kinase in infected cells is suggested by the observation that infection with some herpes viruses (Bittlingmaier _et al_., 1977) can increase the intracellular concentration of cyclic AMP.

Another S6 kinase in BHK cytosol was identified as cellular protein kinase C (Results, Fig. 3.9). It has recently been suggested that the phosphorylation of S6 that occurs in the exocrine pancreas following secretory stimuli, is mediated by this kinase (Padel and Söling, 1985). The studies presented here, involving comparison of the stoichiometry and phosphopeptide patterns of S6 phosphorylated in infected cells and by protein kinase C _in vitro_ [Results, section

3.3 (2)], indicate that this enzyme could be directly responsible for the phosphorylation *in vivo*. However, two main limitations of this sort of analysis should be pointed out. First, because several serine residues can be present in the same tryptic peptide (Wettenhall and Morgan, 1984; Wettenhall and Quinn, 1984), similarity in phosphopeptide pattern does not necessarily mean that identical sites are phosphorylated. Second, it is possible that protein kinase C under the conditions employed *in vitro* phosphorylated more sites than it would phosphorylate when activated in intact cells, even though the additional sites may be physiologically relevant, i.e. can be phosphorylated by some other S6 kinase(s). The example which illustrates this point is the finding that cyclic AMP-dependent protein kianse can phosphorylate *in vitro* (during prolonged incubation or with relatively high amounts of the kinase) some sites, the phosphorylation of which are not increased when the enzyme is activated *in vivo* by glucagon (Wettenhall and Morgan, 1984; Wettenhall *et al.*, 1982). On the other hand, the phosphorylation of these non-physiological sites for cyclic AMP-dependent protein kinase is increased by insulin (Wettenhall *et al.*, 1982). In order to show that protein kinase C is involved in the phosphorylation of S6 during infection of cells with pseudorabies virus, a different kind of evidence is required. This is evidence that the activity of protein kinase C is actually increased in these circumstances. This question has not been examined.

The results presented in section 3.3 (1) leave the possibility that a putative S6 kinase remained undetected. Analysis of fractionated cytosol from cells infected with PRV using ribosomal subunits as substrate *in vitro* (Results, Fig. 3.8) did not reveal the presence

of a kinase which phosphorylated S6 specifically. Such a kinase could have escaped previous characterisation with the more general substrates (histone, protamine and casein). However, if the presence of components other than those included in the assay mixture were required for the activity of a putative S6 kinase, it would not have been detected in these experiments. For example, it has been reported that some calmodulin-dependent kinases can phosphorylate S6 in vitro (Gorelick et al., 1983). A further possibility is that a kinase responsible for increased phosphorylation of S6 was lost during the preparation of the cytosol fraction. Detection of one such S6 kinase activity, which increased up to 25-fold in extracts of cells stimulated by serum, required the presence of phosphatase inhibitors in all extraction buffers (Novak-Hofer and Thomas, 1984). In the light of these recent observations it would be interesting to re-examine the question of the identity of the kinase(s) which mediates the phosphorylation of ribosomal protein S6 in cells infected with pseudorabies virus.

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