DNA SYNTHESIS IN CELLS INFECTED WITH THE PARVOVIRUS MVM

by

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"Everything existing in the Universe is
the fruit of chance and of necessity."

DEMOCRITUS

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ABBREVIATIONS

Abbreviations used in this thesis are as laid down in the Biochemical Journal Instructions to Authors (revised 1976) with the following additions:

BudR	-	5-Bromodeoxyuridine
CNS	-	Central nervous system
cpe	-	Cytopathic effect
CPG	-	Controlled pore glass
DMSO	-	Dimethyl sulphoxide
DNase	-	Deoxyribonuclease
FA	-	Fluorescent antibody
HA(U)	-	Haemagglutination (units)
hpi	-	Hours post-infection
MVM	-	Minute virus of mice
NB cells		SV40-transformed newborn human embryo kidney cells
PBS	-	Phosphate buffered saline
pfu	-	Plaque-forming units
PPLO	-	Pleuro-pneumonia-like organisms
RF	-	Replicative form of DNA
RNase	-	Ribonuclease
SDS	-	Sodium dodecyl sulphate
SSC	-	0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0
SV-15	-	Simian adenovirus 15
SV-40	-	Simian virus 40
TCID/50		Virus dose which infects 50% of a cell population

CONTENTS

SUMMARY

Virus-specific deoxyribonucleic acid (DNA) synthesis in A9 cells infected with the autonomous parvovirus Minute Virus of Mice (MVM) can first be detected 8 - 10 hr after infection. Viral DNA can be extracted from infected cells by the Hirt/Pronase, and other methods, in the form of a double-stranded linear replicative intermediate (RF), of molecular weight about 3×10^6 daltons, twice that of the single-stranded viral genome. In addition, further virus-induced DNA species, sedimenting faster than RF DNA in neutral sucrose gradients are present in Hirt/Pronase extracts. Analysis of this "fast-sedimenting" DNA revealed the presence of both single- and double-stranded DNA, consistent with this class of DNA containing single-stranded progeny molecules, and duplex concatemeric species of viral DNA, reaching at least 4 RF molecules in length.

S_1 nuclease digestion of MVM DNA from purified virions indicated that about 12% of the genome was in duplex form after alkali denaturation. Analysis of Hirt/Pronase extracted DNA by S_1 nuclease digestion and by agarose gel electrophoresis both before and after denaturation suggested that a proportion of the viral DNA molecules was capable of spontaneous renaturation. The majority of this "snap-back" DNA was located in the RF DNA fraction. Furthermore, alkaline sucrose gradient analysis of concatemeric and RF DNA revealed the presence, in each, of DNA strands up to 2 genome equivalents long. These findings suggested that MVM DNA molecules may exist in the form of hairpin structures, comprising covalently-linked viral and complementary strands.

While MVM RF DNA was shown to migrate as a single band on

agarose gels, after cleavage with Endo. R.Eco.R_1 three specific fragments were produced indicating the presence of two sites for the enzyme.

MVM DNA can be extracted from infected cells using triton-X-100 in 2 forms. The first, a 95S DNA-protein complex, distinct from the mature virion, contains viral-sized DNA and, as shown by short term pulse-labelling experiments does not contain replicating DNA. Both of these findings are consistent with the 95S species being a "maturation complex" late in the virus replication cycle. Secondly, a species corresponding to RF DNA, in association with a small amount of protein, was also present.

Short term pulse-labelling experiments revealed that the vast majority of newly-synthesised DNA sequences appeared in RF-sized molecules which, upon denaturation yielded a high proportion of DNA strands which were shorter than unit length MVM DNA.

Pulse-chase experiments were performed using the triton method of extraction, which indicated the presence of further possible intermediates in the replication of MVM.

The mechanisms of parvovirus DNA replication and assembly are discussed.

1. INTRODUCTION

1.1 THE NATURE OF VIRUSES

Viruses are entities whose genome is an element of nucleic acid, either DNA or RNA, which reproduces inside living cells. They are obligate intracellular parasites, using the host cell synthetic machinery to direct the synthesis of progeny virions which transfer the viral genome to other cells (Lwoff, 1957; Luria and Darnell, 1967).

They differ from other living organisms in several fundamental respects, including:-

1) Virions contain only one type of nucleic acid.

2) Virus-specified proteins are synthesised using host cell ribosomes.

3) They do not divide, but are assembled from their constituent parts, which are synthesised separately.

All viruses consist of nucleic acid, surrounded by a protein coat or capsid, which is composed of morphological units of protein called capsomeres. In some cases the capsid is, in turn, enclosed within a lipoprotein envelope, the peplos.

1.2 CLASSIFICATION

Virus classification has been the subject of much debate, owing to the inherent difficulties involved, and no scheme has gained universal acceptance. Most classification schemes are based on specific properties of the virus:-

1) The host range (whether animal, plant or bacterial).

2) The intrinsic properties of the virus:

these include the type of nucleic acid, symmetry of the nucleocapsid, presence or absence of envelope, number of capsomeres (Tournier and Lwoff, 1966). Other characteristics which have been suggested are size, cellular site of virus maturation (Andrewes, 1964) and molecular weight of nucleic acid (Melnick, 1973).

1.3 THE PARVOVIRUS GROUP

Parvoviruses (Mayor and Melnick, 1966; Lwoff and Tournier, 1966; Andrewes, 1970) constitute a group of viruses which was described comparatively recently (Kilham and Oliver, 1959). The group is characterised by having icosahedral naked particles, 18-26 nm in diameter, which contain single-stranded DNA and which are assembled in the cell nucleus. Their densities in caesium chloride solution are relatively high (about 1.40 g/cm^3), owing to their high content of DNA, (20-25% by weight), and they possess considerable heat and acid stability. The viruses infect a wide variety of animal hosts including man, monkeys, cats, dogs, cattle, rodents and birds.

Parvoviruses may be divided into two major groups (Melnick, 1971).

1) Non-Defective

As their name suggests these viruses are capable of autonomous replication within the host cell. Since Minute Virus of Mice (MVM) is a member of this group, the properties of non-defective parvoviruses will be mainly discussed.

2) Defective (see Berns, 1974; Rose, 1974, for reviews)

Viruses in this group comprise the adenovirus-associated viruses (AAV) (Atchison et al., 1965), so called because they are incapable of replication unless the cells are co-infected with a helper adenovirus (Atchison et al., 1965; Hoggan et al., 1966; Smith et al., 1966; Parks et al., 1967a).

The classification and origins of the parvoviruses have been extensively reviewed (Toolan, 1968, 1972; Rose, 1974). Table 1.1 lists some of the viruses which are best characterised, while Table 1.2 shows the origins of some parvoviruses.

It must be pointed out, however, that the classification of some

of the non-defective parvoviruses into their respective groups has posed some problems. Although H-1, HT and HB viruses were apparently originally recovered from human tissues they have been placed tentatively in the rodent group for several reasons. Human antibody to these viruses is rare (Toolan, 1968), while H-1 antibody is frequently found in rats (Kilham, 1966; Cross and Parker, 1972). Furthermore all three viruses are pathogenic in newborn hamsters (Toolan, 1968), a feature not observed with viruses outside the rodent group.

Similarly TVX and Lu-III viruses were originally isolated from human cell lines, while RT virus was recovered from rat fibroblasts. However, since they do not possess any antigenic relationship to the other parvovirus subgroups, they have remained unclassified. It has been suggested (Hallauer et al., 1971) that these three viruses arose as contaminants.

Table 1.1

CLASSIFICATION OF PARVOVIRUSES

1) Non-Defective

Rodent viruses

Rat virus (RV), H-3 virus, X-14 virus, L-S virus, haemorrhagic encephalopathy virus of rats (HER), Kirk virus.

HB virus, H-1 virus, HT virus (tentative).

Minute virus of mice (MVM).

Feline virus

Feline panleukopenia virus (FPV).

Porcine viruses

Porcine parvovirus (PPV), KBSH virus (identical to PPV?).

Bovine virus

Bovine haemadsorbing enteric virus (Haden virus)

Canine virus

Minute virus of canines (MVC).

Unclassified viruses

TVX virus, Lu-III virus, RT virus.

2) Defective

Human and simian Adeno-Associated viruses (AAV)

AAV-1, AAV-2, AAV-3, AAV-4.

(Adapted from Rose, 1974.)

Table 1.2

ISOLATION OF SOME PARVOVIRUSES

Non-Defective

Virus	Source	Primary Natural Host
MVM	Mouse Adenovirus Stock	Mouse
RV	Rat tumour	Rat
H-1	HEp1 cells [1]	Rat ?
Lu-III	Lu 106 cells [2]	?
HER	Rat CNS tissue	Rat
X 14	Rat mammary tissue	Rat
L-S	Rat tumour	Rat

Defective

AAV-1	SV 15 stock	Rhesus monkey ?
AAV-2	Adenovirus-12 stock	Man

1 Transplantable human tumour cells.

2 Continuous human cell lines

(Adapted from Rose, 1974.)

1.4 BIOLOGICAL PROPERTIES OF PARVOVIRUSES

An understanding of the major biological properties of the parvoviruses is essential when considering their method of reproduction. While some of the major biological features will be discussed in the following sections, additional information may be gained from several reviews (Kilham, 1966; Toolan, 1968; Rapp, 1969; Hoggan, 1970; Rose, 1974).

1.4.1 Stability

Parvoviruses are among the most stable of all vertebrate viruses. MVM, however, has been reported to be less stable than the other members of the group (Harris et al., 1974). It has been shown that AAV, H-1 and Lu-III withstand heating at 56°C for 1 hr or more (Greene, 1965; Hoggan et al., 1966; Siegl et al., 1971), while RV and FPV have been shown to survive temperatures of 75-80°C for over 30 min (Kilham and Oliver, 1959; Johnson and Cruickshank, 1966). In contrast, over 99% of MVM infectivity was lost when heated at 45°C for 2 hr (Harris et al., 1974). Similarly, although other parvoviruses have resisted inactivation by ether or chloroform (Kilham and Oliver, 1959; Atchison et al., 1965; Johnson and Cruickshank, 1966; Binn et al., 1968; Siegl et al., 1971), MVM was readily inactivated by a variety of chemical disinfectants including alcohols, formaldehyde and chloroform (Harris et al., 1974). MVM was also shown to be more sensitive than RV to inactivation by ultraviolet light and less stable than other parvoviruses when stored at 4°C (Toolan, 1968; Poole, 1972; Harris et al., 1974).

Further studies have indicated that parvoviruses are resistant to inactivation by trypsin, pepsin, papain (Johnson and Cruickshank,

1966; Rose et al., 1966; Siegl et al., 1971) and they have withstood incubation at pH 3.0 (Greene, 1965; Mayr et al., 1968; Storz and Warren, 1970; Harris et al., 1974). Parvoviruses are resistant to deoxyribonuclease (Vasquez and Brailowsky, 1965; Rose et al., 1966).

1.4.2 Growth, Latency, Assay

Non-defective parvoviruses replicate most efficiently in cultures of actively dividing cells (Johnson, 1967; Mayr et al., 1968; Tennant et al., 1969; Parker et al., 1970b; Hallauer et al., 1972; Tattersall, 1972b). It is often difficult to detect their presence, however, owing to the lack of a pronounced cytopathic effect (cpe). Methods used successfully to uncover parvoviruses have included blind passages both *in vitro* and *in vivo* (Kilham and Oliver, 1959; Toolan et al., 1960; Toolan, 1964; Kilham and Moloney, 1964; Robey et al., 1968), and repeated extraction of cell sheets with a glycine buffer (Hallauer et al., 1971), which extracts virions.

Parvoviruses may also persist in cells, becoming apparent only after their replication is enhanced during passage (Kilham and Oliver 1959; Toolan et al., 1960; Robey et al., 1968), or by certain manipulations such as freeze-storing of cells (Hallauer et al., 1971). Whether parvovirus latency is the result of the non-detection of very low levels of persistent infection, or results from the carriage of viral DNA is not yet clear. However, evidence that the latter may be true for AAV has been reported (Hoggan et al., 1972), and more recently (Berns et al., 1975a), it has been shown that cells latently infected with AAV contain 3-5 AAV genome equivalents per diploid amount of cell DNA. Whether this DNA is integrated into the host cell DNA or exists as a plasmid is unknown.

Virus assays have been carried out using a procedure to detect the virus dose which kills 50% of a cell population ($TCID_{50}$) (Johnson 1967; Mayr et al., 1968; Binn et al., 1970; Hallauer et al., 1972), by haemagglutination activity (HA) titration (Cole and Nathanson, 1969) or by plaquing (Ledinko, 1967; Tennant et al., 1969; Tattersall, 1972b; Bates and Storz, 1973).

The plaque assay for MVM has been developed comparatively recently, owing to the previous lack of a virus-cell system which could provide efficient infection. Tattersall (1972b) has shown that MVM plaque formation requires a dividing cell monolayer and that plaque size decreases as input cell number increases. In contrast to most other viruses (Takemoto, 1966), plaque size is not genetically controlled.

1.4.3 Haemagglutination (HA)

Parvoviruses have the ability to agglutinate the erythrocytes from a wide variety of animal species (Kilham and Oliver, 1959; Crawford, 1966; Toolan, 1967a; Hoggan, 1971; Hallauer et al., 1972). This property has been frequently used to assay and characterise the members of the group. For example, characteristic HA patterns obtained with H-1, H-3 and RV were contributory in showing that they were, indeed, different viruses (Moore, 1962; Toolan, 1964). Since most rodent viruses, including MVM, preferentially agglutinate guinea-pig erythrocytes (Crawford, 1966; Toolan, 1967a), these cells have been used subsequently in HA assays by most workers.

1.4.4 Host Range and Pathology

The host range, both *in vitro* and *in vivo*, of the non-defective parvoviruses is fairly limited. All the rodent viruses can be grown

both in rat and hamster cells (Toolan, 1968; Mirkovic et al., 1971; Hallauer et al., 1972). MVM, however, while growing well in hamster cells (Tattersall, 1972b), is the only virus which can be propagated satisfactorily in mouse cells (Crawford, 1966; Hallauer et al., 1972; Tattersall, 1972b). H-1 and H-3 are capable of replicating in several human and simian cell lines (Toolan and Ledinko, 1965; Hallauer et al., 1972).

H-1 virus exhibits what has been termed "conditional defectiveness" (Melnick and Parks, 1966) in that, although it will not grow in human lung cells, it will multiply in these cells when adenovirus-12 is present as a helper (Ledinko and Toolan, 1968; Ledinko et al., 1969). Stimulations of HA yields of 100 fold for Kirk virus and 4 fold for X14 have been observed on co-infection with adenovirus-7 (Mirkovic et al., 1971). The mechanism of this helper activity is unknown.

The existence of specific antibodies in several animal species has confirmed the occurrence of <u>in vivo</u> infections. RV, H-1 and L-S antibody have been found in rats (Kilham, 1966; Robey et al., 1968; Lum, 1970), MVM antibody in rats and mice (Kilham and Margolis, 1970; Parker et al., 1970b), and rarely, H-1 antibody in man (Toolan, 1964, 1968).

Although rodent viruses are not known to cause disease in the natural environment, they have been shown to do so in experimental animals. RV and H viruses can produce either fatal infection in newborn hamsters or osteolytic lesions which result in a mongoloid-type deformity (Toolan et al., 1960; Kilham, 1961a, b). In addition, H-1 virus injected subcutaneously into pregnant hamsters resulted in congenital deformities of embryos (Toolan, 1961; Ferm and Kilham, 1964). RV and related strains have also been shown to cause lesions

in the central nervous system of newborn hamsters (Kilham and Margolis, 1964; Nathanson et al., 1970), kittens (Kilham and Margolis, 1965) and rats (Nathanson et al., 1970), resulting in cerebellar hypoplasia and ataxia. Inoculation of H-1 into both man (Toolan et al., 1965) and monkey (Toolan, 1966) resulted in viremia with no detectable illness.

The effects of these viruses probably result from their affinity for proliferating generative cells in embryonic and newborn animals.

1.4.5 Interference

The phenomena of homologous interference, in which AAV inhibits its own replication at high multiplicity, and heterologous interference, in which AAV is capable of inhibiting replication of other AAV strains, helper virus, or SV40, have been reviewed in detail (Rose, 1974).

1.4.6 Parvoviruses and Oncogenesis

Since many parvoviruses have been isolated from tumours (Table 1.2), they were obvious candidates for the possession of oncogenic activity. No parvovirus, however has been shown to produce tumours in experimental animals (Kilham and Oliver, 1959; Kilham and Moloney, 1964; Toolan, 1967b; Hallauer et al., 1972).

Several studies, however, have indicated that parvoviruses may, indeed, interfere with oncogenesis. Toolan (1967b) found that the tumour incidence in control hamsters was more than twenty times that observed in mongoloid animals produced by inoculation with H-1. These animals maintain high antibody titres to H-1 throughout their lives (Toolan, 1964). Similarly, the incidence of adenovirus-12 induced tumours in hamsters was decreased by 50% and 66% respectively by co-infection with H-1 (Toolan and Ledinko, 1968) or AAV-1 (Kirschstein et al., 1968). A more recent study has found that pre-infection with

AAV one day prior to adenovirus infection had an even greater interference effect on adenovirus-31 oncogenesis (Mayor et al., 1973), while having no effect on SV40-induced tumours. The interference is therefore virus-specific. AAV has also been shown to inhibit adenovirus transformation of hamster embryo cells in vitro (Casto et al., 1967; Casto and Goodheart, 1972).

1.5 PHYSICOCHEMICAL PROPERTIES OF PARVOVIRUSES

1.5.1 Virion Morphology

A large number of parvoviruses have been visualised in the electron microscope (see Hoggan, 1971; Tinsley and Longworth, 1973). The results indicate that all the viruses have icosahedral symmetry and most are 18-26 nm in diameter. Owing to their small size, detailed analysis of the virion structure has proved difficult. From reconstruction experiments Vasquez and Brailovsky (1965) concluded that the capsid of RV was composed of 32 capsomeres, which agrees with data obtained for other rodent parvoviruses (Karasaki, 1966; Mayor and Jordan, 1966). However, AAV-4 seems to consist of 12 capsomeres (Mayor et al., 1965), while Smith et al. (1966) have concluded that AAV-3 capsids consist of a netlike reticulum similar to that of reovirus (Vasquez and Tournier, 1964). More recently Kongsvik et al. (1974) have reconsidered the structure of H-1, in the light of new evidence and suggested that a capsid containing 12 capsomeres is most likely. The authors propose a capsid structure composed of 12 pentamers of the major capsid protein (B) with a molecule of the minor protein (A) situated at each vertex.

1.5.2 Buoyant Density of Viral Species

Reported buoyant densities in caesium chloride of the different parvoviruses are in the range 1.38 - 1.47 g/cm^3 (McGeoch et al., 1970; Siegl et al., 1971; Hoggan, 1971). Most parvoviruses, however, have densities near 1.40 g/cm^3.

It is a feature of isopycnic centrifugation of parvoviruses that several minor bands are obtained in addition to the major layer of infectious particles. Lighter virus bands are frequently observed

at densities between 1.33 - 1.38 g/cm^3 and 1.30 - 1.32 g/cm^3 (Payne et al., 1964; Crawford, 1966; Robinson and Hetrick, 1969; Torikai et al., 1970; Siegl, 1972; Usategui-Gomez et al., 1969), the latter particles being devoid of DNA (Robinson and Hetrick, 1969; Siegl, 1972). The band of intermediate density (1.33 - 1.38 g/cm^3) contains large proportions of empty particles and has little infectivity associated with it (Payne et al., 1964; Greene and Karasaki, 1965; Crawford, 1966; Robinson and Hetrick, 1969; Usategui-Gomez et al., 1969; Johnson et al., 1971, Tattersall, 1972b). DNA can be extracted from particles in this band but is considerably shorter than genome length (20-30%) (Torikai et al., 1970; Siegl, 1972). Its origin, whether viral or host, is uncertain.

In addition, a band of virus particles more dense than the primary one, is often observed at 1.44 - 1.47 g/cm^3. These particles appear by electron microscopy to be smaller in diameter than main band particles (Hoggan, 1970, 1971) and for AAV and MVM have been shown to be infectious (Johnson et al., 1971; Hoggan, 1971; Clinton and Hayashi, 1975). Furthermore, DNA molecules extracted from heavy particles of AAV, MVM and Lu-III appear very similar to mature viral DNA (Siegl, 1972; Rose; 1974; Clinton and Hayashi, 1975). For MVM, at least, the heavy particles appear to be immediate precursors of main band particles, a change in the ratio of capsid protein species being the only detectable difference (Clinton and Hayashi, 1975). For reference, the physical properties of MVM virions are summarised in Table 1.3.

1.5.3 Viral DNA

The characterisation of parvovirus DNA has presented many technical problems, including the difficulty of obtaining sufficient quantities of purified virus, and the extremely resistant nature of

Table 1.3

PHYSICAL PROPERTIES OF MVM

Particle Weight (daltons)	5.5×10^6	1.
Diameter of Virion (nm)	24	1.
% Weight of DNA	25	1.
Buoyant Density (g/cm^3)	1.43	2.
Sedimentation Coefficient (Svedbergs)	110	3.

1. Tattersall, Ph.D. Thesis University of London (1971).
2. Crawford, 1966.
3. Crawford et al., 1969.

the nucleocapsid, which makes efficient extraction extremely difficult. Reported methods of DNA extraction have ranged from treatment with hot detergent and/or proteolytic enzymes (Crawford, 1966; Rose et al., 1966) to alkaline degradation of the virus and sedimentation of the DNA through CsCl or alkaline sucrose (McGeoch et al., 1970; Koczot et al., 1973). The latter method yields a higher proportion of unbroken molecules (Koczot et al., 1973) and also provides evidence concerning the homogeneity of the DNA.

For the non-defective parvoviruses the physical and chemical properties of their DNA (Table 1.4) indicate that they possess a single-stranded genome. This conclusion has been derived from many experimental approaches including reaction with formaldehyde (Crawford, 1966; Usategui-Gomez et al., 1969; Salzman and Jori, 1970), staining virions with acridine orange (Mayor and Melnick, 1966), digestion with single-strand specific nucleases (Salzman et al., 1971; Siegl, 1973; Tattersall et al., 1973), electron microscopy (Crawford et al., 1969; Robinson and Hetrick, 1969; Salzman et al., 1971; Siegl, 1973), and base composition analysis (McGeoch et al., 1970).

The characterisation of AAV DNA posed a more difficult problem. Staining with acridine orange gave the characteristic "red flame" colour (Mayor and Melnick, 1966) and reaction with formaldehyde produced an increase in extinction at 260 nm, indicating the presence of single-stranded DNA in the virion. However, when AAV DNA was extracted, it was found to be double-stranded (Rose et al., 1966, 1968; Parks et al., 1967b). In a comparative study Crawford et al. (1969) suggested that the AAV genome could not exceed 1.7×10^6 daltons and proposed that the double-stranded DNA was formed, after extraction, by annealing of (+) and (-) strands which had been separately encapsidated. Rose et al.

Table 1.4

PHYSICAL AND CHEMICAL PROPERTIES OF PARVOVIRUS DNA

Virus	ρ CsCl, [1] g/cm^3	Sedimentation Coefficient, S. [2]	Genome Molecular Weight x 10^{-6} [3]	% G + C	Comments
MVM	1.722	-	1.5	40.9	Single-stranded linear
RV	1.726	27	1.6	43.5	Single-stranded linear
H-1	1.720	27.8	1.7	45.2	Single-stranded
AAV-2	1.726	24	1.35	53.0 (-)	Separately encapsidated
	1.714	15		53.1 (+)	(+) and (-) strands which anneal to form duplex molecules

1. Buoyant densities of both duplex and single-stranded AAV-2 are given.
2. Sedimentation coefficients for AAV-2 DNA in both double and single-stranded forms are given. All values determined in 1 M NaCl.
3. Best estimates.

(Adapted from Rose, 1974.)

(1969) tested this hypothesis and found it to be true.

The fact that a single-stranded component is not evident in AAV DNA preparations (Rose et al., 1966, 1968; Parks et al., 1967b) and that when fragmented and denatured, the DNA completely reassociates (Carter et al., 1972), indicates that there is an equivalent synthesis of both strand species in infected cells.

1.5.4 Size and Shape of DNA

The molecular weights of parvovirus DNA's have been determined (Table 1.4) by electron microscopy and sedimentation analysis. Values in the range 1.35 - 1.7 x 10^6 daltons have been reported. Differences, however, may relate to methodology since estimates of AAV DNA size based on sedimentation coefficients are 10-20% greater than those based on electron microscopy (Rose et al., 1966; Parks et al., 1967b).

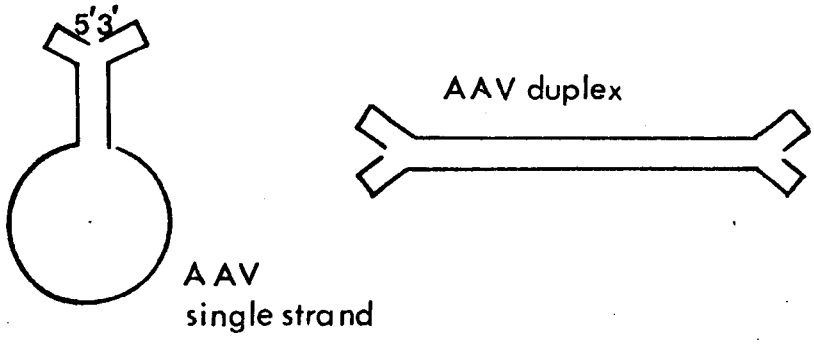
Although DNA from MVM and Lu-III appears to be linear when visualised in the electron microscope (Crawford et al., 1969, Siegl, 1973) up to 40% of Lu-III DNA is resistant to S1 endonuclease before, and about 20% after, denaturation (Siegl and Gautschi, 1976) while 20% of MVM DNA is resistant to exonuclease-I before and 10% after denaturation (Tattersall et al., 1973). It seems likely, therefore, that these molecules possess self-complementary regions, possibly near their terminus (i) which are capable of forming duplex regions of foldback DNA. This feature of parvovirus DNA has been implicated in playing a role in viral DNA replication (Tattersall et al., 1973; Straus et al., 1976), and will be discussed further.

More recently, Denhardt et al. (1976) have used the restriction endonuclease R. Hae-III to study the terminal structure of AAV DNA. Digestion of terminally labelled duplex or single-stranded AAV DNA yielded the same three fragments when analysed by agarose/polyacrylamide

gel electrophoresis. The authors have proposed structures for these terminal fragments and, based on the relationship between mobility and gel pore size, propose that one may have a non-linear ("rabbit ear") conformation.

In detail, the individual single-stranded AAV DNA molecules exist with two different terminal sequences, each of which is self-complementary, and capable of forming "fold-back" structures, but which are not complementary to each other.

eg.

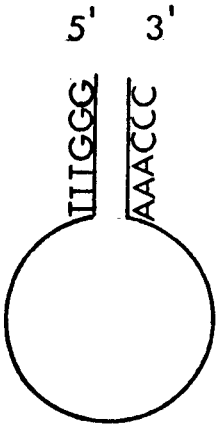


The possible role of such a structure in relation to viral DNA replication will be discussed.

Circular forms of AAV DNA have been visualised using electron microscopy (Vernon et al., 1971; Koczot et al., 1973; Gerry et al., 1973; Berns and Kelly, 1974). Analysis of these and other forms of AAV DNA has been contributory in building up a picture of the complex structure of the AAV genome.

Koczot et al. (1973) have shown that the (+) and (-) strands of AAV-2 DNA contain self-complementary terminal sequences which can anneal to form single-stranded circular molecules closed by relatively short duplex segments.

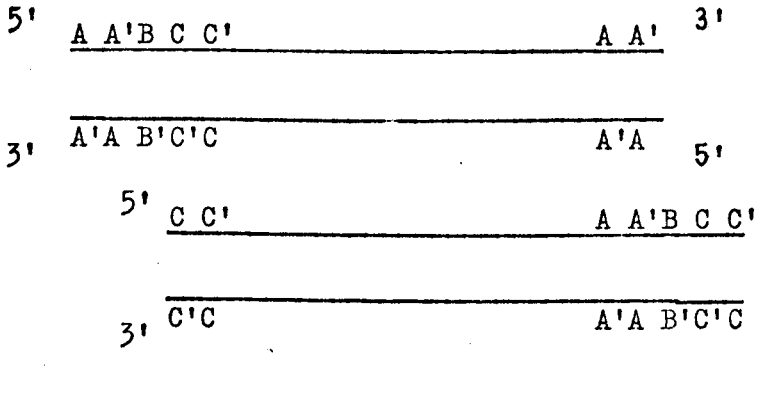
ie.



This means that duplex AAV DNA molecules contain an inverted terminal repetition similar to that described for adenovirus DNA (Garon et al., 1972; Wolfson and Dressler, 1972), in which the base sequence at one end of the molecule is inverted and appears again at the other end (Koczot et al., 1973).

```
      5'                                    3'
          GGGTTT ____________________ AAACCC
ie
          CCCAAA ____________________ TTTGGG
      3'                                    5'
```

Examination of double-stranded AAV DNA in the electron microscope revealed that 5 - 15% of the duplex molecules could form circles closed by hydrogen bond regions (Koczot et al., 1973). Subsequently Gerry et al. (1973) have shown that AAV duplex circles are closed by overlaps of 1.5 - 6% of the genome length. Approximately half the circles could be opened by exonuclease-III while the remaining half could be opened by T5 exonuclease indicating that the overlap region may have either 3' or 5' termini. Since linear AAV DNA single strands were shown not to be randomly circularly permuted, the nucleotide sequence of AAV DNA therefore contains a limited number (possibly only two) of permutations, the start points being confined to a region equivalent to $< 6\%$ of the genome.



A model showing two sequence permutations of AAV DNA. Berns, 1974.

Evidence to support the existence of two nucleotide sequence permutations in AAV DNA, the start points of which have been estimated to be separated by 1% of the genome has been reported (Berns et al., 1975b). They showed that the terminal AAV DNA fragments, produced by cleavage with the restriction endonuclease Hind.II + III could each be resolved into two species differing in molecular weight by about 1% of the genome. The authors, however, make the assumption that a difference in mobility reflects a difference in molecular weight, rather than a conformation change, which may not be valid (Denhardt et al., 1976).

A third type of terminal repetition has been described by Gerry et al. (1973). Duplex linear monomers of AAV DNA could be converted to circles or oligomers after a 1% digestion with exonuclease-III suggesting that AAV DNA molecules may also contain a natural terminal repetition (Thomas and MacHattie, 1967), similar to that present in bacteriophage DNA.

Whether or not all three types of sequence arrangement are present in all AAV DNA strands is not clear, but all these phenomena occur within less than 10% of the genome length at either strand terminus.

There is no evidence that any parvovirus contains a covalently-closed single or double-stranded genome. Therefore, although physical and chemical comparisons between the parvovirus group and bacteriophage

ϕ X 174 have been extremely useful (Crawford et al., 1969), it is likely that there are basic differences in their mechanisms of DNA replication. Thus, although general comparisons may be valid (e.g. replication via a double-stranded intermediate, Sinsheimer et al., 1962), direct comparisons of specific events may not be justifiable.

1.5.5 Infectivity of DNA

The double-stranded form of AAV-1 DNA has been shown to be infectious (Hoggan et al., 1968; Boucher et al., 1971), infectivity being dependent upon the presence of a helper adenovirus. This indicates that the defective step(s) in AAV replication must exist at some stage after uncoating.

It is not known whether infection can be accomplished with purified single-stranded DNA from any parvovirus.

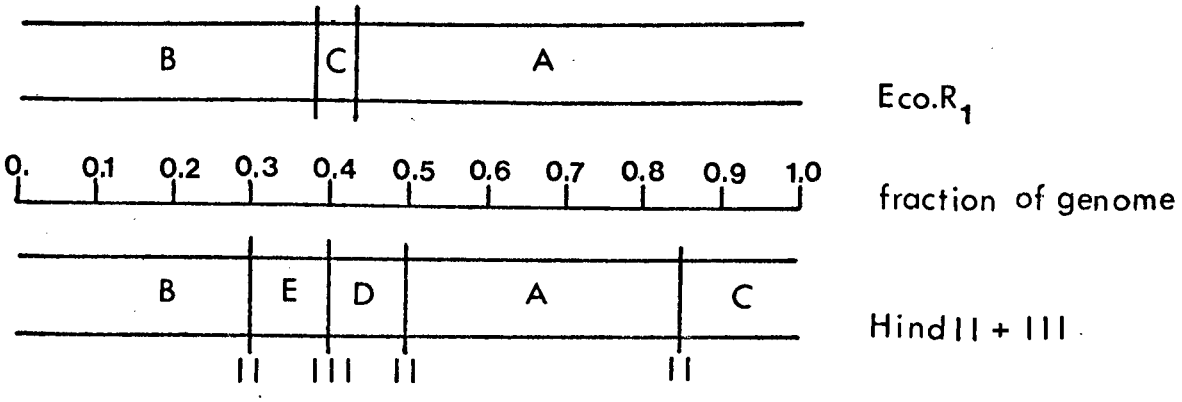
1.5.6 Cleavage of Parvovirus DNA with Restriction Endonucleases

A physical map of AAV-2 DNA has been constructed by analysing fragments of DNA produced after cleavage with the restriction endonucleases R.Eco.R_1 (Carter and Khoury, 1975; Carter et al., 1975), and R. Hind. II + III (Berns et al., 1975b). Carter and Khoury (1975) unambiguously determined the order of endo.R.Eco.R_1 fragments by using a combination of terminal labelling of 5'-ends and substituting the DNA with 5-bromodeoxyuridine (BUdR). After cleavage, the substituted strands of the terminal fragments were separated by isopycnic centrifugation. This enabled the location of the 5' and 3' termini of the (+) and (-) DNA strands.

Berns et al. (1975b)also used terminal labelling, together with complete and partial digestion of DNA with endo.R.Hind.II + III, to determine the order of the fragments. This order was confirmed by

sequential digestion of AAV DNA with endo.R.Eco.R_1 and endo.R.Hind.II + III respectively.

Physical Map of AAV DNA



The physical map has been used to determine the direction of RNA transcription (Carter et al., 1975) and provides a basis for the orientation of further specific fragments produced with other restriction enzymes. The fragment map should also be useful in further analysis of AAV-2 DNA, particularly in determining the origin(s), direction and termination(s) of DNA replication.

1.5.7 Viral Protein

The structural proteins of RV, H-1, Haden virus, MVM and AAV-1, 2 and 3 have been analysed by SDS-polyacrylamide gel electrophoresis (Salzman and White, 1970; Rose et al., 1971; Johnson et al., 1971; Kongsvik and Toolan, 1972; Johnson and Hoggan, 1973; Rose, 1974; Clinton and Hayashi, 1975).

In each case the protein capsid may be resolved into three polypeptide components (A, B, C), Table 1.5, the molecular weights of each being fairly comparable among those viruses studied. The intermediate-sized polypeptide (B) is the major component of RV,

Table 1.5

MOLECULAR WEIGHTS OF PARVOVIRUS POLYPEPTIDES

gel component [1], molecular weight x 10^{-3}

Virus	A	B	C
MVM	92 (9)	72 (54)	69 (8)
RV	72 (9)	62 (60)	55 (10)
H-1	92 (8)	72 (52)	56 (9)
Haden	85.5	76.8	66.8
AAV 1, 2, 3	87 (3.5)	73 (2.7)	62 (52)

[1] Figures in parenthesis represent calculated number of polypeptides per virion.

(Adapted from Rose, 1974).

H-1 and MVM while the smallest polypeptide (C) predominates in Haden virus and AAV. It has been proposed (Rose, 1974) that the major components constitute the identical monomeric protein subunits which join to form the capsomeres, since the number of molecules per virion is about 60 (Table 1.5), the minimum required to construct an icosahedral capsid (Caspar and Klug, 1962). Several factors, however, have recently arisen which complicate the interpretation of data obtained on parvovirus proteins.

1) It has been shown for MVM that the relative amounts of proteins B and C vary inversely, with C increasing during the nuclease digestion step in the virus purification procedure (Rose, 1974). (Virus purification often involves digestion with deoxyribonuclease and ribonuclease.) There is also a quantitative conversion of B to C on treatment of MVM with trypsin (Rose, 1974). Trypsin treatment of empty particles, however, leaves the B protein intact (Clinton and Hayashi, 1975).

2) During the primary cycle of MVM infection the sole product appears to be dense particles (1.47 g/cm^3), with protein B as their major component (Clinton and Hayashi, 1975). However when left for more than one round of replication in the infected culture, these heavy particles are converted to main band virions (1.42 g/cm^3) which contain C as their major protein.

3) Kongsvik et al. (1974) have reported the absence of C protein in H-1 virus isolated from synchronised NB cells (SV40-transformed newborn human embryo kidney cells). When grown in unsynchronised hamster embryo cells, however, C is present.

These data raise the question as to which polypeptide is the primary unit of parvovirus capsids. It seems that the results obtained

may vary according to several factors, including the time of virus isolation, the method of purification and the cell system used. For MVM it appears that the processing of B to C is dependent on a capsid conformation attained only when the particle contains DNA (Clinton and Hayashi, 1975), but this may not be true for all parvoviruses since the relative concentrations of the three AAV proteins are identical in mature virions and empty capsids (Johnson et al., 1975).

It is a feature of parvoviruses that the combined molecular weights of the polypeptides exceeds the coding capacity of the genome. It is possible, therefore, that one or more of the structural polypeptides might be derived from a larger precursor. It has been shown by peptide mapping (Rose, 1974) that there is considerable sequence overlap among all the polypeptides of MVM. Furthermore, information obtained concerning AAV transcription shows that only one messenger RNA species, of molecular weight 9×10^5 daltons, appears to be synthesised (Carter and Rose, 1972; Carter et al., 1972; Carter and Rose, 1974). It would be impossible for this messenger to specify the three observed polypeptides if the sequence of each was unique. These data, together with the fact that a host cell protein is probably not incorporated into MVM virions (Rose, 1974) suggests that parvovirus proteins may be processed after synthesis.

1.5.8 Viral DNA Polymerase

The existence of a DNA polymerase activity associated with purified RV was reported by Salzman (1971), and subsequently purified (Salzman and McKerlie, 1975). For optimal activity the enzyme requires all four deoxynucleoside triphosphates, Mg^{2+} or Mn^{2+}, and exogenous DNA. The polymerase has increased enzyme activity in the presence

of 0.02 M KCl, is more active with activated native DNA than with denatured DNA, has a strong preference for the synthetic DNA polymer, poly[d(A-T)], and differs from the host cell polymerases (Salzman and McKerlie, 1975). Its molecular weight is about 75,000 daltons and it appears to contain endonuclease activity.

In a similar study with H-1 and RV, Rhode (1973) failed to detect any virion-associated DNA polymerase activity. However, it must be noted that, while Salzman propagated his RV in rat nephroma cells (Salzman, 1971; Salzman and McKerlie, 1975), Rhode (1973) used hamster embryo cultures. Further investigation is necessary to determine whether this DNA polymerase plays a role in viral DNA replication.

Recently, however, Dooley et al. (1976) have emphasised the necessity for caution in the interpretation of the role of particle-associated enzymes. They showed that an endonuclease activity associated with purified (by isopycnic centrifugation in CsCl) SV40 virions:-

1) is lost after velocity centrifugation in CsCl,

2) appears to be similar to an enzyme found in the serum in which the cells were grown, and

3) is not necessary for infection.

The authors propose that repeated use of a single technique (e.g. successive isopycnic centrifugation in CsCl) is insufficient to purify virus from contaminants, and suggest that velocity sedimentation should be added to the purification procedure.

1.6 INFECTIOUS CYCLE

Since parvovirus synthesis seems to depend on actively dividing cells, most studies have been performed using non-confluent or tumour cells, or cells induced to divide synchronously by a serum pulse (Tennant et al., 1969; Tattersall, 1972b; Rhode, 1973) or by releasing a block on DNA synthesis (Hampton, 1970; Siegl and Gautschi, 1973a). Multiplicities of infection have ranged from 2 - 200 pfu or $TCID_{50}$/cell, sufficient to provide efficient infections.

1.6.1 Adsorption

The time required for adsorption depends on the virus and cell system used. Within 1 hr 90% of the plaque forming virus particles of an inoculum of RV (5 pfu/cell) had been adsorbed from the medium (Salzman et al., 1972), while with Haden virus about 75% of an 8 pfu/cell inoculum was taken up in 2 hr (Bates and Storz, 1973). For MVM the infectious unit is a single particle (Tattersall, 1972b).

1.6.2 Efficiency of Infection

The efficiency of infection has been measured by estimating the fraction of cells synthesising viral protein as determined by fluorescent antibody (FA) staining. Efficiencies of 30% and 50% have been achieved after infection of dividing rat embryo cells with RV at 5 and 50 pfu/cell respectively (Tennant et al., 1969; Fields and Nicholson, 1972). Similarly, Hela cells infected with Lu-III at 10 $TCID_{50}$/cell were shown to be 95% FA positive (Siegl and Gautschi, 1973a). With RV, at least, the number of virus-synthesising cells, based on FA staining was approximately proportional to the amount of progeny virus synthesised (Tennant and Hand, 1970). However, it should be noted that, even in abortive infections of human lung cells

with H-1, about one third of the cells contain viral antigen, while there is little or no progeny synthesis.

1.6.3 Eclipse and Maturation

After adsorption, there follows a latent period ranging from 10 - 16 hr during which time less than 1% of the infectious input virus can be accounted for (Johnson, 1967; Al-Lami et al., 1969; Cole and Nathanson, 1969; Tennant and Hand, 1970; Fields and Nicholson, 1972; Salzman et al., 1972; Bates and Storz, 1973; Siegl and Gautschi, 1973b).

Virus progeny are assembled in the cell nucleus (Mayor and Jordan, 1966; Mayr et al., 1968; Al-Lami et al., 1969; Siegl et al., 1972) and maturation is usually complete by 24 -30 hr after infection (Johnson, 1967; Tennant and Hand, 1970; Salzman et al., 1972; Siegl and Gautschi, 1973b).

Even late after infection, most of the virus remains in a stable association with the cells (Johnson, 1967; Salzman et al., 1972; Siegl and Gautschi, 1973b; Bates and Storz, 1973).

A small percentage of total virus synthesised, however, is released into the medium late in infection. By 30 hr, about 0.1% of total RV synthesised in rat nephroma cells was found extracellularly (Salzman et al., 1972) while a similar percentage of Lu-III virus had been released by 48 hr (Siegl and Gautschi, 1973b).

1.6.4 Viral DNA Replication

The onset of viral DNA replication generally occurs 8 - 10 hr after infection (Salzman et al., 1972; Siegl and Gautschi, 1973b; Tattersall et al., 1973; Rhode, 1973). The fate of single-stranded parental RV DNA in rat nephroma cells has been followed by infecting

cells with virus containing radioactive DNA (Salzman and White, 1973). Low molecular weight DNA was selectively extracted using the Hirt (1967) procedure (Hirt extract). Analysis of this DNA indicated that within 1 hr 28 - 42% of the parental DNA had been converted to a double-stranded linear intermediate. The relative proportions of parental DNA to double-stranded DNA remained unchanged up to 20 hr after infection. These experiments suggested that the parental DNA is converted to a linear duplex replicative form (RF) shortly after infection. Attempts to repeat this experiment, however, with H-1 (Rhode, 1974b) and Lu-III (Siegl and Gautschi, 1976) have been less successful, with yields of extracted DNA representing only about 1% of the total input radioactivity. This possibly reflects a higher particle/infectivity ratio in these virus stocks.

The reason why the formation of parental RF precedes the onset of viral DNA replication by 7 - 9 hr is unknown. Such RF molecules, however, may be transcribed before progeny DNA synthesis begins. With RV, viral RNA is synthesised as early as 2 hr after infection (Salzman and Redler, 1974). Virus-specified antigen has been detected in the cytoplasm of HER-, KBSH- and Lu-III-infected cells as early as 4 - 6 hr after infection (Cole and Nathanson, 1969; Siegl et al., 1972; Siegl and Gautschi, 1973a), and, for Lu-III at least, is unaffected by fluorodeoxyuridine (FUdR) or arabinofuranosylcytosine (ara-C) and therefore occurs independently of DNA synthesis.

Double-stranded forms of parvovirus DNA have been detected in vivo for MVM (Tattersall, 1972a; Dobson and Helleiner, 1973; Tattersall et al., 1973), for H-1 (Rhode, 1974a, b) and for Lu-III (Siegl and Gautschi, 1976).

Tattersall et al. (1973) showed that the percentage of total

labelled DNA appearing in Hirt/Pronase extracts of control cells during the first 20 hr after infection is constant (0.8%). In contrast, in MVM-infected cells the proportion increases rapidly from 8 - 14 hr post-infection, reaching 25% at 20 hr. Using hydroxyapatite chromatography this DNA was resolved into single-stranded progeny DNA and double-stranded linear replicative intermediates which were present in approximately equal numbers. Furthermore, after denaturation, between 20 - 40% of the double-stranded DNA renatures spontaneously in a monomolecular fashion indicating that it has a cross-linked or hairpin structure. The authors speculated that MVM DNA synthesis might be initiated by a self-priming mechanism in which the 3' end of one strand is used as a primer for the synthesis of the complementary strand.

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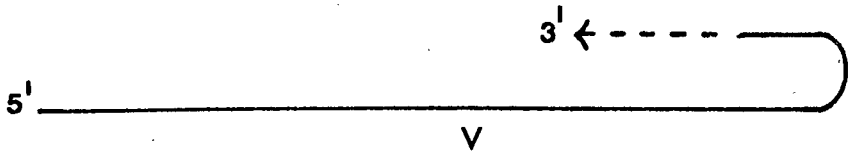
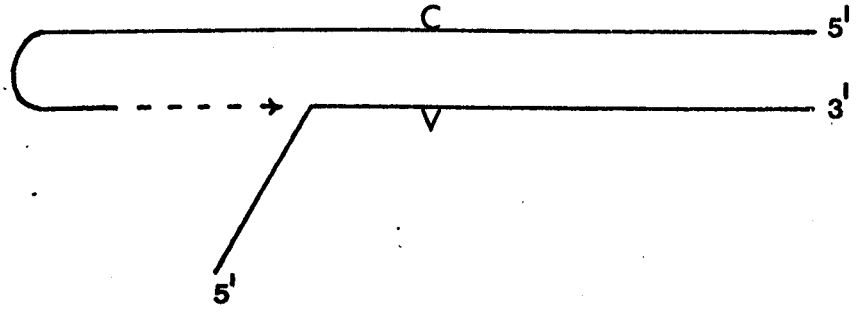


V

It was also shown (Tattersall et al., 1973) that a short ^{3}H-thymidine pulse-label, late in infection, predominantly labelled double-stranded DNA molecules, with single-stranded regions, suggesting that they were true replicative intermediates. In a similar study (Rhode, 1974a), rapidly-labelled H-1 DNA was shown to consist of molecules which sedimented faster than unit length duplex RF DNA in neutral sucrose gradients, while Siegl and Gautschi (1976), working with Lu-III, have described a further replicative intermediate which contains single-stranded side chains. This DNA species appears to

represent a concatemeric form of RF, being three times the length of unit length duplex RF. The length(s) of the contributory single-strands, however, remain unknown since this DNA species was not analysed under denaturing conditions.

The separation of the complementary strands of H-1 RF DNA has been effected (Rhode, 1974a, b) by density labelling with BUdR. Since the viral strand has the greater thymidine content, it becomes relatively more dense than the complementary strand, and can be easily separated by isopycnic centrifugation in Cs_2SO_4. When the cells are labelled with ^{3}H-BUdR late in infection and the RF DNA strands separated, there is an asymmetric distribution of ^{3}H-BUdR, predominating in the viral strand. These data are consistent with the conservation of unsubstituted complementary strands in the RF pool, with displacement of viral strands to yield single-stranded progeny DNA (Rhode, 1974b). A model of this type was proposed by Tattersall et al. (1973) for MVM DNA replication.



1.6.5 Viral RNA Synthesis

Among the non-defective parvoviruses RNA synthesis has been studied for RV (Salzman and Redler, 1974). Viral RNA is synthesised as early as 2 hr after infection, and only two species appear to be present. The predominant species has a molecular weight of about 7×10^5 daltons, accounting for 40 - 50% transcription of the single-

stranded genome, while a small amount of higher molecular weight viral RNA, possibly representing transcription of the entire genome, is also synthesised. Whether the complementary strand of the RV RF DNA is transcribed is unknown.

AAV RNA synthesis has been studied in greater detail. AAV-2 RNA is first detected 10 - 12 hr after infection (Rose and Koczot, 1971) and appears to consist of a single species, of molecular weight 9×10^5 daltons (Carter and Rose, 1972; Carter and Rose, 1974; Carter, 1974), as shown by sedimentation in sucrose-DMSO gradients and electrophoresis in polyacrylamide gels containing 98% formamide.

This stable RNA species is transcribed wholly from the (-) DNA strand and represents about 70% of its entire length (Rose and Koczot, 1971; Carter and Rose, 1972; Carter et al., 1972). The location and direction of transcription has been determined on the physical map (Carter et al., 1975) and it appears that the self-complementary regions (see section 1.5.4) are not transcribed (Carter et al., 1972). The RNA contains polyA (Rose, 1974) and, in contrast to adenovirus RNA, is not cleaved after synthesis (Carter and Rose, 1974).

The AAV message, therefore, may only specify coat protein(s) since its size is about that required to code for the largest structural polypeptide (see section 1.5.7).

1.6.6 Viral Protein Synthesis

The direct detection of parvovirus-specific proteins within infected cells is often hampered by high background levels of cellular protein synthesis. For this reason viral protein synthesis has mostly been studied using FA staining techniques, and by following the formation of viral haemagglutinin in infected cells.

It has been shown that the major capsid component of RV (protein

B) strongly haemagglutinates (Salzman and White, 1970), and haemagglutinin therefore probably represents capsid proteins.

Using FA staining, early cytoplasmic antigen can be detected in HER-, KBSH- and Lu-III-infected cells at 4 - 6 hr after infection, which is before the onset of DNA synthesis. Late nuclear antigen appears 8 - 10 hr after infection (Cole and Nathanson, 1969; Siegl et al., 1972; Siegl and Gautschi, 1973a). In contrast, cytoplasmic antigen is first detected in RV-infected cells at 8 hr post-infection (Fields and Nicholson, 1972) and in MVM-infected cells at 14 hpi, 2 hr after nuclear antigen can be detected (Parker et al., 1970a). As HER, RV and Lu-III infection proceeds, the number of cells with cytoplasmic antigen decreases while the number having nuclear antigen increases. This possibly represents migration of capsid precursors to the nucleus for virus assembly (Cole and Nathanson, 1969; Fields and Nicholson, 1972). As expected the appearance and increase of both nuclear antigen and cell-associated haemagglutinin parallels the rise in infectious titre (Mayr et al., 1968; Siegl and Gautschi, 1973b; Bates and Storz, 1973; Rhode, 1973).

The synthesis of viral protein is probably linked to the synthesis of both cellular and viral DNA (see section 1.6.7) and therefore inhibitors of DNA synthesis completely block the formation of both nuclear antigen and haemagglutinin (Parker et al., 1970a; Siegl and Gautschi, 1973b; Rhode, 1973). Such inhibitors, however, did not block the appearance of early cytoplasmic antigen in Lu-III-infected cells (Siegl and Gautschi, 1973b), but 75% inhibition was produced when RNA synthesis was blocked. Several explanations of these data are possible:-

1) Input single-stranded DNA is transcribed,

2) Early cytoplasmic antigen is host specified and modified, or induced, after infection.

It has been suggested that early cytoplasmic antigen represents antigen from input virus particles.

In any case, the production of this antigen is not dependent on the physiological state of the host cell (Siegl and Gautschi, 1973a).

Kongsvik et al. (1974) have studied the synthesis of H-1-induced proteins in synchronised NB cells. Two virus-specific proteins, corresponding to capsid proteins A and B were detected as early as 8 hr after infection in nuclei from H-1-infected cells.

1.6.7 Physiological Requirement for Virus Multiplication

Experiments using infected synchronised cells have indicated that multiplication of non-defective parvoviruses requires one or more cellular function(s) expressed during the S or G2 periods of the cell cycle (Tennant et al., 1969; Hampton, 1970; Tennant and Hand, 1970; Tattersall, 1972b; Siegl and Gautschi, 1973a; Rhode, 1973). It has been concluded that the required cell function(s) is not expressed before late S phase (Hampton, 1970; Tattersall, 1972b; Rhode, 1973; Siegl and Gautschi, 1973a).

Rhode (1973) has shown that initiation of DNA synthesis, on which subsequent H-1 viral haemagglutinin synthesis is dependent (HA-DNA), occurs more than 5 hr after the onset of S phase. It has been suggested that HA-DNA may represent parental RF (Rhode, 1974a). Since initiation of HA-DNA probably coincides with that of progeny DNA synthesis, and occurs late in S phase, it seems likely that one or more components of the cell DNA synthesising apparatus are utilised for the replication of viral DNA. Since S phase alone is not sufficient for initiation of viral DNA synthesis (Rhode, 1973), Rose (1974) has

further speculated that the required cell factor(s) may be involved in regulating the synthesis of a specific portion of the cell DNA, which is made only in late S phase.

In a study involving the use of inhibitors, Rhode (1974b) showed that initiation of RF replication requires RNA and protein synthesised in late S phase, but that concomitant protein synthesis is not required for the continuation of RF synthesis. In a subsequent study (Rhode, 1976) involving temperature-sensitive H-1 mutants, it was concluded that H-1 requires one of the capsid proteins for the synthesis of single-stranded progeny DNA.

1.6.8 Effects of Parvovirus Infection on Cellular Functions

It has been shown that FPV, RV, Lu-III and MVM are potent inhibitors of cell mitosis (Johnson, 1967; Hampton, 1970; Tennant, 1971; Siegl and Gautschi, 1973a; Tattersall et al., 1973).

After RV infection, inhibition of mitosis can be detected within 2 hr and occurs even in cells which do not immediately synthesise virus (Tennant, 1971). The inhibition of mitosis appears to depend on the time of infection, in relation to the cell cycle. Using synchronised cells, Siegl and Gautschi (1973a) found that after infection with Lu-III in early S phase mitosis was completely prevented, whereas late S phase infection only delayed its onset. Infection with UV-inactivated virus in early S phase only caused delayed mitosis. These results indicate that capsid protein alone may be capable of delaying cell division, but that for complete inhibition, viral DNA synthesis may be necessary.

The effects of viral infection on cellular DNA synthesis have been studied for RV and MVM. The results, however, are conflicting. Following RV infection, total DNA synthesis in rat nephroma cells was

increased between 10 - 24 hr post-infection (Salzman et al., 1972) but was decreased in rat embryo cells during the same period (Tennant, 1971). Infection of rapidly-dividing mouse embryo cells with MVM did not greatly affect their rate of DNA synthesis (Tattersall, 1972b) while a slight depression of total DNA synthesis was observed in A9 cells late in infection (Tattersall et al., 1973).

The effect of RV infection on total RNA and protein synthesis was not pronounced (Tennant, 1971; Salzman et al., 1972) but a selective decrease in 28S RNA and a stimulation of 4S RNA has been observed following H-1 infection of NB cells (Fong et al., 1970).

Aim of the Project

As stated in the Introduction, the precise nature of the viral DNA intermediates in parvovirus-infected cells, and the sequence of events in the DNA replication process are not yet clear.

The aim of the present work is to further characterise the intracellular forms of MVM DNA and to determine their relationship to one another, with a view to understanding the steps involved in viral DNA replication and viral assembly.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Virus

MVM was plaque-purified (Tattersall, 1972b) from the original isolate described by Crawford (1966), and supplied by Dr. P. Tattersall, Department of Molecular Virology, Imperial Cancer Research Fund Laboratories, London.

2.1.2 Cell Cultures

The cultures used were either BHK-21/C13 cells, a continuous line of baby hamster kidney fibroblasts (Macpherson and Stoker, 1962), or A9 cells, a derivative of the mouse L cell line (Littlefield, 1964).

2.1.3 Radiochemicals

All isotopically labelled compounds were obtained from the Radiochemical Centre, Amersham, England.

[6 - ^{3}H] thymidine	27 Ci/m mole
[2 - ^{14}C] thymidine	57 mCi/m mole
^{32}P - orthophosphate	10 mCi/ml

2.1.4 Chemicals for Liquid Scintillation Spectrometry

The chemicals for liquid scintillation spectrometry were obtained as follows:-

2,5 diphenyloxazole (PPO)	Koch-Light Laboratories Ltd., Colnbrook, England.
Toluene (AR grade)	"
Triton - X - 114	"
p-Bis (o-methylstyryl) benzene (BIS-MSB)	Kodak Ltd.

Hyamine hydroxide (1 M solution in methanol)	Nuclear Enterprises, Edinburgh

2.1.5 Enzymes

Pronase (B grade)	Calbiochem, Los Angeles, California.
Deoxyribonuclease-I (DNase-I)	Sigma Chemical Co., London.
Ribonuclease-A (RNase-A)	"
Neuraminidase (Type V)	"
Nuclease S_1 from Aspergillus oryzae	Miles Laboratories Inc., Stoke Poges, England.
Endonuclease Eco.R_1	"

2.1.6 Nucleic Acids

Calf-Thymus DNA	Sigma Chemical Co., London.

^{32}P-labelled Hela cell DNA was kindly provided by Mr. M. J. Browne, Department of Biochemistry, University of Glasgow.

^{14}C-thymidine labelled and unlabelled SV40 DNA were kindly provided by Dr. R. Eason, Department of Biochemistry, University of Glasgow.

2.1.7 Other Materials

All other chemicals were, wherever possible, 'AnalaR' reagents, supplied by B.D.H. Chemicals Ltd., Poole, Dorset, except for the following:-

Foetal calf serum	Biocult Laboratories Ltd., Paisley, Scotland.
Amino acids	"
Vitamins	"
Penicillin	Glaxo Pharmaceuticals, London.

Streptomycin	Glaxo Pharmaceuticals, London.
Sephadex G25 (medium)	Pharmacia, Uppsala, Sweden
CsCl	Hopkin & Williams Ltd., Chadwell Heath, Essex.
Agarose	Sigma Chemical Co. London
Thymidine	"
2'-deoxycytidine	"
Sodium deoxycholate	"
Ethidium bromide	Calbiochem, Los Angeles, California.
Plastic petri dishes	Nunc. Ltd., Denmark.
Whatman 3 MM 2.5 cm discs	H. Reeve-Angel & Co. Ltd., London.
Nonidet-NP40	Shell Chemical Co. Ltd.,
Triton-X-100	Packard Instrument Co. Inc., Downers Grove, Illinois.
Formvar-Carbon Coated Grids (3 mm)	LKB - Produkter, Stockholm, Sweden.
20% palladium - 80% platinum wire	Johnson Matthey Metals Ltd., London.

2.2 STANDARD SOLUTIONS

2.2.1 Cell Culture Solutions

A) Balanced Salt Solution (BSS). Earle (1943) BSS consisted of 0.116 M NaCl, 5.4 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$ and 0.002% (w/v) phenol red. The pH of the solution was adjusted to 7.0 with 8.4% (w/v) $NaHCO_3$. Used in preparation of (B).

B) Eagle's Minimal Essential Medium (MEM), Modified

A modification of Eagle's medium (Busby, House and MacDonald, 1964) was used (Table 2.1).

C) EFc10 90% Modified Eagle's MEM + 10% foetal calf serum.

EFc5 95% Modified Eagle's MEM + 5% foetal calf serum.

D) Phosphate Buffered Saline (PBS)

PBS was composed of 3 separate solutions mixed immediately before use:-

PBS (a)	0.17	M	NaCl	
	3.4	mM	KCl	
	10	mM	Na_2HPO_4	
	2.4	mM	KH_2PO_4	pH 7.2
PBS (b)	6.8	mM	$CaCl_2$	
PBS (c)	5.0	mM	$MgCl_2$	

The ratio of mixing was PBS (a) 8

(b) 1

(c) 1

2.2.2 Enzymes

Pronase

Pronase (B grade), 20 mg/ml in 0.02 M tris-HCl, 0.02 M EDTA

Table 2.1

Eagle's Minimal Essential Medium (MEM), as used in the Department of Biochemistry, University of Glasgow.

MEM amino acids	mg/litre
L-arginine	126.4
L-cystine	24.0
L-glutamine	292.0
L-histidine-HCl	38.3
L-isoleucine	52.5
L-leucine	52.5
L-lysine	73.1
L-methionine	14.9
L-phenylalanine	33.0
L-threonine	47.6
L-tryptophan	10.2
L-tyrosine	36.2
L-valine	46.9

MEM vitamins	mg/litre
D-calcium pantothenate	2.0
choline chloride	2.0
folic acid	2.0
i-inositol	4.0
nicotinamide	2.0
pyridoxal HCl	2.0
riboflavin	0.2
thiamine HCl	2.0

Inorganic Salts and Other Components

	mg/litre
$CaCl_2\ 6H_2O$	393.0
KCl	400.0
$MgSO_4\ 7H_2O$	200.0
NaCl	6800.0
$NaH_2PO_4\ 2H_2O$	140.0
glucose	4500.0
$NaHCO_3$	2240.0
phenol red	15.3
streptomycin	100.0
penicillin	100,000 units/litre

pH 8.6 was heated to 80°C for 5 min and autodigested for 6 hr at 37°C to destroy latent DNase activity. It was stored at -20°C.

S_1 Nuclease

S_1 nuclease 320 units/ml, in 0.03 M NaCl, 0.03 M sodium acetate, 3×10^{-4} M $ZnSO_4$ pH 4.5, containing 50% glycerol, was stored at -20°C.

Endonuclease R.Eco.R_1

Endo. R.Eco.R_1, 2.5×10^3 units/ml, in 0.01 M KPO_4, 7 mM 2-Mercaptoethanol, 1 mM EDTA, 0.2 M NaCl, 0.2% (v/v) NP40, 0.01% (w/v) gelatin pH 7.0 was stored at -20°C.

2.2.3 Buffers

"E Buffer" (Hayward, 1972)

"E Buffer", for agarose gel electrophoresis, was 0.03 M tris-HCl, 0.036 M NaH_2PO_4, 0.001 M EDTA pH 7.7.

SSC

0.15 M NaCl, 0.015 M Sodium citrate pH 7.0.

2.2.4 Scintillation Spectrometry Solutions

A) For most experiments a toluene based scintillation fluid was used. This consisted of 0.5% (w/v) PPO in toluene.

B) Triton/toluene scintillation fluid consisted of 0.5% (w/v) PPO, 0.05% (w/v) Bis-MSB, 35% (v/v) triton-X-114 and 65% (v/v) toluene.

2.3 METHODS

2.3.1 Cell Cultures

Monolayer cultures of BHK-21/C13 cells and A9 cells were grown in 80 oz roller bottles according to the method of House and Wildy (1965). The cells were grown in EFc10 at 37°C in an atmosphere of 5% CO_2 in air.

2.3.2 Contamination Checks

The cells were routinely screened for bacterial, fungal or PPLO (mycoplasma) contamination as follows:-

Bacterial Contamination

Aliquots were grown on blood agar plates and heart infusion broth at 37°C. Results were considered negative if no growth was observed after 7 days.

Fungal Contamination

Aliquots were added to Sabouraud's medium and grown at 32°C. No growth in 7 days indicated the absence of fungal contamination.

PPLO Infection

PPLO agar plates were seeded with cells by piercing the agar surface with a charged pasteur pipette. Plates were incubated in an atmosphere of 5% CO_2 in N_2 at 37°C. After 7 days the plates were examined microscopically for the characteristic "fried egg" appearance of PPLO colonies.

2.3.3 Propagation of MVM in A9 cells

A9 cells were seeded in 80 oz roller bottles at 2×10^7 in EFc10. After 24 hr they were infected with MVM at low multiplicity (0.01 pfu/cell) in 15 ml Eagle's medium. Adsorption was allowed to continue for

90 min, after which 100 ml EFc5 was added and the cells grown for 1 week, by which time cpe was advanced. The cells were harvested by shaking into the medium and collected by low speed centrifugation (1,000 g 10 min). Virus stocks were prepared by resuspending the pellet in 5 ml PBS and disrupting the cells by sonic vibration, in an ultrasonic cleaning tank unit (5 min) (Burndept Ltd., Kent). The cell debris was removed by centrifugation at 10,000 g for 30 min and samples of the supernatant assayed for infectivity. The MVM stock used in these experiments had a titre of 10^8 pfu/ml.

2.3.4 Titration of MVM

A9 cells from logarithmically-growing cultures were seeded in EFc10 at 5 x 10^5 in 50 mm plastic petri dishes, and incubated at 37°C for 2 - 3 hr by which time the cells had attached. The medium was removed and the cells infected with 0.2 ml samples of serially-diluted MVM. Adsorption was allowed to continue at 37°C for 90 min, with occasional rocking, after which cultures were overlaid with 5 ml of Eagle's medium containing 5% foetal calf serum in 0.75% Noble agar (overlay medium). After 7 days at 37°C the monolayers were stained by adding 2 ml overlay medium (without serum) containing 0.008% Neutral red. The plaques were counted after further incubation at 37°C for 4 - 6 hr.

2.3.5 Haemagglutination (HA) Assay (Crawford 1966)

Twofold dilutions of virus in 0.2 ml PBS (a) were mixed with an equal volume of 1% guinea-pig red blood cell suspension. The end point of the agglutination was read after incubation for 3 - 4 hr at 4°C. Titres of HA are expressed as haemagglutination units (HAU), defined as the reciprocal of the dilution causing complete agglutination.

2.3.6 Infection of Cells

Logarithmically growing A9 or BHK-21/C13 cells were seeded in EFc10 at 2 x 10^6/dish in 90 mm plastic petri dishes and grown at 37°C in an atmosphere of 5% CO_2 in air for 1 - 2 days, by which time confluence had not been reached. The cells were infected in 1 ml PBS, with MVM at a multiplicity of 5 pfu/cell. After 90 min adsorption at 37°C, the cultures were overlaid with 10 ml EFc5. Control cells were mock-infected with 1 ml PBS.

2.3.7 Pulse-Labelling of Cells

At the appropriate time after infection, the medium was removed and replaced with EFc5 containing ^{3}H-thymidine (10 μCi/ml) or ^{14}C-thymidine (1 μCi/ml).

2.3.8 Pulse-Chase Experiments

After the period of pulse-labelling the monolayers were washed twice with Eagle's medium and overlaid with 10 ml EFc5 containing unlabelled thymidine (100 μg/ml) and 2'-deoxycytidine (10 μg/ml) and further incubated at 37°C.

2.3.9 (a) Preparation and Purification of MVM Virus

Sub-confluent cultures of A9 cells in 80 oz roller bottles were infected with MVM at a multiplicity of 0.01 pfu/cell and grown in EFc5 for 1 week, by which time cpe was pronounced. For preparation of radioactively labelled virus the medium contained either ^{3}H-thymidine (10 μCi/ml) or ^{14}C-thymidine (2 μCi/ml) at a final thymidine concentration of 4 x 10^{-6} M, or ^{32}P-orthophosphate (10 μCi/ml). The cells were harvested by scraping into the medium and collected by low speed centrifugation (1,000 g for 10 min). The cell pellet was

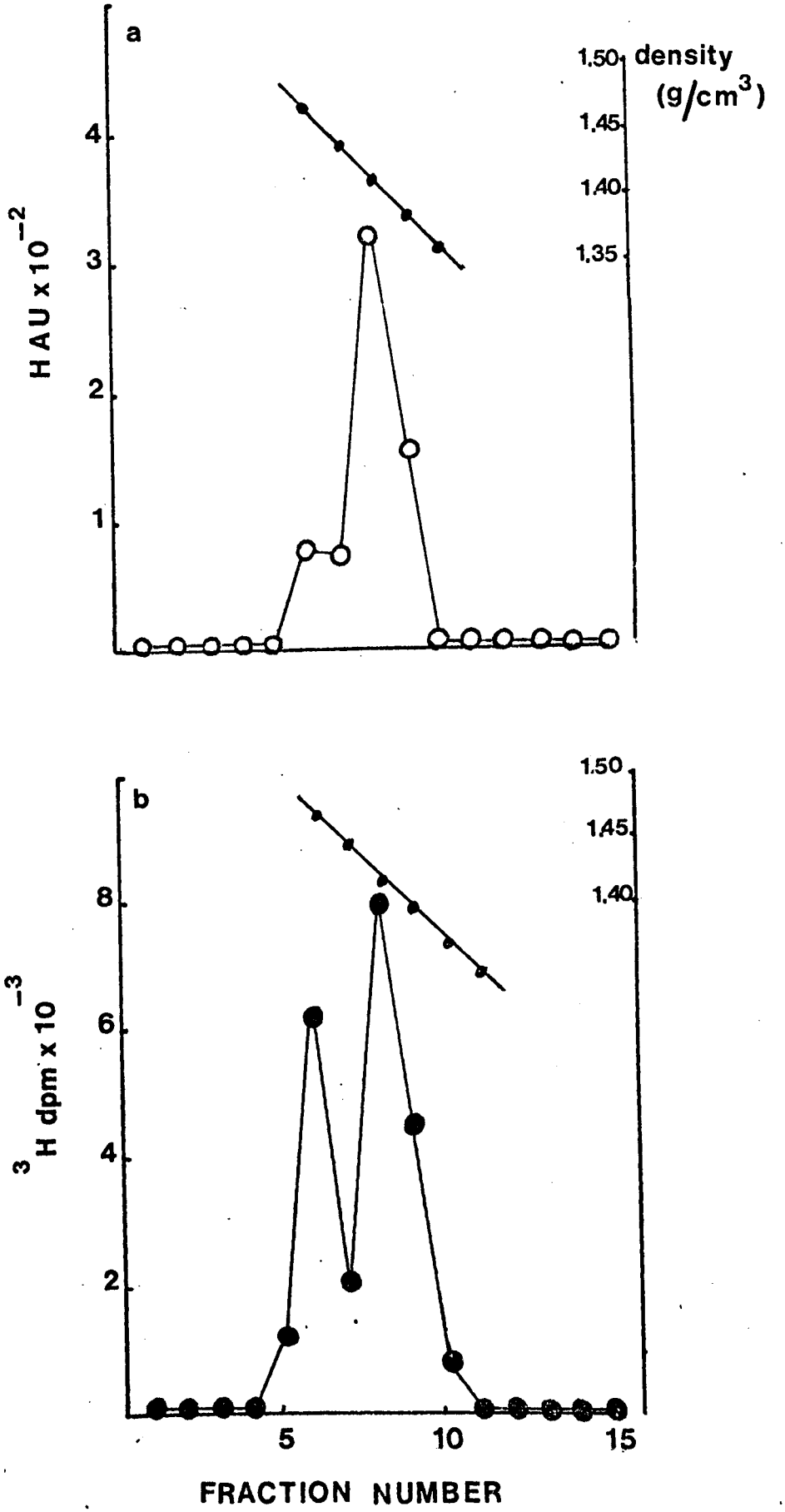
resuspended in 5 ml PBS (a) and disrupted by sonic vibration for 5 min. The suspension was treated with sodium deoxycholate (0.25%), neuraminidase (0.5 μg/ml), DNase-I (50 μg/ml) and RNase-A (100 μg/ml) for 1 hr at 37°C. After removal of cellular debris by centrifugation (10,000 g for 30 min) 1.5 ml aliquots of supernatant were layered on to 4 ml CsCl solution (density 1.37 g/cm^3) in 0.05 M tris-HCl pH 7.5, and centrifuged for 16 hr at 100,000 g (average) in a Spinco SW50 rotor at 20°C. Fractions were collected by direct tube puncture and the refractive index of each determined using an Abbe refractometer. Density was calculated using the relationship of Ifft et al. (1961). Fractions of density greater than 1.4 g/cm^3 were pooled and 1 ml samples mixed with 3 ml CsCl solution containing 0.05 M tris-HCl pH 7.5. The density was adjusted to 1.4 g/cm^3 and the samples centrifuged at 100,000 g (average) for 20 hr at 20°C in a Spinco SW50L rotor. Fractions were collected by direct tube puncture and their HA titres and densities determined. Virus banding at 1.42 g/cm^3 was desalted into 0.01 M tris-HCl pH 7.5 and stored at -20°C.

A typical profile of ^{3}H-thymidine labelled virus is shown in Fig. 2.1. The major viral species bands at a density of 1.42 g/cm^3 and this virus has been used throughout the present study. The minor "heavy" species, which occurs at a density of 1.47 g/cm^3 has about a fourfold lower HA/^{3}H-thymidine ratio. This species has been described in detail (see section 1.5.2; Clinton and Hayashi, 1975).

2.3.9 (b)

MVM was also purified by the method of Tattersall (personal communication, 1975). Infected cells (as in 2.3.9 a) were washed in hypotonic buffer (0.05 M tris-HCl, 0.005 M EDTA pH 8.7) and resuspended

Fig.2.1


a
HAU x 10^{-2}
4
3
2
1
1.50
1.45
1.40
1.35
density
(g/cm^3)
b
^{3}H dpm x 10^{-3}
8
6
4
2
1.50
1.45
1.40
5
10
15
FRACTION NUMBER

in 5 ml of the same buffer. The cells were disrupted by homogenisation using a Potter-Elvehem homogeniser with a close-fitting teflon pestle and glass tube (Sireica, Jamaica, New York). The suspension was checked for cell breakage by phase-contrast microscopy and re-homogenised if necessary. Nuclei were removed by low speed centrifugation (800 g for 10 min), followed by centrifugation at 10,000 g for 30 min to remove cell debris. $CaCl_2$ was added to a final concentration of 0.025 M and the solution stored on ice for 16 hr. 3.5 ml samples were layered on top of a 0.5 ml cushion of CsCl (density 1.7 g/cm^3) and centrifuged at 100,000 g for 3 hr at 20°C. Material from the interphase was collected, desalted into 0.001 M tris-HCl, pH 7.4, and $MgCl_2$ added to a final concentration of 0.005 M. The solution was incubated for 1 hr at 37°C in the presence of DNase-I (50 µg/ml) and RNase-A (100 µg/ml). 1 ml samples of virus suspension were centrifuged to equilibrium in CsCl solution (average density 1.4 g/cm^3) as in (a). The virus band at 1.42 g/cm^3 was collected and desalted into 0.05 M tris-HCl, 0.005 M EDTA pH 8.7 and stored at -20°C. This method yielded greater amounts of purified virus than method (a).

2.3.10 Desalting

Samples of virus and DNA (0.3 - 0.5 ml) were routinely desalted by gel filtration using a 180 mm x 6 mm Sephadex G25 (medium) column. The column was calibrated by passing through a mixture of blue dextran and potassium chromate to ensure complete separation. Before use the column was equilibrated with the appropriate buffer.

Virus was detected by measuring extinction at 260 nm or by assaying HA activity. Labelled DNA was detected by assaying 5 µl

samples of each fraction for acid-insoluble radioactivity, and unlabelled DNA by measuring the extinction at 260 nm.

2.4 BIOCHEMICAL TECHNIQUES

2.4.1 Fractionation of Infected Cell DNA

a) Hirt/Pronase Method

Viral DNA was selectively extracted using a modification of the Hirt (1967) procedure (Tattersall et al., 1973). Infected cells from duplicate 90 mm plastic petri dishes were washed with 0.02 M tris-HCl, 0.15 M NaCl, 0.01 M EDTA pH 7.5, resuspended in 1 ml of the same buffer, and added carefully to an equal volume of 1.2% SDS in 0.02 M tris-HCl, 0.01 M EDTA pH 7.5. Self-digested pronase was then added to a final concentration of 2 mg/ml and the lysate incubated for 1 hr at 37°C. After digestion, 5 M NaCl was added to a final concentration of 1 M and mixed in, by gently inverting the tube 10 times. After standing on ice for at least 8 hr, the lysate was centrifuged at 27,500 g for 30 min at 0°C in a Sorvall SS-34 rotor and the supernatant fluid (Hirt/Pronase extract) removed and stored on ice. The pellet was washed with 70% ethanol in 0.1 x SSC and solubilised by adding 1 ml of 1 N NaOH and heating to 70°C for 1 hr. Samples of supernatant material and solubilised pellet were spotted on Whatman 3 MM discs and assayed for acid-insoluble radioactivity as described below.

b) Hirt Method

Low molecular weight DNA was extracted as in (a), apart from the fact that no pronase was added during the incubation period of 1 hr at 37°C.

c) Whole Cell Lysate Method

Total labelled DNA from infected cells was analysed using a

modification of the method used by Cheevers et al. (1972).

Duplicate 90 mm dishes of A9 cells were washed twice with SSC and resuspended in a volume of 1.8 ml SSC/dish. 0.18 ml samples of cell suspension were gently mixed with 20 µl of 10% SDS in SSC. After 15 min at 20°C the DNA extracts were layered onto a 3.6 ml linear sucrose density gradient (15 - 30% (w/v) in 0.25 M NaCl, 0.001 M EDTA, 0.01 M tris-HCl, pH 7.4 containing 0.5% SDS) formed over a 0.2 ml cushion of 70% sucrose. The gradients were centrifuged in a Spinco SW56 rotor at 220,000 g (average) for 3 hr at 25°C. The gradients were harvested from the top using an MSE gradient harvester. 6 drop fractions were collected on Whatman 3 MM discs and acid-insoluble radioactivity determined.

d) Triton-X-100 method Green at al. (1971)

Duplicate 90 mm dishes of infected cells were washed twice with PBS and 1 ml of 0.25% triton-X-100 in 0.01 M tris-HCl, 0.01 M EDTA pH 7.9 added to each dish. After incubation at 20°C for 1 hr NaCl was added to a final concentration of 0.2 M. The resulting lysates were carefully scraped into centrifuge tubes and centrifuged at 1500 g for 30 min at 4°C. The supernatant fluid (triton extract) was stored on ice. The pellet was solubilised as in (a) and aliquots of both supernatant material and solubilised pellet assayed for acid-insoluble radioactivity.

2.4.2 Preparation of Viral DNA

MVM DNA was prepared from purified virus by the method of Koczot et al. (1973). Samples of purified virus were made 0.3 N with respect to NaOH and layered directly on to a 5 - 20% (w/v) linear sucrose gradient containing 0.3 N NaOH, 0.7 M NaCl, 0.001 M EDTA. Centrifugation

was carried out in pollyallomer tubes in a Spinco SW56 rotor for 5 hr at 120,000 g (average) at 4 °C. This method has been shown to produce minimal strand breakage (Koczot et al., 1973). Fractions from the gradient containing DNA were pooled and desalted into 0.01 M tris-HCl, 0.01 M EDTA pH 7.4 and stored at -20°C.

The concentration of DNA was estimated by measuring the extinction at 260 nm, assuming an E260 nm of 1 corresponds to 28 μg/ml which has been found to be characteristic for the single-stranded DNA of bacteriophage ϕ X 174 (Sinsheimer, 1959).

2.4.3 Centrifugation Techniques and Isotope Measurements

a) Velocity Sedimentation

Neutral sucrose (high ionic strength)

5 - 20% (w/v) linear sucrose gradients were prepared containing 0.01 M tris-HCl, 0.01 M EDTA, 1 M NaCl pH 7.9.

Neutral sucrose (low ionic strength)

5 - 20% (w/v) linear sucrose gradients were prepared containing 0.001 M tris-HCl, 0.001 M EDTA, pH 7.9.

Alkaline sucrose

Samples were made 0.25 N with respect to NaOH and, after 15 min at 20°C, were layered on to a 5 - 20% (w/v) linear sucrose, and 0.25 - 0.5 N NaOH gradient containing 1 M NaCl, 0.001 M EDTA at a final pH of greater than 12.5.

The volumes of gradients and sample were as follows:-

Spinco SW56 Ti	Gradient	3.6 ml	Sample	0.2 ml
Spinco SW50 L		4.8 ml		0.2 ml

The conditions under which the velocity sedimentation runs were carried out are detailed in the legend to each figure. Approximate

sedimentation coefficients were calculated by comparison of the distance sedimented relative to a marker DNA (Burgi and Hershey, 1963).

b) Equilibrium CsCl

A 0.2 ml sample of DNA was added to 3 ml CsCl solution in 0.05 M tris-HCl, 0.002 M EDTA pH 7.4. The final density of the solution was 1.70 g/cm^3. Centrifugation was carried out in a Spinco SW50L rotor at 80,000 g (average) for 60 hr at 20°C.

All analytical gradients were harvested by direct tube puncture and fractions collected on 2.5 cm Whatman 3 MM filter discs. The discs were washed three times in ice-cold 5% trichloroacetic acid (10 ml per disc) and dried in ethanol and ether. 0.5 ml hyamine hydroxide was added to each disc in a scintillation vial and, after incubation at 60°C for 20 min, 5 ml of toluene/PPO scintillator was added. Radioactivity was determined in a Philips liquid scintillation spectrometer.

For preparative gradients, fractions were collected in test-tubes and 10 µl samples spotted on to discs and acid-insoluble radioactivity determined as above. Pollyallomer tubes were used at all times.

2.4.4 Agarose Gel Electrophoresis of DNA (Hayward, 1972)

Electrophoresis of low molecular weight DNA was performed in 1.5% (w/v) agarose gels set in cylindrical perspex tubes of 6 mm internal diameter and 13 cm in length. Agarose was dissolved in "E. buffer", boiled for 10 min, cooled to about 40°C, and poured into the tubes. After allowing the gels to set, the top few mm were cut off to ensure a level surface. The lower ends of the tubes were covered with perforated dialysis tubing to prevent the gels from

sliding out. Electrophoresis was carried out for 2 - 3 hr at 100 V in a vertical tank.

For analysis of native DNA, 50 - 100 µl samples in 0.01 M tris-HCl, 0.01 M EDTA pH 7.9 containing 10% sucrose, were layered on top of the gel column.

For analysis of denatured DNA, samples were made 0.3 N with respect to NaOH for 15 min at 20°C before analysis.

Analysis of Gels Containing Radioactive DNA

After electrophoresis the gels were sliced into 1 mm fractions using a Mickle gel slicer, placed in scintillation vials, and 0.5 ml of hydrogen peroxide (100 volumes) added. The vials were tightly capped and heated at 60°C until the gel slices had completely dissolved (about 12 hr). 5 ml triton/toluene scintillator was added to each vial and radioactivity determined in a Philips liquid scintillation counter.

Staining of Gels (Sharp et al., 1973)

Gels containing unlabelled SV40 DNA were fully immersed in "E. buffer" containing 0.5 µg/ml ethidium bromide. After 30 min the gels were viewed under short-wave UV light and the positions of the bands containing DNA measured.

2.4.5 Cleavage of DNA with Endonuclease R.Eco.R_1

DNA samples were desalted into 0.1 M tris-HCl, 0.05 M NaCl, 0.01 M $MgCl_2$ pH 7.5. 95 µl samples of DNA were mixed thoroughly with 5 µl of endonuclease R.Eco.R_1 solution and incubated for 1 hr at 37°C. As a control, DNA samples were incubated in parallel, but with the enzyme omitted. The reaction was stopped by adding 20 µl of a solution of 5% (w/v) SDS, 25% (v/v) glycerol, after which the samples were

heated to 65°C for 10 min. After thorough mixing 50 µl samples of the reaction products were analysed directly by agarose gel electrophoresis.

To ensure that the enzyme cleavage of MVM DNA went to completion, SV40 form-I DNA was cleaved in a parallel reaction. Under the conditions employed there was quantitative conversion of form-I DNA (supercoil) to form-III DNA (linear).

2.4.6 Digestion of DNA with Nuclease S_1 (Sutton, 1971)

For digestion of DNA with the single-strand specific nuclease S_1, the reaction mixtures contained 0.03 M sodium acetate, pH 4.5, 0.1 M NaCl, 3×10^{-5} M $ZnSO_4$, 16 µg/ml sonicated heat-denatured calf-thymus DNA, 10% glycerol and 40 units of nuclease S_1. Reactions were carried out in duplicate in a volume of 0.5 ml for up to 4 hr at 37°C. The reactions were stopped by chilling in ice-water and, after thorough mixing, two 0.2 ml samples were spotted on Whatman 3 MM discs and acid-insoluble radioactivity determined as before.

Under the conditions employed, about 95% of heat-denatured ^{32}P-Hela cell DNA was rendered acid-soluble while less than 5% of native ^{32}P-Hela DNA or ^{14}C-SV40 DNA was digested, during incubation for 4 hr.

2.4.7 Heat-Denaturation of DNA

Samples of DNA were heat denatured in 0.1 x SSC by heating at 100°C for 5 min and quench cooling on ice-water.

2.4.8 Displacement-Hybridisation

Samples of labelled DNA were mixed with known amounts of unlabelled MVM DNA from purified virus in 0.1 x SSC, and heat-denatured in sealed glass tubes in a volume of 100 µl. 5 M NaCl was added to a final

concentration of 0.3 M and reannealing allowed to take place at 65°C for 60 hr. After reannealing, the samples were digested with S_1 nuclease for 4 hr at 37°C in a final volume of 0.5 ml, as previously described. After S_1 digestion, two 0.2 ml samples were spotted on Whatman 3 MM discs and acid-insoluble radioactivity determined. The results are expressed as the percentage of labelled DNA rendered acid-soluble after S_1 digestion.

2.4.9 Electron Microscopy

Samples of purified MVM virus and 95S "complex", purified by neutral sucrose gradient centrifugation were desalted into 0.01 M tris-HCl pH 7.5 and spotted on to Formvar-carbon coated grids. Excess liquid was drained using filter paper and the grids dried by successive washing in 50%, 75% and 90% ethanol. The grids were rotary shadowed with 20% palladium - 80% platinum wire using an A.E.I. vacuum coating unit and viewed in an A.E.I. EM6B electron microscope.

3. RESULTS

3.1 COMPARISON OF DNA EXTRACTION PROCEDURES

3.1.1 Yields of Extracted DNA

In a preliminary experiment, several different DNA extraction procedures were compared, both for total yield of DNA and species of DNA extracted from virus-infected cells.

Monolayers of A9 cells were infected with MVM at a multiplicity of 5 pfu/cell and labelled with ^{3}H-thymidine from 18 - 22 hpi, after which they were extracted using the following procedures, which are described in detail in the Methods section.

(1) The Hirt Method

(2) The Hirt/Pronase Method

These methods are based on the fact that host cell DNA is selectively precipitated by SDS at $0^{o}C$ in the presence of 1 M NaCl, leaving low molecular weight DNA in the supernatant fraction. A major limitation of these procedures is that some DNA is discarded, in the form of a pellet, before analysis. It is possible, therefore, that certain species of viral DNA, which may be intermediates in the replication process, do not appear in the Hirt supernatant. The following extraction procedure was therefore included as a control.

(3) Whole-cell lysate Method

This involves direct lysis of infected cells using 1% SDS and immediate analysis of the whole-cell lysate by neutral sucrose gradient centrifugation. Since no DNA is discarded, this method has the power to analyse total labelled intracellular DNA, from infected cells.

(4) Triton-X-100 Method, which involves solubilisation of the cytoplasmic and nuclear membranes with the non-ionic detergent Triton-X-100, and subsequent leeching out of low molecular weight DNA. This procedure was included since DNA-protein interactions may be preserved (White and Eason, 1971; Green et al., 1971), yielding viral DNA in the form of nucleoprotein complexes.

Table 3.1 shows the relative amounts of acid-insoluble ^{3}H-thymidine extracted by these methods. It can be concluded that the most efficient procedures are the Hirt/Pronase and whole-cell lysate methods. Omission of the pronase digestion step from the Hirt method effectively halves the yield of low molecular weight DNA. The non-ionic detergent, triton-X-100 is the least efficient. Samples of DNA extracted by each method were analysed by velocity sedimentation in neutral sucrose gradients.

3.1.2 Analysis of Extracted DNA

The DNA species extracted by the four different methods were compared, using neutral sucrose gradient centrifugation.

0.2 ml samples of DNA extracted by the Hirt and Hirt/Pronase methods were mixed with ^{14}C-SV40 form-I DNA (21S) and analysed by centrifugation in 5 - 20% linear sucrose gradients containing 1 M NaCl. The results are shown in Figs. 1 a and b. In each case there is a major peak of DNA, with a sedimentation coefficient of about 15S at peak maximum. In addition, faster-sedimenting DNA is present. Since the two profiles are almost identical, it seems likely that the same species of low molecular weight DNA are extracted by both methods, the only difference being the higher yield of DNA obtained using pronase digestion.

Table 3.1. Comparison of DNA Extraction Procedures

Extraction Procedure	Relative amount of acid-insoluble ^{3}H-thymidine extracted/culture
Hirt/Pronase	1.0
Hirt	0.5 - 0.65
Whole Cell Lysate	0.8 - 0.9 †
Triton-X-100	0.3 - 0.4

Duplicate cultures of A9 cells were infected with MVM (5 pfu/cell), labelled with ^{3}H-thymidine (5 μCi/ml) from 18 - 22 hpi and extracted by the Hirt/Pronase, Hirt, Whole Cell lysated or triton method, as described in Methods section. 0.1 ml samples of each extract (excluding whole cell) were assayed for acid-insoluble radioactivity and total yields per culture calculated.

† Calculated by comparison of radioactivity incorporated into low molecular weight DNA in Hirt/Pronase and whole cell extracts as determined by velocity gradient centrifugation.

Results of two separate experiments are expressed as a fraction of the Hirt/Pronase yield.

Fig. 1

Sedimentation analysis of DNA extracts from MVM-infected and mock-infected A9 cells.

Preparation of the extracts is described in the legend to Table 3.1. Centrifugation of Hirt (a), Hirt/Pronase (b) and triton (f) extracts on 5 - 20% neutral sucrose gradients containing 1 M NaCl was for 5 hr at 120,000 g (average) in a Spinco SW56 rotor at 4°C.

Centrifugation of triton extract (e) in a 5 - 20% neutral sucrose gradient containing 1 M NaCl was for 2 hr at 100,000 g (average) in a Spinco SW50L rotor at 4°C.

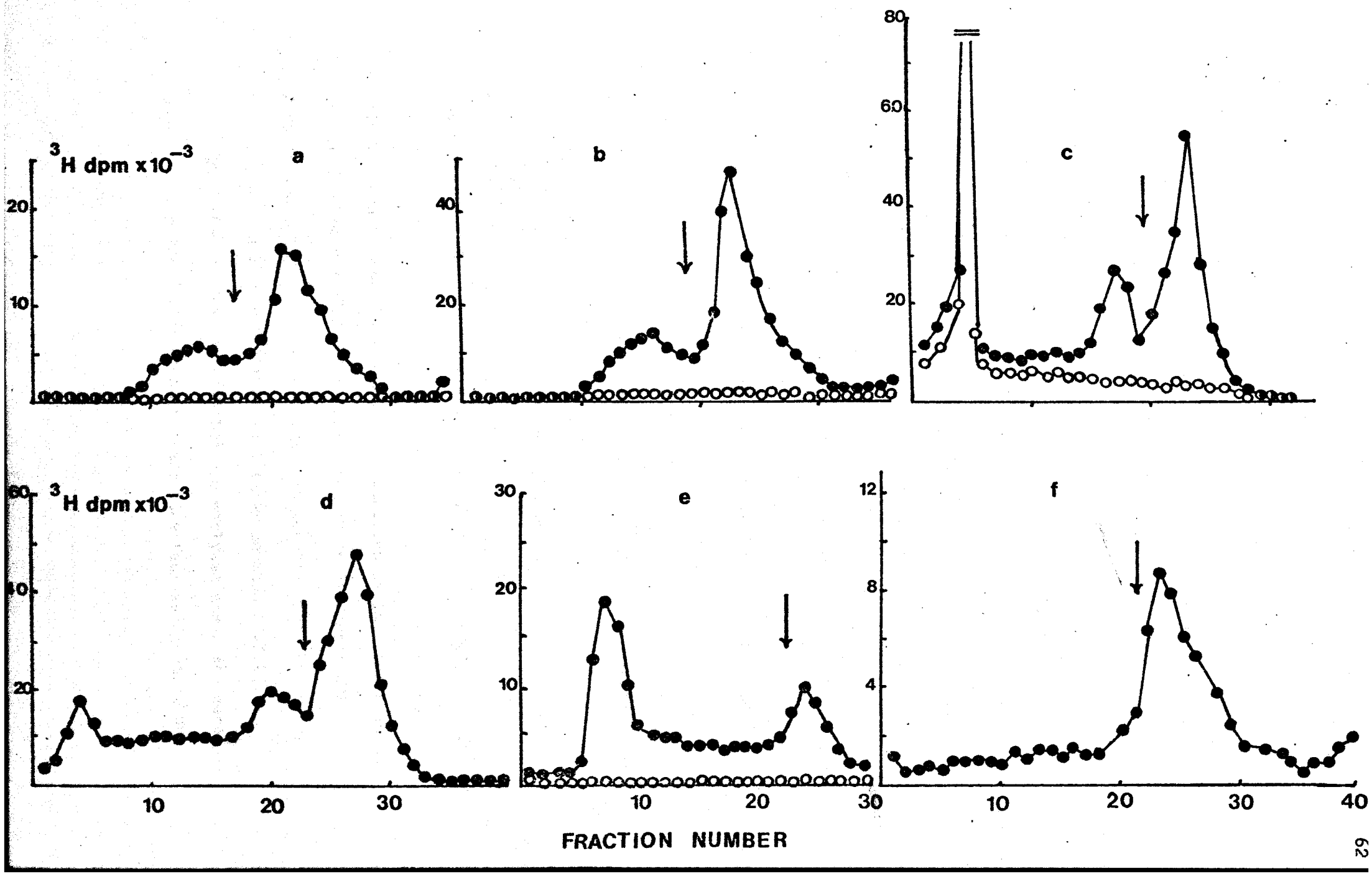
Centrifugation of whole cell lysate (c) and Hirt/Pronase (d) extracts on 15 - 30% sucrose gradients containing 0.5% SDS was for 3 hr at 220,000 g (average) in a Spinco SW56 rotor at 25°C.

The vertical arrows denote the position of ^{14}C-SV40 form-I (21S) DNA in the same gradient.

●—●—● Infected

o—o—o Mock-infected.

Unless otherwise stated, all 5 - 20% neutral sucrose gradients used throughout this work were of high ionic strength, as described in Materials and Methods.


^{3}H dpm x10^{-3}
a
b
c
d
e
f
FRACTION NUMBER

Comparison of DNA species obtained by the Hirt/Pronase method from A9 and Cl3 cells was hampered by the high level of cellular DNA contamination in Cl3 cell extracts. This was true for both virus-infected and control cells.

0.18 ml of infected A9 cell suspension in SSC was mixed gently with 20 µl of 20% SDS in SSC. ^{14}C-SV40 form-I DNA was added and the sample analysed by centrifugation in a 15 - 30% neutral sucrose gradient containing 0.5% SDS (Fig. 1c). As a control a 0.2 ml sample of DNA extracted by the Hirt/Pronase method was similarly analysed (Fig. 1d). It can be seen that the two methods extract similar species of low molecular weight DNA. The major species sediments around 15S while a distinct peak in the 24S region of the gradient can be detected. The ratio of these low molecular weight species of DNA, however, appears to be different in each extract. The whole-cell lysate method extracts more of the minor fast-sedimenting component than the Hirt/Pronase method, indicating the preferential loss of this species in the Hirt/Pronase pellet.

When triton-extracted DNA was analysed on 5 - 20% sucrose gradients (Fig. 1e), the major DNA-containing species has a sedimentation coefficient of ~95S (calculated by comparison with MVM virus, see section 3.4.2b), while the minor component sediments slightly behind SV40 form-I DNA. The position of this DNA species in relation to the SV40 DNA varied slightly in different DNA preparations, sometimes sedimenting coincidently with the marker. This feature will be considered in a later section (3.4.2).

When the triton-extracted DNA was analysed under the same conditions as the Hirt/Pronase extract, the fast-sedimenting DNA

species pelleted. Figure 1f shows the results of such analysis. When compared to the profile obtained with Hirt/Pronase extracted DNA, it can be seen that there is a much smaller proportion of DNA sedimenting faster than 21S. It can be concluded that this fast-sedimenting DNA in Hirt/Pronase extracts is either tightly bound within the cell, or is present in some other form since it is not released by the triton method.

Further analysis of these triton-extracted DNA species is left until a later section (3.4).

Since none of the above-mentioned species of DNA is present in extracts from mock-infected cells (Fig. 1), it can be concluded that each one is virus-induced.

3.2 TIME COURSE OF DNA SYNTHESIS IN MVM-INFECTED AND MOCK-INFECTED CELLS

3.2.1 Rate of DNA Synthesis

In order to determine the overall rate of DNA synthesis, and the temporal order of virus-induced DNA species, virus-infected (5 pfu/cell) and mock-infected A9 cultures were pulse-labelled with ^{3}H-thymidine in regular 2 hr intervals up to 24 hpi. At the end of each pulse-label the monolayers were subjected to the Hirt/Pronase method of extraction.

Aliquots of both supernatant material and solubilised pellet were assayed for acid-insoluble radioactivity as described in the Methods section, and total yields of ^{3}H-thymidine incorporated into DNA calculated. By summation of yields from both supernatant and pellet, total ^{3}H-thymidine incorporated into DNA per culture was obtained. Figure 2a illustrates that up to 12 hpi the total amount of radioactivity incorporated into infected cells and controls is identical, while in the 12 - 24 hr time period the amount of ^{3}H-thymidine incorporated into infected cells is slightly depressed compared to mock-infected ones. In two separate experiments the amount of inhibition of total DNA synthesis in infected cells at 24 hpi was found to be 13% and 20%. Tattersall et al. (1973) have suggested that this inhibition of total DNA synthesis in MVM-infected cells late in infection might be due to the fact that the cells do not divide after infection with MVM at a multiplicity of 5 pfu/cell (see introduction 1.6.8).

Figure 2b shows that, while the amount of labelled DNA appearing in the Hirt/Pronase extract of infected cells in the period 0 - 8 hpi

Fig. 2

Incorporation of ^{3}H-thymidine into total DNA, and low molecular weight DNA in MVM-infected and mock-infected cells.

MVM-infected and mock-infected A9 cultures were pulse-labelled with ^{3}H-thymidine (10 μCi/ml) in regular 2 hr intervals up to 24 hpi and low molecular weight DNA extracted by the Hirt/Pronase method. 0.1 ml samples of supernatant material and solubilised pellet were assayed for acid-insoluble radioactivity as described in Materials and Methods. Yields of total and supernatant acid precipitable ^{3}H-thymidine incorporated per culture were calculated. Cumulative amounts of acid-insoluble ^{3}H-thymidine incorporated at various times after infection were obtained by summation of yields from successive cultures.

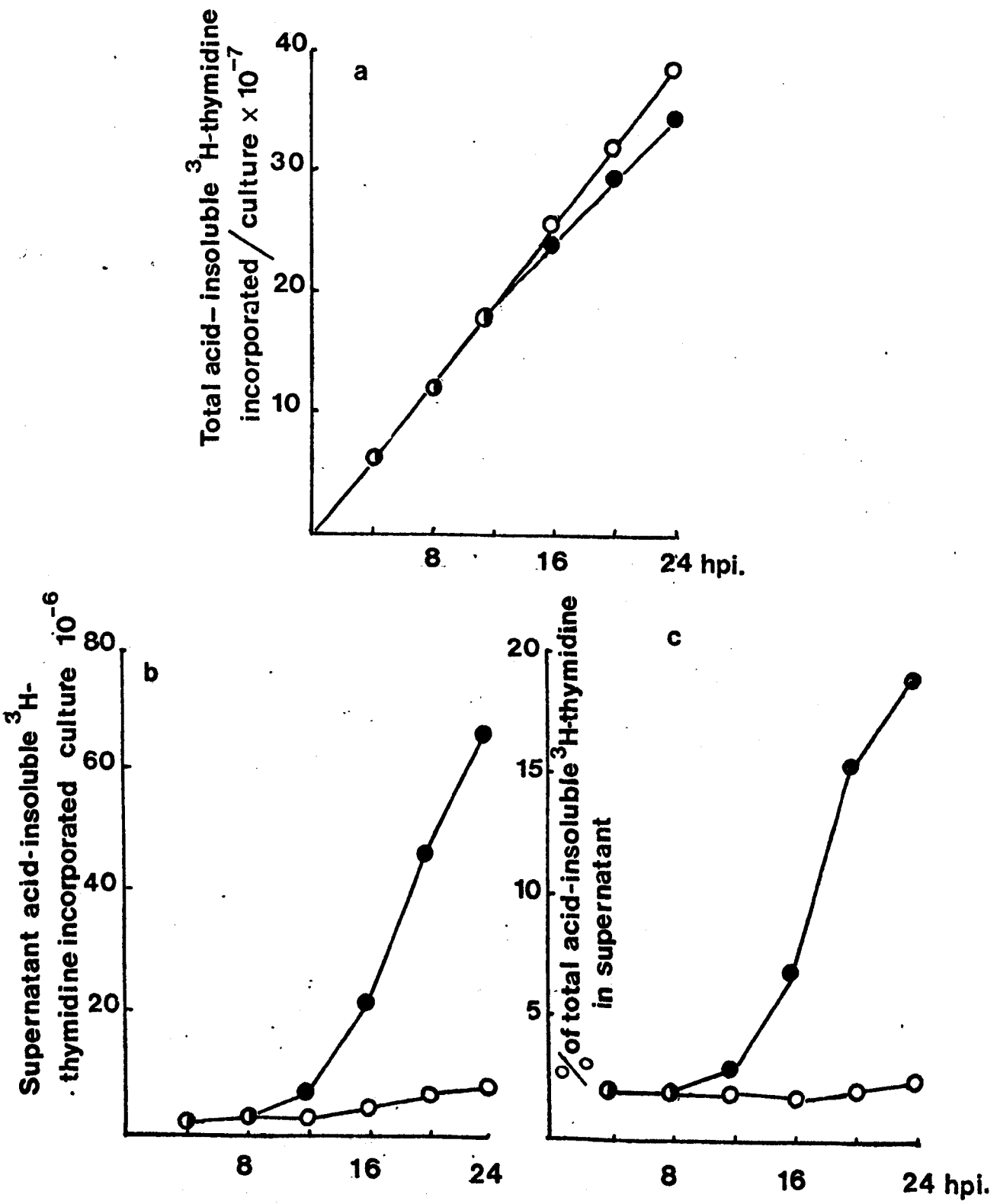
(a) Total cumulative yield of acid precipitable ^{3}H-thymidine incorporated per culture.

(b) Supernatant cumulative yield of acid-insoluble ^{3}H-thymidine incorporated per culture.

(c) Percentage of total acid-insoluble ^{3}H-thymidine appearing in Hirt/Pronase supernatant.

●—●—● Infected

o—o—o Mock-infected.


a
Total acid-insoluble ^{3}H-thymidine incorporated / culture x 10^{-7}
40
30
20
10
8
16
24 hpi.
b
Supernatant acid-insoluble ^{3}H-thymidine incorporated culture 10^{-6}
80
60
40
20
8
16
24
c
% of total acid-insoluble ^{3}H-thymidine in supernatant
20
15
10
5
8
16
24 hpi.

closely parallels that in control cells, a dramatic difference is seen later in infection. In virus-infected cells there is a considerable increase in incorporation of ^{3}H-thymidine into the Hirt/Pronase supernatant DNA from 8 - 24 hpi, reaching eight times that in control cells by 24 hr. When the amount of labelled DNA in the Hirt/Pronase supernatant is expressed as a percentage of total DNA synthesised, the value for mock-infected cells is relatively constant at about 2% (Fig. 2c). Tattersall et al. (1973) obtained a lower figure of 0.8%. The difference is probably due to the fact that suspension cultures of A9 cells were used in their experiments, while monolayers were employed in this study. The percentage of total labelled DNA appearing in extracts from infected cells increases rapidly from 8 - 24 hpi, reaching 19% by 24 hr.

The nature of the DNA present in Hirt/Pronase extracts throughout infection was studied by neutral sucrose gradient centrifugation.

3.2.2 Analysis of Extracted DNA Species

When Hirt/Pronase extracted DNA was analysed by neutral sucrose gradient centrifugation in 1 M NaCl (Fig. 3a), profiles of DNA synthesised in infected and control cells were similar up to 8 hpi. This DNA is heterogeneous and probably constitutes a low background level of cellular DNA contamination in the Hirt/Pronase supernatant. DNA extracted from infected cells pulse-labelled from 8 - 10 hpi shows a distinct peak in the 14 - 16S region of the gradient, the position expected for a unit length duplex form of MVM DNA (molecular weight $\sim 3 \times 10^{6}$ daltons). The appearance of this species of DNA coincides with the increase in low molecular weight DNA appearing in the Hirt/Pronase supernatant fraction (Figs. 2b, c). As infection proceeds,

Fig. 3a

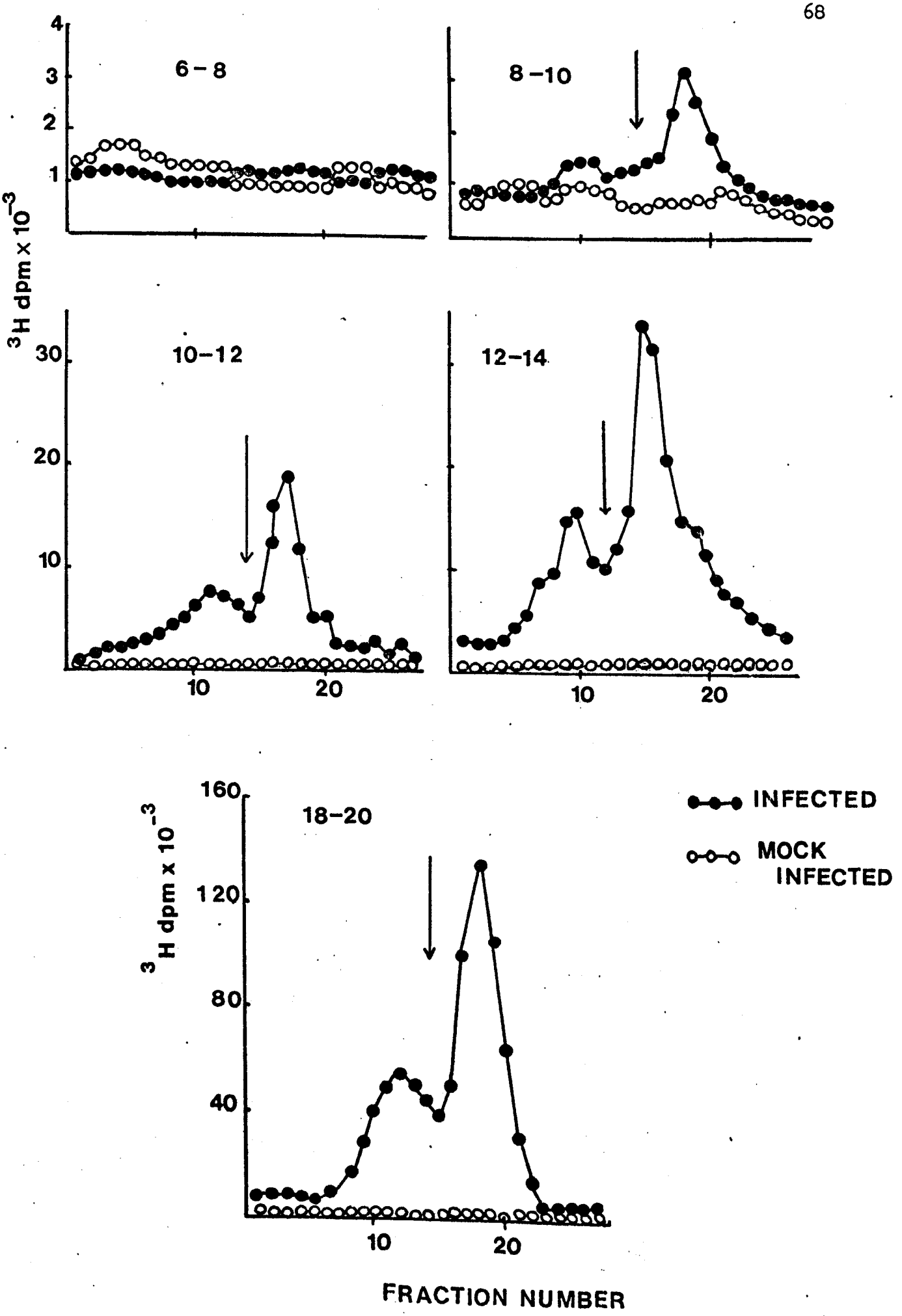
Sedimentation analysis of low molecular weight DNA extracted from MVM-infected and control cells throughout infection.

0.2 ml samples of Hirt/Pronase supernatant DNA, labelled and isolated as described in the legend to Fig. 2 were sedimented in 5 - 20% neutral sucrose gradients. Centrifugation was carried out in a Spinco SW56 rotor at 120,000 g (average) for 5 hr at 4°C. Examples of profiles obtained at different times after infection are shown.

The vertical arrows show the position of ^{14}C-SV40 form-I (21S) DNA in the same gradient.

●—●—● Infected

o—o—o Mock-infected.


6-8
8-10
10-12
12-14
18-20
^{3}H dpm x 10^{-3}
INFECTED
MOCK INFECTED
FRACTION NUMBER

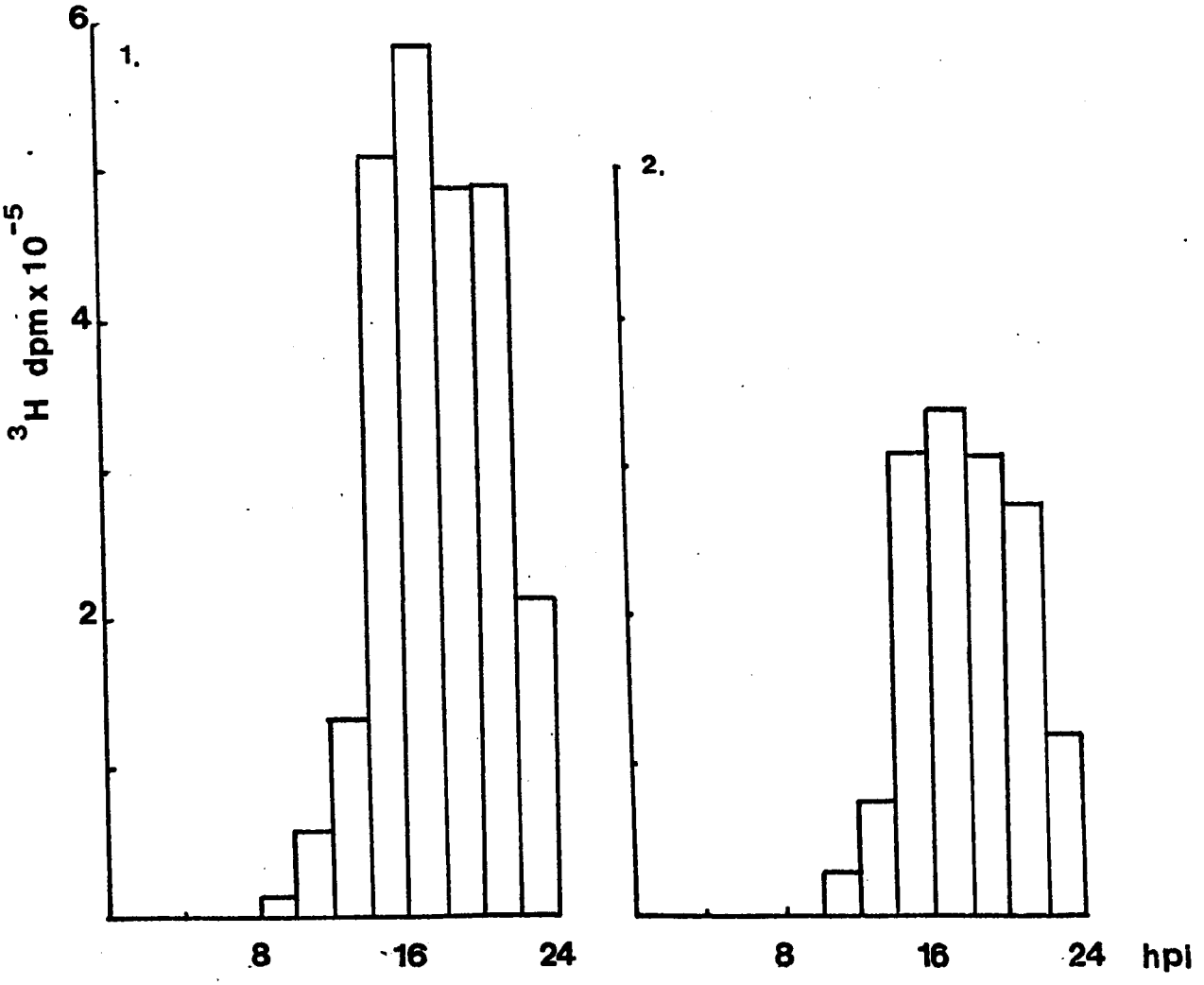


Fig. 3b

Rate of synthesis of the DNA species extracted by the Hirt/ Pronase procedure, throughout infection.

The yields of "15S" and "fast-sedimenting" DNA in the 2 hr intervals up to 24 hpi were estimated by summation of acid-insoluble ^{3}H-thymidine sedimenting in the 14 - 16S region (1) and > 16S region (2) of neutral sucrose gradients, described in the legend to Fig. 3a.

3.3 FURTHER CHARACTERISATION OF VIRAL DNA INTERMEDIATES EXTRACTED BY THE HIRT/PRONASE METHOD

3.3.1 Alkaline Sucrose Gradient Analysis of DNA

To determine the size of the DNA extracted from MVM-infected cells by the Hirt/Pronase method, infected A9 cultures were labelled from 12 - 24 hpi with ^{3}H-thymidine, and the Hirt/Pronase supernatant DNA analysed by sedimentation in neutral sucrose gradients (Fig. 4a). The DNA sedimenting to the bottom of the gradient was present in varying amounts in different DNA preparations. Since it was also present in mock-infected cell extracts it probably represents low levels of cellular DNA contamination.

Material from the 24S and 15S regions of the gradient were pooled as shown (Fig. 4a) and desalted into 0.01 M tris-HCl, 0.01 M EDTA pH 7.9. Samples of 24S, 15S and total Hirt/Pronase DNA were separately mixed with ^{14}C-SV40 form-II (nicked) DNA and analysed by alkaline sucrose gradient centrifugation. As a size control ^{3}H-MVM DNA from purified virions was analysed in parallel.

Under the conditions employed the single-stranded circular (18S) and linear (16S) components of SV40 form-II DNA were not resolved and therefore precise S values cannot be determined (Limit of resolution 16S = 18S).

Figure 4b shows the results of analysis of viral DNA. MVM DNA sediments as a sharp peak, slightly behind the SV40 DNA marker. There was no evidence for a slower-sedimenting shoulder in this MVM DNA preparation, as reported by Tattersall et al. (1973). This probably reflects less DNA strand breakage and agrees with the results obtained by Koczot et al. (1973) who prepared AAV DNA with minimal strand

Fig. 4

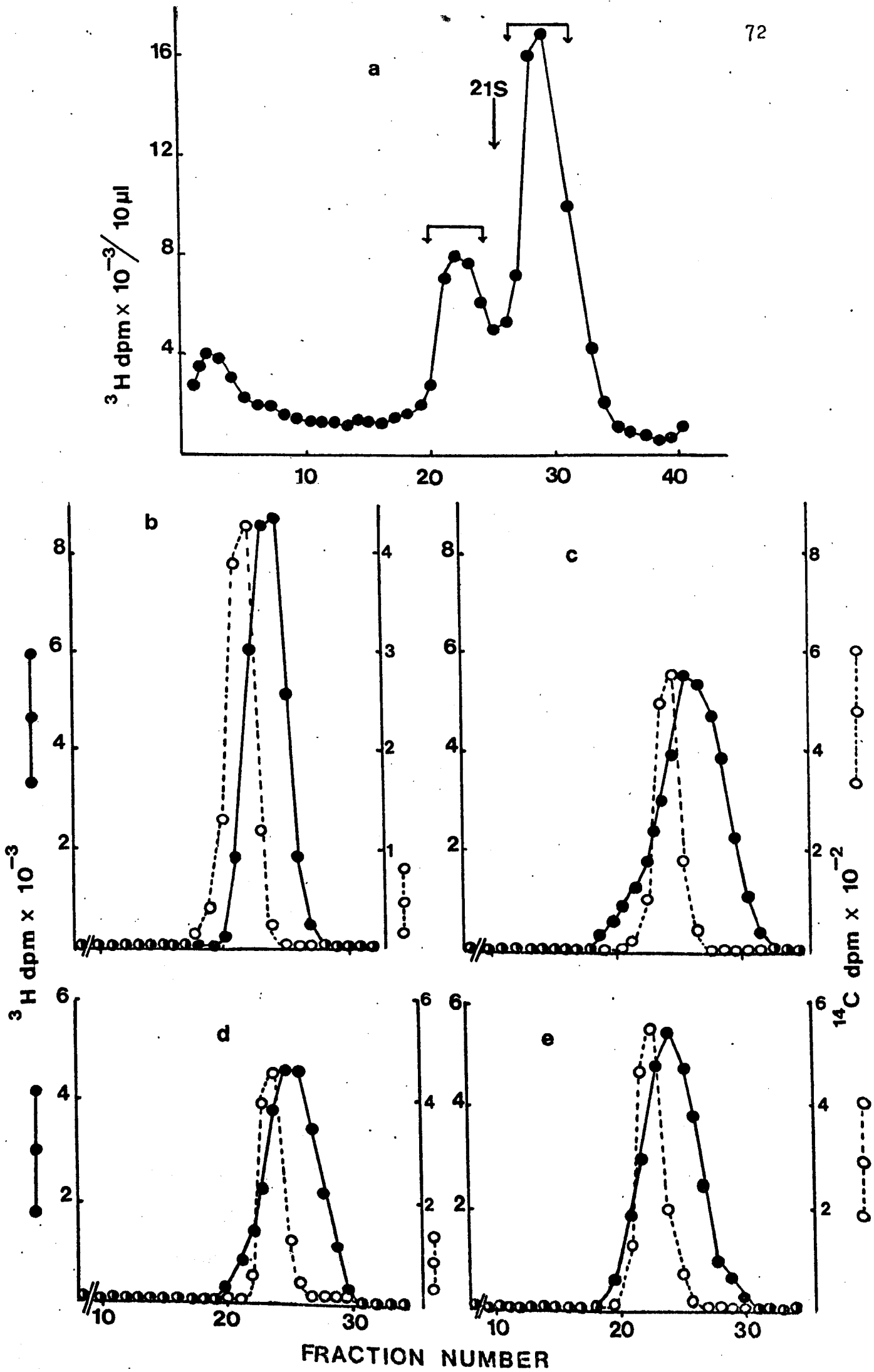
Alkaline sucrose sedimentation of DNA extracted by the Hirt/ Pronase method.

MVM-infected A9 cells (5 pfu/cell) were labelled with ^{3}H-thymidine (10 μCi/ml) from 12 - 24 hpi and extracted by the Hirt/ Pronase method. A 0.2 ml aliquot of the extract was sedimented in a 5 - 20% neutral sucrose gradient at 100,000 g (average) for 5 hr in an SW50L rotor at 4°C (a). Fractions from the 15S and 24S region of the gradient were pooled as shown (a), and desalted into 0.01 M tris-HCl, 0.01 M EDTA, pH 7.9.

Samples of ^{3}H-thymidine labelled MVM virus (b), total Hirt/ Pronase extracted DNA (c), 24S DNA (d) and 15S DNA (e) in 0.01 M tris-HCl, 0.01 M EDTA, pH 7.9 were made 0.25 N with respect to NaOH for 15 min at 20°C. Centrifugation was carried out in 5 - 20% alkaline sucrose gradients for 5 hr at 120,000 g (average) in an SW56 rotor at 4°C.

^{14}C-form II SV40 DNA was added to each gradient as a marker.

(o---o---o)


a
21S
^{3}H dpm x 10^{-3}/ 10µl
b
c
d
e
^{3}H dpm x 10^{-3}
^{14}C dpm x 10^{-2}
FRACTION NUMBER

breakage in this manner. Figure 4c shows the profile obtained when total Hirt/Pronase extracted DNA was analysed.

Although the peak of DNA has a similar mean S value to that of MVM DNA, there is a greater distribution in the size of DNA species present.

When the distribution of ^{3}H-thymidine is compared to that of ^{14}C-marker for MVM DNA (Fig. 4b) and Hirt/Pronase DNA (Fig. 4c), it can be seen that there is a proportion of DNA in the Hirt/Pronase extract sedimenting faster than MVM DNA. At least some of these fast-sedimenting molecules will consist of cellular DNA which sediments to the bottom of the neutral sucrose gradient (Fig. 4a).

When DNA from the 24S (Fig. 4d) and 15S (Fig. 4e) is similarly analysed, however, it can be seen that there are molecules in each of these fractions which are longer than unit length viral DNA.

3.3.2 Isopycnic Gradient Analysis of DNA

To determine the buoyant density of the DNA species extracted by the Hirt/Pronase method, samples of DNA from the 24S and 15S regions of the neutral sucrose gradient (Fig. 4a) were separately mixed with ^{14}C-SV40 DNA and centrifuged to equilibrium in neutral CsCl solution. The results are shown in Fig. 5.

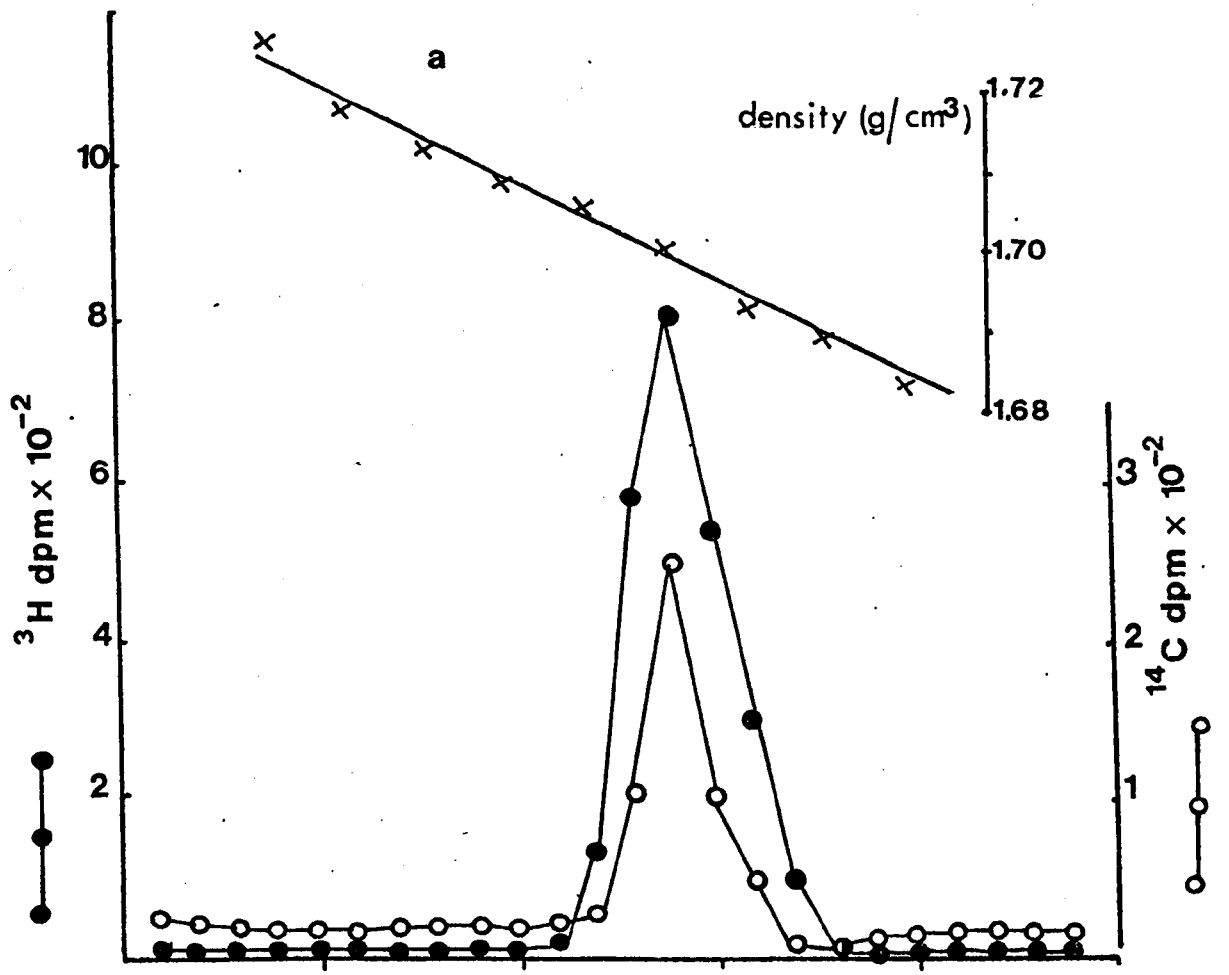
All the 15S DNA banded with the marker SV40 DNA (Fig. 5a) at a density of about 1.7 g/cm^{3} which is consistent with this DNA being double-stranded, with a G + C content of about 40% (Schildkraut et al., 1962). This value agrees with that calculated for a duplex replicative form (RF) of MVM DNA, from the base analysis of the viral strand (Crawford et al., 1969).

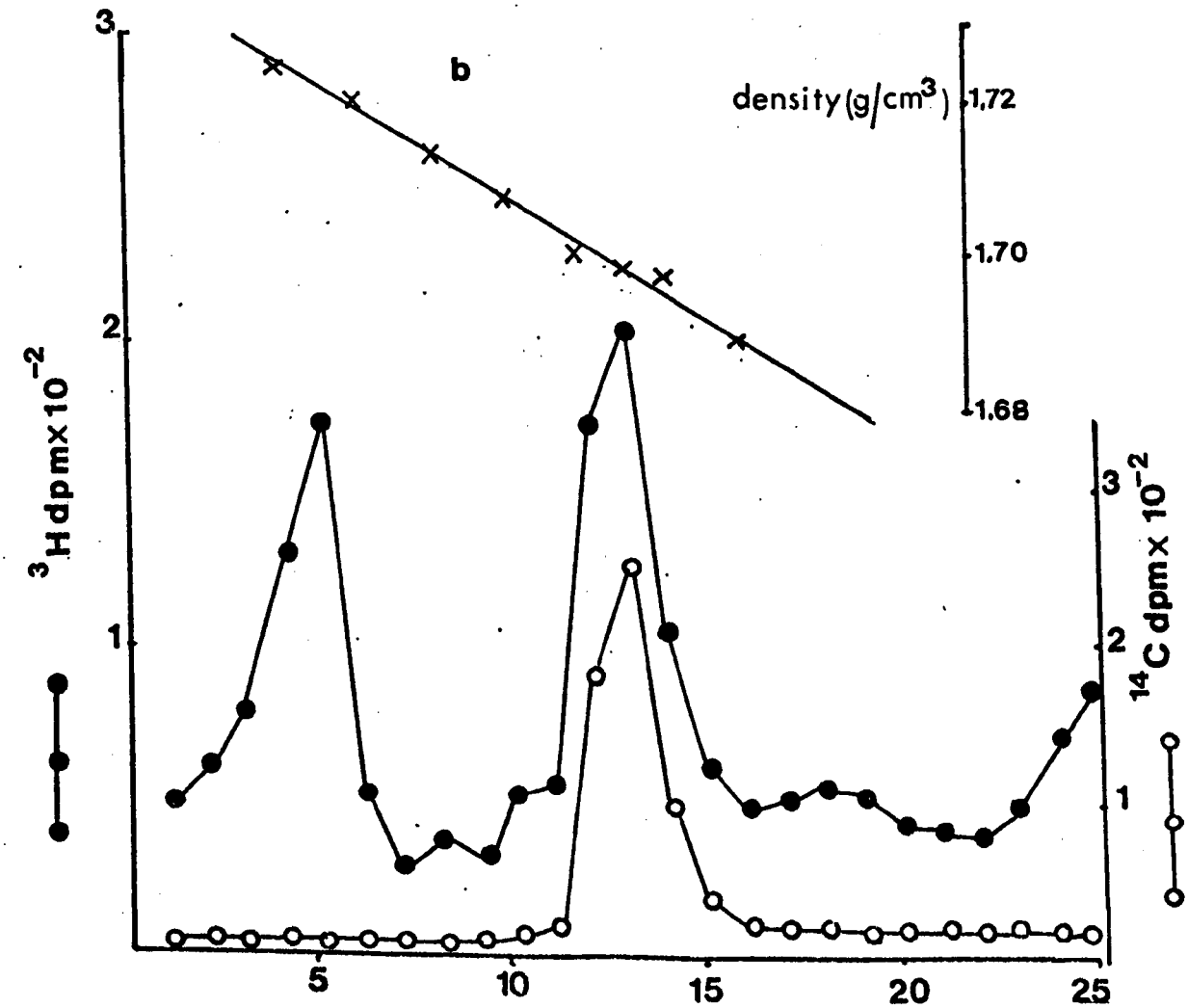
Although the DNA extraction procedure involves SDS and digestion

Fig. 5

Isopycnic gradient centrifugation of DNA extracted by the Hirt/Pronase method from MVM-infected cells.

0.2 ml samples of ^{3}H-thymidine labelled "15S" and "24S" DNA, prepared as described in the legend to Fig. 4a, were separately mixed with ^{14}C-SV40 DNA and centrifuged in CsCl solution (density 1.70 g/cm^{3}) containing 0.05 M tris-HCl, 0.002 M EDTA, pH 7.4. Centrifugation was performed in a Spinco SW50L rotor for 60 hr at 80,000 g (average) at 20°C.


a
density (g/cm³)
1.72
1.70
1.68
10
8
6
4
2
³H dpm x 10⁻²
3
2
1
¹⁴C dpm x 10⁻²


b
density (g/cm³)
1.72
1.70
1.68
3
2
1
³H dpm x 10⁻²
3
2
1
¹⁴C dpm x 10⁻²
5
10
15
20
25

with pronase, it does not rule out the possibility that a proportion of the fast-sedimenting species contains DNA which is associated with other macromolecular components. Several workers have shown that newly-synthesised DNA is often associated with membranes, and is complexed with other macromolecules (Ben-Porat et al., 1962; Tremblay et al., 1969; Pearson and Hanawalt, 1971).

The results of the CsCl equilibrium centrifugation of "24S" DNA (Fig. 5b) shows that the vast majority of this fast-sedimenting DNA is not complexed with other macromolecules. The DNA is resolved into two main species, present in approximately equal amounts. One species bands at the same position as SV40 while the other bands at the position expected for single-stranded MVM DNA (about 1.722 g/cm^3). There is also a certain amount of DNA which bands heterogeneously throughout the gradient, some of which appears on the top of the gradient and may represent complexed DNA.

These results indicate that the fast-sedimenting DNA extracted from MVM-infected cells using the Hirt/Pronase procedure contains both double- and single-stranded DNA.

3.3.3 Low Ionic Strength Sucrose Gradient Analysis of DNA

In an attempt to further resolve the DNA species present in the Hirt/Pronase supernatant fraction, the DNA was analysed by low salt sucrose gradient centrifugation. While variation in ionic strength has little effect on the sedimentation characteristics of native double-stranded DNA, it produces a significant change in the sedimentation coefficient of single-stranded or denatured DNA (Studier, 1965). Single-stranded DNA at neutral pH is contracted by intrastrand base-base interactions which increase with increasing

ionic strength, resulting in a large increase in S value in the ionic strength range 0.01 - 0.5 (Studier, 1965).

In 1 M NaCl MVM DNA sediments at 24S. Reduction of ionic strength should effect the separation of double- and single-stranded DNA sedimenting in the 24S region of a high salt gradient. Salzman and White (1973) have shown that KRV DNA sediments at approximately 6S in 0.01 M NaCl and 24S in 1 M NaCl.

A sample of Hirt/Pronase supernatant DNA was desalted into 0.001 M tris-HCl, 0.001 M EDTA pH 7.9 and analysed by velocity sedimentation in a sucrose gradient containing 0.001 M tris-HCl, 0.001 M EDTA pH 7.9 (Fig. 6b). As a control, a sample of the same DNA was sedimented in a sucrose gradient containing 1 M NaCl (Fig. 6a).

When the two profiles are compared, several differences can be observed. Firstly, the amount of DNA sedimenting faster than the main 15S peak is substantially reduced (by about 35%) at low ionic strength (Fig. 6b), indicating the movement of single-stranded DNA to lower S values. Secondly, the mean sedimentation coefficient of the remaining fast-sedimenting DNA was slightly reduced. Thirdly, at low ionic strength, a DNA species sedimenting in the 3 - 4S region of the gradient (Fig. 6b) was detected. It can be concluded that this DNA is single-stranded, and probably represents viral DNA which would have a sedimentation coefficient of < 6S under these conditions, while sedimenting at 24S in high salt gradients.

The remaining fast-sedimenting DNA in the low ionic strength gradients is essentially double-stranded, and could represent longer than unit length MVM duplex DNA. Such concatemeric species, of three times unit length duplex DNA have been found in Hirt-extracted DNA from Hela cells infected with the parvovirus Lu-III (Siegl and Gautschi,

Fig. 6

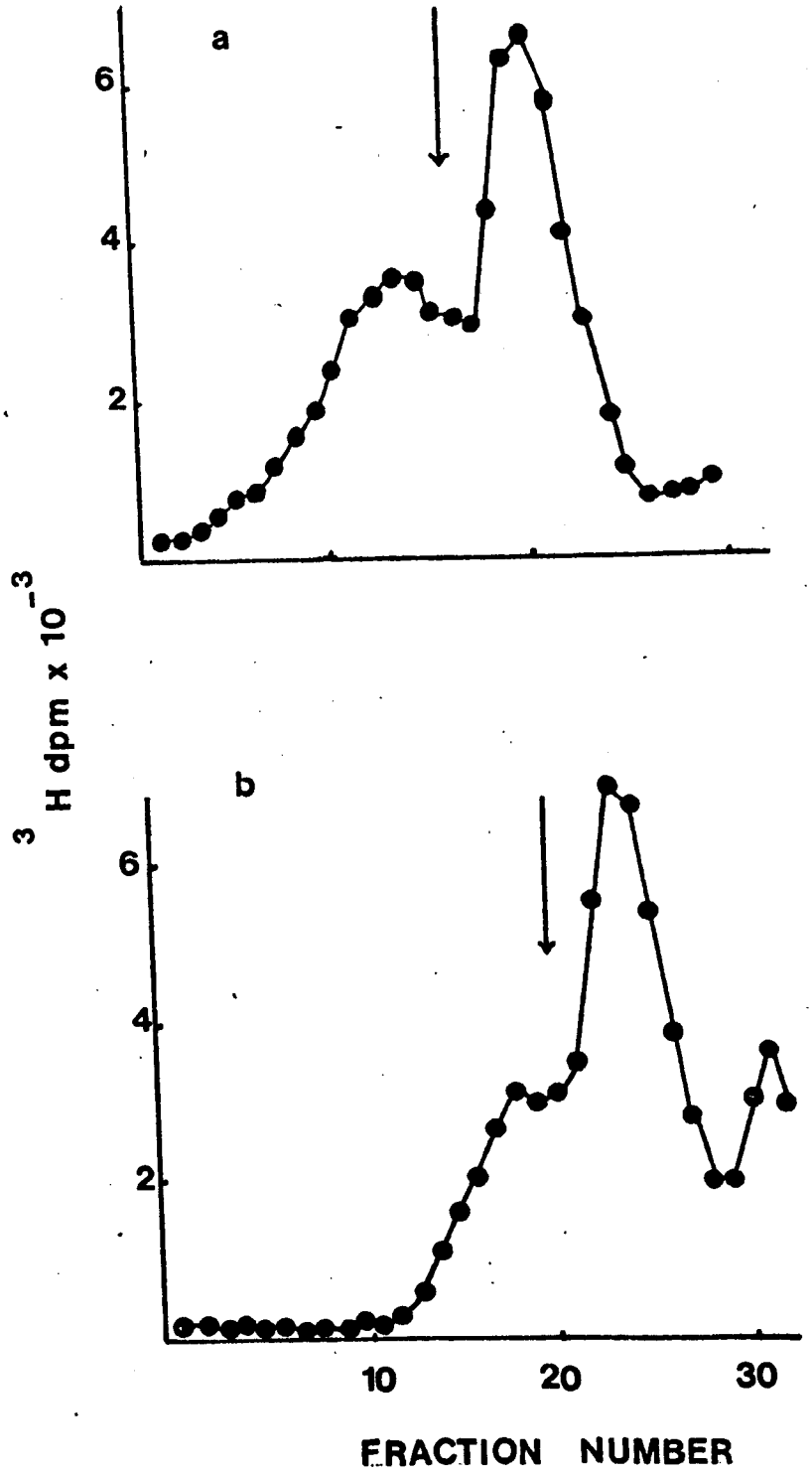
Sedimentation analysis of Hirt/Pronase extracted DNA in low ionic strength sucrose gradients.

A sample of Hirt/Pronase extracted DNA from MVM-infected A9 cells, labelled from 18 - 22 hpi with ^{3}H-thymidine was desalted into 0.001 M tris-HCl, 0.001 M EDTA, pH 7.9, as described in the Methods section.

(a) A 0.2 ml aliquot was sedimented in a 5 - 20% neutral (1.0 M NaCl) sucrose gradient at 120,000 g (average) for 5 hr in a Spinco SW56 rotor at 4°C.

(b) A 0.2 ml aliquot was sedimented in a 5 - 20% neutral sucrose gradient containing 0.001 M tris-HCl, 0.001 M EDTA, pH 7.9. Centrifugation was performed as in (a).

Vertical arrows denote the position of ^{14}C-SV40 form-I DNA in the same gradient.


a
b
6
4
2
6
4
2
^{3}H dpm x 10^{-3}
10
20
30
FRACTION NUMBER

1976). Tattersall et al. (1973) also observed "fast-sedimenting" MVM double-stranded DNA and concluded that it consisted of complexes of unit length duplex DNA.

3.3.4 a) S_1 Nuclease Analysis (MVM DNA)

It has been reported that, although MVM DNA appears to be single-stranded when viewed in the electron microscope, it is partially resistant to the single-strand specific nucleases, exonuclease-I and endonuclease S_1 (Tattersall et al., 1973).

Samples of ^{3}H-thymidine labelled MVM DNA, prepared from purified virions by alkaline sucrose gradient centrifugation, were digested with 40 units of nuclease S_1 for up to 2 hr at 37°C as described in the Methods section. As a control, ^{32}P-Hela cell DNA was similarly digested both before and after heat denaturation. The results are shown in Fig. 7.

While native Hela cell DNA is almost completely resistant to nuclease S_1 digestion, 94% of the ^{32}P label in denatured Hela cell DNA was rendered acid soluble after 2 hr, indicating that the nuclease specifically digests single-stranded DNA sequences (Fig. 7a).

When MVM DNA was digested under the same conditions (Fig. 7b), about 12% of the ^{3}H-thymidine remained acid-insoluble. This indicates that a proportion of the MVM DNA is in duplex form. Since this DNA had been denatured by exposure to alkali during purification, the resistant fraction represents DNA sequences which are able to renature when the DNA is neutralised.

S_1 nuclease was used to analyse the DNA extracted from infected cells using the Hirt/Pronase method.

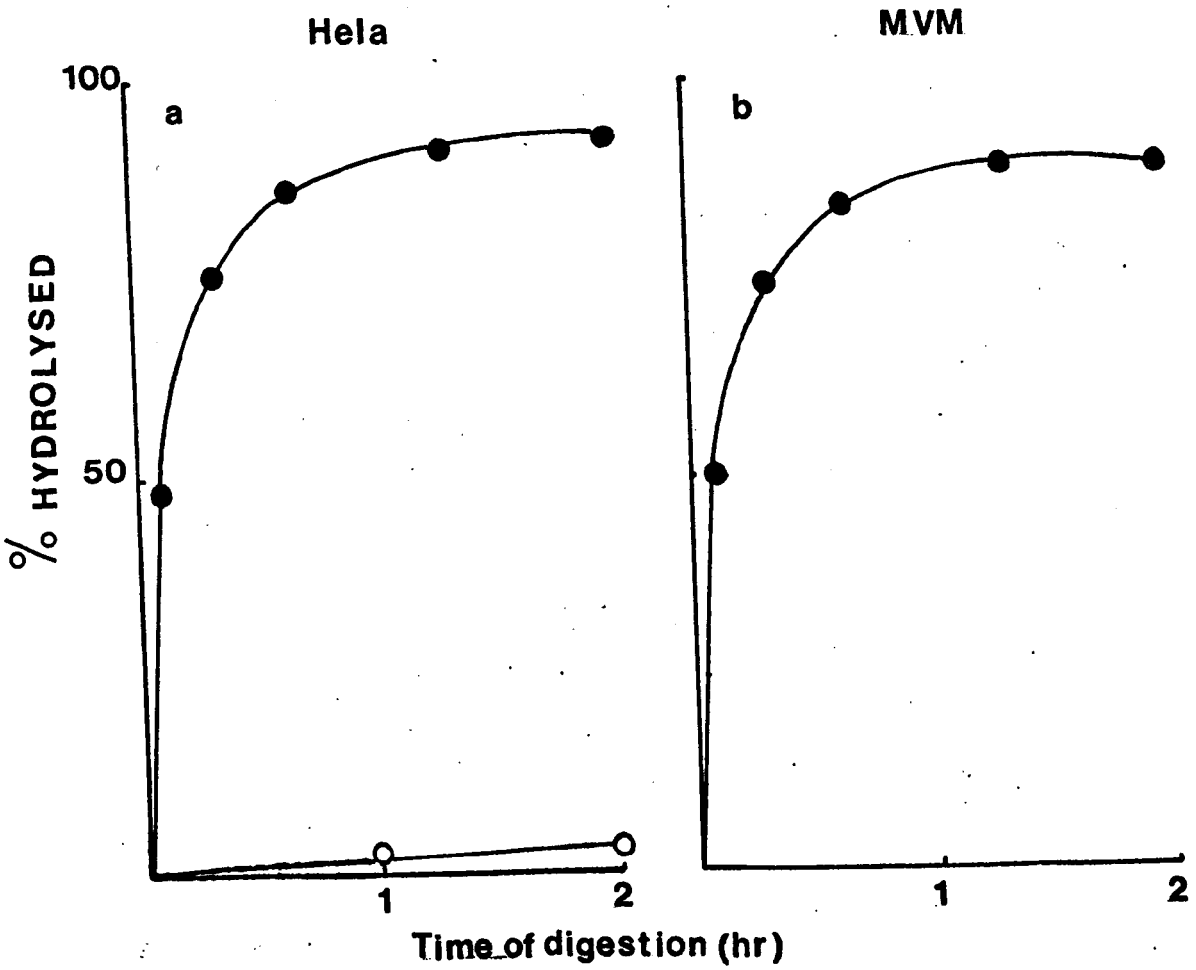


Fig. 7

Digestion of MVM DNA and Hela cell DNA with S_1 nuclease.

Samples of native (●—●—●) and heat-denatured (o—o—o) ^{32}P-Hela cell DNA (a) were digested with 40 units of S_1 nuclease for up to 2 hr at 37°C as described in Materials and Methods.

Samples of ^{3}H-thymidine-labelled MVM DNA, prepared from purified virions by alkaline sucrose gradient centrifugation as described in Materials and Methods were similarly analysed (b). Results are expressed as percentage of radioactive DNA rendered acid soluble (hydrolysed) after digestion for the time specified.

3.3.4 b) S_1 Nuclease Analysis (Hirt/Pronase DNA)

Infected A9 cells were labelled with ^{3}H-thymidine from 18 - 20 hpi, the DNA extracted by the Hirt/Pronase method, and samples analysed by velocity sedimentation in high salt sucrose gradients (Fig. 8a). The DNA in the 24S (pool I), trough (pool II) and 15S (pool III) regions of the gradient was pooled as shown (Fig. 8a). Equal volumes of DNA from each pool were digested with 40 units of nuclease S_1 for 4 hr under standard conditions. It has been shown in the previous section that the nuclease is specific for single-stranded DNA and that the digestion reaction has levelled off by 2 hr under the conditions employed.

The results show a disproportionate distribution of S_1-sensitive DNA sequences throughout the gradient (Fig 8b). The major DNA species, sedimenting between 12 - 18S (pool III) contained 10% single-stranded DNA and was therefore highest in double-stranded content. The DNA obtained from the trough in the 18 - 21S region (pool II) was again mostly double-stranded, having only 15% of the DNA sequences sensitive to S_1 digestion. However, when the DNA from the 22 - 28S region (pool I) was similarly digested, 36% was found to be single-stranded.

To further characterise the DNA, samples from pools I, II and III were heat-denatured for 5 min at 100°C, quench cooled in ice-water, and subjected to S_1 nuclease digestion. The results are shown in Table 3.2. Even after heat denaturation, a proportion of each pool of DNA is resistant to hydrolysis by S_1. When the resistant fraction is expressed as a percentage of the double-stranded DNA present in each fraction before denaturation, the value obtained for DNA sedimenting faster than 18S (pools I and II) is about 20%. DNA sedimenting in the 12 - 18S region (pool III) contains 30% non-

Fig. 8

Digestion of Hirt/Pronase-extracted DNA with nuclease S_1.

Infected A9 cells were pulse-labelled with ^{3}H-thymidine from 18 - 20 hpi. and Hirt/Pronase supernatant DNA extracted. A 0.2 ml aliquot was sedimented in a 5 - 20% neutral sucrose gradient at 120,000 g (average) for 5 hr in an SW56 rotor at 4°C (a). Fractions were collected and pooled as shown (I, II, III). After desalting, samples of each pool were assayed for acid-insoluble radioactivity both before (total DNA) and after digestion of equal volumes with 40 units of nuclease S_1 for 4 hr at 37°C (S_1 resistant DNA). The results are expressed as the percentage of total DNA which is S_1 resistant (i.e. double-stranded) or S_1 sensitive (single-stranded) (b).

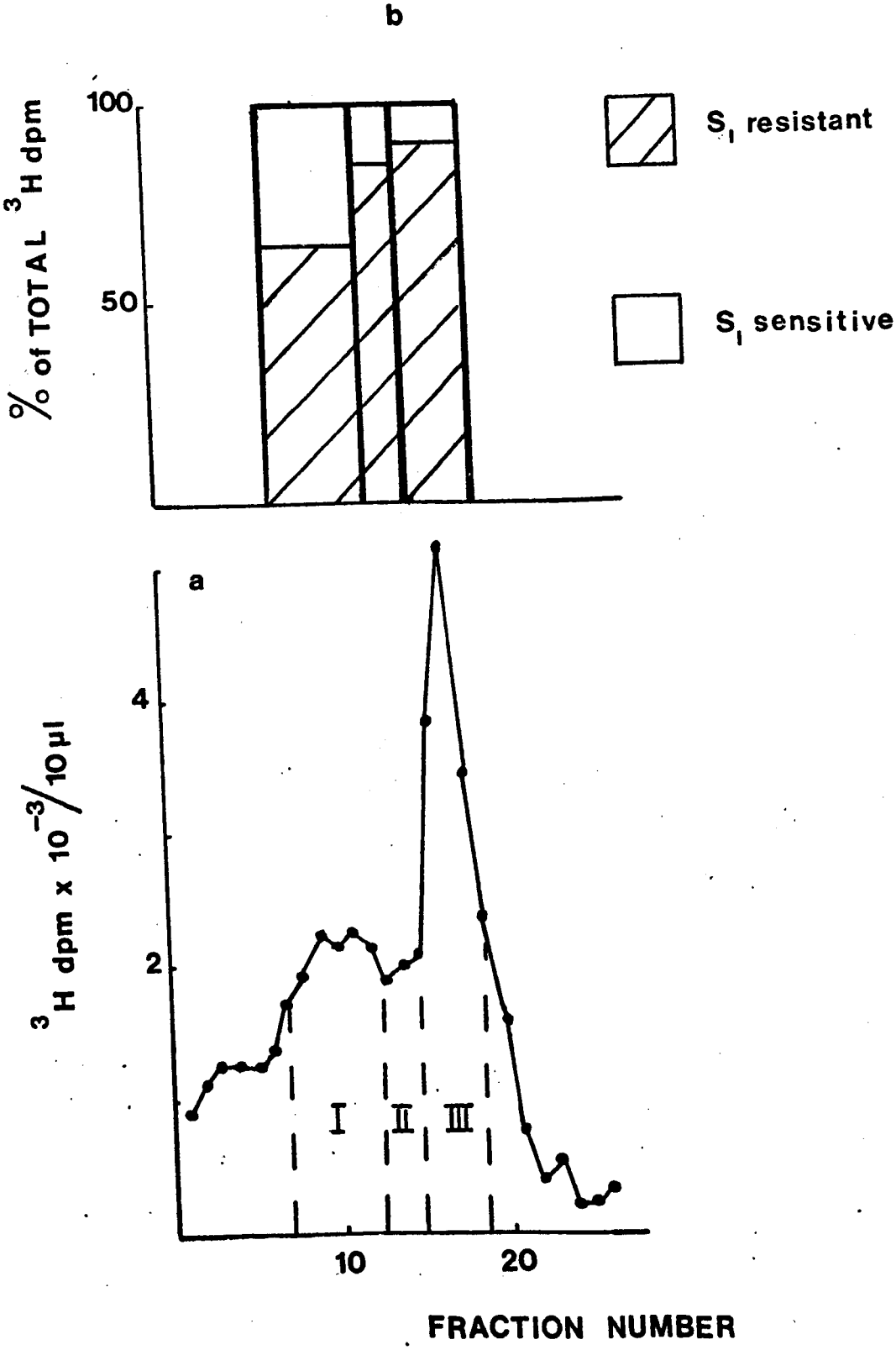

b
100
50
% of TOTAL ^{3}H dpm
S$_1$ resistant
S$_1$ sensitive
a
4
2
^{3}H dpm x 10^{-3}/10µl
I
II
III
10
20
FRACTION NUMBER

Table 3.2 S_1 Nuclease Digestion of DNA Extracted by the Hirt/Pronase Method

Pool	% DNA Hydrolysed by S_1		% Double-stranded DNA Resistant to Denaturation
	Native	Denatured	
I	36	87	20.3
II	15	83	20
III	10	73	30

Samples of DNA from pools I, II and III (Fig. 8a) were digested with 40 units of S_1 nuclease for 4 hr, both before and after heat-denaturation, as described in the Methods section. Mean results of two separate experiments on the same preparation of DNA are expressed as the percentage of DNA rendered acid soluble (hydrolysed) by S_1 nuclease. From these results, the percentage of double-stranded DNA remaining S_1-resistant after denaturation was calculated.

denaturable DNA sequences. These figures may be an overestimate of spontaneously-renaturing duplex DNA since MVM single-stranded DNA has been shown to be partially resistant to nuclease S_1 (section 3.3.4 a) and exonuclease I (Tattersall et al., 1973), even after denaturation.

Such spontaneously-renaturing MVM DNA has been reported (Tattersall et al., 1973). They found that between 20 - 40% of the total double-stranded DNA extracted by the Hirt/Pronase method was still in duplex form after heat-denaturation, as determined by hydroxyapatite chromatography. They concluded that this was a result of intramolecular renaturation. The precise nature of such molecules, however, and their size-distribution on neutral sucrose gradients before or after denaturation was not determined.

The results presented here show that the majority of the renaturing DNA occurs in DNA species sedimenting between 12 and 18S in neutral sucrose gradients. Since the mean S value of this DNA is 15S, these results are consistent with a proportion of MVM unit length duplex DNA molecules existing in the form of a hairpin structure as suggested by Tattersall et al. (1973). A smaller proportion of fast-renaturing molecules is found in the 18 - 21S and 22 - 28S regions of the gradient.

3.3.5 Displacement Hybridisation

To determine the proportion of 12 - 18S DNA which contains viral DNA sequences, samples of pool III (Fig. 8a) were subjected to displacement hybridisation in the presence of unlabelled MVM DNA. A large excess of the unlabelled DNA will displace all labelled strands containing viral DNA sequences, rendering them sensitive to nuclease S_1 digestion, after reannealing is allowed to take place.

Samples of pool III DNA were mixed with 0, 50, 200 and 500 ng of purified unlabelled MVM DNA, heat-denatured, and allowed to reanneal in 0.3 M NaCl for 60 hr at 65°C. After reannealing, the DNA samples were each digested with 40 units of S_1 nuclease for 4 hr under standard conditions. The results are shown in Table 3.3. In the absence of viral DNA the double-stranded DNA in pool III was totally resistant to S_1 nuclease and had therefore reannealed completely. In the presence of increasing amounts of MVM DNA, however, increasing amounts of labelled duplex DNA were rendered acid soluble after nuclease digestion. In the presence of 500 ng of competing viral DNA 37% of the labelled double-stranded DNA is sensitive to S_1 nuclease. The theoretical maximum amount of displacement is 50% since the virus only contains one strand, the labelled complementary strand remaining in duplex DNA. Therefore, at least 74% of the 12 - 18S DNA is virus-specific. Since it has been shown that this DNA contains about 30% non-denaturable DNA sequences (section 3.3.4), which might not be competed out by the unlabelled viral DNA, it is possible that most, or perhaps all, of the 12 - 18S DNA contains viral sequences.

3.3.6 Agarose Gel Electrophoresis (Native DNA)

Agarose gel electrophoresis was used in an attempt to increase the resolution between the DNA species present in the Hirt/Pronase extract.

Infected cultures were pulse-labelled from 16 - 22 hpi with ^{3}H-thymidine and the DNA extracted by the Hirt/Pronase method. The supernatant DNA was analysed by neutral sucrose gradient centrifugation and the fractions pooled as indicated (Fig. 9a). MVM unit length duplex DNA was obtained from the 15S peak fraction of a similar

Table 3.3 Displacement Hybridisation

Amount of Competing MVM DNA (ng)	% Double-stranded DNA [1] rendered S_1 sensitive
0	0
50	30
200	35
500	37

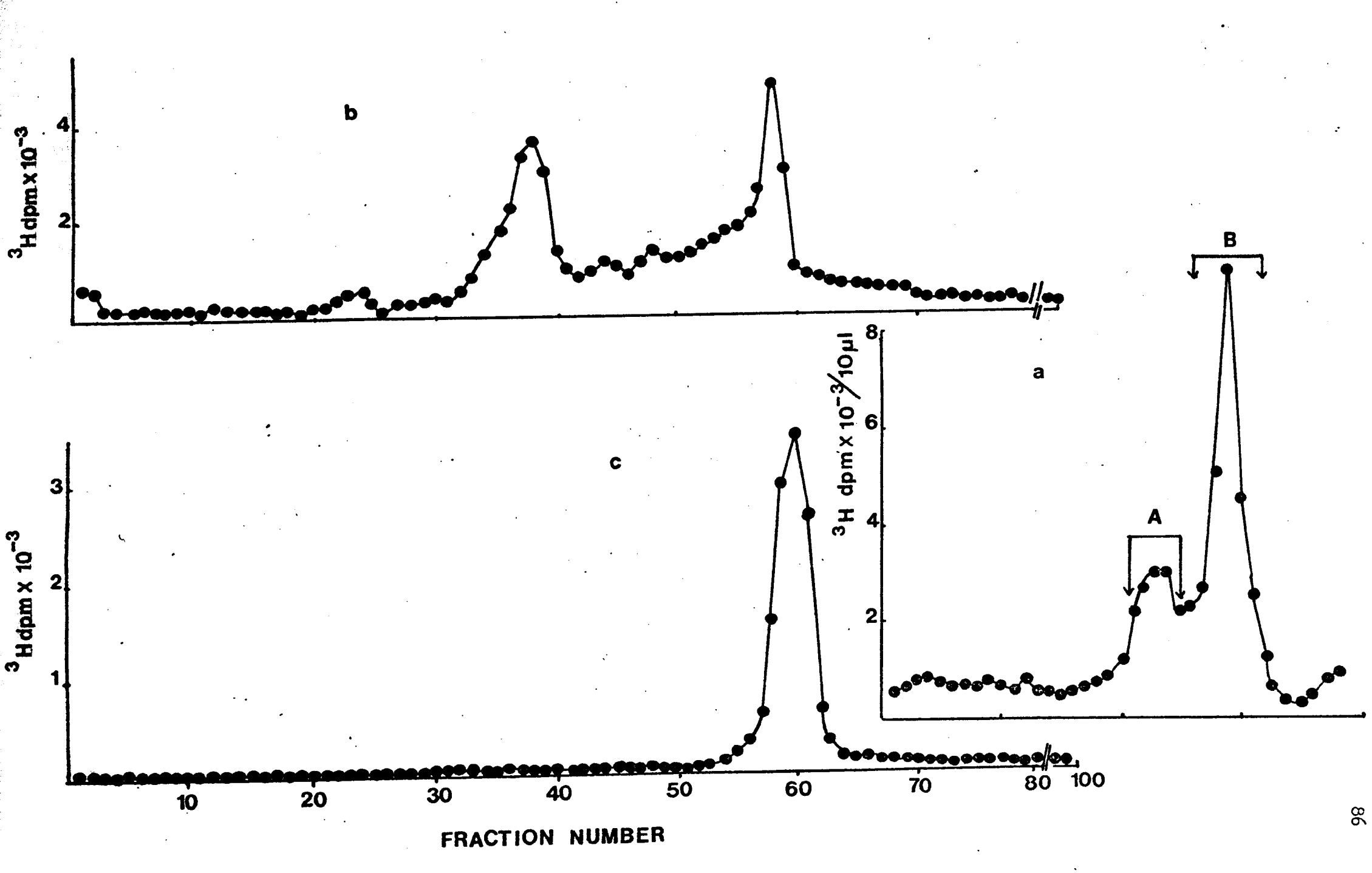
Displacement of labelled molecules from the double-stranded DNA of pool III (Fig. 8a). Aliquots of pool III DNA were heated to 100°C for 5 min in the presence of indicated amounts of unlabelled MVM DNA, quenched in ice-water, and adjusted to 0.3 M with NaCl. Reannealing took place at 65°C for 60 hr. The results are expressed as the percentage of radioactivity in double-stranded DNA rendered acid soluble after digestion of the reannealing product with 40 units of S_1 nuclease during 4 hr at 37°C.

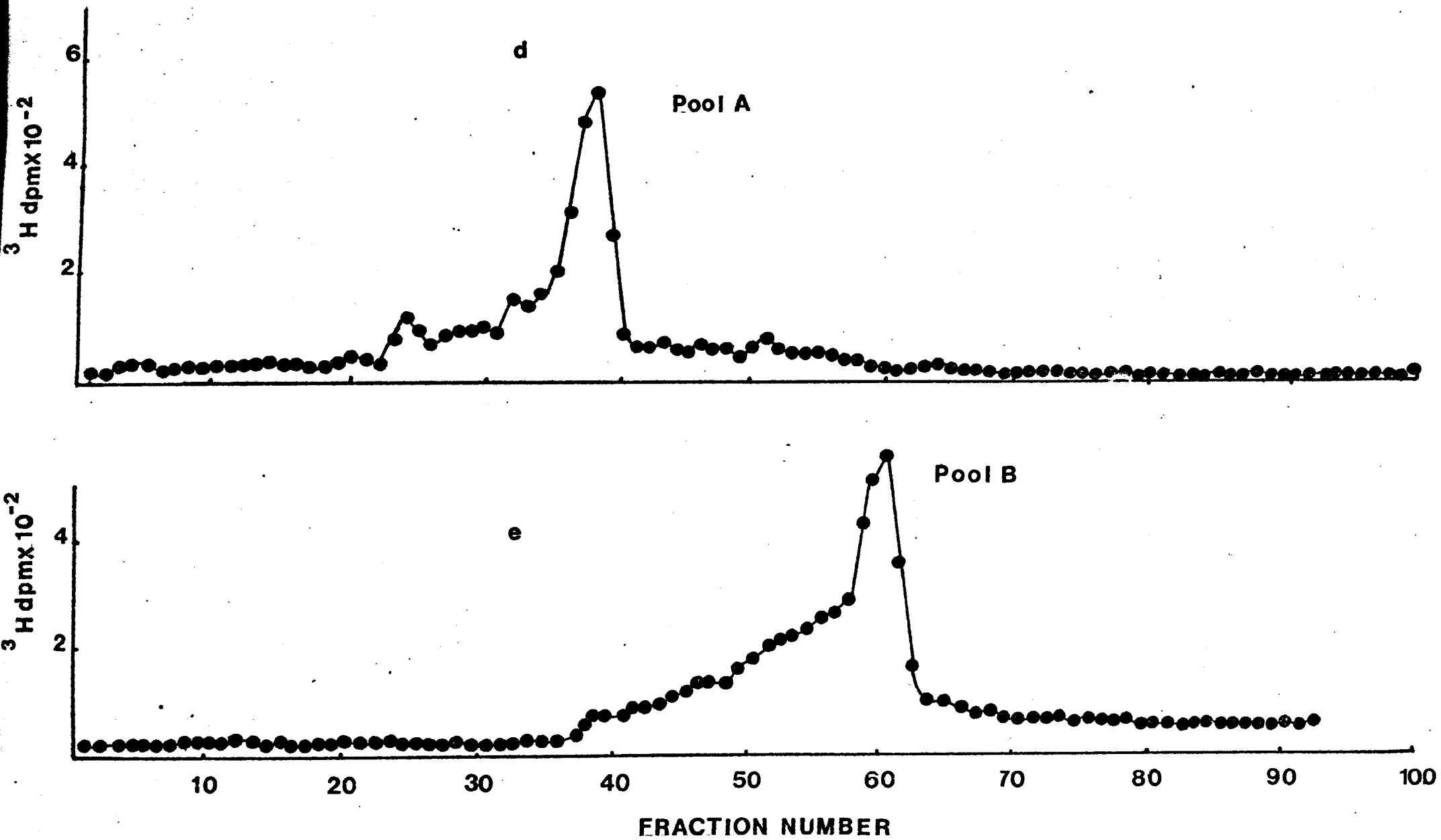
[1] Mean value of two experiments.

Fig. 9

Agarose gel electrophoresis of Hirt/Pronase supernatant DNA.

Infected A9 cultures (5 pfu/cell) were pulse-labelled with ^{3}H-thymidine (10 μCi/ml) from 16 - 22 hpi and low molecular weight DNA extracted by the Hirt/Pronase method. Two 0.2 ml samples of supernatant material were sedimented in neutral sucrose gradients (a) as described in the legend to Fig. 8. Fractions from one gradient were collected and pooled as shown (A, B), while only the 15S peak fraction from the other gradient was used. The DNA samples were desalted into 0.01 M tris-HCl, 0.01 M EDTA, pH 7.9 and 50 μl aliquots analysed by agarose gel electrophoresis as described in Materials and Methods for 3 hr at 100 V. Electrophoretic analysis of total Hirt/Pronase supernatant DNA (b), "15S" peak fraction DNA (c), pool A DNA (d) and pool B DNA (e) are shown.


b
^{3}H dpm X 10^{-3}
4
2
a
^{3}H dpm X 10^{-3}/10µl
8
6
4
2
A
B
c
^{3}H dpm X 10^{-3}
3
2
1
10
20
30
40
50
60
70
80
100
FRACTION NUMBER


d
Pool A
^{3}H dpmX10^{-2}
6
4
2
e
Pool B
^{3}H dpmx 10^{-2}
4
2
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

gradient. Total extracted DNA, 15S DNA and DNA from pools A and B (Fig. 9a) were separately analysed by agarose gel electrophoresis.

It can be seen from the analysis of total extracted DNA (Fig. 9b) that the resolution of DNA species is significantly better than that obtained using sucrose gradient centrifugation (Fig. 9a). Total DNA is resolved into two major species (Fig. 9b) which are present in approximately equal amounts. The faster-migrating species corresponds to the position of MVM unit length duplex DNA from the 15S peak (Fig. 9c). There is also an appreciable amount of heterogeneous DNA, migrating between the two major peaks. One further minor species, migrating slower than the rest of the DNA, is also observed (fractions 23 and 24).

The DNA from the 24S region of the sucrose gradient (pool A, Fig. 9a) contains the slower-migrating major DNA component as well as the minor component (fraction 24) (Fig. 9d). This fast-sedimenting DNA has been shown in the previous sections (3.3.2 - 4) to contain both double-and single-stranded DNA. It is possible that under the conditions employed these species may not be resolved.

The ratio of the two major peaks resolved by gel electrophoresis clearly differs from the ratio of the two species resolved by sucrose gradient centrifugation (Fig. 9a). When pool B DNA is analysed (Fig. 9e), there is a major band at the position of MVM unit length duplex DNA. In addition, there is a considerable amount of heterogeneous DNA migrating more slowly. Since this DNA has been shown to be mainly double-stranded (section 3.3.4), it probably represents longer than unit length duplex DNA or branched molecules, which have not been resolved from unit length duplex under the conditions of centrifugation employed.

Clearly, the existence of this heterogeneous DNA in the 13 - 19S region of the gradient, explains the difference in ratios of major DNA species between gradients and gels.

3.3.7 Cleavage of DNA by Endonuclease R.Eco.R_1

The physical maps of several viral DNA's, including AAV-2 DNA, have been constructed using cleavage with restriction endonucleases (Danna et al., 1973; Mulder et al., 1974; Berns et al., 1975b; Carter et al., 1975). These maps have proved useful in a number of different respects including the determination of the extent and direction of transcription of the viral genome (Khoury et al., 1973; Carter et al., 1975) and determination of the origin, termination and direction of DNA replication (Danna and Nathans, 1972; Tolun and Pettersson, 1975). There have been no reports, however, on the cleavage of duplex DNA from a non-defective parvovirus using restriction endonucleases.

In a preliminary study, therefore, ^{3}H-thymidine labelled 15S duplex MVM DNA, prepared from the peak fraction as previously described, was incubated with endo. R.Eco.R_1 for 1 hr at 37°C. As controls, SV40 form-I DNA (supercoil) was cleaved in a parallel reaction and 15S MVM duplex DNA was incubated without enzyme.

Endo. R.Eco.R_1 cleaves the DNA sequence:-

(T/A) G↓AATT C (A/T)

(A/T) C TTAA↑G (T/A)

by introducing staggered single-strand scissions at the positions indicated by the arrows (Hedgpeth et al., 1972), producing one specific cleavage in SV40 DNA (Morrow and Berg, 1972). Under the conditions used, SV40 form-I DNA was quantitatively converted to form-III DNA (linear).

The results of agarose gel electrophoresis of duplex MVM DNA are shown in Fig. 10a. The DNA migrates as a single sharp peak, slightly faster than SV40 form-III DNA (molecular weight 3.6×10^6 daltons; Tai et al., 1972). The positions of SV40 forms-I and II (nicked) DNA, analysed in parallel, are indicated as controls (Fig. 10a). Incubation without enzyme had no effect on the migration properties of MVM duplex DNA.

Figure 10b shows the pattern obtained after MVM duplex DNA is cleaved. It can be seen that endonuclease R.Eco.R_1 produces three specific fragments, A, B and C, indicating that unit length duplex MVM DNA has two sites for the enzyme. Since the DNA was not uniformly labelled (^{3}H-thymidine as opposed to ^{32}P) the molecular weight of each species cannot be determined.

It has been shown that endo. R.Eco.R_1 produces three fragments with AAV-2 double-stranded DNA, prepared from purified virions (Carter and Khoury, 1975; Carter et al., 1975; Berns et al., 1975b; see section 1.5.6). The larger fragments A and B occur at the termini, with C internal. The order of MVM DNA fragments has not been determined.

3.3.8 Agarose Gel Electrophoresis (Denatured DNA)

MVM-infected A9 cells were labelled from 12 - 24 hpi with ^{3}H-thymidine and low molecular weight DNA was extracted by the Hirt/Pronase method. The DNA was subjected to neutral sucrose gradient centrifugation (Fig. 11a) and MVM unit length duplex DNA was prepared from the 15S peak fraction of a similar gradient.

The total Hirt/Pronase extracted DNA, as well as 15S DNA were analysed by electrophoresis in 1.5 % agarose gels both before and after alkali denaturation (0.3 N NaOH 15 min 20°C). The results of

Fig. 10

Cleavage of 15S MVM RF DNA with restriction endonuclease R.Eco.R_1.

A sample of ^{3}H-thymidine labelled 15S DNA, described in the legend to Fig. 9, was incubated with endo. R.Eco.R_1 as detailed in Materials and Methods. SV40 form-I DNA was cleaved in a parallel reaction. 50 µl samples of untreated 15S DNA (a) and restricted 15S DNA (b) were analysed by agarose gel electrophoresis for 2 hr at 100 V. The position of SV40 forms-I and II DNA, and the position of SV40 form-III DNA which was produced by restriction of form-I DNA with endo. R.Eco.R_1, analysed in parallel gels are shown by arrows (a). The position of these unlabelled SV40 DNA species was determined by staining with ethidium bromide, as described in Materials and Methods.

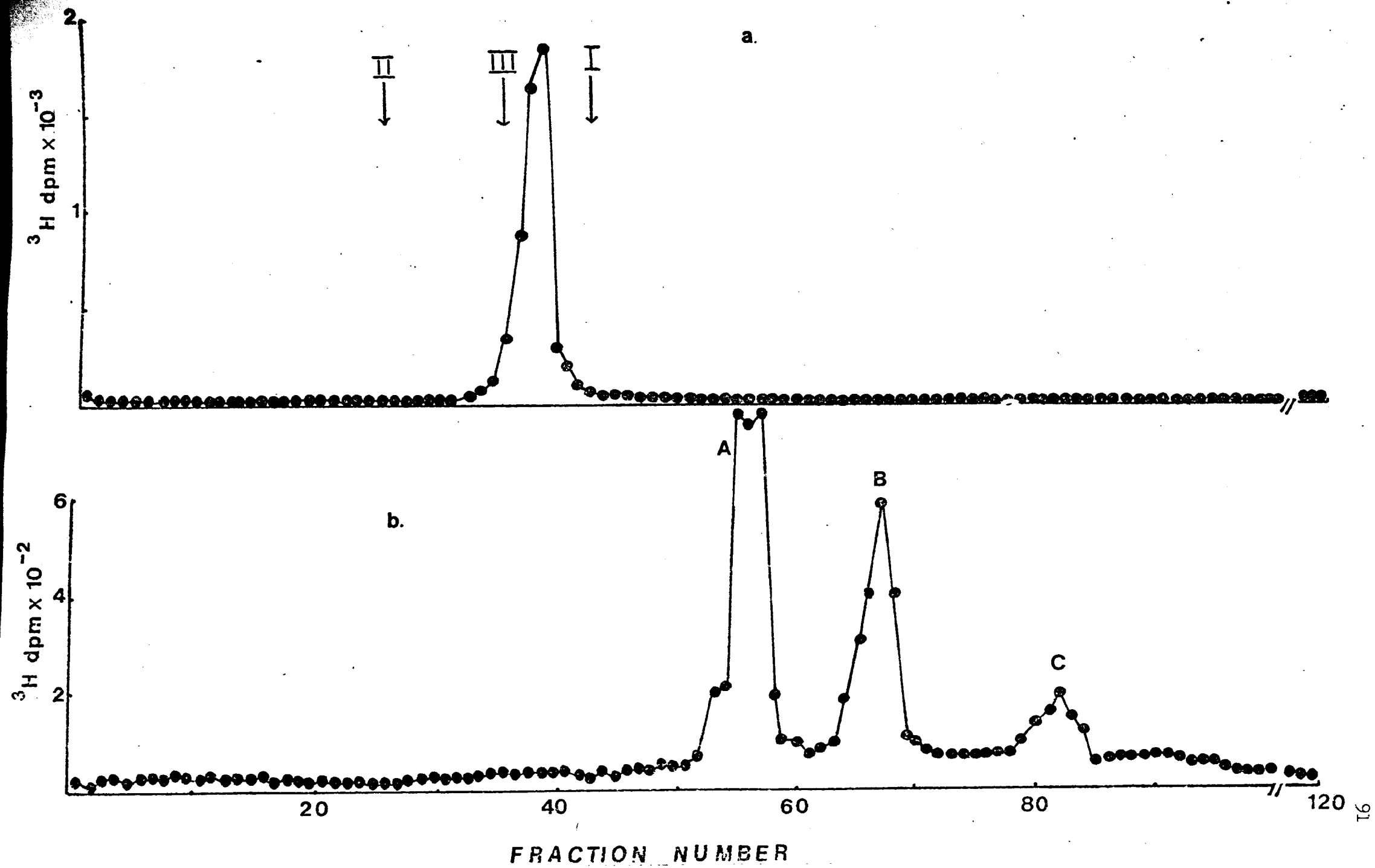

a.
b.
II
III
I
A
B
C
^{3}H dpm x 10^{-3}
^{3}H dpm x 10^{-2}
2
1
6
4
2
20
40
60
80
120
FRACTION NUMBER

Fig. 11

Analysis of Hirt/Pronase-extracted DNA by agarose gel electrophoresis, before and after alkali denaturation.

MVM-infected A9 cells were labelled from 12 - 24 hpi with ^{3}H-thymidine and low molecular weight DNA extracted by the Hirt/Pronase method. (a) A 0.2 ml sample of extracted DNA was sedimented in a 5 - 20% neutral sucrose gradient as described in the legend to Fig. 8a. "15S" DNA was prepared from the peak fraction of a similar gradient.

Samples of "15S" and total extracted DNA were desalted into 0.01 M tris-HCl, 0.01 M EDTA, pH 7.9, and analysed by agarose gel electrophoresis before and after alkali denaturation as described in Materials and Methods. The gels were run at 100 V for 3 hr.

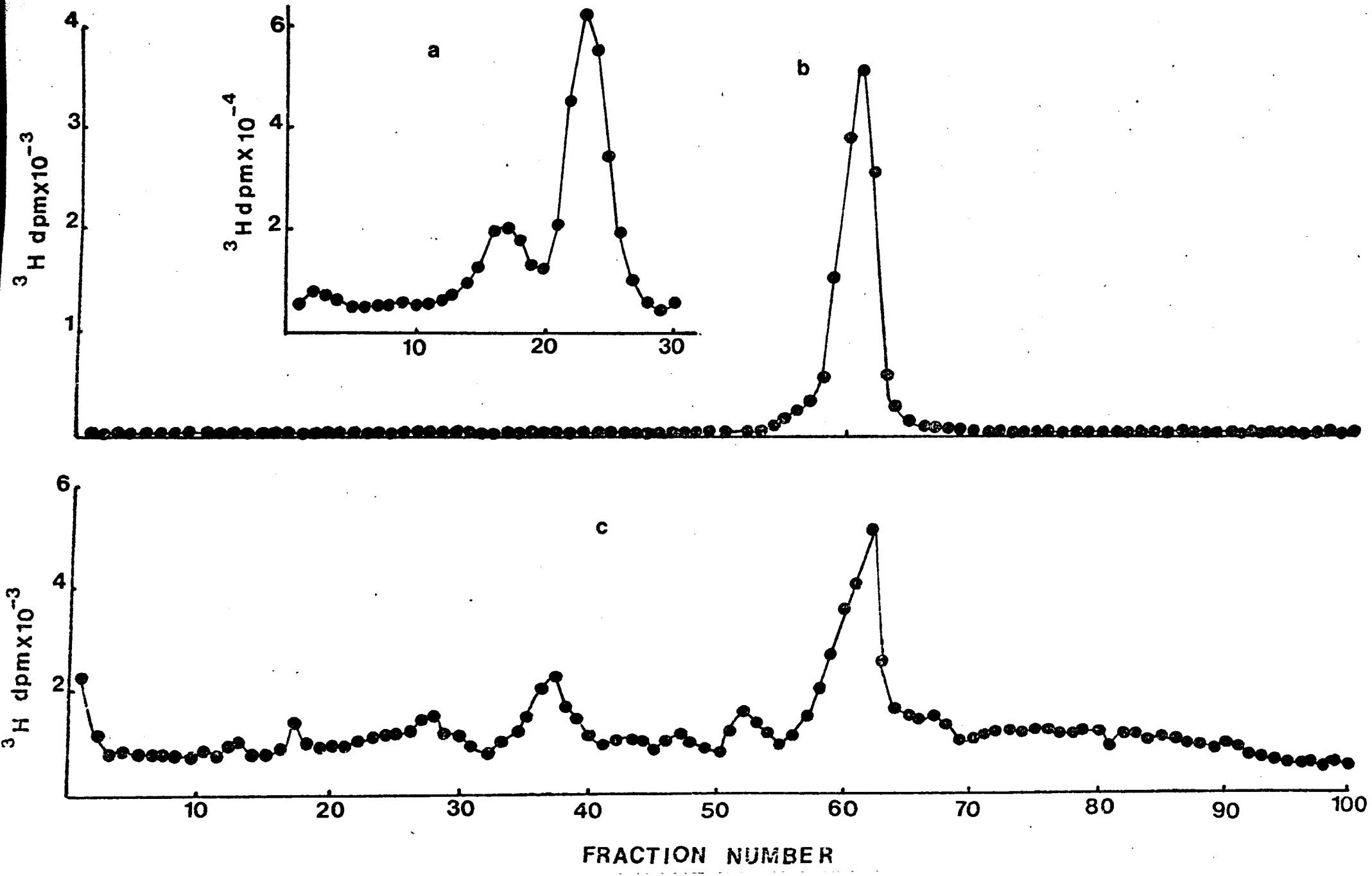
(b) Native "15S" DNA.

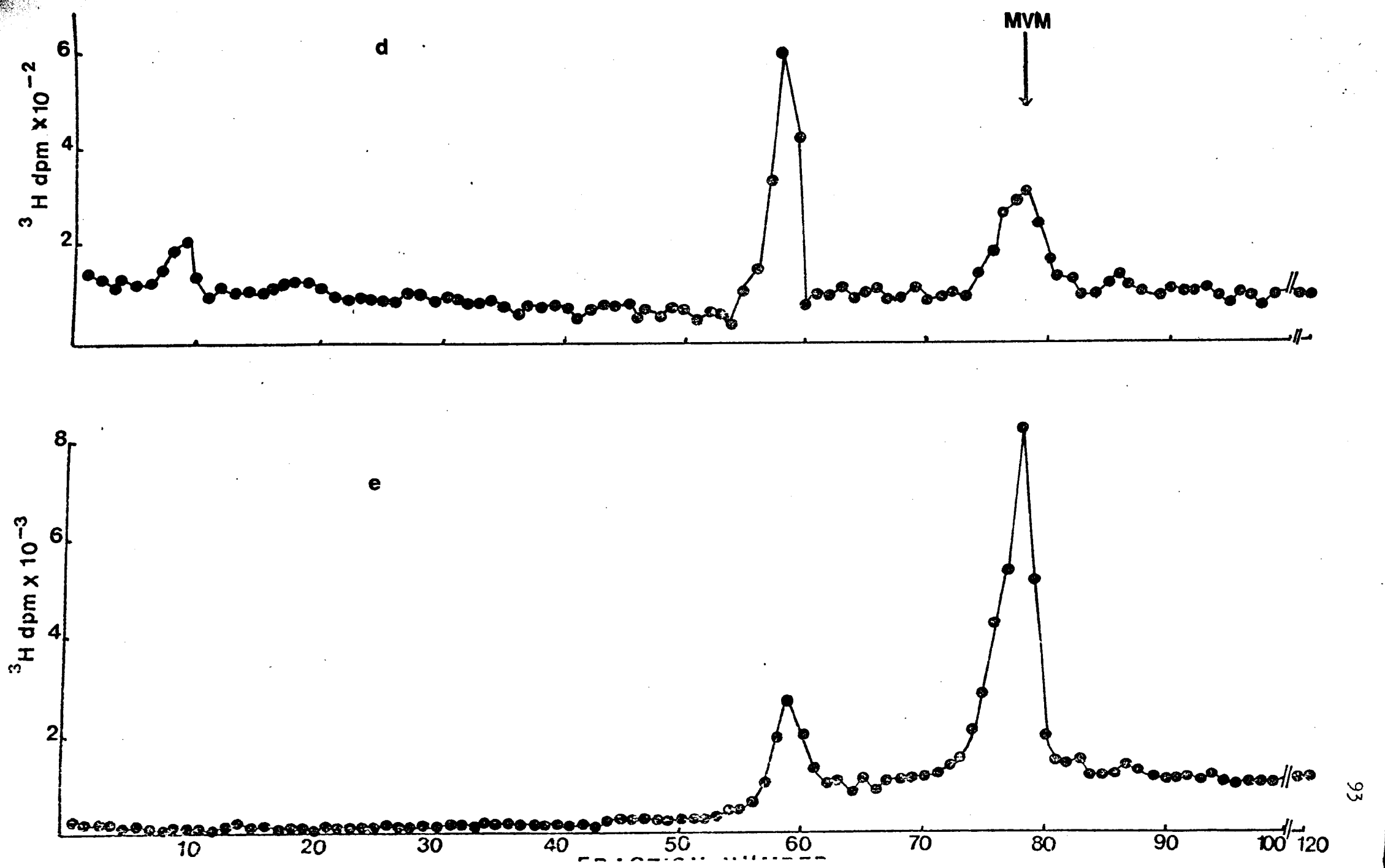
(c) Native Hirt/Pronase extracted DNA.

(d) Denatured "15S" DNA.

(e) Denatured Hirt/Pronase extracted DNA.

The position of ^{32}P MVM-DNA released from purified virions by treatment with 0.3 N NaOH for 15 min at 20°C prior to electrophoresis is indicated by the vertical arrow (d).


a
b
c
^{3}H dpm X 10^{-4}
^{3}H dpm X 10^{-3}
^{3}H dpm X 10^{-3}
FRACTION NUMBER


d
MVM
^{3}H dpm X 10^{-2}
6
4
2
e
^{3}H dpm X 10^{-3}
8
6
4
2
10
20
30
40
50
60
70
80
90
100
120

agarose gel electrophoresis of native DNA are shown in Figs. 11b and c. It can be seen that the 15S DNA migrates as a single band (Fig. 11b). Total extracted DNA, however (Fig. 11c), is more heterogeneous, having a major band at the position of unit length duplex DNA and several minor bands migrating more slowly.

It is a feature of agarose gel electrophoresis of denatured DNA that a small amount of material is found in the position of native double-stranded DNA (Hayward and Smith, 1972). This is probably due to incomplete denaturation, or to reannealing during electrophoresis since the gel does not contain a denaturing component.

Unit length MVM duplex DNA (15S) and total extracted DNA were denatured by exposure to 0.3 N NaOH for 15 min at 20°C. Such treatment has been shown to effect complete separation of DNA strands (Davison, 1966). Samples were analysed directly by agarose gel electrophoresis. The 15S unit length duplex DNA is resolved into two components (Fig. 11d). The faster-migrating component, which comprises about 50% of the total DNA, corresponds to the position of ^{32}P-single-stranded DNA released by alkali from purified virions.

When similarly analysed (Fig. 11e), total DNA is resolved into two species. In this case, however, the component corresponding to MVM single-stranded DNA represents about 80% of the total DNA.

The nature of the slowly-migrating DNA has not been unambiguously determined. It may represent the viral complementary strand which possibly behaves differently from the viral strand when analysed by agarose gel electrophoresis (Hayward, 1972). Since the vast majority of the total DNA, however, behaves as duplex DNA before denaturation (Figs. 11a and c), it is highly unlikely that the ratio of viral strand to complementary strand after denaturation could be 4:1

(Fig. 11e). Furthermore, the relative mobilities of the DNA species would imply a gross conformational difference between the strands, which is unlikely for complementary polynucleotide chains. This explanation is therefore ruled out.

Alternatively, the slowly-migrating DNA may represent longer than unit length single-stranded DNA which could contain covalently linked viral and complementary strands, capable of spontaneous renaturation during electrophoresis. This suggestion is strengthened by the results of nuclease S_1 analysis of denatured Hirt/Pronase extracted DNA (3.3.4) which indicated that a proportion of MVM DNA molecules was capable of spontaneous renaturation.

These data are consistent with the fact that 50% of the 15S unit length MVM duplex DNA and 20% of the total Hirt/Pronase extracted DNA exist in the form of a unit length duplex hairpin structure. The existence of similar molecules, which appear to be intermediates in AAV DNA replication, has been recently reported (Straus et al., 1976).

3.4 EXTRACTION OF VIRAL DNA USING THE TRITON METHOD

3.4.1 Comparison of DNA Species from C13 and A9 Cells

Up to this point the Hirt/Pronase method of extraction has been used exclusively to characterise the intracellular forms of viral DNA. Since this procedure causes total lysis of the infected cell and disruption of protein-DNA interactions, it does not have the power to yield information on the precise form in which the DNA exists within the cell. Non-ionic detergents, however, including triton-X-100, have been used successfully to extract SV40 and polyoma DNA from infected cells (White and Eason, 1971; Green et al., 1971). This DNA was found to exist in the form of a nucleoprotein complex, distinct from the mature virion.

An experiment was designed to compare the products obtained by triton extraction of MVM-infected A9 and C13 cells.

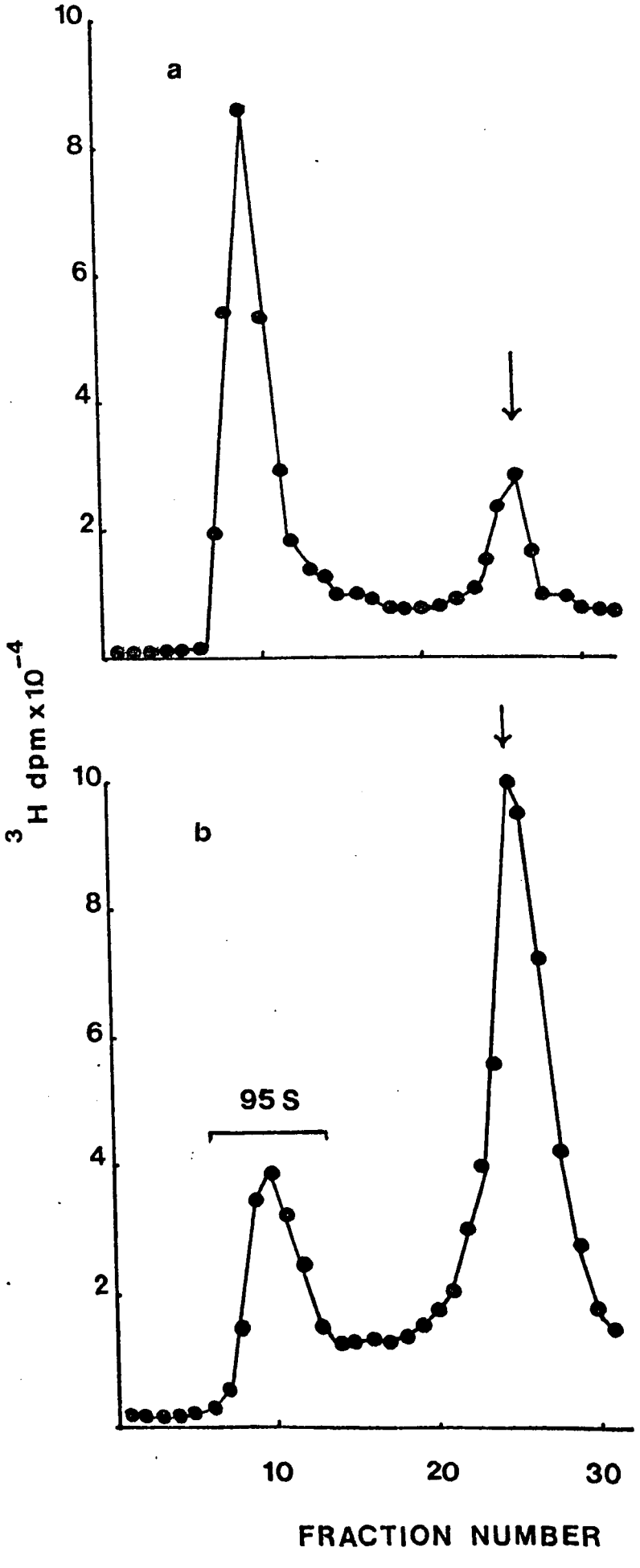
Infected and mock-infected cultures were labelled with ^{3}H-thymidine from 16 - 22 hpi and extracted by the triton method. Figure 12 shows the results of neutral sucrose gradient analysis of the DNA species obtained from both A9 (Fig. 12a) and C13 (Fig. 12b) cells. In each case there are two components, sedimenting around 95S and 21S. The fast-sedimenting species of DNA is the major product from the A9 cells while the 21S component predominates in the C13 extract. This difference in relative amounts of DNA species extracted from infected A9 and C13 cells occurs throughout the infectious cycle. It is possible that this difference in extractability of DNA species reflects the different response of the two cell lines to the triton extraction procedure. On treatment of A9 cells with 0.25% triton, nuclei remain firmly attached to the culture vessel, whereas the majority of C13

Fig. 12

Sedimentation analysis of DNA-containing species extracted from A9 (a) and C13 (b) cells using the triton method.

Cultures of A9 and C13 cells were infected with MVM at a multiplicity of 5 pfu/cell, exposed to ^{3}H-thymidine (10 μCi/ml) from 16 - 22 hpi, and DNA extracted by the triton method. 0.2 ml samples of the triton extracts were centrifuged in a 5 - 20% sucrose gradient at 100,000 g (average) for 2 hr in an SW50L rotor at 4°C.

Similar profiles were obtained by sedimentation analysis in sucrose gradients containing either 1 M NaCl or 0.2 M NaCl.


a
b
95 S
^{3}H dpm x 10^{-4}
FRACTION NUMBER
10
8
6
4
2
10
20
30

nuclei become detached.

The nature of these two DNA components, and their relationship to DNA extracted by the Hirt/Pronase method, and to MVM DNA, was examined.

3.4.2 a. Relationship between Triton-Extracted DNA and Hirt Extracted DNA

Triton extract, prepared from Cl3 cells in the previous experiment was digested with pronase (20 µg/ml) for 3 hr at 37°C and analysed by neutral sucrose gradient centrifugation in 1 M NaCl. The results are shown in Fig. 13. Digestion with pronase had two effects. Firstly, the sedimentation coefficient of the major DNA species was slightly reduced, to a mean S value of about 15. This corresponds to the position of duplex MVM DNA extracted by the Hirt/Pronase method. These results are consistent with the fact that the triton method extracts double-stranded MVM DNA in association with a small amount of protein. This is substantiated by the results shown in Fig. 14. Pronase-treated DNA from the 15S region of the gradient was pooled as shown (Fig. 13), desalted into 0.01 M tris-HCl, 0.01 M EDTA pH 7.9, and subjected to both equilibrium centrifugation in CsCl solution (Fig. 14a) and alkaline sucrose gradient centrifugation (Fig. 14b). The results are identical to those obtained with Hirt/Pronase extracted 15S DNA.

The second consequence of pronase digestion is that the S value of the fast-sedimenting DNA component is considerably reduced (Fig. 13), yielding a DNA species which sediments around 24S. Further pronase digestion did not alter the sedimentation characteristics of either DNA species. The latter result is consistent with the DNA in the fast-sedimenting component being MVM single-stranded DNA, which

Fig. 13

Sedimentation analysis of triton extract after digestion with pronase.

Triton extract from C13 cells, described in the legend to Fig. 12, was digested with pronase at a final concentration of 20 μg/ml for 3 hr at 37°C. After digestion, a 0.2 ml sample of triton extract was sedimented in a 5 - 20% sucrose gradient in an SW50L rotor at 100,000 g (average) for 2 hr at 4°C.

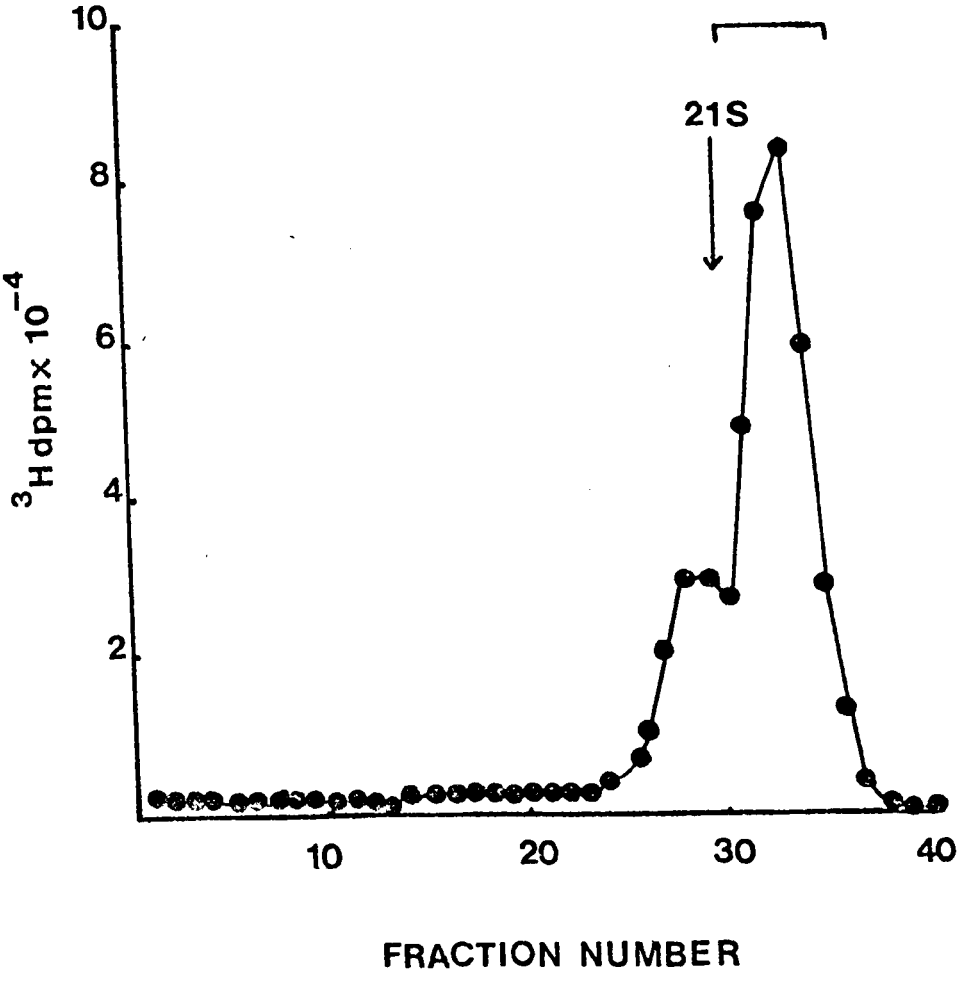

10
8
6
4
2
21S
^{3}H dpm x 10^{-4}
10
20
30
40
FRACTION NUMBER

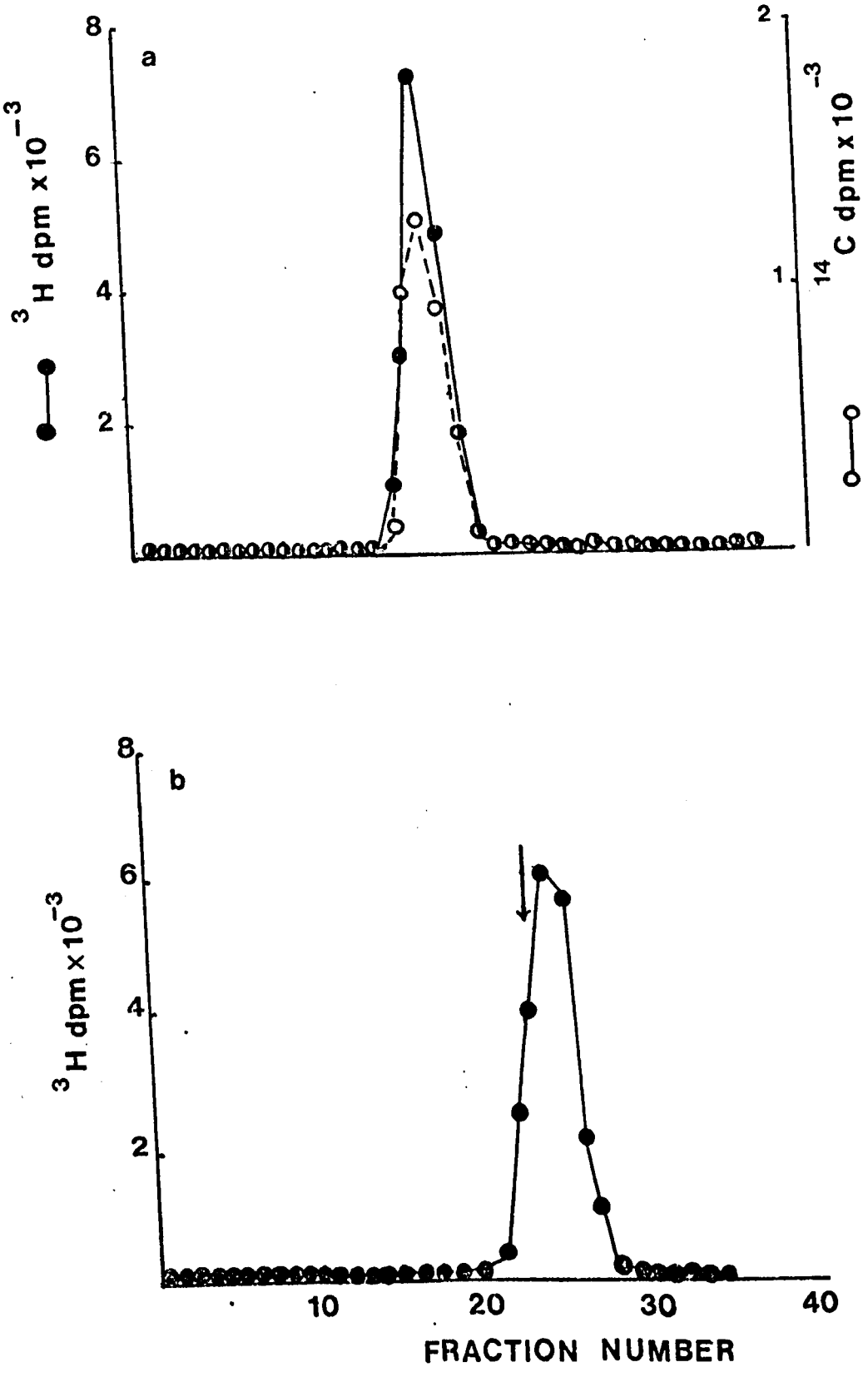
Fig. 14

Sedimentation analysis of 15S DNA prepared by pronase digestion of triton extract from C13 cells.

^{3}H-thymidine-labelled triton extract was treated with pronase and sedimented in a neutral sucrose gradient as described in the legend to Fig. 13. Fractions containing 15S DNA were pooled as shown (Fig. 13) and desalted into 0.01 M tris-HCl, 0.01 M EDTA, pH 7.9.

(a) A 0.2 ml sample of extract was mixed with ^{14}C-SV40 DNA and sedimented to equilibrium in CsCl solution as described in the legend to Fig.5.

(b) A 0.2 ml sample of extract was sedimented in an alkaline sucrose gradient as described in the legend to Fig. 4b. The vertical arrow denotes the position of SV40 form-II DNA in the same gradient.


a
b
^{3}H dpm x10^{-3}
^{14}C dpm x10^{-3}
8
6
4
2
2
1
10
20
30
40
FRACTION NUMBER

sediments at 24S under the conditions employed. The 95S species was purified by neutral sucrose gradient centrifugation (see Fig. 12b), desalted into 0.01 M tris-HCl, 0.01 M EDTA and analysed by alkaline gradient centrifugation (Fig. 15). The DNA sediments in an identical manner to that released from purified virions (see Fig. 4b), substantiating the presence of MVM DNA.

3.4.2 b. Comparison of Triton-Extracted DNA and MVM

The results of pronase digestion and alkaline sucrose gradient centrifugation are consistent with the fact that the fast-sedimenting component in triton extracts contains single-stranded MVM DNA. It is possible, therefore, that this species represents mature MVM virus. Experiments were designed to clarify this point.

A 0.2 ml sample of freshly-prepared ^{3}H-thymidine labelled triton extract from Cl3 cells was mixed with ^{14}C-labelled purified MVM virus and analysed by neutral sucrose gradient centrifugation (Fig. 16a). As a control, ^{3}H-thymidine labelled MVM was treated with 0.25% triton-X-100 in 0.01 M tris-HCl, 0.01 M EDTA, pH 7.9 for 1 hr at 20°C, after which the salt concentration was adjusted to 0.2 M using 2 M NaCl. A 0.2 ml sample was mixed with purified ^{14}C-MVM and analysed as above.

The results indicate (Fig. 16b) that the ^{3}H-triton-treated and ^{14}C-untreated MVM co-sediment. The ^{3}H-triton extracted component from Cl3 cells, however, sediments significantly behind the ^{14}C-MVM marker (Fig. 16a). This result was obtained for triton-extracted components from both A9 and Cl3 cells. Assuming a sedimentation coefficient of 110S for MVM (Crawford et al., 1969), the fast-sedimenting component in triton extracts has an S value of about 95S.

Samples of the 95S component, purified by neutral sucrose

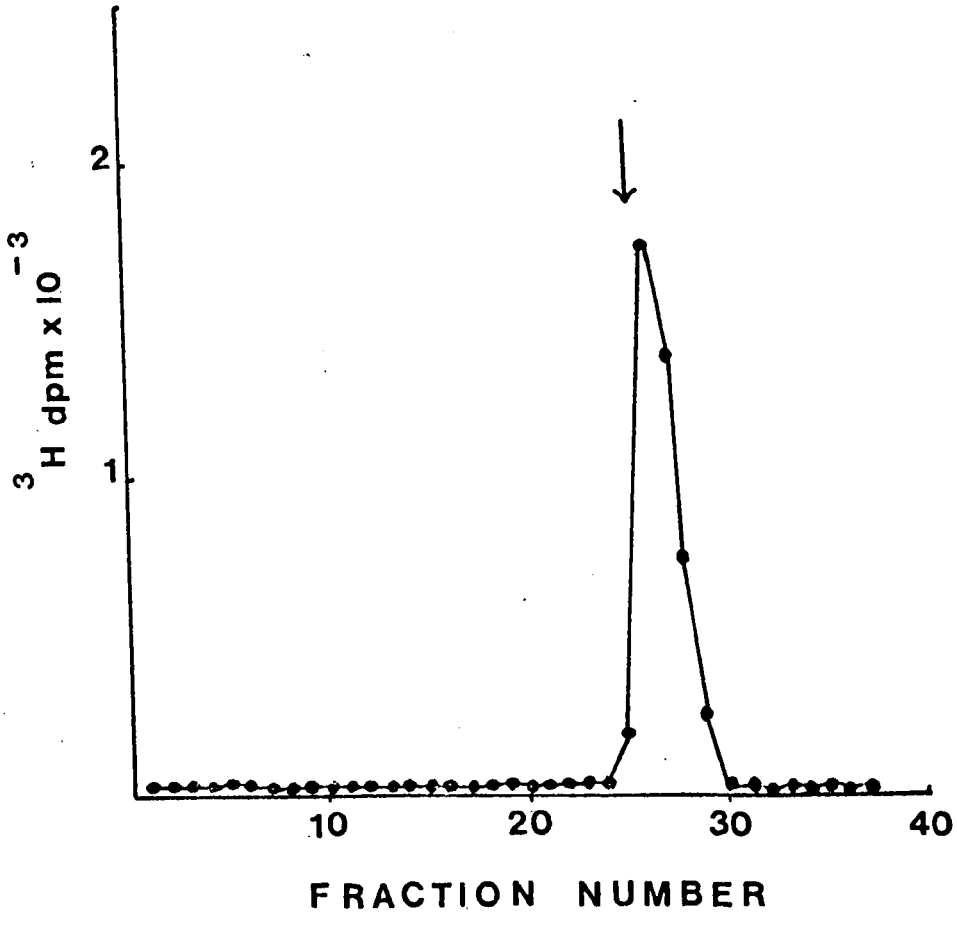


Fig. 15

Alkaline sucrose sedimentation analysis of the 95S component in triton extracts.

^{3}H-thymidine triton extract from MVM-infected C13 cells, labelled from 16 - 22 hpi, was sedimented in a neutral sucrose gradient and the fractions in the 95S region pooled as shown in Fig. 12b. After desalting into 0.01 M tris-HCl, 0.01 M EDTA pH 7.9, a 0.2 ml sample was analysed by alkaline sucrose gradient centrifugation as described in the legend to Fig. 4b.

The arrow shows the position of ^{14}C-SV40 form-II DNA in the same gradient.

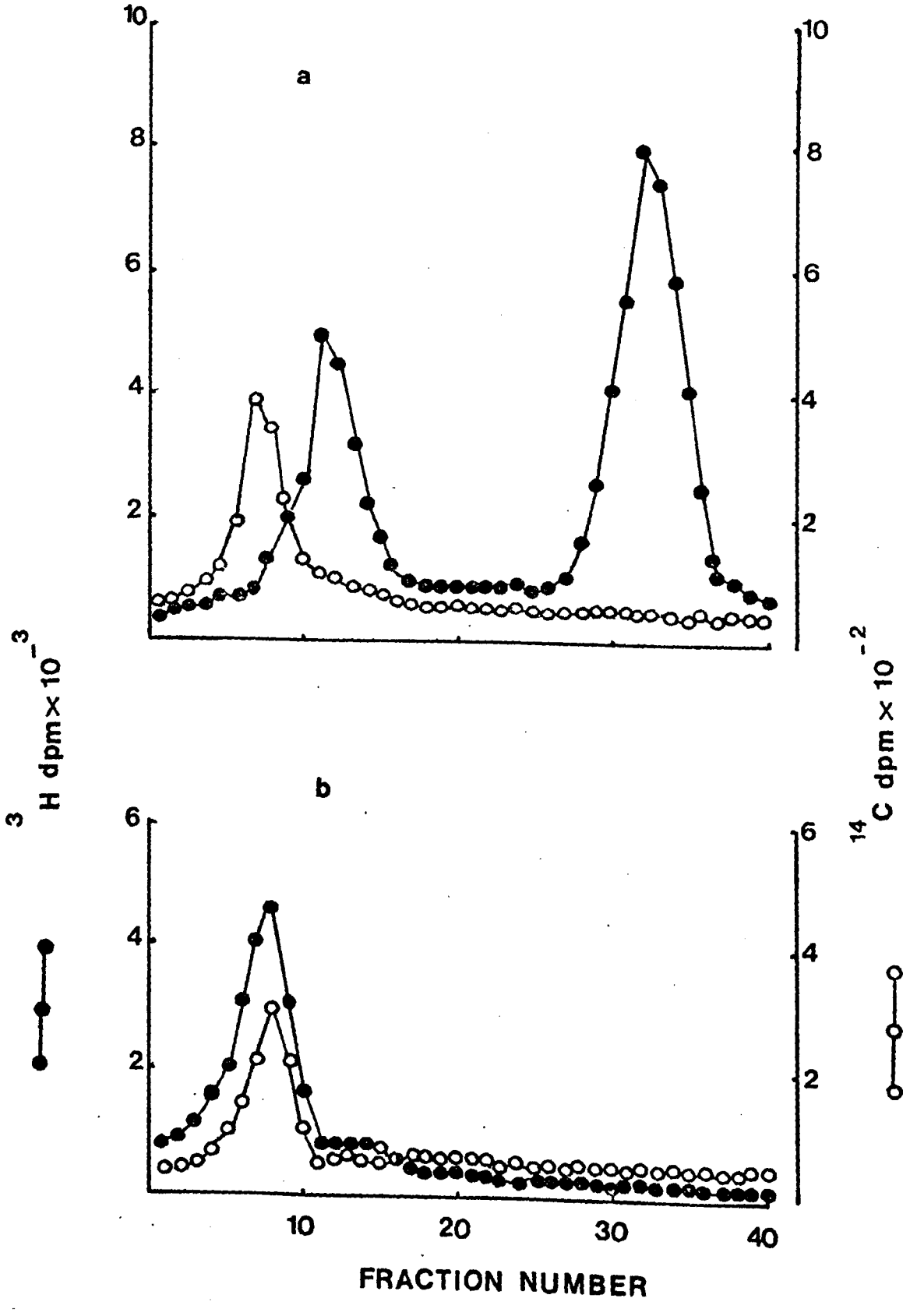
Fig. 16

Comparison of the "fast-sedimenting" component in triton extracts of infected cells with purified MVM virus, by velocity sedimentation.

(a) ^{3}H-triton extract was mixed with ^{14}C-thymidine purified MVM virus and sedimented in a 5 - 20% neutral sucrose gradient at 100,000 g (average), for 2 hr in an SW50L rotor at 4°C.

(b) ^{3}H-thymidine labelled purified MVM virus was treated with 0.25% triton-X-100 in 0.01 M tris-HCl, 0.01 M EDTA, pH 7.9. After 1 hr at 20°C, the salt concentration was adjusted to 0.2 M with 2 M NaCl. A 0.2 ml sample of triton-treated virus was mixed with ^{14}C-MVM virus and sedimented as in (a).

The triton extract was prepared as described in the legend to Fig. 12b.


a
b
10
8
6
4
2
10
8
6
4
2
6
4
2
6
4
2
10
20
30
40
FRACTION NUMBER
^{3}H dpm X 10^{-3}
^{14}C dpm X 10^{-2}

gradient centrifugation, and purified MVM virus, were prepared for electron microscopy as described in the Methods section. The results are shown in Figure 17.

Virus particles (Fig. 17a) can be easily distinguished. They are uniform in size with approximately hexagonal outlines, which are densely shadowed. In contrast, particles of the 95S component (Fig. 17b) have irregular shapes, shadow less densely, and appear to be less tightly packaged. In some preparations of triton extract, virus particles could be seen, but represented a very small percentage of the total number of particles. Triton treatment of MVM had no effect on the appearance of the virion. A characteristic of triton-extracted 95S particles in some preparations was the appearance of material extruding from their centre.

Since this 95S species has been shown to contain unit length MVM DNA it is possible that it represents a "maturation complex" of MVM, distinct from the mature virion.

3.4.3 Time Course of Synthesis of DNA Extractable by Triton

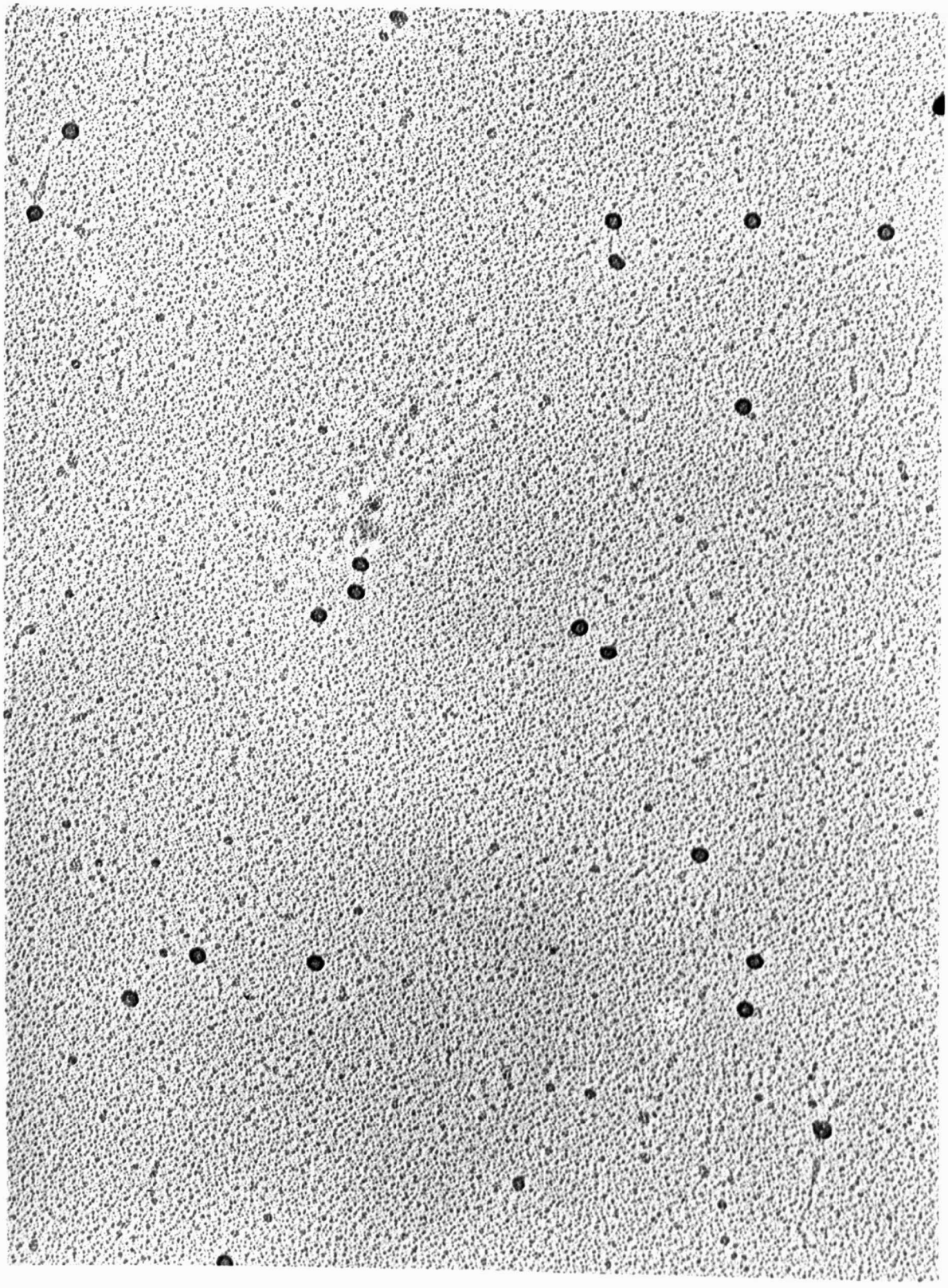
In order to determine the temporal order and rate of synthesis of DNA species extractable by the triton method, infected and mock-infected A9 cells were pulse-labelled with ^{3}H-thymidine in regular 2 hr intervals up to 24 hpi. This experiment was done in parallel with the Hirt/Pronase time course (see section 3.2). At the end of each pulse-label, the cultures were subjected to the triton method of extraction. Aliquots of supernatant and solubilised pellet were assayed for acid-insoluble radioactivity.

Figure 18 shows the time course of appearance of low molecular weight DNA in triton extracts. It can be seen that, up to 12 hpi, the amount of DNA appearing in triton extracts of virus-infected and control cells is almost identical while, between 12 - 24 hpi there is

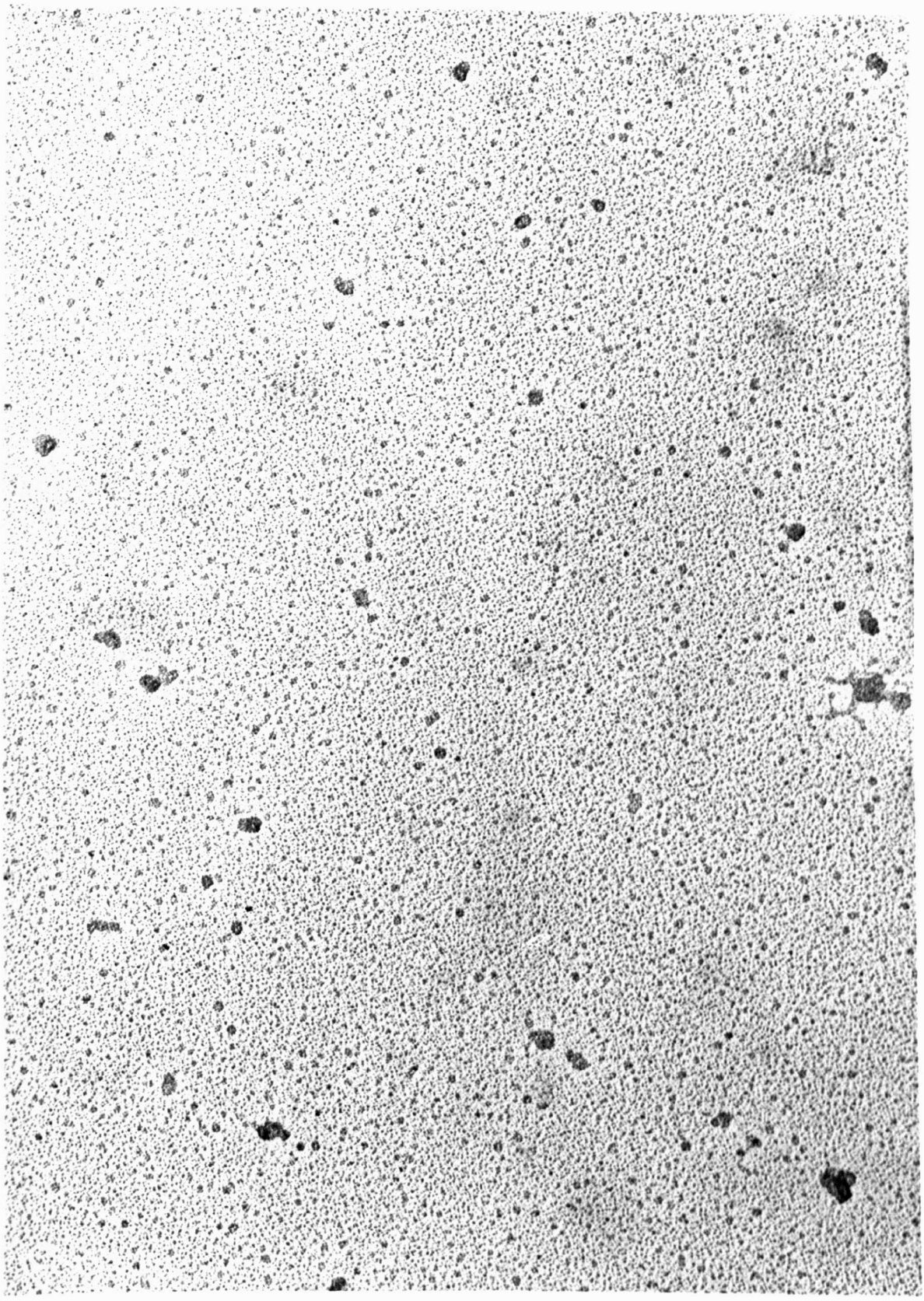
Fig. 17

Electron microscopy of the 95S triton-extracted component and MVM virus.

Samples of the 95S component were prepared and purified by neutral sucrose gradient centrifugation as detailed in the legend to Fig. 15. Samples of purified MVM virus (a) and 95S component (b) were prepared for electron microscopy as described in Materials and Methods.



17 a



17 b

Fig. 18

Incorporation of ^{3}H-thymidine into triton extracts of MVM-infected and mock-infected A9 cells at different times after infection.

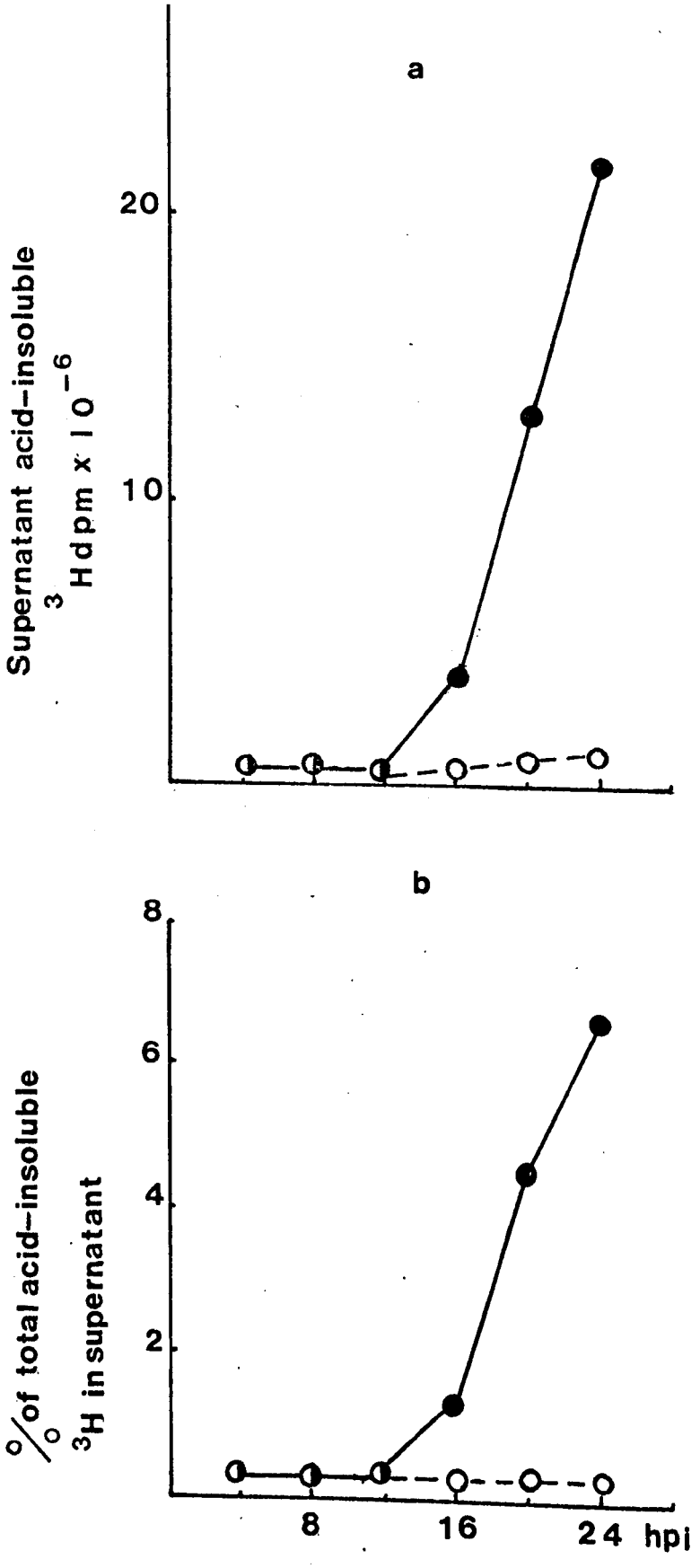
Infected cultures of A9 cells were pulse-labelled with ^{3}H-thymidine (10 μCi/ml) in regular 2 hr intervals up to 24 hpi. Yields of acid-insoluble ^{3}H-thymidine incorporated into total DNA/culture and into triton-extracted DNA/culture during each 2 hr period were calculated by assaying 0.1 ml samples of solubilised pellet and supernatant material. Cumulative yields per culture were calculated as described in the legend to Fig. 2.

(a) Cumulative yield of acid-insoluble ^{3}H-thymidine in triton extracts/culture.

(b) Percentage of total acid-precipitable ^{3}H-thymidine appearing in triton extract.

●—●—● Infected

o—o—o Mock-infected.


a
Supernatant acid-insoluble
^{3}H dpm x 10^{-6}
20
10
b
% of total acid-insoluble
^{3}H in supernatant
8
6
4
2
8
16
24 hpi

a rapid increase in the amount of DNA extracted from infected cells (Fig. 18a). The percentage of total labelled DNA extracted from mock-infected cells (Fig. 18b), is constant throughout infection (0.3%), while the percentage extracted from infected cells increases from 12 hpi, reaching 7% by 24 hpi (Fig. 18b).

When these results are compared to those obtained using the Hirt/Pronase method of extraction (Fig. 2b, c), two major differences can be observed:-

1) The onset of the increase in extractable DNA was delayed in the triton method (from 8 to 12 hpi).

2) Less DNA was extracted, throughout infection, by the triton method (about 30% of the yield obtained using the Hirt/Pronase procedure).

The nature of the DNA extracted throughout infection was characterised by neutral sucrose gradient centrifugation.

3.4.4 Analysis of Triton-Extracted DNA at Different Times During Infection

When the triton-extracted DNA is analysed by neutral sucrose gradient centrifugation, the DNA profiles obtained for infected and control cells from 0 - 10 hpi are identical, very low levels of DNA being extracted (Fig. 19a). Analysis of triton-extracted DNA from cells labelled from 10 - 12 hpi indicates the appearance of the 95S component, which has been shown in previous sections to contain viral DNA. MVM duplex DNA was not extractable by the triton method until 12 - 14 hpi (Fig. 19a).

Figure 19b shows the amount of ^{3}H-thymidine incorporated into 95S "complex" and duplex DNA throughout the course of infection.

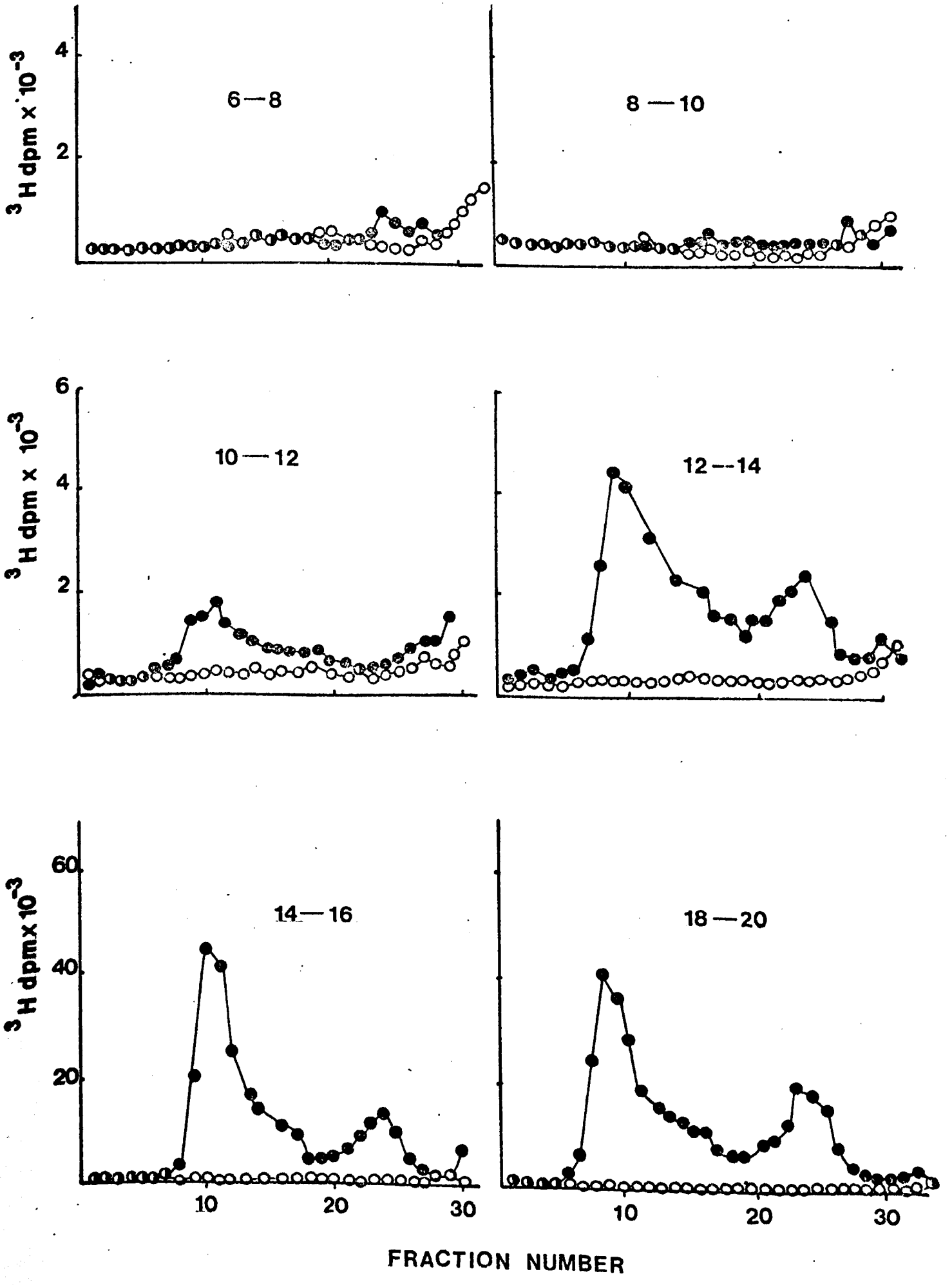
Fig. 19a

Sedimentation analysis of triton extracted DNA from MVM-infected and mock-infected A9 cells throughout infection.

0.2 ml samples of triton extracts prepared as described in the legend to Fig. 18 were sedimented in 5 - 20% neutral sucrose gradients for 2 hr at 100,000 g (average) in a Spinco SW50L rotor at 4°C. Examples of profiles obtained at different times after infection are presented.

●—●—● Infected

o—o—o Mock-infected.


6—8
8—10
10—12
12—14
14—16
18—20
^{3}H dpm x 10^{-3}
^{3}H dpm x 10^{-3}
^{3}H dpmx10^{-3}
FRACTION NUMBER

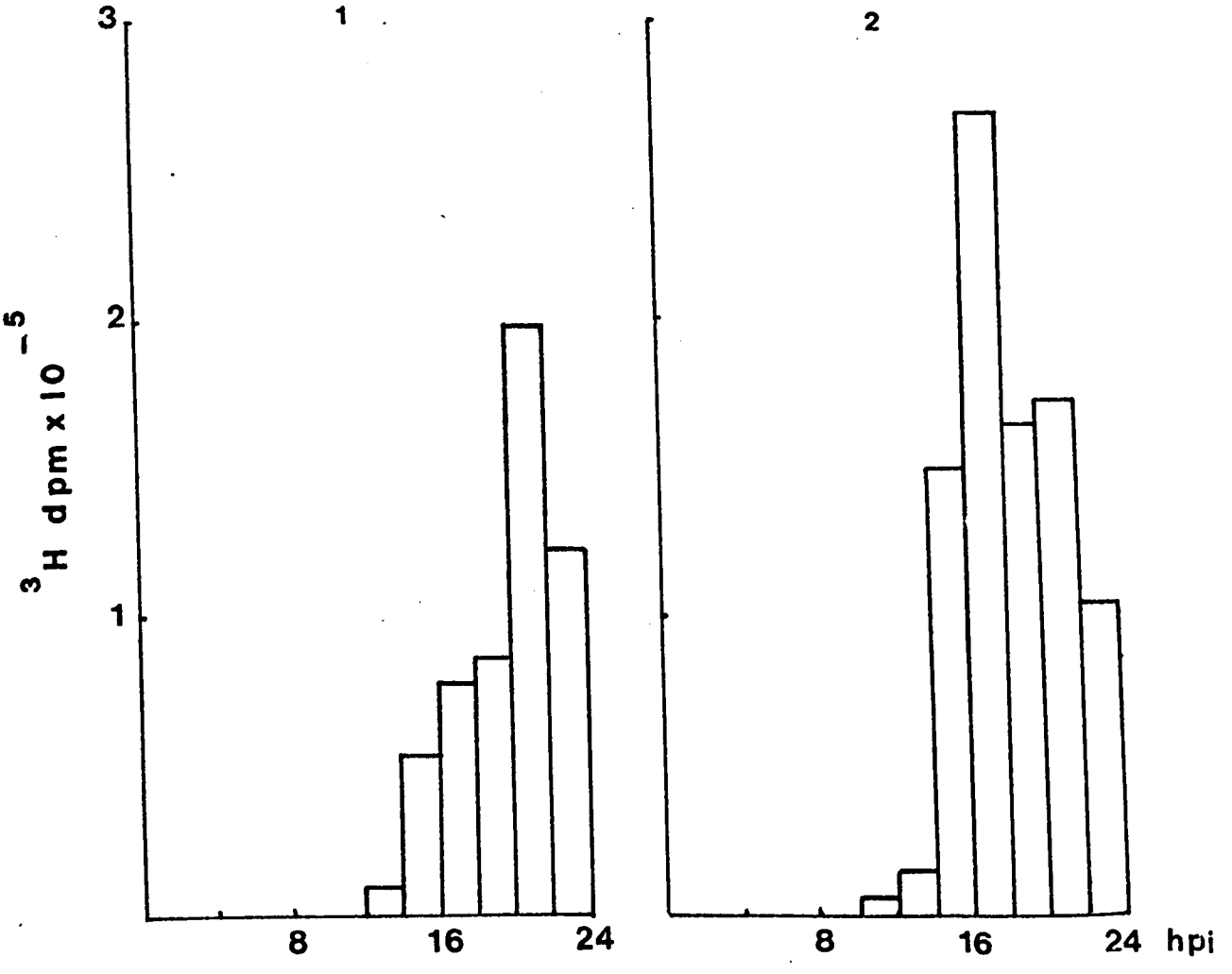


Fig. 19b

Yields of DNA components extractable by the triton method throughout infection.

The yields of DNA-containing species extracted by the triton method from infected A9 cells throughout infection were estimated by summation of the acid-insoluble ^{3}H-thymidine sedimenting in the 18 - 21S region (1) and 95S region (2) of sucrose gradients. Conditions of sedimentation are detailed in the legend to Fig. 19a.

The rate of synthesis of 95S "complex" (MVM DNA) (Fig. 19b) increases from 10 hpi, reaches a maximum in the 16 - 18 hr time period and falls off later in infection. The amount of duplex MVM DNA extracted by the triton method increases from 12 - 22 hpi (Fig. 19b).

The reason why the fast-sedimenting "complex, which has been shown to contain single-stranded progeny DNA, should appear before MVM duplex DNA in triton extracts, is unknown. It probably reflects the inefficiency of the triton method for the extraction of duplex DNA from A9 cells.

3.5 KINETIC STUDIES

3.5.1 Rapidly-Labelled DNA extracted by the Hirt/Pronase Method

Newly-synthesised MVM DNA was studied by pulse-labelling infected cultures with ^{3}H-thymidine for short time periods, and analysing the products obtained by Hirt/Pronase extraction on sucrose gradients.

Infected A9 cells were pulse-labelled with ^{3}H-thymidine for 2 min at 16 hpi, and low molecular weight DNA extracted by the Hirt/Pronase method. The results of neutral sucrose gradient analysis of extracted DNA are shown in Fig. 20. It is clear that the distribution of ^{3}H-thymidine throughout the gradient differs from that obtained when infected cells are labelled for longer times (see Fig. 3a). There is a major peak of DNA with a sedimentation coefficient of about 15S at peak maximum, which is skewed slightly towards the fast-sedimenting side. In addition there is a small amount of DNA remaining on the top of the gradient. The proportion of DNA sedimenting faster than 19S is, however, greatly reduced when compared to the profile of DNA extracted from cells labelled for 2 hr (Fig. 3a). This indicates that the vast majority of the newly-synthesised DNA sequences are associated with DNA sedimenting between 13 - 19S. There is therefore a lag between the synthesis of "15"S and "24"S DNA.

Infected A9 cells were labelled from 0 - 16 hpi with ^{14}C-thymidine (1 μCi/ml) after which the medium was removed, the cultures washed with PBS, and pulse-labelled for 4 min with ^{3}H-thymidine. The cells were subjected to Hirt/Pronase extraction and the low molecular weight DNA analysed by neutral sucrose gradient centrifugation (Fig. 21a). The ^{14}C-thymidine was incorporated into the DNA species in

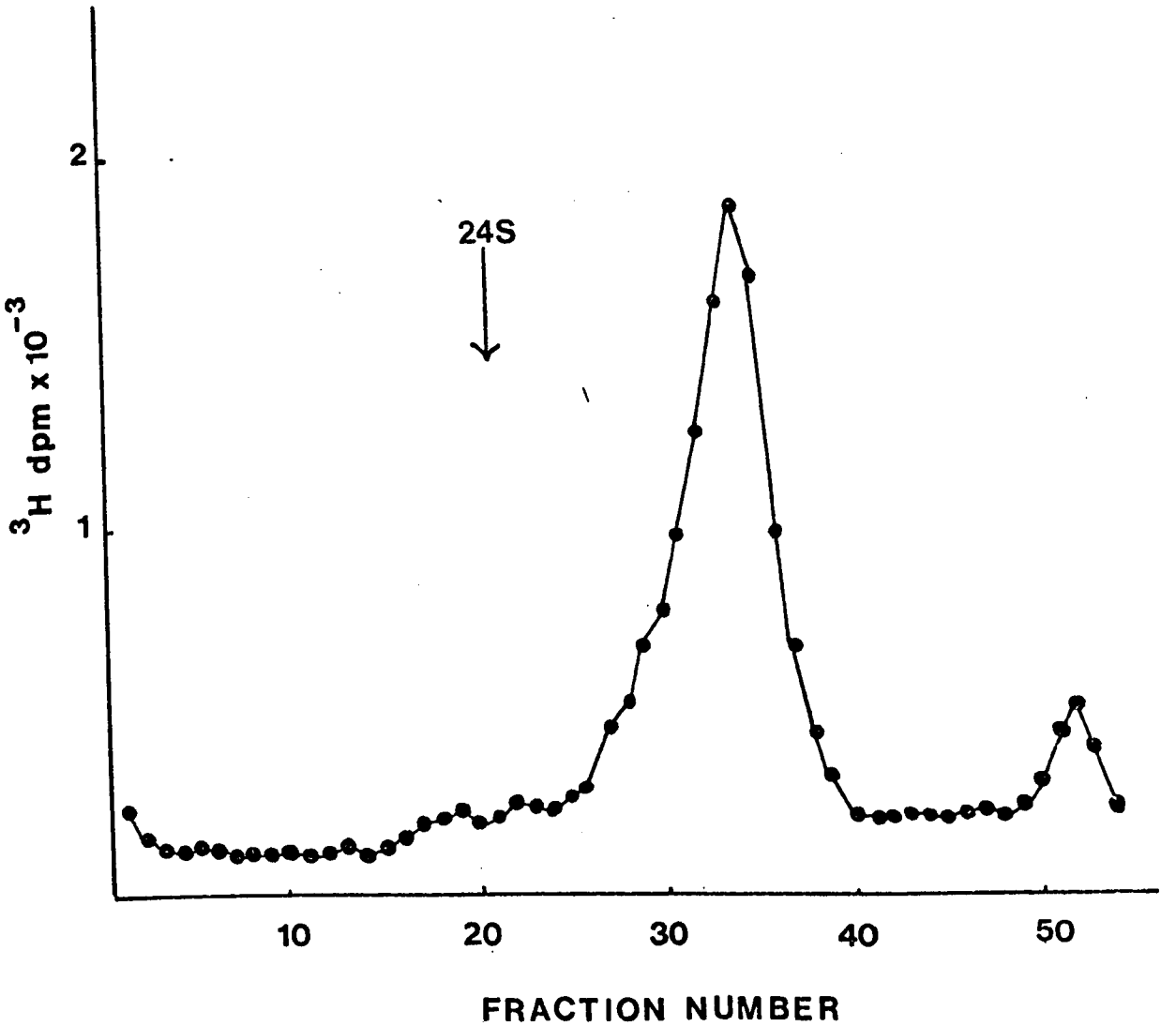


Fig. 20

Sedimentation analysis of rapidly-labelled DNA extracted from MVM-infected cells by the Hirt/Pronase method.

Monolayer cultures of A9 cells were infected with MVM at a multiplicity of 5 pfu/cell. At 16 hpi the cells were exposed to ^{3}H-thymidine (10 μCi/ml) for 2 min and low molecular weight DNA extracted by the Hirt/Pronase method. A 0.2 ml sample of supernatant DNA was sedimented in a 5 - 20% neutral sucrose gradient at 120,000 g (average) for 5 hr at 4°C in an SW56 rotor.

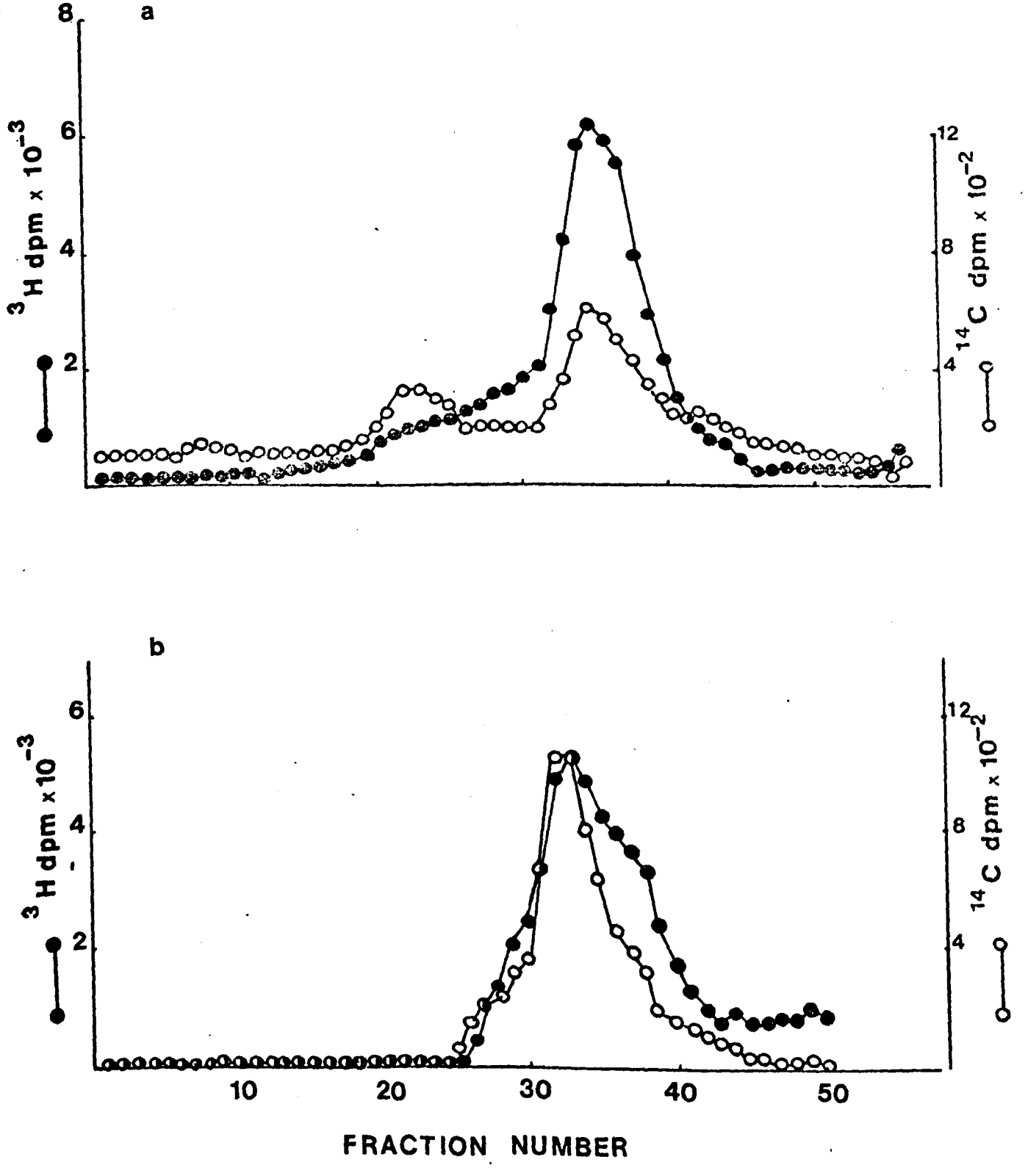
Fig. 21

Sedimentation analysis of double-labelled DNA extracted by the Hirt/Pronase method.

Cultures of A9 cells were infected with MVM (5 pfu/cell) and, after the period of adsorption, labelled with ^{14}C-thymidine (1 μCi/ml). At 16 hpi, the medium was removed, the cells washed with PBS, and labelled with ^{3}H-thymidine (10 μCi/ml) for 4 min. Low molecular weight DNA was extracted by the Hirt/Pronase method.

(a) A 0.2 ml sample of extracted DNA was sedimented in a 5 - 20% neutral sucrose gradient at 120,000 g (average) for 5 hr at 4°C in an SW56 rotor.

(b) A 0.2 ml sample of extracted DNA was analysed by alkaline sucrose gradient centrifugation in an SW56 rotor at 120,000 g (average) for 5 hr at 4°C.


a
b
^{3}H dpm x 10^{-3}
^{14}C dpm x 10^{-2}
FRACTION NUMBER

approximately the same proportions as found previously under long labelling regimes (Fig. 3a). The ^{3}H-thymidine, however, was preferentially incorporated into the slow-sedimenting DNA species.

When analysed by alkaline gradient centrifugation (Fig. 21b) the peaks of ^{14}C-DNA and ^{3}H-DNA sediment coincidently. The ^{3}H-DNA profile, however, has a distinct shoulder sedimenting slower than the main peak, indicating that the nascent DNA contains a higher proportion of molecules which are shorter than unit length MVM DNA.

3.5.2 Pulse-Chase Kinetics - Hirt/Pronase Method

To investigate the relationship between the species of DNA extracted by the Hirt/Pronase method, pulse-chase experiments were designed. If the 15S duplex MVM DNA is a necessary intermediate in the synthesis of virus progeny single-stranded DNA, pulse-chase experiments should reveal the displacement of labelled viral DNA from the RF pool, with concomitant accumulation of progeny DNA molecules.

8 monolayer cultures of A9 cells were infected with MVM at a multiplicity of 5 pfu/cell. At 16 hpi the medium was removed and replaced with 2 ml of EFc5 containing ^{3}H-thymidine (10 μCi/ml). After 2 min at 37°C the medium was removed and low molecular weight DNA extracted from two cultures by the Hirt/Pronase method. The remaining cultures were washed with Eagle's medium and overlaid with 10 ml EFc5 containing unlabelled thymidine (100 μg/ml) and 2'-deoxycytidine (10 μg/ml) and further incubated at 37°C. Duplicate cultures were extracted by the Hirt/Pronase method at 30 min, 3 hr and 6 hr after the removal of radioactive thymidine. 0.2 ml samples of the extracted DNA were analysed by neutral sucrose gradient centrifugation in 1 M NaCl.

The results are shown in Fig. 22.

Fig. 22a indicates that a 2 min pulse-label at 16 hpi preferentially labels DNA in the 13 - 19S region of the gradient as already described (3.5.1). When the cells were pulse-labelled for 2 min and chased with medium containing excess unlabelled thymidine for 30 min (Fig. 22b) there was an increase of about 2-fold in the total amount of labelled DNA sedimenting around 15S. Since the medium contained 100 fold excess unlabelled thymidine this probably reflects slow equilibration between extracellular and intracellular pools of thymidine. There was, however, a significant increase in the amount of DNA sedimenting faster than the main peak. One major species can be detected around 24S with a minor species sedimenting faster. The proportion of DNA sedimenting faster than the main 15S peak after the 30 min chase is increased, indicating that these species of DNA were preferentially labelled during the chase.

With increasing the time of chase, the amount of radioactivity in the main 15S peak decreased, indicating displacement of labelled DNA strands from the RF pool (Fig. 22c, d). There was no concomitant increase, however, in the amount of fast-sedimenting DNA to indicate the accumulation of progeny DNA. The most logical explanation is that, even with pronase digestion, the Hirt method is inefficient at releasing encapsidated viral DNA.

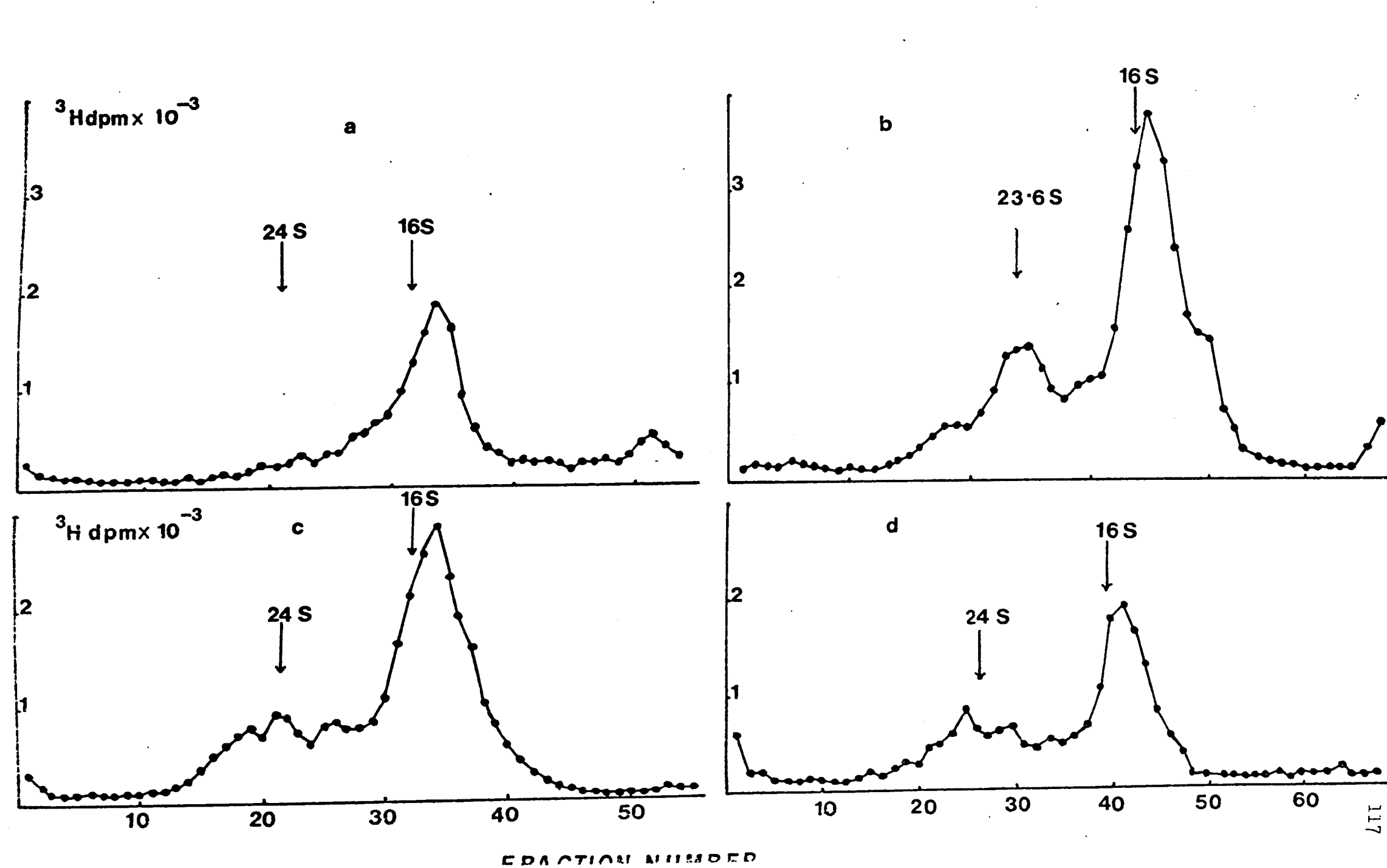
3.5.3 Rapidly-Labelled DNA Extracted by the Triton Method.

Infected C13 cultures were pulse-labelled with ^{3}H-thymidine for 2 min at 16 hpi and the cells extracted by the triton method. The results of neutral sucrose gradient analysis of the triton extract

Fig. 22

Pulse-chase kinetics - Hirt/Pronase extraction of infected cell DNA.

8 monolayer cultures of A9 cells were infected with MVM at a multiplicity of 5 pfu/cell. At 16 hpi, the medium was removed and replaced with 2 ml of EFc5 containing ^{3}H-thymidine (10 μCi/ml). After 2 min at 37°C the medium was removed and low molecular weight DNA extracted from 2 cultures by the Hirt/Pronase method (a). The remaining cultures were washed with Eagle's medium and overlaid with 10 ml of EFc5 containing unlabelled thymidine (100 μg/ml) and 2'-deoxycytidine (10 μg/ml), and further incubated at 37°C. Duplicate cultures were extracted using the Hirt/Pronase method at 30 min (b), 3 hr (c), and 6 hr (d) after the removal of radioactive thymidine. 0.2 ml samples of each extract were analysed by neutral sucrose gradient centrifugation as described in the legend to Fig. 21.


^{3}Hdpm x 10^{-3}
a
24 S
16S
b
16 S
23·6 S
^{3}H dpm x 10^{-3}
c
16S
24 S
d
16 S
24 S
10
20
30
40
50
60
FRACTION NUMBER

are shown in Fig. 23. It is clear that all the newly-synthesised DNA sequences are associated with the slow sedimenting duplex DNA component. No radioactivity is detected in the 95S region of the gradient. A similar experiment was performed using A9 cells.

Infected A9 cells were labelled from 0 - 16 hpi with ^{14}C-thymidine (1 μCi/ml), after which the medium was removed, the cultures washed with PBS, and the cells pulse-labelled for 4 min with ^{3}H-thymidine. DNA was extracted by the triton method, and a 0.2 ml sample analysed by neutral sucrose gradient centrifugation (Fig. 24). The distribution of ^{14}C-thymidine is similar to that found previously in triton extracts of A9 cells early in infection (see Fig. 19a). The vast majority of the ^{14}C-label appears in the 95S region of the gradient, with a minor peak at the position of duplex DNA. The bulk of the ^{3}H-thymidine, however, is associated with the slow-sedimenting duplex DNA. There is also a small peak of ^{3}H-thymidine sedimenting faster than 95S. No ^{3}H-thymidine is found sedimenting coincidently with the ^{14}C-"complex". These results indicate that the 95S "complex" does not contain replicating DNA, substantiating the suggestion that it represents a maturation product.

3.5.4 Pulse-Chase Kinetics - Triton Method

Since the Hirt/Pronase method of DNA extraction does not seem to be very efficient at extracting single-stranded progeny DNA, pulse-chase experiments were performed using the triton extraction method. It has been shown that this method extracts single-stranded viral DNA in the form of a 95S DNA-protein complex. In this way, the accumulation of single-stranded progeny DNA can be monitored.

8 monolayer cultures of each of A9 and Cl3 cells were infected

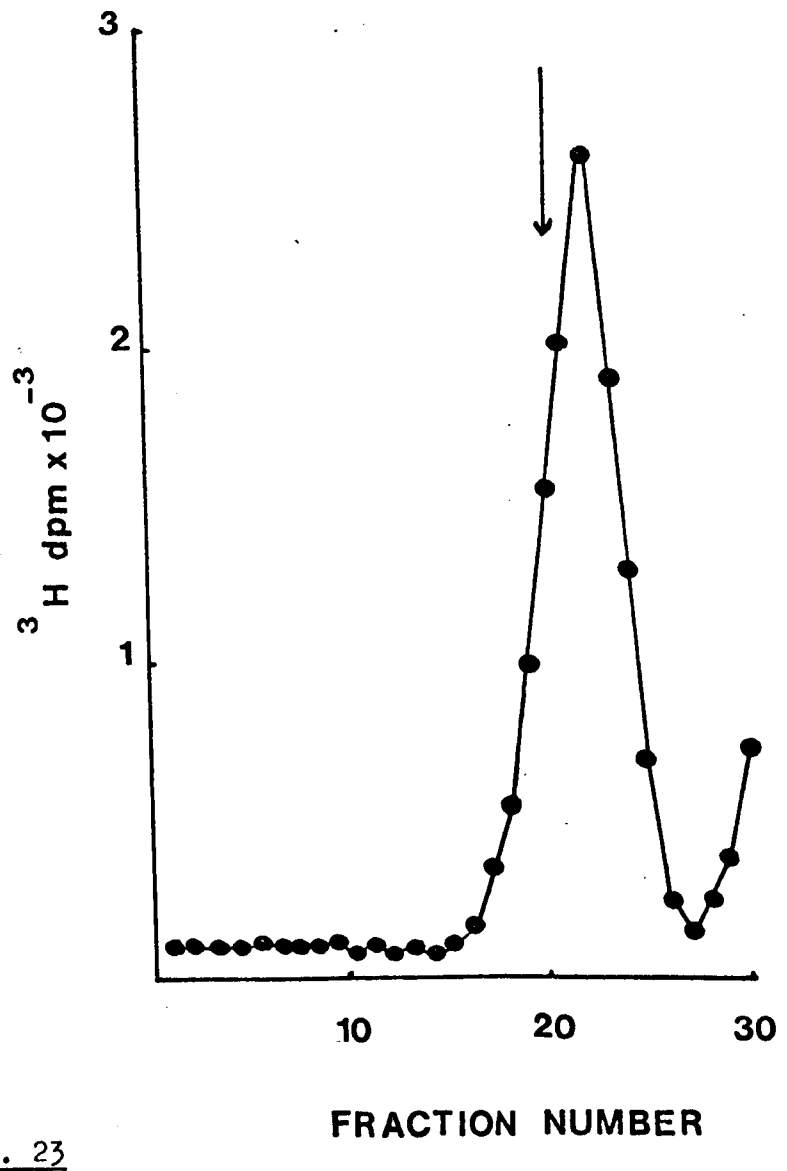


Fig. 23

Sedimentation analysis of rapidly-labelled DNA extracted from MVM-infected cells by the triton method.

Infected C13 cells were pulse-labelled with ^{3}H-thymidine for 2 min at 16 hpi as described in the legend to Fig. 20, and DNA extracted by the triton method. A 0.2 ml sample of triton extract was sedimented in a 5 - 20% neutral sucrose gradient at 100,000 g (average) for 2 hr in an SW56 rotor at 4°C.

The vertical arrow denotes the position of 21S ^{14}C-SV40 form-I DNA.

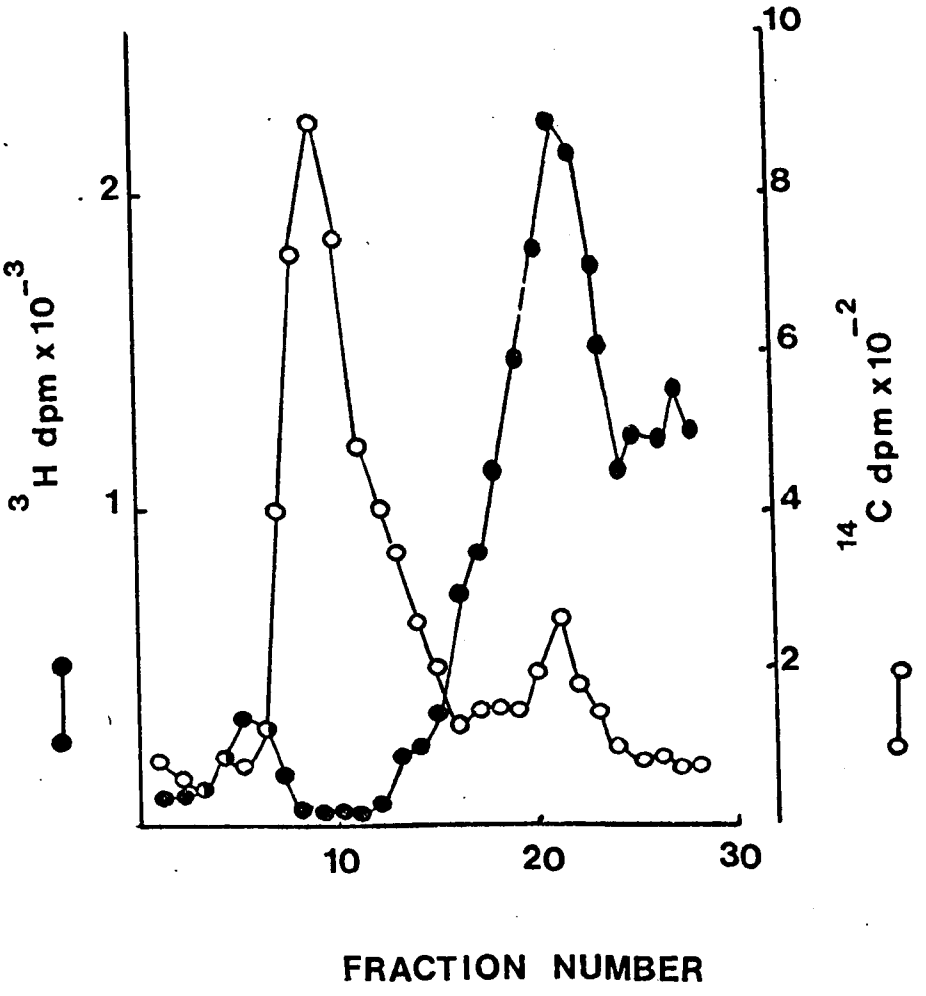


Fig. 24

Sedimentation analysis of double-labelled DNA extracted from infected cells using the triton method.

Infected A9 cells were labelled from 0 - 16 hpi with ^{14}C-thymidine and pulse-labelled for 4 min with ^{3}H-thymidine as described in the legend to Fig. 21. DNA was extracted by the triton method and a 0.2 ml sample of triton extract sedimented in a 5 - 20% neutral sucrose gradient at 100,000 g (average) in an SW56 rotor for 2 hr at 4°C.

with MVM at 5 pfu/cell. At 16 hpi the cultures were pulse-labelled for 2 min with ^{3}H-thymidine (10 μCi/ml) as described in section 3.5.2, and two dishes each of A9 and C13 cells triton extracted. The remaining dishes were overlaid with 10 ml EFc5 containing excess unlabelled thymidine and 2'-deoxycytidine. Duplicate cultures of each of A9 and C13 cells were triton extracted at 30 min, 3 hr and 6 hr after the removal of radioactive thymidine.

Fig. 25 shows the results of neutral sucrose gradient analysis of the triton extracts from A9 cells. The triton method extracts very little labelled DNA from A9 cells pulse-labelled for 2 min (Fig. 25a). There is a small amount of material sedimenting to the bottom of the gradient and a small peak in the position expected for duplex MVM DNA. When chased for 30 min, however (Fig. 25b), a ^{3}H-thymidine peak appears in the 70S region of the gradient, with a distinct shoulder on the fast-sedimenting side (up to about 90S). Since there was also an increase in the amount of duplex DNA the chase was not completely effective, reflecting the slow equilibration of the thymidine pools. When chased for 3 hr (Fig. 25c) the fast-sedimenting material was shifted to higher S values, the main peak corresponding to 95S "complex", with an appreciable amount of trailing material. On increasing the time of chase to 6 hr (Fig. 25d), the vast majority of the fast-sedimenting radioactivity appears in the 95S "complex" peak.

These results indicate that, after a 2 min pulse-label with ^{3}H-thymidine, labelled species sedimenting between 60 - 90S are progressively chased into 95S "complex", in which single-stranded viral progeny DNA accumulates.

Fig. 26 shows the results of sucrose gradient analysis of the

Fig. 25

Pulse-chase kinetics - triton extraction of infected A9 cells.

Cultures of infected A9 cells were pulse-labelled with ^{3}H-thymidine for 2 min at 16 hpi and DNA extracted from duplicate cultures by the triton method before (a) and after chasing for 30 min (b), 3 hr (c) and 6 hr (d) in the presence of unlabelled thymidine and 2'-deoxycytidine. Precise conditions were as described in the legend to Fig. 22.

0.2 ml samples of triton extracts were sedimented in 5 - 20% neutral sucrose gradients in an SW56 rotor for 2 hr at 100,000 g (average) at $4^{\circ}C$.

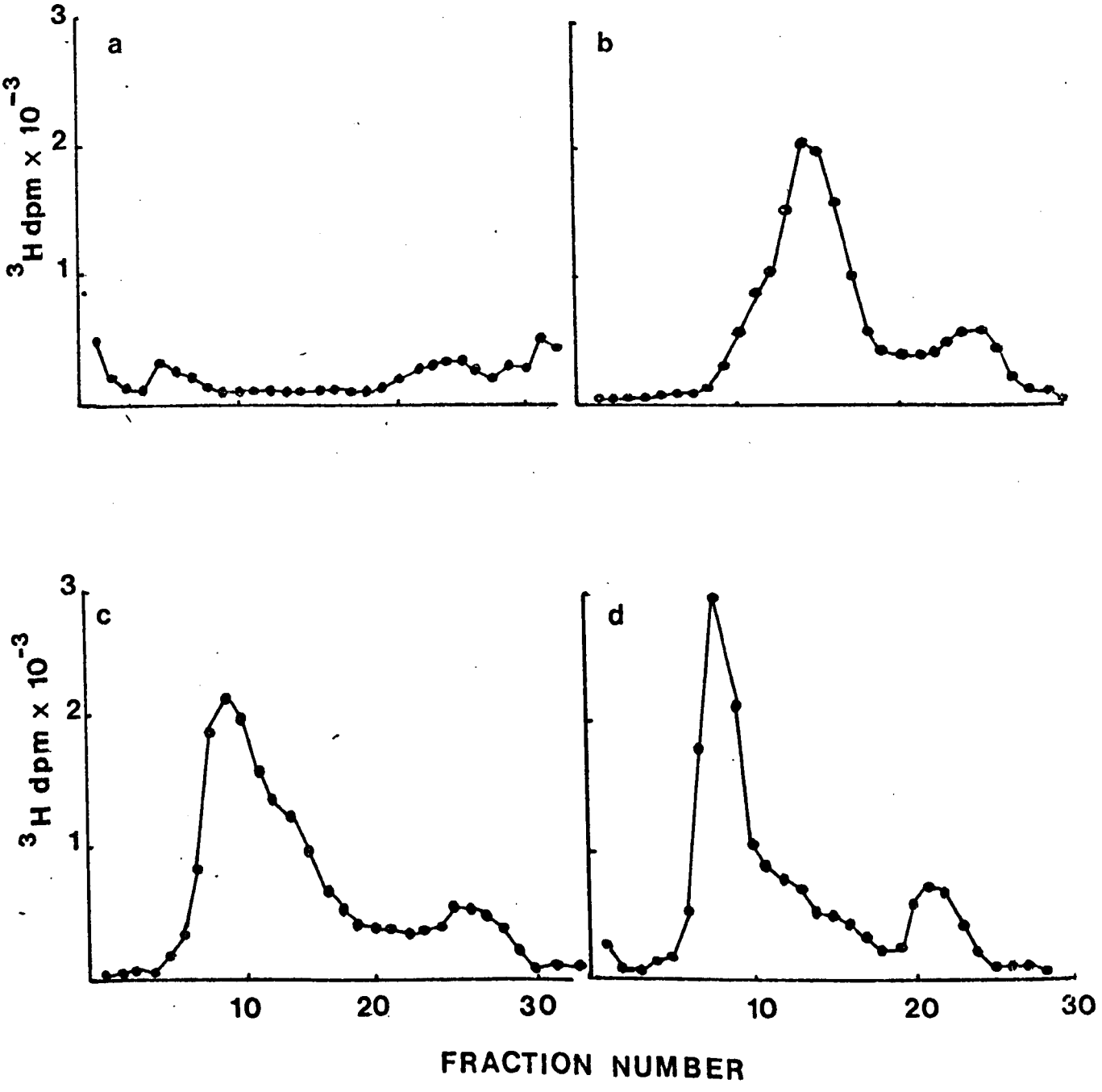
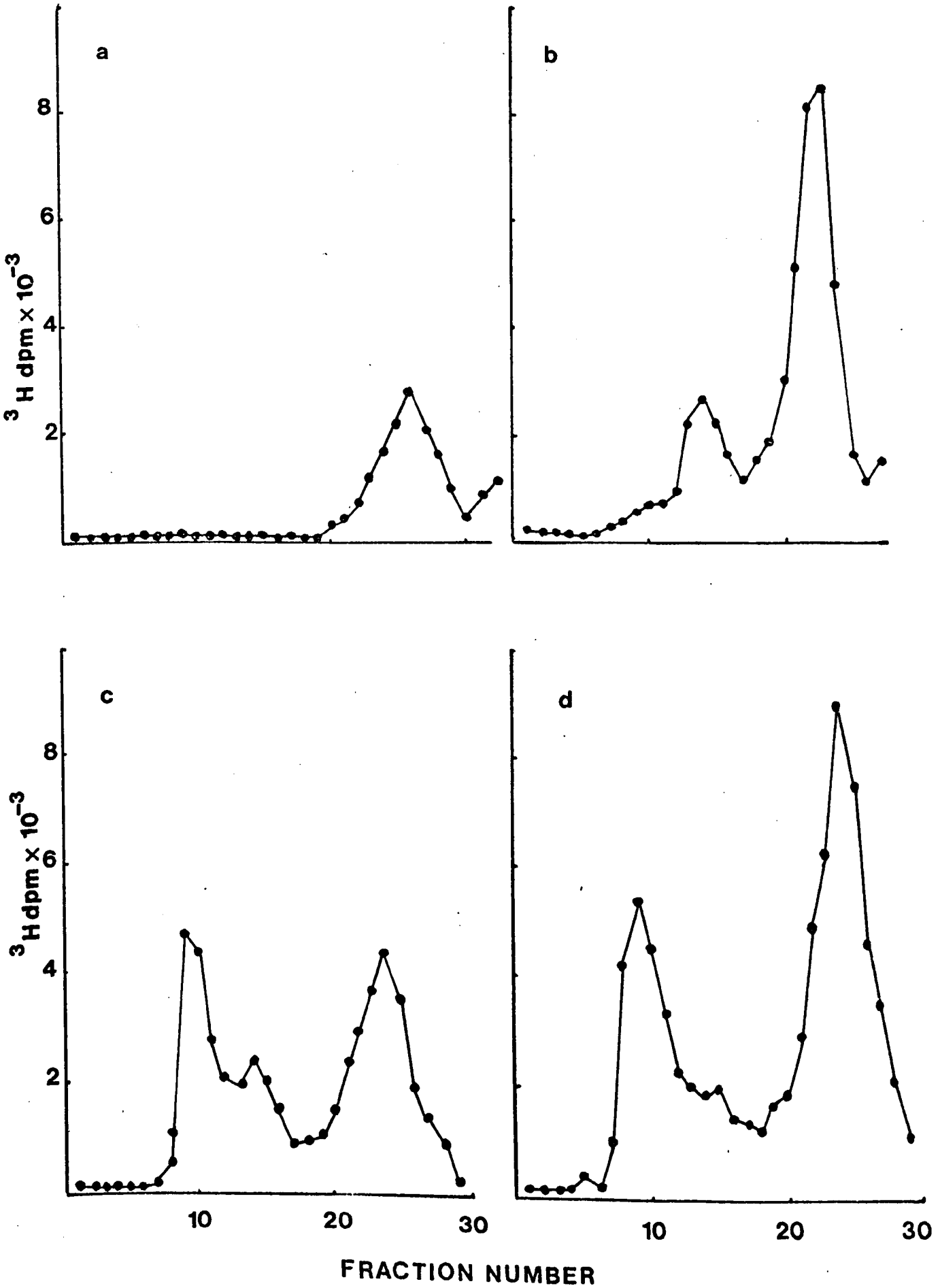

a
b
c
d
^{3}H dpm x 10^{-3}
1
2
3
10
20
30
FRACTION NUMBER

Fig. 26

Pulse-chase kinetics - triton method of DNA extraction from infected Cl3 cells.

This experiment was performed as detailed in the legend to Fig. 25 except that infected cultures of Cl3 cells were used, rather than A9 cells.

Results of sucrose gradient analysis of triton extracts from cells pulse-labelled with ^{3}H-thymidine at 16 hpi for 2 min (a) and chased for 30 min (b), 3 hr (c) and 6 hr (d) in medium containing unlabelled thymidine and 2'-deoxycytidine are shown.


a
b
c
d
8
6
4
2
^{3}H dpm x 10^{-3}
10
20
30
FRACTION NUMBER

triton extracts from Cl3 cells. A 2 min pulse-label incorporates ^{3}H-thymidine exclusively into slow-sedimenting duplex DNA (Fig. 26a). After a 30 min chase there was an increase in the amount of label incorporated into duplex DNA (Fig. 26b). In addition, a distinct peak, similar to that found in infected A9 cell extracts, appears around 70S, with a small amount of material sedimenting faster (Fig. 26b). When incubated in the presence of unlabelled thymidine for 3 hr (Fig. 26c) and 6 hr (Fig. 26d), triton extracts contained a distinct peak of 95S "complex". Since there was a certain amount of fluctuation in the total amount of DNA extracted from each dish, exact quantitation of DNA species and their relationship to one another is not clear.

The results presented in this section suggest the presence of further intermediates in the replication process of MVM.

4. DISCUSSION

The study of viral DNA replication in vivo is limited by the techniques used to extract the DNA from the infected cell. Methods used successfully to recover parvovirus DNA from infected cells have included CPG chromatography (Rhode, 1974a), the selective extraction procedure of Hirt (1967) (Dobson and Helleiner, 1973; Rhode 1974a) and a modification of the Hirt method involving pronase digestion (Salzman and White, 1973; Tattersall et al., 1973). These workers reported that parvovirus DNA could be extracted from infected cells in a double-stranded form which was postulated to be a replicative form (RF) of DNA.

In this study, the Hirt, Hirt/Pronase, whole cell lysate and triton methods were compared both for yields and species of viral DNA extracted from MVM-infected A9 cells. No quantitative comparison of any of these extraction methods has been previously reported. The Hirt/Pronase and whole-cell lysate methods proved to be most efficient in extracting labelled DNA, while the triton method was the least. Neutral sucrose gradient analysis indicated that the Hirt, Hirt/Pronase and whole cell lysate methods extracted similar species of low molecular weight DNA. In each case the major DNA component had a sedimentation coefficient of about 15S, corresponding to the position expected for a unit length duplex RF of MVM DNA. This DNA species was shown, in this work, by displacement hybridisation, to be composed chiefly, and perhaps exclusively, of MVM DNA sequences. "Fast-sedimenting" DNA was also present. There was a greater proportion of fast-sedimenting DNA in the whole-cell extract, indicating that the Hirt/Pronase method is less efficient at extracting these DNA species. Triton extracts contained ^{3}H-thymidine-labelled species sedimenting at 95S and

around 17 - 19S, and these will be discussed later in this section.

The time course of DNA synthesis in A9 cells was studied using the Hirt/Pronase method. When A9 cells were infected with MVM at 5 pfu/cell, the percentage of total labelled DNA appearing in the Hirt/Pronase extracts increased rapidly from 8 hpi, reaching about 19% by 24 hpi, while in control cell extracts it remained relatively constant (about 2%) throughout infection. The time course of DNA synthesis was similar to that obtained by Tattersall et al. (1973) for MVM.

MVM RF DNA was first detected 8 - 10 hpi which is in agreement with the results obtained by Rhode (1974b) for H-1, and Siegl and Gautschi (1976) for Lu-III. On the other hand, within 1 hr of infection of rat nephroma cells with ^{3}H-thymidine-labelled RV (Salzman and White, 1973), 28 - 42% of the parental single-stranded DNA was converted to a double-stranded linear RF. Attempts to repeat this experiment with H-1 (Rhode, 1974b) and Lu-III (Siegl and Gautschi, 1976) were less successful. Very low levels of recovery of prelabelled viral DNA as putative RF were obtained, probably due to a high particle/infectivity ratio. Since the technique used in this present study was less sensitive in detecting small quantities of labelled viral DNA owing to the background levels of cellular DNA contamination, the exact time of synthesis of parental RF, in MVM-infected cells, has not been determined.

As infection proceeds the rate of RF DNA synthesis increases rapidly, reaching a maximum between 16 - 18 hpi. The time course of synthesis of "fast-sedimenting" DNA closely parallels that of RF DNA, the ratio of these two classes of DNA remaining relatively constant from 10 - 24 hpi.

Alkaline sucrose gradient analysis of both "fast-sedimenting" and RF DNA indicated that there was a proportion of each, containing DNA strands longer than unit length MVM DNA. Their maximum length, however, was estimated to be not more than about two genome equivalents. A similar result was obtained by Tattersall et al. (1973) for total double-stranded MVM DNA and by Mayor and Jordan (1976) for X-14 DNA. Recently, however, Straus et al. (1976) have isolated AAV DNA strands of up to 4 genomes long. These findings will be discussed in relation to the mechanism of DNA replication.

Isopycnic gradient centrifugation of the extracted DNA indicated that RF DNA banded as one peak at a density of 1.7 g/cm^3, the position expected for a double-stranded DNA having 40% G + C. On the other hand, the fast-sedimenting DNA contained both double-stranded DNA (1.70 g/cm^3) and single-stranded DNA (1.722 g/cm^3). This was further substantiated by analysis of Hirt/Pronase extracted DNA by centrifugation in low ionic strength sucrose gradients. About 35% of the fast-sedimenting DNA was shifted to the top of the gradient, suggesting that it was single-stranded viral DNA, while the remaining fast sedimenting DNA was found in a position expected for concatemers of 2 - 4 times unit length RF.

Using the Hirt extraction procedure little or no single-stranded progeny DNA was released from cells infected with H-1 (Rhode, 1974a) or Lu-III (Siegl and Gautschi, 1976). Using the Hirt/Pronase method, however, Tattersall et al. (1973) and Straus et al. (1976) recovered MVM and AAV single-stranded DNA. Pronase digestion, therefore, effects the release of progeny DNA.

Double-stranded concatemeric forms of parvovirus DNA have been described. Tattersall et al. (1973) showed that some of the MVM

duplex molecules found in infected cells seemed to be concatemers up to about ten genome equivalents long. These molecules, however, contained no single strands longer than two genome equivalents, and on denaturation and reannealing formed predominantly monomer length duplexes. Concatemers of up to three genome equivalents have been observed in Lu-III-infected cells (Siegl and Gautschi, 1976). Some of these molecules bore small side chains at a regular distance of one RF molecule apart.

The possible role of such molecules in the replication process will be discussed.

It has been reported that, although MVM DNA appears as a linear single-stranded molecule in the electron microscope, it is partially resistant to the single-strand specific nucleases S_1 and exonuclease-I. The results presented here indicate that, even after alkali denaturation, about 12% of the DNA remains S_1 resistant, indicating that the MVM genome contains self-complementary regions, capable of duplex formation.

When native Hirt/Pronase extracted DNA was digested with nuclease S_1, RF DNA (12 - 18S) was found to be 10% single-stranded while DNA from the 18 - 21S region was 15% single-stranded. A larger proportion (36%) of DNA sedimenting between 22 - 28S was S_1 sensitive. These results confirm the previous conclusions derived from isopycnic centrifugation and low ionic strength sucrose gradients, on the distribution of double- and single-stranded DNA sequences in the Hirt/Pronase extracted DNA species.

After heat denaturation, a proportion of each type of DNA remained resistant to digestion by S_1 nuclease. The majority of this spontaneously-renaturing DNA was found in the 12 - 18S region of the

gradient. Analysis of alkali-denatured DNA by agarose gel electrophoresis indicated that 50% of purified MVM RF DNA and about 20% of total Hirt/Pronase extracted DNA was capable of spontaneous renaturation. Renatured DNA migrated as a single peak on agarose gels. These data suggested that a proportion of MVM DNA exists in the form of a "hairpin loop", composed of covalently-linked viral and complementary strands. Such molecules would be expected to sediment faster than unit length viral DNA in alkaline sucrose gradients. Molecules sedimenting in such a manner, both from RF DNA and "fast-sedimenting" DNA, have already been described. The presence of such hairpin molecules suggests that MVM DNA synthesis might be initiated by a self-primed intermediate.

One criticism of such an approach is that, under the conditions employed, the separated strands of MVM duplex DNA may be capable of reannealing. At the concentration of DNA used (< 0.1 µg/ml) and assuming a $Cot_{\frac{1}{2}}$ of 7.4×10^{-4} (determined for AAV DNA in 0.14 M phosphate buffer at 60°C; Carter et al., 1972), it is extremely unlikely that the complementary strands of MVM DNA would reanneal to 50% under the conditions employed for electrophoresis (0.03 M tris-HCl, 0.036 M NaH_2PO_4, 0.001 M EDTA, pH 7.7, 20°C). Furthermore, on analysis of alkali-denatured bacteriophage λ DNA (at a concentration of 20 µg/ml) under similar conditions as used in this work (Hayward, 1972; Hayward and Smith, 1972), very little renaturation was observed.

Such spontaneously-renaturing molecules have been reported in preparations of MVM DNA (Tattersall et al., 1973), and X-14 DNA (Mayor and Jordan, 1976). Recently, Straus et al. (1976) have isolated spontaneously renaturing DNA from AAV-infected cells.

These molecules consist of covalently-linked (+) and (-) strands which can reach at least four times the size of the AAV genome. The most abundant concatemeric species is a dimer which presumably exists in vivo as a unit length hairpin. Pulse-chase experiments indicated that most of the radioactivity in unit length hairpins moves into progeny strands, and therefore these hairpin molecules are directly converted to templates for displacement synthesis. These authors proposed a model for AAV DNA replication, in which DNA synthesis is initiated by a self-priming terminal sequence. Models for the mechanism of DNA replication will be presented later.

Agarose gel electrophoresis was shown to provide better resolution of DNA species extracted by the Hirt/Pronase method than sucrose gradient analysis. This technique was capable of resolving unit length duplex MVM DNA from heterogeneous DNA, both sedimenting between 13 - 19S, and poorly resolved on sucrose gradients. Since the vast majority of this DNA was shown to be double-stranded, and to be mainly composed of viral-specific sequences, these slower-migrating DNA species were postulated to comprise viral duplex molecules of longer than unit length, or branched DNA molecules. Electron microscopy is necessary to determine the precise nature of these DNA species. The quantity of DNA available, however, has made this technically difficult. Branched DNA molecules, with similar sedimentation properties as the species described here, have been reported for Lu-III (Siegl and Gautschi, 1976). Resolution of DNA species sedimenting around 24S was not achieved using gel electrophoresis.

Agarose gel eletrophoresis also provided information on the homogeneity of the unit length duplex MVM DNA, purified from the 15S peak fraction of a sucrose gradient. This DNA migrated as a

single band, slightly faster than SV40 form-III DNA. On cleavage of this DNA with endonuclease R.Eco.R_1, three fragments were produced (A, B, C). A similar result was obtained for AAV-2 DNA (Carter et al., 1975). No other non-defective parvovirus DNA has been studied by restriction endonuclease cleavage. Ordering of the specific fragments will provide a physical map of the MVM genome which may be useful in determining the origin(s) and termination(s) of DNA replication and the direction and extent of transcription of the viral genome.

When MVM-infected A9 or C13 cells were exposed to ^{3}H-thymidine and extracted with the non-ionic detergent triton-X-100, two DNA-containing species, sedimenting around 95S and 18 - 21S, may be distinguished. Analysis of the slow-sedimenting species after pronase digestion, by neutral and alkaline sucrose gradient centrifugation, and by isopycnic centrifugation in CsCl, indicated that this DNA behaved exactly like MVM RF DNA. Since pronase digestion decreased the S value of this DNA only slightly (18 - 21S → 15S) these results suggested that the RF DNA had very little protein associated with it. This unit length MVM RF DNA, therefore, is probably not complexed with viral structural proteins. This is in contrast to triton-extracted DNA from cells infected with the papovaviruses SV40 and polyoma. All the DNA extractable by the triton method exists in the form of viral DNA-protein complexes (White and Eason, 1971; Green et al., 1971), which have been shown to contain viral-specific proteins (McMillen and Consigli, 1974; Sen et al., 1974; Meinke et al., 1975). These complexes contain replicating DNA (White and Eason, 1971; Hall et al., 1973; Seebeck and Weil, 1974) and have been postulated to play a role in the

assembly of virions (Meinke et al., 1975).

Very little triton-extracted DNA was found sedimenting around 24S, the region shown previously to contain both concatemeric and single-stranded viral DNA extracted by the Hirt/Pronase method. This finding suggests that progeny DNA is rapidly encapsidated after synthesis as shown by Siegl and Gautschi (1976) for Lu-III, and that the concatemeric DNA may be tightly bound within the cell.

The MVM 95S component was shown, by neutral sucrose gradient analysis after pronase digestion, and by alkaline sucrose sedimentation, to contain single-stranded viral length DNA. Co-sedimentation of ^{3}H-triton extract and ^{14}C-MVM virus suggested that the 95S species was distinct from the mature virion. This was confirmed by electron microscopy. Since triton-X-100 had no effect on either the sedimentation properties or the appearance of mature virions, the 95S component is not a degradation product of the virus. It is unlikely that the 95S component represents "heavy" (1.47 g/cm^{3}) MVM virions since they have been shown to sediment co-incidently in sucrose gradients (Clinton and Hayashi, 1975). The fact that 95S particles contain mature viral DNA and not replicating DNA, in contrast to SV40 and polyoma complexes, suggests that they may represent "maturation complexes" in the process of virus assembly. It would be of interest to analyse the proteins present in these "complexes" to see if all the viral proteins are present. Owing to the difficulty of obtaining "complexes" containing radio-labelled protein of sufficiently high specific activity this has so far not been achieved.

The time course of appearance of viral DNA species in triton extracts of A9 cells indicates that the onset of synthesis of the 95S "complex" occurs 10 - 12 hpi, about 2 hr after MVM RF can be

detected in Hirt/Pronase extracts. The time course of synthesis of the "complex", which contains progeny DNA, is comparable to that found for Lu-III progeny (Siegl and Gautschi, 1973b) and for RV (Salzman et al., 1972). It must be noted, however, that only the most recently synthesised progeny DNA is likely to be present in the 95S complex and therefore the time course does not take mature virus particles into consideration.

The efficiency of extraction of MVM duplex DNA from A9 cells early in infection by the triton method is extremely low, representing about 10% of total RF DNA extractable by the Hirt/Pronase method. As infection proceeds, however, a greater proportion of total RF DNA is solubilised by the triton method, which may indicate a higher percentage of free RF molecules late in infection.

After infected cells were pulse-labelled for short time periods (2 - 4 min) at 16 hpi with ^{3}H-thymidine, newly-synthesised DNA was found sedimenting in the region of MVM unit length RF DNA, in both Hirt/Pronase and triton extracts. Very little nascent DNA extracted by the Hirt/Pronase method sedimented in the 24S region of the gradient. These results are in contrast to those reported by Rhode (1974a) who found that a short pulse of ^{3}H-thymidine (5 min at 25°C) at 16 hpi, preferentially labelled DNA sedimenting faster than 17S H-1 RF DNA. In Rhode's study, however, using longer labelling regimes, little or no DNA was found sedimenting in this region. Since pronase digestion was not used this can be partially accounted for by the lack of single-stranded progeny DNA. However, in the present experiments, "fast-sedimenting" DNA has been shown to contain a large proportion ($\sim$ 60%) of concatemeric double-stranded DNA, which is absent from Rhode's H-1 DNA preparations.

The results presented here indicate that this concatemeric DNA is not preferentially labelled during a short time of exposure to ^{3}H-thymidine. On the contrary the site of MVM DNA replication appears to be in duplex molecules of approximately RF size. Furthermore, analysis of double-labelled Hirt/Pronase extracted DNA by alkaline sucrose gradient centrifugation indicated that the nascent DNA contained a proportion of molecules of shorter than unit length MVM. A mechanism by which such molecules are synthesised during MVM DNA replication must therefore be considered.

Pulse-chase experiments using the Hirt/Pronase procedure alone failed to produce conclusive evidence of a precursor-product relationship between MVM unit length RF DNA and "fast-sedimenting" DNA. The "24S" DNA was shown to be preferentially labelled during a 30 min chase with medium containing excess unlabelled thymidine but, during longer chase periods (3 hr and 6 hr), when labelled DNA strands were being displaced from the RF pool, no accumulation of "24S" DNA was observed. A possible explanation is that labelled DNA strands were being displaced from both unit length RF DNA and concatemeric double-stranded DNA, and that progeny strands were rapidly encapsidated. Since the Hirt/Pronase method is inefficient at recovering single-stranded progeny DNA, no accumulation would be observed.

Pulse-chase experiments with infected A9 cells, using the triton method of extraction, produced evidence for further possible intermediates in the replication of MVM. After a 2 min pulse-label with ^{3}H-thymidine very little RF DNA was solubilised by the triton method. Comparison with DNA extracted from a parallel culture by the Hirt/Pronase method, however, indicated the presence of an appreciable amount of RF DNA. After a 30 min chase, a major DNA-containing species appeared in

triton extracts, sedimenting around 70S, with a shoulder on the side of higher S value. These species were successfully chased into 95S complex and were therefore concluded to be precursors of complex. Similar experiments performed with Cl3 cells confirmed the existence of these intermediate species. Although these components have not been characterised, their high sedimentation coefficient in neutral sucrose gradients suggests that they probably comprise DNA-protein complexes. It would be of interest to examine the DNA present in these species to see if they may represent "replicating complexes", analogous to those found in SV40 and polyoma-infected cells.

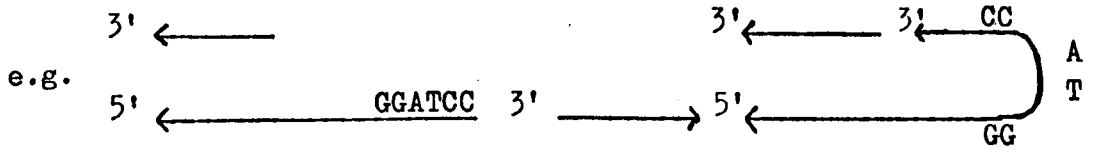
Mechanism of Parvovirus DNA Replication

DNA polymerases have the capacity to elongate polynucleotide chains by adding mononucleotide units to the 3'-OH terminus, but have not been shown to be capable of initiating new ones (Gefter, 1975; Kornberg, 1974). Initiation of new strands is effected by the synthesis of short RNA primers by an RNA polymerase, which are later excised by nucleases (Wickner et al., 1972; Kornberg, 1974). The apparent 3' → 5' growth in the absence of an enzyme that adds mononucleotides to the 5' ends can be explained by the observation that transiently formed short pieces of DNA (Okazaki pieces) themselves primed by RNA (Sugino et al., 1972) are synthesised in the 5' → 3' direction ahead of the main daughter strand (Okazaki et al., 1968). These short pieces are later joined to the main body of the growing chains by DNA ligase. This provides a satisfactory explanation for the mechanism of semi-conservative replication of circular DNA molecules.

For linear DNA molecules which do not replicate via a circular

intermediate, a fundamental problem remains. The excision of the RNA primer from the 5'-termini results in gaps, which cannot be filled because of the lack of a polymerase with 3' → 5' activity. No parvovirus DNA has been shown to exist in the form of a covalently-closed single- or double-stranded circular molecule and therefore an alternative method of replication, which allows completion of 5' termini, must be invoked. Several models have been proposed to effect the completion of the 5' termini of linear DNA molecules.

Watson (1972) proposed that linear DNA molecules must be terminally redundant and must replicate either by circular intermediates (as bacteriophage λ DNA) or via concatemeric replicative intermediates (as T7 DNA). Cavalier-Smith (1974) proposed an alternative method involving a palindromic terminal sequence which is able to form a terminal base-paired loop, capable of self-priming DNA synthesis.



This model has subsequently been modified by Bateman (1975) who suggested that the termini may be covalently continuous (cross-linked) as has been found in vaccinia virus DNA (Geshlin and Berns, 1974).

The results presented in this thesis suggest that MVM DNA replication occurs via a double-stranded linear replicative intermediate (RF) of molecular weight about 3×10^6 daltons, twice that of the viral genome. The fact that MVM DNA is partially resistant to S_1 nuclease, and that viral DNA may be recovered from infected cells in the form of a hairpin molecule composed of covalently-linked viral and complementary strands, suggests that DNA synthesis is mediated by a self-primed intermediate as proposed by Tattersall et al. (1973).

A model for the synthesis of MVM DNA, similar to that proposed by Straus et al. (1976) for AAV DNA is shown in Fig. 4.1. The model is similar to that proposed by Cavalier-Smith (1974), and involves a 3' palindrome which can fold back on itself to form a short duplex region (Fig. 4.1). This serves as a primer for the synthesis of the complementary strand, generating a hairpin molecule (Fig. 4.1.1). A specific endonuclease nick (Fig. 4.1.2) allows the viral strand to be completed by 5'→ 3' synthesis (Fig. 4.1.3) using the complete terminal sequence of the complementary strand as template. A viral progeny strand could then be generated from the duplex by displacement synthesis, which again involves self-priming (Fig. 4.1.5). This step also generates a hairpin molecule which could be converted into a template for displacement synthesis by a combination of nicking and gap-fill synthesis as before (Fig. 4.1, 6, 7). This endonuclease cleavage would occur near the 3'-terminus of the complementary strand, while the previous scission occurred at the 3' end of the viral strand (Fig. 4.1.2). Since there is no detectable relationship between the ends of MVM DNA (Tattersall and Ward, 1976), this may imply the requirement for different endonucleases. These endonucleases may be specific for certain DNA sequences occurring close to a hairpin loop, thus protecting the linear duplex intermediate from similar cleavage, which would generate fragmented viral progeny DNA.

Such a model requires palindromic sequences at each end of the parental viral DNA. For non-defective parvoviruses it also requires a mechanism to ensure that only the viral strand is displaced and encapsidated.

The appearance of concatemeric DNA molecules up to 4 times unit length AAV DNA led Straus et al. (1976) to propose that the 3' end of

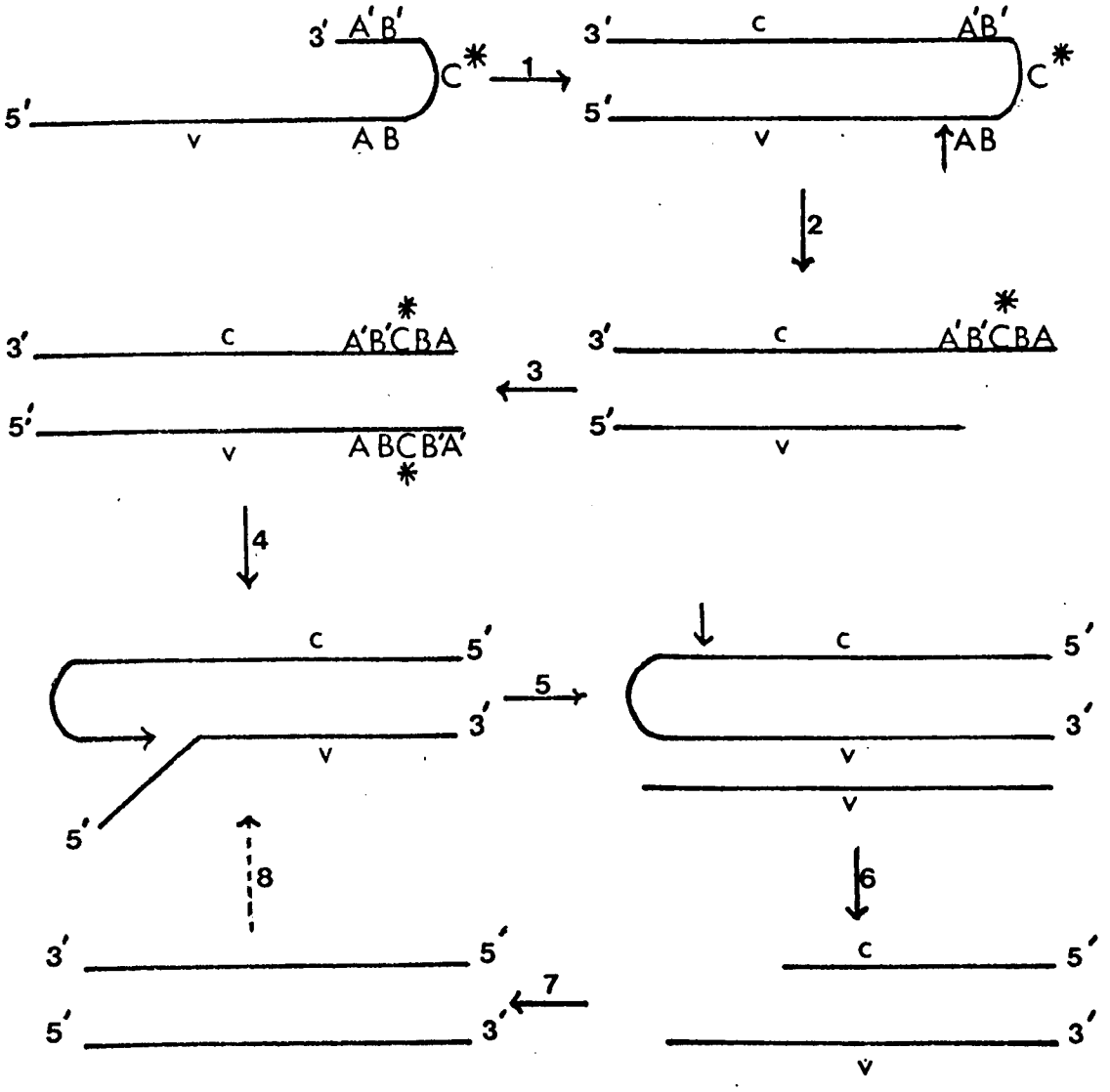


Fig. 4.1

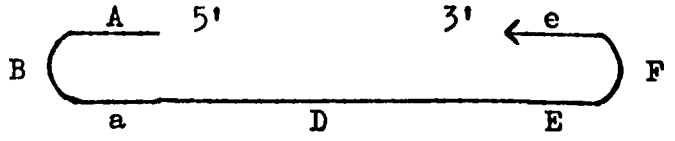
Model for parvovirus replication involving unit length hairpin molecules as the template for progeny strand synthesis (adapted from Straus et al., 1976)

1. 3'-hairpin-primed gap-fill synthesis generating unit length hairpin. 2. Specific endonuclease cleavage (↑). 3. 5'→ 3' synthesis giving complete viral strand. 4, 5. Displacement synthesis generating hairpin and progeny strand. 6, 7. Processing of hairpin into a template for displacement synthesis by nicking (↓) and gap-fill synthesis.

Model requires palindromes at each end of the genome. To conserve terminal sequences the single-strand loops (e.g. C*) must be palindromic e.g. GGTACC.

the unit length hairpin could displace the 5' end of its complementary strand, priming the synthesis of a hairpin twice unit length, or longer (Fig. 4.2.1). Unit length RF molecules, and hairpins, could be generated from concatemers by introducing staggered nicks as proposed by Watson (1972) for T7 DNA. Separation of the unit length molecules would either occur spontaneously (Fig. 4.2.4) or require displacement synthesis (Fig. 4.2.2-3) as proposed by Watson (1972) for T7, depending on the length of the overlapping sequences. In each case the final products would be a unit length duplex molecule, with complete ends, and a hairpin molecule which could be further processed as already described (Fig. 4.1.6-7). Each of these molecules could then act as templates for displacement synthesis of progeny single strands.

Tattersall and Ward (1976) have studied the structure of the MVM genome by S_1 nuclease digestion and by using the DNA as a substrate for several DNA polymerases. They have proposed a structure as follows:



in which Aa represents a duplex of about 130 base-pairs, D is totally single-stranded and represents 95% of the total genome, and EFe represents a hairpin of unknown length. Based on these and other data they have proposed a model for parvovirus DNA replication, similar in some respects to the rolling circle model proposed for ØX174 replication (Gilbert and Dressler, 1968). The main features of their "rolling hairpin model" are described in Figures 4.3 and 4.4.

Briefly, the mechanism involves the synthesis of a unit length hairpin (Fig. 4.3.1-3), followed by an enzymic rearrangement of

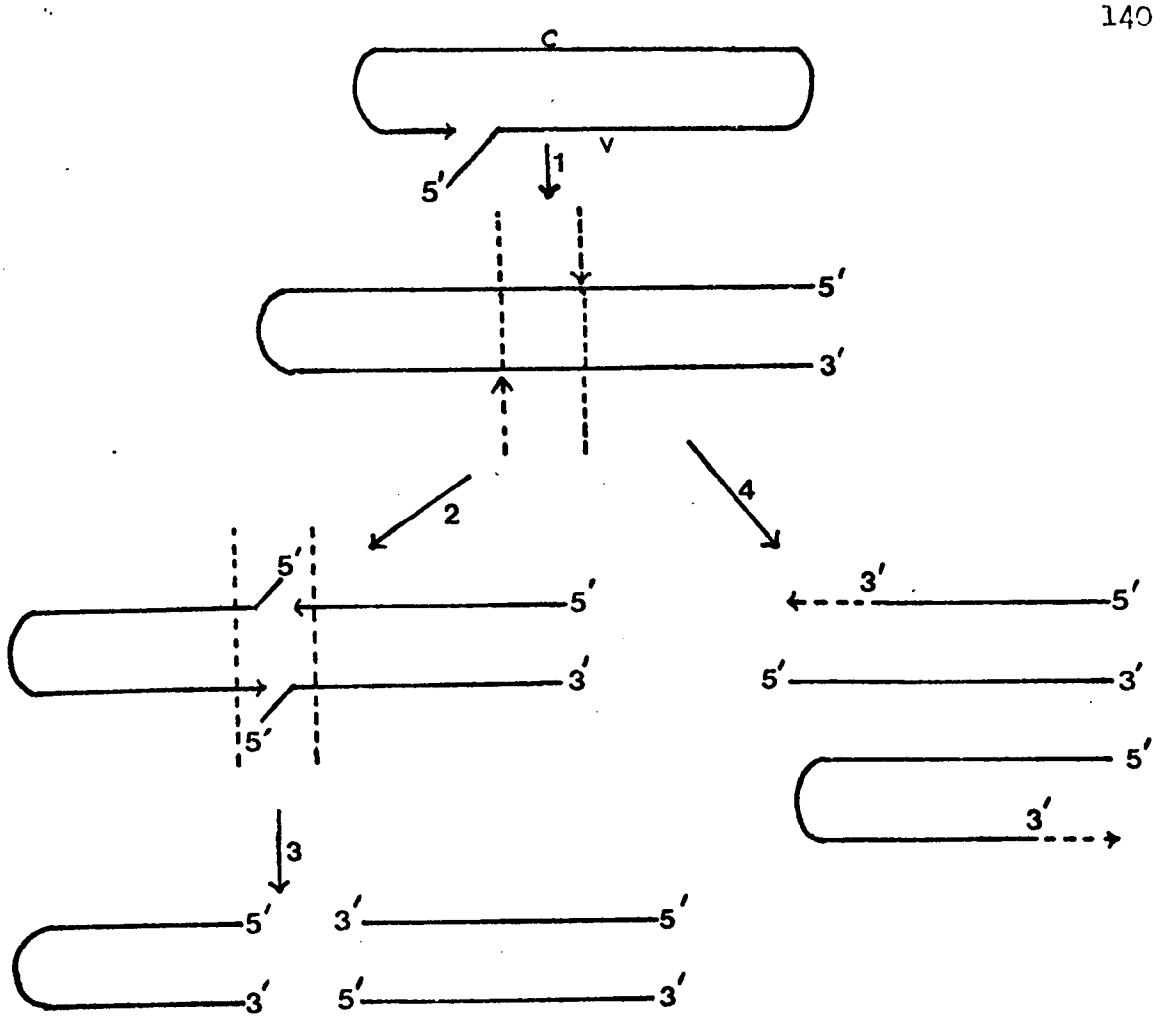


Fig. 4.2

Unit length RF DNA replication via concatemers (adapted from Straus et al., 1976).

1. Displacement synthesis in the absence of endonuclease cleavage, generating dimer hairpin.

2, 3. Introduction of staggered nicks and displacement synthesis (Watson, 1972) to generate hairpin and linear duplex with complete ends.

4. Alternatively the staggered scissions may effect the separation of the molecules.

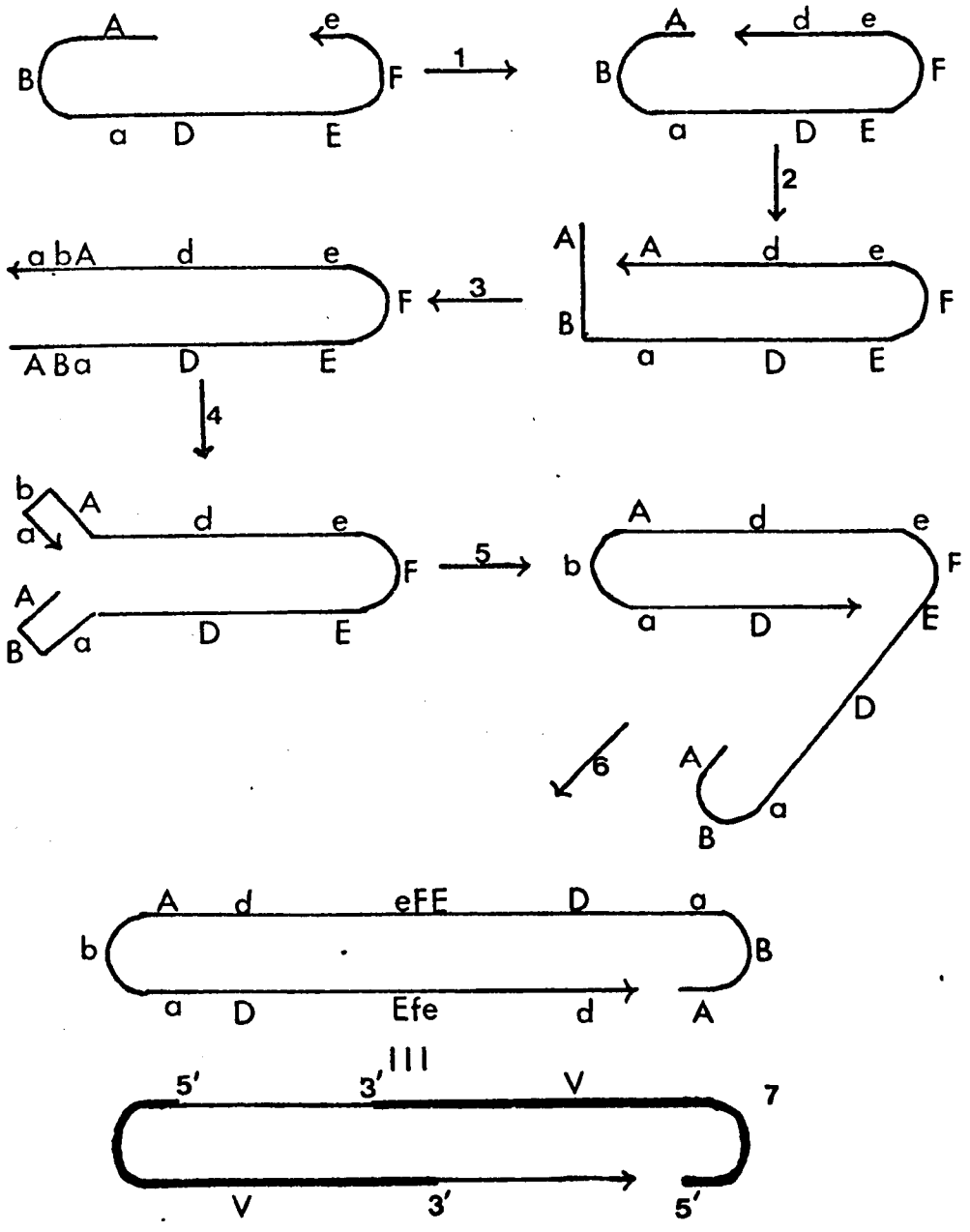


Fig. 4.3

Rolling Hairpin Model for parvovirus DNA synthesis (Tattersall and Ward, 1976).

1 - 3 Gap-fill synthesis followed by displacement synthesis generating unit length hairpin.

4. Enzymic re-arrangement of terminal palindromes.

5, 6. Displacement synthesis, generating dimer.

7. Dimer showing position of viral strands.

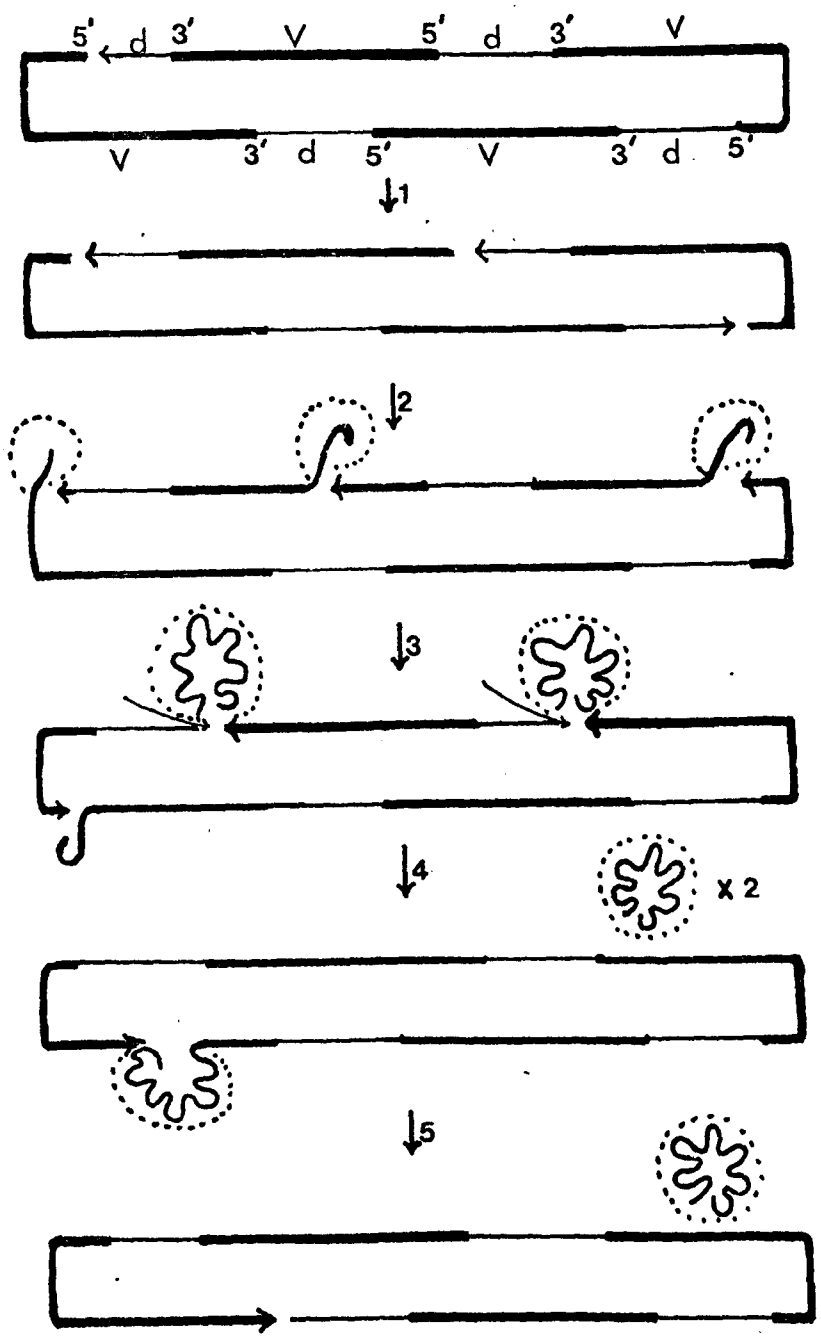
To conserve terminal sequences the single strand loops B and F would have to be palindromic, as mentioned in the legend to Fig. 4.1.

Fig. 4.4

Proposed mechanism for parvovirus progeny DNA synthesis, showing a tetramer intermediate (Tattersall and Ward, 1976).

The progeny DNA strand V is represented by the heavy line throughout, d represents the 4,100 nucleotides of the complementary strand which is not packaged by autonomous parvoviruses. The dotted circle represents the capsid of an immature virion.

1. Endonuclease cleavage at 5' end of viral strands initiates synthesis.
2. Displacement of progeny strand by 5'→ 3' synthesis.
3. Packaging of complete genome and cleavage of 3'-OH end of genome (arrow).
4. Release of virion and repair of 3'-OH end of newly synthesised progeny strand to the 5'-end of its adjoining complementary strand.
5. Regeneration of tetramer.


5' d 3' V 5' d 3' V
V 3' d 5' V 3' d 5'
1
2
3
4
x 2
5

terminal palindromes to form a "rabbit eared" structure (Fig. 4.3.4), as already described for AAV DNA (Denhardt et al., 1976). This recreates the hairpin at the 5' terminus of the genome and creates a copy of this hairpin on the 3' end of the complementary strand, which can serve as a primer for the synthesis of the progeny strand. This new 3' hairpin eventually becomes the 5' hairpin of that future progeny genome (Fig. 4.3.5 and 7). This has been termed "hairpin transfer" and is the novel feature of the model. The dimer length duplex is completed by displacement synthesis (Fig. 4.3.6). In the absence of endonuclease cleavage larger concatemers may be synthesised (Fig. 4.4). The introduction of single-strand scissions at the 5' end of a viral genome within the concatemer (Fig. 4.4.1), and displacement synthesis (Fig. 4.4.2-4) is the essence of the mechanism for generating progeny strands. Tattersall and Ward (1976) have suggested that displacement synthesis may be driven by concomitant packaging of the progeny strand since little or no free single-stranded DNA can be detected during infection (Rhode, 1974a; Mayor and Jordan, 1976; Siegl and Gautschi, 1976). This theory is supported by the report that H-1 progeny DNA synthesis requires a viral protein which is not necessary for ongoing RF replication (Rhode, 1976).

While the two models are similar in some respects, they have basic differences. Concatemers are a pre-requisite for single-stranded progeny DNA synthesis in the rolling hairpin model while their role in the mechanism proposed by Straus et al. (1976) is to effect the replication of unit length RF molecules. The template for displacement strand synthesis in the rolling hairpin model is a concatemer, while the other involves unit length hairpins.

At present it is not possible to distinguish between the two models. Both would appear to explain the presence of hairpin molecules and concatemers (Tattersall et al., 1973; Siegl and Gautschi, 1976; Straus et al., 1976), found in this study, and the presence of branched DNA molecules (Tattersall et al., 1973; Siegl and Gautschi, 1976). If progeny strand synthesis were driven, in each model, by the recognition of the 5' terminal sequence/structure, of only the viral strand, and its subsequent packaging (Tattersall and Ward, 1976), asymmetric displacement synthesis could be accounted for.

Results presented in this thesis, obtained by pulse-labelling infected cells for short times, suggested that most of the newly-synthesised DNA sequences were associated with unit length duplex DNA, but this conflicts with similar experiments described by Rhode (1974a) for H-1. Straus et al. (1976), however, have reported that unit length duplexes appear to be immediate precursors of AAV progeny strands. It has been shown here that during short pulse-labelling regimes, MVM DNA molecules of shorter than unit length are preferentially labelled. It is therefore possible that strand displacement synthesis may be re-initiated before an intact viral strand has been displaced.

The results obtained using the triton extraction procedure suggest that virus maturation may occur via a 95S DNA-protein complex, and that other DNA-containing intermediates may be involved in virus replication. The models proposed for parvovirus replication should prove useful in designing future work to elucidate the precise steps involved in parvovirus replication.

The implications of such models on the mechanism of replication

of the ends of eukaryotic chromosomes have not gone unnoticed. Tattersall and Ward (1976) have proposed that the ends of host cell chromosomes may be replicated by the method of "hairpin transfer" as described, which is essentially a modification of Cavalier-Smith's (1974) method. They have further speculated that the late S phase function(s) required for autonomous parvovirus replication may be the expression of an enzyme system required to effect the replication of the ends of the host chromosome, which the linear parvovirus DNA uses to replicate its own ends.

Terminal palindromes, indeed, may be a pre-requisite for the completion of linear DNA molecules. Several viral DNA's have been shown to contain such sequences, including AAV (Denhardt et al., 1976) and herpesvirus (Wadsworth et al., 1976). Covalently cross-linked terminal structures have been found in vaccinia virus DNA (Geshlin and Berns, 1974) and, more recently chromosome-size yeast DNA has been shown to contain 1 - 4 naturally-occurring cross-links per molecule (Forte and Fangman, 1976). Whether any of these occurs at a terminus, however, has not been established. Similarly, the fact that T7 DNA can be terminally cross-linked in vitro (Weiss, 1970; Sadowski et al., 1974) suggests that it contains a terminal palindrome, which agrees with the finding that rapidly-renaturing T7 DNA of longer than unit length can be isolated from infected cells (Barzilai and Thomas, 1970).

Finally, the results presented in this thesis, obtained using the triton method, which suggest the presence of further MVM replicative intermediates, emphasise the importance of maintaining viral DNA-protein interactions during replication. Indeed, since the proposed models involve such intermediates this approach should be

especially useful. Characterisation of these intermediates should play a significant role in the understanding of parvovirus reproduction.

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