PROTEINS INDUCED IN BHK CELLS

BY PSEUDORABIES VIRUS

by

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THE MICROBE

The Microbe is so very small
You cannot make him out at all
But many sanguine people hope
To see him through a microscope.
His jointed tongue that lies beneath
A hundred curious rows of teeth;
His seven tufted tails with lots
Of lovely pink and purple spots,
On each of which a pattern stands,
Composed of 40 separate bands;
His eyebrows of a tender green;
All these have never yet been seen -
But scientists, who ought to know
Assure us that they must be so .....
Oh! Let us never, never doubt
What nobody is sure about.

Hilaire Belloc

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ABBREVIATIONS

These are laid down in the Biochemical Journal Instructions to authors (revised 1972) with the following additions :

PrV	pseudorabies virus
HSV	Herpes simplex
IP	Induced protein (pseudorabies)
SV40	Simian virus 40
PPLO	pleuropneumonia-like organism
PI	post-infection
EDTA	ethylenediaminetetraaceticacid (versene)
EGTA	ethyleneglycol-bis-βaminoethyl-ether-NN' tetraacetic acid
DTT	dithiothreitol
TCA	trichloracetic acid
SDS	sodium dodecyl sulphate
PPO	2,5 diphenyloxazole
βME	β-mercaptoethanol

CONTENTS

SECTION 1 INTRODUCTION

1.1 THE NATURE OF VIRUSES

Viruses are infective nucleoprotein entities which do not grow or undergo binary fission. They are completely parasitic, replicating within the cell of a susceptible host where they make use of the cellular systems for energy production and protein synthesis (Lwoff, 1957; Luria & Darnell, 1967). They possess only one type of nucleic acid, DNA or RNA, which contains the genetic information required for replication.

Apart from the cell they are completely inert and respond to external stimuli as would an inanimate object of similar size and shape. However in the presence of a suitable host they enter a vegetative phase, undergoing a replicative growth cycle comparable to that of other organisms. This, and the fact that they are susceptible to mutation gives them the right to be termed living.

All viruses consist basically of a core of nucleic acid material enclosed within a protective coat or capsid which is made up of repeating units of protein, the capsomeres. On the outside there is sometimes a lipoprotein envelope, the peplos.

1.2. CLASSIFICATION

Viruses can be classified in accordance with various properties :

(a) the host infected (whether animal, plant or bacterial)

(b) clinical features of the disease caused (e.g. Burkitt lymphoma virus, tobacco mosaic and equine abortion virus)

(c) properties of the virus per se : these include the type of nucleic acid, symmetry of the nucleocapsid, presence or absence of an envelope, and the number of capsomers (Tournier & Lwoff, 1966). Other characteristics which have been suggested are size and cellular-site of virus multiplication and maturation (Andrewes, 1964).

Under (c), the herpesviruses are defined as a group of enveloped deoxyriboviruses with an icosahedral capsid of 162 capsomeres. They are large (150 - 200 mμ in diameter) and replicate within the nucleus of the infected cell.

Some of the more closely related, DNA-containing viruses are classified in Fig 1.2, as reference to various of their properties may be made in comparison with those of the herpesviruses.

1.3 THE HERPESVIRUS GROUP

This group contains the following animal viruses : pseudorabies (PrV), herpes simplex (HSV) types I & II, equine abortion virus (EAV), B-virus, Varicella-zoster, Lucké virus and Epstein-Barr virus (EBV). A growing number of herpesviruses have been shown recently to have oncogenic properties, a fact which has been suspected for many years due to the long-term latent infections produced in many species, a characteristic of all herpesviruses. They have a wide host-range but perhaps surprisingly, few antigenic properties in common. They are large, morphologically similar and induce the formation of eosinophilic

Figure 1.2 Properties of Several Deoxyriboviruses (adapted from Fenner 1968)

	POXVIRUS	HERPESVIRUS	ADENOVIRUS	PAPOVIRUS
Model virus	Vaccinia	Herpes simplex and pseudorabies	Adenoviruses types 2 & 5	Polyoma & SV40
Molecular weight of DNA x 10^{-6}	160 - 200	54 - 92	20 - 25	3 - 5
Envelope	-	+	-	-
Symmetry of nucleocapsid	complex	icosahedral	icosahedral	icosahedral
Number of cistrons	∿ 400	∿ 120	∿ 50	6 - 8
Effects on host macromolecular synthesis :				
DNA	inhibited early	inhibited early	inhibited late	stimulated early
RNA	inhibited early	inhibited early	inhibited late	no effect
protein	inhibited early	inhibited early	inhibited late	no effect
Site of assembly of capsid	cytoplasm	nucleus	nucleus	nucleus

nuclear inclusions, a property which is thought to be related to their assembly within the nucleus.

Most of the published work on the herpesviruses refers to herpes simplex and pseudorabies viruses, and the properties of these two, in particular the latter, will be mainly considered.

1.3 (a) Structure

(i) Core : the genome of pseudorabies consists of a single molecule of double-stranded DNA of molecular-weight 68-70 x 10^6 daltons (Russell & Crawford, 1964; Ben-Porat & Kaplan, 1962; Kaplan & Ben-Porat, 1964). Its buoyant density in caesium chloride (CsCl) gradients is 1.732/3, slightly higher than that for HSV-I, and the G + C content calculated from this is 74% (Russell & Crawford, 1964; Subak-Sharpe et al., 1966). This is slightly lower than the value calculated from base composition analysis, 77% G + C (Graham et al., 1972). The large difference between these values and those for the DNA of mammalian cells 40 - 44% G + C (Schwartz, Trautner & Kornberg, 1962) means that host and viral DNA can be easily separated by gradient centrifugation. This has proved invaluable for studying the infective cycle. The information content of herpesvirus DNA is thought to be sufficient to specify 140,000 amino-acid residues, only 10 - 20% of which can be accounted for by the structural viral proteins (Roizman, 1969).

(ii) Capsid : this is a triple-layered structure consisting solely of protein (Toplin & Schidlovsky, 1966; Roizman & Spear, 1971). It is made up of individual structural units, the capsomeres of molecular weight around 500,000 (d) which are in turn thought to be built from

sub-units of molecular weight 100,000 (d) (Wildy & Horne, 1960). A large protein of about this size has been identified in PrV preparations (Shimono et al., 1969) and seems to form the main capsid protein. A number of minor components are probably present but have not, as yet, been characterised (Shimono et al., 1969). These must all be viral-coded as serological evidence suggests that capsids contain no host proteins (Watson & Wildy, 1963).

It appears that aggregation of the protein sub-units into capsid is not dependent on initial formation of the DNA-containing 'core'. In infected cultures treated with 5-fluoro-uracil, viral DNA synthesis is inhibited but non-infective particles are still produced which have the same external structure as intact virus but lack the electron-dense core (Reissig & Kaplan, 1962).

(iii) Envelope : this is composed of lipids, glycolipids and glycoproteins arranged in two layers, each showing the repeating-unit structure characteristic of host membranes (Epstein, 1962 (a), (b)); it varies in thickness. Its function is not clear but it is probably protective, and has been shown to be related to infectivity; enveloped particles are more readily adsorbed to susceptible cells, although the envelope is not essential for attachment to take place (Holmes & Watson, 1961; Watson et al., 1964; Spring & Roizman, 1968). Unlike the capsid, this does show antigenic properties of the host, and the outer layer has, in fact, been proposed to arise from the inner lamella of the nuclear membrane (Siegert & Falke, 1966; Schwartz & Roizman, 1969; Roizman, 1969).

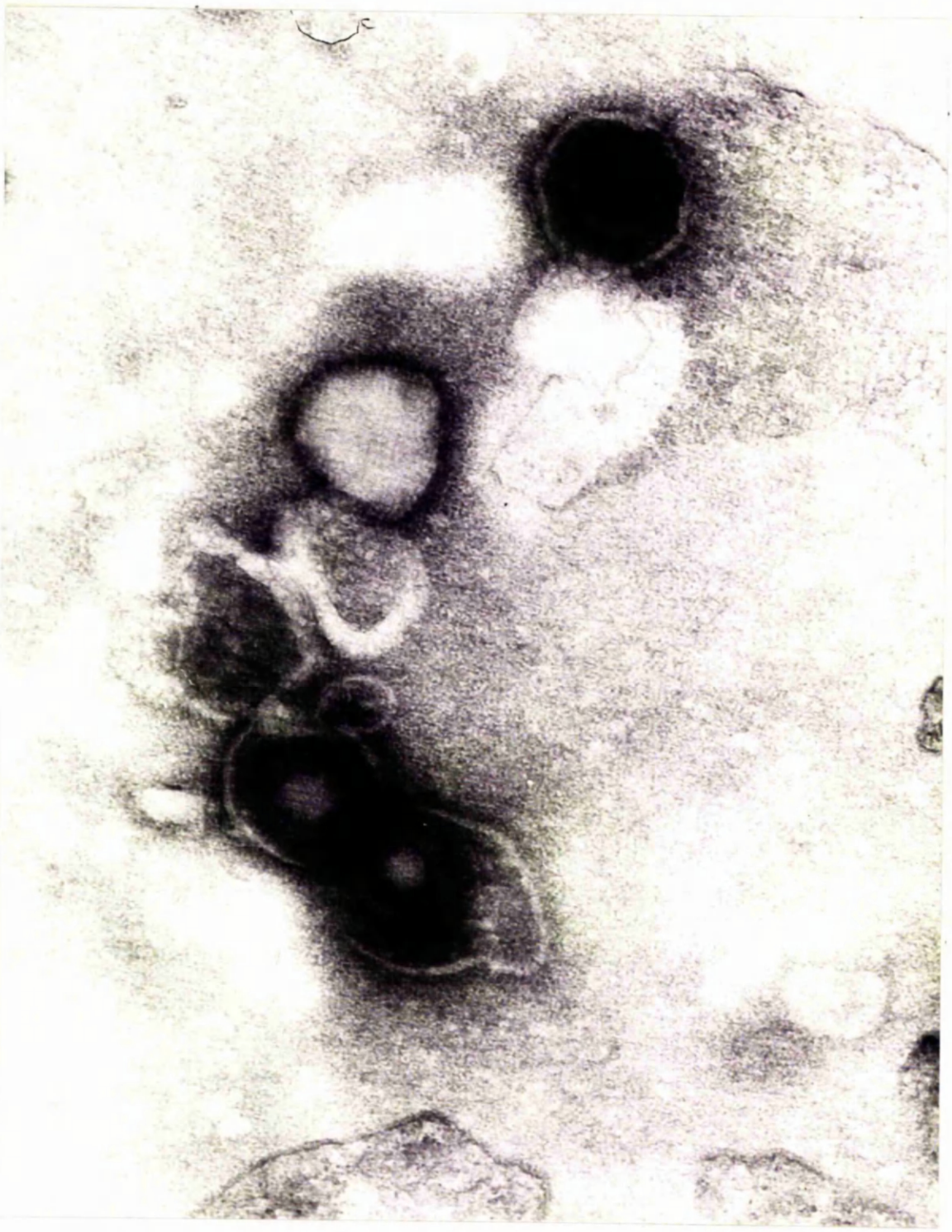


Figure 1.3 : Electronmicrograph of a supernatant preparation of pseudorabies virus.

Magnification : 128,000 fold.

1.3 (b) The Infective Cycle

The growth cycle of pseudorabies can be divided into a number of distinct phases :

(i) adsorption (ii) penetration and uncoating
(iii) eclipse (iv) release.

(i) Adsorption : this appears to be unaffected by temperature but requires the presence of electrolytes in the medium and is therefore probably an electrostatic attraction (Farnham & Newton, 1959). The time required for adsorption varies between different cell types and virus strains. For PrV with rabbit kidney (RK) cells, 50% is adsorbed in 30 mins (Kaplan & Vatter, 1959), and for herpes simplex (HFEM) or PrV in BHK/21 cells, 90% in 45 mins (Holmes & Watson, 1963).

(ii) Penetration and Uncoating : penetration is probably an enzymic reaction as it is dependent on temperature (Huang & Wagner, 1964). Kaplan (1962, 1969) has further shown a requirement for energy in the form of ATP, as cyanide inhibits penetration although adsorption can still occur. The mechanism is thought to be similar to phagocytosis whereby the viral particles are taken into cytoplasmic vacuoles (Holmes & Watson, 1963; Epstein, Hummeler & Berkaloff, 1964). Here the envelope is removed and the naked nucleocapsids then enter the cytoplasm. Alternative mechanisms for penetration have, however, been proposed (Morgan, Rose & Mednis, 1968). The fate of the capsid is uncertain but it is probably shed in the cytoplasm before transport of the nucleic acid moiety to the nucleus. This is the start of the eclipse period.

(iii) Eclipse phase : this is the period during which virus particles can not be identified within the infected cells. It represents a phase of intense biochemical activity during which the viral components are made, and accumulate before assembly takes place. It is also characterised by the inhibition of many cellular events - host DNA, RNA and protein synthesis (Roizman & Spear, 1969). The length of the eclipse period depends on the multiplicity of infection which suggests that although one virion is sufficient, several viral particles can participate in the infective process. For pseudorabies it lasts 3 - 4 hours (Sydiskis, 1969).

After assembly the progeny virus is enveloped by the inner lamella of the nuclear membrane as it exits from the nucleus (Siegert & Falke, 1966).

(iv) Release : the completion of the cycle, release of mature virions, seems to occur by a 'leakage' mechanism spread over a period of time. For HSV this has been proposed to be by 'reverse-phagocytosis' (Nii, Morgan & Rose, 1968) or to occur by passage along tubules continuous with the outer lamellae of the nuclear membrane and the cytoplasmic membrane (Schwartz & Roizman, 1969). Finally the cells lyse with further release of particles.

1.4 EFFECTS OF PSEUDORABIES INFECTION

One of the first observable signs of infection is a dissolution of nucleoli, and margination of the chromosomes (Munk & Sauer, 1964); the chromatin network becomes granular in appearance and is displaced to the periphery of the nucleus leaving a clear central zone

of nucleoplasm. Nuclear inclusions are found in this central region, and by 6 hrs post-infection they have RNA, DNA and non-histone proteins associated with them (Love & Wildy, 1963; Reissig & Kaplan, 1962).

Study of the biochemical effects of animal virus infection has been greatly simplified by the advent of cell-culture techniques. Even then, there are difficulties as the pattern observed is the result of two concomitant reactions - a gradual 'switch-off' of host DNA, RNA and protein synthesis while the corresponding virus-directed reactions are put into action.

1.4 (a) DNA Synthesis

When exponentially growing cells are infected with PrV, there is a decline in total DNA synthesis as measured by incorporation of labelled thymidine (Kaplan & Ben-Porat, 1963; Ben-Porat & Kaplan, 1965). This is in contrast to stationary cells where host DNA synthesis is at a minimum, and infection causes a stimulation of DNA replication corresponding to the synthesis of viral DNA (Ben-Porat & Kaplan, 1963). Similar effects have been found for herpes simplex virus (Roizman & Roane, 1964; Roizman, 1969). The type of DNA being synthesised at any time after infection can be conveniently studied by labelling for the appropriate period, separating cellular and viral DNA by isopycnic centrifugation in CsCl gradients and examining the counts associated with each. Using this type of technique, it has been shown that there is a gradual decrease in host DNA production after infection until between 7 and 8 hours post-infection it has been completely arrested (Kaplan & Ben-Porat, 1963). The mechanism for this is unknown, and there is

controversy as to whether the inhibitor is a structural component of the virion (Newton, 1968) or a product made after infection (Ben-Porat & Kaplan, 1965).

Viral DNA synthesis occurs predominantly between 4 and 7 hours post-infection, in the nucleus (Newton & Stoker, 1958; Sydiskis & Roizman, 1966; Roizman, 1969) within which the site of thymidine incorporation appears to correspond topologically to the inclusion body (Roizman, 1969). There is evidence that viral DNA synthesis and the 'switch-off' of host DNA synthesis are in some way related (D.K. Howard, personal communication) and of interest in this respect is the fact that both are thought to require protein synthesis (Kaplan & Ben-Porat, 1967; Ben-Porat & Kaplan, 1965). In fact the synthesis of viral DNA has been proposed to be a general regulatory event in herpesvirus infected cells (Kamiyama et al., 1964, 1965).

In a series of experiments between 1963 and 1967 Kaplan and Ben-Porat reported that :

(i) Replication of PrV DNA is semi-conservative.

(ii) Less than half the DNA not integrated into virions and presumably available to function as template is actually replicating.

(iii) Withdrawal of DNA from the DNA pool into virions is slow and inefficient.

(Kaplan & Ben-Porat, 1963, 1964, 1966; Ben-Porat & Kaplan, 1963).

Several of the enzymes involved in DNA synthesis in cells infected with pseudorabies or herpes simplex virus differ in activity,

physical properties and immunological reactivity from the corresponding enzymes in uninfected cells. These include thymidine kinase (Klemperer et al., 1967; Kit et al., 1967; Hamada et al., 1966), DNA polymerase (Keir et al., 1966 b), DNA nucleotidyltransferase (Keir et al., 1966 a; Keir, 1968) and deoxyribonuclease (Keir, 1968). This ties in with the requirement for de novo protein synthesis before viral DNA replication can occur.

1.4 (b) RNA Synthesis

(i) Cell-specific : infection of cells with the herpesviruses leads to a gradual decrease in the rate of incorporation of labelled precursors into RNA (Flanagan, 1967; Hay et al., 1966; Kaplan & Ben-Porat, 1960; Wagner & Roizman, 1969). However, although host RNA synthesis is markedly inhibited there is evidence that some cell-specific RNA is synthesised even at later stages of infection (Flanagan, 1967; Rakusanova, 1972). In addition, this cellular RNA accumulates in the infected cells indicating that the virus also interferes with the processing and turnover of these species (Rakusanova, 1972); that is, heterogeneous nuclear RNA which normally turns over in uninfected cells is adenylated and may be transferred to the cytoplasm, although it can not take part in protein synthesising reactions.

Effects on ribosomal precursor RNA have also been found for both HSV and PrV-infected cells (Wagner & Roizman, 1969; Rakusanova, 1972); the reduction in 45 s RNA synthesis seems to be less marked than that of 18 s and 28 s RNA and it appears that there is abortive processing of precursor to oligonucleotides (Rakusanova, 1971). This

interference in processing is not dependent on virus-induced protein synthesis and still occurs in cycloheximide treated cells. This is in contrast to the inhibition of cell-specific mRNA synthesis which does seem to require viral protein synthesis (Ben-Porat et al., 1971).

These findings are similar to those obtained with T_4-infection of E. coli where chloramphenicol was used to show that protein synthesis is necessary to shut off host-specific RNA synthesis (Nomura et al., 1965; Hayward & Green, 1965).

(ii) Virus-specific : the presence of virus-specific RNA in cells infected with HSV was first identified by Hay et al., (1966), in the form of a heterogeneous RNA species with a sedimentation coefficient of about 20 s. This was defined as viral messanger as it was found associated with polysomes (Wagner & Roizman, 1969). In addition a nuclear RNA believed to be a high molecular weight precursor-mRNA has been identified which is thought to be cleaved in a manner analogous to the host system (Wagner & Roizman, 1969; Roizman et al., 1970). Of interest in this respect is the discovery by Rakusanova et al., (1972) that viral messanger contains regions rich in poly A - thought to be important in the normal processing of HnRNA to cytoplasmic messanger in uninfected cells (Darnell, Wall & Tushinski, 1971; Edmonds, Vaughan & Nakazato, 1971).

There is little information available on the mechanism of transcription of viral DNA. Lando & Ryhiner (1969) suggested that the virus utilises host DNA-dependent RNA polymerase for the initial transcription of viral DNA. This agrees with studies by Rakusanova et al., (1972) on virus-specific RNA synthesis in cycloheximide-treated infected

cells. They found that only 25% of the RNA sequences normally transcribed before the onset of viral DNA synthesis were transcribed when protein synthesis was inhibited. This seemed to represent repeated transcription of a small section of the viral genome coding for certain 'early' proteins required before further transcription could occur.

Transport of viral mRNA to the cytoplasm occurs after a lag of 10 - 25 minutes, and differs from the corresponding cellular system in that it is arrested by cycloheximide, suggesting that virus-induced proteins may also be involved in transport mechanisms (Roizman et al., 1970).

1.4 (c) Protein Synthesis

(i) Cell-specific : unlike the synthesis of DNA and RNA in infected cells, the overall rate of protein synthesis does not alter substantially. However, there is inhibition of cellular protein production which starts immediately after infection and continues progressively throughout the infectious cycle (Hamada & Kaplan, 1965; Kaplan et al., 1970). This inhibition appears to be non-specific, i.e. all cellular proteins are affected, and more or less equally. In the early stages it is thought to be due to a rapid dissolution of cellular polysomes post-infection (Sydiskis & Roizman, 1967); thereafter, the inhibition of host DNA and RNA synthesis undoubtedly contribute. The mechanism of polysome dissolution has not been elucidated but the evidence of Sydiskis & Roizman (1967) that HSV loses its capacity to disaggregate Hep-2 cell polysomes and inhibit protein synthesis after u/v irradiation is of interest in this respect. In addition Ben-Porat

et al., have evidence from inhibitor studies that transcription and translation of the viral genome are required before the reduction in cell-specific polysomes occurs.

Similar results with regard to inhibition of cellular protein synthesis have been found for many other animal viruses including poliovirus-infected HeLa cells (Penman & Summers, 1965) and vaccinia virus (Shatkin, 1963; Salzman & Sebring, 1967). Comparisons can also be drawn with the much simpler system of T-4 phage infected E. coli where the overall rate of protein synthesis again remains unchanged after infection (Cohen, 1949; Hershey et al., 1953) and similar inhibition of cellular protein synthesis occurs (Stent, 1963). However, here there is additional evidence for selective inhibition of cell-specific polysome formation after infection, believed to be due to protein-constituent changes in cellular ribosomes (Hsu & Weiss, 1969; Smith & Haselkorn, 1969). There is no evidence for similar changes in cells infected with animal viruses.

(ii) Virus-specific : by 2.5 hrs post-infection a new class of polysomes can be identified in infected cells (Sydiskis & Roizman, 1966, 1968), and the mRNA associated with these has been shown to hybridise preferentially with viral DNA (Wagner & Roizman, 1969 (a) (b)). In association with this, the pattern of proteins on polyacrylamide gel electrophoresis has also altered by 2 - 3 hrs post-infection. These proteins have been studied very rigorously (Hamada & Kaplan, 1965) using the highly sensitive serological method devised by Gerloff, Hoyer & McLaren (1963) and Scharff & Levintow (1963).

The results show that two types of protein are induced in RK cells after PrV infection :

1. precursor structural proteins which are eventually incorporated into the viral particles
2. non-structural proteins synthesised early in the virus growth cycle.

1. There is still no precise information as to the exact number of structural proteins in pseudorabies or herpes simplex virus due to the difficulty in preparing very pure preparations of the herpesviruses. The early estimates of 8 or 9 constituents (Shimono et al., 1969; Olshevsky & Becker, 1970) may prove to be very low as evidenced by a recent paper by Spear & Roizman (1972) who found up to 24 components in extensively purified preparations of herpes simplex.

Although structural proteins are made early in the infectious cycle, their assembly into virions does not occur till after a lag phase; in addition, they appear to be made in excess, as 35% remains non-sedimentable late on in infection, at centrifugal forces large enough to bring down viral particles (Hamada & Kaplan, 1965). Similar results have been reported for herpes simplex virus (Russell et al., 1964).

2. A considerable number of non-structural proteins have also been identified; their production seems to preceed that of the structural proteins although they may be made till fairly late on in infection (Hamada & Kaplan, 1965). These are thought to include the various enzymes required to permit virus replication to proceed, and also, possibly, several inhibitors of host metabolism.

All the present evidence indicates that viral proteins are made in the cytoplasm using the host machinery - ribosomes, t-RNA's, etc. There has been a suggestion that virus-specific t-RNA's might be produced but this has never been shown conclusively (Subak-Sharpe & Hay, 1965; Subak-Sharpe et al., 1966; Morris et al., 1970). After synthesis, many of the viral proteins migrate to the nucleus, as shown by the 'pulse-chase' experiments of Fujiwara & Kaplan (1967). In addition, there is evidence that this migration is selective, as Spear & Roizman (1968) have identified 3 proteins which appear to be restricted to the cytoplasm. The rate of migration seems to be relatively slow and inversely proportional to size (Olshevsky et al., 1967; Spear & Roizman, 1968; Ben-Porat et al., 1969). It does not seem to be dependent on the synthesis of viral DNA, as in the presence of the DNA synthesis inhibitors, Ara-C or 5-fluoro-uracil, not only does protein migration occur but assembly also, into non-infective viral particles which are morphologically indistinguishable from infective ones under the electron-microscope (Reissig & Kaplan, 1962; Ben-Porat et al., 1969). Kaplan has proposed the existence of an arginine-rich protein which allows transport to occur. This will be discussed in greater detail in Section 5.

These viral proteins have not been well-characterised but several features are worth mentioning :

(a) Some are glycoproteins. Ben-Porat & Kaplan (1970) have reported that two types of glycoproteins are found in PrV infected RK cells :

1. Several found only in the cytoplasmic fraction, which are excreted in large amounts into the extracellular fluid and

may therefore be involved in the process of release of mature virions.

2. Others which are found associated with nuclei of infected cells and appear to be identical to those present in partially purified preparations of PrV. The virus has been proposed to bring about modifications in the nuclear membrane of infected cells - this may be involved in transport of structural proteins to the nucleus, and also in the 'budding' of virus into the cytoplasm. In this way the viral glycoproteins could be incorporated into the viral envelope believed to be obtained at this time.

(b) Several are basic. Stevens et al., (1969) identified five virus-induced proteins in acid extracts of whole nuclei which were produced by 4 hrs post-infection. These aroused interest as basic proteins (histones) and are believed to be involved in the regulation of transcription in non-infected cells. They further showed that all five contained appreciable amounts of tryptophan and that four could be identified in preparations of pseudorabies virions. The fifth was non-structural and associated with chromatin by 6 hrs post-infection.

The presence of small basic proteins in viral preparations is very common among the animal viruses. They have been identified in adenovirus (Russell, Laver & Sanderson, 1968; Russell, 1971) polyoma (Fine, Mass & Murakani, 1968; Frearson & Crawford, 1972) SV40 (Anderer et al., 1967) and poliovirus (Maizel, 1963; Maizel, Phillips & Summers, 1967). Again, there is a correlation with the phage-infected bacteria as the original discovery of a basic viral internal protein was made for phage T-2

(Hersey, 1957; Levine, Barlow & Van Vunakis, 1958). The functional significance of the internal protein is presently unknown; however, its basic nature, as well as the fact that it is normally found in the viral core suggests that it is involved in the wrapping up of viral DNA in a suitable form for encapsidation.

Further points of interest include (i) the suggestion by Mantyjarvi & Russell (1969), and Russell, Laver & Sanderson (1968) that the synthesis of adenovirus basic proteins may be linked with viral DNA replication in a manner analogous to histones and cellular DNA (Robbins & Borun, 1967), (ii) the possibility that the basic components of polyoma and SV40 have a host origin (Frearson & Crawford, 1972), and finally, related to this, the fact that pseudorabies and adenovirus both inhibit cellular histone synthesis and produce their own basic proteins, whereas polyoma and SV40 promote the synthesis of host histones and appear to utilise some of this material for assembly of their viral particles.

1.5. HISTONE FUNCTION IN NORMAL CELLS

It is therefore apparent that several basic proteins found in virus-infected cells have properties in common with cellular histones. For this reason, the functions of histones in uninfected cells will be briefly considered, as far as is known at the present time.

Histones are found associated with DNA in all higher organisms. Their function in chromatin is not fully understood although they undoubtedly help to counteract the highly acidic nature of DNA and

thereby stabilise the nucleoprotein complex in its confined state within the nucleus. Whether this is their sole function or whether they have additional specific properties in controlling transcription and allowing cellular differentiation to occur is not clear.

Huang & Bonner (1962, 1963) made the initial observation that histones annealed to DNA in vitro and thereby inhibited transcription. In addition, it has been shown that extraction of certain classes of histone from chromatin stimulates template activity to RNA polymerase (Georgiev, Ananieva & Kozlov, 1966). Apparently histones can repress DNA transcription by merely precipitating the chromatin (Sonnenberg & Zubay, 1965); this may be an important physiological mechanism as it is known that dispersed chromatin (euchromatin) is much more metabolically active than condensed or heterochromatin. However there is definite evidence that soluble chromatin can also be repressed (Huang, Bonner & Murray, 1964) and in this case histones must either affect the initiation of RNA chain growth or its elongation, as the binding efficiency of polymerase to the template does not seem to alter (Marushige & Bonner, 1966).

There are several factors which argue against histones having a specific function in regulation of transcription. Firstly the lack of heterogeneity - only 3 main classes of histones are found, lysine-rich, slightly lysine-rich and arginine-rich. Although there is limited heterogeneity within each group, this is not sufficient to make histones likely contenders for the role of specific repressors. Secondly, the similarity in histones which is found, not only from tissue to tissue within a single organism (Fambrough, Fujimara & Bonner, 1968) but also

between species. Almost perfect conservation of the amino-acid sequence of histones during evolutionary development has been shown by the analysis of histone IV of calf thymus and pea-seedling (Delange et al., 1968). Amino-acid substitutions were found in only 2% of the molecule, as opposed to in 30% of cytochrome c molecules from a plant and animal species (Stevens et al., 1967; Yasunoba et al., 1963). This not only suggests that it is unlikely that histones are involved in differentiation of cells, but also that their function is very closely linked to their structure - possibly due to their mode of attachment to DNA. Finally, there is no evidence for histones having the ability to recognise base sequences and they seem to be dispersed randomly along the length of the DNA chain (Johns & Butler, 1964; Kurashina et al., 1970).

It is therefore probable that the histones themselves act as non-specific repressors as proposed by Paul & Gilmour (1968, 1969). Specificity could be obtained by interaction of histones with non-histone proteins (Wang & Johns, 1968; Paul & Gilmour, 1968), RNA (Huang & Bonner, 1965, 1964) or by chemical modification of the histones in the form of phosphorylation (Stevely & Stocken, 1966; Langan, 1967), acetylation (Allfrey, 1969; Pogo et al., 1968) or methylation (Allfrey et al., 1964; Paik & Kim, 1971).

To summarise therefore, histones play an important structural role as components of chromatin which is probably related to their rigidly conserved primary sequence. They undoubtedly act as non-specific repressors of transcription but at the moment it is not known how much specificity they can exhibit. These are important facts to remember

when considering the functions of the small basic proteins which will be described in this thesis.

1.6 SUMMARY

Infection of a cell by a virus results in a gradual inhibition of cellular metabolism, while the host protein-synthesising machinery is directed towards producing viral progeny. This seems to be a general phenomenon whether one is considering phage-infected bacteria or the infection of mammalian cells by animal viruses like pseudorabies.

A number of the inhibitory reactions, including the switch-off of host DNA and RNA synthesis, are thought to require viral transcription and translation. In addition various early non-structural viral proteins are necessary to allow viral DNA replication and transcription to occur normally; in pseudorabies, these proteins may be arginine-rich (Rakusanova et al., 1972). Furthermore basic proteins seem to be found fairly generally as internal proteins associated with the DNA core of viruses. These proteins are thought to have similar functions to histones in eukaryotic nucleoprotein, and in fact some viruses like polyoma and SV40 appear to incorporate cellular histones into their DNA core.

This project is mainly concerned with the study of three basic proteins present in pseudorabies-infected cells. These are found in the acid-extractable (histone) fraction of chromatin by 3 hrs post-infection and in view of this may provide a possible mechanism for the

process of host repression. Alternatively they may form part of a virus-synthesising complex caught up in the chromatin network, and be involved in virus assembly.

SECTION 2 BASIC PROTEINS OF CHROMATIN IN INFECTED CELLS

2.1. INTRODUCTION

Very little work has been done on studying specific virus-induced proteins in mammalian systems. This is mainly due to the difficulty in distinguishing viral from host proteins, and a lot of earlier work was merely on antigenic studies to characterise the total spectrum of proteins produced post-infection and to identify those proteins which were destined to become part of the viral particles.

In pseudorabies-infected cells, several 'early' proteins were found to be non-structural (Hamada & Kaplan, 1965) and were suggested to include a number of enzymes which were believed to be virus-induced. In addition, these early proteins were proposed to be involved in the inhibition of host-function which occurs shortly after infection.

This thesis is based on the discovery of several virus-induced proteins which are in close association with host deoxynucleoprotein. These include three small basic proteins which extract with acid along with cellular histones. They can be readily identified by electrophoresis of acid extracts of chromatin on 15% polyacrylamide pH 4.0 gels which separates them from large molecular weight material unable to penetrate the gel and also from histones which have a higher mobility under these conditions. This system has allowed extensive characterisation of these proteins to be carried out, both in terms of time-course of production and amino-acid content.

In addition, several virus-induced proteins have been discovered in the urea or SDS (acidic nuclear protein) fractions of chromatin. These will only be briefly described (Section 4).

2.2. BASIC PROTEINS PRODUCED 1-6 HOURS POST-INFECTION

BHK/21 cells in the exponential phase of growth were infected with pseudorabies virus (PrV) at a multiplicity of 20 pfu/cell or mock-infected, in EC10. At 1 hour post-infection the medium was changed to EC10 $\frac{1}{5}$ Met and 200 μc's of (^{3}H-methyl)-methionine were added. The cells were harvested at 6 hours post-infection and chromatin was prepared as in Materials and Methods (Appendix 2 II (i)). The chromatin was well-washed by homogenisation in buffer and 1 mM HCl and it is concluded that any material thereafter extracted must have a fairly strong affinity for the nucleoprotein complex.

Basic proteins extracted with 250 mM HCl were analysed on 15% polyacrylamide gels (pH 4) both for their staining pattern, and their radioactive profile. On staining, both control and infected samples were identical (Fig 2.2(a)) and it was only on slicing and counting the gels that differences could be seen (Fig 2.2(b)).

Thus by 6 hours post-infection it appears that a new radioactive species is present in the histone fraction of infected cells. Its concentration must be very low as it does not show up on staining, and correspondingly, its specific activity quite high. This suggests it may be a viral protein produced post-infection. In addition, a comparison of Fig 2.2(b) control and infected, indicates that the

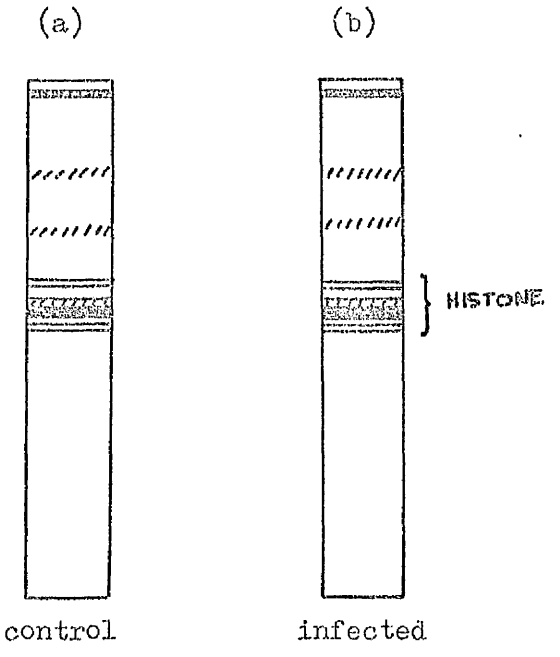
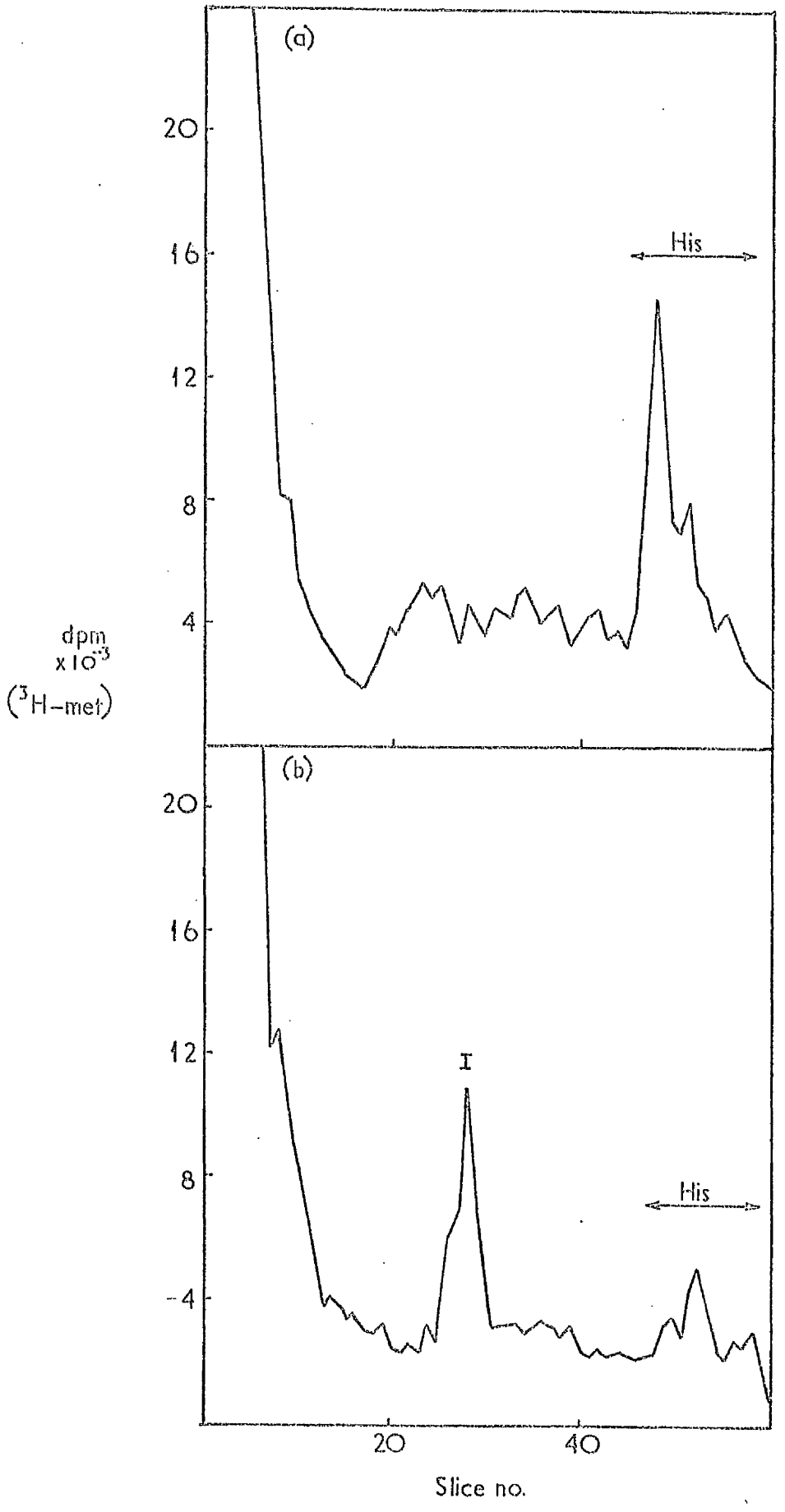


Fig 2.2(a) : 15% polyacrylamide pH 4 gels of acid extracts of control and infected chromatin, stained with naphthalene black. Both gels are identical and above the main region of histone material are pale bands believed to be dimers and trimers of f_1. At the top of the gels is a dark band of aggregated histone material.

Figure 2.2(b) Electropherogram of Basic proteins produced 1 - 6 hours post-infection

BHK/21 cells in the exponential phase of growth were mock-infected, or infected with PrV at 20 pfu/cell. At 1 hour post-infection the medium was changed to ECl0 $\frac{1}{5}$ Met and the cells were labelled from 1 - 6 hours with (^{3}H-methyl)-methionine. Chromatin was prepared and extracted with 250 mM HCl for 5 hours. Samples were dialysed against 0.02M tris pH 7.8 before electrophoresis on 15% polyacrylamide gels.

The region where histones run has been marked and the new peak which has appeared in infected samples is arrowed I.


(a)
20
16
12
8
4
His
dpm
x10⁻³
(³H-met)
(b)
20
16
12
8
-4
I
His
20
40
Slice no.

labelling of histone species is markedly inhibited as found by other workers (Stevens et al., 1969).

2.3. TIME-COURSE OF PRODUCTION OF IP BETWEEN 1 AND 6 HOURS

The time of appearance of this viral-induced peak in chromatin acid extracts was then examined by labelling for one hour periods between 1 and 6 hours after infection. The conditions used were identical to those in Exp. 2.2 and are detailed in Materials and Methods. The picture obtained is shown in Fig 2.3.

There is no sign of any induced protein in a 1 - 2 hour label, and the only difference between control and infected samples is an apparent reduction in histone synthesis. This is difficult to interpret due to the considerable amount of histone aggregation represented by the high counts at the top of the gel. High molecular weight material which does not enter 15% polyacrylamide gels is found in all infected samples and some of this undoubtedly represents virus-specified material. However most of the counts in the first few slices of control gels could be got rid of by extracting and storing the samples in 1% β-mercapto-ethanol, and seemed to represent aggregation of cellular histones.

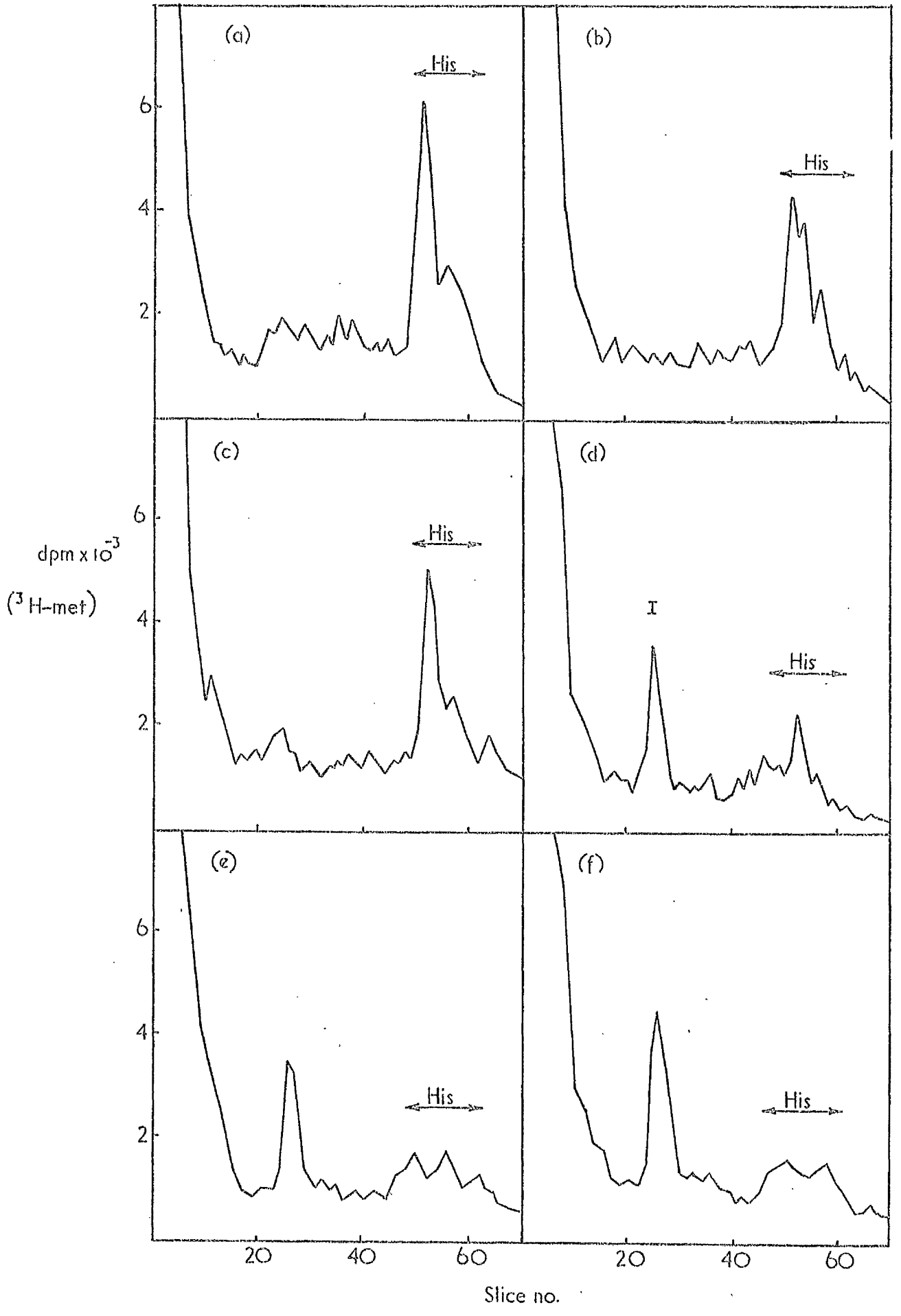
The viral peak appears between 2 and 3 hours post-infection and is still being made in substantial amounts in the 5 - 6 hour label. Its time of appearance is thus in accordance with it being one of the 'early' proteins, i.e. proteins produced prior to the onset of DNA synthesis (Hamada & Kaplan, 1965).

Figure 2.3 Time-course between 1 and 6 hours post-infection

BHK/21 cells were labelled with (^{3}H-methyl)-methionine for one hour periods between 1 and 6 hours after infection. Basic proteins in chromatin were extracted as before and dialysed against buffer (T.M.E.), before electrophoresis. A control for each labelling period was done, but only one is shown as they were all very similar. The induced protein is marked I and the histone region is also indicated.

(a) control (b) 1 - 2 hour (c) 2 - 3 hour

(d) 3 - 4 hour (e) 4 - 5 hour (f) 5 - 6 hour


(a)
His
(b)
His
(c)
His
(d)
I
His
(e)
His
(f)
His
6
4
2
dpm x 10⁻³
(³H-met)
20
40
60
Slice no.

2.4. TIME-COURSE OF IP PRODUCTION BETWEEN 1 AND 12 HOURS POST-INFECTION

As this induced protein is still being made in substantial amounts at 6 hours post-infection, the time-course was extended to 12 hours by which time 80% of the progeny viral particles are in the cytoplasm (Sydiskis, 1970). Control and infected cells were labelled for each of the time periods 1 - 3 hr, 3 - 5 hr, 5 - 7 hr, 7 - 10 hr and 10 - 12 hr after infection. The result is shown in Fig 2.4.

The most noticeable feature is the presence of a second peak in the 3 - 5 hr pulse. The time of appearance of this protein (IP II) has been found to vary considerably, particularly between stock preparations of pseudorabies virus used for infection. Thus in experiments 2.2 and 2.3 there was no sign of IP II by 6 hr post-infection whereas in most subsequent experiments, it has been produced by 5 hours. Note also that a third peak has been marked, IP III running in the histone region of the gels. At first this was thought to represent incomplete inhibition of histone synthesis, but the fact that it was still being synthesised at 10 - 12 hours post-infection suggested that a third viral protein was present. This has since been shown to be the case.

2.5. PRE-LABELLING OF CELLS

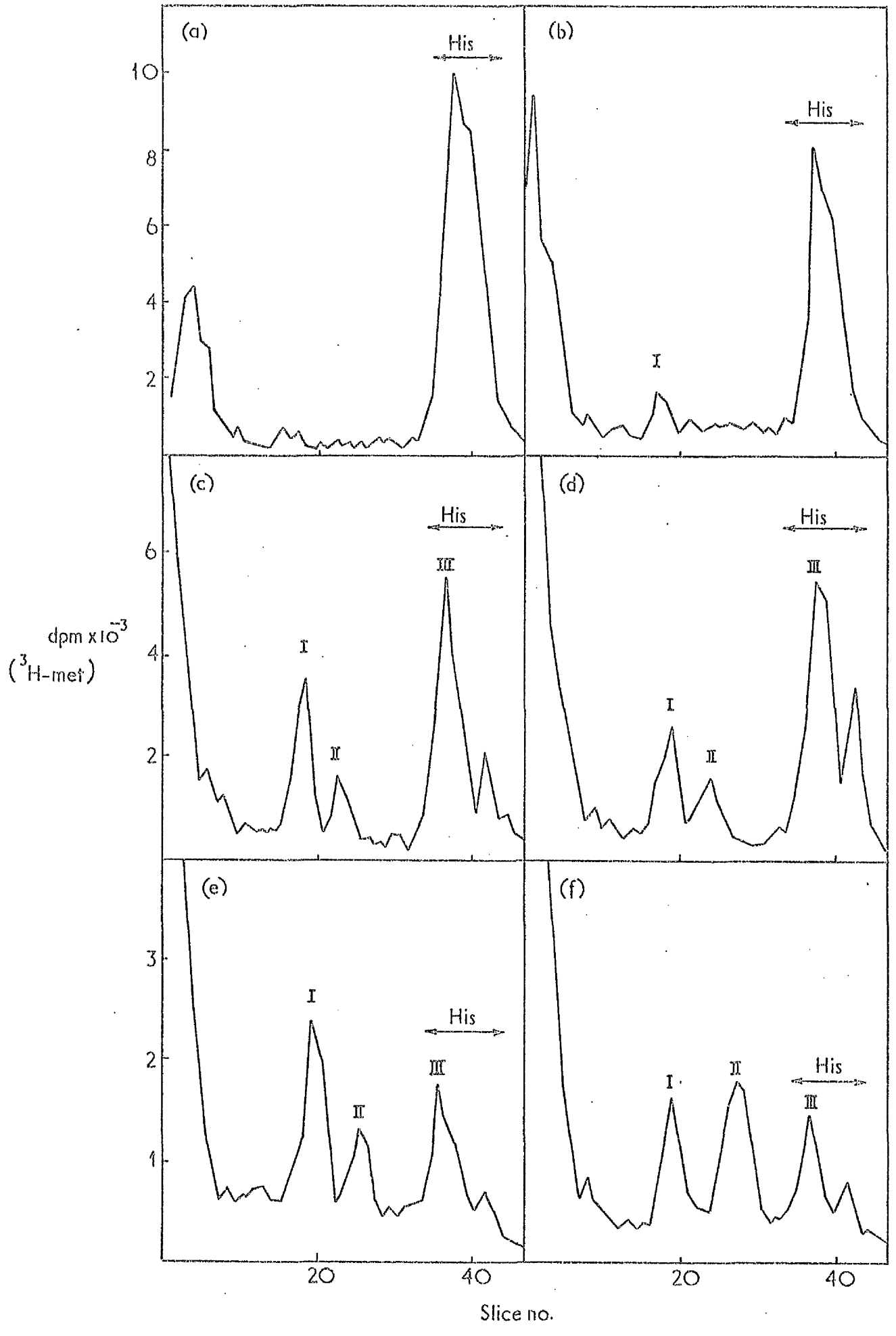
The 3 induced proteins present in acid-extracts of infected cells may represent de novo synthesis of virus-coded proteins or alternatively a viral modification of cellular histones. This latter reaction would be of interest as modification of histones, whether

Figure 2.4 Time-course of IP production between 1 and 12 hours post-infection

Control and infected burlers of BHK/21 cells were labelled with (^{3}H-methyl)-methionine for the time periods indicated below. The acid-extractable material from chromatin was then run directly on 15% polyacrylamide gels. No dialysis was carried out to help prevent aggregation of histone. Controls for each time period were done, but only one is shown. Viral proteins are marked I, II and III. For further details, see text.

(a) control (b) 1 - 3 hour (c) 3 - 5 hour

(d) 5 - 7 hour (e) 7 - 10 hour (f) 10 - 12 hour


(a)
His
(b)
His
I
(c)
His
I
II
III
(d)
His
I
II
III
(e)
I
His
II
III
(f)
I
II
His
III
dpm x10⁻³
(³H-met)
Slice no.

chemical, or by interaction with non-histone chromosomal proteins, has been suggested as the mechanism for controlling DNA transcription in uninfected cells (see Section 1.5). A similar viral alteration could thus provide a mechanism analogous to the host system for the virus to switch off host RNA synthesis, and could result in the change in electrophoresing properties of the histone molecule giving rise to 2 virus-induced peaks, on 15% gels. This modification could be in the form of methylation of the histone molecule as the compound used for labelling was (^{3}H-methyl)-methionine, or by interaction of a virus-induced peptide with the histone. These possibilities were examined with a prelabelling experiment in which host proteins were labelled prior to infection.

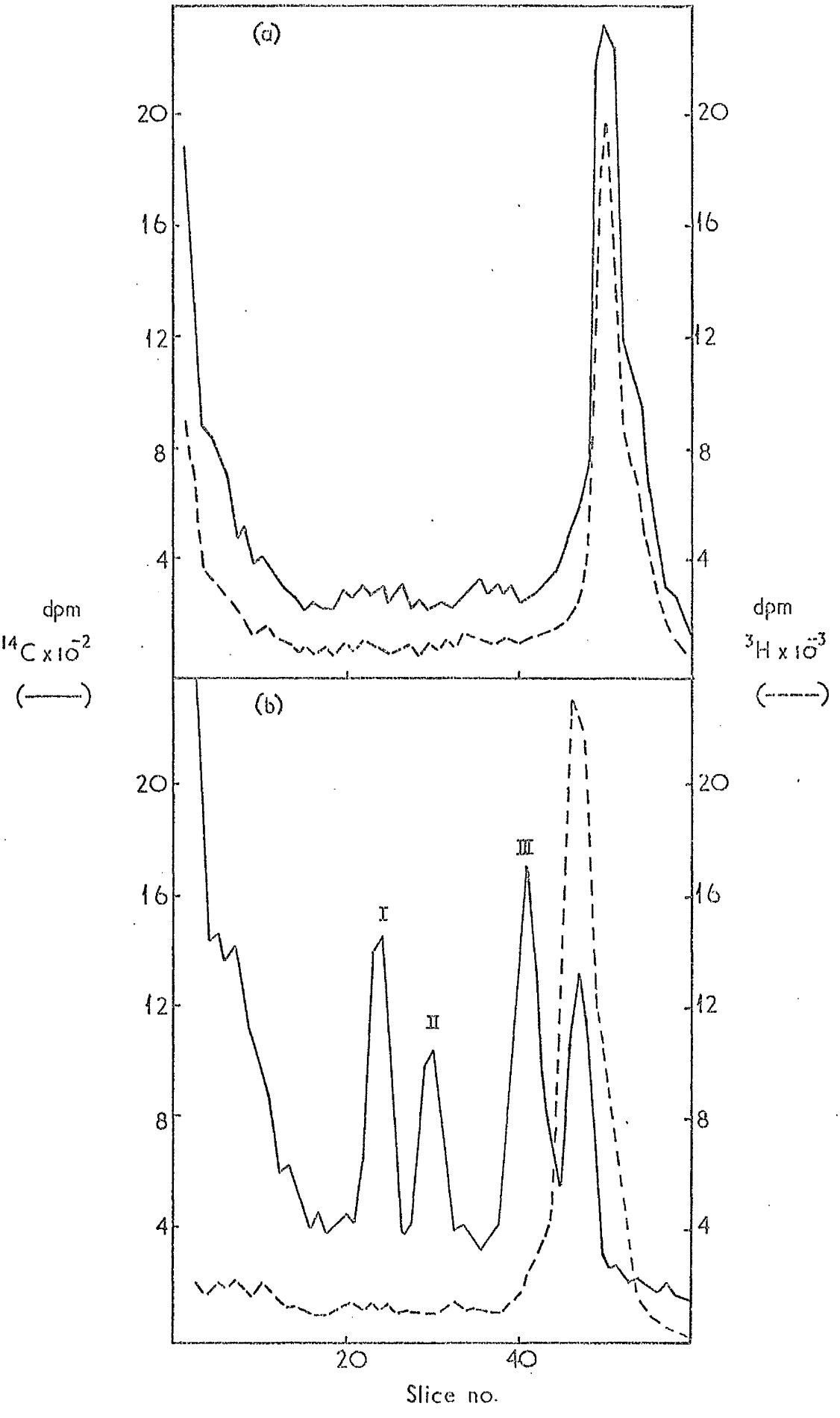
BHK/21 cells were labelled for 5 hours in the early exponential phase with (^{3}H-methyl)-methionine, then the radioactive medium was replaced with normal ECl0 and the ^{3}H-label diluted out by growing for a further 18 hours. The cells were then mock-infected or infected with PrV in ECl0 and at 1 hour post-infection the medium was changed to ECl0 $\frac{1}{5}$ Met. The cells were then labelled 3 - 8 hours after infection with (^{14}C-methyl)-methionine. Chromatin was prepared and extracted as usual with 250 mM HCl/1% βME and the extract examined on 15% gels (Fig 2.5).

It is clear that none of the ^{3}H-labelled host proteins are converted to virus-induced peaks and these must therefore represent de novo synthesis of proteins produced only post-infection and therefore most likely virus-coded. This experiment also shows very clearly the presence of IP III in the histone region of the gels.

Figure 2.5 Pre-labelling of cells

The cellular proteins of BHK/21 cells were labelled for 5 hours in the early exponential phase with (^{3}H-methyl)-methionine. The ^{3}H-activity in the medium was then diluted out by changing to normal EC10 and growing for a further 16 hours. The cells were then mock-infected, or infected with PrV and labelled 3 - 8 hours post-infection with (^{14}C-methyl)-methionine. Those proteins which are ^{14}C-labelled and contain no ^{3}H-counts are therefore viral proteins produced post-infection.

(a) control (b) infected


(a)
(b)
20
16
12
8
4
I
II
III
20
40
Slice no.
dpm
$^{14}C \times 10^{-2}$
(——)
dpm
$^{3}H \times 10^{-3}$
(- - - -)

2.6. LABELLING OF IP'S WITH THE BASIC AMINO-ACIDS

(i) arginine (ii) lysine

In all previous experiments (^{3}H-methyl)-methionine was used as the labelling compound to initially characterise these induced proteins discovered while examining changes in histone methylation in pseudorabies-infected cells. However, to check that their existence was not due to some isotope effect, the labelling pattern of several other radioactive amino-acids was studied. The amino-acids arginine and lysine were initially chosen due to the basic nature of the induced proteins.

Tankersley (1964) had already shown several interesting effects of the deletion of basic amino-acids from the growth medium on the infectivity of herpes simplex virus. He found that whereas deprivation of arginine or histidine drastically inhibited viral replication, a deletion of lysine seemed to potentiate viral yields. Similar results have been found for pseudorabies by Stevens et al., (1969). This suggests that a number of arginine/histidine rich proteins may be important in the infective process and that lysine must form a minor component of most, if not all, viral proteins. In agreement with this, an analysis of relative amino-acid incorporation into proteins before and after infection has shown that virus-induced proteins contain more arginine but less lysine, methionine, tyrosine, isoleucine and phenylalanine relative to leucine than proteins produced prior to infection (Kaplan, Shimono & Ben-Porat, 1970).

Also of interest is the finding of Stevens et al., (1969) that

the five proteins which extract with acid from the whole nuclei of infected cells do not label at all with lysine under conditions where histones are readily labelled. However all contain appreciable amounts of arginine.

(i) Arginine-label : BHK/21 cells were infected and labelled from 1-5 hours post-infection in EClO $\frac{1}{5}$ Arg containing 4 μc's/ml ^{3}H-arginine. The labelling pattern of basic chromosomal proteins is shown in Fig 2.6 (a) and (b). IP II is the major labelled species under these conditions indicating that it has a high arginine/methionine ratio. IP III is also arginine-rich whereas IP I, which might be expected to be the least basic of the IP's from its gel-running properties, does indeed label poorly with this amino-acid.

(ii) Lysine-label : as it was expected that there would be few counts in the IP's in cells labelled with radioactive lysine, a double-label experiment was carried out using (^{3}H-methyl)-methionine and ^{14}C-lysine. BHK/21 cells were grown in medium containing half the normal concentration of both these amino-acids, and the labelling period was from 3 - 8 hours post-infection. The result is shown in Fig 2.6. The gels were run for $5\frac{1}{2}$ hours instead of the usual 4 hours, and clear separation of f_1 (the lysine-rich histone) from the others was obtained. This is shown very clearly by the ^{14}C-lysine counts. All 3 IP's do appear to contain small amounts of lysine, though proportionately less so (relative to methionine) than the histones. IP II, the most arginine-rich induced protein, also seems to contain more lysine than the other two.

Figure 2.6 Labelling pattern of induced proteins with (i) ^{3}H-arginine and (ii) ^{14}C-lysine and (^{3}H-methyl)-methionine

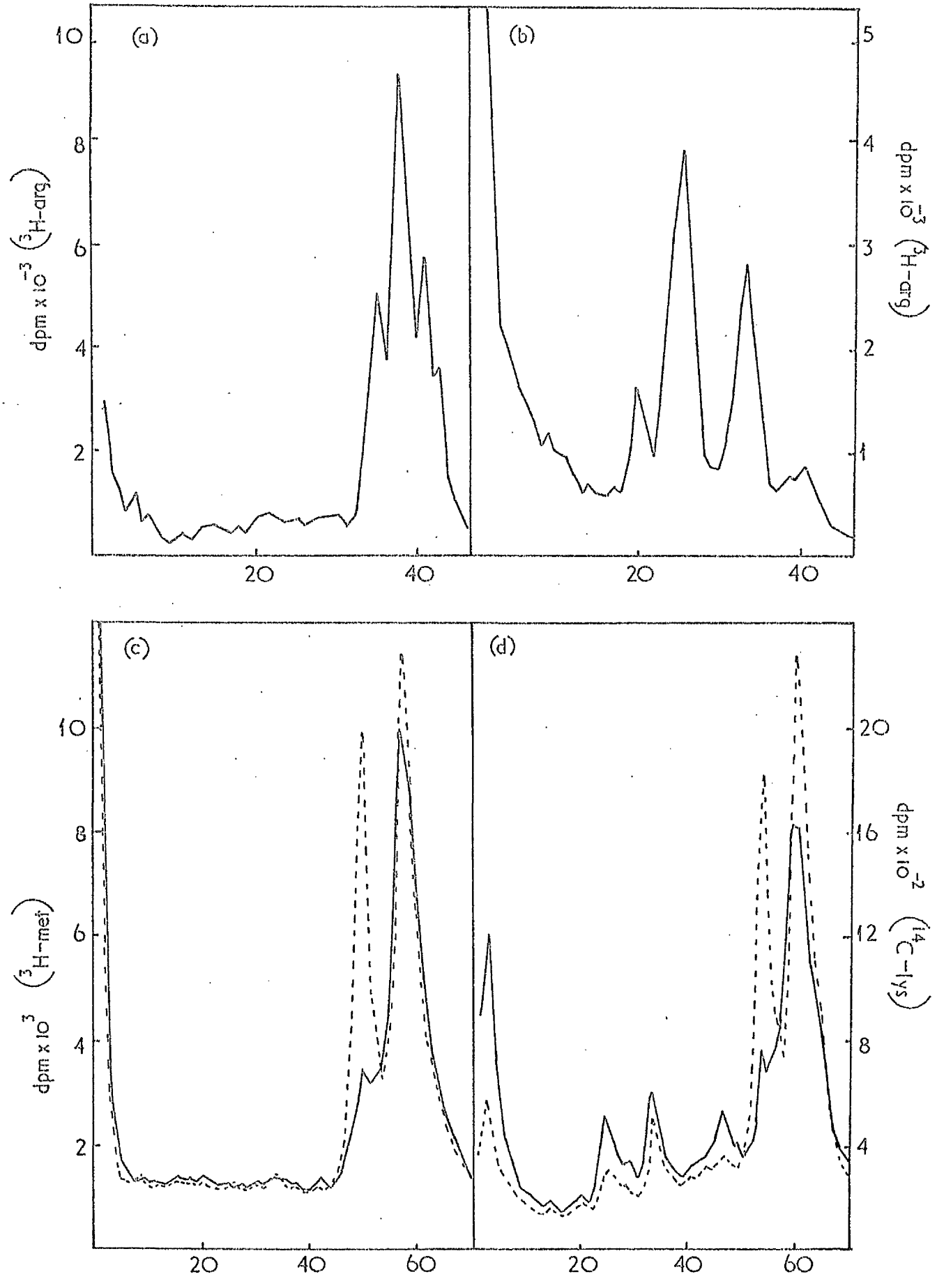
(i) BHK/21 cells were labelled with ^{3}H-arginine 1 - 5 hours after infection with PrV, or in the case of the control, after mock-infection. Basic chromosomal proteins were examined on 15% gels, and the radioactive profile of these is shown. Induced proteins are indicated I, II and III.

(a) control (b) infected.

(ii) Control and infected burlers were double-labelled with 200 µc's(^{3}H-methyl)-methionine and 50 µc's ^{14}C-lysine in EC10 containing half the normal concentration of these two amino-acids.

Acid extracts of chromatin were examined on 15% polyacrylamide pH 4 gels and IP's I, II and III are marked.

(c) control (d) infected.


(a)
(b)
(c)
(d)
dpm x 10^-3 (3H-arg)
dpm x 10^-3 (3H-arg)
dpm x 10^3 (3H-met)
dpm x 10^-2 (14C-lys)
20
40
60

2.7. ARGININE TIME-COURSE

The previous time-course using (^{3}H-methyl)-methionine indicated that the synthesis of IP I and III had commenced by 3 hr PI whereas IP II was not made till the 3 - 5 hour pulse or occasionally till after 5 hr post-infection. However the arginine-label showed IP II to be the predominant labelled species in a 1 - 5 hour pulse. This suggested that IP II might be made much earlier than initially concluded, and simply contain very little methionine, or none at all, but be methylated some time after synthesis. To examine this a ^{3}H-arginine time-course was carried out. The labelling periods chosen were 1 - 3 hour, 3 - 5 hour, 5 - 7 hour, 7 - 10 hour and 10 - 12 hour as before. Only two controls were included, labelled from 1 - 5 hours and 5 - 10 hours respectively (Fig 2.7).

This experiment shows that IP II is indeed made early on in infection, and has a very high arginine-methionine ratio. The possibility that it contains no methionine but is methylated after synthesis may be subsequently examined.

2.8. TRYPTOPHAN LABEL

The absence of tryptophan is one of the characteristics used to identify histones in normal cells. A labelling experiment with ^{3}H-tryptophan was therefore done to see if the IP's are virus-induced histones by this definition. Cells were infected in the exponential phase and labelled from 1 - 4 and 4 - 8 hours post-infection. The profile of radioactive chromosomal basic proteins is shown in Fig 2.8.

Figure 2.7 Arginine time-course

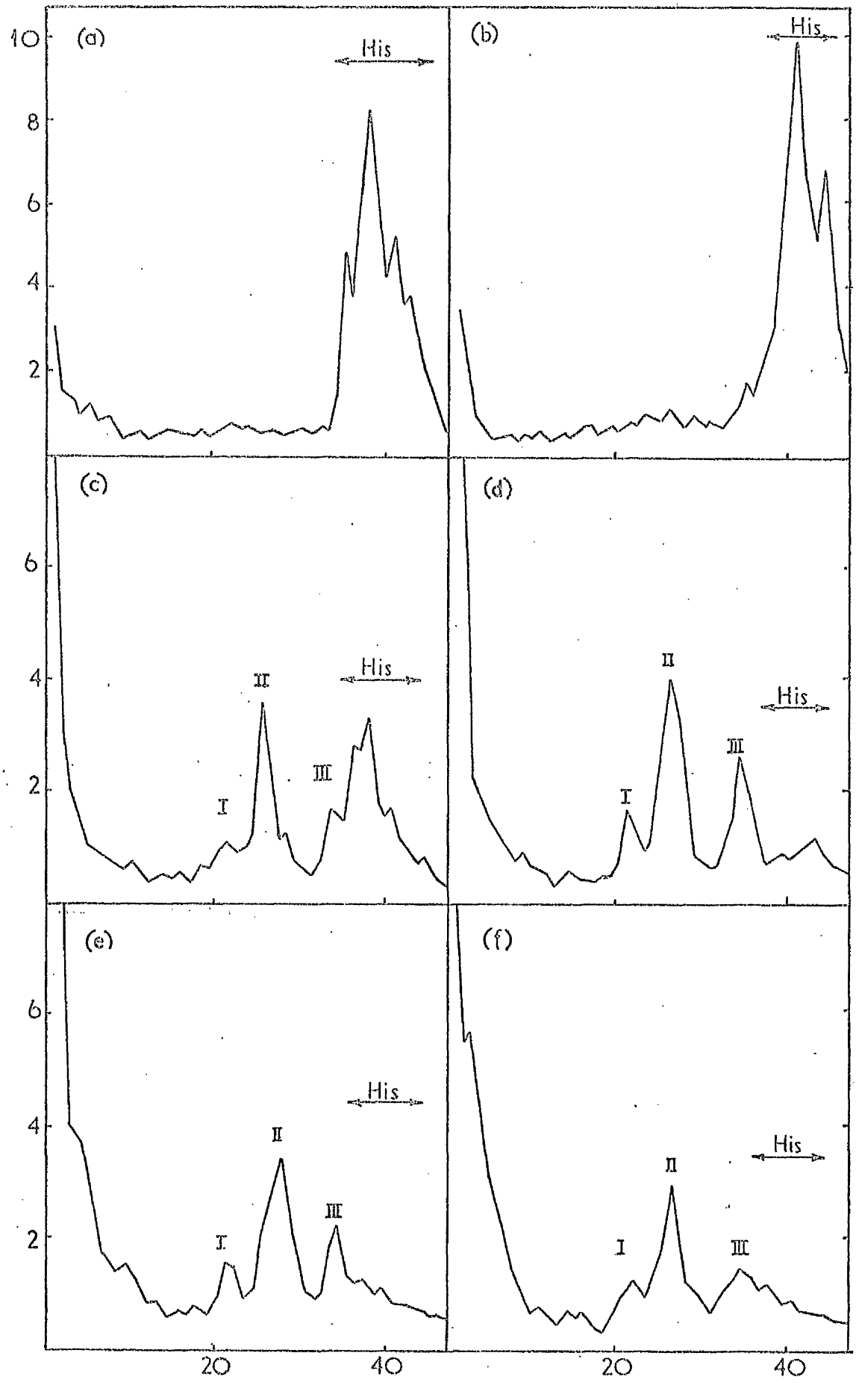
The previous (^{3}H-methyl)-methionine time-course was repeated only using ^{3}H-arginine to label the cells. The labelling times for infected samples are shown below and extend only till 10 hours post-infection. In this experiment only 2 control burlers were included, a 1 - 5 hour label (a) and a 5 - 10 hour label (b).

(c) 1 - 3 hour infected

(d) 3 - 5 hour infected

(e) 5 - 7 hour infected

(f) 7 - 10 hour infected


(a)
His
(b)
His
(c)
I
II
III
His
(d)
I
II
III
His
(e)
I
II
III
His
(f)
I
II
III
His
10
8
6
4
2
6
4
2
6
4
2
20
40
20
40
dpm x 10^3
(3H-arg)
Slice no.

Both IP II and IP III contain appreciable amounts of tryptophan whereas the counts in the IP I region are back-ground level and it must contain very little if any tryptophan. The control gels have a higher back-ground level than expected and it appears that some contamination with non-histone material has occurred; the presence of low amounts of tryptophan-containing material in histone fractions has been reported by other workers.

All five basic proteins identified by Stevens et al., (1969) in acid extracts of whole nuclei contained appreciable amounts of tryptophan. They also mentioned in an addendum that two of these I and V were found to be associated with chromatin by 6 hrs post-infection and these may therefore be identical to IP II and III described here.

2.9. ^{14}C-AMINO-ACID MIXTURE

Although the previous labelling experiments have given much interesting information on the nature of the virus-induced proteins, they have not provided an estimate of the amounts of the 3 proteins produced. For this reason, a ^{14}C-protein hydrolysate was used to label infected cells 3 - 8 hours post-infection in order to determine the relative quantities of each synthesised during this period.

The result is shown in Fig 2.9, and shows that with a generalised label IP I is the predominant species, IP II and IP III being present in roughly equal amounts. If this diagram is compared with similar labelling patterns for (^{3}H-methyl)-methionine and ^{3}H-arginine (Figs 2.4 and 2.7), it can be seen that the picture with

Figure 2.8 Tryptophan label

Control and infected burlers of BHK/21 cells were labelled with ^{3}H-tryptophan 1 - 4 and 4 - 8 hours post-infection. The pattern of labelled chromosomal basic proteins is shown opposite. Histones do not contain tryptophan and so can not be identified but the induced proteins are marked I, II and III. The counts in IP I are back-ground level but the region where this protein would be expected to run has been indicated.

(a) control 1 - 4 hour

(b) infected 1 - 4 hour

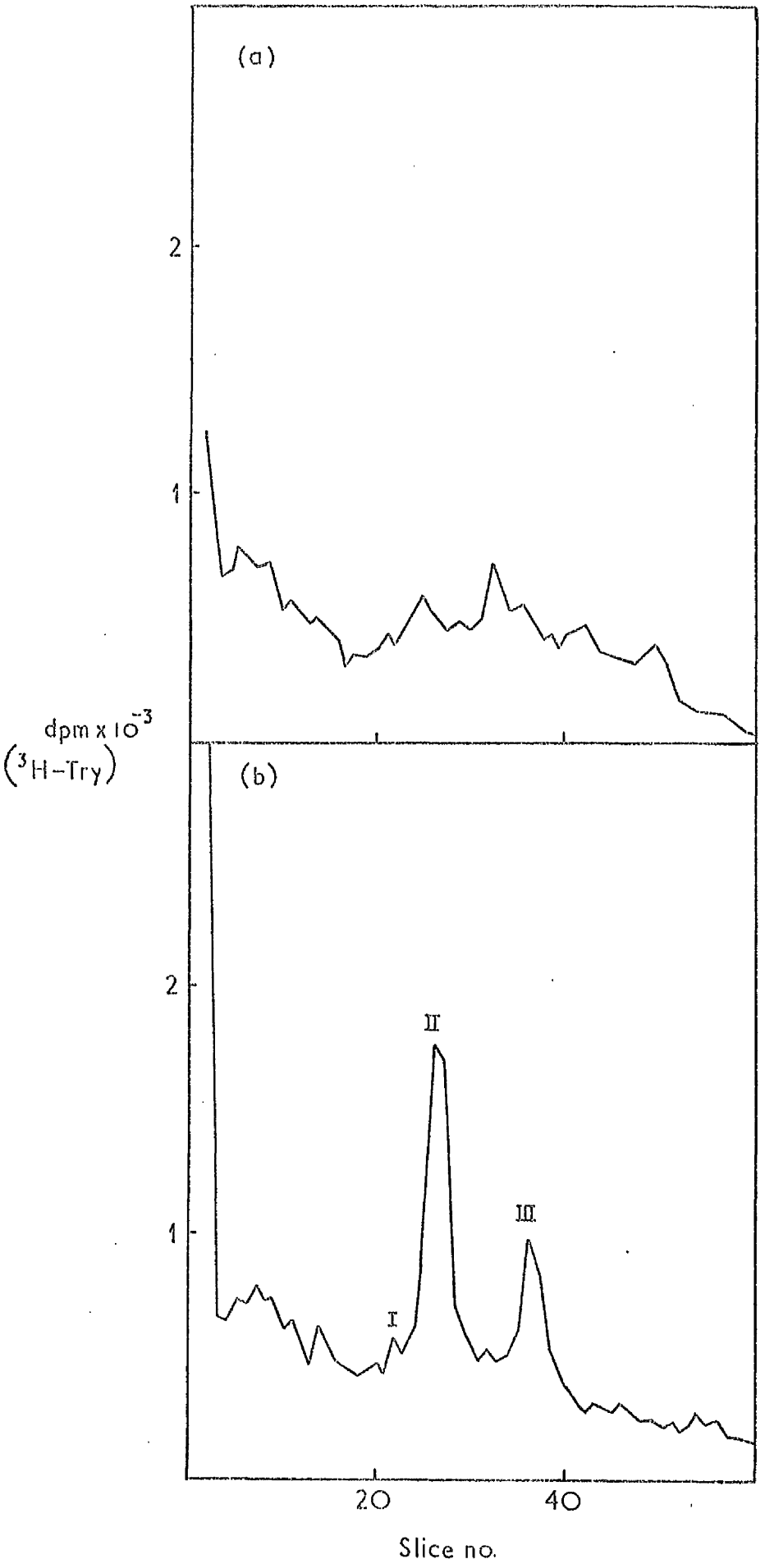
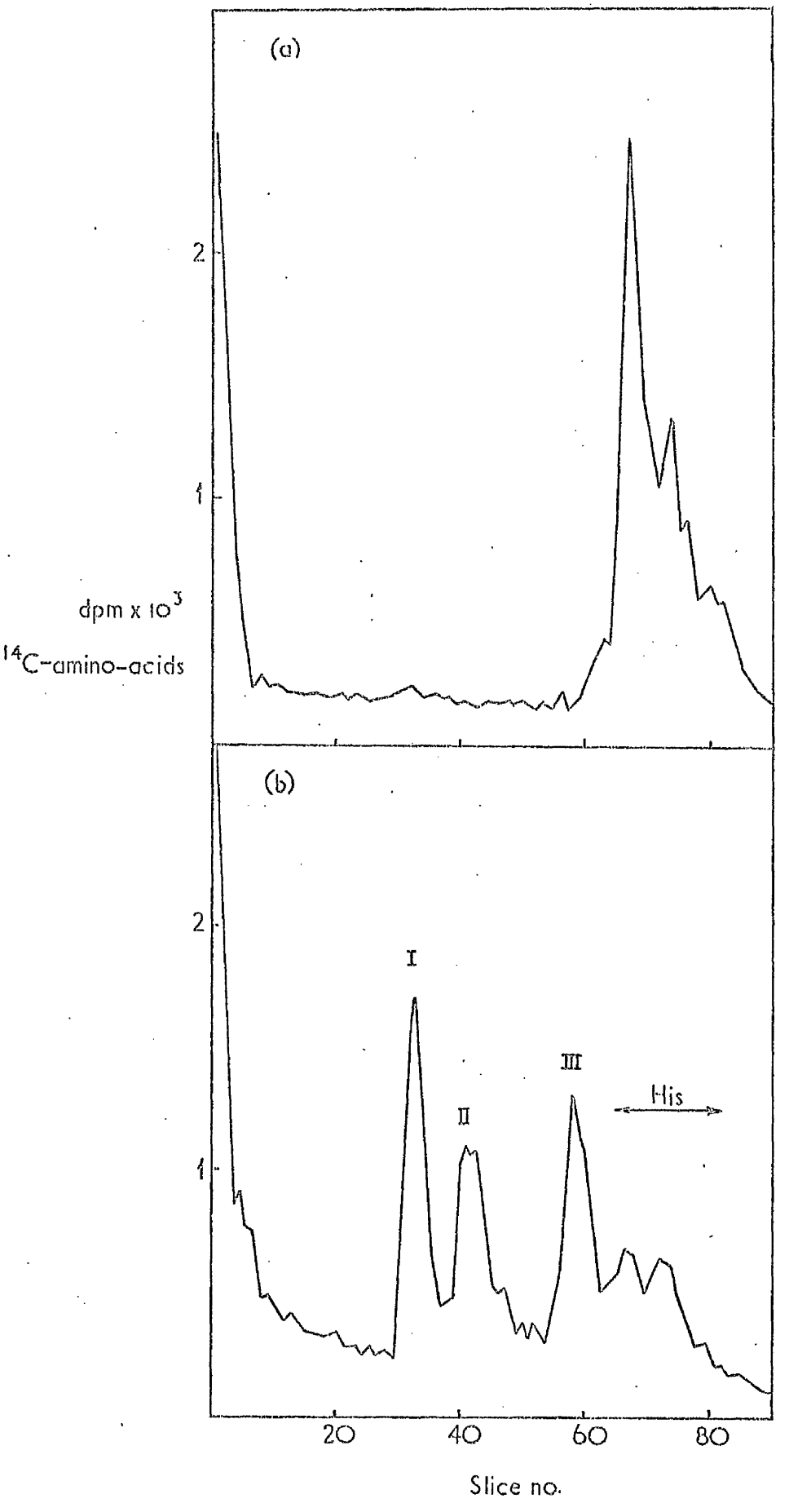

(a)
2
1
dpm x 10⁻³
(³H-Try)
(b)
2
1
II
III
I
20
40
Slice no.

Figure 2.9 Labelling pattern with a ^{14}C-amino-acid hydrolysate

Exponential BHK/21 cells were mock-infected or infected with PrV as usual. They were then labelled 3 - 8 hours post-infection with a ^{14}C-amino-acid protein hydrolysate to give a generalised label of the induced proteins. The radioactive profile of a 15% polyacrylamide gel is shown opposite.

(a) control (b) infected


(a)
2
1
dpm x 10³
¹⁴C-amino-acids
(b)
2
1
I
II
III
His
20
40
60
80
Slice no.

methionine is very similar to that for the amino-acid mixture, and for that reason, a lot of later labelling experiments have been done with this amino-acid.

2.10. DISCUSSION

All three acid-extractable proteins in pseudorabies-infected BHK chromatin are thus synthesised by 3 hours post-infection. Two, IP II and III appear to be arginine-rich but only IP II contains appreciable amounts of lysine. This is of interest with regard to the effects of deletion of these amino-acids noticed by Tankersley (1964) for herpes simplex, and Stevens et al., (1969) for pseudorabies. Their basic nature, and size as judged from their ability to run on 15% pH 4 polyacrylamide gels, as well, of course, as their presence in chromatin preparations, indicates their similarity to cellular histones. However they are not produced by viral modification of these molecules and IP II and IP III contain appreciable amounts of tryptophan. Their time of synthesis indicates that they may be 'early' proteins i.e. proteins produced before the onset of DNA synthesis.

It should be remembered that viral-directed protein synthesis is thought to be required -

(a) to switch off host DNA synthesis, transcription and translation

(b) to allow viral DNA transcription to occur normally.

In an experiment examining the effects of cyclohexamide inhibition on RNA synthesis in infected cells, Rakusanova et al., (1971) showed that under these conditions, a small portion of the viral DNA was repeatedly

transcribed into RNA which, on removal of the inhibitor, coded for a number of arginine-rich proteins. These seemed to be required before further transcription could take place. In addition, Kaplan has proposed the existence of an arginine-rich condensing protein which allows transport of viral structural proteins to the nucleus to occur (Mark & Kaplan, 1971). Both the time of production and arginine content of the induced proteins is in accordance with them having functions of this kind. However their presence in chromatin and similarity in size and change to histones, indicates that they may rather be involved in 'switching-off' host function. If they are indeed concerned with viral transcription and assembly, this suggests that there must be a virus-synthesising complex in close association with the host deoxynucleoprotein. Finally, the possibility that they are viral-structural proteins which become non-specifically adsorbed to chromatin due to their basic nature, can not be ignored.

SECTION 3 STRUCTURAL VIRAL PROTEINS

3.1. INTRODUCTION

Basic proteins are known to be structural components of a number of animal viruses; see Section 1.4 (c). In most cases, they have been identified as forming part of the core, where they are thought to have a similar function to histones in chromatin. However there is a report of a basic component in capsid preparations of SV40 (Anderer et al., 1967).

In general the composition of viral capsids has been found to be remarkably simple. Harris and Hindley (1965) showed that the coat of turnip yellow mosaic, an icosahedral RNA plant virus, consisted of 180 identical polypeptide chains. F2 coliphage has also been shown to have only one protein constituent (Konisberger, Weber, Notani and Zinder, 1966), while two members of the picornavirus group, poliovirus (Maizel, 1963) and mouse encephalitis virus (Rueckert & Duesberg, 1966) appear to have two capsid proteins. More closely related to the herpesviruses are the DNA-containing, nuclear replicating adenovirus and papovavirus groups. The latter includes the viruses polyoma and SV40, which are small and contain coding information for only 5 - 10 proteins. Polyoma capsid consists of sub-units which migrate on gel electrophoresis as one sharp peak. However, whether this peak contains one or several structural polypeptides is uncertain as Anderer et al., (1967) found that SV40 consisted of 3 different polypeptide chains with the same average molecular weight which co-electrophoresed under

similar conditions. The adenoviruses, in comparison, have a much greater coding capacity, and correspondingly, a more complicated structure. The capsid consists mainly of 3 proteins, the fiber and base proteins of the penton and the hexon but a number of minor components may also be present (Maizel, 1966; Russell & Knight, 1967).

The herpesviruses are large and enveloped containing coding information for 140,000 amino-acids or about 200 average size proteins (Kaplan & Ben-Porat, 1968); they might therefore be expected to have a relatively complex structure. Several reports of their protein composition have been published :- Kaplan's group have studied pseudorabies virions prepared by sucrose gradient centrifugation, and also by a method involving serological precipitation of virus during purification (Shimono et al., 1969; Ben-Porat et al., 1970). They found the virus to consist principally of one peak of molecular weight 120,000 daltons, along with a number of minor components. These minor peaks were found to vary in size between preparations, and may represent contamination with cellular material. However, they were found to be very much reduced in preparations of so-called 'empty' particles, and this may mean that some, at least, are core proteins. In later preparations, they identified two major species in approximately equal quantities, the second of which appeared to have properties related to membranous material and may be the main constituent of the viral envelope. These later results compare reasonably favourably with similar studies by Stevens et al., (1969) who also identified only 2 or 3 major components of the virus.

In the case of herpes simplex virus, Robinson & Watson (1971) using virus extensively purified by fluorocarbon treatment, ultra-centrifugation and chromatography on calcium phosphate, again identified one major compound peak, as well as a number of minor components.

Earlier work by Spear & Roizman (1968) and Olshevsky & Becker (1970), also suggested that herpes simplex consisted of a relatively small number of main constituents. However in a recent paper, the former authors claim to have identified 24 proteins and glycoproteins in highly purified preparations of enveloped virions. In addition, they describe protein components of molecular weight 275 K daltons, over twice the size previously recorded (Spear & Roizman, 1972).

In conclusion, it seems clear that the herpesviruses may have a more complex composition than has been found for many of the other animal viruses. The capsid is composed mainly of a protein, or group of proteins of molecular weight 106,000 daltons (M. Walker, personal communication). The figure of 120,000 d quoted by Shimono et al., (1969) is based on a standard curve incorporating the value of 50,000 for the sub-unit of aldolase, rather than the true value of 42,000 (Castellino & Baker, 1968). On correcting this error, their data agrees well with the value reported in this laboratory. In addition to this main species, a few minor components are probably present although the exact number of these is uncertain. At least six glycoproteins are found in the envelope and thus the proportion of enveloped to non-enveloped particles markedly changes the electrophoretic profile of the structural proteins. Finally there are probably a number of minor components in the core which are absent in 'empty' particles. None of these have been definitely identified.

In order to find out whether the 3 pseudorabies-induced peaks described in Section 2 are viral structural proteins, an examination of the protein content of pseudorabies virions was carried out.

3.2. PURIFICATION OF PSEUDORABIES VIRUS

The purification of all members of the herpesvirus group has proved very difficult for a number of reasons which stem from the site of maturation and complexity of the virion. The viruses acquire an envelope during their passage from nucleus to cytoplasm which is believed to arise from the inner lamella of the nuclear membrane. This makes it difficult to distinguish between host proteins forming a constitutive part of the envelope and extraneous contamination with cellular membrane material. However, Spear & Roizman (1972) believe that the membranes which ultimately give rise to the envelope are either made de novo after infection or re-organised to the extent that all host proteins are ejected. If true, this means that any host material found in viral preparations is present as contamination, a proposal which is supported by antigenic evidence. In addition to problems with host contamination, the herpes virion is very susceptible to aggregation and disruption in salt solutions.

Several precautions can be taken in virus purification to increase yields and decrease the amount of host contamination. Firstly, Kaplan has found that high speed centrifugation prior to sedimentation in sucrose gradients causes extensive aggregation of viral particles.

In addition, the choice of cellular fraction is important, in that cytoplasmic virus has been shown to contain less membranous and host DNA contamination than the virus prepared from whole cells or nuclei. However ribosomal species and cytoplasmic membranes can prove as much of a problem. Ideally, use of the supernatant fraction results in minimal cellular contamination, but as it was hoped to prepare empty, as well as full viral particles, this fraction was not used in these experiments.

The method used by B. Jacquemont (1971) in purifying the herpesvirus from nasopharyngeal carcinoma, was the one used in these experiments; experimental details are given in Materials and Methods. The radioactive profile of a 12-52% sucrose gradient of virus labelled till 16 hours post-infection with (^{3}H-methyl)-methionine is shown in Fig 3.2. Only 1 peak is seen, in contrast to the two distinct ones found by Kaplan and coworkers representing infective and empty particles. Electron micrographs have shown that the main peak contains both complete and DNA-deficient particles, and thus no separation is obtained under the conditions used here. Subsequent experiments have indicated the co-sedimentation of ^{3}H-thymidine with this protein peak. In order to prepare a virus sample for protein analysis, the fractions marked were pooled, the sucrose diluted and the virus pelleted by centrifugation at 105,000 g.

3.3. PROTEIN COMPOSITION OF PSEUDORABIES VIRIONS

A portion of the viral pellet was incubated overnight in 2% SDS at 37°C before being applied to a 7.5% SDS polyacrylamide gel.

Figure 3.2 Purification of PrV by Sucrose gradient Centrifugation

Samples for gradient purification were prepared by labelling burlers with (^{3}H-methyl)-methionine till 16 hours post-infection with pseudorabies virus. Whole cell, or nuclear extracts were thoroughly sonicated until homogeneous then layered on to a 30 ml 12 - 52% preformed sucrose gradient and spun 105,000 g for 40 mins. 1 ml fractions were collected and 0.1 ml of each withdrawn for radioactivity measurements. The profile of counts through the gradient is shown opposite. A broad peak with 2 shoulders has been marked I, and subsequent work has shown the co-sedimentation of ^{3}H-thymidine label with this protein peak.

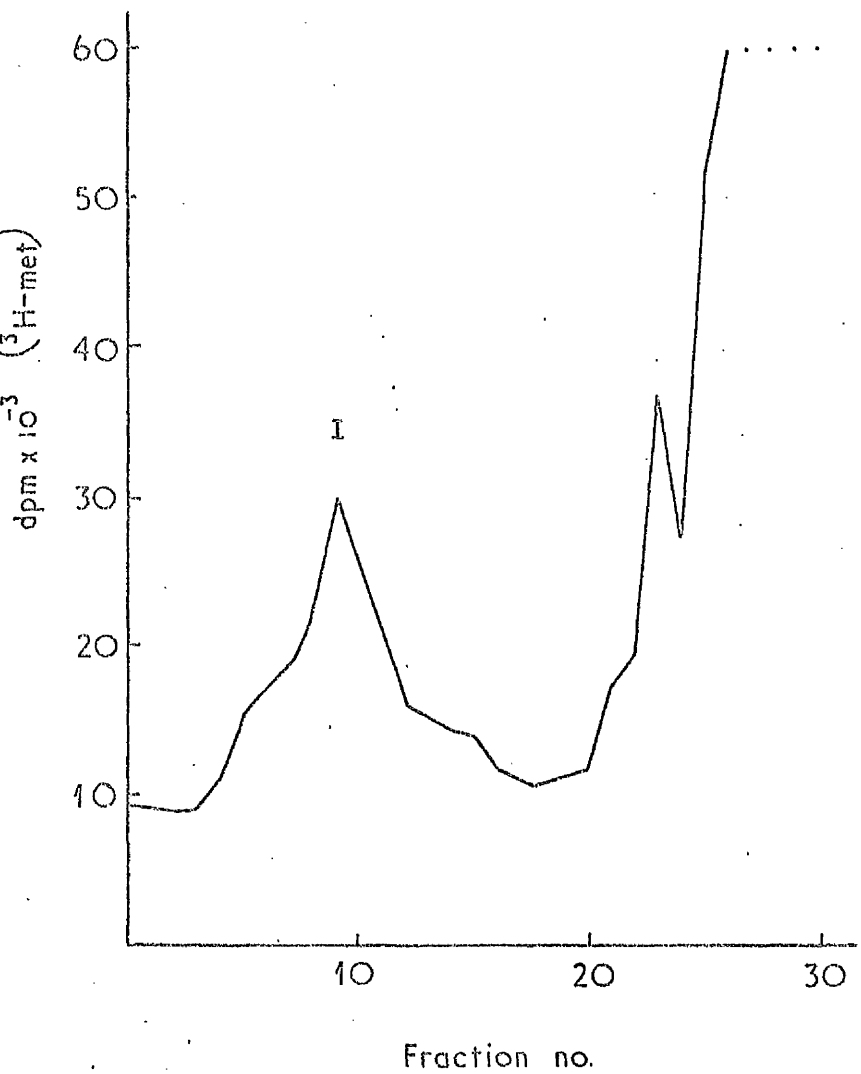

60
50
40
30
20
10
dpm x 10^{-3} (^{3}H-met)
I
10
20
30
Fraction no.

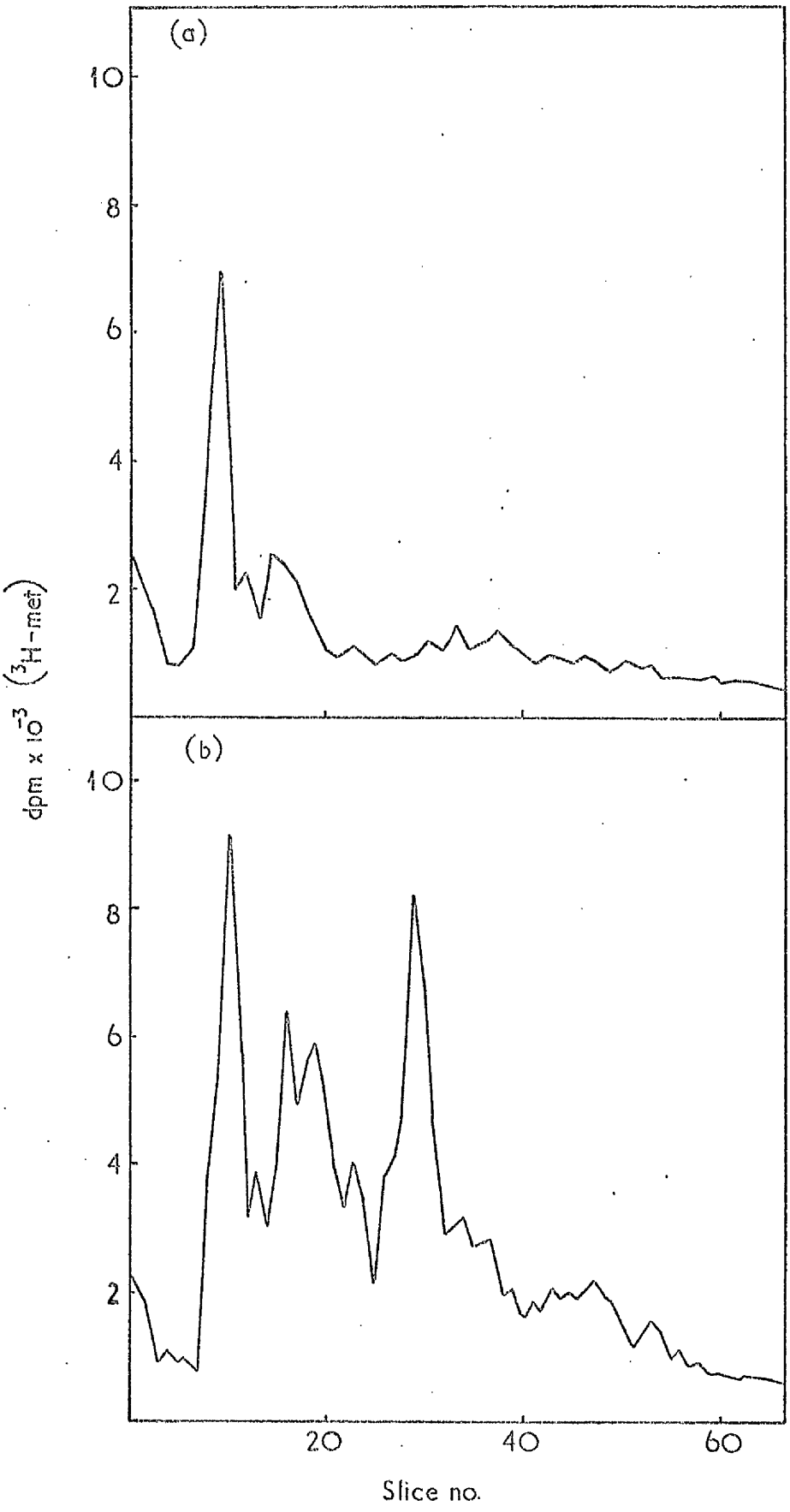
Figure 3.3 (a) shows the electrophoretic profile of structural components of the virus prepared from isolated nuclei. In comparison Figure 3.3 (b) is the picture for virus obtained from whole cells. In agreement with the work of others, pseudorabies virus was seen to consist of one major protein with a varying amount of other components. The increase in protein constituents in (b) is undoubtedly partly due to the presence of enveloped particles in whole cell preparations. In addition there may be a greater proportion of empty particles in nuclear preparations resulting in an enrichment of capsid relative to core proteins in these samples.

3.4. BASIC PROTEINS IN PSEUDORABIES PREPARATIONS

In order to examine the basic components of pseudorabies virions, a viral pellet was taken up in 250 mM HCl, sonicated until fairly well dispersed, then freeze-thawed three times. Acid-soluble material was extracted for 5 hours then analysed on 15% polyacrylamide pH 4 gels (Fig 3.4 (i)). It can be seen that small amounts of 3 basic components are present which appear to run in approximately the same positions as IP's I, II and III. A co-electrophoresis was therefore done of ^{3}H-labelled viral basic proteins with a ^{14}C-acid extract of chromatin to examine just how similar these proteins are, and this is shown in Fig 3.4 (ii). The two sets of proteins can be seen to electrophorese together under these conditions which suggests very strongly that they are identical species, as in contrast to the SDS system, these gels separate molecules on the basis of size and charge.

Figure 3.3 Protein Composition of PrV

7.5% SDS gels of the protein constituents of pseudorabies virions prepared from (a) isolated nuclei and (b) whole cell extracts. Samples were incubated overnight at 37°C in 2% SDS before being applied to the gels. The extra proteins in whole cell preparations may represent, at least partially, constituents of the viral envelope.


(a)
(b)
10
8
6
4
2
dpm x 10^-3 (3H-met)
20
40
60
Slice no.

It is noteworthy that the amount of these basic proteins is very low and does not approach the value of 10% of the total viral proteins found for the basic components of polyoma and SV40 (Frearson & Crawford, 1972; Anderer et al., 1967). This may mean that the induced proteins are present as part of contaminating chromatin species and not constituents of the virus at all. Alternatively, if they are core proteins, they may not be extracted very efficiently by 250 mM HCl; this seems unlikely as there is evidence from EM studies that the virus is disrupted under the conditions used.

Perhaps the most likely explanation is that the virus preparations contain a large number of empty particles, and so are greatly enriched with capsid, relative to core proteins. There is a certain amount of evidence for this from electron micrographs.

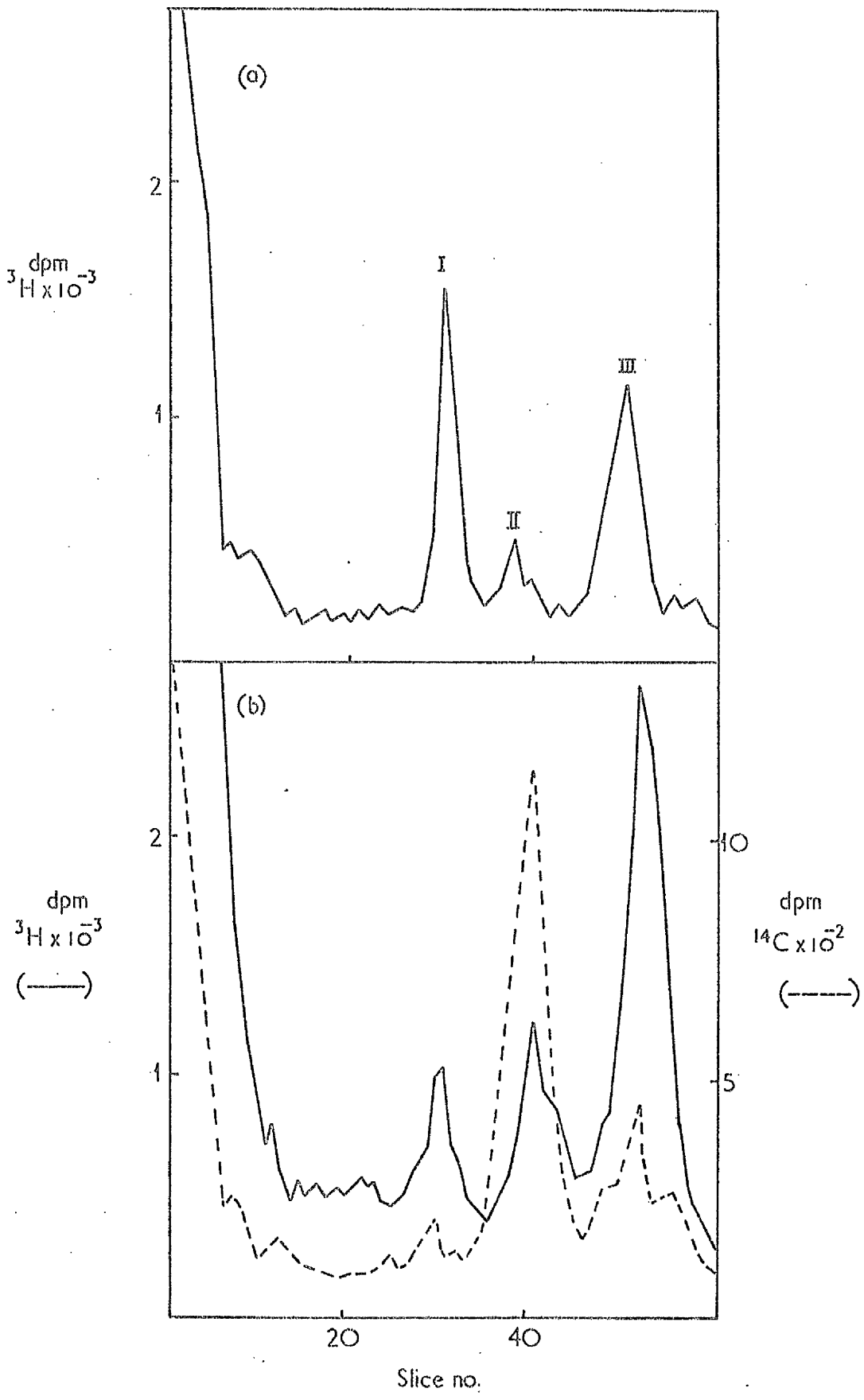
3.5. DISCUSSION

Fig 3.5 shows an electron micrograph of washed chromatin prior to acid extraction. It can be seen that there is a large amount of contamination of host chromatin with viral particles. At first this was thought possibly to mean that the IP's were present in chromatin merely as part of contaminating virions. However the fact that total virus purified from 1 burler of cells contains so much less IP than is found in the chromatin prepared from those cells suggests that the IP's must be present in vast excess in chromatin. Whether this merely represents the excess of viral proteins synthesised which is not required for virus production (Hamada & Kaplan, 1965) and becomes associated with

Figure 3.4 Basic Proteins in pseudorabies preparations

(i) 15% polyacrylamide gel of the acid-extractable material from partially purified PrV.

(ii) Co-electrophoresis of a ^{14}C-labelled chromatin extract labelled 3 - 5 hours post-infection with the ^{3}H-labelled extract of purified virus (Fig 3.4 (i)).
Note that peak I has diminished greatly in size relative to II and III, probably due to breakdown on storage of the material.


(a)
2
1
dpm
$^{3}H \times 10^{-3}$
I
II
III
(b)
2
1
dpm
$^{3}H \times 10^{-3}$
(——)
10
5
dpm
$^{14}C \times 10^{-2}$
(– – –)
20
40
Slice no.

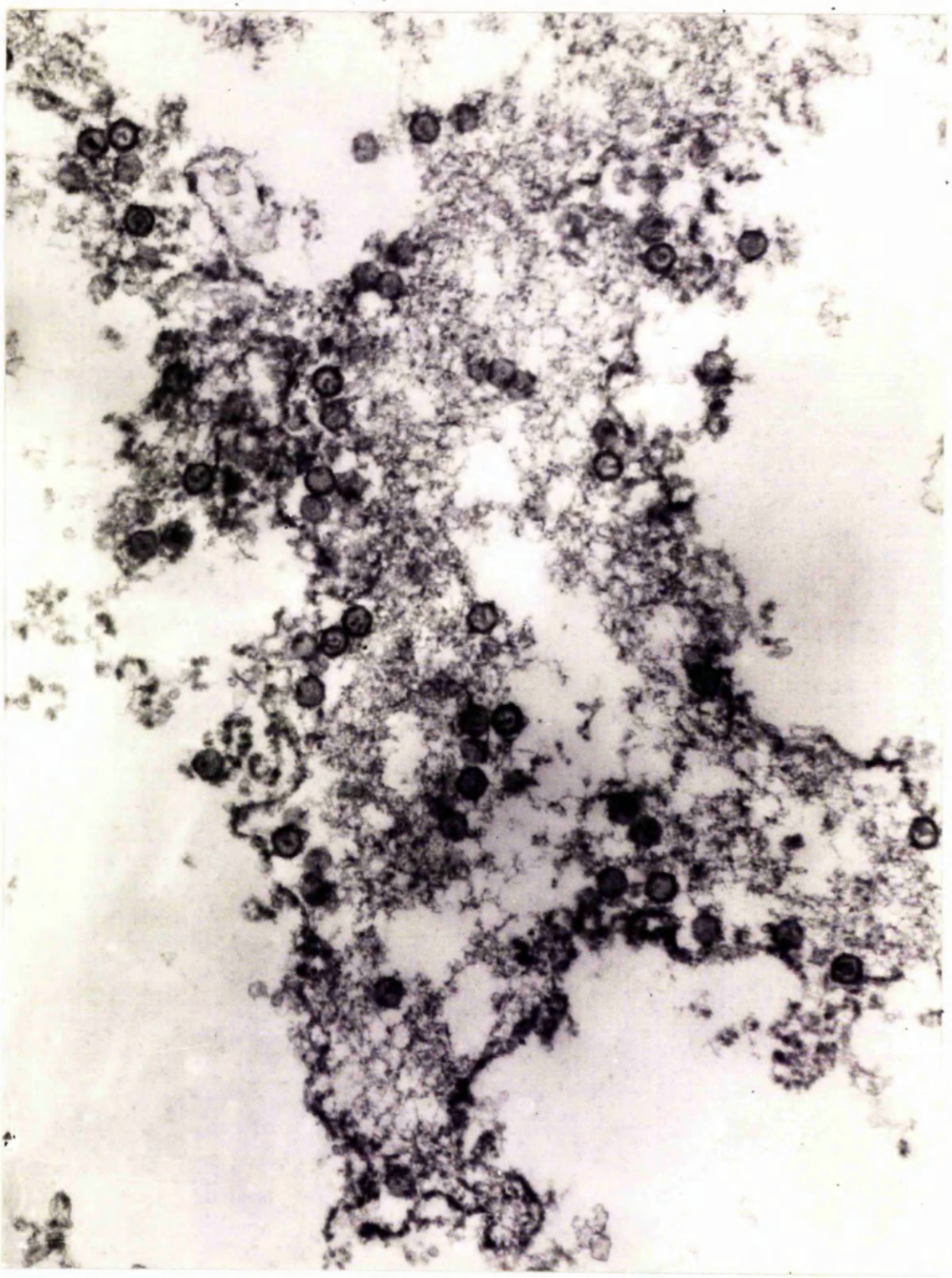


Figure 3.5 : Electronmicrograph of a preparation of washed chromatin from pseudorabies infected cells.

Magnification : 50,000 fold.

the deoxynucleoprotein complex due to its basic nature, is unclear. However the proposal for structural viral proteins having inhibitory functions on host metabolism is not unique - Levine & Ginsberg (1968) have claimed to show inhibition of both host DNA and RNA synthesis *in vitro* by the adenovirus fiber and hexon proteins.

In conclusion, it is at present impossible to state unequivocally whether the IP's are viral structural proteins. If so, it seems clear that they are minor components found in the deoxynucleoprotein core. They are certainly present in relatively high amounts in chromatin preparations, and as such are in a suitable position to be involved in one or both of the reactions of virus assembly and repression of host function.

SECTION 4 CELLULAR DISTRIBUTION OF IP'S

4.1. INTRODUCTION

An examination was made of various cell fractions to determine the cellular distribution of the induced proteins. Samples were analysed on both SDS and pH 4 gel systems in order to characterise the total spectrum of proteins produced in infected cells, as well as more specifically, the basic components. This is important when comparing these results with those described by other research groups.

4.2. CYTOPLASMIC PROTEINS

The profile of all cytoplasmic proteins on 7.5% polyacrylamide SDS gels is shown in Figure 4.2 (i). A large number of species can be identified in control samples (a), the synthesis of some of which seems to be repressed more substantially than others after infection (b). In addition two species appear to be induced in infected samples CP 1 and 2, one and probably both of which do not co-electrophorese with cellular material. Other viral proteins whose presence is masked by host proteins are very likely present, and two peaks CP 3 and 4 have been indicated which certainly represent protein made in considerable quantity post-infection, whether host or virus-specified.

The small basic proteins present in the cytosol were also examined by acidifying the samples, respinning, and then running on 15% pH 4 gels. The result of this is shown in Fig 4.2 (ii). As would be

Figure 4.2 (i) CYTOPLASMIC PROTEINS

7.5% SDS gels of the total cytoplasmic proteins in control (a), and infected (b) cells.

CP 1, 2, 3 and 4 have been indicated in infected samples as probably representing virus-induced material.

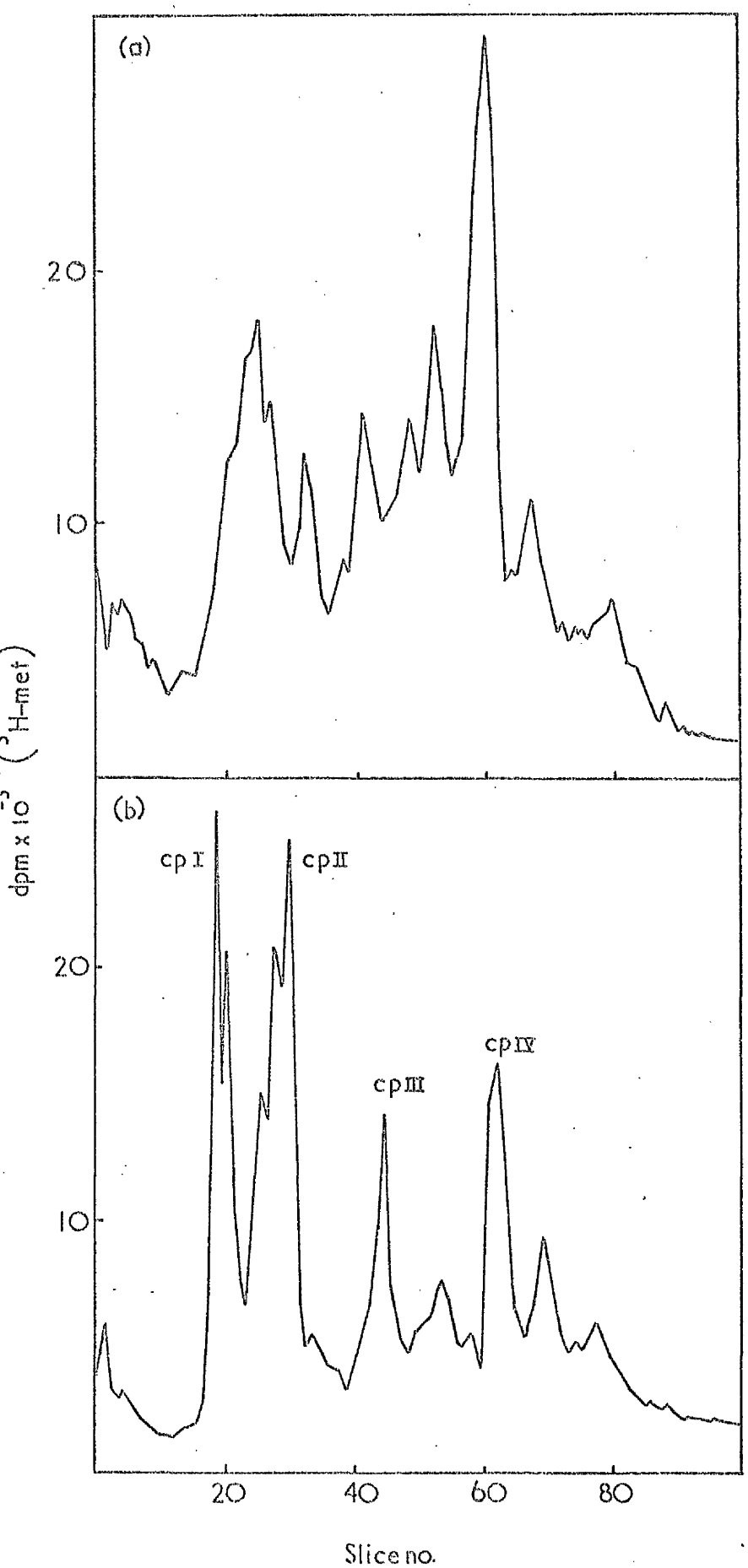

(a)
(b)
cp I
cp II
cp III
cp IV
20
10
20
10
20
40
60
80
dpm x 10^-3 (3H-met)
Slice no.

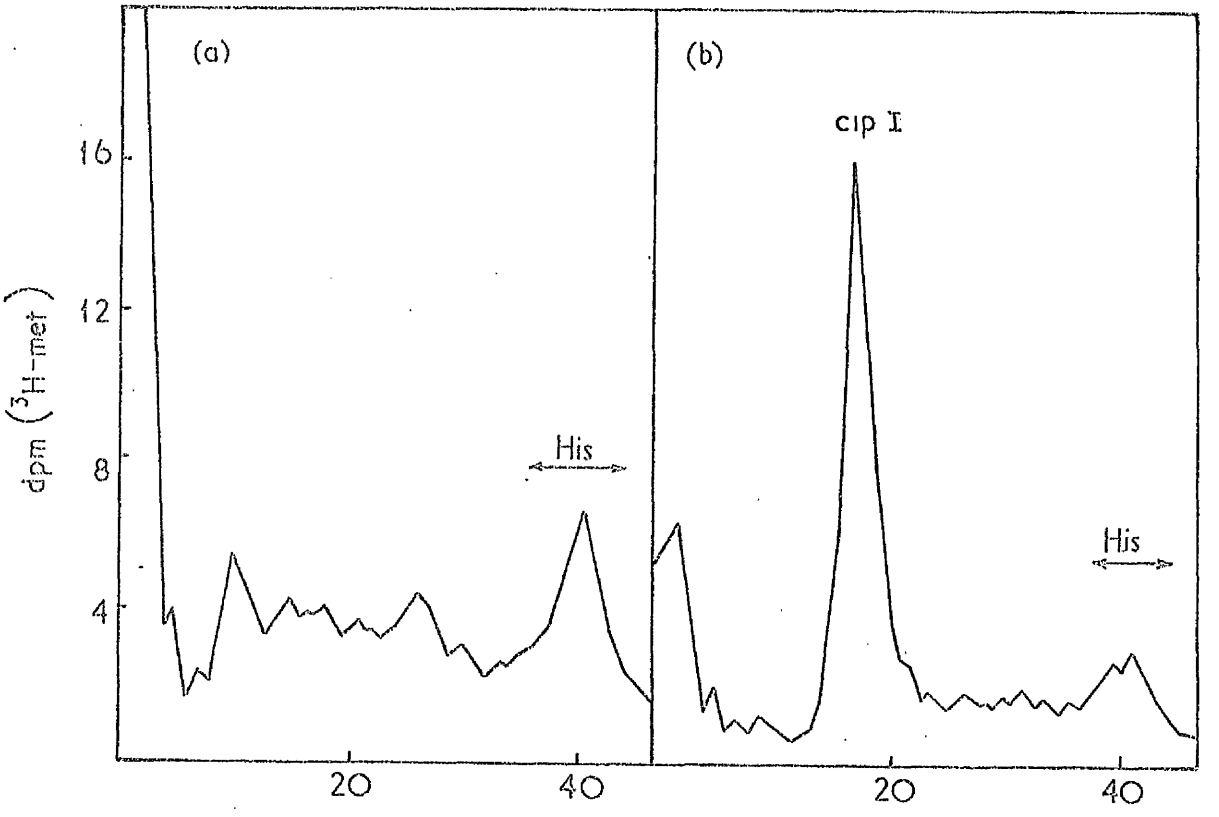
Figure 4.2 (ii) BASIC CYTOPLASMIC PROTEINS

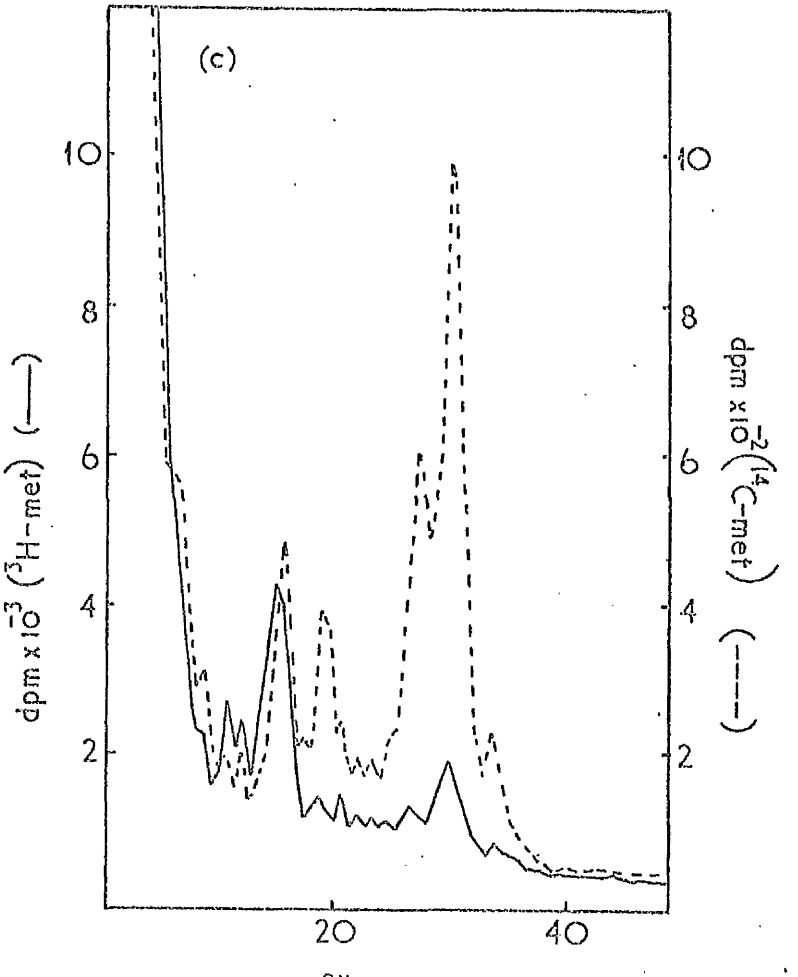
1.5% pH 4 gels of basic cytoplasmic proteins prepared by acidifying the cytosols from control and infected cells and spinning at 105,000 g for 1 hour to pellet membranous and acid-insoluble material. The supernatants were then analysed for protein content.

(a) control (b) infected

One major induced species is found in infected extracts and has been designated CIP I.

(c) is a co-electrophoresis of cytosol (^{3}H-labelled) with a (^{14}C-methyl)methionine labelled acid extract of infected chromatin.


(a)
(b)
cip I
His
His
dpm (^{3}H-met)
16
12
8
4
20
40
20
40


(c)
dpm x10^{-3} (^{3}H-met) (——)
dpm x10^{-2} (^{14}C-met) (– – –)
10
8
6
4
2
20
40
Slice no.

expected, a substantial amount of material does not enter the gel, and the counts in the first two slices are high. The background level ir control gels is also high but few defined species can be identified apart from material in the histone region. This probably represents histone species on route to the nucleus but has not been definitely identified as such. In this system, only one major virus-induced peak can be recognised which has been labelled CIP I. This migrates to approximately the same region as IP I and II and a co-electrophoresis of ^{3}H-labelled cytoplasm with a ^{14}C-acid extract of chromatin was run in order to compare these species (Figure 4.2 (ii) c).

It can be seen that CIP I and IP I run very close together, their maxima being separated by only one slice. There is therefore the possibility that they are the same species which becomes modified, for example by phosphorylation, on entry to the nucleus.

4.3. BASIC PROTEINS IN ISOLATED NUCLEI

Stevens et al., (1969) identified 5 basic proteins in acid extracts of whole nuclei of pseudorabies-infected cells, only two of which seemed to be associated with host chromatin. Similar extracts were made from nuclei treated with Tween 80 to remove cytoplasmic contamination. These were treated with SDS overnight and run on 7.5% polyacrylamide gels. The radioactive profiles obtained (Fig 4.3) are very similar to those found by Stevens, with 5 main regions of induced material, several of which most likely consist of more than one species. The control nuclei were found to contain no major peaks but material

could be identified in the histone region in addition to a large number of poorly defined species of higher molecular weight. In comparison with these (c) and (d) are similar gels of acid extracts of washed chromatin :- it can be seen that substantial purification has been obtained in this preparative procedure. The control gels contain histone species alone and only two induced peaks can be identified in infected samples. It thus appears that 2 of the IP's co-electrophorese under these conditions.

The whole nuclear acid extracts were also run on 15% pH 4 gels as shown in Fig 4.3 (ii). Four main species can be identified, one of which appears to co-migrate with a similar peak in uninfected cells. This extra protein IP IV, which is larger, or less basic than the IP's is not found in nucleoplasmic fractions, nor in the chromatin washes and may therefore be associated with the inner nuclear membrane. Virus-induced alterations of the nuclear membrane are known to occur (Ben-Porat & Kaplan, 1970; Spear, Keller & Roizman, 1970). The fifth peak identified in the SDS system is not distinguishable on these gels; it may represent a protein which can not enter the 15% gels, or it may co-electrophorese with IP IV above, which has appeared as a double peak on some occasions.

4.4. NUCLEOPLASMIC PROTEINS

The patterns of 7.5% SDS gels, and 15% pH 4 gels is shown in Fig 4.4. As would be expected, there are a large number of species present in control cells, the synthesis of most of which is greatly

Figure 4.3 (i) BASIC PROTEINS IN ISOLATED NUCLEI

Nuclei were isolated from control and infected burlers and washed in Tween 80 to remove cytoplasmic contamination. Acid extracts were made and analysed on 7.5% SDS gels.

(a) control (whole nuclei) (b) infected (whole nuclei)

In comparison, the profiles obtained for chromatin acid extracts on this gel system are shown below

(c) control (chromatin) (d) infected (chromatin).

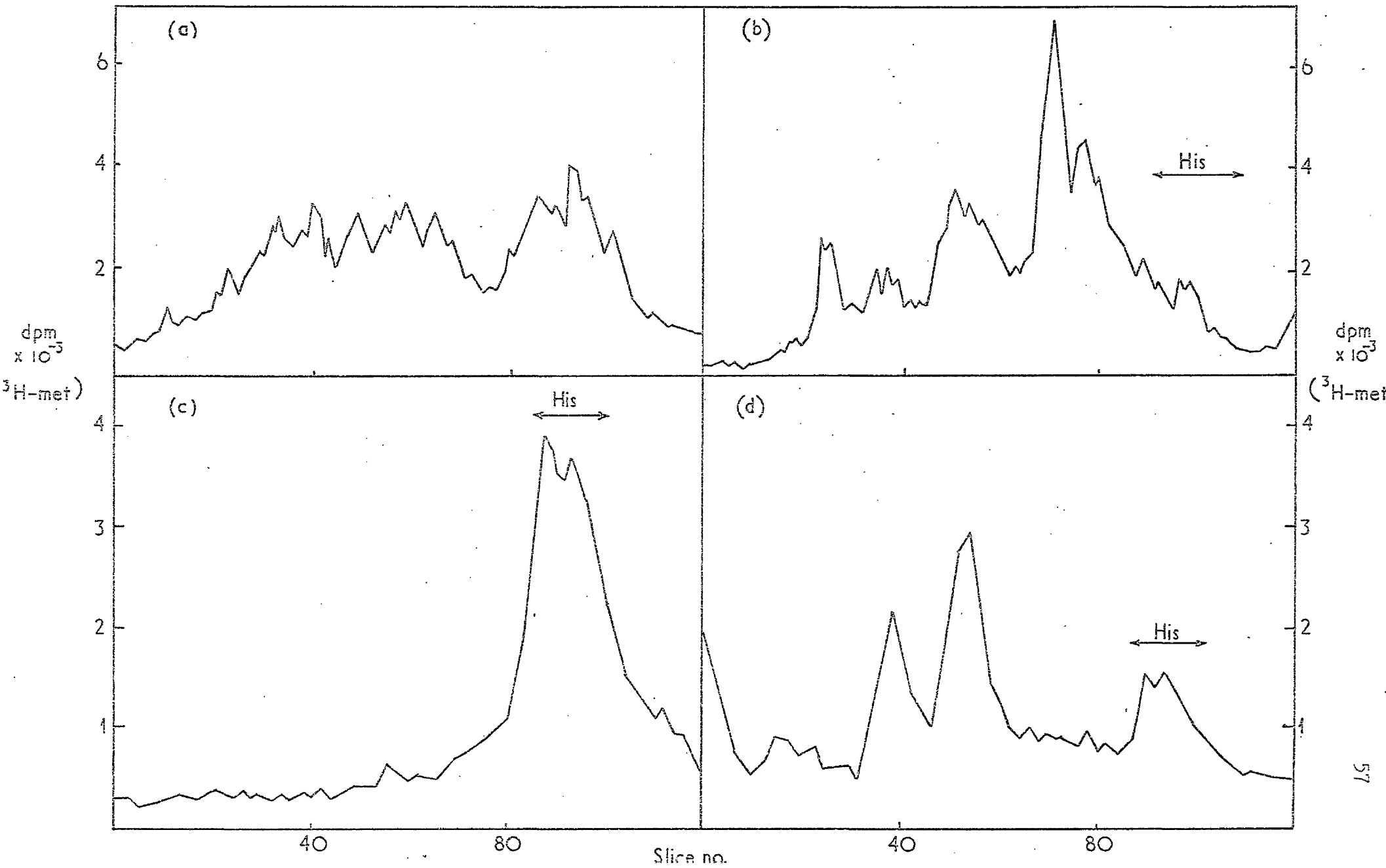
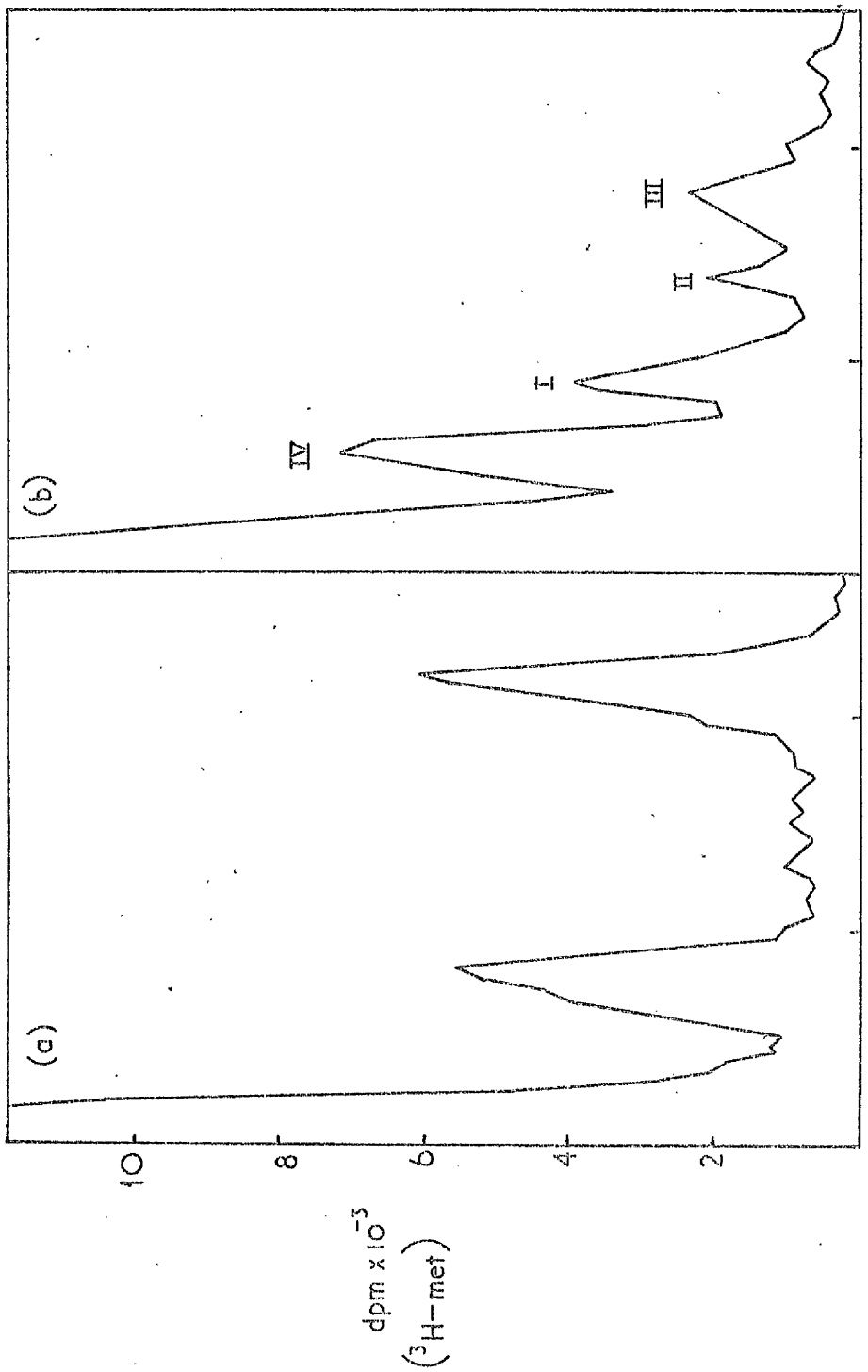

(a)
(b)
(c)
(d)
His
His
His
6
4
2
4
3
2
1
40
80
40
80
dpm x 10⁻³ (³H-met)
dpm x 10⁻³ (³H-met)
Slice no.

Figure 4.3 (ii) BASIC PROTEINS IN ISOLATED NUCLEI

15% pH 4 gels of the acid extractable material from whole, Tween 80-treated nuclei. One extra peak is found in both control and infected samples but these run in slightly different regions. The additional peak in infected extracts has been labelled IP IV.

(a) control (b) infected


(a)
(b)
I
II
III
IV
2
4
6
8
10
20
40
20
40
dpm x 10^-3
(3H-met)
Slice no.

depressed after infection. A small number of proteins are found in infected nucleoplasms which may represent continued synthesis of certain cellular molecules or alternatively, may be viral structural proteins (c.f. Fig 3.3). However none of these run on pH 4 gels, (c) and (d) which does suggest that any basic molecule present in nuclei will become associated with chromatin whether specifically or non-specifically.

4.5. NON-HISTONE CHROMOSOMAL PROTEINS

After extraction of the basic chromosomal proteins, the solution is clarified by spinning at 1500 g. The pellet obtained contains DNA and a number of non-histone chromosomal proteins. In normal, uninfected cells these proteins exhibit more tissue-specificity than their basic counterparts (Elgin & Bonner, 1970; Loeb & Creuzet, 1970), turn over rapidly (Sadgopal & Kabat, 1969; Hnilica *et al.*, 1965) and are localised in chromatin in areas active in RNA synthesis (Marushige & Dixon, 1969; Grunicke, Potter & Morris, 1970; Littau *et al.*, 1964). For these reasons they have been proposed as specific activators of chromatin in a system where the histones would act as non-specific repressors (Paul & Gilmour, 1968; Spelsberg, Wilhelm & Hnilica, 1972).

It was therefore decided to be of interest to identify any viral-induced proteins present in this fraction. However, the number of residual proteins in uninfected cells (Benjamin & Gelhorn, 1968; Huang & Bonner, 1962) and the difficulties which have been encountered in separating them (Spelsberg & Hnilica, 1969; Langan, 1967; MacGillivray *et al.*, 1971; etc) made it unlikely that it would be found

Figure 4.4 NUCLEOPLASMIC PROTEINS

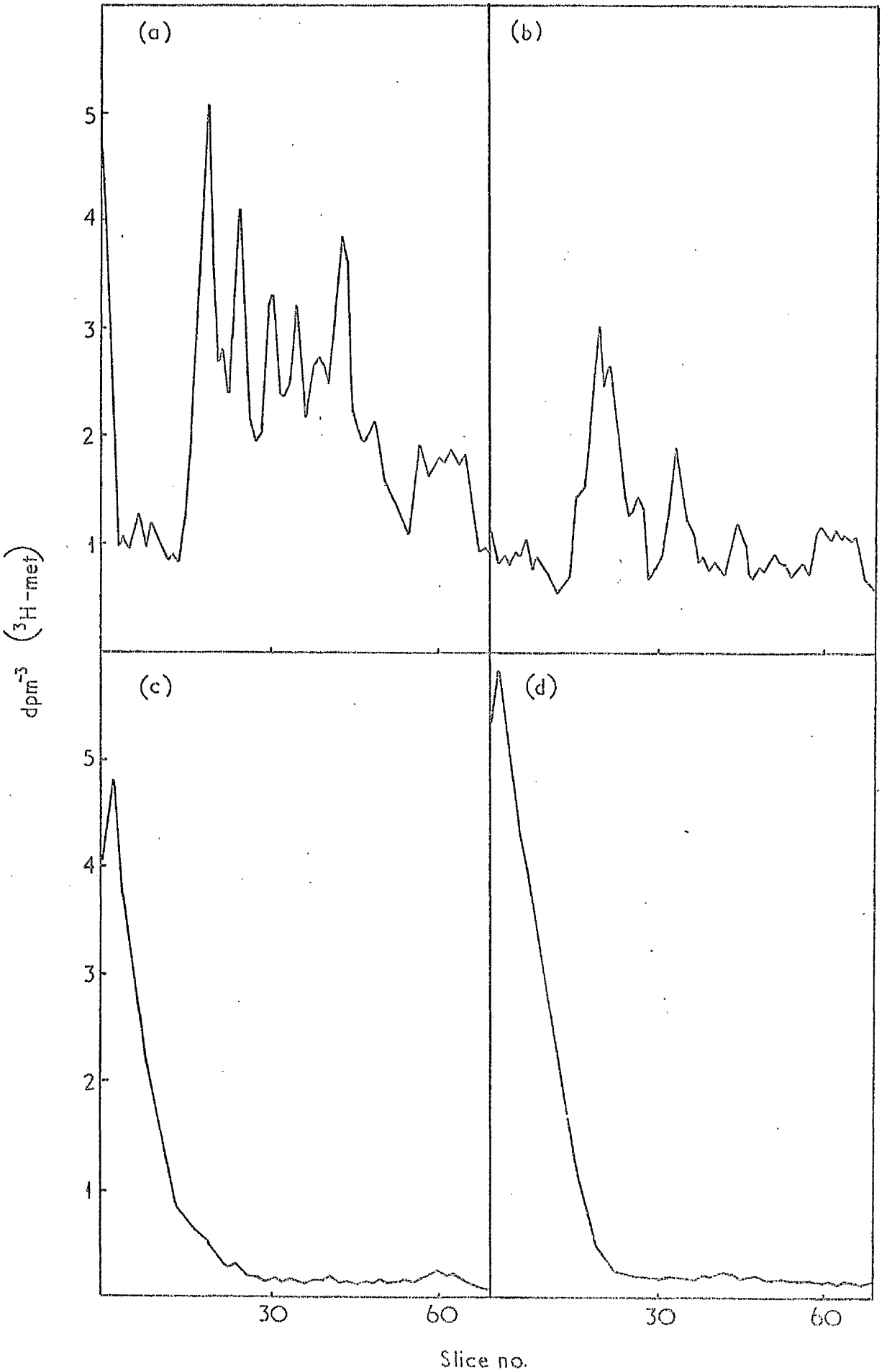
Nucleoplasmic fractions were prepared by breaking open "clean" nuclei and spinning to remove chromatin material. The supernatants were analysed on 7.5% SDS gels:

(a) control (b) infected

and also on the 15% pH 4 gel system

(c) control (b) infected

Several proteins can be identified in the infected nucleoplasms but none of these run on the low pH gels. There are therefore no small basic proteins in this fraction.


(a)
(b)
(c)
(d)
5
4
3
2
1
30
60
dpm^{-3} (^{3}H-met)
Slice no.

possible to do any more than initially characterise the species present.

The chromatin pellets were therefore first extracted with urea, respun and finally taken up in SDS. Both fractions were solubilised with SDS overnight and run on 7.5% SDS gels (Fig 4.5).

Several viral proteins are present in the urea-soluble fraction including one of similar molecular weight to the main capsid protein. Again in the final SDS samples very high quantities of a 'capsid'-sized protein are found. On comparison of the two gel profiles it is apparent that very similar protein species are present in each and differences appear to be mainly in the quantities extracted in each fraction. The reason why some molecules are more tightly bound in the chromatin network is at present unknown.

4.6. 0.35M NaCl EXTRACT

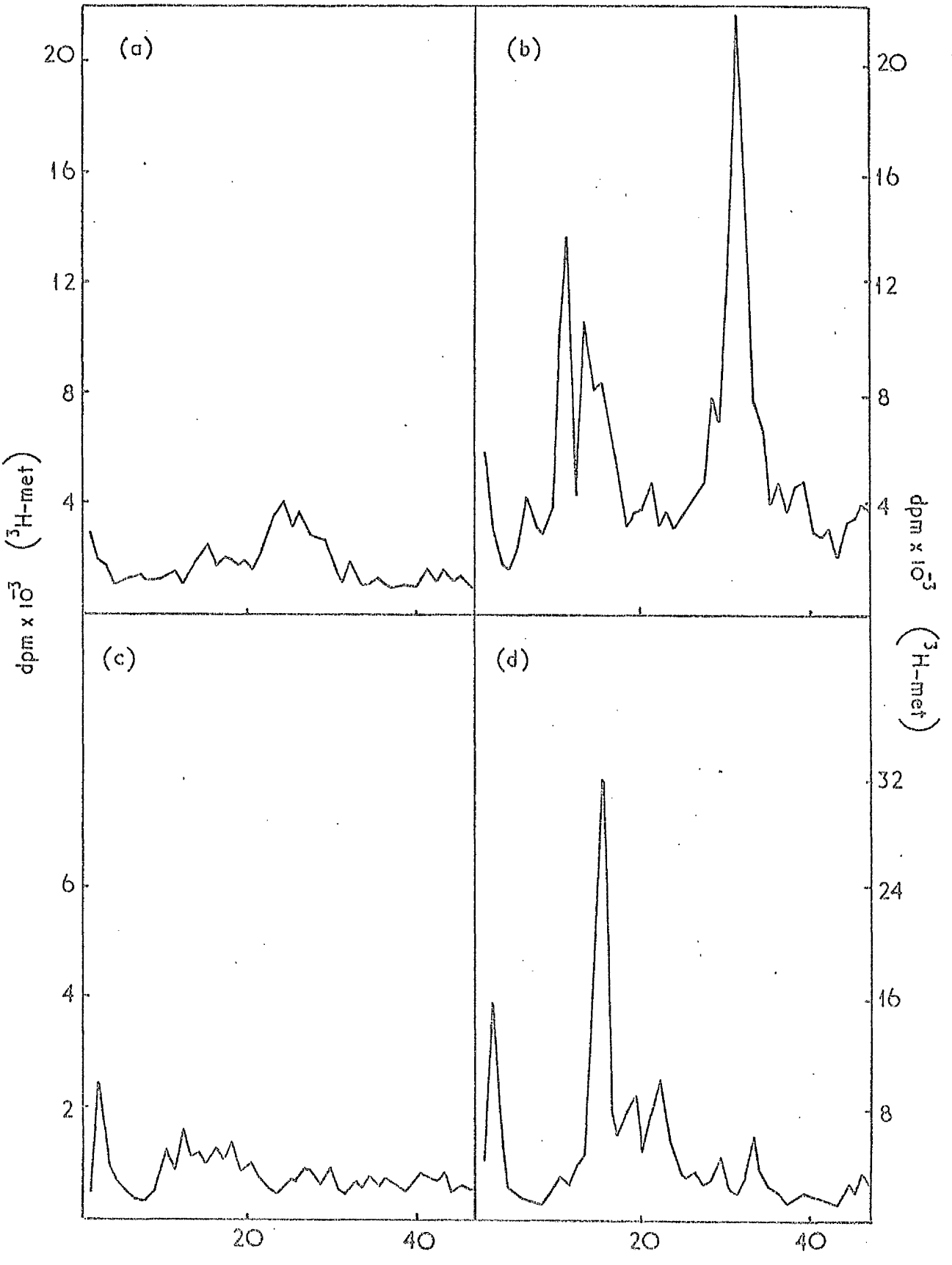
The absence of any basic proteins in the nucleoplasmic fraction suggests that any such material will become adsorbed specifically or non-specifically to the deoxynucleoprotein complex during extraction procedures, if not *in vivo*. Johns has claimed that proteins which attach non-specifically to chromatin can be removed by treatment with 0.35 M NaCl without the true chromosomal proteins being affected (Johns & Forrester, 1969). An experiment was therefore performed to see if the 3 induced proteins would be removed under these conditions. This might be expected if they are structural viral proteins made in excess, with no specific function and therefore binding site in chromatin.

Figure 4.5 NON-HISTONE CHROMOSOMAL PROTEINS

The chromatin pellet, after acid extraction, was first treated with 8M urea, spun down again and finally taken up in 2% SDS. The protein constituents of the urea and SDS fractions were examined on 7.5% SDS gels.

(a) Urea control (b) Urea infected

(c) SDS control (d) SDS infected


(a)
(b)
(c)
(d)
20
16
12
8
4
6
2
32
24
20
40
dpm x 10⁻³ (³H-met)

Chromatin was prepared as usual and washed twice in tris-HCl, but not with 1 mM HCl. It was then extracted for 5 hours with buffered 0.35M NaCl. The solution was spun at 1500 g to bring down the chromatin pellet which was extracted with 250 mM HCl. Fig 4.6 (i) shows the electrophoretic profile of material extracted with 0.35M NaCl (a) and (b) and also the basic components remaining in the chromatin pellet (c) and (d). It appears that only a small amount, if any, of the induced proteins are removed by this washing procedure. However it is perhaps of interest that on 7.5% SDS gels, the 0.35M NaCl extracts can be shown to contain a proportion of material which migrates to the same position as the main capsid protein of pseudorabies virions. This has been reported to be fairly rich in arginine (Spring, Roizman & Spear, 1969) and the excess, not involved in virus assembly, may adsorb non-specifically to the deoxynucleoprotein complex.

4.7. SONICATED CHROMATIN

Several methods have been described for the separation of chromatin into fractions which differ in their DNA and protein content. These have included mild DN'ase treatment followed by salt precipitation (Marushige & Bonner, 1971), sonication and differential centrifugation (Frenster, Allfrey & Mirsky, 1963) and sonication with separation by column chromatography (Reeck, Simpson & Sober, 1972). The result is the production of two or more fractions which vary in their melting temperatures (Reeck *et al.*, 1972), appearance under the electron-microscope (Frenster *et al.*, 1963), protein constituents and ability to be transcribed into RNA (McConaughty & McCarthy, 1972).

Figure 4.6(i) 0.35M NaCl EXTRACT

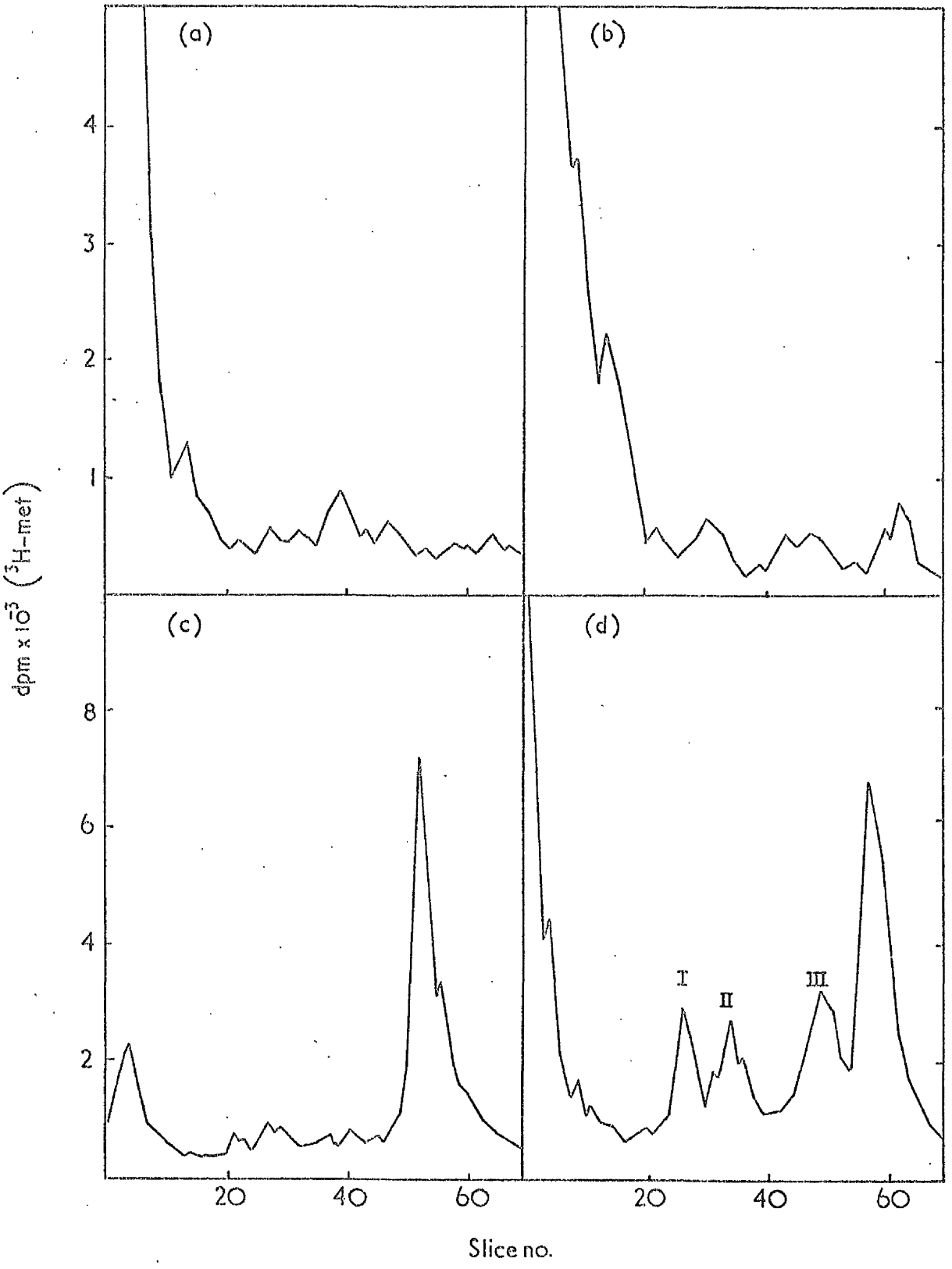
Chromatin prepared in the usual manner was washed twice in Tris-HCl (pH 7.8) then once with homogenisation, in 0.35M NaCl. Proteins loosely adsorbed to the deoxynucleoprotein complex were allowed to extract for 3 hours at $0^{o}C$.

The chromatin was then pelleted and extracted with 250 mM HCl for 3 hours.

All extracts were examined on pH 4 gels.

(a) 0.35M NaCl (control) (b) 0.35M NaCl (infected)

(c) 250 mM HCl (control) (d) 250 mM HCl (infected)


(a)
(b)
(c)
(d)
I
II
III
dpm x 10^{-3} (^{3}H-met)
Slice no.

The methods have thus been proposed to provide a separation of regions of repressed heterochromatin from the diffuse euchromatin which is active in transcription. This could be important in determining the factors necessary for repression or activation of the genome *in vivo*, and of interest in this respect is the finding that the diffuse region appears to be relatively rich in several non-histone proteins and correspondingly deficient in histones (Littau *et al.*, 1964; Bonner *et al.*, 1968).

The distribution of induced proteins between condensed and diffuse chromatin was therefore examined using the fractionation procedure of Frenster *et al.*, (1963) involving mild sonication and differential centrifugation. Experimental details are given in Materials and Methods and the fractions obtained from both control and infected nuclei were extracted with acid and run on 15% pH 4 gels.

The result is shown in Figure 4.7; no differences in distribution of the induced proteins between dense and diffuse chromatin were found. However it is interesting that IP II and IP III are very much reduced relative to IP I and seem to be preferentially lost under this treatment. This will be discussed later.

4.8. DISCUSSION

A large number of virus-specified proteins can thus be identified in all cellular fractions. Few of these are basic, and thus acid-extraction immediately greatly reduces the number of proteins under examination which is very useful when purification of particular species is to be attempted.

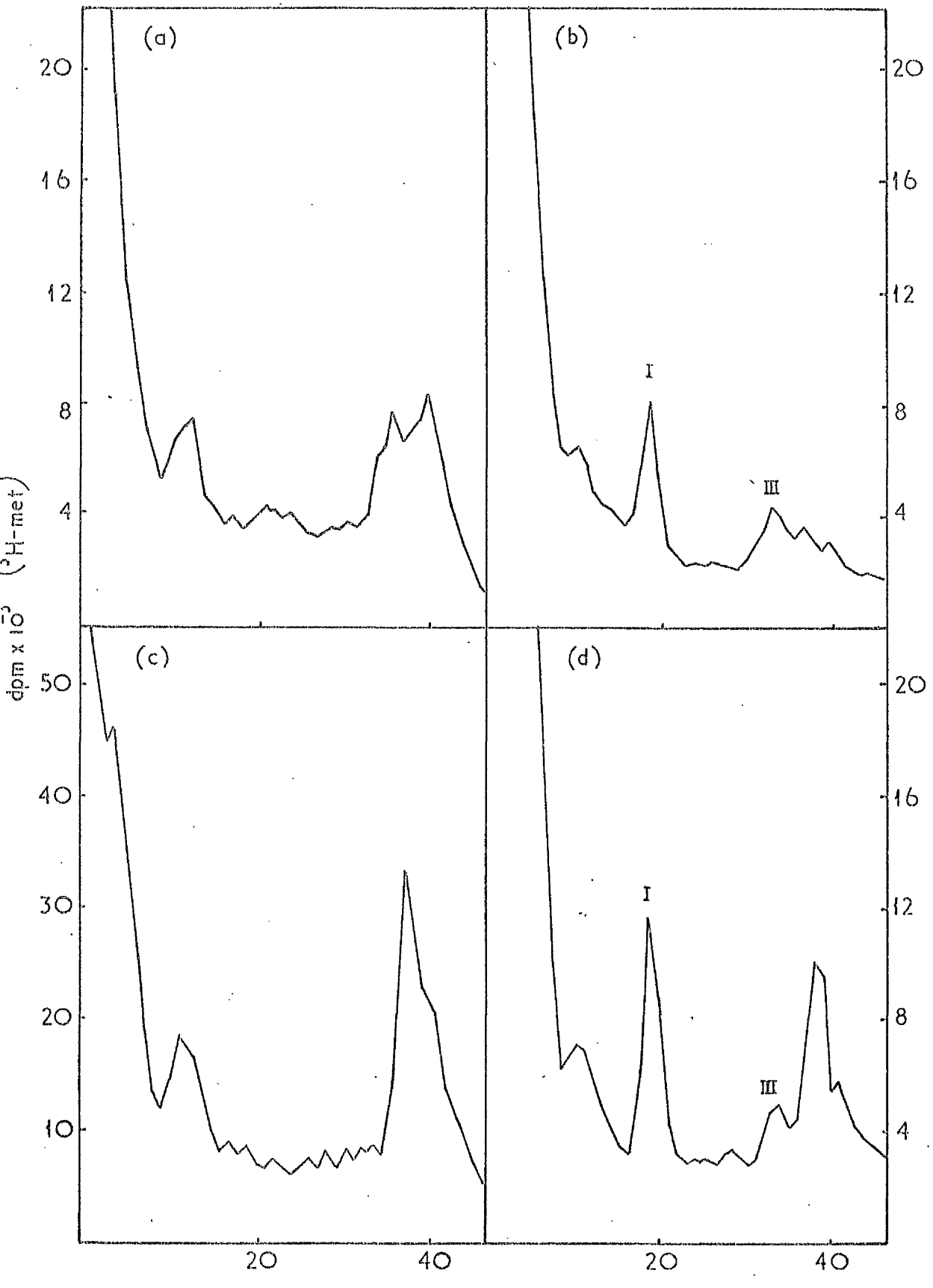
Figure 4.7 SONICATED CHROMATIN

Diffuse and dense portions of chromatin were prepared from cells labelled with (^{3}H-methyl)methionine by sonication followed by differential centrifugation. These were then extracted with 250 mM HCl and analysed for basic proteins on pH 4 gels.

(a) control (dense) (b) infected (dense)

(c) control (diffuse) (d) infected (diffuse)

IP's I and III are marked. IP II appears to have been lost during this treatment.


(a)
(b)
(c)
(d)
I
III
I
III
dpm x 10⁻³ (³H-met)
Slice no.
20
16
12
8
4
50
40
30
20
10
20
40

Two of the proteins IP II and III can only be identified in the chromatin fraction. Their absence from the cytoplasm under conditions where histones and IP I (as CIP I) can be recognised is interesting; as they do not migrate as far as histones in pH 4 gels, they are probably larger, or less basic than these host molecules. Their passage to the nucleus would thus be expected to be slower, not more rapid than that of the histones (Spear & Roizman, 1968) and this may therefore suggest that they are made intranuclearly. There is some evidence for protein synthesis in isolated nuclei (Gallwitz and Mueller, 1969; Anderson, Slavik & Ellebute, 1972) as well as within the chromosome structure itself (Cave, 1968).

It should be remembered that Kaplan has proposed the existence of an arginine-rich protein made intranuclearly for the role of assembly of structural viral proteins into mature virions in pseudorabies-infected cells (Mark & Kaplan, 1971). Either IP II or III could fulfill a function of this sort.

No differences in distribution of the IP's between dense and diffuse chromatin were found. This was disappointing in view of the differences in template activity of the two fractions; it was hoped to correlate the presence of the possible transcription inhibitor(s) with the active portions of chromatin. It would seem wasteful for the virus to 'switch off' areas of the genome already being repressed by the cell. Although rather difficult to interpret, the results may indicate a fairly general distribution of binding-sites on the genome.

The presence in chromatin of a molecule which migrates to the same region as the main capsid protein of pseudorabies virions, lends

support to the proposal for a virus-synthesising complex existing within the chromosomal network. This protein is partially removed by 0.35M NaCl or 250 mM HCl but is also found in substantial amounts in the chromatin pellet after acid extraction. It is known to be made in excess, and the molecules not being used in virus assembly may be loosely adsorbed to the deoxynucleoprotein complex and readily removed by homogenisation in mild salt or acid.

The fact that the 3 induced proteins are not removed by 0.35M NaCl does suggest that they are bound within the chromatin network by a fairly strong electrostatic attraction. Also of interest is the almost complete loss of IP II and III from sonicated chromatin. This may indicate a difference in function of these two species from the other induced protein. For example, IP II and III could be involved in virus assembly as the 'condensing' proteins proposed by Mark & Kaplan (1971), which might be removed along with the rest of a virus-synthesising complex, on disruption of the chromatin network by sonication, leaving IP I to fulfill some function within the genome itself.

SECTION 5 EFFECTS OF ARGININE DEPRIVATION

5.1. INTRODUCTION

Arginine is an essential amino-acid in tissue-culture and hence the effects of arginine depletion can be studied very easily by removing it from the growth medium. This amino-acid has been shown to be particularly important in determining the infectivity of a number of animal viruses : herpes simplex (Tankersley, 1964), adenovirus (Rouse et al., 1963), SV40 (Goldblum et al., 1968) and polyoma (Winters and Consigli, 1969). These are all DNA-containing viruses which replicate in the nucleus and are capable of forming latent infections. In comparison, the poxviruses (DNA-cytoplasmic) and poliovirus (RNA cytoplasmic) show no such dramatic inhibition of virus maturation under conditions of arginine deprivation, although Ackermann et al., (1966 a & b) have shown that poliovirus loses its ability to switch off HeLa cell DNA synthesis under these conditions.

In the case of adenovirus, Bonifas (1967) claimed that arginine was required for synthesis at the structural viral proteins, whereas Rouse & Schlesinger (1967) found that synthesis of various early antigens and capsid antigens still occurred and it was the assembly of structural components into mature virions which was inhibited. More specifically Russell & Becker (1968) claimed that the step blocked was the synthesis of a component of the P antigen which is known to be arginine-rich, and to be found within the virion (Russell & Knight, 1967). They suggested it might react with the viral DNA causing it to fold in a specific manner, thus allowing encapsidation to occur.

Goldblum, Ravid and Becker (1968) found that in the case of SV40 replicating in BSC, cells, arginine was required for some lat· step in virus development, and at least partly for the synthesis of structural viral proteins. Several early functions were unaffected, in particular the synthesis of the 'T'-antigen, an early protein whose function in viral oncogenesis is not known (Pope & Rowe, 1964; Rapp et al., 1964). They suggested that control of arginine concentration in the infected cell may constitute the mechanism whereby late functions are regulated, and may also be a deciding factor between oncogenic and productive infections; with regard to this, Rogers & Moore (1963) found an elevated concentration of arginine in Shope-papilloma induced tumours in rabbit.

A considerable amount of work has also been done on the effect of deprivation of various amino-acids on the herpes virus group. Tankersley (1964) made a detailed study of herpes simplex in human cells and found that the basic amino-acids, histidine and arginine were much more dramatic in their inhibitory effects than any other amino-acids. Inglis (1968) studying herpes simplex in RK 13 cells showed that adsorption, penetration and eclipse of the virus occurred as normal. However no synthesis of virus-induced proteins was detected and neither intranuclear nor cytoplasmic granules were formed. Becker et al., (1967) found similar results in a line of MK cells, and in addition reported that the synthesis of viral DNA and inhibition of cellular nucleic acid synthesis were unaffected. The viral DNA formed was found to remain in the nucleus in a DN'ase sensitive form, and could be converted to mature virus on removal of the arginine block. However the effect of

arginine deprivation on viral DNA synthesis was found by Gönczöl to vary between cell types.

In contrast to these results, Courtney et al., (1970) reported the presence of cytoplasmic (CF and IF) antigens, but no nuclear ones in HSV infected BHK/21 cells. Finally Spring, Roizman and Spear (1969) complete the picture by claiming that, not only are viral proteins synthesised in HSV infected Hep 2 cells, but also that transport of these to the nucleus does occur and it is only assembly which is inhibited. These differences are probably due to variation between cell lines; thus in certain primary cultures, arginine deprivation has been shown to have no effect at all on virus multiplication (Jeney et al., 1967), a finding which may reflect the size of the arginine pool (Piez & Eagle, 1958; Gönczöl et al., 1967) or the presence of mycoplasma which degrade arginine in the continuous cultures (Rouse et al., 1963).

With regard to pseudorabies virus, Mark & Kaplan have studied the effects of arginine-depletion on viral protein synthesis and virus-induced alterations in the nuclear membrane. In agreement with the work on HSV by Courtney et al., (1970), and Olshevsky & Becker (1970) they found that the major viral proteins were still being made at a reduced rate and accumulated in the cytoplasm. The normal mechanism for migration of these proteins to the nucleus is, as yet, unknown, but is not dependent on the synthesis of viral DNA. They proposed the existence of an arginine-rich condensing protein to effectively reduce the concentration of unassembled viral proteins in the nucleus, and allow further migration to occur against the concentration gradient.

They also suggested that this protein may be made intranuclearly to prevent aggregation of the structural proteins in the cytoplasm.

Recent experiments by Winters & Russell (1971) have suggested that nuclear membranes may be important in the assembly of adenovirus in vitro. For this reason, Mark & Kaplan (1972) examined the effect of arginine deprivation on the characteristic alterations of the nuclear membrane in pseudorabies-infected cells in the hopes of correlating this with the lack of structural protein migration to the nucleus. They found that although the non-structural glycoproteins were still being made in arginine-deprived cells, they were not incorporated into the membrane. However the relationship between these results and the lack of virus assembly is not clear.

In conclusion, although considerable variation in the effect of arginine-deprivation has been reported for several animal viruses, it seems clear that absence of this amino-acid does have a more marked effect on the maturation of DNA-containing viruses which replicate within the nucleus, than a deficiency in any other amino-acid. The variation in the results found probably reflects differences in host cell type as well as in the experimental procedure used; that is, whereas some groups deprived their cells of arginine for 24 hours or more prior to infection, others used only a 2 hour starvation period. This would markedly affect the size of the arginine pool. The interpretation that Spring et al., (1969) put on their results seems fair; that under conditions of arginine-starvation, the probability of a given protein being completed is inversely proportional to its size and arginine-content. It appears that arginine-deprivation may be a useful

procedure for selectively inhibiting a small number of arginine-rich proteins whose function in the infected cell can thus be examined. The present results suggest that one or several proteins which are not made in infected cells in the absence of arginine, are involved in the transport of structural viral proteins to the nucleus, and in assembly of infective virions.

5.2. CHROMOSOMAL BASIC PROTEINS IN ARG$^-$ CELLS

Initial studies indicated that the effect of deprivation of arginine for 2 hours and 24 hours was essentially the same, except that the total counts incorporated were further reduced in the longer starvation period. For this reason, subsequent work was done with 2-hour deprivation periods as the cells were healthier under these conditions. In order to remove all traces of arginine from the growth medium, this amino-acid was left out of the Eagles Stock (ES x 10), and in addition, extensively dialysed calf serum was used in preparing EC10.

After the 2 hour starvation period, control and infected burlers were grown, both in the presence and absence of arginine. The cells were labelled with (^{3}H-methyl)methionine in EC10 ($\frac{+}{-}$Arg)($\frac{1}{5}$ Met), 1 - 4 hours and 4 - 8 hours post-infection or mock-infection. At 4 hours, the infected burlers Arg$^+$ showed extensive CPE although the corresponding Arg$^-$ cells were unchanged. This was even more dramatically demonstrated at 8 hours showing that virus maturation in the arginine deprived cells was not just slowed down, but appeared to be completely arrested up to 8 hours at least.

Figure 5.2 CHROMOSOMAL BASIC PROTEINS IN ARG$^-$ CELLS

All cells were deprived of arginine for 2 hours, then, at the time of infection, arginine was replaced in the media of half the burlers. Thus control and infected burlers were grown in the presence (a), (b), (c) and (d) or absence (e), (f), (g), (h) of arginine, and were labelled with ^{3}H-methyl methionine in EC10 ($\frac{1}{5}$ Met) (+/- Arg) from 1 - 4 and 4 - 8 hours post-infection.

Acid extracts of chromatin were analysed on 15% pH 4 gels as before.

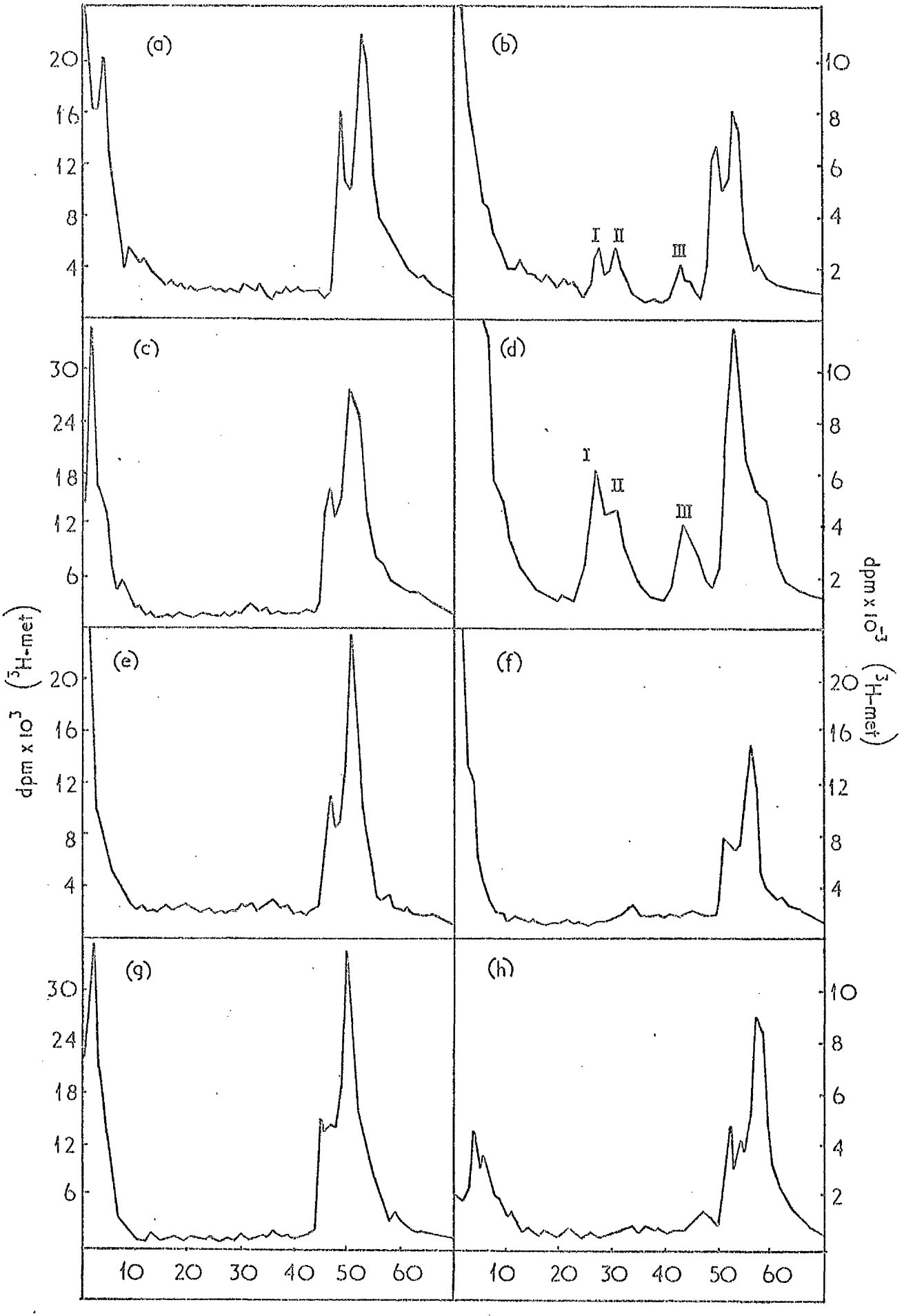
(a) 1 - 4 Arg$^+$ control (b) 1 - 4 Arg$^+$ infected

(c) 4 - 8 Arg$^+$ control (d) 4 - 8 Arg$^+$ infected

(e) 1 - 4 Arg$^-$ control (f) 1 - 4 Arg$^-$ infected

(g) 4 - 8 Arg$^-$ control (h) 4 - 8 Arg$^-$ infected

No sign of the IP's was found in Arg$^-$ cells although histones were still being made even in infected cultures.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
I
II
III
dpm x 10³ (³H-met)
dpm x 10⁻³ (³H-met)
10 20 30 40 50 60
Slice no.

Acid extracts of chromatin were prepared as usual and run on 15% pH 4 gels (Fig 5.2). With respect to the burlers supplemented with arginine after the 2 hour starvation period, it can be seen that incorporation of arginine seems to be reduced between 1 and 4 hours relative to the 4 - 8 hour label. This probably is due to replenishing of the arginine pools during this time. However IP's I, II and III can be readily identified in both the 1 - 4 and the 4 - 8 hour infected samples.

In comparison the Arg^- controls are very similar to the corresponding Arg^+ samples showing that, although histone synthesis is reduced under conditions of arginine starvation, it is by no means completely inhibited. However there is very little sign of any of the 3 induced proteins in the infected samples deprived of arginine either between 1 - 4 or 4 - 8 hours after infection. This again indicates that the lack of arginine does more than retard the effects of virus-infection.

5.3. BASIC CYTOPLASMIC PROTEINS IN ARG^- CELLS

Cytoplasmic fractions were also examined to see if accumulation of viral proteins had occurred, as reported by Mark and Kaplan (1971). Figure 5.3 shows the profile of basic proteins on 15% pH 4 gels. It can be seen that there is very little difference between Arg^+ and Arg^- samples, although total protein synthesis seems to be reduced to about half in the arginine-deprived cells. Thus CIP I is still being made under conditions whereby there is no sign of IP I in chromatin fractions. If these two proteins are the same species (see

Figure 5.3 BASIC CYTOSOLIC PROTEINS IN ARG$^-$ CELLS

The cytoplasmic fractions from control and arginine-deprived, infected and uninfected cells were acidified, spun at 105,000 g, then run on 15% pH 4 gels.

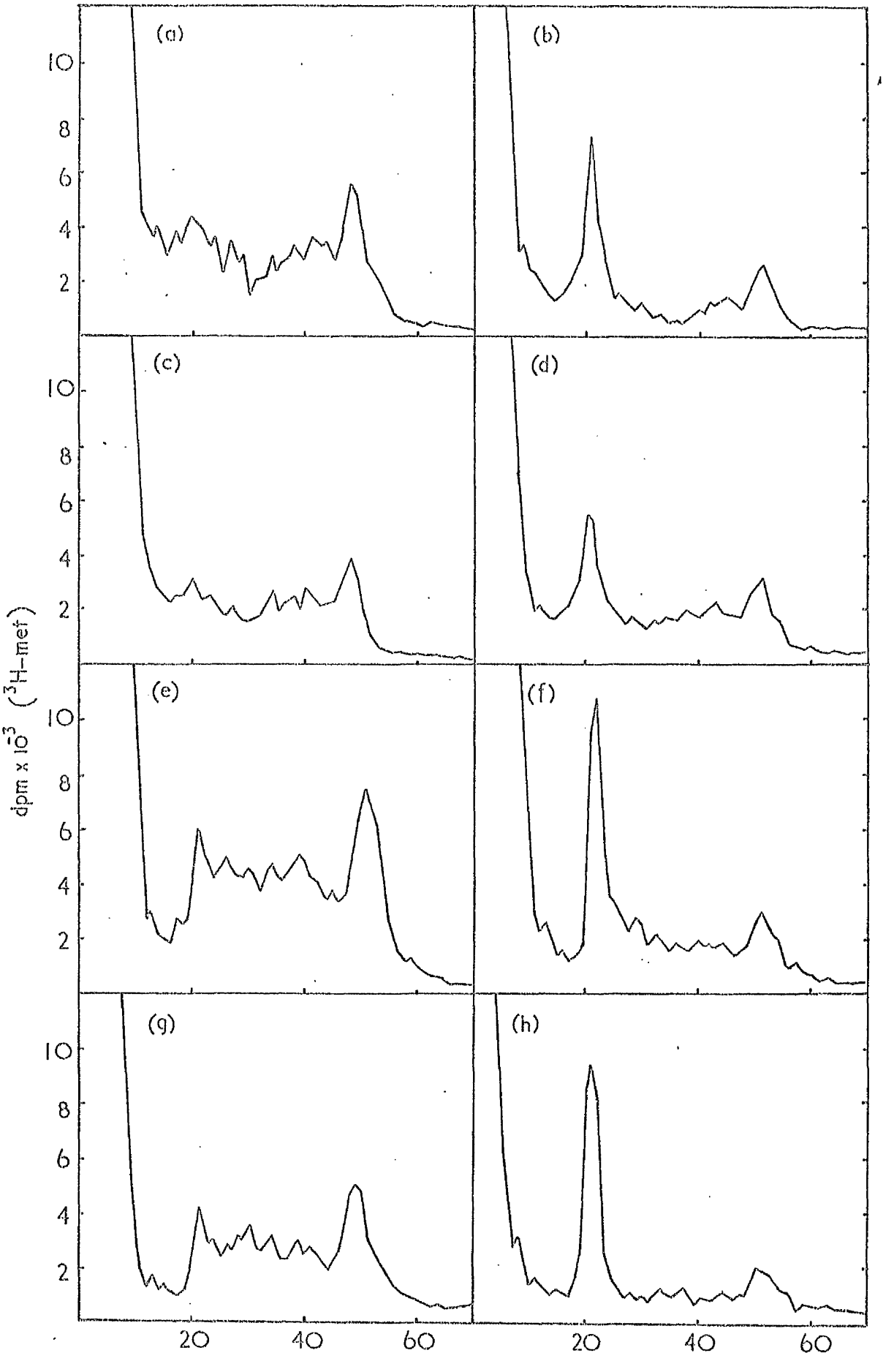
The presence of reduced amounts of CIP I in arginine deficient cells is shown in (d) and (h).

(a) 1 - 4 Arg$^+$ CON (b) 1 - 4 Arg$^+$ VI

(c) 1 - 4 Arg$^-$ CON (d) 1 - 4 Arg$^-$ VI

(e) 4 - 8 Arg$^+$ CON (f) 4 - 8 Arg$^+$ VI

(g) 4 - 8 Arg$^-$ CON (h) 4 - 8 Arg$^-$ VI


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
10
8
6
4
2
20
40
60
dpm x 10^{-3} (^{3}H-mef)
Slice no.

Section 4), this may suggest that transport of viral proteins to the nucleus is being inhibited.

No sign of either IP II or III was found in any of the cellular fractions. As histones are still being made, this suggests that the IP's should be synthesised, at least in small amounts if also made in the cytoplasm. The fact that they are not found, may again suggest that IP II and IP III are made intranuclearly where the effects of arginine starvation on pool size may be more drastic.

5.4. ACIDIC NUCLEAR PROTEINS IN ARG$^-$ CELLS

The synthesis of basic nuclear proteins would be expected to be more substantially inhibited than that of their acidic counterparts under conditions of arginine deprivation. However Mark & Kaplan (1971) have claimed that although many viral proteins are made in the absence of arginine in the growth medium, their transport to the nucleus is completely inhibited. This is disputed by Spring et al., (1969). An examination was therefore made of the urea and SDS soluble fractions of the chromatin pellet after acid extraction, to see if any viral proteins could be identified in the absence of IP's I, II and III.

(i) Urea-extracted (Figure 5.4 (i))

Both the 1 - 4 and 4 - 8 hour control samples show that the synthesis of host proteins in this fraction is not drastically inhibited by arginine deprivation. In infected cells, four main peaks of activity can normally be identified, two of which (indicated by broken arrows) may represent continued synthesis of host proteins. These species can

Figure 5.4 (i) ACIDIC NUCLEAR PROTEINS IN ARG$^-$ CELLS

The chromatin pellets after acid extraction from cells grown in the presence or absence of arginine (Exp 5.2) were treated with 8M urea, and the proteins extracted examined on 7.5% SDS gels.

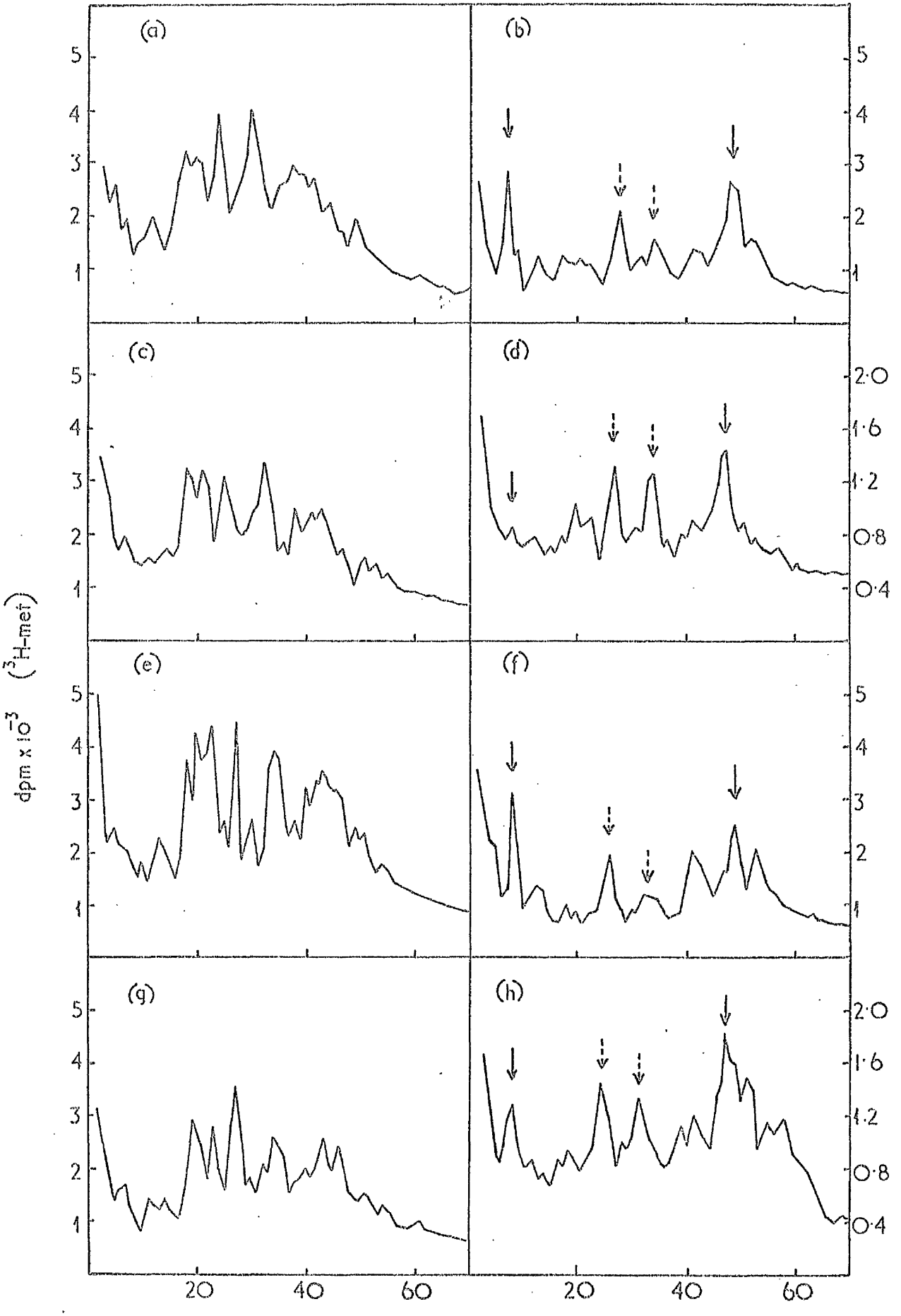
Four main peaks are present, two of which (indicated by dotted arrow) may represent continued synthesis of host proteins.

(a) 1 - 4 Arg$^+$ control (b) 1 - 4 Arg$^+$ infected

(c) 1 - 4 Arg$^-$ control (d) 1 - 4 Arg$^-$ infected

(e) 4 - 8 Arg$^+$ control (f) 4 - 8 Arg$^+$ infected

(g) 4 - 8 Arg$^-$ control (h) 4 - 8 Arg$^-$ infected


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
dpm x 10⁻³ (³H-mei)
20
40
60
20
40
60

all be recognised in Arg^- cells and their synthesis, as with host proteins, seems to be reduced only to about half. Thus no evidence for inhibition of transport of viral proteins to the nucleus in conditions of arginine deficiency was found.

(ii) SDS fraction (Figure 5.4 (ii))

Two main peaks are normally present in this fraction 1^1 and 7^1 together with a number of minor components. These can be identified in (b) and (f). In the 1 - 4 hour infected Arg^- sample (d), it is apparent that the synthesis of both of these is substantially inhibited but to different extents. Thus while there is almost 3 times the quantity of 1^1 as 7^1 in infected Arg^+ cells, in Arg^- cells the amounts are roughly equal. Again in the 4 - 8 hour labelled Arg^- samples the two peaks are reduced approximately to the same size, although for this time interval, in the presence of arginine, protein 1^1 is produced in 6 times the amount of protein 7^1. This substantially greater reduction in protein 1^1 relative to 7^1 at least partly reflects its large size and therefore diminished chances of being completed in conditions of arginine deficiency (Spring et al., 1969). However as Mark and Kaplan (1971) found that the cytoplasmic synthesis of the main capsid protein, which is of similar size, was reduced to only 50% in arginine deficiency, this may also indicate a selective inhibition of transport to the nucleus.

5.5. DISCUSSION

The metabolism of BHK/21 cells does not seem to be drastically altered by depriving the cells of arginine for up to 24

Figure 5.4 (ii) SDS FRACTION OF ARG$^-$ CELLS

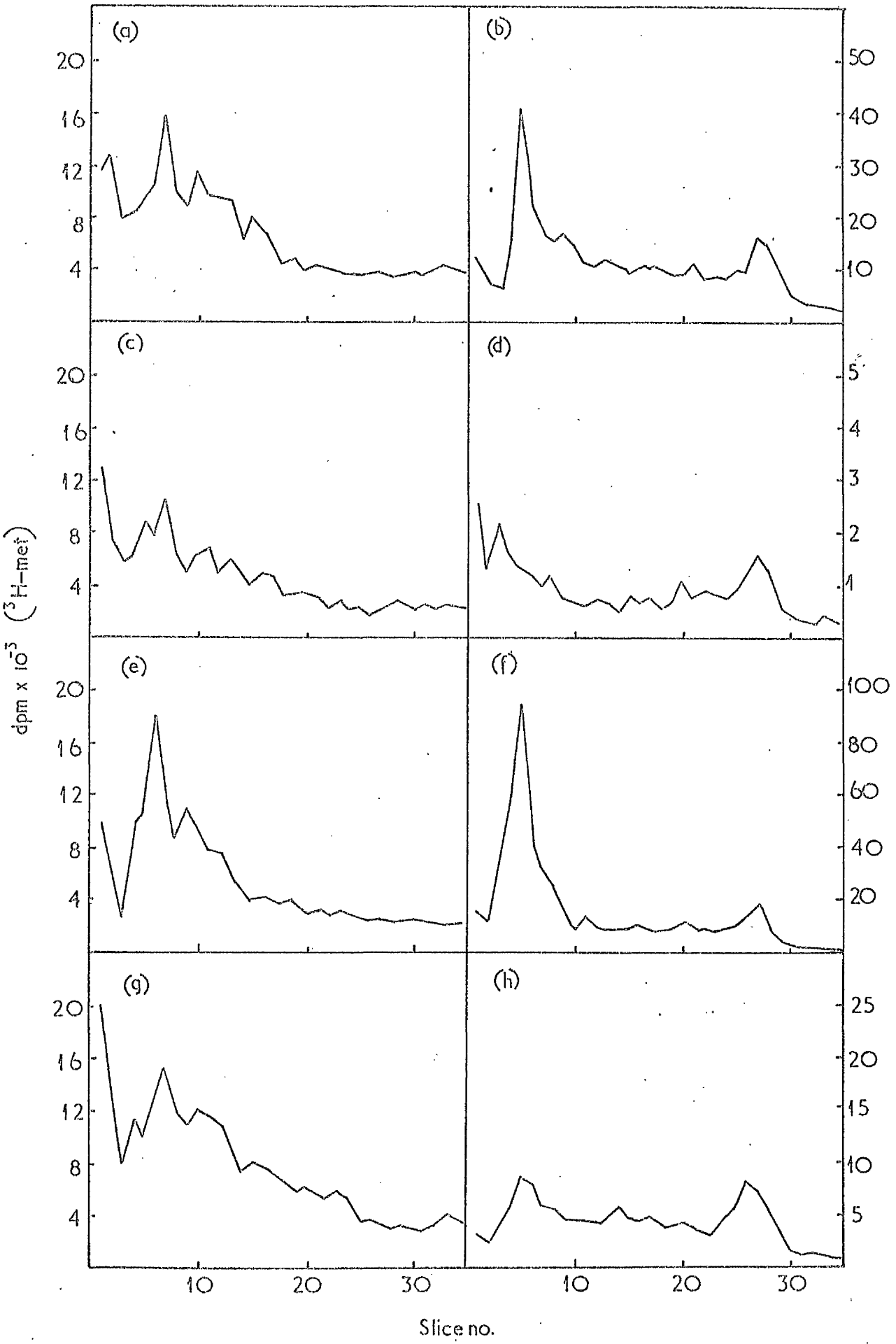
After extraction of the urea-soluble proteins, the DNA was spun down then taken up in 2% SDS and incubated overnight. The remaining proteins are thus solubilised and examined on 7.5% SDS gels. The main capsid protein SDS 1 is the predominant species in this fraction and can be seen to be present in very low amounts in arginine-deprived cells.

(a) 1 - 4 Arg$^+$ control (b) 1 - 4 Arg$^+$ infected

(c) 1 - 4 Arg$^-$ control (d) 1 - 4 Arg$^-$ infected

(e) 4 - 8 Arg$^+$ control (f) 4 - 8 Arg$^-$ infected

(g) 4 - 8 Arg$^-$ control (h) 4 - 8 Arg$^-$ infected


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
dpm x 10^{-3} (^{3}H-met)
Slice no.

hours, although the rate of protein synthesis is restricted to about half that in control cells. However the growth of pseudorabies in cells deficient in arginine is markedly affected. No cytopathic changes could be seen even at 8 hours post-infection indicating that the infective process had not merely been slowed down but was completely arrested under these conditions.

Examination of the various cellular fractions for the presence of virus-induced proteins showed that infection had occurred and various viral functions were being carried out. Thus CIP I was found in reduced amounts in the cytosol, and small quantities of all the nuclear acidic proteins could be identified in chromatin extracts. The notable exceptions were the 3 basic IP's which were absent from chromatin and could not be identified in any other cellular fraction, with the possible exception of IP I, as CIP I in the cytoplasm. This may further suggest that selective inhibition of transport of viral proteins to the nucleus does occur but perhaps more likely indicates that CIP I and IP I are separate species. Certainly, there was no evidence for accumulation of the other viral proteins in the cytoplasm, and when synthesised, these seemed to find their way to the nucleus as usual.

Unfortunately, an examination of the synthesis of viral and host DNA under conditions of arginine deficiency was not made. It would have been interesting to see whether the 'switch-off' of host DNA synthesis occurred as usual, as found by Becker et al., (1967), in the absence of the 3 induced proteins. This system could also prove useful for examining in vivo, possible functions of the IP's in the synthesis and transcription of viral DNA. Their absence under conditions where

virus assembly is completely inhibited is certainly circumstantial evidence for their being involved in this process.

SECTION 6 COMPARISON WITH HERPES SIMPLEX VIRUS

6.1. INTRODUCTION

The two viruses, herpes simplex and pseudorabies, have a large number of properties in common. These include their effects on host macromolecular synthesis and very largely their reproductive cycles. In many review articles (e.g. Roizman & Spear, 1971), no differentiation is made in discussing features of one or other of the two species, and the evidence for properties of one is assumed to hold for the other. Although largely true, this is undoubtedly dangerous as several differences have been found. For example, HSV in BHK/21 cells stimulates its own enzymes deoxycytidine kinase and thymidine kinase whereas PrV in the same cell system uses, and stimulates production of the host enzyme (J.P. Durham, personal communication).

In addition the 2 viruses induce the synthesis of quite different spectra of proteins on gel electrophoresis, and have different antigenic properties (Kaplan & Vatter, 1959) although there may be one common antigen (Watson et al., 1967). However it is clear that in terms of biochemical activity the similarities between HSV and PrV are very strong.

In view of the close correlation between the viruses in the inhibition of host macromolecular synthesis, which it is hoped to correlate with the presence of induced proteins, an examination was made of chromatin in herpes simplex infected cells in comparison with the results found for pseudorabies.

6.2. ACID-EXTRACTABLE MATERIAL IN CHROMATIN OF HSV-INFECTED CELLS

Experimental conditions were identical to those used for pseudorabies. BHK/21 cells were infected at 20 pfu/cell, virus was adsorbed for 1 hour, then the cells were labelled 4 - 8 hours post-infection with ^{3}H-arginine in EC10 $\frac{1}{5}$ Arg. A control, uninfected burler was treated identically. Chromatin was prepared and extracted for 5 hours with 250 mM HCl/1% βME. The basic proteins were then examined on 15% pH 4 gels as before (Fig 6.2).

Two distinct peaks can be identified, the second of which almost certainly consists of 2 species. Therefore it is probable that 3 HSV-induced proteins very similar in size and basicity to the pseudorabies proteins are present. It is also interesting that HSV-IP I is arginine-rich relative to HSV-IP II a and b whereas IP II and III are the arginine-rich species in PrV-infected cells.

6.3. COMPARISON OF THE HSV AND PrV-INDUCED PROTEINS

Burlers infected with either herpes simplex or pseudorabies virus (20 pfu/cell for each) were labelled 3 - 8 hour post-infection with ^{3}H or ^{14}C-methionine in EC10 $\frac{1}{5}$ Met. Acid extracts of chromatin were prepared and examined on pH 4 gels to ensure that production of the induced proteins had occurred as usual. Co-electrophoreses were then run of ^{3}H-HSV chromatin with ^{14}C-PrV chromatin and vice versa. The result is shown in Figure 6.3 :- only the ^{14}C-single label gel patterns are shown as the ^{3}H samples gave very similar results. It is noteworthy that in

Figure 6.2 Basic Chromosomal Proteins in HSV-infected cells

BHK/21 cells were infected with herpes simplex (MP 17) under the same conditions used for pseudorabies, and were labelled 4 - 8 hours post-infection with ^{3}H-arginine in EC10 $\frac{1}{5}$ Arg. The acid-soluble material of chromatin was prepared and examined on 15% pH 4 gels.

Three main virus-induced peaks have been indicated HSV-I and HSV-II a and b.

(a) control (b) HSV-infected

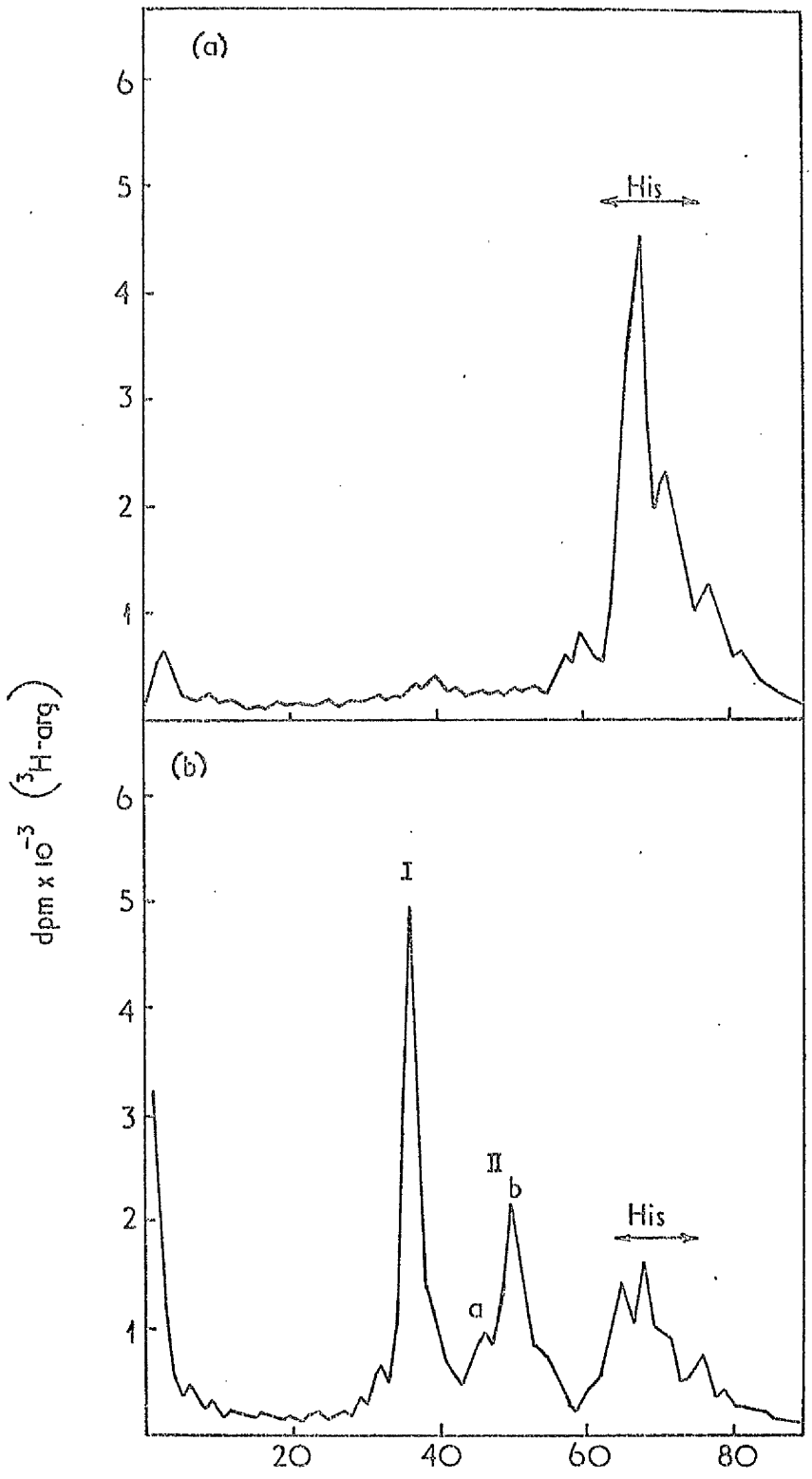

(a)
His
(b)
I
II
a
b
His
dpm x 10⁻³ (³H-arg)
Slice no.

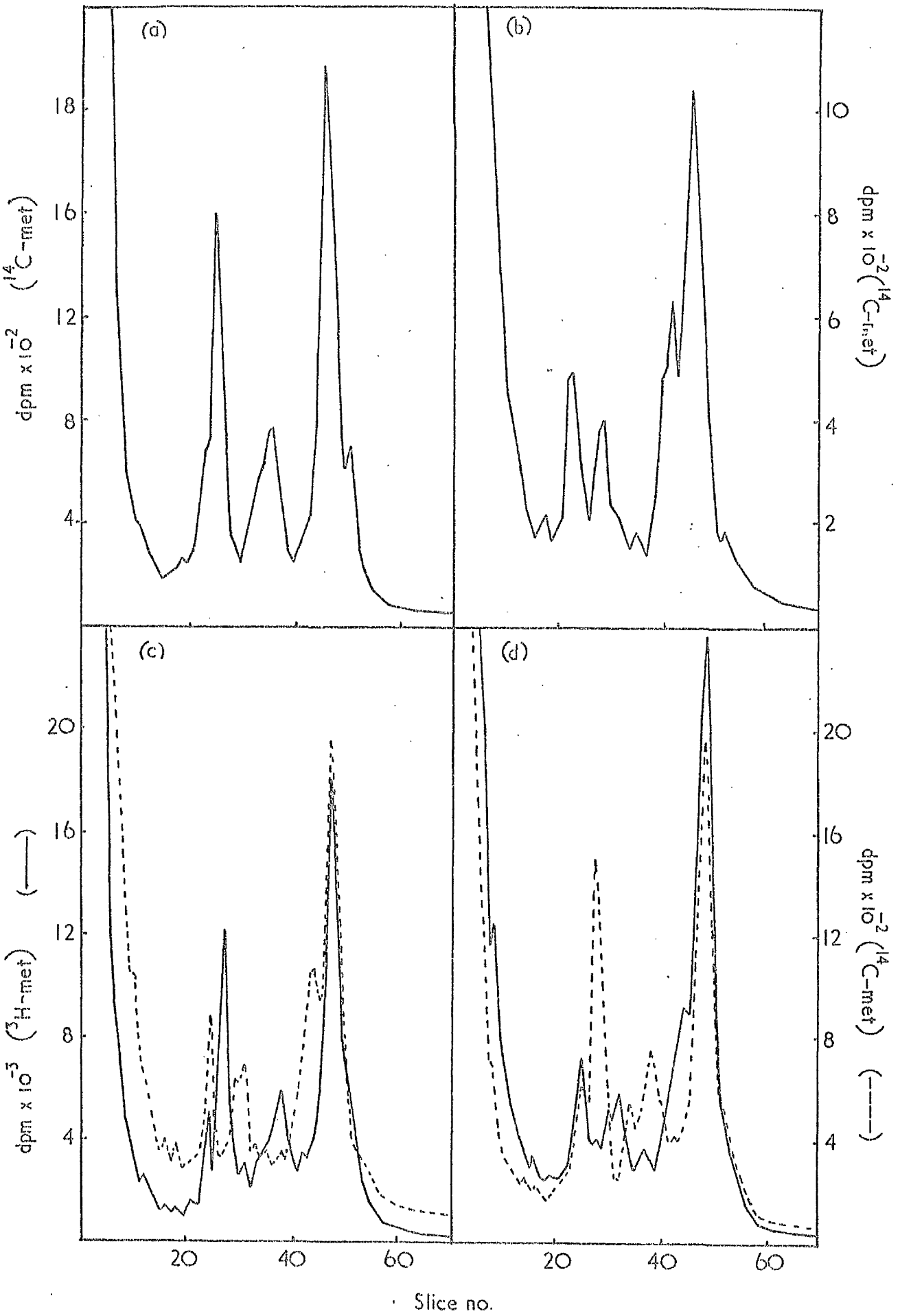
Figure 6.3 Comparison of the HSV and PrV Induced Proteins

^{14}C and ^{3}H-methyl methionine-labelled samples of both HSV and PrV-induced basic proteins were prepared, and electrophoresed separately (a) and (b), and with the oppositely labelled sample of the other virus (c) and (d).

Only IP I and HSV-I(a) appear to co-electrophorese.

(a) ^{14}C-HSV (b) ^{14}C-PrV

(c) ^{3}-HSV and ^{14}C-PrV (d) ^{14}C-HSV and ^{3}H-PrV.


(a)
(b)
(c)
(d)
dpm x 10⁻² (¹⁴C-met)
dpm x 10⁻³ (³H-met) (——)
dpm x 10⁻² (¹⁴C-met) (– – –)
Slice no.

these methionine-labelled HSV samples, there is an additional peak which runs just behind HSV-IP I, either as a shoulder (a), or as a separate peak (b) and (c). This peak, HSV-IP Ia co-electrophoreses with PrV-IP I and like this pseudorabies protein, appears to have a low Arg/Met ratio. None of the other proteins co-electrophorese - thus PrV IP II and III and HSV-IP Ib, IP II a and b seem to be virus specific.

6.4. BASIC CYTOPLASMIC PROTEINS

In pseudorabies-infected cells, a cytoplasmic protein CIP I has been identified which runs very close to IP I on pH 4 gel electrophoresis. The two do not co-electrophorese and a suggestion was made that CIP I may be modified, for example by phosphorylation, on reaching the nucleus (See Section 4.2). The other two induced proteins could not be identified even in small amounts in the cytosol and were tentatively suggested to be made intranuclearly.

A comparison of these results with samples from HSV-infected cells was carried out in a reverse isotope experiment where both ^{3}H and ^{14}C cytosols were co-electrophoresed with the oppositely labelled acid extract of chromatin. This is shown in Fig 6.4. It is interesting that again the major species is a protein which runs very near to HSV-IP I(a) (which in turn co-electrophoreses with IP I). In this case it is difficult to tell whether the two protein peaks are separated by one slice, as in the pseudorabies case, as HSV-IP Ia forms a shoulder on the HSV-IP Ib peak and the maximum is difficult to differentiate. However it appears that the two may co-electrophorese exactly in this case.

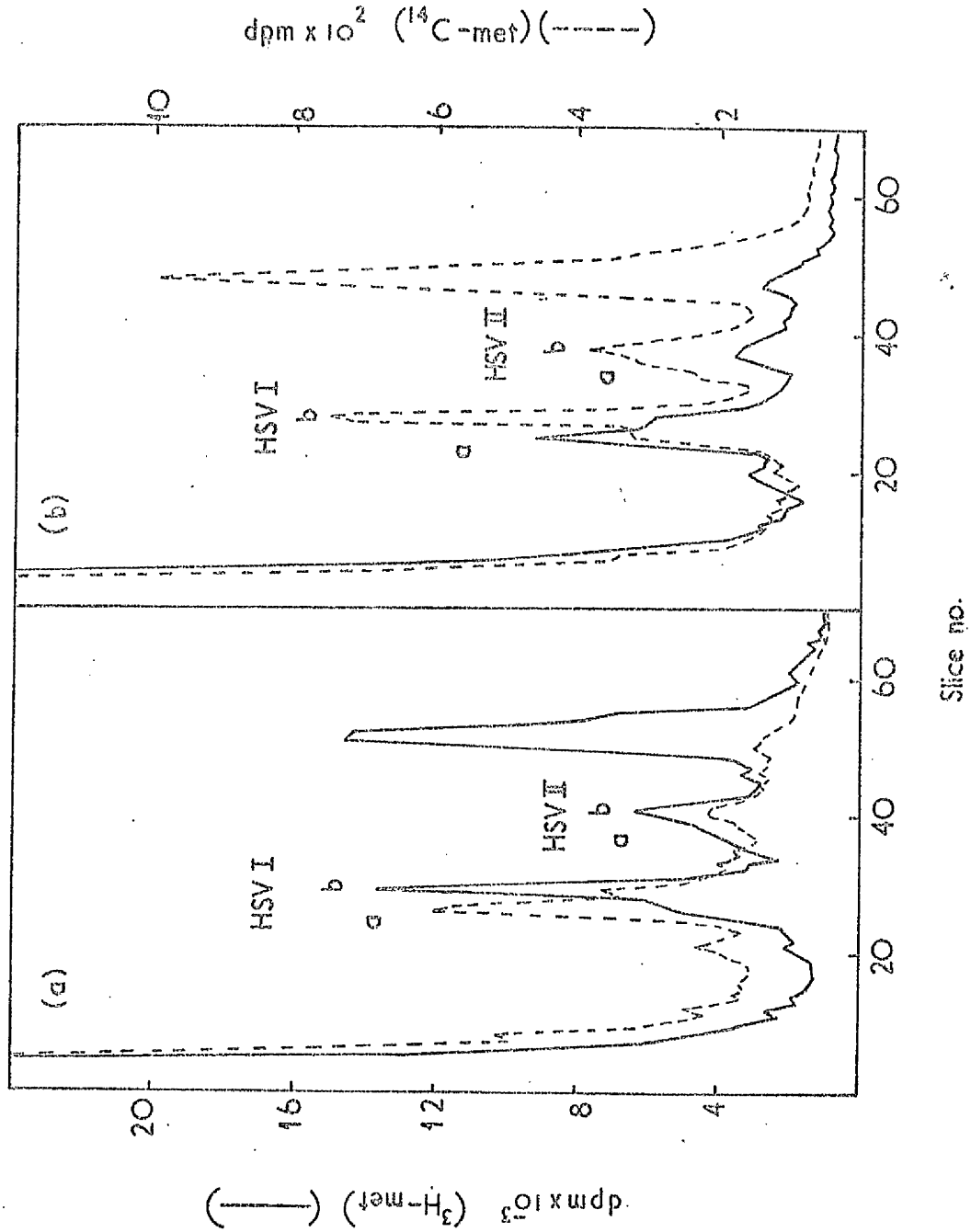
Figure 6.4 Comparison of Basic Proteins of Cytosol and Chromatin in HSV-infected cells

Two burlers were labelled with either (^{3}H or ^{14}C-methyl)-methionine and cytoplasmic and chromatin fractions were prepared. The small basic proteins in each were compared by co-electrophoresing ^{3}H-labelled cytosols with ^{14}C-nuclear fractions and vice-versa.

The major species in the cytosol is a protein which co-electrophoreses with HSV-I a but the other herpes proteins HSV-I b and HSV-II a and b can also be identified in small amounts.

(a) HSV ^{3}H-chromatin (————————) + ^{14}C-cytoplasm (- - - -)

(b) HSV ^{14}C-chromatin (- - - - - -) + ^{3}H-cytoplasm (————)


dpm x 10² (¹⁴C-met) (– – –)
dpm x 10³ (³H-met) (——)
(a)
(b)
HSV I
HSV II
a
b
Slice no.

In addition, the other HSV-induced chromosomal proteins (Ib, IIa and b) can be identified in the cytosol, along with histones in quantities sufficient to account for their synthesis in the cytoplasm.

6.5. SDS-GEL ANALYSIS OF HSV-INFECTED (a) cytosol and (b) nucleoplasm

In order to examine the total spectrum of HSV-induced proteins in the cytosol and nucleoplasm, these fractions were analysed on 7.5% SDS gels. The patterns obtained resemble those found for PrV-infected samples (See Section 4) with the main exception that no species comparable to CP 2 (PrV) is found for herpes simplex. As the antigenic properties of these two viruses are so different, it is perhaps the similarity between the patterns of proteins induced by the two viruses which is striking, rather than the differences.

The cytosol contains a high molecular weight species of approximately the same size as the main capsid protein, along with a number of other peaks which may be host proteins still produced in considerable quantities after infection. In the nucleoplasm, only one distinct species can be identified, and it is apparent that most viral proteins do become associated with the deoxynucleoprotein pellet.

6.6. DISCUSSION

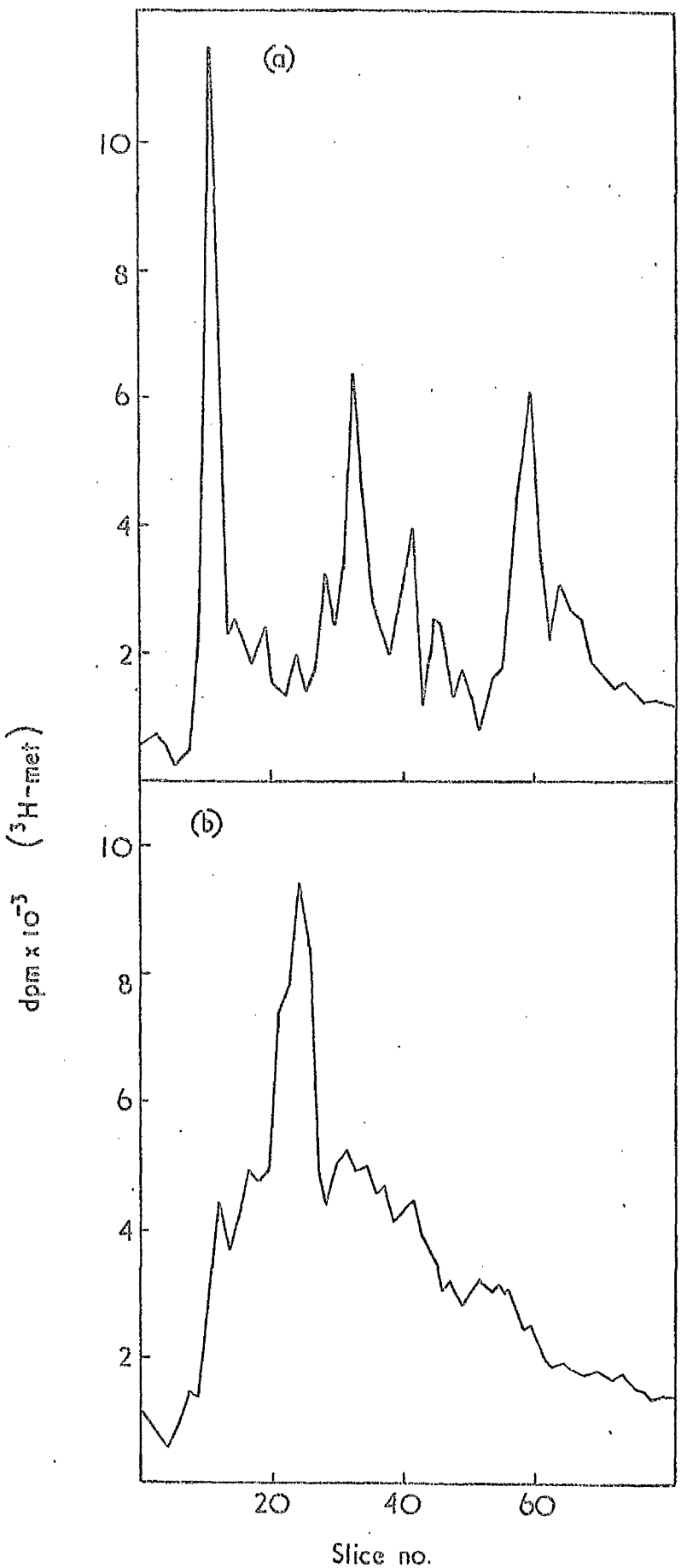
In contrast to the results of Kaplan et al., (1970) the spectrum of proteins in all cellular fractions was found to be similar for herpes simplex and pseudorabies infected BHK/21 cells, although with

Figure 6.5 SDS Gels of Cytosol and Nucleoplasm

Cytosolic and nucleoplasmic fractions of HSV-infected cells were incubated in 2% SDS overnight then analysed on 7.5% SDS gels.

The pictures shown opposite should be compared with the results found for control, and PrV-infected cells shown in Fig 4.2 (i) and Fig 4.4.

(a) control (b) HSV-infected.


(a)
(b)
10
8
6
4
2
dpm x 10⁻³ (³H-met)
20
40
60
Slice no.

the exception of IP I and HSV-Ia, co-electrophoresis was not shown to occur.

The presence of small basic proteins in both HSV and PrV-infected chromatin suggests that this may be a general phenomenon in herpesvirus-infected cells. This finding is of particular interest as both viruses inhibit the synthesis and transcription of host DNA soon after infection, reactions believed to require viral-directed protein synthesis (Ben-Porat & Kaplan, 1965; Sydiskis & Roizman, 1967). In addition, the similar effects of arginine deprivation on virus assembly for either virion suggests the involvement of comparable arginine-rich proteins in both cases.

Apart from the species present in chromatin acid extracts, a further similarity was found in the presence of a basic cytoplasmic protein of very similar size to IP I and HSV-I (a) (which themselves co-electrophorese). Whether either of these cytoplasmic proteins is identical to its nuclear counterpart is an open question at the present time. However it is of interest that all the herpes simplex nuclear basic proteins were found in sufficient quantities in the cytoplasmic fraction to account for their synthesis there, in contrast to the results found for pseudorabies.

In conclusion, it appears that the similarities between herpes simplex and pseudorabies extend to the spectra of proteins they induce in infected cells, although the functional significance of this finding is at present unclear.

SECTION 7 PURIFICATION AND FUNCTIONAL STUDIES

7.1. INTRODUCTION

Purification of the three IP's at least from host cell material, if not from each other, was essential before *in vitro* studies on their function could be carried out. Unfortunately due to their similarity in size and basicity, not only to each other but also to histones, this has proved very difficult. However eventually a reproducible method was obtained for separating all 3 IP's from histone material with very little contamination. Although far from ideal, this has enabled preliminary studies to be made on the combined effects of the 3 proteins on DNA and RNA synthesising reactions *in vitro*.

7.2. COLUMN CHROMATOGRAPHY

CM cellulose is an ion-exchanger which adsorbs positively charged molecules at neutral pH. Any acidic proteins pass straight through the column, which is useful as the acid extracts of chromatin are known to contain small amounts of non-histone material. Basic proteins can then be eluted by increasing the salt concentration, either as a gradient, or in step-wise fashion, and the position of proteins in the eluate recorded by collecting and counting fractions. Both methods of elution were tried but the gradient was found to dilute the samples too much - material being eluted over a wide salt range without any defined peaks being apparent. The step-wise procedure was more successful and

sharp peaks of varying size were obtained for each of the salt concentrations used. Attempts to concentrate the fractions before analysing them on 15% pH 4 gels met with limited success. 'Lyphogel', a polyacrylamide hydrogel which adsorbs 5 times its own weight of water and other low molecular weight substances, was tried, and also concentration in a Buchler evapomix at 25°C, but the samples were either almost completely lost, or were denatured or aggregated and thus did not penetrate the 15% gels. Eventually, proteins in the 'peak' tube(s) for each eluant were analysed and the rest of the material was discarded.

The initial columns were run with the following eluants (i) buffer (tris-HCl pH 7.8) (2) 0.1M NaCl (3) 0.25M NaCl (4) 0.37M NaCl (5) 0.5M NaCl (6) 1M NaCl and (7) 250mM HCl. All salt solutions were buffered in tris (0.02M pH 7.8) and contained 1% β-mercaptoethanol to prevent aggregation of histones. On analysis by gel-electrophoresis it was found that most of the induced proteins had been removed by the 0.1M and 0.25M NaCl concentrations. Both of these fractions were found to contain all 3 IP's though in varying amounts while the later fractions contained histone species. For this reason, future columns were eluted with just three solutions. Buffer to remove acidic protein contamination, 0.25M NaCl to elute almost all the 3 IP's along with a varying but very low amount of histone, and 0.25M HCl to remove the bulk of histone material. This is shown in Fig 7.2 (i) and the gel pattern for each fraction in Fig 7.2 (ii).

All <u>in vitro</u> functional studies were done on eluate 2.

7.3. <u>CHROMATIN ASSOCIATION</u>

Before examining the effects of the IP's on host DNA and

Figure 7.2 (i) CM Cellulose Chromatography

Chromatin acid extracts dialysed against column buffer were passed through a 7 cm CM-cellulose column equilibrated with 0.02M tris-HCl pH 7.8, 1% β-mercaptoethanol. Material was eluted with three solutions

(i) buffer

(ii) 0.25M NaCl

(iii) 250 mM HCl

1 ml fractions were collected and counted to record the position of eluted radioactive proteins. The pattern of elution is shown opposite :

Peak 2 contains the induced proteins, and Peak 3 the histones (see Figure 7.2 (ii)).

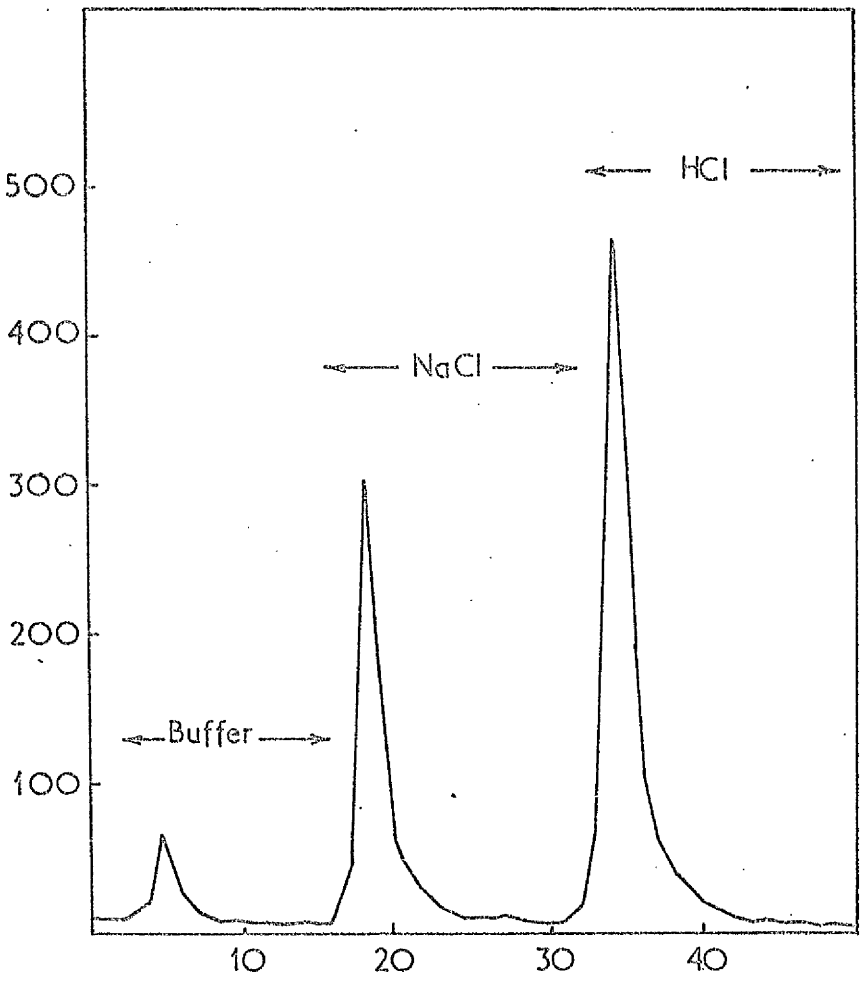

500
400
300
200
100
HCl
NaCl
Buffer
10
20
30
40
Fraction number

Figure 7.2 (ii) Gel analysis of Column Fractions

15% polyacrylamide pH 4 gels of :-

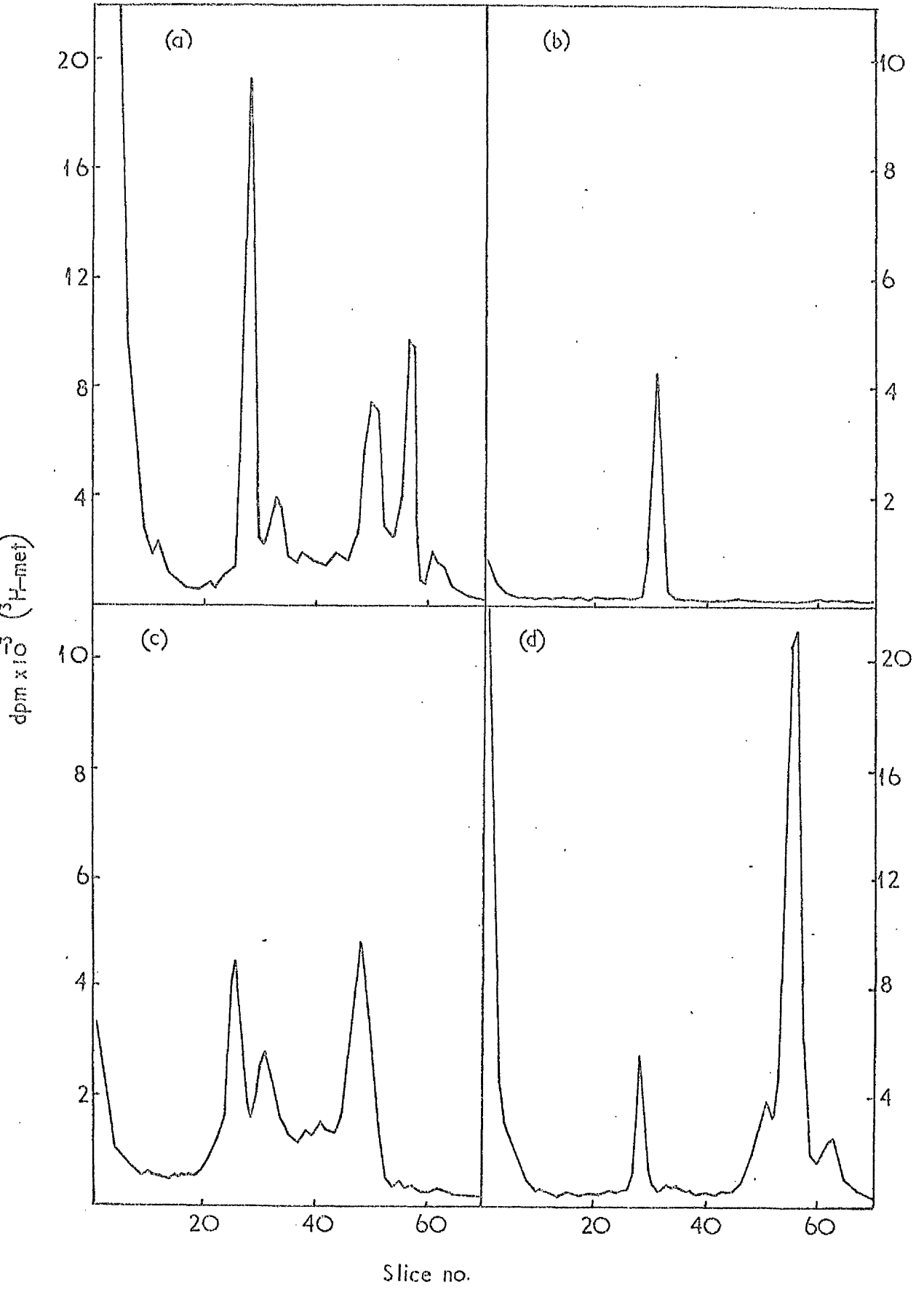
(a) chromatin acid extract applied to the column

(b) Peak 1 (see Fig. 7.2 (i))

(c) Peak 2 "

(d) Peak 3 "

Analysis of the peak tubes for each eluant shows that the IP's are in Peak 2 and the histones in Peak 3.


(a)
(b)
(c)
(d)
dpm x 10⁻³ (³H-met)
Slice no.
20
16
12
8
4
10
6
2
40
60

	Total Counts (dpm)	% counts
Supernatant	286,400	76
Pellet	91,262	24

Table 7.3

RNA polymerising activities, the ability of the viral proteins to associate with chromatin in vitro was examined. This was carried out by first dispersing chromatin in 2M NaCl, then mixing this with a preparation of ^{3}H-labelled IP's, also in high salt. DNA and protein were then reassociated by the gradient dialysis method of Bekhor, Kung & Bonner (1969) (see Appendix 2.II (iv)).

After washing the chromatin pellet in buffer and 1 mM HCl, it was extracted with 250 mM HCl and a portion of this counted along with a fraction of the pooled supernatants. The result is shown in Table 7.3. Over 20% of the counts were found to be in association with the DNA; this compares favourably with work done on histone reassociation studies.

7.4. DNA POLYMERASE

One of the first biochemical effects of pseudorabies infection is the switch-off of host DNA synthesis. This reaction has been proposed, by Ben-Porat & Kaplan (1965), to require viral-induced protein synthesis, although there is evidence in the case of herpes simplex virus for the inhibitor being a structural component of the virion (Newton, 1968).

The adenoviruses have also been shown to inhibit host DNA

synthesis after infection and here there is more conclusive evidence for the inhibitor being a structural component in the form of the fiber antigen (Ginsberg et al., 1967). In contrast to this, two members of the papova virus group, polyoma and SV40 appear to induce cellular DNA synthesis under certain conditions. In the case of polyoma, both types of DNA are made (Basilco, Marin & DiMayorca, 1966) unless high multiplicities of infection are used (Sheinin, 1966) but positive induction can also be identified in cases (e.g. contact inhibited cells) where cellular DNA synthesis is at a minimum prior to infection. SV40 exhibits both inhibition and stimulation of cellular DNA synthesis depending upon the cell-type infected (Sauer, Fischer & Munk, 1966; Hatanaka & Dulbecco, 1966). However SV40 DNA synthesis can occur in the absence of detectable host DNA synthesis, a finding not yet reported for polyoma (Gershon, Sachs & Winocour, 1966).

The switch-off of host DNA synthesis in pseudorabies infected cells may therefore be due to a structural viral protein, or a product of infection. With regard to the 3 IP's, this is interesting as they may be core proteins of the virus (see Section 3) as well as early proteins present in the cell by 3 hours post-infection (Section 2). This may explain the discrepancy in published results in that the initial inhibition of cellular DNA synthesis may be brought about by viral structural proteins, and this later augmented by further molecules synthesised after infection.

7.4 (i) Supernatant Polymerase Activity

The in vitro effects of the IP's on DNA synthesis were therefore examined. A ^{14}C-acid extract was dialysed against TME and

SAMPLE	Counts incorp. at 30' (dpm)	Mean (dpm)	Counts incorp. at 60' (dpm)	Mean (dpm)
CONTROL	1207 1158	1182	1967 2015	1991
50 µl IP	1714 1583	1648	2243 2067	2155
100 µl IP	1840 1869	1854	2220 2416	2318
50 µl HIS	2159 1913	2036	2437 2770	2604
100 µl HIS	2313 1984	2148	2884 2754	2819

Table 7.4 (i)

fractionated on CM cellulose. The peak tubes were analysed on 15% pH 4 gels (see Fig 7.2 (ii)) and the IP's as well as the histone fraction dialysed against assay buffer (TMK). ^{14}C-labelled proteins were used as the assay involves the incorporation of ^{3}H-TTP into DNA.

The enzyme tested was the cytoplasmic polymerase prepared by homogenising cells in TMK and spinning at 105,000 g; sperm DNA was used as primer. The results are shown in Table 7.4 (i). Samples were removed in duplicate at 30' and 60' of incubation time, and two concentrations of both IP's and histone were tested.

Instead of an inhibition of polymerase activity, both IP's and histones were found to induce the reaction. This was unexpected as histones at least are known to inhibit DNA replication *in vitro* (Gurley *et al.*, 1964). The explanation of the results may lie in the fact that the enzyme preparation used was unpure, and the added protein may act in another way quite apart from the DNA-binding reaction expected. However as the amount of protein added in the case of the IP's is so small relative to the total protein present, the result can not be satisfactorily explained.

7.4 (ii) Endogenous DNA polymerase activity in Isolated nuclei

In the hopes of simulating the in vivo system more closely, the effect of added IP's and histones on the endogenous DNA polymerase activity of isolated BHK nuclei was examined. The method used was essentially that of Winnacker, Magnusson & Reichard (1972) for polyoma infected mouse fibroblasts (for details see Appendix 2.II (vii)). This system requires the addition of exogenous ATP and Mg^{2+} ions - EGTA being added to complex other divalent ions. A preparation of cold IP's and histones was used to eradicate possible errors introduced by double-

Sample	Counts (dpm) incorporated	Mean	% stimulation	% inhibition
Control	3809 3524 3617	3650	-	-
10 µl IP	3406 3877 3562	3615	-	-
20 µl IP	4401 3945 4312	4219	15.5	-
50 µl IP	4648 4921 4576	4715	29	-
10 µl HIS	3359 2944 3107	3137	-	14
20 µl HIS	3097 3413 2976	3162	-	13
50 µl HIS	2735 2970 3469	3058	-	16

Table 7.4 (ii)

label counting.

The results of a typical assay are shown in Table 7.4 (ii). In accordance with the results found for supernatant polymerase with added template, the IP's were found to stimulate the incorporation of ^{3}H-TTP into DNA. In contrast, the histones gave a slight inhibition of polymerase activity which was not increased by further addition of histone material.

The exact significance of these findings is not clear. The result for histones is certainly more promising than that obtained with the supernatant assay and perhaps places more reliability on the stimulation effect found for the IP's in both cases. Certainly, there was no evidence for the inhibition of DNA polymerase by IP's as had been hoped.

7.5. RNA POLYMERASE

The DNA-dependent synthesis of RNA, *in vitro*, has also been shown to be inhibited by histones (Hnilica, 1967; Bonner *et al.*, 1968). This inhibition is not only brought about by association of the histones with template, but also by complex formation between arginine-rich histones and polymerase enzymes if these are mixed prior to the addition of DNA template. Lysine-rich histones can also combine with RNA polymerase but in this case the complex is dissociated by DNA (Spelsberg *et al.*, 1969; Spelsberg & Hnilica, 1969). No such complexes of histone with DNA polymerase have been identified. In addition to these results, experiments on the selective removal of histones from chromatin have again implicated mainly the arginine-rich fraction, rather than the lysine-rich, in template restriction (Spelsberg & Hnilica, 1971).

The situation in animal cells is further complicated by the presence of 3 distinct RNA polymerase activities, of which two are nucleoplasmic (polymerase II and III), and one nucleolar (polymerase I) (Roeder & Rutter, 1969; Lindell et al., 1970). By assaying the enzymes with and without salt ($(NH_4)_2SO_4$), in the presence and absence of α-amanitin (a toxin which specifically inhibits polymerase II), all 3 activities can be independently evaluated. However in the experiments reported here, only the effects of histones and IP on polymerase I (no salt) and polymerase II

Sample	No Salt Count incorp (Mean) dpm.	% stimulation (S) or % inhibition (I)	0.2M Salt Counts incorp (Mean) dpm	% stimulation (S) or % inhibition (I)
control	1613	-	2895	-
10 µl IP	1793	11% S	2851	1.5% S
20 µl IP	1927	19.5% S	3279	13% S
50 µl IP	2100	30% S	3762	30% S
10 µl HIS	1242	23% I	2930	-
20 µl HIS	1161	28% I	2499	14% I
50 µl HIS	983	39% I	1899	34% I

Table 7.5

and III (0.2M $(NH_4)_2SO_4$) were assessed. In this system, the ammonium sulphate would be expected not only to provide a selective assay for the nucleoplasmic polymerases, but also to stimulate the reaction by preventing complex formation between arginine-rich histones and the polymerase enzymes.

The assay involved measuring the incorporation of ^{14}C-uridine into RNA in nuclei treated with Tween 80 to remove cytoplasmic contamination, then spun through sucrose. Experimental details are given in Appendix 2.II (vi). In the initial experiments ^{3}H-labelled samples of IP's and histones which could be analysed on gel-electropherograms were used. However latterly, 'cold' preparations were tested to check that errors were not being introduced by double-label counting. The results in both cases were found to be the same (Table 7.5).

The results shown for the presence and absence of $(NH_4)_2SO_4$ are not directly comparable as different nuclear preparations were used in each case (up to five-fold stimulation by salt was found in controls when the same preparation was used). The activities of nucleolar polymerase I and the nucleoplasmic polymerases (II and III) were both stimulated by IP and inhibited by added histone.

7.6. DISCUSSION

The stimulation of both endogenous RNA and DNA polymerising activities in isolated nuclei by the IP's was unexpected. However as histones in the same system do give inhibition, the result is probably valid. Taken individually, the effect on each enzyme might suggest the

presence of an inducer of that type of polymerase activity in the IP preparation. However the fact that both enzymes are stimulated, and by about the same amount, points rather to an alteration of the chromatin template. As stimulation of host DNA and RNA production is not found in infected cells, it can only be assumed that other factors must operate under these conditions, perhaps to allow IP's to stimulate the viral DNA and RNA polymerase activities rather than the host ones.

In conclusion no evidence in favour of the IP's being inhibitors of host macromolecular synthesis was obtained and it appears they may rather be involved in viral replication and transcription.

SECTION 8 CONCLUSIONS

In view of the results presented in the previous sections, it seems most likely that the IP's are involved in virus assembly. The evidence for this comes from their characterisation as small basic proteins which would be capable of interaction with DNA, their presence in partially purified preparations of virus again indicating their association with viral DNA, and the finding of similar proteins in cells infected with herpes simplex virus which has a similar mechanism for producing progeny virus. The most conclusive evidence however, is found in the study of the effects of arginine deprivation. Under these conditions, no mature virions are found although most viral proteins, including the main capsid protein, are still produced in diminished quantities. The notable exception to this was found to be the three basic IP's which were completely absent from chromatin under these conditions. An examination of cytoplasmic and nucleoplasmic fractions to see whether accumulation of the IP's had occurred elsewhere in the cell was negative, indicating that it was their synthesis that had been inhibited, not their association with the chromatin complex. From these results it is proposed that the IP's are involved in the production of progeny virions.

This proposal requires that a virus 'factory' exists within the chromatin preparation, and in agreement with this, several other viral proteins are found in the urea and SDS fractions, including a molecule of similar size to the main capsid protein. These results were unexpected; it had been thought that the viral structural proteins

would be found in the nucleoplasm, and that only basic proteins with an affinity for deoxynucleoprotein would attach either specifically or non-specifically to the chromatin complex. At the time, it was hoped that these basic molecules might explain the inhibition of host DNA and RNA synthesis which occurs around the same time as their synthesis but no evidence to substantiate this has been obtained.

In contrast to the expected results, however, few viral-induced species were identified in the nucleoplasm and it does appear that the viral proteins are somehow contained within the chromatin preparation. An interesting comparison of these results with those obtained from examinations of the assembly process by electron microscopy can be made.

Various research groups have studied the infective process of herpesviruses by electron microscopy (Morgan et al., 1959; Watson et al., 1964; Felluga, 1963), and although it has proved impossible to state unequivocally how the processes of assembly and release occur, several features are worth mentioning. Soon after infection there is a dissolution of nucleoli and margination of the chromatin to the periphery of the nucleus. Dense granular aggregates then appear and these are thought to be the sites of viral DNA synthesis (Roizman, 1969). These bodies also tend to be near the periphery of the nucleus in association with chromatin, although smaller aggregates are found in the central matrix. The appearance of naked particles has been suggested to occur in close association with these granular bodies which have thus been proposed as 'template' sites (Morgan et al., 1959; Felluga, 1963). However in a later paper, Morgan's group have claimed that the first viral particles

found are scattered throughout the matrix and that if assembly does occur at prescribed foci the virus must be rapidly dispersed (Morgan et al., 1968). This is in agreement with results obtained in this laboratory (Figure 8). Whether or not virus assembly does occur in association with 'template sites' is thus uncertain. However it seems clear that the dense granular aggregates are closely caught up in the host deoxynucleoprotein complex and probably sediment with it to form part of the chromatin pellet. Thereafter it would be impossible to differentiate between basic proteins extracted from chromatin and those removed from the granular aggregates induced by the virus.

Further evidence in favour of the IP's being associated with viral assembly sites comes from the study by Russell et al., (1971) on the position of arginine-rich proteins in adenovirus-infected cells by a cytochemical staining technique. They showed that various nuclear inclusions identified by electron-microscopy were highly stained with phenanthrenequinone and thus represented the sites of accumulation of arginine-rich basic proteins.

Finally, the probable presence of the IP's in assembly complexes which are also the sites of synthesis of viral DNA and RNA, may further indicate functions for them in regulating viral replication and transcription. These possibilities are of interest with respect to the in vitro effects of the IP's found on DNA and RNA polymerase reactions. Control of transcription would be necessary to give the 'early' and 'late' phenomenon of Hamada and Kaplan (1965), where certain proteins are not made till the start of synthesis of viral DNA. This control is also a feature of many other viral systems - for example, bacteriophage

Figure 8 : Electronmicrograph of isolated nuclei (Tween 80 treated) of Pseudorabies infected cells.

Magnification : 12,500 fold.

(McCorquodale et al., 1967; Levinthal et al., 1967; Siegel & Summers, 1970), vaccinia virus (Jungworth & Joklik, 1965; Esteban and Metz, 1973) and herpes simplex (Wagner, 1972). All of these have an internal basic protein associated with their viral DNA.

In conclusion, the most likely function for the IP's is to associate with newly synthesised viral DNA. This may cause the DNA to enfold more tightly and allow encapsidation to occur and may also be the mechanism whereby the virus controls its own replication and transcription.

SECTION 9 SUMMARY

Three virus-induced proteins are found in the acid-extractable (histone) fraction of chromatin in pseudorabies infected cells. These are similar in size and basicity to histones but two, at least, differ from the host molecules in containing tryptophan. All three are produced early in the infectious cycle at a time when viral inhibition of host macromolecular synthesis is occurring. This suggests that the IP's may act in a similar way to histones in control cells in repressing host DNA replication and transcription. However no evidence has been found to support this proposal.

In order to establish whether pseudorabies virions contained the three IP's, purification of the virus was attempted on sucrose gradients. The preparations thus obtained were found to possess small amounts of 3 basic proteins which co-electrophoresed with the IP's. This suggested that they might be minor components of the virus and in view of their basic nature, probably constituents of the deoxynucleo-protein core. However the possibility of contamination of the virus preparation with chromatin material could not be ruled out.

An analysis of all other cellular fractions identified over twenty protein species produced in considerable quantities after infection, although some of these could represent continued synthesis of host molecules. In contrast, only 5 basic proteins small enough to run on 15% polyacrylamide gels were found - the three in chromatin, plus a fourth in acid extracts of whole nuclei and a species CIP I in the cytosol. This electrophoreses to almost the same position as IP I and

may represent the same molecule which becomes chemically modified on reaching the nucleus, a possibility being studied at the present time. The absence of the other IP's from the cytoplasm under conditions whereby histones can be identified on route to the nucleus, gave rise to the tentative proposal that they are made intranuclearly.

In addition to the 3 basic IP's, a number of other viral proteins were found in chromatin, in the nuclear acidic protein (urea or SDS) fractions. These included a molecule of similar molecular weight (> 100 x 10^3 daltons) to the main capsid protein of pseudorabies virions, a finding which suggested that a virus-synthesising complex might exist in the chromatin network. Within this, the IP's could act as assembly proteins either causing structural viral proteins to condense into capsids (c.f. Mark and Kaplan, 1971) or 'wrapping-up' the DNA in a suitable form for encapsidation to occur (c.f. Fine et al., 1968).

Studies on pseudorabies infection in arginine-deprived cells gave further evidence for involvement of the IP's in virus assembly. Under these conditions, viral maturation is completely inhibited although there is evidence that synthesis of viral DNA is almost completely unaffected (Becker et al., 1967) and that of the structural proteins reduced to only about half. Various research groups have therefore proposed that arginine-rich proteins may play a part in assembly of structural components into mature virions. As two of the IP's (II and III) have a high arginine content, an examination was made of their synthesis in infected cells deficient in this amino-acid. The result was quite striking. Whereas other viral proteins could still be identified in reduced amounts under these conditions, for example, the

arginine-rich CIP I in the cytoplasm and the various acidic proteins including small amounts of the capsid protein, in the nucleus, no sign of any of the 3 IP's was found in chromatin fractions. This is strong circumstantial evidence for their involvement in the assembly process.

A comparison of the proteins induced by herpes simplex MP17, with the patterns obtained for PrV showed strong similarities between the 2 viruses in contrast to the results of Shimono et al., (1969). In particular, HSV was also shown to induce small basic proteins in infected cells, one of which was shown to co-electrophorese with IP I of pseudo-rabies. This is of interest with respect to the similarity between the 2 viruses in their modes of inhibiting host function as well as in their mechanisms for assembling progeny virions.

Finally, a procedure was devised for separating all 3 IP's together from host material, and preliminary in vitro studies on their functions were carried out. First the proteins were shown to associate with DNA in chromatin reassociation experiments, and then their effects on host DNA and RNA polymerases in isolated nuclei were examined. Both reactions were found to be stimulated by the IP's under conditions where added histones gave inhibition. The function of this property in vivo is unclear.

In conclusion, although substantial evidence was found in favour of the IP's being involved in viral replication and assembly, no support was obtained for the proposal that they may take part in the inhibition of host macromolecular synthesis.

APPENDIX 1 MATERIALS

1. Virus

Pseudorabies virus (PrV) was originally derived from a stock preparation (Kaplan & Vatter, 1959) and has subsequently been plaque-purified three times.

Herpes simplex MP17 (Glasgow Strain 17) is a type 1 strain obtained from the Institute of Virology, Glasgow (S.M. Brown, D.A. Ritchie, J.H. Subak-Sharpe, 1973).

2. Tissue Culture Cells

A continuous line of baby hamster kidney fibroblasts BHK/21 (C13) was used for all experiments (Macpherson & Stoker, 1962), which had been adapted to grow in the absence of tryptose phosphate (C13A).

3. Radiochemicals

All isotopically labelled amino-acids were obtained from the Radiochemical Centre, Amersham, Bucks.

4. Chemicals for Liquid Scintillation Counting

2,5 diphenyloxazole (PPO) was purchased from Koch Light Laboratories Ltd., Colnbrook, Bucks; toluene, 2-methoxyethanol and hydrogen peroxide (100 vol) from BDH.

5. Polyacrylamide gel materials

Acrylamide, NN' methylenebisacrylamide and Temed (NNN'N' tetramethylethylenediamine) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Ammonium persulphate was obtained from BDH, Poole.

6. Other materials

All other chemicals used were 'Analar' grade or its equivalent and were purchased mainly from BDH, Biochemicals Ltd., Poole, Dorset, or Sigma Chemical Co., London. In particular -

Tween 80	Sigma
Naphthalene black	George T. Curr Ltd., London, S.W.6
Bromophenol blue	BDH
Lyphogel	Hawksley & Sons Ltd., Lancing, Surrey
Polyethylene glycol	BDH
CM and DEAE cellulose	Whatman Biochemicals.

7. Enzyme Assay Materials

^{14}C-dUTP and ^{3}H-dTTP were purchased from the Radiochemical Centre, Amersham, and deoxynucleotide triphosphates and nucleotide triphosphates from SIGMA, London. Salmon sperm DNA, as the highly polymerised sodium salt (type III) was obtained from SIGMA, London, and EGTA from Calbiochem.

8. Media and Solutions

(i) Eagles Minimal Essential Medium (MEM) (modified)

A modification of Eagle's medium (Busby, House and Macdonald, 1964) containing 100 units/ml penicillin and 100 µg/ml streptomycin (Glaxo Laboratories) with 0.02% (w/v) phenol red to indicate acidity (Figure 8). Vitamins (X 100) and amino-acids (X 10) were obtained from Flow Laboratories, Irvine, Ayrshire.

(ii) EC10

90% modified Eagles MEM + 10% calf serum (v/v) from Flow Laboratories.

(iii) ETC

80% modified Eagles MEM, 10% tryptose phosphate broth (2.95% w/v in distilled water) + 10% calf serum.

(iv) Balanced Salt Solution (BSS) - Eagle 1943

Stock solutions contained 68 g NaCl, 4 g KCl, 2 g $MgSO_4.7.H_2O$, 1.4 g NaH_2PO_4, 3.93 g $CaCl_2.6H_2O$, 15 ml 1% phenol red, diluted to 1 litre. This was further diluted ten-fold before use.

(v) Tris-buffered Saline (TBS)

0.01M tris-HCl pH 7.2 containing 0.15M NaCl and 0.01% β-mercaptoethanol.

(vi) Phosphate-buffered Saline (PBS) Dulbecco & Vogt, 1954

0.17M NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.

(vii) Tris-mercaptoethanol (TME)

0.02M tris-HCl pH 7.8, 1% β-mercaptoethanol.

(viii) Trypsin/Citrate

0.25% (w/v) trypsin, 10.5 mM NaCl, 1.0 mM sodium citrate and 0.002% phenol red adjusted to pH 7.8 with NaOH.

(ix) Versene

0.6 mM EDTA in PBS.

(x) Versene/Trypsin

Versene 4 parts, trypsin/citrate 1 part (v/v).

(xi) TKM

50 mM Tris pH 7.4 containing 25 mM KCl and 5 mM $MgCl_2$.

(xii) RNA polymerase Assay Buffer

250 mM Tris-HCl pH 7.9, 20% glycerol, 100 mM β-mercaptoethanol.

(xiii) TGMED

0.05M Tris-HCl pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 25% glycerol (v/v).

(xiv) Hepes Assay Buffer

120 mM Hepes pH 8.0, 160 mM NaCl, 20 mM $MgCl_2$, 1.6 mM $CaCl_2$, 800 mM sucrose, 3 mM DTT.

(xv) Isotonic Hepes pH 8.0

0.05M Hepes pH 8.0, 0.22M sucrose, 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM $CaCl_2$.

(xvi) Hypotonic Hepes pH 8.0

0.02M Hepes pH 8.0, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM DTT.

Appendix Table 8 Eagles Minimal Essential Medium (modified)

AMINO-ACIDS	mg/l
L-arginine HCl	126.4
L-cystine	24.0
L-glutamine	292.0
L-histidine HCl	41.9
L-isoleucine	52.5
L-leucine	52.5
L-lysine HCl	73.1
L-methionine	14.9
L-phenylalanine	33.0
L-threonine	47.6
L-tryptophan	10.2
L-tyrosine	36.2
L-valine	46.8

VITAMINS	mg/l
D-calcium pantothenate	2.0
Choline chloride	2.0
Folic acid	2.0
Inositol	4.0
Nicotinamide	2.0
Pyridoxal - HCl	2.0
Riboflavin	0.2
Thiamine HCl	2.0

INORGANIC SALTS and OTHER COMPONENTS

	mg/l
$CaCl_2 \cdot 2H_2O$	393
KCl	400
$MgSO_4 \cdot 7H_2O$	200
NaCl	6800
$NaH_2PO_4 \cdot 2H_2O$	140
$NaHCO_3$	2200
Phenol red	17.0
Dextrose	4500
Penicillin	10^5 units
Streptomycin SO_4	10^5 µg/l

APPENDIX 2 METHODS

I CELL CULTURE TECHNIQUES

(i) Propagation of Cells

BHK/21 cells, an established line of hamster fibroblasts (Macpherson and Stoker, 1962) were routinely cultured as monolayers in 80 oz Winchester bottles (burlers) according to the method of House and Wildy (1965). The burlers were gassed to give an atmosphere of 5% CO_2/95% air and 0.02% phenol red was used to indicate acidity.

For serial passaging, the cells were removed from the glass by treatment with trypsin/versene, were suspended in ECl0, counted and dispensed into sterile burlers containing 200 ml of ECl0, in aliquots of $20 - 25 \times 10^6$. Cells were not used beyond a passage number of 15 (Sheddon & Wildy, 1966). These cultures were maintained by the staff of the Wellcome Cell Culture Unit of this department.

(ii) Contamination Checks

All sterile media, and passaged cells were checked regularly for bacterial, fungal or PPLO infection as follows:

Bacterial Contamination : aliquots were grown on blood agar plates and brain-heart infusion broth at $37^{\circ}C$. Results were considered to be negative if no growth was seen within 7 days.

Fungal contamination : a small portion of the sample to be tested was added to Sabouraud's medium and incubated at $32^{\circ}C$. No growth in 7 days

was assumed to indicate the absence of fungal contamination.

PPLO infection : agar plates were seeded with passaged cells by piercing the agar surface with a charged pasteur pipette. The plates were grown in an atmosphere of 5% CO_2 in N_2 at 37°C.

Infected cultures resulted in the occurrence of the characteristic 'fried-egg' appearance of PPLO colonies on examination of the plates under the microscope. These cultures were discarded.

(iii) Propagation of Virus

Monolayer cultures of BHK/21 cells (about 10^8 cells per burler) maintained in ETC were infected at a multiplicity of 1 plaque-forming unit (pfu) per 300 cells in 20 ml medium. Virus was allowed to adsorb for 1 hour, then the inoculum was removed and replaced with 100 ml ETC. The cultures were rolled at 37°C for 36 hours, then harvested aseptically by shaking the bottle to dislodge cells into the medium, transferring to centrifuge bottles and spinning at 600 g for 10 mins. This pellets the cells, and the supernatant was then spun at 15,000 g for 2 hours to bring down the virus. This supernatant virus was resuspended in PBS (1 ml for every original burler used) and gently sonicated to make the suspension uniform. Aliquots were stored at -70°C and thawed only once before use.

Cell-associated virus, prepared by resuspending and sonicating the cell pellets, gives a low titre and was used only for production of virus stocks.

Plaque-assays were carried out on monolayer cultures of BHK/21 cells and normally gave titres of 10^9 - 10^{11} pfu/ml.

(iv) Plaque assay for PrV

Confluent monolayers of BHK/21 cells in 50 mm Petri dishes, which had been seeded at 4 x 10^6 cells/dish 18 hours previously, were infected with serial dilutions of PrV in 0.2 ml EC10. After 1 hour adsorption at 37°C, the excess was poured off, 4 ml EC10 was added and incubation continued. After a further hour, 50 µg/ml heparin was added to the medium to prevent vertical transmission of the virus. 28 hours after infection incubation was arrested, the cell sheet was washed with PBS and fixed with 3 ml formol-saline per dish (30 min at room temperature). Plates were then stained with 0.5 ml Giemsa stain/dish for 1 hour at room temperature, excess stain was washed off very gently with H_2O and plaques were counted under a low-power microscope.

(v) Growth of Cells for Experimental Use

Cells were normally seeded at 20 x 10^6/burler and incubated at 37°C for 2 days in 5% CO_2 until the cell-density had reached approximately 60 - 80 x 10^6. Monolayer cultures are at a cell-density of 10^8/burler, therefore the cells used were still in the exponential phase. This was true both for the cultures used for infection and those used to prepare enzymes (DNA and RNA polymerase), chromatin, nuclei, etc.

(vi) Infection of Cells

Exponential cells were infected at 20 pfu/cell in 20 ml slightly acid medium as adsorption is more efficient at lower pH's. After 1 hour, the excess was decanted, and 50 ml of medium deficient in the amino-acid to be used for labelling added. The radio-active isotope was added at the appropriate time and at the end of the labelling period, the cells were harvested.

(vii) Harvesting of Cells

a. Mechanical : the growth medium was poured off, or decanted into an isotope-container if radio-active, then the cell sheet was washed once in ice-cold BSS. Cells were removed from the glass into 20 ml BSS by means of a rubber scraper, transferred to a 50 ml centrifuge tube and pelleted by centrifugation at 800 g for 5 mins at 2°C. The pellet was washed once in TBS before being stored at -70°C.

b. Trypsin/Versene : where cells were to be reseeded they were removed enzymically from the glass. The cell sheet was washed twice in 50 ml BSS then 5 ml of trypsin/versene were added, and the burlers rotated for a few minutes. On standing them upright, the cells could be seen to slide to the bottom, and trypsinisation was then arrested by diluting with EC10.

(viii) Preparation of Labelled Virus

a. Whole Cell Preparation : Monolayer cultures of BHK/21 were infected at 5 pfu/cell and maintained for 16 hours in EC10 $\frac{1}{5}$ Arg + 4 µc's/ml of ^{3}H-arginine. Cells were shaken off the glass into the medium and this was spun at 15,000 g to pellet total virus + cells. The pellet was taken up in 1.5 ml EC10 and was sonicated until a smooth paste was produced. This was layered on top of a 12 - 52% (w/w) preformed sucrose gradient and spun for 40 min at 105,000 g (Jacquemont, 1972). The gradient was then fractionated into 1 ml portions and 0.1 ml of each counted for radioactivity. The fractions containing the viral peak were pooled, the sucrose diluted to around 5% and virus pelleted at 100,000 g for 2 hours. The pellet was taken up in 1 ml 250 mM HCl + 1% βME,

homogenised briefly by hand, freeze-thawed twice and extracted for 5 hours at $0^{o}C$. This was then spun again at 100,000 g leaving a clear supernatant for gel electrophoresis.

b. Nuclear Preparation : In order to obtain unenveloped virus, nuclei were first prepared in the usual way and washed in Tween 80. This nuclear preparation was then sonicated till homogeneous and layered on to a 12 - 52% sucrose gradient as before.

II BIOCHEMICAL TECHNIQUES

(i) Cell Fractionation

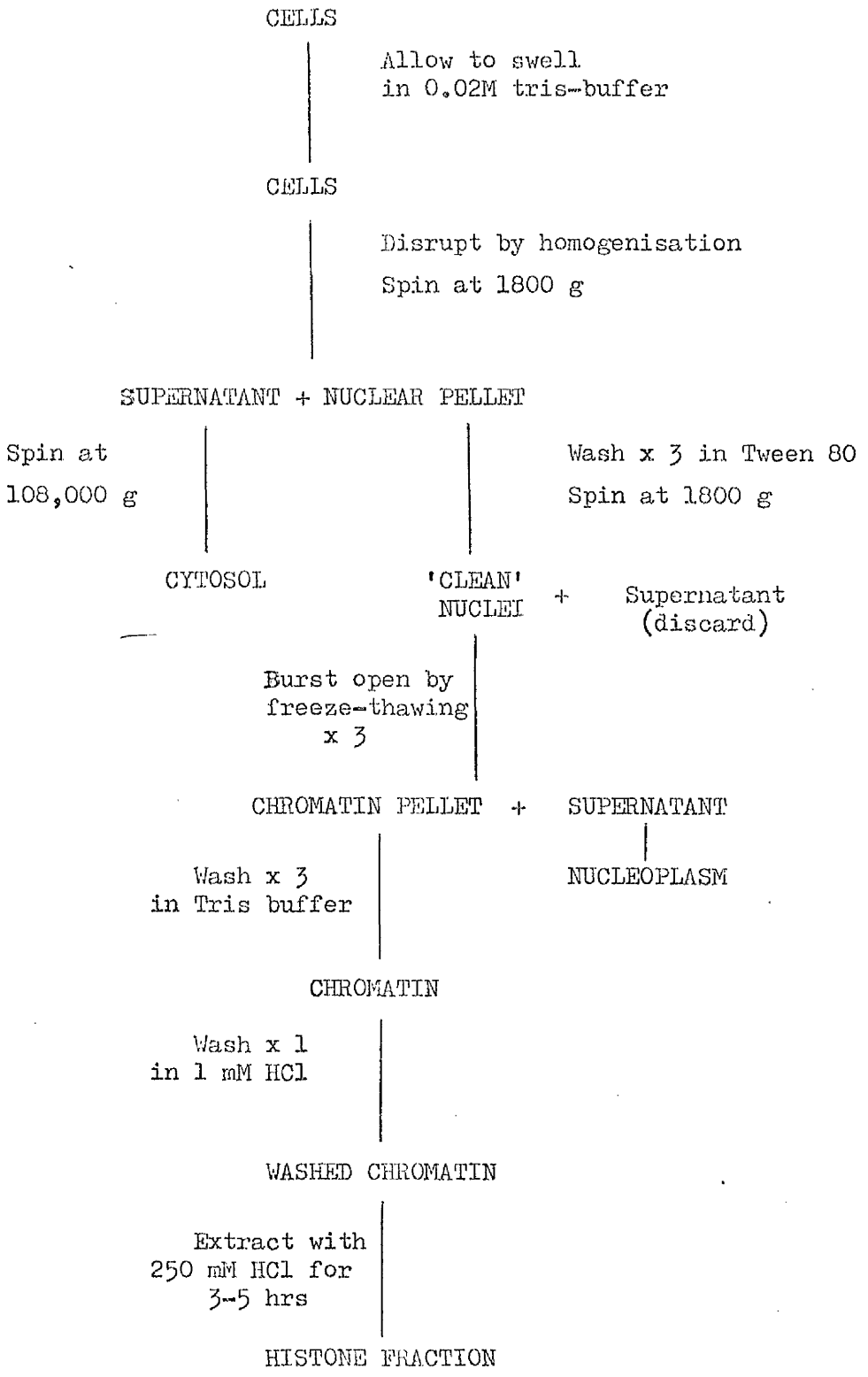
Cytosol, whole nuclei, nucleoplasm and chromatin were to be examined for protein content. These fractions were prepared as follows (also see Fig II (i)).

Labelled cell pellets were taken up in TME pH 7.8 and allowed to swell for 10 - 20 min. The cells were then disrupted with 3 strokes in a tight-fitting Dounce homogeniser, causing minimal nuclear damage. Whole nuclei were spun down at 1800 g and the supernatant further centrifuged at 105,000 g for 1 hour to give the cytoplasmic fraction. The nuclear pellet was washed at least 3 times in 1% Tween until no obvious cytoplasmic contamination could be identified under a low power microscope, and were then burst by taking up in TME and freeze-thawing. Chromatin was spun down at 1800 g and the supernatant formed the nucleoplasmic preparation.

The pellet was washed 3 times in TME with homogenisation, and was then thoroughly dispersed in 1 mM HCl using a pipette. After centrifuging the pellet of chromatin was extracted for 5 hours at $0^{\circ}C$ with 250 mM HCl/1% βME. DNA and residual proteins were spun down at 1800 g to form the pellet for urea and SDS extraction. In some cases the HCl-extract was dialysed overnight against TME pH 7.2.

Where whole nuclei were to be used, the procedure was stopped after the 3 Tween washes, centrifuged and the pellet used as the nuclear preparation.

Figure II (i) Preparation of Cellular Fractions



(ii) Diffuse and Dense Chromatin - Fractionation of Chromatin

The method was essentially that of Frenster, Allfrey and Mirsky (1963).

A nuclear pellet was suspended in 0.25M sucrose, allowed to stand for 10 mins at 0°C then treated ultrasonically for 1 min at 1.5 amps and 20,000 cycles/sec, in an MSE sonicator. Dense chromatin was then pelleted at 1000 g for 10 mins in an MSE major centrifuge. The supernatant was further centrifuged for 30 mins at 3000 g, the pellet in this case being discarded (intermediate chromatin) and the supernatant spun for 60 mins at 80,000 g (35,000 rpm) in a Spinco model L centrifuge to bring down the diffuse chromatin.

Both pellets of control and infected diffuse and dense chromatin were then extracted with acid and the basic protein examined on 15% pH 4 gels.

(iii) Column Chromatography

Carboxy-methyl-cellulose (CM-32) was precycled as recommended in the Whatman manual, then equilibrated in tris-buffer pH 7.2. A 7 cm column was poured and allowed to settle at 0°C, then was washed with 100 ml of column buffer. To apply samples, the buffer was allowed to run down to the top of the column, the sample was layered on and allowed to run in, then more buffer was pipetted carefully on top without disturbing the surface of the column. As only 15 - 20 ml of each eluant was to be used, a closed system was considered unnecessary and reasonable flow rates were obtained by simply pipetting the appropriate eluant over the column and allowing it to run through, before applying the next.

Samples for chromatography were dialysed against column buffer, and the 1% βME was found to be essential to stop aggregation of histones which prevented them from being eluted, even by 250 mM HCl. 1 ml fractions were collected by a L.K.B. Ultrorac fraction collector and were counted for radioactivity.

(iv) Chromatin Association

Chromatin was prepared in the usual manner from uninfected burlers of BHK/21 cells, and washed thoroughly in TME. Attempts were made to reassociate this with preparations of IP (Eluate 2 from CM cellulose columns) by the gradient dialysis method of Bekhor, Kung & Bonner (1969). Chromatin was first dissociated in 1 ml 2M buffered NaCl and mixed with 1 ml IP previously dialysed against 2M buffered NaCl, then dialysed against decreasing salt concentrations until DNA and protein had recombined. This involved 2 hour dialysis steps against 1M, 0.8M, 0.6M buffered NaCl, followed by an overnight dialysis versus 0.4M. Prolonged incubation at this ionic strength is thought to minimise non-specific aggregation of DNA and protein, and aid correct orientation of the protein molecules within the nucleoprotein complex (Huang, Bonner & Murray, 1964). Finally the chromatin was dialysed against 0.2M NaCl and then buffer alone (2 hours each). The precipitated complex could then be spun down at 1800 g and washed thoroughly in TME and 1 mM HCl. It was then extracted with 250 mM HCl and a portion of this counted along with a fraction of the pooled supernatants.

(v) DNA Polymerase Assay

Enzyme: Supernatant polymerase was prepared by dispersing

the cell pellet from 1 burler of cells in TMK, and homogenising at 40 x 8 in a tight-fitting Dounce homogeniser. The solution was then spun at 105,000 g (40K) in an MSE Spinco ultracentrifuge, and the pellet discarded.

Cocktail : A mixture of triphosphates dGTP, dATP, dCTP at concentrations of 15 mM together with ^{3}H-dTTP (sp. activity - 15 Ci/ m mole) formed the cocktail.

Assay : Enzyme, cocktail, sperm DNA as primer, and IP's or histone were incubated at 37°C for 30 minutes. 50 µl was removed, then the remaining solution incubated further till 60' and another 50 µl removed. The samples were applied to Whatman No. 1 paper discs and plunged into ice-cold 10% TCA + pyrophosphate. They were washed thoroughly twice in this then in 10% TCA alone, and finally in ethanol and ether before air-drying. DNA was eluted with hyamine hydroxide before scintillant was added and the samples counted.

IP or histone labelled with ^{14}C-amino acids were used (so that the column eluate could be detected), and so ^{3}H-thymidine incorporation into DNA was estimated using channel settings on a Packard scintillation counter to minimise overflow of ^{14}C into the ^{3}H channel.

(vi) RNA Polymerase

Enzyme : endogenous enzyme and primer were used in the form of isolated nuclei. This system has the advantage of simulating the *in vivo* situation most closely but unfortunately does not result in as high incorporation of ^{14}C-UTP as when using isolated enzyme systems with added primer.

The Blobel & Potter method for preparing rat liver nuclei was not found to work for BHK/21 cells and the method finally adopted involved the normal preparation of whole nuclei treated once with 1% Tween 80, then spun through 0.33M sucrose at 1800 g. The nuclear pellet was taken up in TGMED to give a final volume of 1 ml nuclear suspension for use in the assays.

Both amanitin sensitive and insensitive RNA polymerase activities were examined in the presence and absence of $(NH_4)_2SO_4$. After incubating nuclei with 0.1 mM each adenosine, guanosine and cytidine tri-phosphates ^{14}C-UTP (0.02 mM sp. activity - 43 mCi/m mole), 2 mM $MnCl_2$, and IP or histone in low or high salt buffer (0.2M $(NH_4)_2SO_4$) at 37°C for 30 mins, the reaction was terminated on ice. 50 µl were pipetted on to Whatman No. 1 discs which were then treated in an identical manner to the DNA polymerase discs for the estimation of radioactivity.

(vii) DNA Polymerase in Isolated Nuclei

The endogenous DNA polymerising activity in isolated nuclei was also examined using the method of Winnacker et al., (1972). In this case nuclei were prepared as follows :-

The cell pellet was washed once in isotonic hepes, then the cells were allowed to swell in hypotonic hepes pH 8.0. After disrupting the cells by homogenisation, the suspension was made up to 15 ml with hypotonic hepes and an equal volume of 0.6M sucrose was immediately added. Nuclei were pelleted by spinning at 2000 g for 30 mins, and were then taken up in 2 vols of isotonic hepes for the assay.

Assay : nuclei were incubated for 30 mins in the presence

of 40 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM ATP, 0.1 mM each of dATP, dGTP and dCTP, 0.02 mM ^{3}H-dTTP (sp. activity - 15 Ci/m mole), 200 mM sucrose, 30 mM Hepes pH 8.0, 0.8 mM DTT and 0.4 mM $CaCl_2$.

The reaction was stopped by placing the tubes on ice and adding 0.5 ml 10 mM EDTA in 50 mM Hepes pH 8.0. 100 µl 10% SDS were then added and the tubes incubated at 37°C for a further 30 mins. DNA was then precipitated by making the solutions 5% with respect to TCA and then collected on to Whatman glass-fibre filters, washed with 5% TCA + pyrophosphate, then dried in ethanol and ether. DNA was solubilised in hyamine hydroxide before being counted in toluene-PPO as before.

(viii) Gel Electrophoresis

15% pH 4 gels : 7 cm gels contained 15% acrylamide (w/v), 0.1% NN methylene bis acrylamide, 2% TEMED (tetramethylethylene diamine) 0.7% ammonium persulphate. They were subjected to pre-electrophoresis for at least 30 mins in glycine-acetate buffer pH 4.0 (McAllister, Wan & Irvin, 1963) at 3 m.a./tube then run for 4 hours at 4 - 5 ma/tube. Samples for electrophoresis were applied to the gels in glycerol. After electrophoresis, the gels were stained with naphthalene black for 30 mins, then left overnight in 7% acetic acid. Destaining was completed electrophoretically at 7 ma/gel. The gels were frozen in Drikold and ½ mm slices made using a Mickle gel slicer. These were then counted for radioactivity.

7.5% SDS gels : the method used was essentially that of Weber & Osborn (1969). 12 cm gels contained 7.5% acrylamide, 0.25% NN methylenebis acrylamide, 0.05M phosphate buffer, 0.1% SDS, 0.1% temed, 0.1% persulphate. Samples for electrophoresis (<100 µl) were incubated

overnight in 2% SDS, 1% β-mercaptoethanol in 0.04M phosphate buffer, before being applied to the gels in glycerol. Electrophoresis was carried out for 8 hours, then the gels stained for 1 hour in Coumassie Brilliant Blue. Destaining was done in methanol/acetic acid. The gels were frozen in Drikold and sliced in 1 mM portions for radioactivity estimation.

(ix) Radioactivity Measurements

Gels : 0.5 mm or 1 mm slices were dissolved in 0.3 ml 100 vol hydrogen peroxide at 40 - 50°C overnight. 10 ml of scintillant (toluene-PPO-methoxyethanol) were added and the samples counted in a Phillips scintillation spectrometer (Counting efficiencies ^{3}H - 35%, ^{14}C - 70-80%, ^{3H} double-label - 30%, ^{14}C double-label - 60%).

Assays : Whatman No. 1 discs from DNA or RNA polymerase assays were eluted with hyamine hydroxide at 37°C for 20 mins, then counted in toluene-PPO scintillator.

Column eluate : 0.1 ml of each 1 ml fraction was counted in toluene-PPO-methoxyethanol.

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